Cuticular Wax Biosynthesis of *Lycopersicon esculentum* and Its Impact on Transpiration Barrier Properties during Fruit Development

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von
Jana Leide
aus Grevesmühlen

Würzburg 2008

Eingereicht am: ……...…….…….02.12.2008

Mitglieder der Promotionskommission:

Vorsitzender: Prof. Dr. Martin Müller

Gutachter: Prof. Dr. Markus Riederer

Gutachter: Prof. Dr. Wolfram Hartung

Tag des Promotionskolloquiums: ……...…….…….26.02.2009

Doktorurkunde ausgehändigt am: …….....……

Meinen Eltern

CONTENTS

INTRODUCTION

Aerial organs of higher plants are covered by a continuous extracellular membrane, the cuticle. Due to its position, the cuticular membrane plays a key role in the survival of terrestrial plants. Representing a dynamic and selective barrier, one of its main functions is to prevent desiccation by limiting non-stomatal water loss from primary plant surface (Lendzian and Kerstiens, 1991; Kerstiens, 1996; Edwards *et al*., 1996; Riederer and Schreiber, 2001; Burghardt and Riederer, 2006). Other major functions of the cuticle as an interface between plants and their surrounding environment include protection against pathogens, herbivores, UV light, freezing temperatures, wind and mechanical damage (Schönherr, 1982; Eigenbrode and Espelie, 1995; Post-Beittenmiller, 1996; Becraft, 1999; Jenks and Ashworth, 1999; Heredia, 2003; Bargel *et al*., 2004). Moreover, it plays a critical role for cell-to-cell communication including pollen-pistil interaction and organ separation processes (Pruitt *et al*., 2000; Sieber *et al*., 2000; Krolikowski *et al*., 2003). These multiple roles underlining the physiological and ecological importance of plant cuticles can only be understood on the basis of their compositional arrangement and their biosynthetic origin.

The cuticle is heterogeneous in nature. Structurally, the cuticular membrane is composed of an insoluble polymer matrix (cutin) and associated solvent-soluble lipids (cuticular waxes) that can be divided into two spatially distinct layers: the epicuticular wax coating the outer surface and the intracuticular wax embedded within the cutin matrix (Holloway, 1982; Baker, 1982; Walton, 1990; Figure 1).

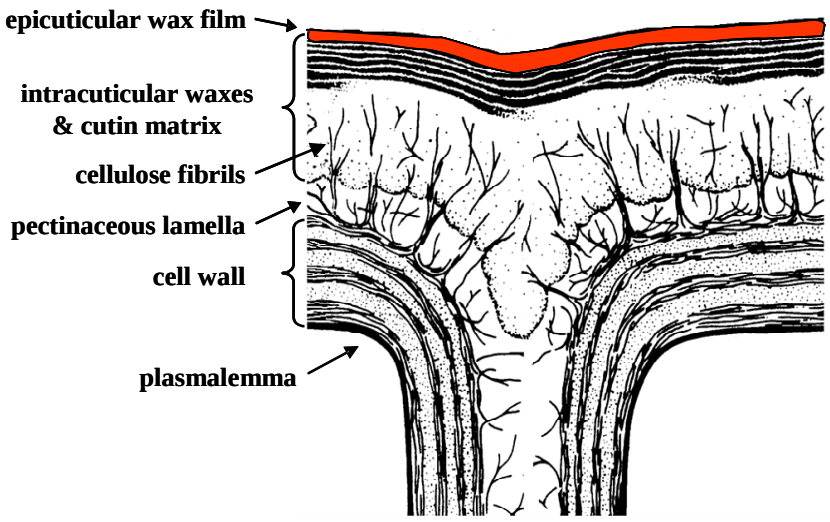

Figure 1. Schematic assembling of a cross section of the plant cuticle. The cuticular membrane is composed of the cutin matrix and cuticular waxes, overlapping the cell wall of epidermal cells (according to Jeffree, 1986).

Each of these layers is distinctly defined by its thickness, ultrastructure and chemical composition, which can vary dramatically across plant species and even within individual plants (Jeffree, 1996; Post-Beittenmiller, 1996; Kerstiens *et al.*, 1996; Jenks and Ashworth, 1999; Jetter *et al.*, 2000; Riederer and Schreiber, 2001; Kolattukudy, 2001; Marga *et al.*, 2001; Guhling *et al*., 2005).

The permeability of the cuticle is not necessarily correlated with its thickness or wax coverage. Its interfacial properties are more likely determined by the chemical composition and/or the assembly of its compounds (Riederer and Schreiber, 1995; Baur *et al*., 1999; Riederer and Schreiber, 2001; Kerstiens, 2006). Though cuticular membranes are mainly considered as a lipid barrier, hydrophilic structures like non-esterified hydroxy and carboxy groups within the cutin polymer and polysaccharides like pectin and cellulose are also present (Schönherr und Huber, 1977; Holloway, 1982; Jeffree, 1996; López-Casado *et al*., 2007). Currently, two parallel and independent pathways for diffusion across the plant cuticle are discussed: (i) a lipophilic pathway accessible for non-ionic hydrophobic molecules composed of lipophilic cutin and wax domains and (ii) a hydrophilic pathway for inorganic ions and small uncharged and charged organic molecules, which is postulated to consist of a continuum of polar materials across the cuticle (Schönherr, 1976, 1982; Popp *et al.*, 2005; Schönherr, 2006; Schreiber, 2006). There is evidence that water as a small uncharged polar molecule may have access to both pathways of transport (Schönherr and Merida, 1981; Niederl *et al.*, 1998; Riederer and Schreiber, 2001; Schlegel *et al.*, 2005; Riederer, 2006).

The formation of cuticular components in the plant epidermal cells involves several biosynthetic pathways: acyl lipids, isoprenoids and phenylpropanoids (Hunt and Baker, 1980; Luque *et al*., 1995; Bauer *et al*., 2004a, 2004b; Suh *et al*., 2005; Jetter *et al*., 2006; Mintz-Oron *et al*., 2008).

Acyl-precursors of cutin and wax monomers up to a C_{18} skeleton are *de novo* synthesized by a plastid-localized fatty acid synthase (FAS) system, which utilizes intermediates bound to acyl carrier proteins (ACP; Stumpf, 1980; Browse and Somerville, 1991; Slabas and Fawcett, 1992; Ohlrogge *et al.*, 1993; Aharoni *et al*., 2004; Figure 2).

The sequential linkage of two carbon units of acetyl coenzyme A (CoA) begins with the condensation of malonyl-ACP to form a C_4 β-ketoacyl-ACP product. This condensation reaction is catalyzed by a β-ketoacyl synthase III (KASIII). The enzyme β-ketoacyl synthase I (KASI) is involved in the stepwise condensation of a fatty acyl-moiety to produce C_6 to C_{16} β-ketoacyl-ACP products. The final condensation reaction, which is the

conversion of C_{16} to C_{18} β-ketoacyl-ACP product, is catalyzed by β-ketoacyl synthase II (KASII). Each elongation cycle involves three supplementary enzymatic steps in addition to the condensation reaction: The β-ketoacyl-ACP product is reduced to β-hydroxyacyl-ACP, dehydrated to enoyl-ACP, and finally reduced to acyl-ACP. Subsequently, the fully reduced fatty acyl-ACP reaction product serves as substrate for the next cycle of elongation (Somerville *et al*., 2000).

The plastidic pool delivering *de novo* synthesized long-chain fatty acyl-precursors divides into various metabolic pathways, whereas both cutin and cuticular wax biosynthesis are supposed to be dominant sinks after a conversion of fatty acyl-ACP to fatty acyl-CoA as acyl donor (Post-Beittenmiller, 1996; Kolattukudy, 2001; Nawrath, 2002).

The cutin monomers are formed by multiple hydroxylation and epoxidation reactions of predominantly $C_{16:0}$ and $C_{18:1}$ fatty acyl-CoA that are three-dimensionally cross-linked into the polyester cutin matrix contributing to the mechanical strength of the whole cuticle (Kolattukudy, 1980, 1981; Blée and Schuber, 1993; Wellesen *et al*., 2001; Reina and Heredia, 2001; Nawrath, 2006; López-Casado *et al*., 2007; Li *et al*., 2007).

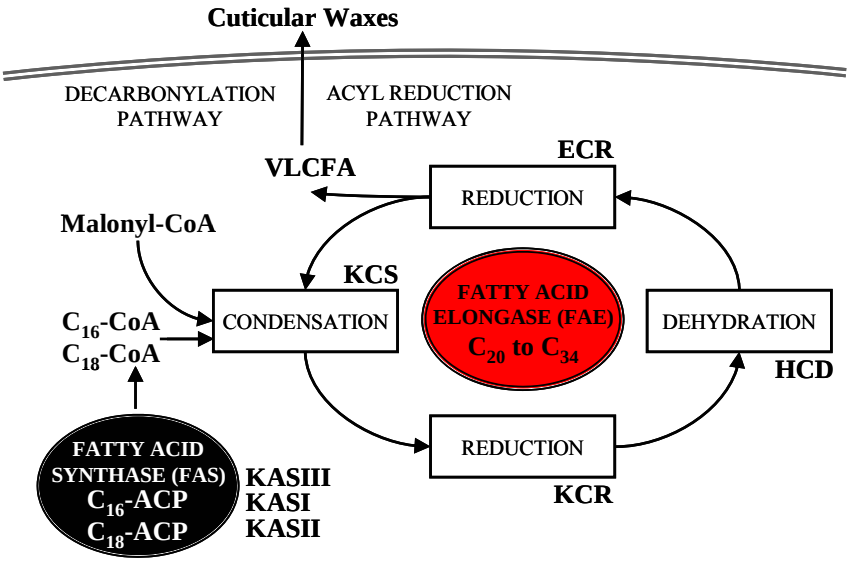

Figure 2. A simplified scheme of biosynthetic pathways of aliphatic wax compounds in plants. The biosynthesis in epidermal cells comprises a plastidic synthase complex of long chain fatty acids and a microsomal elongase complex of very-long-chain fatty acids, which yields wax precursors for different compound classes (according to Kunst and Samuels, 2003; Costaglioli *et al*., 2005).

In the case of cuticular waxes, the long-chain fatty acyl-precursors are transferred across the plastid envelop to the endoplasmic reticulum, where mainly $C_{18:0}$ fatty acyl-CoA is used to generate very-long-chain carbon skeletons with chain length ranging from C_{20} to

C_{34} (Baker, 1982; Lemieux *et al.*, 1994; Post-Beittenmiller, 1996; Jenks *et al*., 2002; Schnurr *et al*., 2004; Lai *et al.*, 2007).

The biosynthesis of the collectively termed as very-long-chain fatty acids (VLCFA) is a stepwise condensation of two carbon units to fatty acyl-precursor *via* CoA-intermediates. The microsomal fatty acid elongation depends on the activity of a membrane-bound, multienzyme fatty acid elongase (FAE) system to carry out four dissociable enzymatic reactions similar to those described for the plastidic fatty acid biosynthesis (Stumpf and Pollard, 1983; Lessire *et al.*, 1985; Agrawal and Stumpf, 1985; Bessoule *et al.*, 1989; Fehling and Mukherjee, 1991; Cassagne *et al.*, 1994).

The initial condensation reaction in VLCFA biosynthesis, which links two carbon units from malonyl-CoA to the long-chain fatty acyl-CoA-precursor, is catalyzed by different β-ketoacyl-CoA synthases (KCS). Experimental evidence suggests that this first step is substrate-specific as well as rate-limiting in the microsomal elongation pathway (Lassner *et al.*, 1996; Millar and Kunst; 1997). Subsequent reactions comprise a reduction of the β-ketoacyl-CoA, extended by two carbon units, to β-hydroxyacyl-CoA by a β-ketoacyl-CoA reductase (KCR). A β-hydroxyacyl-CoA dehydrogenase (HCD) is responsible for dehydration of β-hydroxyacyl-CoA to form an 2,3-enoyl-CoA. A second reduction of the resulting double bond is catalyzed by 2,3-enoyl-CoA reductase to generate the elongated fatty acyl-CoA (ECR; Fehling and Mukherjee, 1991). Enzymes involving in these latter three reactions are constitutively expressed and present in the same cell as the condensing enzyme (Millar and Kunst, 1997).

The resulting mixture of unbranched, even-numbered VLCFA-CoA esters represents essential precursors of diverse acyl lipid classes found in the cuticular waxes, which mainly consist of homologous series of VLCFA derivatives. VLCFA can either be hydrolyzed to free alkanoic acids, modified by the decarbonylation pathway to yield aldehydes that are further derivatized to alkanes, secondary alkanols and ketones or used to generate wax esters by condensing primary alkanols in the acyl reduction pathway (Harwood, 1980; Cheesbrough and Kolattukudy, 1984; Vioque and Kolattukudy, 1997; Millar *et al*., 1999; Han *et al*., 2001; Kunst *et al.*, 2006; Jetter *et al*., 2006; Rowland *et al*., 2006; Shepherd and Griffith, 2006; Samuels *et al*., 2008).

Cuticular waxes are compositionally characterized by complex mixtures of aliphatic compounds, most commonly composed of very-long-chain alkanoic acids, alkanols, aldehydes, alkanes and esters varying in a species-, organ- and tissue-specific manner (von

Wettstein-Knowles, 1993; Post-Beittenmiller, 1996; Jenks and Ashworth, 1999; Kunst and Samuels, 2003).

Additionally, cuticular waxes often contain non-aliphatic lipid classes for example pentacyclic triterpenoids and sterol derivatives originating from the cytosolic isoprenoid pathway. The triterpenoid production is independent of acyl lipid biosynthesis and starts with the transformation of acetyl-CoA into mevalonate, proceeding *via* the formation of farnesyl pyrophosphate, squalene and 2,3-oxidosqualene with the ensuing cyclization of oxidosqualene, the branch point for the biosynthesis of triterpenoids and sterols in plants (Xu *et al*., 2004; Jetter *et al*., 2006; Murata *et al*., 2008).

Cuticular components are formed in epidermal cells, but there is considerably less information about how wax monomers are exported from the outer layer of the plasma membrane onto the outer surface of the cell wall and assembled into the cuticle. On the one hand, the transfer of cuticular compounds probably occurs *via* diffusion through molecular-scale spaces and channels consisting between the cutin polymer and the cuticular waxes. On the other hand, recent reports provided evidence for an enzymatic transport mechanism that governs the secretion of cuticular components from the epidermal cells towards the cuticular membrane (Yamada, 1992; Pighin *et al*., 2004; Shepherd and Griffith, 2006).

Cuticular waxes were identified as the actual barrier of plant cuticles against transpiration and diffusion of solutes (Schönherr, 1976, 1982; Schönherr and Riederer, 1989; Wijmans and Baker, 1995). The amount, chemical characteristics and spatial arrangement of waxes within the cuticle may have an important effect on properties of the cuticular barrier. On the molecular level, cuticular waxes consist of morphologically distinct fractions of different degrees of order and composition including crystalline and amorphous wax structures (Figure 3).

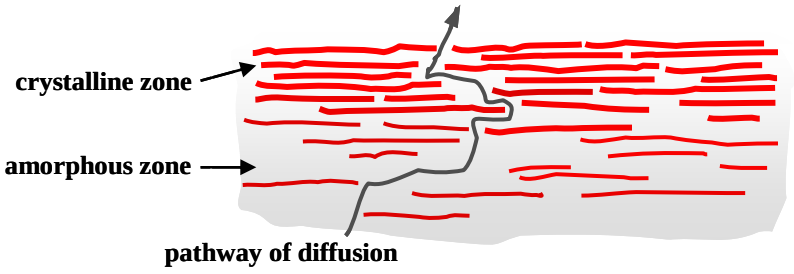

Figure 3. The barrier model of the transport-limiting structure of plant cuticular waxes. The cuticular waxes consist of impermeable flakes of crystalline embedded within a matrix of amorphous material (according to Riederer and Schreiber, 1995).

The crystalline zones of cuticular waxes act as diffusion barriers against water loss, which increase the tortuosity and thus the length of the diffusion pathway (Riederer and

Schreiber, 1995; Buchholz *et al.*, 1998; Riederer and Friedmann, 2006). Nevertheless, the understanding of the relationship between the chemical basis of cuticular waxes and their function as a transpiration barrier remains unclear.

Several wax mutants are known from *Arabidopsis thaliana* (L.) Heynh. and other plant species. At first they were isolated in visual screens *via* the degree of glossiness and later analyzed for alterations in quantity or composition of cuticular waxes (Koorneef *et al.*, 1989; McNevin *et al.*, 1993; Aarts *et al.*, 1995; Jenks *et al.*, 1995, 1996a, 2002; Post-Beittenmiller, 1996). The phenotypic differences in wax composition that can be traced down to the molecular level are discernible for only for a limited number of these *eceriferum* (*cer*) or glossy (*gl*) mutants (Millar *et al.*, 1999; Fiebig *et al.*, 2000; Chen *et al.*, 2003; Pighin *et al.*, 2004; Goodwin *et al.*, 2005; Sturaro *et al.*, 2005; Nawrath, 2006; Rowland *et al.*, 2006; Jung *et al.*, 2006; Qin *et al.*, 2007). Hence, the knowledge on the elements involved in biosynthesis and regulation of wax deposition is still fairly restricted, most notably since the precise functions of many of the so far identified genes involved in cuticular wax biosynthesis still remain to be determined. 'Reverse genetic' approaches based on the knock-out of a target gene can probably bridge the gap between gene sequences and their enzymatic function.

Another obstacle that has to be overcome is the difficulty to correlate the mutant phenotypes with functional aspects (Kerstiens *et al.*, 2006). Particularly with regard to transpirational water loss, *Arabidopsis* with its comparably small and delicate organs is not suitable for the functional analysis of the intact plant cuticle. Furthermore, as described by Kerstiens (1996) a residual stomatal transpiration even under conditions of minimal stomatal conductance cannot be excluded. Therefore, the analysis of astomatous plant surfaces is favorable. A series of recent reports underlined the essential requirement for a suitable model plant system to relate wax composition to cuticular permeability (Kerstiens, 2006; Kerstiens *et al.*, 2006).

Fruits of the solanaceous species tomato (*Lycopersicon esculentum* Mill.), an appropriate plant model system for functional genomic studies, ideally serve this purpose (Fei *et al.*, 2004; Fernie *et al.*, 2004; Rose *et al.*, 2004; Alba *et al.*, 2004, 2005; Moore *et al.*, 2005). Tomato is one of the most important crop species that possesses several features like a relatively small, widely known diploid genome (n = 12 chromosomes; Rick and Yoder, 1988; Hille *et al.*, 1989; Meissner *et al.*, 1997; Emmanuel and Levy, 2002). Presently, a total of 35% of the complete tomato genome is sequenced (www.sgn.cornell.edu/about/tomato_sequencing.pl) corresponding to more than 46849

unique gene sequences in the tentative expressed sequence tag (EST) assembly database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=tomato). A multiplicity of sequence information is available for tomato but the function of only a few genes are known.

The molecular and genetic analysis of tomato fruit development -mainly the climacteric ripening process- as well as pathology and physiology make this fleshy berry fruit a highly interesting model system because of the distinctive feature of such biological mechanisms in plants (Gillaspy *et al.*, 1993; Joubes *et al.*, 1999; Giovannoni, 2004, 2007; Carrari and Fernie, 2006; Dan *et al.*, 2006). Over the years, many studies were performed on ethylene biosynthesis and response, cell wall metabolism, carotenoid accumulation and the production of aromatic compounds during the development of tomato fruits (Oeller *et al.*, 1991; Theologis *et al.*, 1993; Picton *et al.*, 1993; Ayub *et al.*, 1996; Ronen *et al.*, 1999; Giovannoni, 2001, 2004; Alexander and Grierson, 2002; Carrari and Fernie, 2006; Giorio *et al.*, 2008).

The present thesis focuses on wax biosynthesis during tomato fruit development that affects the water balance of fruits and, furthermore, the metabolic homeostasis. The chemical composition of tomato fruit cuticular waxes, which predominantly consist of very-long-chain alkanes and triterpenoids, was the topic of previous investigations (Hunt and Baker, 1980; Baker *et al.*, 1982; Bauer, 2004a; Bauer, 2004b). The composition of the cuticular wax coverage changed in correspondence with an increase of wax deposition as the fruit ripened. At present, it still remains unknown how and to which extent several wax compounds contribute to the physiological function of the cuticle as a transpirational barrier.

The dwarf tomato cultivar MicroTom differs from standard tomato cultivars primarily by only three major dominant genes *miniature* and *dwarf* and *self-pruning*, respectively, conferring the miniature growth habit of all MicroTom organs, with the exception of seeds, in a well-proportioned manner (Scott and Harbaugh, 1989; Meissner *et al.*, 1997, 2000; Emmanuel and Levy, 2002; Yamamoto *et al.*, 2005).

With detailed analyses of different fruit developmental stages of MicroTom according to fruit size and color this study aims to broaden the knowledge of the developmental pattern of tomato fruit wax accumulation in combination with functional analysis of cuticular water loss barrier properties, while focusing on the effects caused by the specific β-ketoacyl-CoA synthase deficiency in the *lecer6* mutant plants. *Lecer6* is a defined Activation (*Ac*)/Dissociation (*Ds*) transposon-tagged wax mutant of MicroTom deficient in

a β-ketoacyl-CoA synthase, an essential element of a VLCFA elongase complex (Meissner *et al.*, 1997, 2000; Emmanuel and Levy, 2002; Yamamoto *et al.*, 2005).

In order to investigate the role of different wax compositions of MicroTom wild type and MicroTom *lecer6*, studies of both, the chemical wax composition and functional genomics a microarray was established. Gene expression studies were performed in order to correlate distinct functional and chemical aspects of wax biosynthesis with transcriptional alterations during different fruit developmental stages.

The accumulation of the cuticular waxes is known to depend on several aspects like the genetic background, the developmental stage and environmental conditions such as internal factors including water deficit, which ultimately involves oxidative stress, and external factors like temperature, light, wind (Thomas and Barber, 1974; von Wettstein-Knowles *et al.*, 1979; Bengtson *et al.*, 1979; Gordon *et al.*, 1998; Jetter and Schäffer, 2001; Hooker *et al.*, 2002; Cameron *et al.*, 2006; Shepherd and Griffiths, 2006). The presently available data on tomato fruit wax development are restricted to mature fruits or reflect developmental changes with respect to fruit size only (Haas, 1974; Baker *et al.*, 1982; Bauer *et al.*, 2004b). Extensive studies on the wax biosynthesis of tomato fruits are still needed with respect to both different developmental stages and stress stimuli, especially under confined and controlled conditions.

The knowledge about cuticular barrier properties is fundamental for the understanding of general shifts that are linked to the metabolic changes occurring during fruit development. CHAPTER I to CHAPTER IV address these questions by critically evaluating the current state of knowledge (Figure 4).

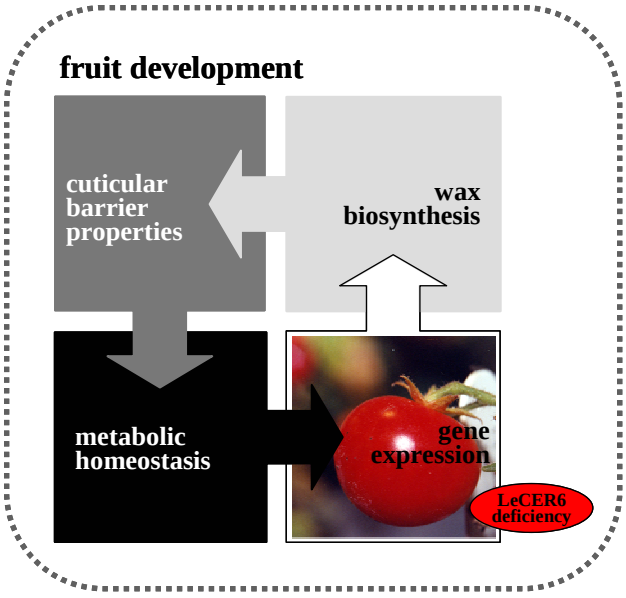

Figure 4. Schematic overview of experiments in CHAPTER I to CHAPTER IV: different aspects of cuticular wax biosynthesis of tomato and its impact on transpirational barrier properties during fruit development. The impact of LeCER6 deficiency on cuticular wax accumulation was analyzed.

Attached tomato fruits receive water from their parent plant maintaining vascular continuity in a source-sink relationship. Except the objectives detailed above, the present study was also intended to address the *de novo* synthesis of a barrier of detached tomato fruits.

During harvest the tomato fruit incurs wounding, particularly at the stem scar region. Hence, a rapid 'wound healing' including the formation of a hydrophobic barrier to reduce water evaporation and/or transpiration is pivotal in preventing the tomato fruit from dehydration and, additionally, shields against pathogenic invasion and strengthens the fruit integrity during post-harvest fruit development. However, knowledge on the nature, time course, efficiency and regulation of 'wound healing' processes at the tomato stem scar tissue is very limited.

Suberin is a cell wall-associated biopolymer that in many cases acts as an interface separating plant cells from their hostile environment (Zimmermann *et al.*, 2000; Hose *et al.*, 2001; Kolattukudy, 2001; Schreiber *et al.*, 2005). Suberin is a high-molecular-weight lipid polyester consisting of a polyaliphatic domain in association with a polyaromatic domain that is derived from ferulic acid (Lulai and Corsini, 1998; Moire *et al.*, 1999; Bernards *et al.*, 2004). The polyaliphatic suberin domain is predominantly based on long-chain α,ω-alkandioic acids and ω-hydroxyalkanoic acids interesterified in a glycerol-bridged network (Bernards, 2002; Moon *et al.*, 2005; Graça and Santos, 2006, 2007; Franke and Schreiber, 2007). Both the suberin and the cutin polymer are closely related in function and monomeric composition but differ in chain length and substitution patterns of their fatty acid constituents (Post-Beittenmiller, 1996; Le Bouquin *et al.*, 2001; Nawrath, 2002; Aharoni *et al.*, 2004; Pollard *et al.*, 2008). The assembly of the monomeric units at a macromolecular level still remains hypothetical.

The exact qualitative and quantitative composition of suberin monomers varies in different species. In aerial parts of higher plants, suberin deposition, which impregnates cell walls, is considered to be a component of the plant response to mechanical damages by wounding or pathogenic attack and, thus, protecting plant surfaces (Kolattukudy, 1981; Mohan *et al.*, 1993; Lulai and Corsini, 1998; Nawrath, 2002). Wound-induced suberization of tomato fruit surfaces was described by Dean and Kolattukudy (1976). However, this process is complex and still poorly understood.

A rapid reduction in permeance for water at the wound surface is suggested to be essential to keep cells viable to allow for the subsequent formation of an efficient transport barrier. So far, it is not known how wounding induces suberization of cells. Several studies

on potato tuber tissue suggested that the stress-regulatory molecule abscisic acid (ABA) might be involved in the regulation of 'wound healing' (Soliday *et al.*, 1978; Cottle and Kolattukudy, 1982; Peña-Cortés *et al.*, 1989, 1996; Herde *et al.*, 1996). In plants, ABA is known to integrate environmental constraints linked to changes in water availability with metabolic and developmental programs. Plants that are challenged by different osmotic stresses recruit ABA as an endogenous signal to initiate adaptive responses (Zhu, 2002; Himmelbach *et al.*, 2003). Upon wounding, ABA levels of tomato leaves were preferentially increased near the wound site (Birkenmeier and Ryan, 1998). As a result, ABA was suggested to accumulate upon wounding because of dehydration. Nevertheless, the role of ABA in wound-healing of tomato fruits remains to be elucidated.

The research scope of this present study was therefore broadened. As a second major aim biochemical aspects were examined that comprise the wound response of the tomato fruit stem scar with respect to its function as an impervious barrier restricting water loss and entry of plant pathogens at the site of injury. In this regard, the post-harvest course of gene expression activity in the stem scar tissue and the role of ABA in the regulation of 'wound healing' were investigated and presented in CHAPTER V (Figure 5).

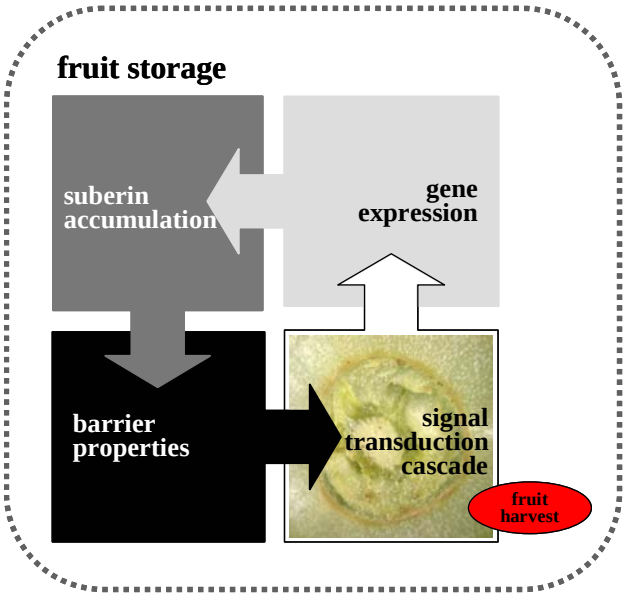

Figure 5. Schematic overview of experiments in CHAPTER V: post-harvest formation of the suberized stem scar tissue during storage of tomato fruits. Visual, transcriptional, chemical and physiological changes at the stem scar tissue of detached, mature green fruits were investigated.

CHAPTER I

Developmental Pattern of Tomato Fruit Wax Accumulation and Its Impact on Cuticular Transpiration Barrier Properties: Effects of a Deficiency in the β-Ketoacyl-CoA Synthase LeCER6

RESULTS

The cuticle is the outermost layer of the terrestrial plants contributing as boundary against their environment. Its main component cutin provides a matrix for cuticular waxes. These waxes are either embedded within the cutin biopolymer framework and form the intracuticular wax or are loaded outside of the cuticle membrane generating the epicuticular wax layer. This study is referring to the functional impact of these layering in relation to the transpirational barrier property of the cuticle. Thereby the fruit development of MicroTom wild type and MicroTom *lecer6* serves as an excellent model system for investigation of both increased amounts of cuticular waxes, which are synthesized and transferred to the fruit surface, and the relation of cuticular waxes to the water flux through the astomatous fruit cuticle. The characteristic chemical composition of the cuticular wax depending on the way and the rate by which different constituents are exuded through the fruit cuticle occupied the central issue.

Cuticular Water Permeability during Development of Tomato Wild Type and LeCER6-Deficient Fruits

For a comparative experimental design fruits of MicroTom wild type and MicroTom *lecer6* were divided according to their developmental stage by means of fruit size and color into seven categories. On the average, fruits of the *lecer6* mutant remained slightly smaller than their wild type counterparts of comparable age and pigmentation (Figure 1).

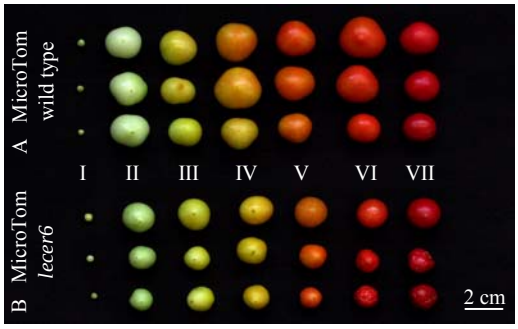

Figure 1. Assignment of representative MicroTom wild type (A) and MicroTom LeCER6-deficient (B) fruits to the developmental categories. Fruits were arranged into seven groups according to size and color: I immature green, II mature green, III early breaker, IV breaker, V orange, VI red ripe, VII red overripe.

MicroTom wild type fruits assigned into fruit developmental category I (immature green) exhibited virtually the same cuticular water permeance as the LeCER6-deficient fruits of the same developmental stage (Table I). In category II, representing mature green fruits, *lecer6* fruits had significantly higher permeance values (2.8-fold) when compared to the wild type. Following the transition from category I to category II wild type fruits showed a decrease in cuticular water permeance of about 96%, whereas mutant fruits exhibited a reduction of permeance values by about 89%. In categories III and IV, covering early breaker and breaker fruit developmental stages, water permeance was further reduced by another 2% of the initial value for MicroTom wild type fruits, but no more for the *lecer6* mutant.

Table I. Permeance for water (x 10^{-5} m s^{-1}) of MicroTom wild type and MicroTom LeCER6-deficient fruits of seven developmental categories of untreated, gum arabic-stripped, chloroform-extracted and peeled tomato fruits. Within each line, treatments were compared with a Kruskal-Wallis ANOVA and subsequent multiple comparison of ranks: significant differences are marked with different letters ($P < 0.05$). Statistical differences between MicroTom *lecer6* and MicroTom wild type was tested with Mann-Whitney *U*-tests (n.s. = not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Data are shown as means ± SD ($n = 10$).

category		I	II	III	IV	V	VI	VII
untreated	wild type	46.0 ± 15.7^{A}	1.8 ± 0.3^{B}	0.9 ± 0.2^{B}	1.1 ± 0.2^{B}	1.1 ± 0.3^{B}	0.9 ± 0.2^{B}	0.9 ± 0.2^{B}
	lecer6	47.5 ± 25.4^{a}	5.1 ± 1.6^{a}	7.9 ± 2.3^{a}	9.0 ± 2.8^{a}	9.0 ± 4.4^{a}	7.2 ± 1.6^{a}	7.8 ± 1.6^{a}
	ratio	*1.0*$^{n.s.}$	*2.8****	*8.4****	*8.1****	*8.2****	*8.2****	*8.5****
gum arabic	wild type	96.5 ± 26.4^{A}	6.7 ± 2.7^{B}	2.2 ± 0.6^{B}	3.3 ± 1.1^{B}	2.5 ± 0.5^{B}	3.0 ± 0.9^{B}	3.5 ± 0.8^{B}
	lecer6	76.9 ± 28.9^{a}	21.6 ± 8.5^{a}	19.1 ± 4.6^{a}	17.4 ± 5.8^{a}	16.2 ± 5.7^{a}	13.5 ± 4.5^{a}	11.7 ± 2.9^{a}
	ratio	*0.8*$^{n.s.}$	*3.2****	*8.5****	*5.2****	*6.6****	*4.5****	*3.4****
chloroform	wild type	232.2 ± 93.8^{A}	79.3 ± 8.3^{AB}	65.0 ± 6.4^{ABC}	51.2 ± 6.7^{BC}	45.3 ± 13.6^{BC}	27.6 ± 7.0^{C}	24.4 ± 3.9^{C}
	lecer6	224.2 ± 77.2^{a}	111.4 ± 10.1^{a}	90.8 ± 12.3^{ab}	75.9 ± 8.0^{abc}	68.6 ± 11.2^{abc}	48.6 ± 7.9^{bc}	53.8 ± 9.0^{b}
	ratio	*1.0*$^{n.s.}$	*1.4****	*1.4****	*1.5****	*1.5****	*1.8****	*2.2****
peeled	wild type	-	133.8 ± 17.0^{A}	141.3 ± 22.3^{A}	138.8 ± 37.9^{A}	129.5 ± 17.4^{A}	132.3 ± 14.5^{A}	159.2 ± 47.5^{A}
	lecer6	-	173.9 ± 15.5^{a}	217.4 ± 17.0^{a}	213.8 ± 38.1^{a}	182.7 ± 33.1^{a}	184.9 ± 16.2^{a}	203.6 ± 34.2^{a}
	ratio	-	*1.3****	*1.5****	*1.5****	*1.4****	*1.4***	*1.3****

Orange to red overripe fruits, assigned to categories V to VII, exhibited only minor changes concerning cuticular water permeance in the further course of fruit maturation. The water permeance of wild type fruits finally decreased to 2% of the category I value, whereas the permeance of the *lecer6* fruits was reduced to only 16% of the initial value in category I. Hence, in categories III to VII, *lecer6* mutant fruits exhibited an eightfold increased water loss per unit time and fruit surface area when compared to the wild type.

Data on permeance for water within categories II to VII differed significantly between both lines.

Effects of Wax Extraction and Different Removal Techniques

To analyze the individual effects of epi- and intracuticular waxes on water permeance, mechanical fractionation of epi- and intracuticular waxes and chloroform extraction of total cuticular waxes were applied using MicroTom wild type and MicroTom *lecer6* fruits of all selected developmental categories I to VII (Table I). The relevance of the outermost epicuticular waxes on cuticular water permeance was analyzed after selective removal of this wax layer with gum arabic, a solvent-free water-based polar organic adhesive. Gum arabic treatment resulted in a two- to fourfold increase of water permeance for both wild type and *lecer6* mutant fruits. As with untreated fruits, significant differences between wild type and *lecer6* became discernible in category II. Hence, mechanical removal of the epicuticular waxes with gum arabic did not abolish initially measured differences in permeance between wild type and *lecer6*. In later stages of fruit development (categories IV to VII) the differences between wild type and *lecer6* permeances were slightly decreased. In category II gum arabic treatment of *lecer6* fruits resulted in a fourfold increase of permeance values, whereas fruits of category VII showed only 1.5-fold elevated permeances in comparison to untreated mutant fruits. In line with this, *lecer6* fruits exhibited slightly decreasing permeance values during the course of fruit ripening (categories III to VII), whereas the wild type values remained more or less constant in the respective developmental stages.

Chloroform dipping, however, increased permeances in fruits of category I by a factor of five. This effect was distinctly enhanced with fruits of category II showing a 44-fold increase of permeance for the wild type and a 22-fold increase for *lecer6* compared to the respective values of untreated fruits. After chloroform dipping, both wild type and *lecer6* fruits exhibited a distinct decrease of permeance values during the course of fruit

development. However, this decrease was more pronounced with wild type than with *lecer6* fruits.

Even the complete removal of the fruit cuticle resulted in distinct permeance values of wild type and *lecer6* fruits. Peeled *lecer6* fruits continuously exhibited 1.3- to 1.5-fold elevated permeance values when compared to wild type fruits of the respective developmental categories II to VII. Nevertheless, a distinct decrease of permeance values in the course of fruit development was no longer observed with fruits fully devoid of their cuticular transpiration barrier.

According to a simplifying model proposed by Schönherr (1976), epicuticular (ECW) and intracuticular waxes (ICW) and the dewaxed cutin matrix (DCM) can be considered to act, in analogy to electrical circuits, as diffusion resistances in series. Resistances are the reciprocal of water permeances. Therefore, the relative contribution of wax layers to total resistance may be estimated from the equation $r_{CM} = r_{WAX} + r_{DCM} = r_{ECW} + r_{ICW} + r_{DCM}$, where r_{CM}, r_{WAX}, and r_{DCM} correspond to the resistances of the cuticular membrane (CM), the wax layers (ECW, ICW), and the dewaxed cutin matrix (DCM), respectively (Knoche *et al.*, 2000). By applying this model to the data (Figure 2), the distinctly increasing diffusion resistances during the first three categories of fruit development became evident in untreated wild type fruits (r_{CM}), whereas *lecer6* fruits exhibited a significant increase only during the transition from category I to II.

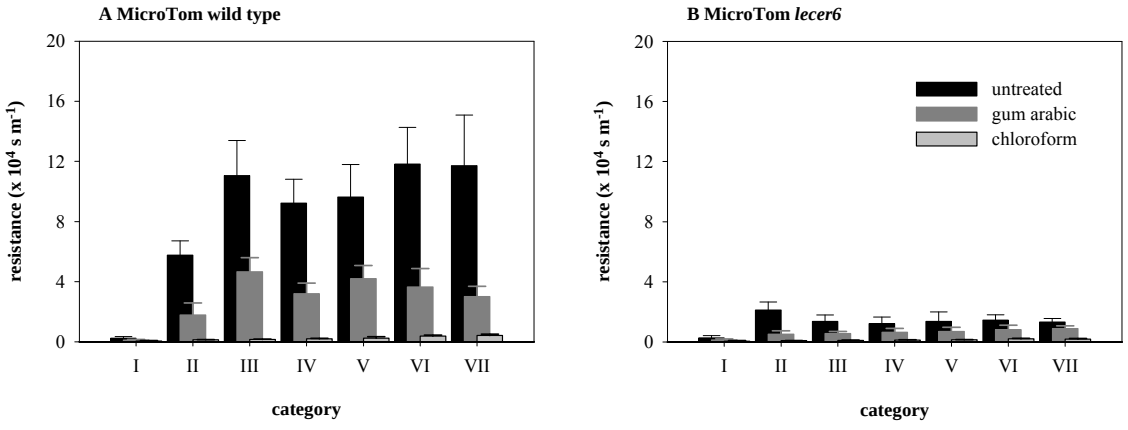

Figure 2. Cuticular resistances of tomato wild type (A) and LeCER6-deficient (B) MicroTom fruit cuticles of different developmental categories against water loss. Resistances were calculated as the reciprocals of water permeances of untreated, gum arabic-stripped and chloroform-extracted tomato fruits. Data are shown as means ± SD (n = 10).

For wild type fruits, gum arabic stripping exerted a particularly pronounced effect on cuticular resistance, resulting in a significant decrease. Resistances largely followed the pattern observed for untreated fruits in the time course of development. Chloroform dipping further reduced cuticular resistance paralleled by a continuous increase of resistance over the seven categories of fruit development in wild type and *lecer6* mutant.

To substantiate differences between different wax extraction methods, hitherto present chloroform treatment was compared to the usage of tert-butylmethylether:methanol (TBME:methanol) as organic solvent. TBME:methanol treatment in combination with ultra sonic allows the extraction of an identical spectrum of cuticular wax components as with chloroform, exhibiting only minor quantitative differences (data not shown). In addition to cuticular fruit waxes, the TBME:methanol extracts contained compounds like the cuticular membrane derived flavonoids naringenin and naringenin-chalcone, which were only barely extractable with chloroform.

Nonetheless, exemplary for red ripe MicroTom wild type fruits (category VI) TBME:methanol dipping did not result in significant differences in comparison to the chloroform treatment (Figure 3). A threefold gum arabic removal of epicuticular waxes resulted in an increase of permeance for water inherently by a factor of 3.4 compared with intact MicroTom wild type fruits. Both extractions of total cuticular waxes with chloroform and with TBME:methanol, respectively, boosted the water permeance between 8.8- to 20.3-fold. Whereas rather the immersion efficiency, the extraction time and usage of ultra sonic than the extracting agent exerted influence on the permeance for water of the fruit.

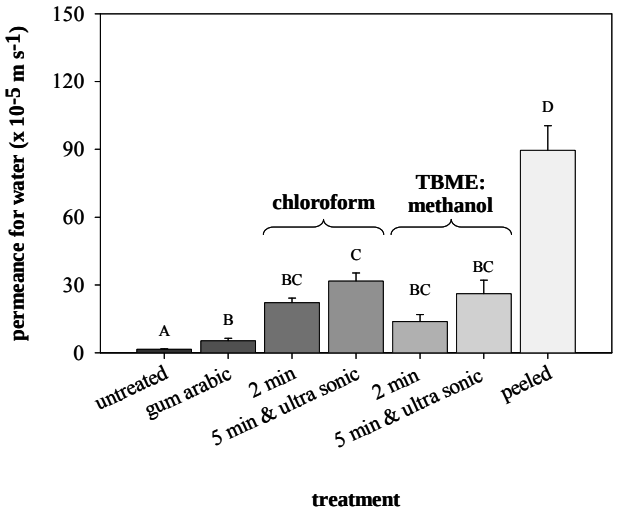

Figure 3. Effects of 3x gum arabic treatment, chloroform and tert-butylmethylether:methanol (TBME:methanol) dipping and epidermis peeling on cuticular permeance for water of red ripe MicroTom wild type fruits in category VI. Different treatments were compared with a Kruskal-Wallis ANOVA and subsequent multiple comparison of ranks: significant differences are marked with different letters ($P < 0.05$). Data are given as means ± SD (n = 10 to 20).

In case of fruit dipping for 2 min in chloroform or TBME:methanol no solvent penetrated the tomato fruit at the stem scar area. This can not be guarantied for the 5 min extraction time combined with ultra sonic as described in detail by Bauer (2002). Additionally, an advanced entering of the solvent extracted compounds derived from deeper fruit layers. However, the permeance for water measured for fruits totally devoid of the cuticle layer was not reached with none of the four different extraction procedures.

Cuticular Wax Coverage and Compound Classes

MicroTom fruit cuticular waxes, derived from chloroform extracts, mainly consisted of *n*-alkanes, pentacyclic triterpenoids, sterol derivatives, primary alkanols, and alkanoic acids. This composition was analyzed over categories I to VII during fruit development, both in wild type and *lecer6* mutant. Immature green fruits (category I) of wild type and *lecer6* mutant did not exhibit major differences regarding to total cuticular wax compound class composition (Figure 4). Nonetheless, in category I wild type fruits (3.98 ± 0.88 μg cm^{-2}; mean ± SD) possessed an almost twofold higher overall wax load as compared to *lecer6* mutant fruits (2.33 ± 0.37 μg cm^{-2}; Student's *t*-test $P = 0.01$, $t = 3.8$). In category II, both lines accumulated a similar amount of cuticular waxes (wild type: 8.06 ± 1.36 μg cm^{-2}, *lecer6*: 10.02 ± 1.66 μg cm^{-2}; $P = 0.03$, $t = 2.6$). Therefore, the increase of wax accumulation between categories I and II was most striking for *lecer6* mutant fruits. Moreover, in category II, significant differences in wax composition between mutant and wild type became apparent, which, in particular, concerned the deposition of aliphatics, especially *n*-alkanes ($P < 0.001$, $t = 15.3$), and cyclic compounds like triterpenoids and sterol derivatives ($P < 0.001$, $t = 5.6$). When compared to the wild type fruit wax composition, LeCER6-deficient fruits exhibited a sharp decrease in the proportion of aliphatic compounds, which was paralleled by a distinct accumulation of triterpenoids. Peaking in category II, the proportion of triterpenoids in wild type wax (approximately 53% of total wax) exhibited a continuous relative decline following the time course of further fruit ripening (category VII: approximately 21% of total wax), whereas it remained more or less constant in *lecer6* mutant fruits grouped into categories II to VII (approximately 80% of total wax). The relative proportion of the *n*-alkanes, another major component class present in wild type wax, was 45% in the total cuticular wax of category I fruits, decreased in category II to approximately 33%, and finally contributed 55% to the total wax composition in category VII.

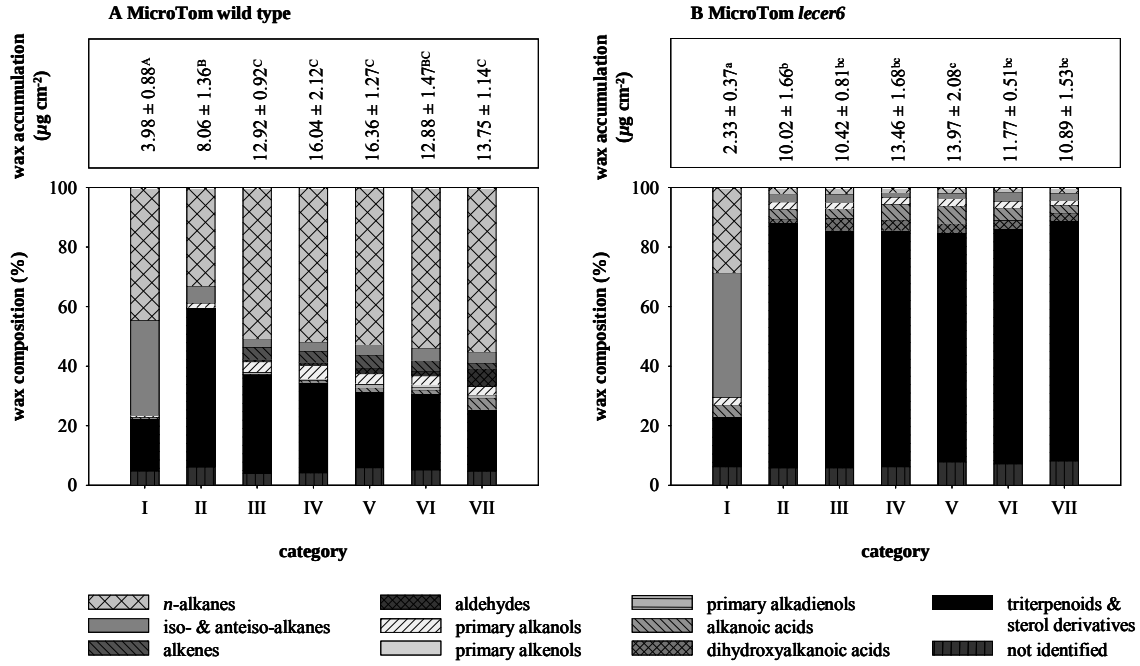

Figure 4. Total cuticular wax quantities and relative wax compositions of wild type (A) and LeCER6-deficient (B) tomato fruits of seven different developmental categories. Wax quantities of different categories within each line were compared by one-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SD ($n = 3$ to 6).

Branched alkanes (iso- and anteiso-alkanes) accumulated to comparable relative amounts in category I fruit cuticles (wild type: 32%, *lecer6*: 42% of total wax), followed by a sharp decrease in category II (wild type: 6%, *lecer6*: 3% of total wax). The deposition of long-chain aldehydes (C_{24}, C_{26}, C_{32}) was exclusively seen with the wild type wax, starting in category II and reaching its maximum in category VII. The total wax load, which peaked for both *lecer6* and wild type fruits in category V, slightly declined in categories VI and VII. However, the strongest augmentation of wax accumulation was marked by the transition from category I to category II. During fruit development, the total soluble fruit wax of both lines increased by a factor of up to six, whereas the final cuticular wax load of the *lecer6* fruits was only slightly, although significantly, decreased in comparison to wild type fruits (wild type: 13.75 ± 1.14 μg cm^{-2}, *lecer6*: 10.89 ± 1.53 μg cm^{-2}; $P = 0.01$, $t = 3.5$).

Compositional Changes of Specific Wax Constituents during Fruit Development

A closer, more detailed look at the specific composition of the different compound classes allowed a distinct evaluation of differences between wild type and *lecer6* mutant, as well as amongst the seven fruit developmental categories (Figure 5). In the wild type and the *lecer6* mutant, the most striking change in wax composition occurred between category I and category II, where cyclic triterpenoids and sterol derivatives accumulated. The amyrins (α-, β- and δ-amyrin; Figure 5: composition number 1 to 4) accounted for about 76% to 91% of the cyclic wax constituents in all categories. In both lines, β-amyrin and δ-amyrin occurred in category II as the most prominent triterpenoids. During transition from category II to III, only wild type fruits exhibited a twofold reduction of the α- and δ-amyrin coverage from 0.82 μg cm^{-2} to 0.42 μg cm^{-2} and from 1.40 μg cm^{-2} to 0.77 μg cm^{-2}, respectively. Only β-amyrin accumulated to about 2.68 μg cm^{-2}. In category II fruits of the *lecer6* mutant α- and β-amyrin reached values of 1.53 μg cm^{-2} and 2.17 μg cm^{-2}, respectively, whereas δ-amyrin accumulated to 2.92 μg cm^{-2}.

The sterols cholesterol (Figure 5: composition number 5), lanosterol (6), multiflorenol (7), stigmasterol (9), taraxasterol and ψ-taraxasterol (10), and lupeol derivative I (13) were identified as common constituents of wild type and *lecer6* wax. Exclusively in the wild type fruit, β-sitosterol (8) and taraxerol (11) were detected, whereas the occurrence of lupeol (12), lupeol derivative II (14), and of an unidentified triterpenoid compound (15) was restricted to the cuticular wax of *lecer6* fruits. The amounts of lanosterol, multiflorenol, taraxasterol and ψ-taraxasterol, and of lupeol derivative I were distinctly increased in the *lecer6* mutant fruit cuticular wax in categories II to VII.

During the early stages of fruit development (categories I to III), *n*-alkanes with chain lengths of C_{29} to C_{31} accumulated in the wild type, whereas this was not the case in the *lecer6* mutant. On the contrary, the total quantities of *n*-alkanes in the *lecer6* wax were strikingly reduced during the transition from category I (29% of total wax) to category II (2% of total wax), which was in parallel with the sharp decline of iso- and anteiso-alkanes in both the MicroTom wild type and *lecer6* mutant.

Figure 5. (continued) 7 multiflorenol, 8 β-sitosterol, 9 stigmasterol, 10 taraxasterol & ψ-taraxasterol, 11 taraxerol, 12 lupeol, 13 lupeol derivative I, 14 lupeol derivative II, 15 unknown triterpenoid. Carbon chain lengths are shown for *n*-alkanes, iso- & anteiso-alkanes, alkenes, aldehydes, primary alkanols, primary alkenols, primary alkadienols, alkanoic acids, and dihydroxyalkanoic acids. Data represent means ± SD (n = 3 to 6).

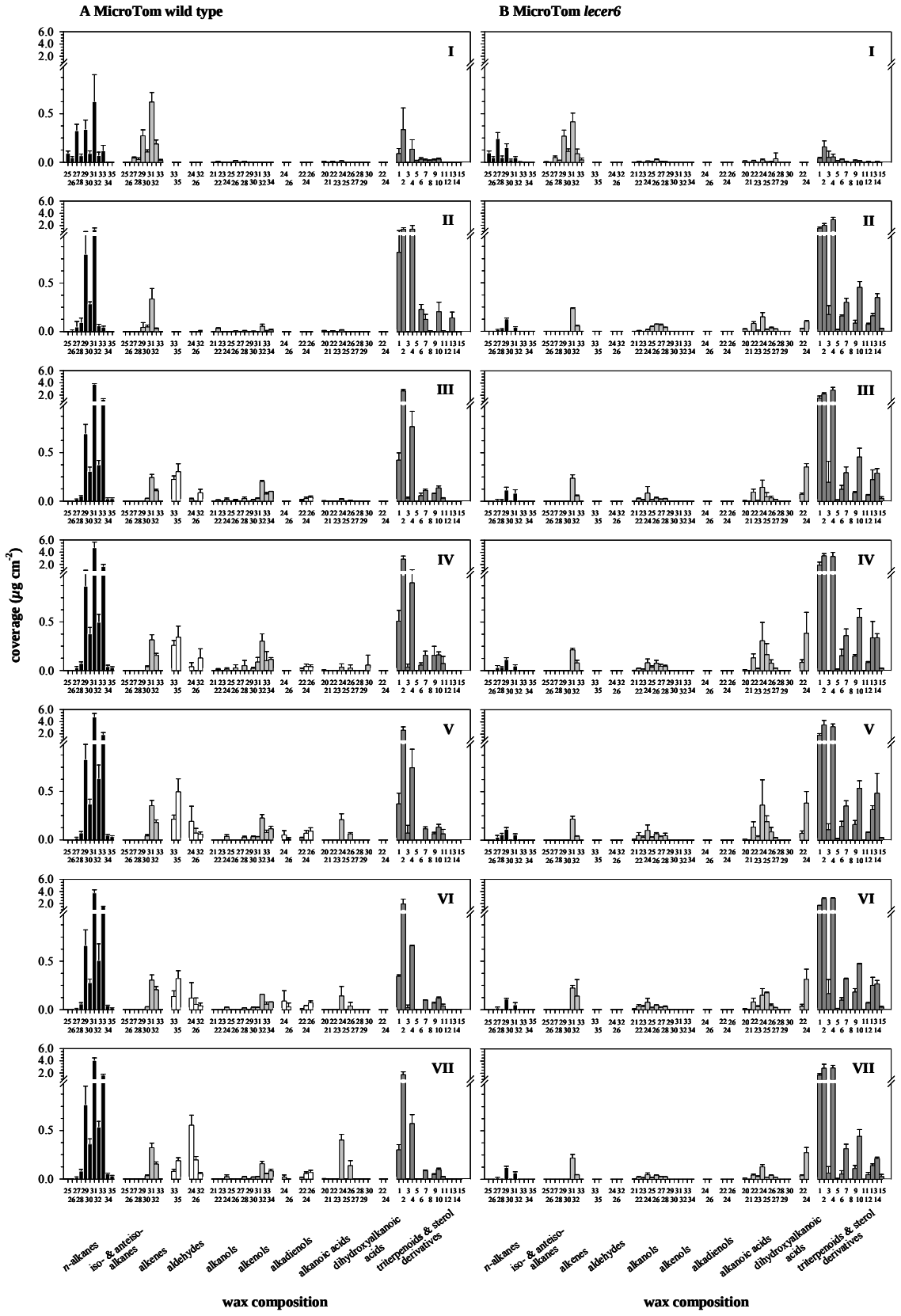

Figure 5. Cuticular wax constituents of MicroTom wild type (A) and LeCER6-deficient (B) fruits assigned to seven developmental categories extracted by chloroform dipping. Triterpenoids and sterol derivatives are numbered as follows: 1 α-amyrin, 2 β-amyrin, 3 β-amyrin derivative, 4 δ-amyrin, 5 cholesterol, 6 lanosterol,

In category III, a qualitative change was mirrored by the increase of *n*-alkanes in the wild type wax with chain lengths of C_{32} and C_{33}, whereas the *n*-alkane composition of the *lecer6* mutant remained unaffected. In parallel, alkenes (C_{33} and C_{35}), which were not detected in the wax of *lecer6,* were present in wild type wax. Similarly, primary alkanols started to accumulate in the wild type in category III, whereas the cuticular deposition of their mutant wax counterparts remained unchanged. Unlike the wild type, *lecer6* fruit cuticles accumulated dihydroxyalkanoic acids (C_{22} and C_{24}) in categories II to III.

Long-chain alkanoic acids (C_{20} to C_{30}) were apparent in the fruit cuticular wax of both lines in categories II to VII. Throughout categories I to VI, the overall amount of alkanoic acids was increased in the *lecer6* cuticular wax when compared to the wild type. Most strikingly, the spectrum of alkanoic acids occurring was narrowed to mainly C_{24} and C_{26} in the wild type, whereas the *lecer6* mutant cuticle contained alkanoic acids with chain lengths from C_{20} to C_{27} in different amounts. In the wild type, alkanoic acids started to accumulate in category IV, peaking in category VII, whereas the *lecer6* mutant exhibited elevated levels already in category II, peaking in category V.

A Spearman's correlation of ranks resulted in a significant negative correlation between permeance for water and the cuticular accumulation of *n*-alkanes (R^2 = -0.58, $P < 0.05$) throughout all developmental categories of MicroTom wild type and MicroTom *lecer6* fruits. A non-significant correlation coefficient was obtained for the correlation between water permeance and amounts of triterpenoid and sterol derivatives (R^2 = 0.23).

Chemical Composition of the Cutin Polymer Matrix during Fruit Development

The chemical composition of the cutin polymer was followed over the selected developmental stages of MicroTom wild type and *lecer6* mutant fruits (Figure 6). The relative proportions of different cutin monomers *lecer6* and wild type cuticles were largely comparable. The dominating cutin monomers in both lines were ω-dihydroxyalkanoic acids. In category VII, they comprised 82% of wild type and 78% of *lecer6* cutin. For both, the major compound was 9 (10), ω-dihydroxyhexadecanoic acid contributing 61% to 80% to the wild type and 67% to 76% to *lecer6* cutin. During the course of fruit development, the proportion of ω-dihydroxyalkanoic acids increased by 16% in wild type and 8% in *lecer6*. Likewise, the proportion of α, ω-alkandioic acids decreased from category I to VII by about 50% in wild type and *lecer6*. The overall proportion of phenolic compounds, like coumaric acid and naringenin, did not exhibit vast changes during fruit ripening in both lines.

Whereas coumaric acid decreased during fruit ripening, naringenin started to accumulate in early breaker fruits (category III). Between wild type and *lecer6* cuticles, a striking difference became discernible regarding the total weight of enzymatically isolated fruit cuticles. Throughout all developmental stages, *lecer6* cuticles exhibited higher weights per fruit surface area (up to fourfold in category I) than wild type cuticles. Nevertheless, for both lines, the cuticle weights increased to the same extent from category I to II and remained more or less constant during fruit maturation.

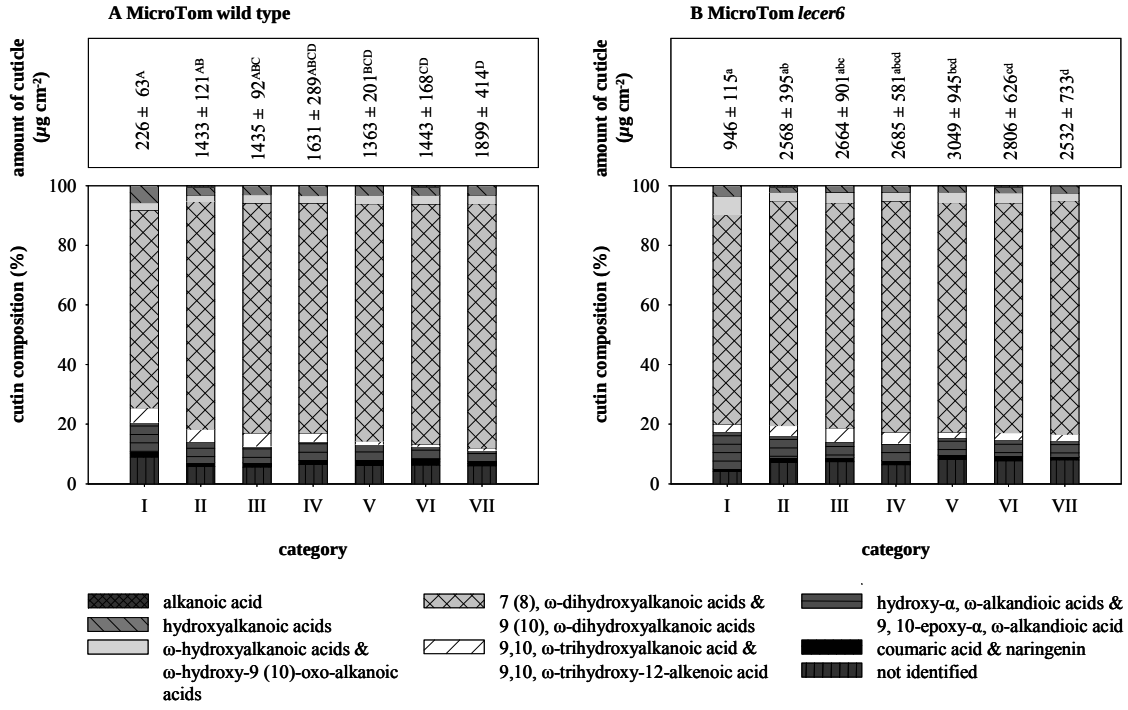

Figure 6. Total amount of cuticle and relative cutin compositions of MicroTom wild type (A) and LeCER6-deficient (B) fruits of seven different developmental categories. Cuticle amounts of different categories within each line were compared with a Kruskal-Wallis ANOVA and subsequent multiple comparison of ranks: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SD ($n = 6$).

DISCUSSION

A number of previous studies focused on the relationship between wax characteristics and transpirational barrier properties of the cuticle. Geyer and Schönherr (1990), as well as Riederer and Schneider (1990), analyzed the effects of various growth conditions on the composition of *Citrus aurantium* L. leaf cuticles and their water permeability. Hauke and Schreiber (1998) demonstrated ontogenetic and seasonal modifications of wax composition

and cuticular transpiration properties in ivy (*Hedera helix* L.) leaves. Jetter and Schäffer (2001) studied ontogenetic changes in the composition of *Prunus laurocerasus* L. adaxial leaf epicuticular waxes. Whereas the overall amounts of *Prunus* leaf waxes increased steadily, several distinct shifts in wax composition occurred in the course of leaf growth and maturation. The authors concluded from their data that such dynamic changes possibly reflect a defined developmental program that regulates biosynthesis and accumulation of cuticular wax constituents. Jenks *et al.* (1996b) analyzed leaf and stem epicuticular wax compositions of *Arabidopsis* wild type and of different wax mutants during development. Because a reliable astomatous plant model system was lacking, which would allow the functional analysis of cuticles from plants with defined genetic modifications, all attempts to correlate water permeance with the qualitative or quantitative composition of plant cuticular waxes failed so far (Riederer and Schreiber, 1995, 2001).

In a recent study Bauer *et al.* (2004b) demonstrated that the quantitative composition of tomato fruit waxes considerably depends on the cultivar assayed and the stage of ripening. To elucidate the quantitative and qualitative modifications of cuticular wax composition during fruit maturation and its functional impact on the fruit cuticular water barrier properties, the composition of fruit waxes of the dwarf tomato cultivar MicroTom and its *lecer6* mutant was followed over seven distinct stages of fruit development. The developmental categories employed corresponded to stages of the ripening process that could easily be differentiated visually according to fruit size and color.

Cuticular Water Permeability and Wax Component Accumulation during Tomato Fruit Development

In the present study, the cuticles of MicroTom fruits were found to have cuticular water permeances between 47.5 ± 25.4 x 10^{-5} m s^{-1} in immature green fruits of category I and 0.9 ± 0.2 x 10^{-5} m s^{-1} (vapor based) in red overripe fruits of category VII. Values obtained for wild type and *lecer6* fruits of categories II to VII were in a similar range as those for isolated mature tomato fruit cuticles from previous investigations, ranging from 2.7 x 10^{-5} m s^{-1} to 14.0 x 10^{-5} m s^{-1} (Schönherr and Lendzian, 1981; Becker *et al.*, 1986; Lendzian and Kerstiens, 1991; Schreiber and Riederer, 1996). Generally, category I fruits exhibited clearly higher values. The overall cuticular water permeance of MicroTom wild type fruit cuticles decreased during fruit development, reaching minimum values in early breaker to red overripe fruits (categories III to VII). Similar developmental patterns concerning the cuticular transpiration were described for the tomato cultivars Bonset and Hellfrucht 1280

(Haas, 1974). Likewise, permeances for *lecer6* fruits remained more or less constant in categories III to VII. In wild type and *lecer6* fruits, the decrease of permeance in categories I to III was paralleled by an increase of total extractable waxes. Considering wax amounts and water permeances, the results suggest the existence of a negative correlation between water permeance and wax coverage in MicroTom fruits. Nonetheless, wax extractions of red ripe (category VI) wild type and *lecer6* fruits yielded comparable amounts of total extractable waxes, although the water permeance of *lecer6* cuticles was increased eightfold when compared to the wild type. The data indicate that, like in other systems, cuticular wax quality rather than its quantity predominantly affects the barrier properties of MicroTom fruit cuticles (Riederer and Schreiber, 2001).

In accordance with these results, Bauer *et al.* (2004b) demonstrated a continuous increase in wax coverage in the developmental course from mature green to red ripe fruits in the tomato cultivar RZ 72-00. In an earlier publication, Baker *et al.* (1982) sampled tomato fruits (cultivar Michigan Ohio) of distinct sizes, ranging from immature green to firm red. Their data indicate a continuous increase in total waxes, except in the mature green stage in which a transient decrease was observed. However, this decrease could not be corroborated with MicroTom fruits used in the present study. Surprisingly, the amounts of extractable total cuticular waxes in the late ripening categories VI and VII were slightly diminished in wild type and mutant fruits and did not result in any increase of water permeance values. This further supports the suggested predominant significance of wax composition rather than amount for cuticular barrier properties of MicroTom fruits. In addition, almost identical permeance levels of *lecer6* were paralleled by approximately twofold higher wax amounts present in the wild type cuticle of category I fruits. Thus, more wax does not necessarily result in reduced permeance levels. The relation of wild type to *lecer6* fruit water permeance values changed dramatically in developmental category II, accompanied by a significant increase in total wax coverage of both lines. In wild type fruits, this increase was largely caused by increasing amounts of *n*-alkanes and pentacyclic triterpenoids, whereas the *lecer6* mutant, though accumulating cyclic triterpenoids in even higher quantities, exhibited a distinct reduction in *n*-alkanes and a slight increase in primary alkanols, alkanoic acids, and dihydroxyalkanoic acids. This emerging reduction of *n*-alkanes per fruit surface area in *lecer6* fruits during transition from category I to II, possibly caused by the considerable surface expansion of the tomato fruits between these particular categories, might be responsible for the observed switch in water permeance characteristics, resulting in elevated permeance values for the *lecer6*

mutant. Regardless of wax composition, a decrease of cuticular water permeance may nonetheless be negatively correlated with the overall cuticular wax amount.

In category III, wild type fruit wax exhibited a continuous accumulation of *n*-alkanes (now mainly C_{31} and C_{33}) and a distinct reduction of water permeance. Commencing with category IV, the amounts and specific compositions of *n*-alkanes in the wild type wax, as well as the respective water permeance characteristics, remain more or less stable. According to this dataset, the barrier properties of the tomato fruit cuticle is supposed to be already largely determined in early breaker fruits (fruit developmental category III in this study), although the overall fruit wax coverage has not yet reached its maximum values in both wild type and *lecer6* plants. Considering the chemical and functional differences between the wild type and *lecer6* mutant, it can be inferred that in MicroTom fruits the developmentally regulated distinct rise of cuticular *n*-alkane constituents is one of the major determinants of cuticular water permeance properties.

Impact of LeCER6 Deficiency on Cuticular Wax Component Accumulation

Biosynthesis of aliphatic wax constituents, starting with C_{18} fatty acid precursors, proceeds *via* sequential elongation steps followed by modification of functional groups (von Wettstein-Knowles, 1993; Hamilton, 1995; Kolattukudy, 1996; Post-Beittenmiller, 1996; Kunst and Samuels, 2003; Kunst *et al.*, 2006). The MicroTom *lecer6* mutant is defective in a β-ketoacyl-CoA synthase (LeCER6) activity, responsible for the elongation of very-long-chain fatty acids. In *lecer6* fruit wax of category II, the largely missing increase of *n*-alkanes with chain lengths > C_{28} indicates that the range of substrate specificity of LeCER6 could actually encompass molecules with chain lengths > C_{29} and therefore might underestimated analyzing mature red fruits only. The *Arabidopsis* AtCER6 β-ketoacyl-CoA synthase, encoded by a gene highly homologous to *Lecer6*, is responsible for the elongation of very-long-chain fatty acids larger than C_{26} (Millar *et al.*, 1999; Fiebig *et al.*, 2000; Hooker *et al.*, 2002). The occurrence of branched iso- and anteiso-alkanes and *n*-alkanes with chain lengths of C_{25} to C_{33} in *lecer6* mutant cuticles of category I suggests that, in early stages of fruit ontogeny, a β-ketoacyl-CoA synthase different from LeCER6 must be involved in the biosynthesis of these particular constituents. In later developmental stages the loss of LeCER6-function is obviously not complemented by functionally redundant enzymes. In the case of *lecer6* fruit wax, the cuticular accumulation of triterpenoids and sterol compounds, as well as the increased weight of the cuticular membrane, may serve as compensations for the reduced amounts of *n*-alkanes. The

biosynthetic apparatus of *lecer6* fruits may not be flexible enough to respond to the loss of LeCER6 function in a way different from increasing the amounts of pentacyclic triterpenoids and sterol derivatives in categories II to V. Nevertheless, this increase mainly of amyrins is obviously not fully sufficient to functionally complement the lack of *n*-alkanes, finally resulting in an up to eightfold increased cuticular water permeance.

Apart from the dramatic differences concerning the accumulation of *n*-alkanes, the specific knock-out of LeCER6 function is suggested to completely prevent the formation of alkenes (C_{33}, C_{35}), aldehydes (C_{24}, $C_{26,}$ C_{32}), alkenols (C_{24}, C_{26}), and alkadienols (C_{22}, C_{24}, C_{26}) in *lecer6* wax, most probably due to missing common precursor molecules. The differential accumulation of alkanoic acids with chain lengths of up to C_{27} in the *lecer6* mutant might reflect the accumulation of molecules that otherwise might serve as potential substrates for the missing LeCER6. As already deduced from the water permeance analyses, the data on the developmental sequence of cuticular wax composition indicate that major effects caused by the deficiency in the LeCER6 β-ketoacyl-CoA synthase are possibly not displayed until the fruit entered the transition from fruit developmental category I to II.

Contribution of Different Cuticular Layers and Wax Constituents to the Transpiration Barrier

Most wax analyses and functional studies in the past did not differentiate between cuticular substructures. Recently, methods were developed that allow the selective sampling of exterior (epicuticular) and interior (intracuticular) wax layers for chemical analyses (Jetter *et al.*, 2000; Jetter and Schäffer, 2001). The permeability properties of epi- and intracuticular waxes might differ in relation to the strong chemical distinctions between these layers. Moreover, the cutin polymer not only serves as a matrix, but may also contribute to the transport characteristics of the cuticle. Therefore, it is desirable to assess to what extent the different cuticular compartments contribute to the overall transpiration barrier. Gum arabic treatment of fruit surfaces allows to selectively remove the outermost epicuticular wax layer and assessment of its relevance for the permeance properties of tomato fruit cuticles *in vivo* (Jetter and Schäffer, 2001).

The composition of tomato fruit epicuticular waxes distinctly differs from intracuticular waxes as exclusively aliphatic components were detected in gum arabic samples obtained from wild type and *lecer6* fruits (data not shown). Because the increase of permeance following chloroform wax extraction was much greater than after gum arabic stripping of

epicuticular waxes, it may be concluded that tomato fruit epicuticular waxes might play a smaller role in determining the cuticular barrier properties in comparison to the intracuticular wax fraction. However, the contribution of the epicuticular wax layer to the permeance properties of tomato fruit cuticles for water becomes more conspicuous when the cuticular resistance model is applied (Schönherr, 1976; Kerstiens, 2006). The resistance of the epicuticular wax layer (r_{ECW}) of MicroTom red ripe wild type fruits (category VI) contributes roughly 70% to total cuticular resistance (r_{CM}), whereas the epicuticular waxes of *lecer6* fruits of the same developmental stage account only for 44%. In line with this finding, the epicuticular waxes of sweet cherry fruit (*Prunus avium* L.) contribute to a similar extent (72%) to the total cuticular resistance (Knoche *et al.*, 2000). According to this model, the striking difference in epicuticular resistance between wild type and *lecer6* fruits of category VI cannot simply be explained by the 2.5-fold reduced epicuticular wax coverage of *lecer6*. Hence, factors other than wax amount must be responsible for the decreased contribution of *lecer6* epicuticular waxes to total cuticular resistance. In category I, the major portion of cuticular wax constituents found with ripe tomato fruits is not yet present. This might explain the almost identical permeance values for wild type and *lecer6* fruits of category I, not only after gum arabic stripping.

Nevertheless, the distinctly elevated permeance values after chloroform or TBME:methanol dipping underline the significance of the intracuticular waxes of MicroTom fruit cuticles as important factors determining fruit water retention capacities.

Impact of the Cutin Matrix

Gum arabic treatment and chloroform dipping resulted in converging permeance values, distinctly reducing the ratio between permeances of wild type and *lecer6* fruits. This clearly reflects the functional significance of cuticular wax quality for the transpirational barrier properties of tomato fruit cuticles. The remaining differences between wild type and *lecer6* fruits after chloroform extraction are only in part attributable to the remaining 20% of the fruit surface area not subjected to chloroform extraction. Therefore, other additional factors must be involved in determining the transpiration barrier properties of the tomato fruit cuticle.

This view is supported by the decreasing permeance values of wild type and *lecer6* fruits after chloroform dipping in the course of fruit maturation. Supposing that the extraction efficiency of chloroform remains constant throughout the selected categories of fruit development, this effect might be explained with distinct modifications of cutin

polymer composition and/or polymer cross-linking during fruit growth and maturation. However, the compositional analysis of MicroTom cutin monomers revealed that both wild type and *lecer6* cuticles exhibited a similar compositional pattern of cutin polymer constituents, when fruit cuticles of the same developmental category were compared. This was not surprising since the loss of LeCER6 activity was not expected to exert direct effects on cutin matrix composition.

During fruit development, similar compositional changes were detected for wild type and *lecer6* cuticles. The results are largely in accordance with data obtained from cuticles of the cultivar Michigan Ohio (Baker *et al.*, 1982). In contrast, Haas (1974) reported that the cutin monomer composition of two different tomato cultivars remained virtually constant during fruit development. Nevertheless, maturation of tomato fruit cutin may entail structural changes resulting in lowered polarity and/or higher cross-linking and, therefore, reduced permeability to water (Schmidt *et al.*, 1981). Although this decrease in categories III to VII becomes evident exclusively after chloroform extraction, the distinct compositional changes of the cutin polymer during fruit maturation might play a role as well. Obviously, in intact wild type and *lecer6* fruits, modifications of the cutin polymer are largely masked by the more effective barrier properties of epi- and intracuticular waxes. Even the distinctly higher weights of *lecer6* fruit cuticles, most probably reflecting an increased amount of cutin, are probably not sufficient to largely affect transpiration barrier properties of the mutant cuticle. After complete removal of the cuticle, wild type and *lecer6* fruits still exhibited significantly distinct permeance values. The dimension of these differences was largely comparable to those observed after chloroform dipping. This suggests that the distinct cuticular permeance of wild type and *lecer6* fruits after removal of the cuticular waxes by chloroform treatment might be largely cuticle independent.

Contribution of Flavonoids

In some cases, additional secondary metabolites, mostly phenylpropanoid derivatives such as the flavonoids, also constitute the plant cuticle (Muir *et al*., 2001; Bovy *et al*., 2002; Verhoeyen *et al*., 2002; Schijlen *et al*., 2007). A striking difference between mature green and red ripe fruit developmental stages is the presence of the flavonoids naringenin and naringenin-chalcone in the cuticular membrane of red ripe fruits (Luque *et al.*, 1995). Both phenolics represent 5% to 10% of total cutin weight and 10% to 40% of the epicuticular waxes of tomato fruits (Hunt and Baker, 1980; Baker *et al.*, 1982).

According to Bauer *et al.* (2004a) total cuticular wax extracts of TBME:methanol contained up to 70% naringenin and naringenin-chalcone, which were detected only in traces when cuticular waxes were extracted with chloroform. Moreover, unhinging of compounds belonging to the inner of the fruits can in this case not be excluded. Likewise, an increasing stringency of the extraction process might lead to extraction artifacts. Nevertheless, no significant differences in the permeance values after chloroform or TBME:methanol dipping were documented. Hence, the additional elution of more polar cuticular compounds like flavonoids such as naringenin by TBME:methanol dipping does not significantly affect water permeance properties of tomato fruit cuticles.

Wax Composition and Cuticular Barrier Properties

Geyer and Schönherr (1990) suggested that the molecular structure of cuticular waxes might be a much more important determinant of permeability than either wax amount or wax composition. Therefore, a model for the molecular structure of plant cuticular waxes was developed (Riederer and Schneider, 1990; Reynhardt and Riederer, 1994; Riederer and Schreiber, 1995). According to this model, the wax barrier consists of impermeable platelets of crystalline zones embedded in a matrix of amorphous material. Water diffusion occurs only in the amorphous volume fractions, whereas crystallites, increasing the tortuosity of the diffusion path, are inaccessible (Buchholz, 2006). Based on x-ray diffraction studies it was postulated that triterpenoids are localized exclusively in the amorphous zones (Casado and Heredia, 1999). Hence, for *lecer6* fruits, a reduction in *n*-alkanes together with the increase in triterpenoids, should create amorphous zones at the expense of crystalline domains. This shift in overall crystallinity might be the actual reason for the increase in cuticular transpiration. Nevertheless, this model exclusively considers the lipophilic path of transport and does not integrate compositional changes either of the cutin matrix or of other constituents of the cuticle. In a previous study, Haas (1974) suggested that the cuticular transpiration of tomato fruits might be strongly promoted by hydrophilic constituents of the cuticle, like cellulose or other polysaccharides. Such compounds might be involved in the formation of polar pores and therefore enhance cuticular transpiration. Previous studies predicted that, in general, more polar constituents embedded in the cuticle could increase its permeability (Goodwin and Jenks, 2005). In fact, several cuticular constituents could provide polar functional groups, which may form the basis of polar aqueous pores. However, the nature of such pores remains to be elucidated (Schreiber, 2006). Nevertheless, the overriding role of cuticular waxes for the

transport-limiting barrier of the tomato cuticular membrane is evident because the cuticular water permeability was increased by one order of magnitude after chloroform dipping of intact fruits. This role holds true for *lecer6*, although the contribution of the largely modified cuticular waxes was significantly reduced. Even Haas (1974) argued that predominantly epi- and intracuticular waxes are responsible for the observed reduction of cuticular water permeability during the course of fruit growth and ripening. With regard to the hydrophilic pathway, cuticular waxes might block potential polar paths and the removal of waxes might increase the pore area or decrease the tortuosity of the hydrophilic pathway, thereby increasing water permeability (Popp *et al.*, 2005). Chloroform extraction of astomatous pear leaf cuticular membranes (*Pyrus communis* L.), however, resulted in only two- to threefold increased rates of penetration for calcium chloride, being restricted to the polar pathway. This showed clearly that the aqueous pores present in extracted cuticles were not covered or plugged up by cuticular waxes (Schönherr, 2000). As yet, it can only be speculated, that individual, eventually more polar, constituents of cuticular waxes might interact with polar pores and, in turn, affect the transpiration characteristics of the cuticle.

CHAPTER II

Transcriptional Changes Related to the Wax Biosynthesis of Tomato during Fruit Development: Effects of a Deficiency in the β-Ketoacyl-CoA Synthase LeCER6

RESULTS

The formation of plant cuticular waxes is a complex mechanism involving wax component biosynthesis, wax modification and associated transport processes that are part of the developmental program of tomato fruits. This present study aimed at elucidating putative gene expression activities relevant in wax formation and deposition. Several transcriptional patterns of MicroTom wild type and MicroTom *lecer6* exhibit differences in gene expression during fruit development between both MicroTom lines or reveal discrepancies between different fruit tissues. This comparative and simultaneous analysis of gene expression activity in fruits of MicroTom wild type and its *lecer6* mutant was done using an oligonucleotide microarray, whereas the examinations mainly focused on the epidermal fruit tissue, where the wax biosynthesis is assumed to be localized. This 'reverse genetic' approach correlates events in gene expression activity pointing at wax biosynthesis with alterations in qualitative and quantitative wax composition (i) and its functional impact as efficient barrier against transpirational water loss (ii) with reference to fruit developmental aspects (iii) as a dynamic process.

Basic Requirements of a Microarray Analysis on Tomato Fruits

As a functional genomic approach, samples of MicroTom wild type and *lecer6* mutant fruits were used for oligonucleotide microarray experiments. The deficiency of the β-ketoacyl-CoA synthase LeCER6 resulted in a different fruit wax coverage affecting both the water loss barrier properties and, consequently, the water balance of MicroTom fruits. These characteristics were investigated during different fruit developmental stages (Figure 1).

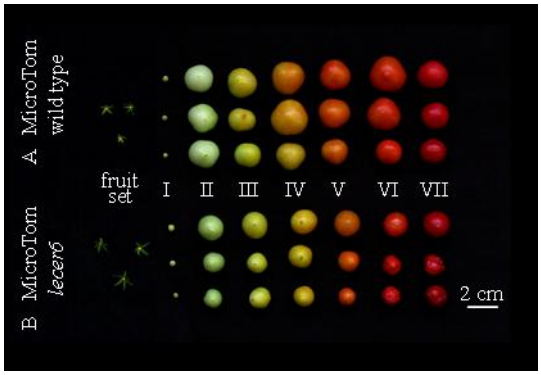

Figure 1. Assignment of representative MicroTom wild type (A) and MicroTom LeCER6-deficient (B) fruits to the developmental categories. Fruits were arranged into eight groups according to size and color: 'fruit set', I immature green, II mature green, III early breaker, IV breaker, V orange, VI red ripe and VII red overripe.

The quantitative wax coverage determined from a series of chloroform extractions was similar between MicroTom wild type and MicroTom *lecer6* in all fruit development categories (Figure 2). In category I, the wax accumulation initiated at a level of 3.98 ± 0.88 μg cm^{-2} (mean ± SD) for the wild type and 2.33 ± 0.37 μg cm^{-2} for the *lecer6* mutant, respectively, and reached a maximum in category V. Exclusively, in category I a difference of 1: 1.7 was found between MicroTom mutant and wild type wax load.

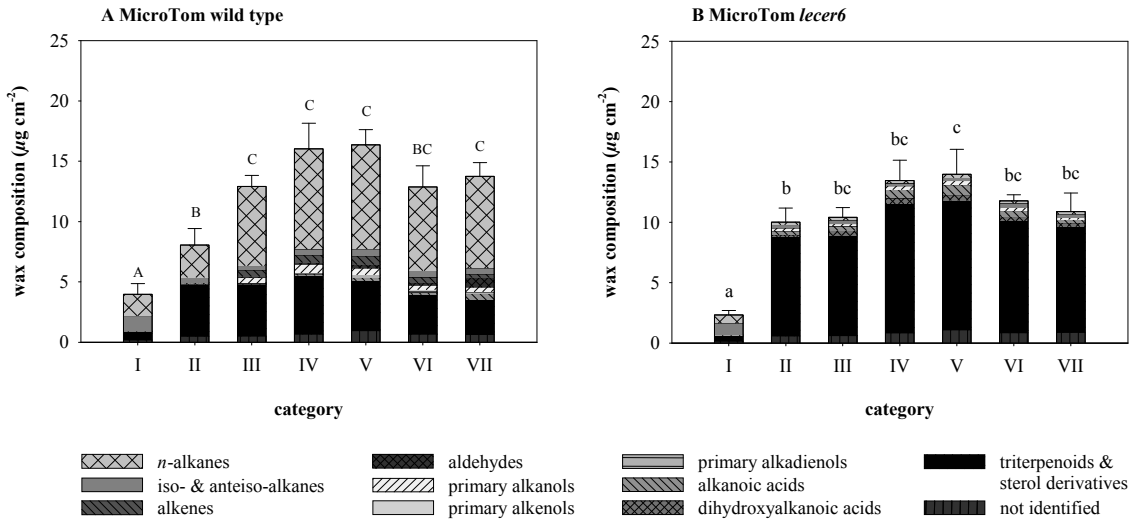

Figure 2. Cuticular wax composition of MicroTom wild type (A) and MicroTom LeCER6-deficient (B) fruits of seven different developmental categories. Wax quantities of different categories within each line were compared by one-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SD ($n = 3$ to 6).

A characteristic shift in the cuticular wax composition occurred during the transitions of category I to category III. In category I the wax composition of both MicroTom lines included three major substance classes: *n*-alkanes, iso- and anteiso-alkanes, and triterpenoids and sterol derivatives. However, the wild type wax in category III and all

further categories mainly consisted of *n*-alkanes (51% to 55% of total wax) as well as triterpenoids and sterol derivatives (21% to 33% of total wax), while the mutant wax was composed of triterpenoids and sterol derivatives (77% to 82% of total wax). From category III on, the percentage of iso- and anteiso-alkanes did not exceed 5% of total wax in both MicroTom lines.

Simultaneous changes were shown for water permeance and water content of MicroTom wild type and *lecer6* mutant fruits (Figure 3 and Figure 4). Within category I the permeance for water was almost equal for MicroTom wild type and LeCER6-deficient fruits. Nevertheless, significant differences were found in the further course of fruit development. After a drastic decrease of the water loss rate from category I to category II in both MicroTom lines the values of permeance for water ranged eightfold higher in the *lecer6* mutant than in the wild type fruits within the later developmental stages.

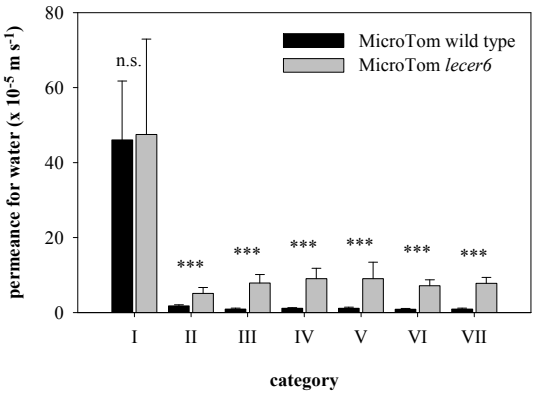

Figure 3. Cuticular permeance for water of MicroTom wild type and MicroTom LeCER6-deficient fruits of seven developmental categories. Statistical differences between MicroTom wild type and MicroTom *lecer6* were analyzed with Mann-Whitney *U*-test (n.s. = not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Data are shown as means ± SD ($n = 10$).

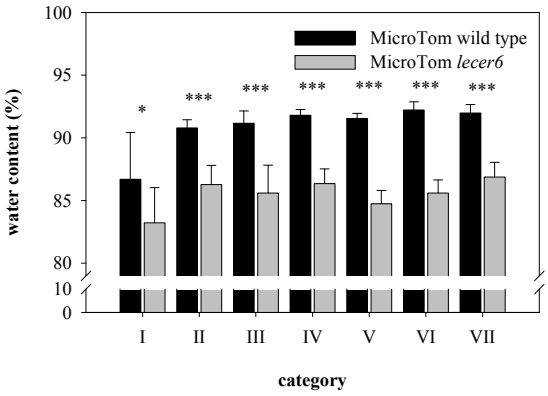

Figure 4. Water content of MicroTom wild type and MicroTom *lecer6* fruits divided into different developmental categories. Statistical differences between MicroTom wild type and MicroTom *lecer6* were analyzed with Mann-Whitney *U*-test (n.s. = not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Data are shown as means ± SD ($n = 10$).

With exception of category I fruits the water content of MicroTom wild type fruits averaged about 92% and about 86% in *lecer6* mutant fruits. The significant difference between both MicroTom lines increased from 3.5% in category I to 6.8% in category V. A significant negative correlation between the permeance for water and the water content throughout all developmental categories of MicroTom wild type and MicroTom *lecer6* fruits was found ($R^2 = -0.86$, $P < 0.05$; Spearman's correlation of ranks).

Design of Oligonucleotide Microarray Experiments Related to Cuticular Wax Biosynthesis

In order to compare the transcriptional differences between equivalent fruit tissues of MicroTom wild type and MicroTom *lecer6* nine microarray experiments were performed. Each experiment represents an identical fruit developmental stage corresponding to the seven fruit categories known from the chemical and functional analyses. In addition, the developmental stage 'fruit set' was investigated. Mature green MicroTom fruits of category II were analyzed twice: on the one hand, as whole fruit without seeds like in category 'fruit set' and category I and, on the other hand, as fruit peel tissue equally used for category III to VII.

The amount of transcripts differed between MicroTom wild type and MicroTom *lecer6* (Figure 5). The total RNA content in category 'fruit set' was 2.51 ± 0.71 μg g^{-1} fresh weight for the wild type and 3.88 ± 1.07 μg g^{-1} fresh weight for the *lecer6* mutant. Starting at this initial level the RNA content of whole fruits increased distinctly in both MicroTom lines, but more striking for the wild type. However, the fruit peel tissue revealed an apparently higher RNA content in the LeCER6-deficient samples compared to in the wild type. For both, MicroTom wild type and MicroTom *lecer6*, same amounts of RNA were used for the microarray cDNA syntheses to avoid any discrimination.

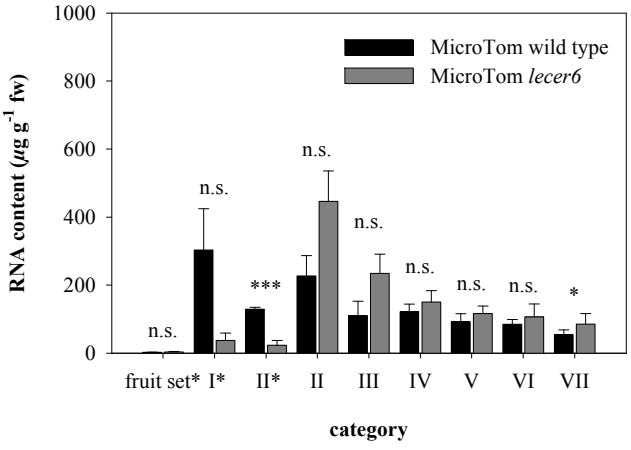

Figure 5. Content of total RNA of MicroTom wild type and LeCER6-deficient MicroTom fruits. Total RNA was isolated of whole fruits without seeds (*) or the fruit peel tissue. Statistical differences between MicroTom wild type and MicroTom *lecer6* were analyzed with Mann-Whitney *U*-test (n.s. = not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Data are shown as means ± SD (n = 3 to 21).

To characterize the spanning course of fruit development of about eighty days in detail, the total expression signal intensities were summarized, which were received from 167 oligomers in different microarray experiments of MicroTom wild type and MicroTom *lecer6* fruits, respectively (Figure 6). These selected 167 oligomers, used as probes in the

tomato wax microarray, represents mainly genes that are related to diverse aspects of wax biosynthesis, linked transport mechanisms, fruit ripening and stress-associated processes.

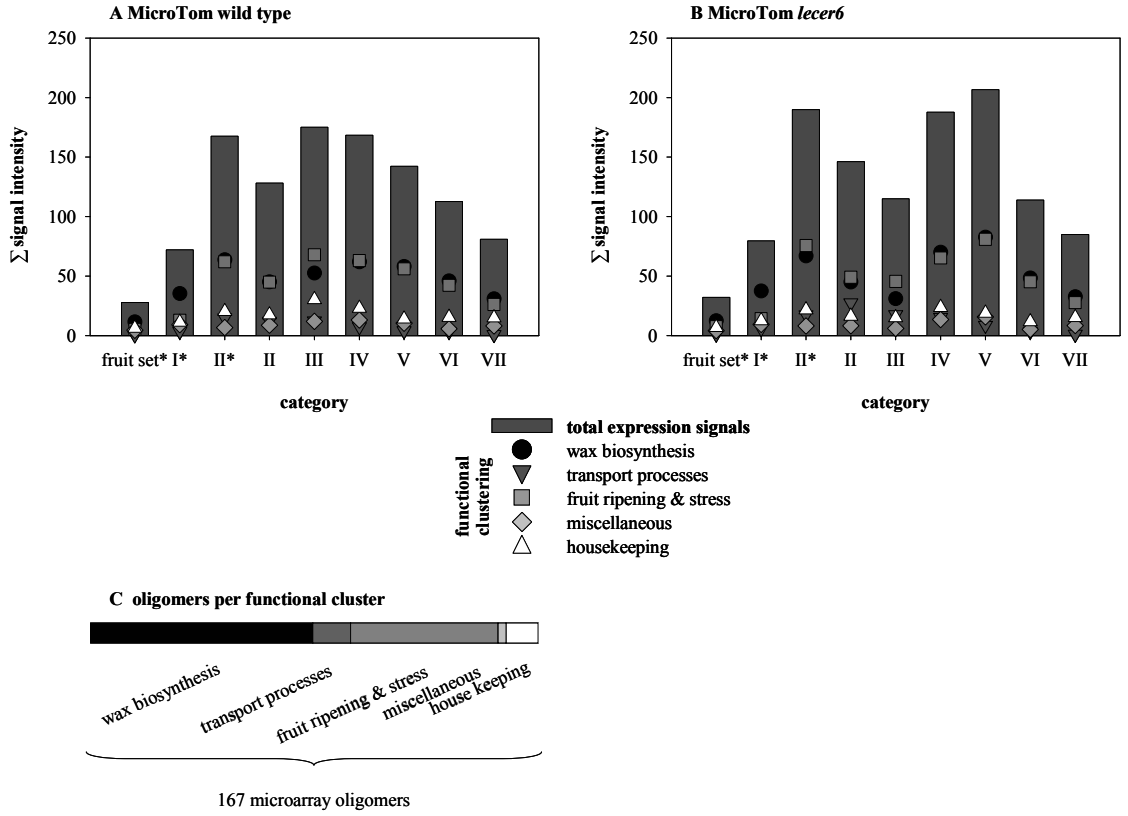

Figure 6. Total expression signal intensities of MicroTom wild type (A) and MicroTom *lecer6* (B) fruits referring to 167 oligomers screened in several microarray experiments and their functional arrangement into different clusters: wax biosynthesis, transport processes, fruit ripening and stress, miscellaneous and housekeeping. Microarray samples of different fruit developmental categories composed of whole fruits without seeds (*) or exclusively the fruit peel tissue. The number of oligomers was represented schematically for each functional cluster (C). Data are shown as means (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

In the course of fruit development the microarray data of the first three developmental categories, which were investigated at the whole fruit level, showed a stepwise 2.5-fold increase of the total signal intensity in both MicroTom lines. In the fruit peel tissues the increase in total signal intensities was temporally retarded in MicroTom *lecer6* in comparison to the wild type. A maximum in expression signal intensity was reached in wild type category III and in *lecer6* mutant category V each followed by a progressive signal reduction up to category VII. Ultimately, the expression signal intensity in category VII was still threefold higher compared to the initial value of category 'fruit set'. However, the highest total signal intensities of the whole fruit samples were similar to those obtained from the fruit peel tissue in both MicroTom lines.

The pattern of signal intensities differed, as the oligomers were arranged in functional clusters. Approximately 50% of all oligomers used in the microarray experiments were related to the wax biosynthesis cluster. In both MicroTom lines the percentage of these wax biosynthesis oligomers ranged between 27% and 49% of the total signal intensity with a distinct peak in fruit category I. The oligomers belonging to the small cluster of transport processes reached a proportion between 1% and 19% of the total signal intensity and exhibited a maximum in the fruit peel tissue of category II. Approximately 30% of all oligomers were arranged in the fruit ripening and stress cluster that total signal intensity highlighted in fruit category III and constituted a value between 18% and 40%. The functional clusters of miscellaneous and housekeeping oligomers reaching maximal signal intensity in category 'fruit set' were represented by 9% of all microarray oligomers.

Examinations of the Transcriptional Activity of Tomato Fruits

Independently of the corresponding functional cluster all expression signals were subdivided into four groups according to their intensity values: no signal, weak signal (≥ 0.2), distinct signal (≥ 0.5) and strong signal (≥ 5.0; Figure 7).

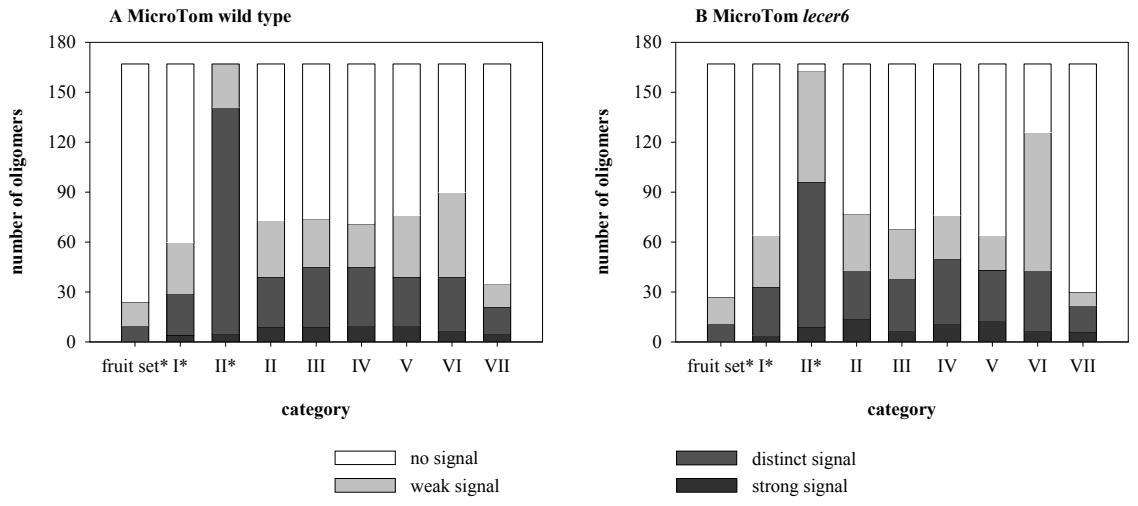

Figure 7. Number of microarray oligomers grouped according to their expression signal intensities during different developmental stages of MicroTom wild type (A) and MicroTom *lecer6* (B) fruits into: no signal, weak signal (≥ 0.2), distinct signal (≥ 0.5) and strong signal (≥ 5.0). Microarray samples composed of whole fruits without seeds (*) or exclusively the fruit peel tissue. Data are shown as means (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

In category 'fruit set' and category VII a minimum of 14% to 21% of the total oligomer number were assigned to have at least weak expression signal intensity. However,

expression signals of nearly all oligomers represented on the microarray slide were found in category II at the whole fruit level. Focusing on the fruit peel tissues a maximum of oligomers displaying detectable expression signal intensities was shown in category VI of both MicroTom lines.

Distinct expression signals were mainly detected in whole fruits of category II (wild type: 84%, *lecer6*: 58% of the total oligomer number), whereas at most 30% of the oligomers were distinctly detectable within the other fruit developmental categories. The strongest expression signals were found in the category II to category V for the fruit peel tissues. The developmental category 'fruit set' did not expose oligomers with strong expression signal intensities. Nevertheless, with exception of whole fruits in category II, more than 25% of the total oligomer number was not detectable in each microarray experiment.

An overview of individual expression signal intensities for all 167 oligomers in the nine experimental set-ups of the microarray analysis is given in Figure 8. The overall extent of the expression signal intensity of MicroTom wild type and MicroTom *lecer6* was comparable, while expression signal intensities within each MicroTom line varied in several orders of magnitude.

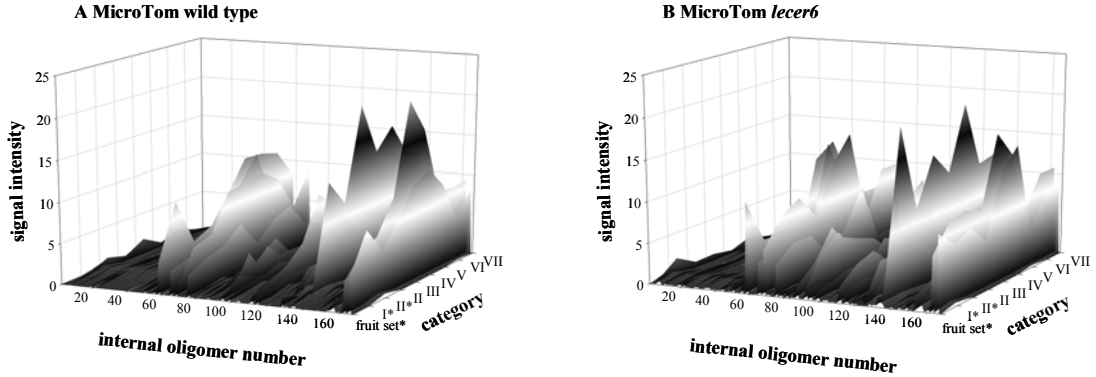

Figure 8. Overview of microarray expression signals of 167 oligomers related to MicroTom wild type (A) and MicroTom *lecer6* (B) fruits. Microarray samples composed of whole fruits without seeds (*) or exclusively the fruit peel tissue. Data are shown as means (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

The most prominent peaks corresponding to high expression signal intensities were found for a fatty acid hydroxylase (represented by internal oligomer number 50), a wax synthase (58), *cer* homologues (65, 68, 75), an 1-aminocyclopropane-1-carboxylate

oxidase (111), abscisic stress ripening proteins (134, 135), a phytoene synthase (136), a glyceraldehyde-3-phosphat dehydrogenase (162) and a hypothetical protein (165; Table I).

Table I. Selection of the highest microarray signal intensities of MicroTom wild type and MicroTom *lecer6* fruits. Microarray samples composed of whole fruits without seeds (*) or exclusively the fruit peel tissue. Changes of expression signal intensities were emphasized with shadings: no signal (white), weak signal (≥ 0.2; light gray), distinct signal (≥ 0.5; gray) and strong signal (≥ 5.0; dark gray). Data are shown as means ± SD (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

oligomer		fruit developmental category							
		fruit set*	I*	II*	II	III	IV	V	VI	VII
50 Fatty acid hydroxylase	wild type	0.4 ± 0.1	1.1 ± 0.6	1.5 ± 0.3	1.6 ± 1.0	1.2 ± 0.2	4.2 ± 2.0	5.0 ± 0.7	4.7 ± 1.9	4.1 ± 0.9
	lecer6	0.4 ± 0.1	1.1 ± 0.6	1.2 ± 0.2	1.4 ± 1.1	0.8 ± 0.2	4.7 ± 1.4	9.5 ± 1.6	6.1 ± 2.0	5.7 ± 2.9
58 Wax synthase	wild type	3.8 ± 1.9	10.3 ± 5.5	4.7 ± 2.0	5.8 ± 2.8	8.9 ± 3.9	12.5 ± 2.7	12.3 ± 1.2	11.7 ± 3.2	8.1 ± 5.7
	lecer6	4.2 ± 2.1	11.1 ± 7.3	5.7 ± 2.8	5.5 ± 2.7	3.7 ± 1.3	13.1 ± 2.7	14.6 ± 1.2	8.8 ± 2.8	6.7 ± 3.5
65 CER1 homologue	wild type	3.0 ± 1.6	3.6 ± 0.6	3.4 ± 0.1	8.3 ± 1.5	10.2 ± 3.6	13.2 ± 2.1	6.2 ± 0.8	5.5 ± 3.6	3.4 ± 1.3
	lecer6	2.8 ± 1.0	4.0 ± 0.3	4.6 ± 0.8	8.7 ± 1.6	5.5 ± 2.5	15.1 ± 1.8	9.8 ± 1.3	4.7 ± 3.3	3.5 ± 2.4
68 CER1 homologue	wild type	0.5 ± 1.1	5.0 ± 3.4	5.7 ± 2.1	10.1 ± 1.3	10.5 ± 3.9	10.9 ± 4.3	9.4 ± 1.3	5.1 ± 3.3	9.0 ± 2.3
	lecer6	0.1 ± 0.2	5.5 ± 3.3	8.6 ± 3.3	10.0 ± 1.0	7.0 ± 2.5	12.8 ± 5.6	15.6 ± 1.7	4.5 ± 3.3	9.7 ± 3.9
75 CER2 homologue	wild type	2.6 ± 1.3	3.8 ± 0.4	4.4 ± 0.8	6.2 ± 1.4	3.1 ± 0.9	5.1 ± 2.0	6.0 ± 1.0	3.1 ± 1.5	2.2 ± 0.5
	lecer6	3.0 ± 1.1	3.9 ± 0.4	5.6 ± 1.3	5.6 ± 1.3	2.4 ± 0.8	4.9 ± 0.7	9.1 ± 1.3	2.2 ± 1.3	2.3 ± 0.9
111 1-aminocyclopropane-1-carboxylate oxidase	wild type	0.9 ± 0.5	1.7 ± 1.1	2.6 ± 0.8	4.6 ± 1.2	9.7 ± 2.5	7.8 ± 3.0	4.9 ± 1.2	1.8 ± 0.5	1.5 ± 0.3
	lecer6	1.1 ± 0.2	2.0 ± 1.2	3.8 ± 1.2	4.5 ± 1.1	2.2 ± 0.7	9.0 ± 2.3	10.7 ± 2.6	3.4 ± 0.6	2.4 ± 1.2
134 Abscisic stress ripening protein (DS2 protein)	wild type	0.2 ± 0.2	0.6 ± 0.4	4.1 ± 0.5	7.7 ± 3.5	2.1 ± 0.7	1.5 ± 0.5	1.1 ± 0.2	3.0 ± 1.3	1.6 ± 0.6
	lecer6	0.4 ± 0.2	0.6 ± 0.4	11.1 ± 1.9	7.9 ± 3.1	1.3 ± 0.7	1.7 ± 0.5	0.9 ± 0.3	0.7 ± 0.4	0.6 ± 0.3
135 Abscisic stress ripening protein	wild type	0.2 ± 0.2	0.4 ± 0.2	13.2 ± 2.4	9.9 ± 2.5	20.8 ± 3.9	14.6 ± 4.8	17.0 ± 1.1	13.1 ± 3.4	12.5 ± 3.8
	lecer6	0.6 ± 0.3	0.5 ± 0.2	20.4 ± 3.8	9.9 ± 2.1	15.6 ± 3.7	12.8 ± 3.0	20.3 ± 0.6	12.3 ± 2.8	12.8 ± 6.6
136 Phytoene synthase	wild type	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.2	0.0 ± 0.0	4.2 ± 0.8	7.1 ± 2.9	7.1 ± 0.7	0.9 ± 0.4	0.7 ± 0.1
	lecer6	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.4	0.0 ± 0.0	1.8 ± 0.4	5.2 ± 1.3	15.1 ± 1.2	2.3 ± 0.7	1.3 ± 0.5
162 Glyceraldehyde-3-phosphate dehydrogenase	wild type	3.7 ± 2.5	6.2 ± 1.8	7.3 ± 1.4	11.0 ± 0.5	21.8 ± 3.4	17.7 ± 2.7	9.5 ± 1.7	9.1 ± 4.8	9.3 ± 3.6
	lecer6	3.7 ± 2.1	6.7 ± 2.2	9.7 ± 2.1	10.3 ± 0.5	9.4 ± 4.6	17.8 ± 2.3	14.7 ± 2.4	7.0 ± 4.9	10.8 ± 7.2
165 hypothetical protein (lipid transfer protein)	wild type	4.4 ± 2.3	9.0 ± 3.8	5.6 ± 2.2	8.7 ± 2.1	11.9 ± 5.2	12.9 ± 2.6	10.3 ± 2.2	5.4 ± 3.1	8.1 ± 2.5
	lecer6	4.2 ± 2.1	9.3 ± 4.1	7.2 ± 2.8	8.3 ± 1.9	5.9 ± 2.7	13.4 ± 3.2	15.8 ± 1.6	4.6 ± 3.1	8.4 ± 3.7

Throughout the first three fruit development categories mainly the wax synthase (58), the glyceraldehyde-3-phosphat dehydrogenase (162) and the hypothetical protein (165) reached high expression signal levels. Having already distinct expression signal intensity in category 'fruit set' their signal intensities continued to rise during the transition of category 'fruit set' to category I and, furthermore, remained very strong in the later fruit developmental stages. The expression signal intensities did not peak until category III.

During the first three developmental stages the expression signals of the fatty acid hydroxylase (50), the CER1 homologue (65), the 1-aminocyclopropane-1-carboxylate

oxidase (111) and the phytoene synthase (136) were distinctly lower when compared to the wax synthase (58), glyceraldehyde-3-phosphat dehydrogenase (162) or hypothetical protein (165). However, the expression signal intensity of these oligomers peaked also in category III to category V. In the earlier developmental stage of category II, the abscisic stress ripening protein (135) accumulated to a great extent representing one of the strongest expression signals during MicroTom fruit development. Simultaneously, the signal intensity of the abscisic stress ripening protein (134) increased, but in comparison to the abscisic stress ripening proteins (135) at a twentyfold lower level in the later fruit developmental categories. Although the *cer* homologues (68, 75) exhibited high expression signal intensities during fruit development of both MicroTom lines, a distinct peak was not found.

Analysis of Transcriptional Changes Due to LeCER6 Deficiency of Tomato Fruits

For a more distinct visualization of the transcriptional differences between both MicroTom lines the ratios of all expression signals of MicroTom *lecer6* versus MicroTom wild type were calculated. A ratio ≤ 0.5 indicated an increased expression for the wild type samples, in contrast a ratio ≥ 2.0 specified a higher transcriptional level in the mutant samples (Figure 9).

Transcriptional differences in more than one fruit developmental category were found for: very-long-chain fatty acid condensing enzymes (internal oligomer numbers 29, 30), CER1 homologues (66, 67), lipid transfer proteins (84, 86, 87, 91, 92), an anionic peroxidase (98), phenylalanine ammonia-lyases (103, 104), an ethylene inducible protein kinase (CTR1; 106), an 1-aminocyclopropane-1-carboxylate synthase (107), an 1-aminocyclopropane-1-carboxylate oxidase (111), a naringenin-chalcone synthase (119), an abscisic acid and environmental stress inducible protein (122), lipoxygenases (123, 124, 125), abscisic stress ripening proteins (134, 135) and a phytoene synthase (136).

Of these 23 oligomers those representing the very-long-chain fatty acid condensing enzymes (29, 30) and the CER1 homologues (66, 67) are related to the functional cluster of wax biosynthesis. The five lipid transfer proteins (84, 86, 87, 91, 92) are linked with the cluster of transport processes and the remaining fourteen oligomers specified above are arranged into the cluster of fruit ripening and stress.

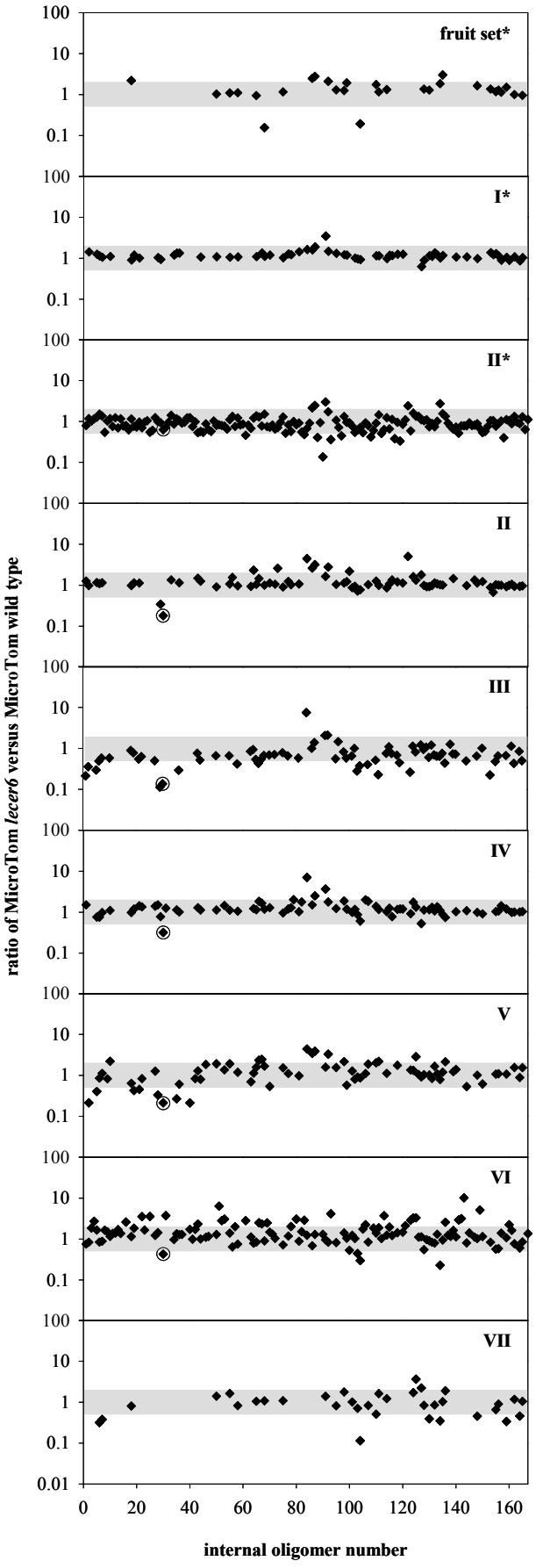

Figure 9. Ratio between the expression signal intensities of MicroTom *lecer6* and MicroTom wild type corresponding to 167 oligomers for nine different microarray experiments. Samples consisted of whole fruits without seeds (*) or exclusively the fruit peel tissue. Gray bars illustrate a ratio ≤ 0.5 and ≥ 2.0 calculated from expression signal intensities of MicroTom wild type and the corresponding mutant fruits. The oligomer with the internal number 30 represents the very-long-chain fatty acid condensing enzyme (*LeCer6*) is marked with a circle. Data are shown as means (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

Temporal Gene Expression Patterns of Tomato Wild Type and LeCER6-Deficient Fruits

Focusing on the microarray data of individual expression signals a sevenfold higher signal intensity became evident for both the very-long-chain fatty acid condensing enzymes (29, 30; Figure 10) in category III of MicroTom wild type fruits in comparison to MicroTom *lecer6*. The very-long-chain fatty acid condensing enzyme (29) showed predominantly in category II, III and V an increased transcriptional level, whereas the signal intensity between MicroTom wild type and *lecer6* for the very-long-chain fatty acid condensing enzyme (30) differed in all fruit peel tissue categories (category II to VII).

The CER1 homologues (66, 67) followed a similar expression pattern concerning to the signal intensity levels as well as the differences between MicroTom wild type and *lecer6* mutant. Both homologues were two times amplified in MicroTom wild type fruits of category III compared to LeCER6-deficient fruits. During the transitions of category IV to category V and VI this discrepancy changed in favor of MicroTom *lecer6*. In the later developmental stages the ratio between MicroTom wild type and MicroTom *lecer6* rose to a factor of approximately 2.3 to 2.5 in the fruit peel tissue.

Apart from different signal intensity levels, an equivalent expression pattern was detected for the 1-aminocyclopropane-1-carboxylate synthase (107) and the 1-aminocyclopropane-1-carboxylate oxidase (111). For both, the 1-aminocyclopropane-1-carboxylate synthase and the 1-aminocyclopropane-1-carboxylate oxidase, the expression signal intensities were more than twofold increased in wild type fruits of category III in comparison to the *lecer6* mutant. The wild type-mutant relationship changed for the 1-aminocyclopropane-1-carboxylate synthase in category IV and V and similarly for the 1-aminocyclopropane-1-carboxylate oxidase in category V and VI. Thus, the transcriptional level was twofold higher in *lecer6* mutant fruits compared to the wild type. The 1-aminocyclopropane-1-carboxylate oxidase reached a maximum of signal intensity of about 10 in category V. The expression signal maximum of the 1-aminocyclopropane-1-carboxylate synthase was also found in category V but at a fourfold lower level than for the 1-aminocyclopropane-1-carboxylate oxidase.

The most striking differences in expression signal intensities were found for lipid transfer proteins (84, 86, 87, 91, 92) in *lecer6* mutant fruits from category II to category V in comparison to the wild type fruits, whereas some lipid transfer proteins (86, 87, 91, 92) exhibited similar expression patterns. In the fruit peel tissue of developmental category II the ratio of signal intensities between both MicroTom lines ranged at most between 1:2 and

1:3 except for the lipid transfer protein (84). In the fruit peel tissues the differences in signal intensities of the lipid transfer protein (84) varied by a factor of 4.4 to 7.4 between MicroTom wild type and *lecer6* mutant.

In the earlier developmental stage of fruit category II the abscisic acid and environmental stress inducible protein (122) showed transcriptional changes between both MicroTom lines in the whole fruit and in the fruit peel tissue, respectively. The expression signal intensity accumulated twofold higher in the *lecer6* mutant than in the wild type fruits. All the other developmental stages revealed only marginal levels of expression signal intensities.

Comparable expression patterns were found for the ethylene inducible protein kinase (CTR1; 106) and the anionic peroxidase (98) at different signal intensity levels exhibiting transcriptional differences only in the later developmental stages of category IV and category VI or category IV and category V.

The expression signals of the lipoxygenases (123, 124, 125) increased delayed in the *lecer6* mutant with regard to the later developmental categories V to category VII. In particular, signal intensities of these lipoxygenases exhibited a ratio of 1:3 in category VI in a comparison of MicroTom wild type and the *lecer6* mutant fruit peel tissues. A similar shift in expression signal intensity was found for the phytoene synthase (136). In the category V to category VII a 1.9- to 2.6-fold increased expression signal of the phytoene synthase (136) was screened in MicroTom *lecer6* compared to wild type fruits.

The abscisic stress ripening protein (134) showed an elevated expression level in the *lecer6* mutant for whole fruits of category II. However, in the fruit peel tissues the expression signal intensity of abscisic stress ripening protein was up to fourfold higher abundant in category V and category VII of MicroTom wild type in comparison to the *lecer6* mutant.

For the phenylalanine ammonia-lyases (103, 104) expression signal intensities were reduced by half in category III and category VI of MicroTom *lecer6* fruits compared to the wild type. A similar pattern was detected for the naringenin-chalcone synthase (119) in category III and V, whereas the naringenin-chalcone synthase (119) showed only weak expression signals within all fruit developmental categories.

In common, the most remarkable transcriptional dissimilarities between MicroTom wild type and MicroTom *lecer6* were documented in the fruit peel tissue of categories III, V and VI. In category 'fruit set' and in category VII due to low expression signal intensities no individual expression signal could be discriminated.

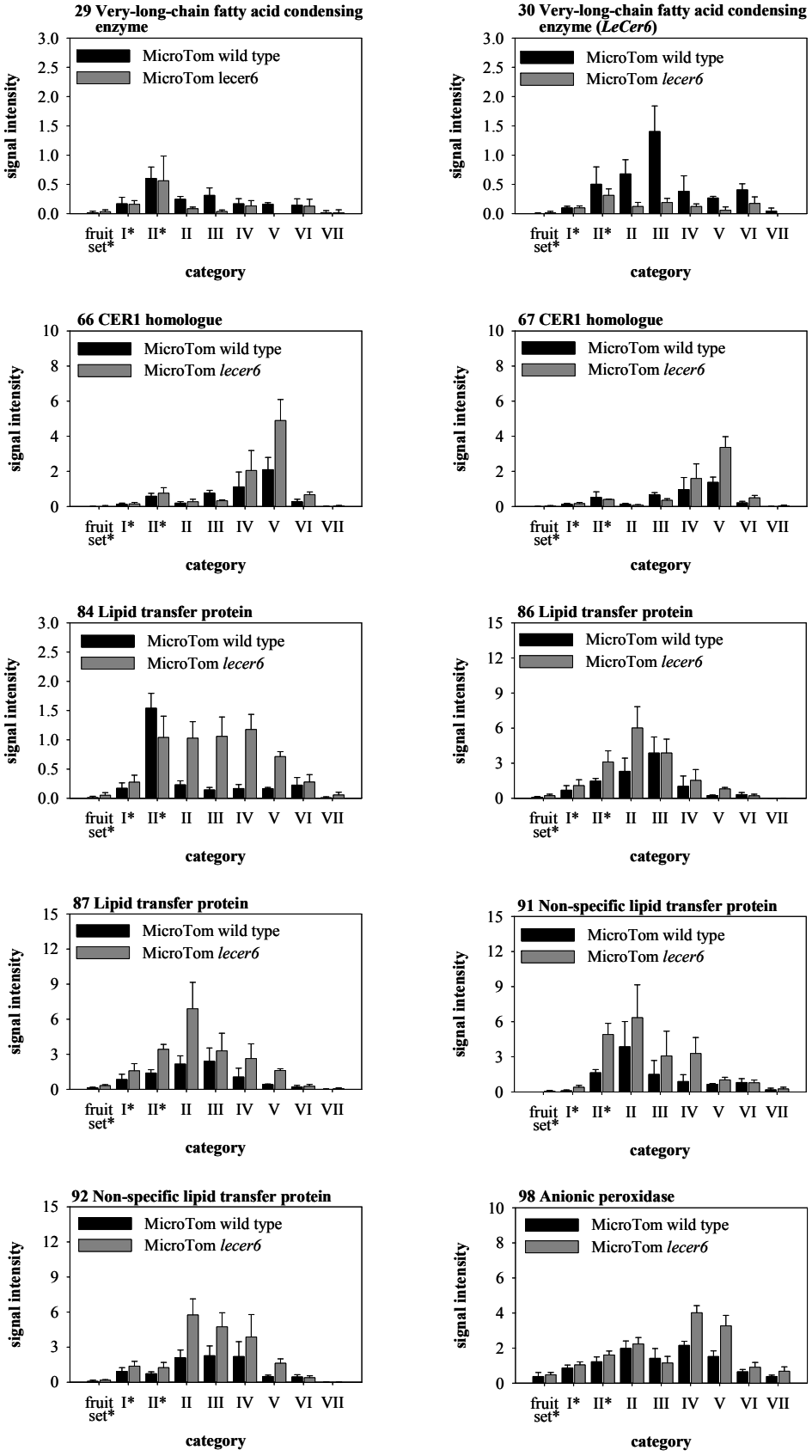

Figure 10. A detailed comparison of microarray expression signals of oligomers revealing different expression patterns between MicroTom wild type and MicroTom *lecer6* fruits. Microarray samples

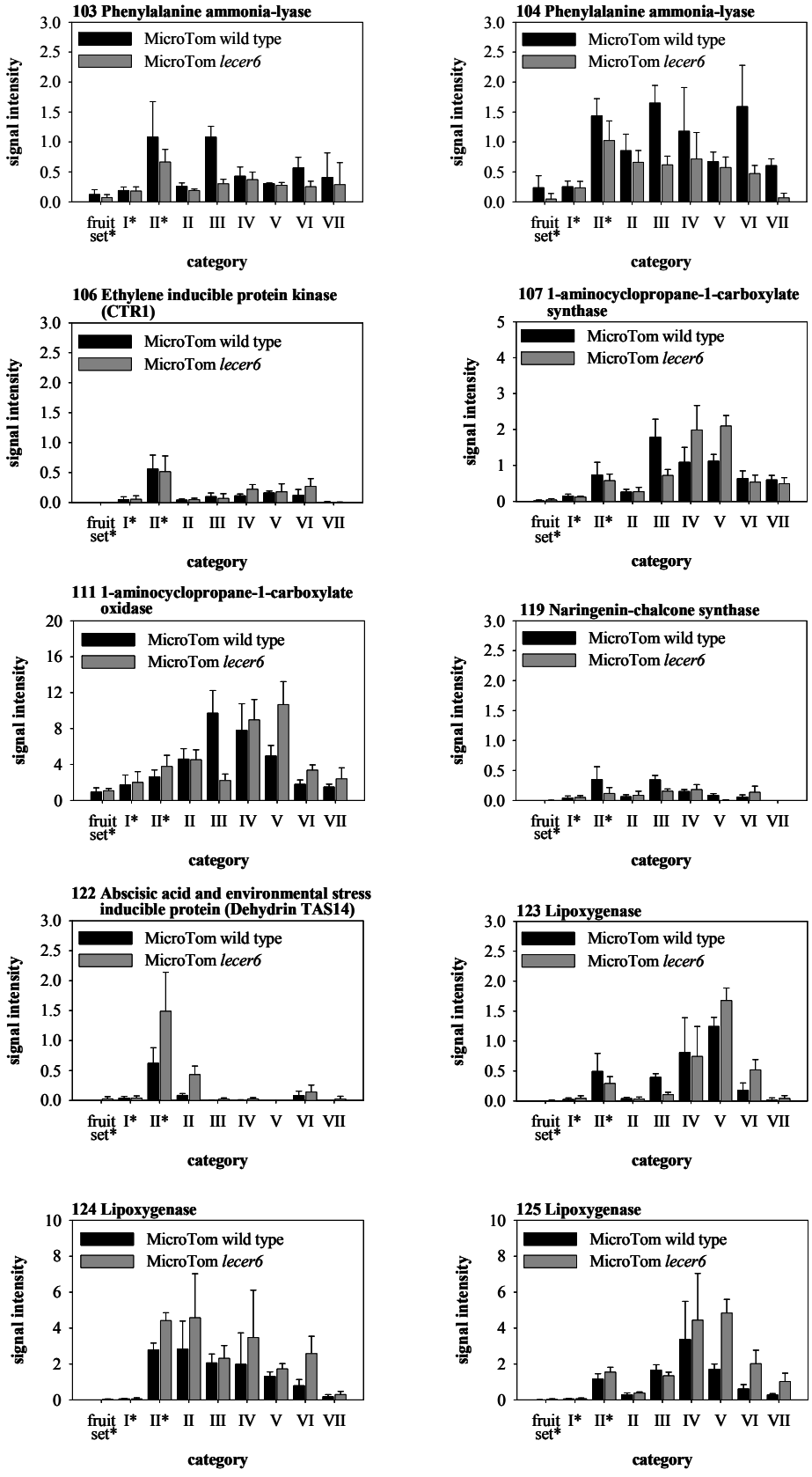

Figure 10. (continued) composed of whole fruits without seeds (*) or exclusively the fruit peel tissue. Data are shown as means ± SD (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

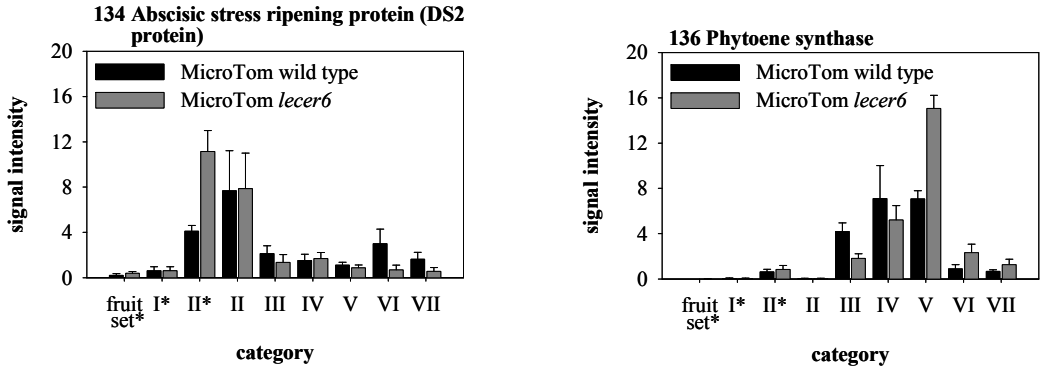

Figure 10. (continued)

Spatial Gene Expression Patterns of Tomato Wild Type and LeCER6-Deficient Fruits

Results of category II allowed for a comparison of transcriptional expression patterns of both, the whole MicroTom fruits versus the isolated fruit peel tissue of an equivalent fruit developmental stage. Compared to the whole fruit level, only a few transcripts were found to accumulate in the fruit peel tissues of MicroTom wild type and MicroTom *lecer6*, which is indicated by a ratio of signal intensities ≥ 2.0 (Figure 11).

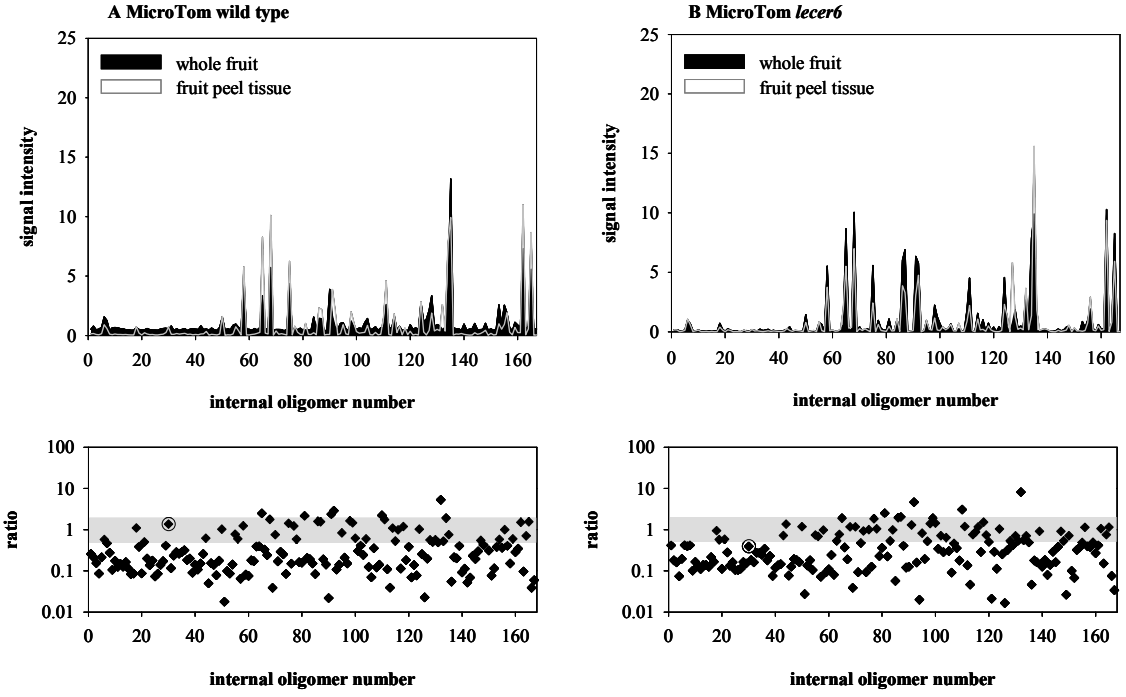

Figure 11. Overview of microarray expression signals of 167 oligomers of MicroTom wild type (A) and MicroTom *lecer6* (B) referring to the whole fruit without seeds or the fruit peel tissue of developmental category II. Gray bars illustrate a ratio ≤ 0.5 and ≥ 2.0 calculated from expression signal intensities of the fruit peel tissue and the whole fruit for both MicroTom lines. The oligomer with the internal number 30 represents the very-long-chain fatty acid condensing enzyme (*LeCer6*) and is marked with a circle. Data are shown as means ± SD (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

A transcriptional gradient between the fruit peel tissue and the whole fruit was found for several lipid transfer proteins (86, 87, 91, 92), which also exhibited differences between MicroTom wild type and the MicroTom *lecer6* (Figure 10). Likewise, a β-amyrin synthase (81), an anionic peroxidase (99), an 1-aminocyclopropane-1-carboxylate oxidase (110) and a CER1 homologue (65; Table II) were more prominent in the fruit peel tissue of both MicroTom lines.

Table II. Microarray expression signals of MicroTom wild type and MicroTom *lecer6* of whole fruits without seeds and the fruit peel tissue, respectively, both representing fruit developmental category II. The ratio of signal intensities between the fruit peel tissue and the whole fruits is marked in italics. Data are shown as means ± SD (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

oligomer		MicroTom wild type			MicroTom *lecer6*		
		whole fruit	fruit peel tissue	*ratio*	whole fruit	fruit peel tissue	*ratio*
65	CER1 homologue	3.4 ± 0.1	8.3 ± 1.5	*2.5*	4.6 ± 0.8	8.7 ± 1.6	*1.9*
81	β-amyrin synthase	0.5 ± 0.3	1.0 ± 0.5	*2.1*	0.4 ± 0.1	1.1 ± 0.6	*2.5*
86	Lipid transfer protein	1.5 ± 0.2	2.3 ± 1.1	*1.6*	3.1 ± 0.9	6.0 ± 1.8	*1.9*
87	Lipid transfer protein	1.4 ± 0.3	2.2 ± 0.7	*1.5*	3.4 ± 0.4	6.9 ± 2.3	*2.0*
91	Non-specific lipid transfer protein	1.6 ± 0.3	3.9 ± 2.1	*2.3*	4.9 ± 1.0	6.3 ± 2.8	*1.3*
92	Non-specific lipid transfer protein	0.7 ± 0.2	2.1 ± 0.7	*2.9*	1.2 ± 0.4	5.8 ± 1.4	*4.6*
99	Anionic peroxidase	0.7 ± 0.3	1.0 ± 0.2	*1.5*	0.7 ± 0.2	1.3 ± 0.2	*1.9*
110	1-aminocyclopropane-1-carboxylate oxidase	0.7 ± 0.3	1.5 ± 0.5	*2.2*	0.6 ± 0.2	1.8 ± 0.8	*3.1*

Validation of Microarray Experiments by Semi-Quantitative RT-PCR Analysis

Different tomato EST or gene sequences screened in the microarray experiments were selected. Subsequent, a RT-PCR analysis as a more sensitive technique was performed to validate the data obtained in the microarray experiments for fruit developmental categories I to category III. The results of the microarray experiments differed distinctly and, furthermore, ranged over several orders of magnitude. An 18S rRNA fragment was investigated as an external control of the RT-PCR analysis (Figure 12).

The expression signal of the very-long-chain fatty acid condensing enzyme (*LeCer6*; 30) was clearly detected in MicroTom wild type fruits of all three categories, investigated by RT-PCR analysis. In contrast, a non-specific lipid transfer protein (92) showed distinct expression signals to the same extent in LeCER6-deficient fruits. Differences in expression signals between both MicroTom lines were detected for CER1 homologue (66) and the lipid transfer protein (87) but only in one fruit developmental stage.

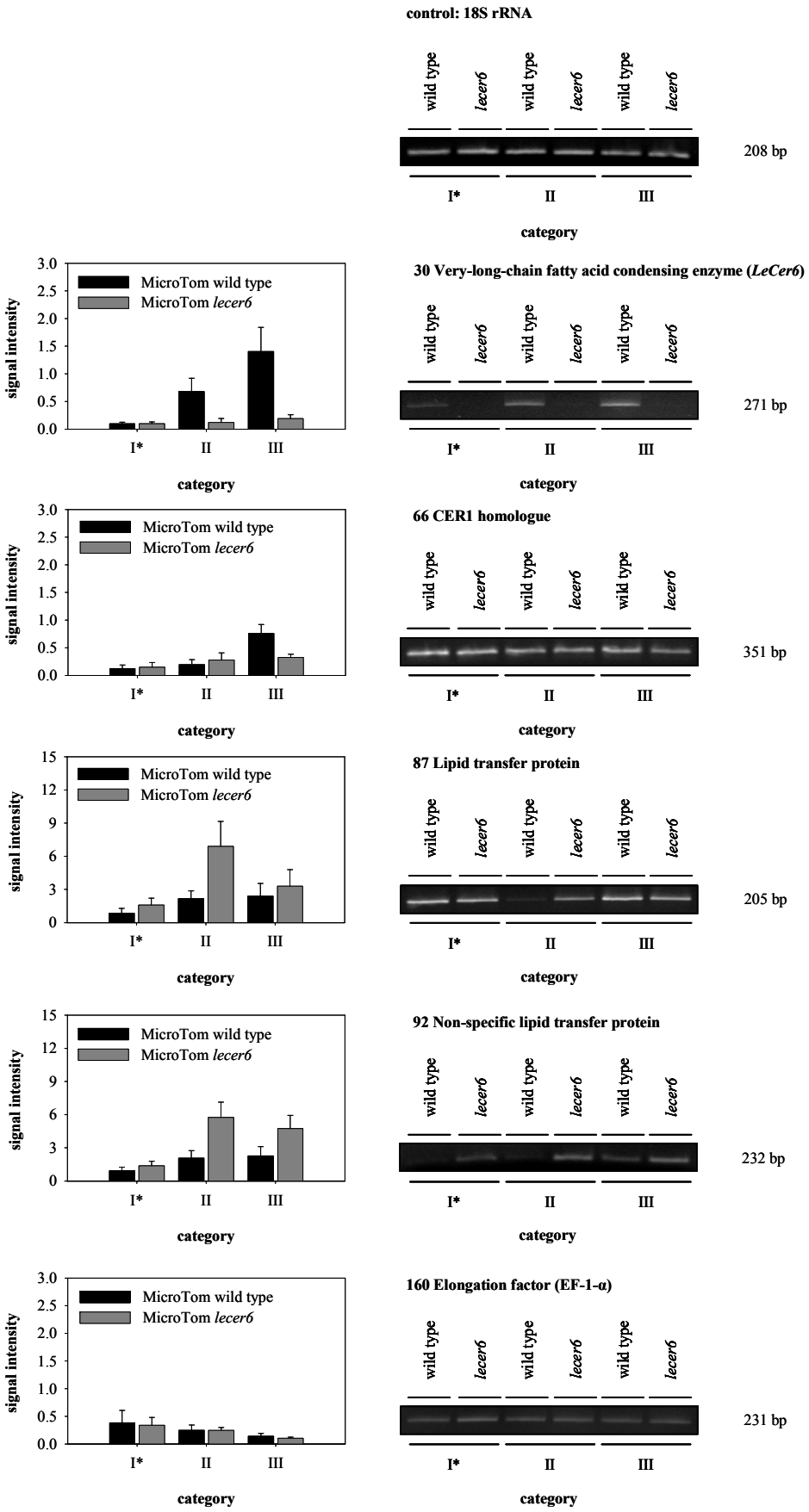

Figure 12. Comparison of expression signals for selective oligomers obtained from microarray experiments and of RT-PCR analysis. Samples composed of whole fruits without seeds (*) or exclusively the fruit peel

In *lecer6* mutant fruits, the expression signal of the CER1 homologue (66) was reduced in category III, whereas in category II the expression signal of the lipid transfer protein (87) was higher accumulated compared to the wild type. Focussing on the elongation factor EF-1-α (160) and the 18S rRNA, no differences were found between MicroTom wild type and MicroTom *lecer6*.

DISCUSSION

Microarray analyses are a valuable tool for dissecting regulatory mechanisms and transcriptional networks that underlie plant processes such as development and stress response (Alba *et al.*, 2004). In recent years microarray studies were performed with plants of different genetic and physiological backgrounds. Thereby, it turned out that a subset of gene sequences representing a selective part of the complete genome is suitable for experimental researches. One benefit is that specific gene sequences can be screened in a large scale concerning a defined issue. Another advantage of such a 'theme' microarray is facility of handling with data. Otherwise, this approach using only a selection of gene sequences is limited in interpretation from perspectives of misrepresenting accurate transcriptional activity. Previously, Seki *et al.* (2002), Lemaire-Chamley *et al.* (2005), Alba *et al.* (2005), Suh *et al.* (2005) and Mintz-Oron *et al*. (2008) published diverse microarray studies. Their reports were concerned either to the developmental progress and distinct tissues of tomato fruits or were related to the cuticle biosynthesis and effects of stress conditions in *Arabidopsis*. Hitherto, a combination of all these factors was not yet intended to be investigated within one plant model system.

In the present study, a tomato microarray for comparative transcriptional analysis was established and used to allow for a correlation of data with respect to changes in cuticular wax accumulation and transpirational water loss in the dynamic view of the tomato fruit development. Thus, a subset of 167 EST or gene sequences, which are potentially involved in wax biosynthesis, wax modification, cuticle related transport processes and stress response, was selected out of approximately 35000 genes of the tomato genome (van der Hoeven *et al.*, 2002). For the experimental design samples of MicroTom wild type and MicroTom *lecer6* fruits were probed according to eight distinct developmental categories starting from the developmental category 'fruit set', one to three days after flowering.

Figure 12. (continued) tissue. The size of the amplified RT-PCR fragments is given. The RT-PCR fragments of 18S rRNA were used as external control. Microarray data are shown as means ± SD (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

The following fruit phase termed as category I was the earliest developmental stage, which was suitable for comparison of chemical and functional analyses. The microarray analysis showed that, with regard to the complete course of tomato fruit development ending with fruit category VII, more than 75% of the oligonucleotides at the microarray slide were regulated during fruit expansion, maturation, ripening and senescence, respectively. A distribution of signal intensities over several orders of magnitude was detected, which represents gene expression activity at different levels.

To interpret gene expression data obtained from microarray experiments it was necessary to normalize the large microarray data sets. For transcript normalization commonly housekeeping genes as internal standard and non-plant genes as external standard were additionally integrated in the microarray experiments. A stable transcriptional level of reference genes is pivotal for normalization of gene expression data, particularly for microarray experiments using a subset of gene-encoding sequences of the whole genome. Genes involved in basic cellular processes are commonly used as internal control, since they are supposed to be expressed constitutively. Some of the well-known reference or housekeeping genes are those of 18S rRNA, α-tubulin (internal oligomer numbers 153 and 154), ubiquitin (155 and 156), actin (158 and 159) and elongation factor-1α (EF-1α; 160; Goidin *et al*., 2001; Bustin, 2002; Kim *et al*., 2003; Andersen *et al*., 2004; Brunner *et al*., 2004; Radonić *et al*., 2004; Jain *et al*., 2006).

The nuclear gene that encodes the cytosolic glyceraldehyde-3-phosphate dehydrogenase (162; GAPDH) was found to be an important housekeeping gene suitable for measuring the gene expression activity in different plant tissues (Iskandar *et al*., 2004). GAPDH, which functions in glycolysis, catalyzes the conversion of glyceraldehyde-3-phosphate to glycerate-1,3-bisphosphate, an important pathway of energy metabolism in plants (Cerff, 1982; Shih *et al*., 1991; Lawlor and Cornic, 2002). Even though GAPDH exhibited a high-level gene expression in MicroTom wild type and MicroTom *lecer6* during all fruit developmental stages, in contrast to the housekeeping genes specified above, the transcriptional level of GAPDH was developmentally regulated.

Unfortunately, the gene expression levels of all potential housekeeping genes investigated by this present microarray analysis varied within both MicroTom lines depending on the fruit developmental stage (Thellin *et al*., 1999; Suzuki *et al*., 2000; Lee *et al*., 2001; Czechowski *et al*., 2005). This agreed well with data from Coker and Davies (2003), who could not identify genes that showed a stable gene expression in tomato fruits across a wide range of developmental conditions and a variety of stress stimuli. Thus, the

present study underlined the importance of external references for transcript normalization and quantification. In addition, transcript abundance was analyzed by reverse transcription PCR analysis to validate gene expression data obtained from microarray experiments.

Gene Expression Pattern of β-Ketoacyl-CoA Synthase LeCER6 Related to Cuticular Wax Biosynthesis

Several plant genes are known to encode β-ketoacyl-CoA synthases that are effective in generating very-long-chain fatty acids (VLCFA; Millar *et al.*, 1999). Amongst others, VLCFA are incorporated into epi- and intracuticular waxes and serve as essential precursors for other aliphatic components within the cuticular waxes of most plant species (Post-Beittenmiller, 1996). The VLCFA condensing enzymes, which are involved in both, decarbonylation and acyl reduction wax biosynthetic pathways, catalyze the initial condensation reaction in the microsomal fatty acid elongation system that links two carbon units from malonyl-CoA to a pre-existing long-chain acyl-CoA primer (von Wettstein-Knowles, 1982; Lessire *et al.*, 1985, 1999; Fehling and Mukherjee, 1991; Lassner *et al.*, 1996; Millar and Kunst, 1997; Ghanevati and Jaworski, 2002).

This present investigation focused mainly on the *LeCer6* gene, encoding a β-ketoacyl-CoA synthase, which plays a key role in very-long-chain fatty acid elongation of the wax biosynthetic pathway in tomato fruits. The gene expression signals of LeCER6 (internal oligomer numbers 29 and 30) were found very early in the course of tomato fruit development. With the onset of the 'fruit set' stage, marginal amounts of LeCER6 transcripts were detected in immature green MicroTom fruits. This transcriptional level was continuously amplified during fruit maturation and highlighted at the early breaker stage after a prominent accumulation. Following this characteristic peak, the LeCER6 gene expression remained on a distinctly reduced transcriptional level throughout the fruit developmental phases breaker, orange and red ripe. Ultimately, LeCER6 gene expression was unverifiable in senescent, red overripe fruits. However, when compared to the total transcriptional signal intensity screened by microarray analysis, the *LeCer6* gene rendered only a weak expression signal intensity probably representing a low gene expression activity, in general.

Similarly to tomato fruits, where the *LeCer6* gene was maximally transcribed in the expanding and maturing fruit stage, β-ketoacyl-CoA synthases were also isolated from other developing plant tissues for example embryos of nasturtium (*Tropaeolum majus* L.; Mietkiewska *et al.*, 2004), leaves, stems, siliques and flower buds of *Arabidopsis* (Hooker

et al., 2002) and during the early fiber development of cotton (*Gossypium hirsutum* L.; Qin *et al.*, 2007).

The results presented here showed that LeCER6 transcripts temporally accumulated at the early fruit developmental stages during the transition of 'fruit set' to the early breaker stage. This corresponds to the specific period, in which VLCFA biosynthesis is required for generating the cuticular wax coverage due to the fact that MicroTom fruits rapidly expanded. The rise in LeCER6 gene expression went simultaneously along with an increased deposition of cuticular fruit waxes. The timing of induction of the fatty acid elongation throughout the earlier fruit developmental stages determined an increase of aliphatic components within the overall cuticular wax layer. At later fruit developmental stages, the cuticular wax load was only marginally elevated. The alterations in the surface area of the still slightly enlarging fruits were more or less compensated. Following the further fruit developmental process, modifications in the major wax composition were not observed.

On the basis of these findings, it can be concluded that the wax biosynthesis is fulfilled in MicroTom within the first four fruit developmental stages: 'fruit set', immature green, mature green and early breaker. After an early maximum LeCER6 transcripts became evidently reduced at the breaker fruit stage. Red overripe fruits did not expand and, consequently, there was almost no need to synthesize cuticular wax components. LeCER6 gene expression was marginal during this developmental stage. A relationship between LeCER6 gene expression and the accumulation of aliphatic wax components on the fruit surface was documented. Derived from its unique temporal expression pattern during fruit maturation, the *LeCer6* gene is suggested to be highly developmentally regulated at the transcriptional level.

Based on results from mature green fruits, which were investigated either as whole fruit or exclusively their fruit peel tissue, a spatial restriction of LeCER6 gene expression was demonstrated. An abundance of LeCER6 transcripts was found in the epidermis-associated tissues. This was consistent with the observation that elongase activity is localized in epidermal plant cells (Fehling *et al.*, 1992; Hooker *et al.*, 2002). In line with Cassagne and Lessire (1978), Kolattukudy (1984), Kolattukudy and Espelie (1985), Schneider *et al.* (1993) and Cassagne *et al.* (1994), who described the wax biosynthesis being restricted to the epidermis of aerial tissues in leek (*Allium porrum* L.) and *Arabidopsis*, LeCER6 gene expression in mature green tomato fruits can be attributed specifically to the epidermal

tissue, the major site of VLCFA biosynthesis, where VLCFA are utilized for the production of cuticular waxes (Kunst and Samuels, 2003).

Impact of Deficiency in β-Ketoacyl-CoA Synthase LeCER6: Transcriptional Changes Associated with Cuticular Wax Coverage

Transgenic plants modified in β-ketoacyl-CoA synthases exhibit altered levels of VLCFA most notably in leaves, fruits and seeds of *Arabidopsis* (James *et al.*, 1995; Millar *et al.*, 1999). To the date, CER6 condensing enzymes, which were annotated as β-ketoacyl-CoA synthases, were studied in *Arabidopsis* and *Gossypium hirsutum* and referred to the production of VLCFA > C_{25} in aerial plant tissues (Fiebig *et al.*, 2000; Hooker *et al.*, 2002; Qin *et al.*, 2007).

In this present study, LeCER6 function was studied by a 'reverse genetic' approach in tomato. The LeCER6 deficiency due to transposon mutagenesis resulted in a strikingly disrupted LeCER6 gene expression activity of MicroTom mutant plants (Emmanuel, 2001). LeCER6-deficient MicroTom fruits possessed similar wax quantities as the wild type fruits but showed an evidently altered wax quantity. The total wax amount differed only significantly within both MicroTom lines over the course of fruit development.

The wax composition of MicroTom wild type and *lecer6* mutant fruits was almost equal in very small, immature green fruits, whereas mature green fruits already revealed immense compositional differences. Thus, the cuticular wax pattern was significantly different in both MicroTom lines at the early breaker fruit stage. Instead of the wild type wax composition consisting mainly of *n*-alkanes in addition to triterpenoids and sterol derivatives, only triterpenoids and sterol derivatives were most predominant in the *lecer6* mutant fruit wax. Concluding from this 'reverse genetic' strategy, the LeCER6 condensing enzyme was characterized on the biochemical level as a key component in VLCFA biosynthetic pathways of tomato fruits determining the amount and the overall chain length of very-long-chain aliphatic wax compounds > C_{29}.

Substrate specificities of β-ketoacyl-CoA synthases play a decisive role in determining the extent of the chain length and the degree of unsaturation of the reaction products (Lassner *et al.*, 1996; Millar and Kunst, 1997; Millar *et al.*, 1998; Cahoon *et al.*, 2000; Han *et al.*, 2001). In jojoba (*Simmondsia chinensis* Nutt.) Bessoule *et al.* (1989) demonstrated that a β-ketoacyl-CoA synthase was active with several acyl-CoA substrates showing highest substrate preference towards saturated $C_{18:0}$- and $C_{20:0}$-CoA and monounsaturated $C_{18:1}$- and $C_{20:1}$-CoA substrates. According to these findings, the LeCER6 protein, most

probably performing a comparable function, might be involved in fatty acid elongation of saturated and monounsaturated C_{28}- to C_{30}-CoA or longer. Nonetheless, it can not be ruled out the possible availability of a β-ketoacyl-CoA synthase, which biochemical activity overlapped with that of LeCER6. There are still open questions about the mechanism of the enzymatic reactions involved in fatty acid elongation (Lessire *et al.*, 1999; Trenkamp *et al*., 2004). Apart from the substrate specificity of elongases, the condensing reaction step in wax formation is also rate-limited by the pool of substrate levels at the site of biosynthesis (Lassner *et al.*, 1996; Millar and Kunst, 1997; Millar *et al.*, 1999).

After a block in the VLCFA biosynthesis precursors for aliphatic wax components were still available in *lecer6* mutant fruits. Substrates of LeCER6 were suspected to accumulate or alternative pathways in wax biosynthesis were probably intensified. There was no formation of alternative aliphatic compounds found in cuticular waxes of LeCER6-deficient MicroTom fruits. Summing up, these findings indicate an essential role for the LeCER6 gene product in formation of the aliphatic domain of cuticular waxes by controlling biosynthesis of VLCFA and its derivatives found in tomato fruits.

Transcriptional Activity with Respect to Cuticular Wax Accumulation: Aspects of Wax Component Biosynthesis and Wax-Related Transport Processes

Many genetic approaches were initiated in order to induce compositional variations in the cuticular wax load, but there is still a lack in information about the molecular events that control wax biosynthesis (Xia *et al.*, 1996, 1997; Xu *et al.*, 1997; St-Pierre *et al.*, 1998; Millar *et al.*, 1999; Todd *et al.*, 1999; Pruitt *et al.*, 2000; Fiebig *et al.*, 2000). Gene expression activity putatively linked to biosynthesis, modification and transport of cuticular wax components was analyzed by using LeCER6-deficient MicroTom fruits in comparison to wild type fruits, which are characterized by a different wax quality. High-abundance transcripts were localized in the fruit peel tissue, the site of wax biosynthesis. More particularly, this present investigation focused on transcripts encoding proteins that might be involved in biosynthetic pathways that generated the *lecer6* mutant phenotype. Thus, relationships between alterations at the transcriptional level and changes in the biochemical composition of the cuticular wax coverage triggered by a block in the very-long-chain fatty acid elongation were highlighted.

Following fruit developmental processes, dynamic changes at the gene expression level in fruits of MicroTom wild type and MicroTom *lecer6* were found for transcripts encoding aldehyde decarbonylases (CER1), a fatty acid elongase (CER2), a wax synthase (WS), a β-

amyrin synthase (AS), a fatty acid ω-hydroxylase (FA hydroxylase) and lipid transfer proteins (LTP), which are discussed in the following paragraph.

Aldehyde Decarbonylase (*CER1*): Sequence homology to decarbonylases suggest that the functional integral membrane protein CER1 represents an aldehyde decarbonylase, which catalyzes the conversion of long-chain aldehydes to *n*-alkanes, a key step in wax biosynthesis (Hannoufa *et al.*, 1993; McNevin *et al.*, 1993; Lemieux *et al.*, 1994; Aarts *et al.*, 1995; Kurata *et al.*, 2003; Rashotte *et al.*, 2004). Otherwise, CER1 might also be an enzymatic component in the transfer of aldehydes to both, the decarbonylation and acyl reduction pathway in wax biosynthesis (Jenks *et al.*, 1995; Kunst and Samuels, 2003; Kunst *et al.*, 2006). Neither of these hypotheses was yet supported by sufficient data. However, CER1 shares high sequence similarity with GLOSSY1 of corn (*Zea mays* L.; Hansen *et al.*, 1997) and rice (*Oryza sativa* L.; Sturaro *et al.*, 2005) as well as WAX2 of *Arabidopsis* (Chen *et al.*, 2003). Both epidermis-specific enzymes are reported to be involved in cuticular wax deposition.

In fruits of MicroTom wild type and MicroTom *lecer6*, transcripts of CER1 homologues (internal oligomer numbers 65, 66, 67 and 68) were found to be highly abundant in the fruit peel tissue. A comparison of both MicroTom lines revealed a developmental regulation of CER1 gene expression but at different transcriptional levels. An increased CER1 gene expression activity in MicroTom *lecer6* might indicate an alternative pathway affecting cuticular wax accumulation. Although the cuticular wax biosynthesis was already finished in the breaker and orange fruit developmental stages, when CER1 showed the highest gene expression activity, CER1 might possibly function in modification or arrangement of the cuticular wax components in MicroTom fruits.

Previously, gene expression of a CER1 homologue was characterized to be specific to the epidermis, developmentally regulated as well as responsive to dehydration stress conditions in *Arabidopsis* (Karaba, 2007). The latter finding could also implicate an elevated CER1 gene expression pattern in response to water stress of MicroTom *lecer6* fruits in comparison to the wild type. This corroborates the hypothesis that enzymes involved in cuticular wax biosynthesis also have a regulatory function in limiting water loss of aerial plant tissues.

Fatty Acid Elongase (*CER2*): Several studies characterized the CoA-dependent acyltransferase CER2, which shows sequence similarity to GLOSSY2 of corn (*Zea mays* L.; Xia *et al*., 1996), either to have a biosynthetic function as membrane-bound component of the fatty acid elongase complex or to play a regulatory role in accumulation of long-

chain cuticular waxes components (Jenks *et al.*, 1995; Negruk *et al*., 1996; Costaglioli *et al*., 2005; Kunst *et al*., 2006; D'Auria, 2006; Raffaele *et al*., 2008; Samuels *et al*., 2008). Somehow unexpected, CER2 gene expression was not inducible by dehydration, a stress factor known to stimulate cuticular wax accumulation in several plant species (Riederer and Schreiber, 1995; Xia *et al.*, 1997). However, the exact biochemical role of CER2 is not clarified yet.

The high gene expression levels found for a CER2 homologue (internal oligomer number 75) in the fruit peel tissue of MicroTom were recently described to be related to the epidermal tissue of plants (Tanaka and Machida, 2006; Mintz-Oron *et al*., 2008). CER2 gene expression was highlighted to be dependent on the fruit developmental stage of MicroTom wild type and MicroTom *lecer6*, respectively, but did not differ considerably between both genotypes. Hence, CER2 gene product might not be involved in surrogate wax biosynthesis processes of LeCER6-deficient MicroTom fruits. The putative functions of CER1 and CER2 in cuticular wax formation and, particularly, in fatty acid elongation of tomato fruits remain to be confirmed.

***Wax Synthase (*WS*)*:** The final step of the acyl reduction pathway in wax biosynthesis is the transfer of an acyl moiety from acyl-CoA to primary alkanols, which mediates the formation of wax esters. This reaction is catalyzed by the integral membrane acyl-CoA:alcohol acyltransferase termed as wax synthase (Lardizabal *et al*., 2000). Although wax synthases are described with regard to their substrate preferences and their intracellular location in several plant species such as broccoli (*Brassica oleracea* L.; Kolattukudy, 1967), jojoba (*Simmondsia chinensis* Nutt.; Wu *et al*., 1981; Metz *et al*., 2000) and *Arabidopsis* (Li *et al*., 2008), very little is known about the biochemical function (Kunst *et al*., 2006). It is presumed that the wax synthase plays a role in the biosynthesis of wax and cutin monomers or/and cross-linking of the cuticular layer (Kunst and Samuels, 2003).

Differences in gene expression patterns of a wax synthase homologue (internal oligomer number 58) were not found between MicroTom wild type and MicroTom *lecer6*. It may be deduced from the high gene expression level of wax synthase and the absence of esters in the cuticular wax composition of tomato fruits that the wax synthase was probably required in the formation of the cutin matrix.

***β-Amyrin Synthase (*AS*)*:** The β-amyrin synthase catalyzes a series of biosynthetic steps that generate the pentacyclic carbon skeleton of β-amyrin for example in pea (*Pisum sativum* L.; Morita *et al*., 2000), licorice (*Glycyrrhiza glabra* L.; Hayashi *et al*., 2001) and

barrel medick (*Medicago truncatula* L.; Kushiro *et al*., 2000; Suzuki *et al*., 2002). The product β-amyrin, which is synthesized from the acyclic precursor 2,3-oxidosqualene, is one of the most commonly occurring triterpenoid found in plant species (Abe *et al*., 1993).

The gene encoding a β-amyrin synthase homologue (internal oligomer number 81) was highly expressed in the fruit peel tissue of MicroTom fruits and might be essential in biosynthesis of the aromatic domain of cuticular waxes. Strikingly, the gene expression of the β-amyrin synthase was also found to be developmentally regulated but at the same transcriptional level in fruits of MicroTom wild type and MicroTom *lecer6*. This finding corresponds to the marginally differing amounts of β-amyrin in the cuticular wax composition of both MicroTom lines. An increased triterpenoid amount that mainly based on an accumulation of α-amyrin and δ-amyrin in the cuticular wax of LeCER6-deficient tomato fruits compared to the wild type can not linked to the transcriptional level of the β-amyrin synthase.

Fatty Acid Hydroxylase (*FA Hydroxylase*): The fatty acid ω-hydroxylase belonging to the cytochrome P_{450} dependent monooxygenases catalyzes the ω-hydroxylation of saturated and unsaturated fatty acids with chain lengths ranging from C_{12} to C_{18} that fulfills different functions during plant development (Pinot *et al*., 1992; Kahn *et al*., 2001). Previously, Tijet *et al*. (1998) and Wellesen *et al.* (2001) found that fatty acid ω-hydroxylase is implicated in the biosynthesis of cutin monomers in common vetch (*Vicia sativa* L.) and *Arabidopsis*.

A homologue of the fatty acid ω-hydroxylase (internal oligomer number 50) revealed a similar gene expression pattern in the fruit peel tissue of MicroTom wild type and MicroTom *lecer6* with a more pronounced gene expression activity in the later fruit developmental stages. Due to the temporal gene expression pattern and the participation in modification of fatty acids the fatty acid ω-hydroxylase is suggested to be involved in cross-linking of aliphatic precursor to generate the cutin matrix but do not interact in the formation of cuticular waxes of tomato fruits (Benveniste *et al*., 1998).

Lipid Transfer Protein (*LTP*): The transfer of cuticular wax components from the outermost layer of the plasma membrane, across the cell wall and the cutin matrix is largely unknown (Shepherd and Griffiths, 2006). Hydrophobic, small LTP, associated with the epidermal cell wall, might be involved in cuticle deposition (Thoma *et al.*, 1994; Pyee and Kolattukudy, 1995; Kader, 1996; Post-Beittenmiller, 1996; Kunst and Samuels, 2003; Renan *et al*., 2003; Yeats and Rose, 2008). To corroborate this hypothesis, Yamada (1992), Bourgis and Kader (1997) demonstrated that LTP, which exhibit an aminoterminal signal

peptide for translocation into the endoplasmic reticulum, *in vitro* have the ability to transport lipids between membrane vesicles (Bernhard *et al.*, 1991).

In this present study, LTP gene expression showed a specific spatial and temporal pattern. Different transcripts of LTP homologues were found most abundantly expressed in the fruit peel tissue as well as differently regulated in MicroTom wild type and *lecer6* mutant fruits (internal oligomer numbers 84, 86, 87, 91, 92 and 165). Based on sequence similarity by performing a BLAST search, these LTP were classified as belonging to distinct gene families: LTP family 1 (84) and LTP family 2 (86, 87, 91 and 92). Both families, LTP1 and LTP2, were reported as carrier of a variety of acyl lipids, whereas LTP2 were proposed to exhibit a broader range of substrate specificity of lipophilic molecules including sterols (Wirtz and Gadella, 1990; Samuel *et al*., 2002; Pons *et al*., 2003; Cheng *et al*., 2004; Hoh *et al*., 2005).

In MicroTom fruits the accumulation of LTP transcripts was largely developmentally regulated peaking in the earlier fruit developmental stages, whereas the gene expression activity was more pronounced in MicroTom *lecer6* when compared to the wild type. Hence, the highly induced LTP gene expression triggered by LeCER6 deficiency might be linked to the increased deposition of triterpenoids and sterol derivatives in the cuticular wax layer or the enhanced cutin accumulation of *lecer6* mutant fruits (CHAPTER I).

The developmental regulation of LTP gene expression in plants may play an important role (Sossountzov *et al.*, 1991; Tsuboi *et al.*, 1992; Torres-Schumann *et al.*, 1992; Thoma *et al.*, 1993). Correspondingly, previous investigations demonstrated that LTP transcripts accumulated, when biosynthesis of the cuticular membrane was required for epidermal cell expansion, and decreased with a reduced demand for cuticular compounds (Sterk *et al.*, 1991; Ma *et al.*, 1995; Treviño and O´Connell, 1998). Furthermore, several studies displayed an epidermis specific gene expression of LTP for example in tobacco (*Nicotiana tabacum* L.; Fleming *et al.*, 1992) and *Arabidopsis* (Thoma *et al.*, 1994; Suh *et al*., 2005). Interestingly, Pyee *et al.*, 1994 reported a LTP to be a major surface wax protein in broccoli leaves (*Brassica oleracea* L.).

LTP gene expression can be triggered by abscisic acid (ABA) and responds to different forms of osmotic stresses for example in leaves of barley (*Hordeum vulgare* L.; Dunn *et al.*, 1991), tobacco (*Nicotiana tabacum* L.; Kawaguchi *et al.*, 2003) and in stems of tomato (*Lycopersicon esculentum* Mill.; Torres-Schumann *et al.*, 1992) and *Arabidopsis* (Hooker *et al.*, 2002). A higher induction of LTP expression in MicroTom *lecer6* fruits could also

indicate in adaptive response to dehydration stress due to an increased water loss rate that based on alterations in cuticular wax composition.

However, the findings are in agreement with the proposed involvement of LTP in cuticle formation, since the cuticular membrane plays an essential role in the plant water balance (Lemieux, 1996; Seki *et al.*, 2002). Following alterations in biosynthesis and carbon fluxes generated by LeCER6 deficiency, the lipophilic monomers might be released and accumulate into the cuticular wax coverage or esterified into the growing cutin matrix. These data provide supporting evidence for a major influence of LTP in cuticle biosynthesis, which is requiring for an effective water barrier, most evident during maturation of tomato fruits.

A reduced level of aliphatic wax proportion did not result in a decreased cuticular wax coverage of MicroTom *lecer6* fruits, since the deposition of the aliphatic wax components was largely substituted by pentacyclic triterpenoids and sterol derivatives. Using microarray technology few transcripts, most notably LTP, were found that might contribute to adaptation and/or compensation reactions displaying the cuticular wax composition of LeCER6-deficient tomato fruits.

Transcriptional Activity with Respect to Stress Adaptation: Aspects of Stress Avoidance and Tolerance

The most important biological function of cuticular waxes is to limit non-stomatal water loss. The increase in very-long-chain aliphatic compounds that was detected in MicroTom wild type fruit wax was missing in *lecer6* mutant fruits. With beginning of the mature green fruit developmental stage the transpirational water loss rate, which is mainly determined by the cuticular wax quality, varied significantly between fruits of both MicroTom lines. As a consequence the water status of LeCER6-deficient MicroTom fruits was affected and dehydration stress might be generated in *lecer6* mutant fruits.

Dehydration is a major stress that triggers important alterations in plant physiology, metabolism and gene expression (Bonhert *et al.*, 1995). Therefore, dehydration stress might influence the development of MicroTom fruits in a complex way (CHAPTER IV). The fruit size of MicroTom *lecer6* was significantly reduced and fruits of the later developmental stages were wrinkled in comparison to MicroTom wild type. The deleterious consequences of diminished water availability can be due to either accumulation of solutes to a toxic level or osmotic effects, which were accompanied by alterations in ionic transport, lipid composition and deterioration of cell membranes.

Stress-induced gene expression in response to dehydration was analyzed by studying different plant species, whereas their functional importance can be divided into several clusters: directly and indirectly protection from stress (Thomashow, 1999; Karaba, 2007). The first cluster includes genes that are probably involved in stress avoidance. The gene products may play a decisive role in fatty acid metabolism in order to influence the lipid composition, repair stress-induced damages or regulate the fluidity of cell membranes (Torres-Schumann *et al.*, 1992). The second cluster includes genes that may interact in stress tolerance due to regulate gene expression, signal transduction in stress response and detoxification (Holmberg and Bülow, 1998; Seki *et al.*, 2002).

Gene expression analysis of MicroTom wild type and LeCER6-deficient fruits revealed several genes, which indicate dehydration stress for example an abscisic acid and environmental stress-inducible protein (Dehydrin TAS14), abscisic stress ripening proteins (ASR), lipoxygenases (LOX), anoinic peroxidases, phenylalanine ammonia-lyases (PAL) and a naringenin-chalcone synthase (CHS).

Abscisic Acid and Environmental Stress Inducible Protein (*Dehydrin TAS14*): The classification of the dehydrin protein family based on structural features, even though dehydrins are commonly induced by different stresses, all of them involving dehydration to some extent (Close, 1996; Allagulova *et al.*, 2003). Dehydrins are widely disturbed in higher plants like pea (*Pisum sativum* L.; Robertson and Chandler, 1994), sunflower (*Helianthus annuus* L.; Giordani *et al*., 1999) and peach (*Prunus persica* (L.) Batsch; Arora and Wisniewski, 1994). Nevertheless, the exact roles in conferring stress tolerance are not yet fully understood. Due to their high water-binding capacity dehydrins are considered as stress proteins that are involved in regulation of the osmotic potential in plants ensuring hydrophobic and hydrophilic interactions with low molecular weight components like sugars and amino acids (Close *et al.*, 1989; del Mar Parra *et al.*, 1996; Allagulova *et al.*, 2003). It is suggested that dehydrins exhibit also chaperon-like activity protecting various macromolecules from denaturation by forming a protective shell and maintaining the integrity of cellular structures in plants (Mayhew and Hartl, 1996; Close, 1996, 1997; Danyluk *et al.*, 1998; Koag *et al.*, 2003; Porat *et al.*, 2004). Putative functions of dehydrins include the stabilization of the transcription machinery as well as an involvement in detoxification during dehydration (Bray, 1993).

Transcripts of the abscisic acid and environmental stress-inducible protein classified as dehydrin TAS14 homologue (internal oligomer number 122). TAS14 transcripts were found in high abundance in both, MicroTom wild type and MicroTom *lecer6*, at the early

stage of mature green fruits, which is characterized by fruit expansion and, therefore, a requirement for cuticular wax biosynthesis. The TAS14 transcriptional level was more prominent in fruits of MicroTom *lecer6* compared to the wild type probably exposing a higher transpirational water loss of LeCER6-deficient fruits. These findings indicate both a developmentally regulated and a stress responsive TAS14 gene expression of tomato fruits. The early and transient induction of the TAS14 gene expression agreed with the previously reported accumulation of dehydrins in water-stressed tomato plants (Godoy *et al.*, 1994). However, TAS14 transcript amplification was not only spatially restricted to the fruit peel tissue but comprised the whole fruit level of both MicroTom lines. TAS14 gene expression highlighted a stress-related impairment whose signal migrates through all tissues of the tomato fruit.

ABA, which is known to occur in water-stressed plant tissues, was demonstrated to be a necessary element in the signaling cascade that leads from the stress perception to the induction of dehydrins in plants (Godoy *et al.*, 1990; Roberton and Chandler, 1992; Lång *et al.*, 1998; Choi *et al.*, 1999; Nylander *et al.*, 2001; Jiang and Huang, 2002; Welling *et al.*, 2002). The regulation of TAS14 gene expression is supposed to be complex mechanism probably involving further factor in addition to ABA as internal signal. Independently of the molecular mechanism being responsible for gene expression induction or its functional importance a correlation concerning dehydration stress found in tomato fruits may point to a regulatory role of TAS14 to stress tolerance.

***Abscisic Stress Ripening Protein (*ASR*)*:** The abscisic stress ripening protein is a member of a small protein family, which is involved in the cross-talk between ABA and sugar metabolism (Dure, 1993; Wang and Cutler, 1995; Carrari *et al.*, 2004). According to a relationship of induction of the ASR gene expression under conditions of dehydration stress in different plant species, Çakir *et al.* (2003) suggests an essential role of the ASR gene product in a transcription-regulating complex.

Distinct ASR genes showed varying expression patterns during development and under stress conditions such as water deficit and abscisic stress (Amitai-Zeigerson *et al.*, 1995; Gilad *et al.*, 1997; Maskin *et al*., 2001; Rom *et al*., 2006). In tomato ASR gene expression was shown to be inducible by water deficit in leaves and during fruit ripening (Picton *et al.*, 1993; Amitai-Zeigerson *et al.*, 1994; Rossi and Iusem, 1994; Frankel *et al*., 2006).

In this present study, transcripts of an ASR1 homologue (internal oligomer number 135) was found to accumulate to high levels in the fruit peel tissue of MicroTom wild type and MicroTom *lecer6* as fruit ripening progressed. Previously, Iusem *et al.* (1993) and Bovy *et*

al., (2002) also demonstrated ASR1 gene expression to be correlated with fruit ripening and, furthermore, regulated by ABA. These findings underlined the assumption that the accumulation of ASR1 transcripts might intact in sugar and ABA signaling during development of tomato fruits (Finkelstein and Gibson, 2001; Rolland *et al.*, 2002).

The drought stress gene, termed as DS2, represents a rare member of the water-stress-inducible and ripening-related ASR family in solanaceous species whose signaling pathway is not primarily linked to sugar and ABA signaling (Wang *et al.*, 1998; Dóczi *et al.*, 2002, 2005). The DS2 gene product probably functions in the protection of cellular structures including the nuclear DNA from the adverse effects of dehydration (Rossi *et al.*, 1998; Faurobert *et al.*, 2007).

A homologue of the DS2 gene (internal oligomer number 134) exhibited high transcript abundance in the fruit peel tissue of both MicroTom wild type and MicroTom *lecer6*. However, the DS2 gene was also found to be differently expressed in both MicroTom lines. During the early developmental stage of mature green fruits, DS2 gene expression activity was clearly enhanced in fruits of MicroTom *lecer6* when compared to the wild type. In the later fruit ripening stages, the DS2 gene expression decreased in MicroTom wild type and *lecer6* mutant fruits, but was found more prominent in the fruit peel tissue of MicroTom wild type.

Dóczi *et al.* (2005) reported that DS2 gene expression of tomato was rapidly and strongly inducible in response to dehydration, but revealed also a developmental control. Therefore, it was speculated that DS2 play a critical role in the mechanisms by which plants translate stress exposure into changes in gene expression activity as a part of the transcription-regulating complex or to participate in the transduction pathway (Silhavy *et al.*, 1995; Dóczi *et al.*, 2005).

Results of this present study indicating distinct patterns of development-and stress-dependent regulation probably pointed a dual function of DS2 gene expression out. Specific to mature green tomato fruits, the ASR transcriptional level might be transiently increased in consequence of dehydration stress accompanying the fruit expansion as described for the dehydrin TAS14. The DS2 gene product might be involved in stress signaling in ripe and over-ripe red tomato fruits possibly in order to protect the transcription/translation machinery.

***Lipoxygenase (*LOX*)*:** LOX enzymes are dioxygenases that catalyze the peroxidation of polyunsaturated fatty acids containing a *cis,cis*-1,4-pentadiene configuration such as linoleic ($C_{18:2}$) and linolenic acids ($C_{18:3}$) (Liavonchanka and Feussner, 2006). Collectively,

indications suggest that these fatty acids, primarily constituents of membrane phospholipids in tomato fruits, were converted within an alternative fatty acid pathway to give fatty acid hydroperoxides and free radicals (Todd *et al.*, 1990; Bowsher *et al.*, 1992; Droillard *et al.*, 1993; Brash, 1999). A precise physiological function of the organ-specific LOX is still unknown, although LOX is implicated in plant growth and development like fruit ripening and senescence, biosynthesis of regulatory molecules such as jasmonic acid, abscisic acid and traumatin, production of volatile compounds, pigment degradation and response to stress (Alexander and Grierson, 2002; Feussner and Wasternack, 2002; Porta and Rocha-Sosa, 2002; Zhu *et al.*, 2005; Andersson *et al*., 2006; Schwab *et al*., 2008). Currently, a family consisting of five genes was described to control tomato LOX activity: TomloxA, TomloxB, TomloxC, TomloxD and TomloxE (Ealing, 1994; Ferrie *et al.*, 1994; Heitz *et al.*, 1997; Chen *et al.*, 2004).

In this present study, LOX transcripts corresponding to TomloxA, TomloxB and TomloxC (internal oligomer numbers 124, 125 and 123) were found to be expressed in fruits of MicroTom wild type and MicroTom *lecer6*. In MicroTom wild type transcripts of TomloxA were maximally expressed during the earlier developmental stage of mature green fruits and decreased continuously as fruit development progressed. In contrast, gene expression of TomloxB and TomloxC was most abundant during the later developmental stages, whereas TomloxC was regulated at a distinctly lower level in comparison to TomloxB. Both gene expression of TomloxB and TomloxC followed a distinct ripening-related pattern with an increased transcriptional level detected at the early breaker fruit stage, peaking at breaker/post-breaker stage and declining thereafter. These transcriptional alterations, which were accompanied by a distinct color change from pale-yellow to yellow, orange and red, correspond to findings of Griffiths *et al.* (1999). These results corroborated that LOX have a development-dependent gene expression but in different ways probably functioning under diverse physiological conditions in tomato fruits. A regulatory influence of the fruit ripening-related ethylene and unknown developmental factor(s) on TomloxB and TomloxC gene expression is discussed (Earling, 1994; Griffiths *et al.*, 1999).

In addition to developmental aspects, LOX gene expression activity was also investigated with respect to stress response. For example, dehydration stress induced LOX transcript accumulation in common bean (*Phaseolus vulgaris* L.; Porta *et al.* 1999) and soybean (*Glycine max* L.; Maccarrone *et al.*, 1995) and an increase in LOX activity of tea (*Camellia sinensis* L.; Takeo and Tsushida, 1980).

MicroTom *lecer6* fruits exhibited a significantly reduced water status in comparison to fruits of MicroTom wild type due to a higher cuticular water loss. Under water deficit conditions of *lecer6* mutant fruits transcripts of TomloxA, TomloxB and TomloxC were found to be higher accumulated compared to MicroTom wild type during the later fruit developmental stages. Although speculative, these findings demonstrated both a dependence of the LOX transcript level on the developmental stage and dehydration stress that was generated by alterations in the cuticle deposition of LeCER6-deficient tomato fruits (CHAPTER I).

A modified LOX gene expression pattern is proposed to have different origins for example membrane deterioration occurring during water deficit, requirement for jasmonic acid biosynthesis in response to dehydration or, alternatively, may be a consequence of reduced plant growth evoked by water limiting conditions (Creelman and Mullet, 1995; Porta *et al.*, 1999).

Maalekuu *et al.* (2005, 2006) showed a relationship between cuticle properties, membrane integrity and LOX activity under water stress conditions of ripe pepper fruits (*Capsicum annuum* L.). These reports revealed that a high rate of water loss was linked to an increased LOX activity accelerating the post-harvest fruit ripening process. A turnover of fatty acids within membrane lipids alters cell membrane properties including integrity, compartmentation and oxidative index (Maccarrone *et al*., 1995). This might be the key event further cascading biochemical reactions that culminate in plant's deteriorative processes, known to facilitate fruit ripening and senescence (Siedow, 1991; Rogiers *et al.*, 1998; Maalekuu *et al.*, 2006).

LOX pathways are supposed to have several functions at particular fruit developmental stages and may contribute to stress response under different physiological situations. An ethylene-dependent and ethylene-independent LOX regulation may orchestrate the multiple characters of LOX with regard to fruit development: on the one hand, defense of premature fruits and reordering of thylakoid membranes during the chloroplast-to-chromoplast transition, on the other hand, generation of flavor and aroma and degradation processes of membranes during fruit ripening and senescence (Thelander *et al.*, 1986; Todd *et al.*, 1990; Ferrie *et al.*, 1994; Kausch and Handa, 1997; Griffiths *et al.*, 1999).

***Anionic Peroxidase*:** The anionic peroxidase of plants belongs to peroxidase enzymes that catalyze the oxidation of several substrates using hydrogen peroxide (H_2O_2) as a source of oxidizing potential (Fry, 1986; Dawson, 1988; Wallace and Fry, 1999; Jouili *et al*., 2008). The catalytic activity of peroxidases is attributed to plant growth and

development, stress tolerance and cell wall biogenesis implicating cross-linking of cell wall compounds, lignification, suberization and cutin deposition (Espelie *et al*., 1986; Fry, 1986; Roberts *et al*., 1988; Ferrer *et al*., 1991; Lagrimini *et al*., 1997; Hatfield *et al*., 1999; Quiroga *et al*., 2000). Due to their spatial distribution peroxidases are discussed in relation to a crucial role in regulation of the cell wall extensibility in fruits, whereas the cross-linking of cell wall components was suggested to be the mechanism by which the peroxidase activity may control fruit growth probably incorporating aromatic components (Lagrimini and Rothstein, 1987). In accordance to Brett and Waldron (1990), Thompson *et al.* (1998), Klotz *et al*. (1998) and Andrews *et al*. (2002) a termination of fruit growth as a result of cell wall strengthening is suggested to be linked with a developmental-dependent increase in peroxidase activity. In spite of this functional importance of peroxidases, little is known about their specific physiological role in plants.

The gene expression of anionic peroxidase 1 and anionic peroxidase 2 homologues (internal oligomer numbers 98 and 99) was highly regulated throughout development of MicroTom wild type and MicroTom *lecer6* fruits, which increased with fruit maturation. In contrast to anionic peroxidase 2, however, the gene expression activity of anionic peroxidase 1 was affected by LeCER6 deficiency of MicroTom fruits after the cuticular wax coverage was completed. Based on these findings a developmental regulation and different stress responsiveness of peroxidase gene expression was highlighted (Botella *et al*., 1994).

The biosynthetic activity of peroxidases contributes to the epoxidation of unsaturated fatty acids in plants (Hamberg and Hamberg, 1990; Blée and Schuber, 1990; Bafor *et al*., 1993). The anionic peroxidase might possibly interact in alterations of cuticle deposition in MicroTom fruits. Since cuticle accumulation was increased in LeCER6-deficient fruits, the anionic peroxidase 1 was possibly involved in the mechanism of cutin cross-linking or the removal of active oxygen species in response to dehydration stress. Both functions are essential in protection of tomato fruits from membrane damages.

***Phenylalanine Ammonia-Lyase (*PAL*)*:** In higher plants the key enzyme PAL catalyzes the first step in the phenylpropanoid pathway by converting L-phenylalanine to *trans*-cinnamate and, therefore, is supposed to be an important branch point between primary and secondary plant metabolism (Olsen *et al*., 2008; Song and Wang, 2008). This reaction leads to a number of different structural and potentially protective compounds such as the cell wall polymer lignin and flavonoid pigments (Dixon *et al*., 1983; Jones, 1984; Hahlbrock and Scheel, 1989).

Differences in gene expression pattern among genes encoding PAL homologous enzymes (internal oligomer numbers 103 and 104) were found between MicroTom wild type and MicroTom *lecer6*. On the one hand, PAL gene expression was developmentally regulated within both MicroTom lines and, on the other hand, varieties in transcript abundance between MicroTom wild type and MicroTom *lecer6* were found. LeCER6-deficient fruits exhibited a reduced PAL gene expression activity in comparison to the wild type. A higher water loss rate in MicroTom *lecer6* fruits correlated negatively with PAL gene expression, whereas the complex mechanism of PAL gene regulation is still unclear.

Previous reports showed that PAL gene expression was highly regulated during plant development and in response to a wide range of stresses such as temperature, UV light and ozone (Gowri *et al*., 1991; Eckey-Kaltenbach *et al*., 1994; Kangasjärvi *et al*., 1994; Tuomainen *et al*., 1996; Anterola and Lewis, 2002; Khan *et al*., 2004; Apel and Hirt, 2004; Olsen *et al*., 2008). Gene expression of PAL was found to be increased by different treatments including ABA and dehydration in red sage (*Salvia miltiorrhiza* Bunge; Song and Wang, 2008). Inconsistent observations were made with respect to dehydration stress in MicroTom fruits. Nevertheless, an altered gene expression of PAL might be related to the regulation of several enzymes associated with phenylpropanoid metabolism possibly involved in stress adaptation of tomato fruits.

***Naringenin-chalcone synthase (*CHS*)*:** CHS, a key enzyme in a branch of the phenylpropanoid metabolism, is the first committed step in the biosynthetic pathway of flavonoid pigments (Grisebach, 1965; Hahlbrock, 1981). Commonly, CHS catalyzes the stepwise condensation of three molecules of malonyl-CoA with the phenylpropanoid *p*-coumaroyl-CoA molecule to form the three-ring structure that is the backbone of the flavonoid biosynthesis (Ibrahim and Varin, 1993; Jez and Noel, 2000). The product of the CHS condensing reaction is the light-yellow colored naringenin-chalcone that isomerizes to the naringenin, from which various end products are derived (Stafford, 1990; Holton and Cornish, 1995; Tanaka *et al*., 1998; Durbin *et al*., 2000; Muir *et al*., 2001). Flavonoids form a large and diverse family of more than 6000 polyphenolic secondary metabolites in plants, which can be structurally divided into different classes (Haslam, 1998; Schijlen *et al*., 2006, 2007). Flavonoids have many physiological functions including pigmentation of flowers, fruits and seeds, regulation of auxin transport, fertilization and protection against oxidative damage (Koes *et al*., 1994; Parr and Bolwell, 2000; Winkel-Shirley, 2001; Wasson *et al*., 2006; Pfündel *et al*., 2006; Bovy *et al*., 2007).

Many of the compounds belonging to the CHS biosynthetic pathway are potential antioxidants that are subjected to a tissue- and developmental-specific regulation and responsive to stress stimuli (Schöppner and Kindl, 1979; Mol *et al*., 1983; Kaulen *et al*., 1986; Feinbaum and Ausubel, 1988; Holton and Cornish, 1995; Shirley, 1996; Pelletier *et al*., 1999; Jandet *et al*., 2002).

In tomato, CHS is encoded by a small multigene family of at least three members (O'Neill *et al*., 1990). Low level gene expression of a CHS B homologue (internal oligomer number 119) was analyzed in MicroTom wild type and MicroTom *lecer6* (Bovy *et al*., 2002, 2007). The CHS B gene expression pattern was found to be developmentally coordinated and, in addition, displayed different transcript abundance in both MicroTom lines. MicroTom *lecer6* fruits exhibited a reduction in CHS gene expression activity when compared to wild type fruits similarly to those of PAL. A co-regulated expression of the flavonoid-related genes PAL and CHS was also documented by Mintz-Oron *et al*. (2008). Based on a modified gene expression of PAL and CHS, belonging to the general phenylpropanoid and flavonoid pathways, quantitative and qualitative differences in the metabolic profile between fruits of both MicroTom lines can be hypothesized (Verhoeyen *et al*., 2002; Bovy *et al.*, 2007).

In general, tomato fruits contain only small amounts of flavonoids localized in the epidermal cell layer mostly naringenin-chalcone, the flavanone naringenin and the flavonols quercetin rutinoside or rutin, which accumulate during fruit development and improve the overall antioxidant capacity of the fruit (Hunt and Baker, 1980; Stewart *et al*., 2000; Bauer *et al.*, 2004a; Giovinazzo *et al*., 2005; Schijlen *et al*., 2006, 2007).

Flavonoids, as secondary metabolites of plants, serve also as structural elements influencing the mechanical properties of the epidermal cell layer of tomato fruits. Consequently, the amount of flavonoid accumulation might affect the spatial arrangement of the cuticular membrane. The flavonoid quantity probably varied between fruits of both MicroTom lines in order to avoid cuticle cracking and/or strengthen the organization and structure of the cuticular layer, since the cuticle deposition was significantly enhanced in *lecer6* mutant fruits (CHAPTER I). A down-regulation of flavonoid branch of the phenylpropanoid metabolism might also accelerate the production of hydroxycinnamate, mainly coumaric acid, as consequence of a common substrate pool (Hemm *et al*., 2004; Skirycz *et al*., 2007). The later is an essential component of the cutin matrix of tomato fruits, which yielded distinctly higher amounts in LeCER6-deficient fruits (CHAPTER I).

This present study suggests that differences at the transcriptional level between MicroTom wild type and MicroTom *lecer6* are part of regulatory and structural adaptations in order to ensure the functional efficiency of LeCER6-deficient tomato fruits. Most significantly, compositional alterations of the cuticular membrane generated a higher water loss rate in *lecer6* mutant fruits compared to MicroTom wild type. Both MicroTom lines showed different levels of water availability with respect to the whole fruit level. At the transcriptional level it can be differentiated between early and late responses to dehydration stress in MicroTom *lecer6* fruits possibly depending on the fruit developmental stage and/or the degree of stress-related impairments.

Strikingly, the dehydrin TAS14 and the abscisic stress ripening protein DS2 showed a higher gene expression activity in the *lecer6* mutant fruit compared to the wild type in the earlier fruit developmental stages putatively having a regulatory function by modulating gene expression when the tomato fruit expands and the wax biosynthesis is not yet completed. During the later fruit development another set of transcripts such as individual LOX and anionic peroxidase, which were predicted to be involved in membrane integrity and detoxification, exhibited transcriptional changes. These genes in addition to PAL and CHS may play an essential structural role in stress tolerance of tomato fruits contributing to protect cell membranes.

The significance of stress-induced or -inhibited gene expression may be related to the highly diverse physiological function of gene products in MicroTom wild type and MicroTom *lecer6* fruits. Commonly, genes encoding stress proteins are important for the stabilization of cellular structures, form protective reactions of the transcription/translation apparatus or are subjected to regulation of the osmotic potential of tomato fruits. Each of these processes is important to stress adaptation of plants. Nevertheless, the exact mechanisms by which plants mediate stress-responsive signaling into changes in gene expression and confer tolerance to adverse stress conditions are very complex and still not fully understood.

Transcriptional Activity with Respect to Fruit Developmental Processes: Aspects of Maturation, Expansion, Ripening and Senescence

In addition to aspects of the cuticular wax biosynthesis and the interacting water loss barrier property, this present study focused on development-associated processes in MicroTom fruits. In general, development of climacteric tomato fruits comprises complex biochemical and physiological changes such as alterations in cell wall metabolism,

chlorophyll degradation together with an increase in respiration, carotenoid accumulation, shift in sugar-acid-balance and biosynthesis of volatiles. These culminating modifications lead to the characteristic color, texture, flavor and aroma of the red ripe, fleshy tomato fruit (Brady, 1987; Gray *et al.*, 1994; Griffiths *et al.*, 1999; Flores *et al.*, 2001; Giovannoni, 2001; Alexander and Grierson, 2002; Alba *et al*., 2005).

Thereby, an important role of auto-catalytic ethylene production in controlling many aspects of fruit development was established. Ethylene biosynthesis and perception was shown to be essential for the later stages of fruit development due to the fact that ethylene promotes the transcription and translation of a number of ripening-related genes (Lincoln *et al.*, 1987; DellaPenna *et al.*, 1989; Rottmann *et al.*, 1991; Oeller *et al.*, 1991; Picton *et al.*, 1993). In addition to the fruit ripening, ethylene is also known to be involved in other plant processes such as response to a variety of stress conditions including dehydration (Kieber *et al*., 1993; Yang and Hoffman, 1984; Abeles *et al.*, 1992; Novikova *et al*., 2000; Bleecker and Kende, 2000; Crozier *et al*., 2000; Gazzarrini and McCourt, 2003).

Tomato fruits exhibit a climacteric increase in respiration with a concomitant burst of massive ethylene production at the onset of ripening. Based on this altered ethylene level, two systems of ethylene biosynthesis regulation were proposed (McMurchie *et al.*, 1972; Klee, 1993; Kende, 1993; Barry *et al.*, 2000): Ethylene synthesis system 1 is ethylene auto-inhibitory and responsible for producing the basal low level of ethylene. It is functional in vegetative plant tissues including green, pre-climacteric fruits and continues until a competence to ripen is attained. At this point a transition occurs probably by a change in ethylene sensitivity due to system 1 ethylene production. The auto-stimulatory ethylene synthesis system 2 initiates. Ethylene synthesis system 2 is classified by the characteristic climacteric rise in ethylene production, which coordinates and accelerates the fruit ripening process by a positive feedback regulation. In turn, the high rate of system 2 ethylene production results in a negative feedback on the ethylene synthesis system 1 pathway.

Various ethylene-dependent and -independent genes were involved in the widely coordinated changes during the fruit development of MicroTom wild type and MicroTom *lecer6*. Some of those genes displayed differences in the gene expression pattern between MicroTom wild type and *lecer6* mutant fruits: ethylene-inducible protein kinase (CTR1), 1-aminocyclopropane-1-carboxylate synthase & oxidase (ACC synthase & ACC oxidase) and phytoene synthase (PSY).

Ethylene-Inducible Protein Kinase (*CTR1*): The mitogen-activated protein kinase kinase kinase (MAPKKK) CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) which is a

negative regulator of the ethylene signal transduction pathway acting downstream of ethylene receptors (Kieber *et al*., 1993; Clark *et al*., 1998; Ichimura *et al*., 2002; Alexander and Grierson, 2002; Huang *et al*., 2003; Adams-Phillips *et al*., 2004a; Giovannoni, 2004). The ETHYLENE RECEPTOR1 (ETR1), type of a two-component receptor kinase, activates CTR1 by forming a signaling complex until ethylene binds to ETR1 (Gao *et al*., 2003; Hong *et al*., 2004). This event causes monomerization of the response regulator and release of CTR1 (Hua and Meyerowitz, 1998; Gamble *et al*., 1998). The absence of CTR1 activation, in turn, releases positive regulators from the negative regulation of CTR1, which allows ethylene response mechanism that can regulate further signal transduction and activate gene expression (Bleecker *et al*., 1998; Gazzarrini and McCourt 2001; Chang and Stadler, 2001; Wang *et al*. 2002).

A CTR1 homologue (internal oligomer number 106) showed high transcript abundance in whole MicroTom fruits in the mature green fruit stage, which specified an important inhibitory regulation of the ethylene signaling pathway at this fruit developmental phase. Low gene expression activity of CTR1 was found in the fruit peel tissue of MicroTom, although the CTR1 transcriptional level slightly increased during the later fruit developmental stages representing periods of high ethylene exposure. Likewise, Zegzouti *et al.* (1999), Leclercq *et al*. (2002) and Adams-Phillips *et al*. (2004b) documented an up-regulation of CTR1 gene expression in response ethylene during fruit ripening, whereas Kiss *et al*., 2007 found a constitutive gene expression of CTR1 in strawberry (*Fragaria* x *ananassa* Duchesne). As part of normal fruit developmental or response processes CTR1 gene expression allows also continued ethylene responsiveness and signal modulation in the climacteric fruit stage (Giovannoni, 2004).

Higher CTR1 gene expression activity of MicroTom *lecer6* in comparison to the wild type suggests a negative feedback regulation that may serve to control the ethylene signal transduction during fruit ripening stages. Acting as a negative regulator of ethylene response, differences in the CTR1 abundance might represent a mechanism for optimizing specificity of ethylene responses or a cross-talk with an ethylene-independent pathway in tomato fruits (Adams-Phillips *et al*., 2004a). CTR1 indicates a negative ethylene response in tomato, but the physiological role in the ethylene signal transduction mechanism is not established (Lin *et al*., 1998; Zegzouti *et al*., 1999; Alexander and Grierson, 2002; Liu *et al*., 2002). With respect to the degraded water loss barrier of LeCER6-deficient tomato fruits in comparison to the wild type, a higher CTR1 transcript abundance possibly

operates in mediating stress response due to the regulation of various pathways, in which ethylene is involved.

1-Aminocyclopropane-1-Carboxylate Synthase & Oxidase (*ACC Synthase & ACC Oxidase*): In higher plants, the ethylene biosynthetic pathway starts with methionine, occurs *via* *S*-adenosyl-L-methionine (SAM) and 1-aminocyclopropane-1-carboxylate (ACC). The key enzyme ACC synthase catalyzes the reaction of SAM to the immediate precursor of ethylene ACC. Subsequently, the ACC oxidase, also termed as ethylene forming enzyme (EFE), carries out the conversion of ACC to ethylene (Yang and Hoffman, 1984). Both ACC synthase and ACC oxidase are encoded by divergent multigene families that generate multiple control points at which ethylene production may be highly regulated throughout fruit development (Gray *et al.*, 1994; Barry *et al*., 1996; Deikman 1997; Johnson and Ecker, 1998; Giovannoni, 2001; Alexander and Grierson, 2002).

In tomato, the role of ACC synthases and ACC oxidases was extensively studied, whereas different gene expression patterns of individual ACC synthases and ACC oxidases were found to be specific to the developmental stage, tissue-dependent and responsive to a variety of stress stimuli, respectively (Lui *et al.*, 1985; Rottmann *et al.*, 1991; Zarembinski and Theologis, 1994; Abel *et al.*, 1995; Fluhr and Mattoo, 1996; Picton *et al.*, 1993; Nie *et al*., 2002; Carrari and Fernie, 2006). Nakatsuka *et al.* (1998) and Barry *et al.* (1996, 2000) showed that the transition from ethylene synthesis system 1 to ethylene synthesis system 2 was correlated with an accumulation of transcripts homologous to ACO1 (internal oligomer number 111) and ACS2 (internal oligomer number 107) and with a burst in ethylene biosynthesis.

In MicroTom fruits, low abundance of ACO1 transcripts was detected during the immature green fruit stage possibly indicating a constitutive gene expression of ACO1 at a basal level during fruit development. At the early breaker stage, the ACO1 gene expression increased significantly representing the beginning of the climacteric fruit phase and the onset of fruit ripening. This time course of ACO1 gene expression found in MicroTom fruits was in agreement with earlier studies of Hamilton *et al.* (1990) and Nakatsuka *et al.* (1998). This present study indicated ACO1 as one of the major genes expressed in ripening tomato fruits (Barry *et al.*, 1996; Alba *et al*., 2004).

In contrast to ACO1 gene expression, which was suggested to be independent of ethylene biosynthesis, the induction of ACS2 gene expression requires ethylene (Olson *et al.*, 1991; Barry *et al.*, 2000). The transcript abundance of ACS2 increased similarly to that

of ACO1 at the early breaker fruit stage but at a considerably lower level. These findings displayed a distinctly transcriptional regulation of ACO1 and ACS2 genes, which are involved in ethylene biosynthesis, in tomato throughout different fruit developmental stages (Kende, 1993; Nakano *et al*., 2003).

Gene expression of an ACO2 homologue (internal oligomer number 110) was mainly restricted to mature green MicroTom fruits. These data are consistent with results prior described in peach (*Prunus persica* (L.) Batsch) revealing ACO2 transcript accumulation only during the earlier fruit developmental stages (Ruperti *et al*., 2001; Rasori *et al*., 2003). Moreover, the regulation of ACO2 gene expression was correlated with cell expansion in seedlings of *Arabidopsis* (Raz and Ecker, 1999). Collectively, these findings highlighted a limitation of ACO2 gene expression to the expanding and maturing MicroTom fruits. The discrepancy in the initiation of gene expression of ACO1 and ACO2 each regulated in a unique way might expose a differently functional importance of both ACC oxidases in tomato fruits.

In MicroTom *lecer6* the gene expression of ACO1 and ACS2, which are linked to large quantities of ethylene, showed comparable patterns of transcript accumulation as the wild type but with a temporal retardation during fruit development (Barry *et al.*, 1996). The gene expression of ACO1 and ACS2 increased clearly with a shift towards the later fruit ripening stages of MicroTom *lecer6* when compared to the wild type. These results might indicate an effect of LeCER6 deficiency on the regulation of these ripening-related genes at the transcriptional level in MicroTom fruits probably due to water loss stress.

Different stress stimuli including water deficit control ethylene biosynthesis in plant tissues beside a development-dependent regulation (Abeles *et al*., 1992; Morgan and Drew, 1997). A delay in the initiation of ACO1 and ACS2 gene expression in MicroTom *lecer6* fruits permitted restriction of the endogenous ethylene level and, further, regulation of the fruit ripening process that is controlled by ethylene. This probably referred either to the structural stability by retarding fruit softening, contributed to maintain active photosynthesis for longer periods or allowed to produce osmoprotectants by inhibition of ethylene biosynthesis (Nakano *et al*., 2003; Manavella *et al*., 2006). However, the physiological role of distinct ACC synthase and ACC oxidase family members in stress response mechanisms is still unknown. A combination of endogenous ethylene level, developmental factors and stress conditions may be required. These findings represent an involvement of ethylene biosynthesis genes in the stress signaling of LeCER6-deficient tomato fruits.

Phytoene synthase (*PSY*): The membrane-associated phytoene synthase catalyzes the first committed step in carotenoid biosynthesis by condensation of two molecules geranylgeranyl diphosphate (C_{20}; GGPP) derived from the isoprenoid pathway to produce phytoene (C_{40}; Goodwin, 1980; Fraser *et al*., 1994; Bartley and Scolnik, 1995; Lindgren *et al*., 2003). The dimerization proceeds through the intermediate pre-phytoene diphosphate (PPDP). Subsequently, a series of four desaturation reactions converts colorless phytoene to red colored lycopene *via* phytofluene, ζ-carotene and neurosporene. Compounds before ζ-carotene (pale yellow) do not absorb light in the visible region of the spectrum (Cunningham and Gantt, 1998; Harker and Hirschberg, 1998; Hirschberg, 2001; Fraser and Bramley, 2004; DellaPenna and Pogson, 2006; Li *et al*., 2007).

The ripening-related reaction of phytoene synthase, a rate-limiting key enzyme in lycopene formation, is viewed as major regulatory step in carotenogenesis (Bartley and Scolnik, 1995; Schofield and Paliyath, 2005). The cyclization of the lycopene molecule is an important branching point in the carotenoid pathways. One route yields β-carotene and its xanthophyll derivatives: zeaxanthin, antheraxanthin, violaxanthin and neoxanthin, whereas the latter two components are precursors in the biosynthesis of ABA, a regulator of stress response in plants (Zhu, 2002; Finkelstein *et al*., 2002; Seo and Koshiba, 2002; Welsch *et al*., 2008). The alternative pathway leads to carotenoids such as α-carotene and lutein.

During plant development lipophilic carotenoids play several roles for example act as accessory light-harvesting pigments and essential photoprotectants that prevent from oxidative damage, function as structural component and fulfill an important purpose as bright yellow, orange or red colorants (Demmig-Adams and Adams, 1996, 2000; Niyogi, 1999; Booker *et al*., 2004; Simkin *et al*., 2004; Auldridge *et al*., 2006; Galpaz *et al*., 2006).

In tomato, phytoene synthases are encoded by two different genes: PSY1 is chromoplast-deduced and responsible for carotenoid formation in ripening fruits, while PSY2, also present at a low level in the chromoplast, is only active in the chloroplast (Bartley *et al*., 1992; Fraser *et al*., 1999; Steinbrenner and Linden, 2001; Giorio *et al*., 2008). According to previous reports, low transcript abundance of a PSY1 homologue (internal oligomer number 136) was found in mature green MicroTom fruits (Fray and Grierson, 1993; Bartley *et al*., 1994; Fraser *et al.*, 1995). Starting at the early breaker stage, MicroTom wild type fruits displayed a significant increase in PSY1 gene expression peaking in the breaker and orange fruit developmental stages and, thereafter, exhibiting a

drastically reduction in red ripe and red overripe fruits. Particularly, PSY1 was one of the main genes expressed at the transcriptional level during ripening of MicroTom fruits.

LeCER6-deficient MicroTom fruits showed also a developmental regulation of the PSY1 gene expression as documented for the wild type, but the increase in PSY1 transcript accumulation of MicroTom *lecer6* was induced delayed as well as more intensified when compared to the wild type. Based on the gene expression patterns both a regulation of PSY1 by developmental mechanisms and in response to stress conditions were demonstrated in fruits of MicroTom wild type and its *lecer6* mutant.

The distinct up-regulation of PSY1 gene expression within the transition of the breaker to the orange developmental stage of tomato fruits was reported to control the carotenoid accumulation, mainly lycopene and β-carotene, independently of ethylene (Bartley *et al.*, 1992; Pecker *et al*., 1992; Bramley *et al.*, 1992; Ray *et al.*, 1992; Gillaspy *et al*., 1993; Ronen *et al.*, 1999; Alba *et al.*, 2000; Lois *et al*. 2000; Fraser *et al*., 1994, 2000, 2007; Giorio *et al*., 2008). Besides a significant carotenoid accumulation and chlorophyll degradation, both affecting epidermal quantities, transgenic plants expressing higher or constitutive levels of PSY1 transcripts exhibited a reduction of growth, a delayed development and an increased amount of ABA (Cheung *et al*., 1993; Grierson and Fray, 1994; Bartley and Scolnik, 1995; Fray *et al.*, 1995; Rodríguez-Concepción *et al*., 2003; Efrati *et al*., 2005; Hörtensteiner, 2006). Thereby, the degree of developmental retardation was positively correlated with an increased level of carotenoids, accompanied by ABA accumulation probably in response to dehydration (Koornneef, 1986; Bartley *et al.*, 1991; Guiliano *et al.*, 1993; do Rêgo *et al.*, 1999; Thompson *et al*., 2000; Steinbrenner and Linden, 2001; Lindgren *et al.*, 2003). These observations support the idea that extensive changes in PSY1 transcript abundance regulating the carotenoid pathway flux played an important role in the stress response of tomato fruits due to their beneficial properties against oxidative stress, their function as structural component or their rate-limiting impact on ABA formation.

The present study led to the assumption that LeCER6 deficiency interfered with fruit developmental mechanisms. The modified cuticular barrier properties of LeCER6-deficient tomato fruits exerted influence on several fruit ripening processes by changes at the transcriptional level of gene expression in an ethylene-dependent or -independent manner. An induction of ripening-related gene expression of CTR1, PSY1, several ACC-synthases and -oxidases and, as aforementioned, individual LOX might indicate a retarded though increased climacteric peak in *lecer6* mutant fruits in comparison to the wild type. The

importance of these transcriptional alterations might be due an involvement of the ethylene signal transduction pathway, biosynthesis of ethylene and ABA and cell wall modifications (Jiang and Fu 2000; de Bruxelles and Roberts, 2001; Hoeberichts *et al*., 2002; Karakurt and Huber, 2004). Delaying or lowering the amount of ethylene production like the inhibition of ethylene biosynthesis was demonstrated to constitute a successful strategy to extend the shelf-life of fruits, since the rise of ethylene was shown for example to induce the accumulation of several cell wall degradative enzymes in ripening tomato fruits (Grierson *et al*., 1986; Ayub *et al.*, 1996; Saladie *et al.*, 2007). A retardation of membrane and cell wall loosening may contribute to a physical support by affecting tomato fruit softening and improve the overall barrier function against uncontrolled water loss. Carrari and Fernie (2006) discussed a relationship among the biosynthesis of ethylene in a putative fruit ripening signal cascade and the interaction with other biosynthetic pathways such as an interaction of ABA with the ethylene reception and response pathway (Ghassemian *et al*., 2000; Beaudoin *et al*., 2000). Temporally coordinated alterations in gene expression might be an integral part of stress response in tomato during fruit development.

Impact of LeCER6: Gene Expression Involved in Cuticular Wax Accumulation

The comparative study of gene expression patterns of tomato fruits was carried out during fruit development, in a comparison between the whole fruit and the fruit peel tissue as well as between tomato wild type and LeCER6-deficient fruits to broaden the knowledge related to the cuticle biosynthesis and its functional impact. Using microarray technology, it was found that the transcriptional regulation of most genes exposed fruit development-dependent initiation, amplification, reduction or fluctuation, which were pinpointed similarly for both tomato wild type and its *lecer6* mutant.

Particularly, biosynthesis and deposition of the cuticular membrane resulted from highly coordinated gene expression activities in the fruit peel tissue and were closely correlated with metabolic changes occurring during fruit maturation.

This study provided conclusive evidence that a block in fatty acid elongation by LeCER6 deficiency led to both changes in the compositional and structural property of the cuticular membrane in addition to fruit developmental aspects. The key role of the β-ketoacyl-CoA synthase LeCER6 in determining the amount and chain length of fatty acids and corresponding derivatives found in fruit cuticular waxes was highlighted by characterizing tomato fruits in a 'reverse genetic' approach. The level of substrate pools altered in LeCER6-deficient tomato fruits compared to the wild type. Compensative or

alternative biosynthetic pathways were promoted for example in cuticle biosynthetic pathways, cross-linking and transport of components involved in cuticle formation.

Concerning the functional importance of the cuticle as an efficient transport barrier against water flux, LeCER6 deficiency caused dehydration stress conditions in tomato fruits. Changes in gene expression provided the basis for stress adaptation mechanisms responsible for the homoeostasis of ripening tomato fruits to extend their shelf-life and, primarily, ensure their productivity. Genes that were differently expressed in both tomato lines encoded whether structural information or signals, which are able to activating or repressing regulatory cascades. In common, these transcriptional differences orchestrate the highly complex nature of stress responses in consequence of an impaired cuticular barrier of tomato fruits. In tomato fruits, stress signaling and adaptation probably involved osmoregulation, detoxification, protection against oxidative damage, isoprenoid/carotenoid metabolism and phenylpropanoid/flavonoid metabolism and is mediated by plant regulatory molecules such as ethylene, abscisic and jasmonic acid.

These findings displayed the participation of complex regulatory network of underlying gene expression patterns, which point at an interaction between dehydration and oxidative stress responses and indicate a delay of a number of ripening processes as indirect effects of modifications in the cuticular membrane. The latter might be a result of altered perception or communication of ripening signals, which may provide an additional benefit to reduce the water loss rate and probably helps to stabilize the integrity of tomato fruits.

Nevertheless, there remains some debate whether gene expression is regulated at the transcriptional, post- transcriptional or post-translational level with respect to an agreement between gene expression activity and gene product accumulation (Bartley and Scolnik, 1995; Nakano *et al*., 2003). These data address further needs to validation at the translational level to gain a better understanding of the mechanisms generating biochemical modifications in tomato wild type and LeCER6-deficient fruits.

This present study demonstrated the potential and the methodical restriction of the comparative gene expression analysis allowing an insight into the genetic make-up of cuticle assembly and its biological function in tomato fruits. Based on deficiency in β-ketoacyl-CoA synthase LeCER6 the pivotal role of an intact cuticle consisting of cuticular waxes embedded in the cutin matrix was emphasized during tomato fruit development. Referring to this, tomato fruits provide a useful model system in order to understand developmental aspects and to learn more about the complex regulatory mechanisms underlying fruit stress responses.

CHAPTER III

Analysis of Various Parameters of the Cuticular Wax Biosynthesis in Tomato

RESULTS

The wax biosynthesis is specific for plant species, their organs and tissues and, in addition, subject to developmental and environmental influences. The complex mechanisms of wax biosynthesis are supposed to interact with the enhancement of water barrier property. Examinations of the present study were focused on differences in wax biosynthesis of MicroTom wild type and its LeCER6-deficient mutant leaves (i). In order to clarify organ specific aspects of wax biosynthesis MicroTom leaves were compared with MicroTom fruits, which act as a model system for cuticular barrier properties. In addition, MicroTom wild type fruits were used to investigate quantitative and qualitative changes of cuticular waxes and their functional significance under water-limited (ii) and mechanical stress conditions (iii). Compensation as well as adaptation reactions of mature green MicroTom fruits to these stress stimuli were studied on both, the biochemical and transcriptional level, since changes in cuticular waxes could be regarded as important consequences of stress resistance that lead to improved water loss barrier properties of surfaces of higher plants.

Impact of LeCER6 Deficiency on Wax Component Accumulation of Tomato Leaves

The tomato leaf is divided into five leaflets and their stomatous surfaces are covered with hairs. In general, fully developed MicroTom *lecer6* leaves were frizzlier than the wild type (Figure 1). However, the LeCER6 deficiency had no significant impact on the leaf surface area. MicroTom leaves had an adaxial plus abaxial surface area of 62.38 ± 18.02 cm^2 (mean ± SD) in the wild type line. MicroTom *lecer6* leaves exhibited a total surface area of 84.90 ± 9.02 cm^2.

Similar to MicroTom fruits, cuticular wax characteristics of leaves of MicroTom wild type and its *lecer6* mutant were studied (CHAPTER I).

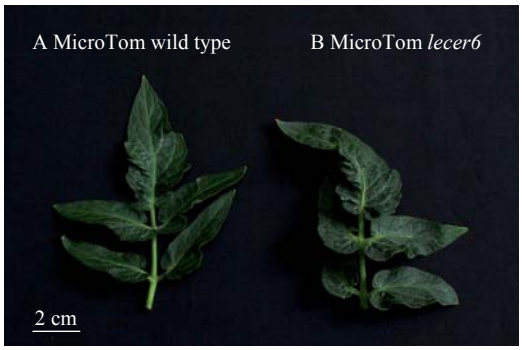

Figure 1. Photograph of MicroTom wild type (A) and MicroTom *lecer6* (B) tomato leaves.

Cuticular waxes were extracted from leaf surfaces of MicroTom wild type and MicroTom *lecer6*. Subsequently, the composition of these leaf waxes was qualitatively and quantitatively assessed by gas chromatography. The analysis revealed that the amount of cuticular wax coverage differed significantly between MicroTom wild type and its *lecer6* mutant. MicroTom wild type leaves exhibited a 2.7-fold higher wax accumulation than MicroTom *lecer6*. The main part of both cuticular leaf waxes of MicroTom wild type and *lecer6* mutant consisted of *n*-alkanes, iso- and anteiso-alkanes as well as triterpenoids and sterol derivatives (Figure 2). The amount of primary alkanols and alkanoic acids in the cuticular leaf wax was found at a distinctly lower level.

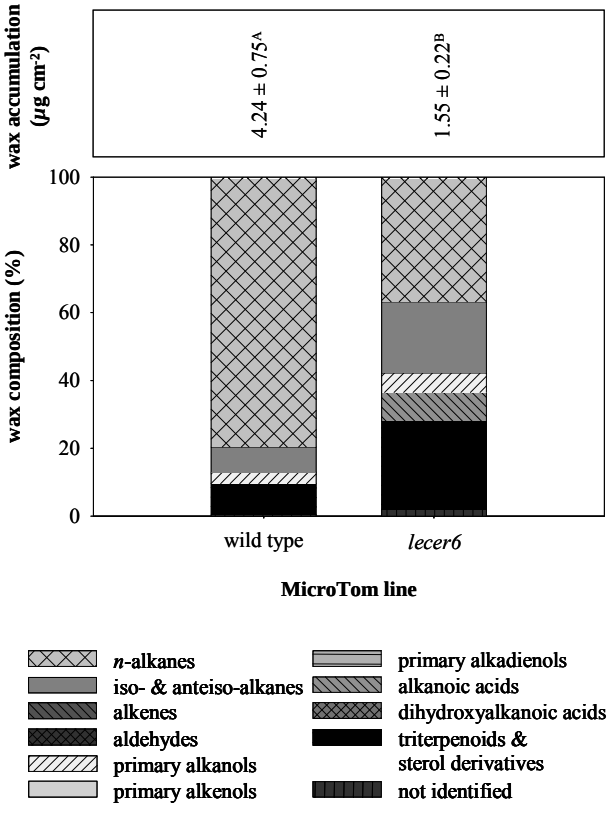

Figure 2. Total cuticular wax quantities and relative wax compositions of fully developed MicroTom wild type and MicroTom *lecer6* leaves. Leaf wax quantities were compared by one-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SD ($n = 4$ to 5).

The alteration in the wax composition of MicroTom *lecer6* is mainly based on a reduced proportion of *n*-alkanes in comparison to the wild type (Figure 3). Whereas the wild type wax showed a proportion of 80% *n*-alkanes, the wax composition of *lecer6* mutant was composed of 37% *n*-alkanes. The chain length distribution of *n*-alkanes was between C_{27} up to C_{35}, whereas *n*-hentriacontane (C_{31}) was found to dominate the wild type leaf wax.

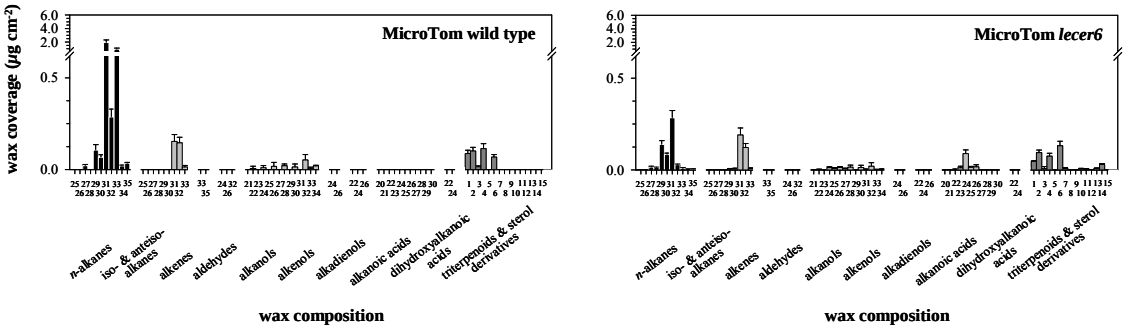

Figure 3. Cuticular wax constituents of fully developed MicroTom wild type and MicroTom *lecer6* leaves. Triterpenoids and sterol derivatives are numbered as follows: 1 α-amyrin, 2 β-amyrin, 3 β-amyrin derivative, 4 δ-amyrin, 5 cholesterol, 6 lanosterol, 7 multiflorenol, 8 β-sitosterol, 9 stigmasterol, 10 taraxasterol & ψ-taraxasterol, 11 taraxerol, 12 lupeol, 13 lupeol derivative I, 14 lupeol derivative II, 15 unknown triterpenoid. Carbon chain lengths are shown for *n*-alkanes, iso- & anteiso-alkanes, alkenes, aldehydes, primary alkanols, primary alkenols, primary alkadienols, alkanoic acids and dihydroxyalkanoic acids. Data are shown as means ± SD (n = 4 to 5).

LeCER6-deficient leaves showed a significantly reduced *n*-alkane accumulation > C_{30} in comparison to the MicroTom wild type wax, while shorter chain *n*-alkanes were not affected. Additionally, an 1.6-fold decrease in alkanols, but, in contrast, accumulation of alkanoic acids were observed in the mutant leaf waxes. Apart from this observation, for both, the MicroTom wild type and the *lecer6* mutant, similar levels of iso- and anteiso-alkanes (0.33 ± 0.01 μg cm^{-2} in MicroTom wild type and 0.31 ± 0.02 μg cm^{-2} in MicroTom *lecer6*) as well as triterpenoids and sterol derivatives (0.40 ± 0.01 μg cm^{-2} in MicroTom wild type and 0.39 ± 0.01 μg cm^{-2} in MicroTom *lecer6*) were identified.

Analogous to MicroTom fruits, gene expression analysis in MicroTom leaves was focused on gene regulation of wax biosynthesis, wax formation associated transport processes and possible dehydration stress responses (CHAPTER II). For this transcriptional analysis total RNA of MicroTom wild type and its mutant leaves were investigated. The total RNA content varied between 406.6 ± 147.9 μg g^{-1} fw in MicroTom

wild type and 473.8 ± 66.0 μg g^{-1} fw in MicroTom *lecer6*, but the total RNA levels in the leaves of both MicroTom genotypes differed not significantly.

Figure 4 shows the few transcripts represented on the wax microarray slide, which were highly expressed in MicroTom leaves of both wild type and *lecer6* mutant. The strongest signal intensities were recorded for a wax synthase (58), two *cer* homologues (65, 68), an abscisic stress ripening protein (DS2 protein, 134), a glyceraldehyde-3-phosphate dehydrogenase (162) and a hypothetical protein (lipid transfer protein; 165). Only in the case of two lipid transfer proteins (86, 87) and a non-specific lipid transfer protein (91) distinct differences in the expression intensity between both MicroTom genotypes were found (Table I).

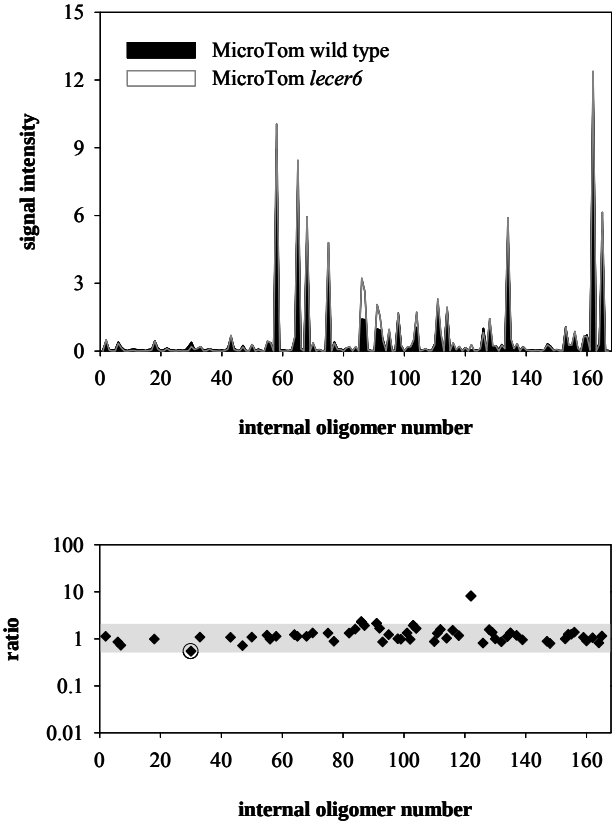

Figure 4. Overview of all microarray expression signals of 167 oligomers related to fully developed MicroTom wild type and *lecer6* leaves. Gray bars illustrate a ratio ≤ 0.5 and ≥ 2.0 calculated for gene expression signals of the wild type and the corresponding mutant leaf tissue. The oligomer with the internal number 30 represents the very-long-chain fatty acid condensing enzyme (*LeCer6*) and is marked with a circle. Data are shown as means (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

Transcripts of the *LeCer6* gene encoding for the very-long-chain fatty acid condensing enzyme (30) were weakly expressed in the leaves of MicroTom wild type. In LeCER6-deficient leaves gene expression signals for this very-long-chain fatty acid condensing enzyme were scarcely detectable.

Table I. Selection of the strongest microarray signal intensities of MicroTom wild type and MicroTom *lecer6* leaves labeled with internal oligomer number. Additionally, changes of expression signal intensities were emphasized with shadings: no signal (white), weak signal (≥ 0.2; light gray), distinct signal (≥ 0.5; gray) and strong signal (≥ 5.0; dark gray). Remarkable ratios of expression signals intensities are marked in bold. Data are shown as means ± SD (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

	oligomer	MicroTom wild type	MicroTom *lecer6*	*ratio*
30	Very-long-chain fatty acid condensing enzyme (*LeCer6*)	0.4 ± 0.1	0.2 ± 0.1	***0.5***
58	Wax synthase	9.0 ± 2.7	10.1 ± 3.7	*1.1*
65	CER1 homologue	7.5 ± 3.7	8.4 ± 4.3	*1.1*
68	CER1 homologue	5.3 ± 2.2	5.9 ± 2.9	*1.1*
86	Lipid transfer protein	1.4 ± 0.5	3.2 ± 1.9	***2.3***
87	Lipid transfer protein	1.4 ± 0.2	2.6 ± 1.2	***1.9***
91	Non-specific lipid transfer protein	1.0 ± 0.2	2.1 ± 0.8	***2.1***
134	Abscisic stress ripening protein (DS2 protein)	5.3 ± 1.8	5.9 ± 3.5	*1.1*
162	Glyceraldehyde-3-phosphate dehydrogenase	11.8 ± 5.6	12.4 ± 4.2	*1.0*
165	Hypothetical protein (lipid transfer protein)	5.3 ± 1.3	6.1 ± 1.7	*1.1*

Effects of Atmospheric Humidity and Soil Moisture on Fruit Wax Accumulation

After a standard cultivation program of six weeks, MicroTom wild type plants were grown under different conditions of atmospheric humidity and soil irrigation. The atmospheric humidity was adjusted to 40% and 80%, respectively. To regulate soil moisture, all potted plants were substrate saturated watered once a week. Furthermore, only a group of these plants was irrigated to assure optimally growing conditions (optimal watering conditions), whereas a second group had to cope with little water supply (no watering conditions). During this weakly procedure the extent of weight loss of all potted plants was recorded (Figure 5).

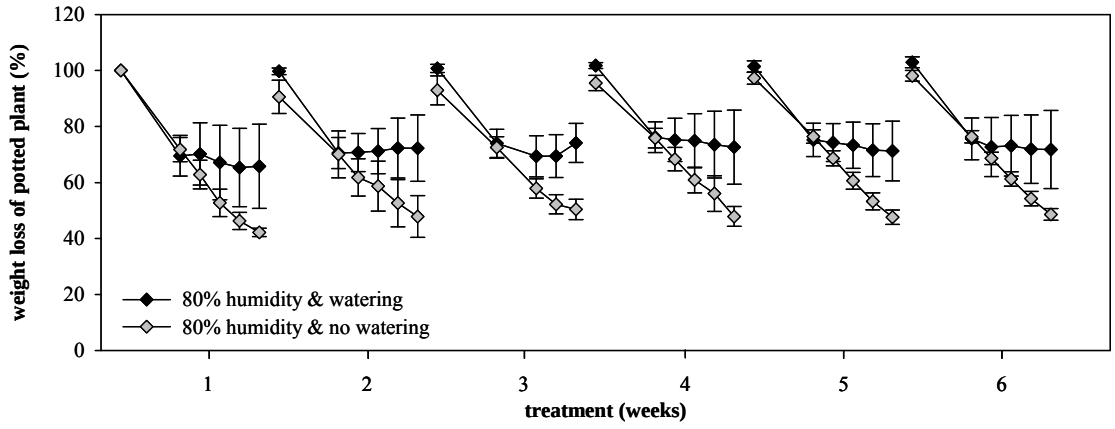

Figure 5. Weight loss of potted MicroTom wild type plants under four different atmospheric humidity and watering conditions. Data are shown as means ± SD (n = 20).

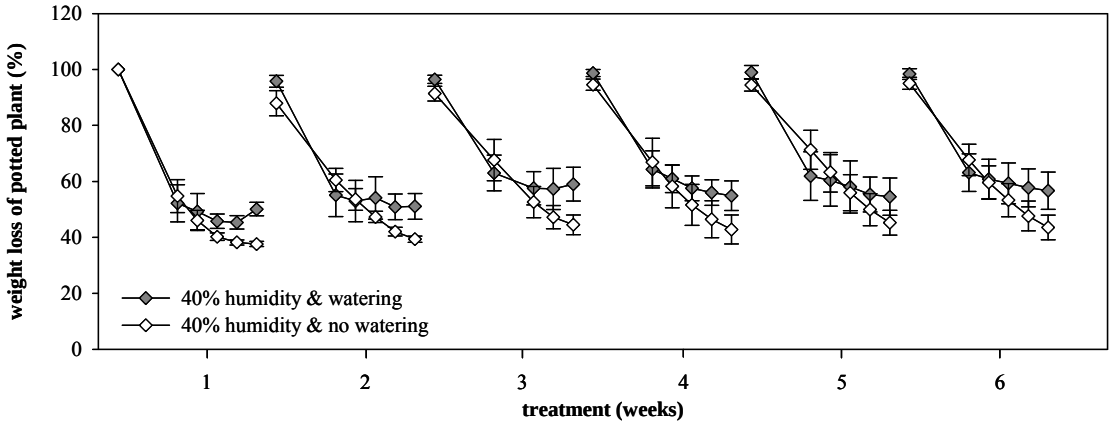

Figure 5. (continued)

Within six weeks the tomato plants flowered and subsequently developed fruits. Mature green fruits of the four different treatments were harvested and investigated with respect to their water content, cuticular wax coverage and transcriptional changes.

At high humidity (80%), mature green fruits exhibited a fruit surface area of 8.23 ± 1.56 cm^2 (optimal watering conditions) and 7.79 ± 1.82 cm^2 (no watering conditions). In contrast, at low humidity (40%) the fruit surface area was significantly decreased to 82% and 70%, respectively, when compared to that fruits of well watered plants grown in high humidity conditions (Figure 6).

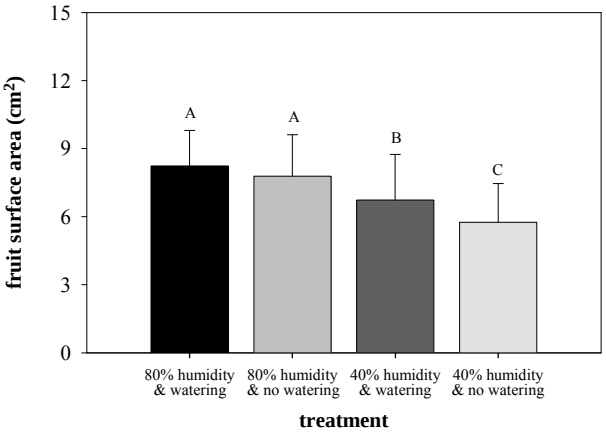

Figure 6. Surface area of mature green MicroTom wild type fruits developed under different atmospheric humidity and watering conditions. Fruit surface area was compared by one-way ANOVA followed by HSD post-hoc test for unequal *n* within each treatment: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SD ($n = 45$ to 48).

Furthermore, the water content of the fruits grown under these four different environmental conditions differed significantly (Figure 7). Mature green fruits grown at 80% humidity and watered daily exhibited the highest water content (90%), whereas the water content of fruits matured at 80% humidity and 'no watering' or at 40% humidity and

'watering' conditions was diminished by 2%. The water content in fruits of 40% humidity and 'no watering' condition was even strongly reduced (by a proportion of 4%).

The permeance for water of these fruits differed also significantly. Fruits cultivated at high humidity (80%) showed the lowest permeance for water of all four treatments (Figure 8). Transpirational rate of fruits, which were not watered on a daily basis, decreased 0.8-fold in comparison to optimally watered fruits. Those fruits matured at low humidity (40%) displayed a similar rate regarding the permeance for water, at a significantly higher level, though. In reference to the highest value at low humidity (40 %) and 'watering' conditions, the permeance for water was diminished by 9% at low humidity (40%) and 'no watering' conditions or by 26% and 42% at high humidity (80%), respectively.

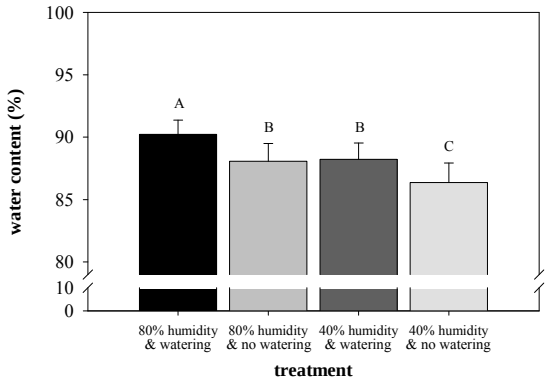

Figure 7. Water content of mature green MicroTom wild type fruits developed under different atmospheric humidity and watering conditions. Water content was compared by one-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SD ($n = 18$ to 20).

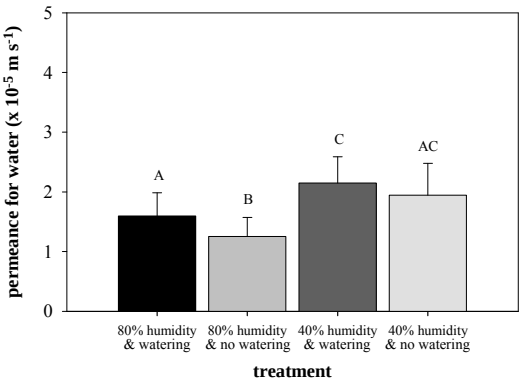

Figure 8. Cuticular permeance for water of mature green MicroTom wild type fruits developed under different humidity and watering conditions. Water permeance was compared by one-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SD ($n = 27$ to 30).

The amount of cuticular wax, which covered these mature green fruits, did not differ significantly between the treatments (Figure 9). However, regardless of the different levels of atmospheric humidity, conditions without daily watering tended to result in an increased wax accumulation on MicroTom wild type fruits.

The mature green MicroTom wild type fruit wax of these four different environmental conditions contained 45% to 51% aliphatic and 42% to 48% aromatic compounds. The aliphatic compounds were represented predominantly by *n*-alkanes, iso- and anteiso-alkanes and primary alkanols. Thereby slight alterations within the relative wax

composition were detected. Fruits harvested at 80% humidity and daily watering exhibited the highest relative amount of primary alkanols in comparison to all others fruits. In contrast to fruits at 40% and the daily watering, they not only revealed the lowest alkanol percentage in the total wax, but also the highest value of iso- and anteiso-alkanes. Fruits grown at high and low humidity, respectively, watered once a week only, varied mainly in the proportion of *n*-alkanes as well as triterpenoids and sterol derivatives. An atmospheric humidity of 80% and 'no watering' resulted in mature green fruits with the highest percentage of *n*-alkanes correspondingly the lowest proportion of triterpenoids and sterol derivatives compared with the other three experimental conditions. In contrast, a reduced humidity of 40% and 'no watering' affected a distinct increase in triterpenoid and sterol derivative accumulation, but a decline of *n*-alkanes and iso- and anteiso-alkanes.

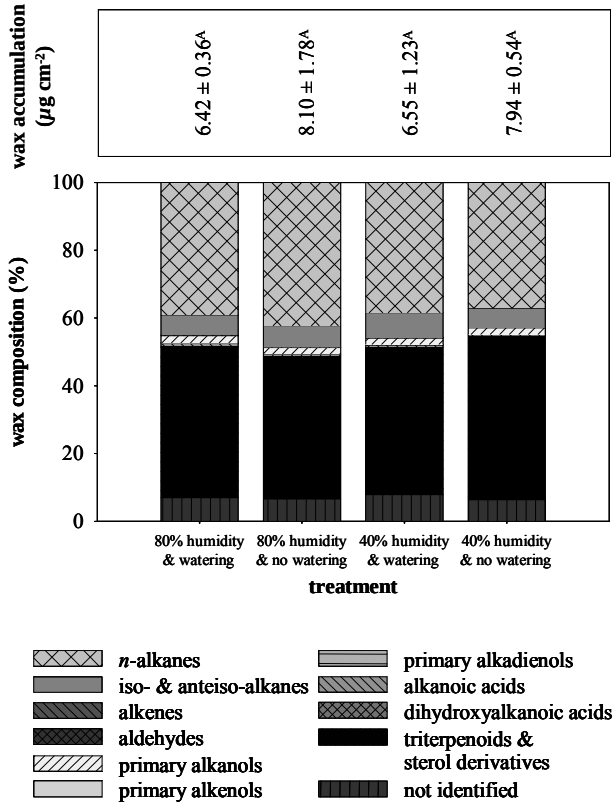

Figure 9. Total cuticular wax quantities and relative wax compositions of mature green MicroTom wild type fruits cultivated under different atmospheric and soil humidity conditions. Wax quantities of fruit grown under different environmental conditions were compared by one-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SD ($n = 4$ to 5).

Considerable changes in the wax composition between fruits grown under different levels of atmospheric humidity and watering regimes were exclusively found for the distinctly lower proportions of alkenes, aldehydes, primary alkenols, primary alkadienols and alkanoic acids (< 1% of total wax).

More detailed studies concerning the wax composition of fruits matured under different atmospheric and soil humidity treatments did not show significant modifications with respect to a distinct increase or reduction of single components or compounds classes (Figure 10). The content of *n*-alkanes within the total wax was increased in fruits of 80% humidity and 'no watering' at 3.35 ± 0.63 μg cm^{-2} in relation to fruits harvested under standard conditions at 80% humidity and 'watering' with an amount of 2.49 ± 0.36 μg cm^{-2} of *n*-alkanes. However, the triterpenoids and sterol derivatives accumulated in the wax coverage of fruits developed at 40% humidity and 'no watering' at 3.81 ± 0.57 μg cm^{-2} compared with 2.86 ± 0.53 μg cm^{-2} of fruits at 80% humidity and 'watering'.

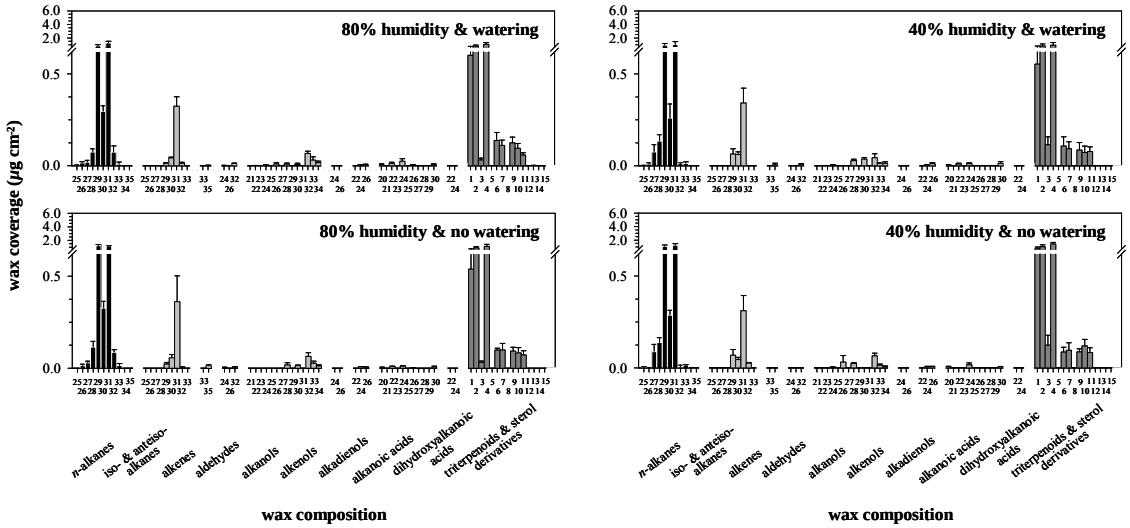

Figure 10. Cuticular wax constituents of MicroTom wild type fruits cultivated under different atmospheric and soil humidity conditions. Triterpenoids and sterol derivatives are numbered as follows: 1 α-amyrin, 2 β-amyrin, 3 β-amyrin derivative, 4 δ-amyrin, 5 cholesterol, 6 lanosterol, 7 multiflorenol, 8 β-sitosterol, 9 stigmasterol, 10 taraxasterol & ψ-taraxasterol, 11 taraxerol, 12 lupeol, 13 lupeol derivative I, 14 lupeol derivative II, 15 unknown triterpenoid. Carbon chain lengths are shown for *n*-alkanes, iso- & anteiso-alkanes, alkenes, aldehydes, primary alkanols, primary alkenols, primary alkadienols, alkanoic acids and dihydroxyalkanoic acids. Data are shown as means ± SD (n = 4 to 5).

Alterations on the transcriptional level in the fruit peel tissue were screened by microarray experiments. Figure 11 shows differences in the total RNA content of the fruits of the four environmental conditions. Fruits of the strongest water limited conditions at 40% atmospheric humidity and 'no watering' had a 2.1-fold higher total RNA level in the fruit peel tissue than in fruits grown under 80% atmospheric humidity and optimal 'watering'. For microarray experiments equal amounts of total RNA were applied.

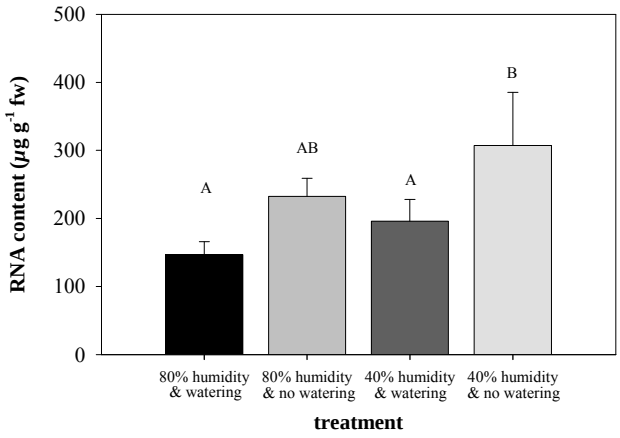

Figure 11. Total RNA content of mature green fruits grown under different humidity and watering conditions. Samples consisted exclusively of the fruit peel tissue. The content of extractable total RNA was compared by one-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SD ($n = 4$ to 6).

Signal intensities of 167 oligomers detected in a microarray analysis using fruits of each treatment are given in Figure 12. Only few transcripts were highly abundant in the peel tissue of mature green fruits cultivated under different experimental conditions: a wax synthase (internal oligomer number 58), a CER2 homologue (75), lipid transfer proteins (86, 91), an 1-aminocyclopropane-1-carboxylate oxidase (111), abscisic stress ripening proteins (134, 135) and a hypothetical protein (lipid transfer protein; 165).

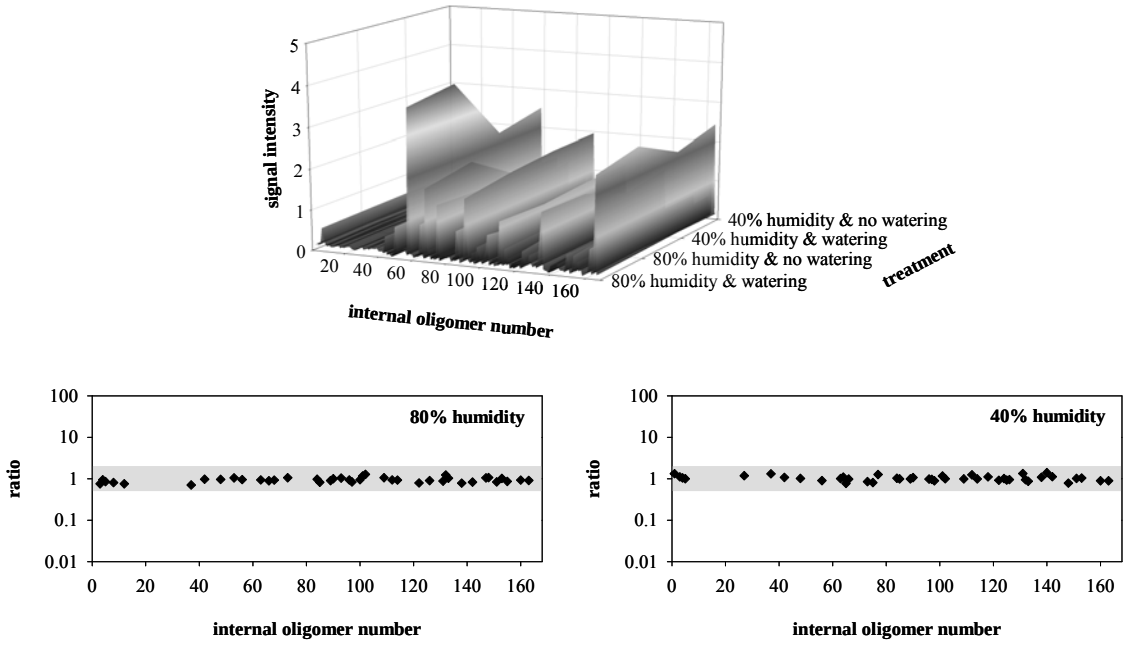

Figure 12. Overview of all microarray expression signals of mature green MicroTom wild type fruits of 167 oligomers. Gray bars illustrate a ratio ≤ 0.5 and ≥ 2.0 calculated for fruits of 'watered' and 'non-watered' plants within the atmospheric humidity of 80% or 40%. The samples consisted exclusively of fruit peel tissue. Data are shown as means ($n = 12$ consisting of 2 microarray experiments with dye switch à 6 spots).

No transcriptional differences were detected between mature green fruits of optimal watered and water stressed plants hold at the same level of atmospheric humidity.

A comparison of both different humidity conditions, regardless whether watered or not, showed down-regulation of abscisic stress ripening protein (DS2 protein, 134) by more than 40% when comparing fruits developed under 80% humidity with fruits of 40% humidity (Table II). In contrast, the expression signal intensities for anionic peroxidase (100) and allene oxide cyclase (116) were more than twofold higher under reduced atmospheric humidity.

Table II. Selection of the strongest microarray signal intensities of mature green MicroTom wild type fruits labeled with internal oligomer number grown under different humidity and watering conditions. The samples consisted exclusively of fruit peel tissue. The ratio was calculated between expression signal intensities of fruits cultivated at 80% humidity versus 40% humidity. Changes of expression signal intensities were emphasized with shadings: no signal (white), weak signal (≥ 0.2; light gray), distinct signal (≥ 0.5; gray) and strong signal (≥ 5.0; dark gray). Remarkable ratios of expression signal intensities are marked in bold. Data are shown as means ± SD (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

oligomer		80% humidity		40% humidity		*ratio*
		watering	no watering	watering	no watering	
58	Wax synthase	3.7 ± 1.0	3.9 ± 1.1	2.3 ± 1.2	2.5 ± 1.4	*0.7*
75	CER2 homologue	1.4 ± 0.8	1.3 ± 0.7	1.0 ± 0.6	1.2 ± 0.9	*0.7*
91	Non-specific lipid transfer protein	1.6 ± 0.5	1.8 ± 0.5	1.9 ± 0.6	2.0 ± 0.5	*1.2*
100	Anionic peroxidase	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	***2.4***
111	1-aminocyclopropane-1-carboxylate oxidase	1.2 ± 0.5	1.1 ± 0.5	1.2 ± 0.5	1.2 ± 0.6	*1.0*
116	Allene oxide cyclase	0.3 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	***2.0***
134	Abscisic stress ripening protein (DS2 protein)	0.9 ± 0.3	0.8 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	***0.5***
135	Abscisic stress ripening protein	1.5 ± 0.9	1.5 ± 0.6	1.0 ± 0.5	1.2 ± 0.7	*0.7*
165	Hypothetical protein (lipid transfer protein)	2.5 ± 1.5	2.7 ± 1.6	2.2 ± 1.2	2.4 ± 1.5	*0.9*

Regeneration of Epicuticular Wax Components on Tomato Fruits after Mechanical Removal

In order to analyze regeneration effects of cuticular waxes different experiments were performed with MicroTom wild type fruits. For total wax extraction the use of different solvents, such as chloroform, negatively affected the epidermal cell layer. Treated fruits colored brownish and wrinkled due to the external disturbance. Based on these experiences, consequently a mild, mechanical removal of cuticular waxes was performed.

During this experiment mature green MicroTom wild type fruits remained on the tomato plant. The fruit surface was treated twice with aqueous gum arabic as a fixative to

remove the epicuticular waxes. Comparing untreated and gum arabic stripped fruits, the mechanical removal of the outermost wax layer resulted in a significant increase in the permeance for water by a factor of 7.0 to 12.45 ± 5.53 x 10^{-5} m s^{-1} (Figure 13). Fifteen days after gum arabic stripping, the water permeance reached the same level again as detected for intact fruits in the beginning of the experiment.

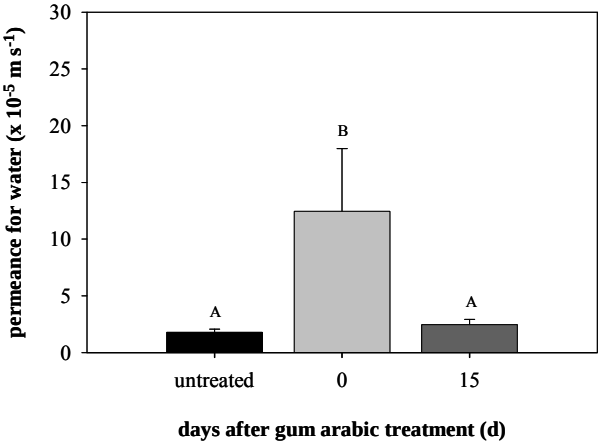

Figure 13. Cuticular permeance for water of mature green MicroTom wild type fruits with or without gum arabic treatment. After gum arabic stripping the fruits stayed on the tomato plant for fifteen days. Water permeance was compared by one-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SD (n = 10 to 15).

Moreover, chemical and gene expression data were documented for this experiment. The wax of untreated, mature green MicroTom wild type fruits contained 62% aliphatic and 32% aromatic compounds (Figure 14). The aliphatic compounds were represented predominantly by *n*-alkanes (48%), iso- and anteiso-alkanes (7%) and primary alkanols (4%). Considerably, lower was the percentage of alkenes, aldehydes, primary alkenols, primary alkadienols and alkanoic acids (<1%).

The removal of epicuticular waxes generated quantitative and qualitative changes in the fruit wax accumulation and wax composition. Gum arabic stripped fruits lost 43% of their total wax coverage compared to the initial value of untreated fruits. In respect to the wax composition mainly the portion of the *n*-alkanes and the iso- and anteiso-alkanes was reduced by more than a third after removal of the epicuticular waxes. In contrast to the triterpenoids and sterol derivatives, their relative proportion of the residual wax increased by a factor of 1.5 caused by heterogeneously layering within epi- and intracuticular fruit wax. The remaining intracuticular wax consisted of 44% aliphatic compounds and 50% triterpenoids and sterol derivatives as aromatic compounds, respectively.

During fifteen days of fruit regeneration and development the wax accumulation significantly heightened to a 4.0-fold of the freshly gum arabic treated fruits and even to a

1.7-fold of the untreated fruits. The distribution of aliphatic compounds, mainly *n*-alkanes, altered distinctly to an account for 69% and of triterpenoids and sterol derivatives to 25% within the wax in the course of fifteen days after gum arabic stripping.

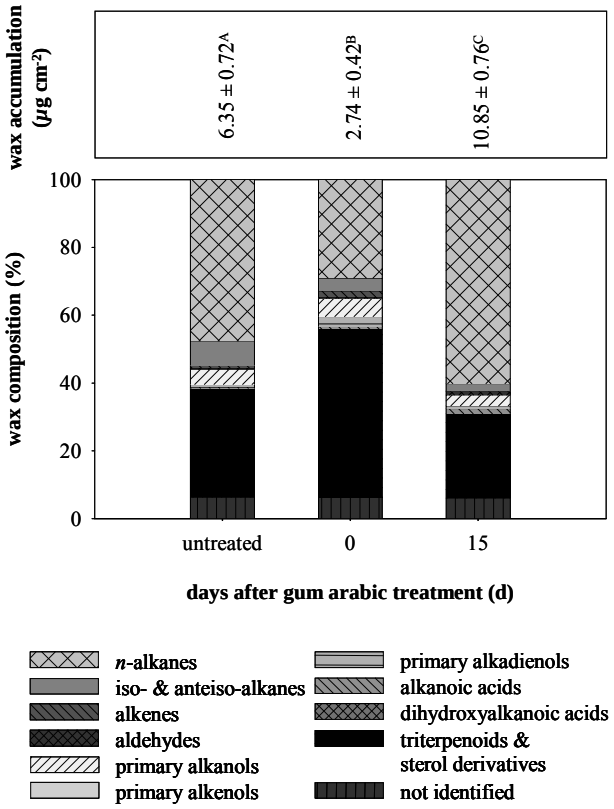

Figure 14. Total cuticular wax quantities and relative wax compositions of mature green MicroTom wild type fruits with or without gum arabic treatment. Fruits were immediately or fifteen days after gum arabic stripping harvested. Wax quantities of different treatments were compared by one-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SD ($n = 4$ to 5).

A detailed analysis of the epicuticular wax removal showed a reduced content of *n*-alkanes with chain length of C_{27} to C_{33} and iso- and anteiso-alkanes with chain lengths of C_{31} to C_{32} in the remaining intracuticular wax coverage of freshly stripped fruits in comparison to intact fruits (Figure 15). The content of triterpenoids and sterol derivatives ranged at the same level.

Fifteen days of regeneration led to a significant increase of most *n*-alkanes with chain lengths of C_{29}, C_{31} and C_{33} and, in addition, the content of aldehydes, primary alkanols and alkanoic acids slightly enhanced. Nevertheless, 3.50 ± 0.44 μg cm^{-2} triterpenoids and sterol derivatives strongly affect the wax composition. In contrast to the regenerated fruits, intact fruits and gum arabic treated fruits without regeneration exhibited 1.80 ± 0.25 μg cm^{-2} and 1.51 ± 0.20 μg cm^{-2} triterpenoids and sterol derivatives. Most notably, the value of α-amyrin, β-amyrin, lanosterol, multiflorenol, taraxasterol and ψ-taraxasterol as well as lupeol derivative I increased distinctly.

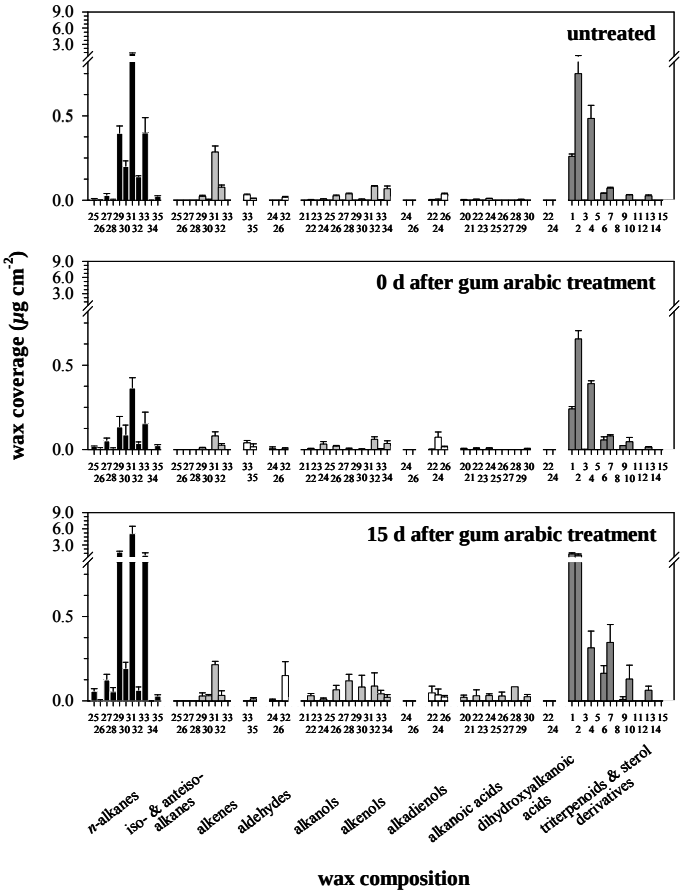

Figure 15. Cuticular wax constituents of intact mature green MicroTom wild type fruits and stripped by a gum arabic treatment. Fruits were immediately or fifteen days after gum arabic stripping harvested. Triterpenoids and sterol derivatives are numbered as follows: 1 ά-amyrin, 2 β-amyrin, 3 β-amyrin derivative, 4 δ-amyrin, 5 cholesterol, 6 lanosterol, 7 multiflorenol, 8 β-sitosterol, 9 stigmasterol, 10 taraxasterol & ψ-taraxasterol, 11 taraxerol, 12 lupeol, 13 lupeol derivative I, 14 lupeol derivative II, 15 unknown triterpenoid. Carbon chain lengths are shown for *n*-alkanes, iso- & anteiso-alkanes, alkenes, aldehydes, primary alkanols, primary alkenols, primary alkadienols, alkanoic acids and dihydroxyalkanoic acids. Data are shown as means ± SD (n = 4 to 5).

The transcriptional examination of these regeneration effects was performed with mature green fruits in a microarray analysis. Gene expression signal intensities of untreated fruits and fruits harvested two days after gum arabic treatment were screened in the fruit peel tissue (Figure 16). Tissue from regenerated fruits two days after gum arabic treatment showed a 2.0-fold increase in total RNA content compared to the untreated fruit sample (Student's *t*-test $P = 0.01$, $t = 3.8$; data not shown). However, for implementation of the microarray experiments equal amounts of total RNA were applied.

A closer examination revealed the highest gene expression for a wax synthase (internal oligomer number 58), *cer* homologues (65, 68, 75), lipid transfer proteins (87, 91, 92), an 1-aminocyclopropane-1-carboxylate oxidase (111), abscisic stress ripening proteins (134, 135) and a hypothetical protein (lipid transfer protein; 165; Table III). The wax synthase (58) and the non-specific lipid transfer protein (92) transcripts showed the strongest accumulation within both samples.

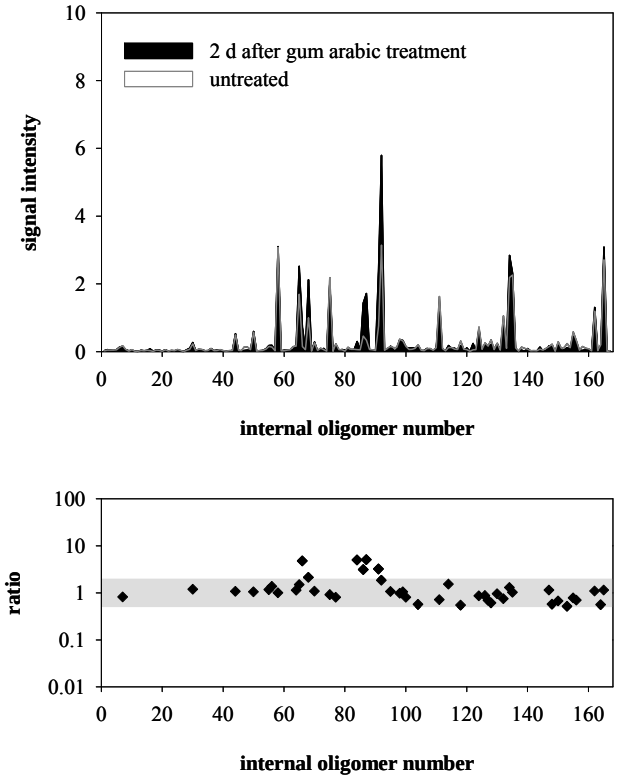

Figure 16. Overview of all microarray expression signals of 167 oligomers related to both, intact fruits and fruits, harvested two days after gum arabic stripping. Gray bars illustrate a ratio ≤ 0.5 and ≥ 2.0 calculated for gene expression signals of intact and treated fruits. The samples consisted exclusively of the fruit peel tissue of mature green MicroTom fruits. Data are shown as means (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

Distinctly differentiated expressed genes for both samples were observed for three lipid transfer proteins (84, 86, 87), two non-specific lipid transfer proteins (91, 92) and, in addition, for a CER1 homologue (68). Thereby, a comparison of these transcripts in both samples revealed exclusively an up-regulation in gene expression during the fruit regeneration process.

Table III. Selection of the strongest microarray signal intensities of mature green MicroTom wild type fruits during the regeneration experiment labeled with internal oligomer number. The samples consisted exclusively of fruit peel tissue. Changes of expression signal intensities were emphasized with shadings: no signal (white), weak signal (≥ 0.2; light gray), distinct signal (≥ 0.5; gray) and strong signal (≥ 5.0; dark gray). Remarkable ratios between expression signal intensities of fruits harvested two days after gum arabic treatment versus fruits without gum arabic treatment are marked in bold. Data are shown as means ± SD (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

oligomer		untreated	2 d after gum arabic treatment	*ratio*
58	Wax synthase	3.1 ± 0.6	3.1 ± 0.5	*1.0*
65	CER1 homologue	1.7 ± 1.1	2.5 ± 1.7	*1.5*
68	CER1 homologue	1.0 ± 0.6	2.1 ± 1.2	***2.1***
75	CER2 homologue	2.2 ± 0.8	2.0 ± 1.0	*0.9*
84	Lipid transfer protein	0.1 ± 0.0	0.3 ± 0.1	***5.0***
86	Lipid transfer protein	0.5 ± 0.2	1.4 ± 0.4	***3.1***
87	Lipid transfer protein	0.3 ± 0.2	1.7 ± 0.5	***5.1***

Table III. (continued)

	oligomer	untreated	2 d after gum arabic treatment	*ratio*
91	Non-specific lipid transfer protein	0.8 ± 0.2	2.4 ± 0.6	***3.2***
92	Non-specific lipid transfer protein	3.1 ± 1.1	5.8 ± 1.7	***1.8***
134	Abscisic stress ripening protein (DS2 protein)	2.2 ± 0.8	2.8 ± 1.0	*1.3*
135	Abscisic stress ripening protein	2.3 ± 0.9	2.3 ± 1.2	*1.0*
165	Hypothetical protein (lipid transfer protein)	2.7 ± 1.5	3.1 ± 1.9	*1.1*

DISCUSSION

The cuticular wax layer is formed of species-, organ- and tissue-specific homologous series of very-long-chain aliphatic compounds with characteristic chain length distribution and varying proportions of cyclic compounds. Qualitative and/or quantitative variations in the composition of cuticular waxes are determined by developmental (documented in CHAPTER I and CHAPTER II) and environmental parameters. Since cuticular waxes play an essential role for the limitation of uncontrolled water loss, changes in the cuticular wax load strongly affect water balance of a plant (Jefferson, 1994; Riederer and Schreiber, 1995; Jenks and Ashworth, 1999; Wang *et al.*, 2001; Goodwin and Jenks, 2005). The absence of cuticular waxes, as an effective water loss barrier, and, furthermore, deficiency of water may cause severe stress to plants, which require various biochemical and physiological mechanisms to avoid or tolerate these stress conditions (Riederer and Schreiber, 2001; Shepherd and Griffith, 2006).

The present study investigated different aspects on wax biosynthesis: the organ-specific wax accumulation in respect to the LeCER6 deficiency in MicroTom leaves and the relationship between cuticular wax load and environmental stress, namely limitation of water as a crucial precondition for plant activity, in MicroTom fruits. In addition, MicroTom fruits were mechanically manipulated in order to clarify the water loss barrier property of their cuticular waxes.

Organ-Specific Character of LeCER6 Deficiency on Cuticular Wax Composition of Tomato

The most appropriate way for studies on the water loss barrier properties of cuticular waxes is to use wax mutants that are characterized by a reduced wax accumulation and/or altered wax composition. In *Arabidopsis*, this approach has enormously contributed to the

identification of components and genes involved in the cuticular water barrier property (Aarts *et al.*, 1995; Millar *et al.*, 1999; Chen *et al.*, 2003). However, mutations in genes related to wax biosynthesis are often responsible for multiple phenotypic variations, which display a certain similarity with some dehydration stress induced modifications in plants such as reduction in organ size, increase in cuticle thickness and untimely wilting (von Wettstein-Knowles, 1995; Post-Beittenmiller, 1996; Kunst and Samuels, 2003; Shepherd and Griffith, 2006).

The β-ketoacyl-CoA synthase, designated LeCER6, is involved in the fatty elongation process for the production of aliphatic wax components and, consequently, confers resistance to cuticular water loss (Hooker *et al.*, 2002). To elucidate the functional impact of LeCER6 on the organ-specific wax biosynthesis and its role for cuticular barrier property in tomato plants, the quantity and quality of the wax layer of leaves of MicroTom wild type and MicroTom *lecer6*, respectively, were studied, and compared to those of MicroTom fruits.

The cuticular leaf wax of MicroTom wild type was dominated by *n*-alkanes with chain lengths from C_{27} to C_{35}. Iso- and anteiso-alkanes, alkanols as well as triterpenoids and sterol derivatives, such as α-amyrin, β-amyrin, δ-amyrin and lanosterol, occurred only in minor amounts. Similar leaf wax compositions were found for other solanaceous species, for example tobacco (*Nicotiana tabacum* L.; Cameron *et al.*, 2006) and potato (*Solanum tuberosum* L.; Szafranek and Synak, 2006). Moreover, the total wax load of tomato leaves (4.24 ± 0.75 μg cm^{-2}) was in a similar range as those for potato leaves: 4.4 μg cm^{-2} to 6.4 μg cm^{-2} (Szafranek and Synak, 2006). According to these results, the surface wax of tomato leaves consists mainly of aliphatic compounds, but exhibits only a small wax layer for limiting water loss in comparison to tomato fruits. However, on the basis of these qualitative and quantitative data, a less importance could not yet attribute to the leaf wax coverage as transpirational and structural barrier with regard to the tomato fruit.

Significantly lower wax quantities were observed in leaves of MicroTom *lecer6* when compared to MicroTom wild type. A comparison of both MicroTom genotypes revealed a reduction of the total amounts of most *n*-alkanes > C_{30} in the LeCER6-deficient mutant. No clear differences between leaf waxes of MicroTom wild type and its *lecer6* mutant were found for iso- and anteiso-alkanes and alkanols, triterpenoids and sterol derivatives. However, the amount of alkanoic acids was slightly increased in the wax composition of *lecer6* mutant leaves. According to these findings, the increase in alkanoic acids should be regarded as result of LeCER6 deficiency, since similar patterns were found for *lecer6*

mutant fruits (CHAPTER I). The threefold decrease in cuticular leaf wax production, mainly based on the reduction of *n*-alkanes, presents a consequence of LeCER6 deficiency. However, alterations in the wax coverage demonstrated in the *lecer6* mutant might result in an increased cuticular water loss in comparison to the wild type leaves.

Apart from a reduction of *n*-alkanes, LeCER6 deficiency in MicroTom fruits resulted in an increase of triterpenoids and sterol derivatives. The latter was not observed in LeCER6-deficient leaves. The absence of very-long-chain *n*-alkanes did not evoke an accumulation of cyclic wax compounds in *lecer6* mutant leaves, which may indicate the existence of different regulation in metabolic pathways in MicroTom leaves and fruits.

However, on an organ-specific level in MicroTom wild type leaf wax *n*-alkanes were the major wax compounds, while *n*-alkanes plus triterpenoids and sterol derivatives were predominant in wild type fruits. MicroTom wild type showed a similar chain length distribution of aliphatic components in leaves and fruits, but with less pronounced maxima in MicroTom leaves. LeCER6 deficiency in tomato plants led to a decreased level of *n*-alkanes with chain lengths > C_{30} in cuticular waxes in leaves, while *n*-alkanes > C_{28} were reduced in fruits. Hence, deduced from the compositional level, the *LeCer6* gene product exhibited a comparable substrate specificity of very-long-chain fatty acids by comparing both MicroTom organs. This unique substrate preference of elongation reactions is attributed to be responsible for the selectivity of each fatty acid elongase system (Millar and Kunst, 1997; Kunst and Samuels, 2003).

A detailed gene expression analysis of MicroTom wild type and MicroTom *lecer6* leaves was performed *via* microarray experiments on the transcriptional level. The revealed gene expression signals were in a comparable range with those detected in MicroTom fruits. In MicroTom fruits and leaves, highest signal intensities were obtained for a wax synthase (58), CER1 homologues (65, 68), an abscisic stress ripening protein (DS2 protein; 134), a glyceraldehydes-3-phosphate dehydrogenase (162) and a hypothetical protein (lipid transfer protein; 165).

Surprisingly, the analysis of expressed genes in fully developed leaves of MicroTom *lecer6* versus wild type showed a small number of genes only, which were differentially induced. The deficiency in the β-ketoacyl-CoA synthase LeCER6 in MicroTom leaves affected an amplified gene expression of lipid transfer proteins (86, 87, 91). The expression of these genes is potentially linked with the transport of wax or cutin components across the epidermal cell wall to the cuticle surface (Yamada, 1992; Post-Beittenmiller, 1996; Bourgis and Kader, 1997; Kunst and Samuels, 2003). Previously, Treviño and O´Connell

(1998) reported that lipid transfer proteins are inducible by ABA and different osmotic stresses. These results reflect gene expression profiles obtained with fruits of MicroTom wild type and MicroTom *lecer6*. According to these findings, increased gene expression signals of lipid transfer proteins may affect changes in the cuticle thickness and/or structure by modified transport process activity or showed response to the altered permeance for water, respectively (CHAPTER II). However, if amplified transcripts of lipid transfer proteins indicate alterations in the cutin matrix of LeCER6-deficient leaves remains speculative.

An assumed water loss stress of *lecer6* mutant was visually detectable only in *lecer6* fruits, which were wrinkled and smaller than fruits of MicroTom wild type plants. Leaves of MicroTom wild type and *lecer6* mutant were visually not distinguishable by their surface structure. No significantly phenotypic symptoms were observed in MicroTom *lecer6*, although alterations of wax accumulation in photosynthetically active leaf tissues may strongly affect the plants' physiology (Jenks *et al.*, 2002). Leaves of MicroTom *lecer6*, which looked only slightly frizzlier than wild type leaves, might also suggest alterations in the biomechanical stability of the whole organ evoked by a reduced wax load. Stress avoidance and tolerance strategies may provide effective regulation mechanisms in balancing water loss on the leaf level. However, apart from a waxy surface, a variety of different parameters might have impact in controlling the water status of tomato leaves.

One should consider that, unlike tomato fruits, the transpiration of tomato leaves is determined by a cuticular plus stomatal water permeability. The interplay between both should be taken into account for estimating the functional impact of the cuticular wax load regarding the water balance of the tomato leaf (Raven, 1977). Cuticular water permeability is affected by physico-chemical attributes of the wax composition, whereas the stomatal transpiration is regulated by stomatal conductance. The latter is largely determined by the water status of the plant tissue (Riederer and Schreiber, 1995; Schreiber and Riederer, 1996; Shepherd and Griffith, 2006). However, the little impact of the cuticular permeance for water in comparison to the stomatal water permeability indicates that the water transfer across the leaf cuticle has only a minor importance for the overall water loss (Burghardt and Riederer, 2003). Thus, the cuticle plays an essential protective role under conditions when stomata are closed, for example under dehydration stress that evokes a drop of the stomatal conductance (Jenks and Ashworth, 1999).

Exclusively in conditions of maximal stomatal closure representing 'minimum conductance', cuticular water permeability is technical measurable, actually. These data correspond to results of experiments with stomata-free systems such as tomato fruits (Kerstiens, 1996). In the present study, the permeance for water of intact, red ripe fruits of MicroTom wild type was determined at 0.9 x 10^{-5} m s^{-1} (category VI; CHAPTER I). Bakker (1991) mentioned the nocturnal permeance for water of adaxial, stomatous leaf surfaces of tomato at 55 x 10^{-5} m s^{-1}, which was in a comparable range of other solanaceous species exhibiting cuticular permeances between 49 x 10^{-5} m s^{-1} and 69 x 10^{-5} m s^{-1} for sweet pepper (*Capsicum annuum* L.), thorn apple (*Datura stramonium* L.), tobacco (*Nicotiana tabacum* L.) and eggplant (*Solanum melongena* L.; Bakker, 1991; Šantrůček, 1991). Thus, in terms of an efficient water loss barrier, the capacity of the tomato leaf in comparison to the fruit appears to be in general distinctly degraded. In wild type plants, the cuticular wax coverage of the leaf was threefold reduced compared to the fruit, whereas the transpirational water loss barrier property was apparently degraded by one order of magnitude. A similarly divergence may be expected for the *lecer6* mutant plant.

The previously described increased cuticular wax load by AtCER6 overexpression in transgenic tomato plants did not correlate with a modified transpirational rate, but might refer to a possible increase in stomatal conductance, which compensates the altered cuticular permeability for water (Karaba, 2007). Consequently, despite lower wax quantities and probable higher cuticular water loss, LeCER6 deficiency in MicroTom leaves may not necessarily provoke dehydration stress. Moreover, the morphology and density of leaf stomata respond to variable stress conditions such as dehydration (Ramos *et al.*, 1992; Schönherr and Baur, 1996; Zinsou *et al.*, 2006).

In addition to leaf stomata, differences in the variety and density of non-glandular and glandular trichomes between leaves of both MicroTom genotypes may influence the transpirational water loss (Luckwill, 1943; Schönherr and Baur, 1996; Roy *et al.*, 1999; Holroyd *et al.*, 2002; Aharoni *et al.*, 2004).

In comparison to fruits, MicroTom leaves do not provide an effective model system for analyses on cuticular wax biosynthesis and its function as water loss barrier. In the present study on MicroTom leaves, the LeCER6 function in VLCFA biosynthesis and organ-specific differences in cuticular wax pattern between tomato fruits and leaves were biochemical characterized. However, investigations on cuticular leaf waxes for its functional role as a protective layer against water loss may be affected by stomata,

trichomes and the character of the leaf cutin matrix, respectively. In contrast to the fruit, the high variability of parameter having an effect on the water loss barrier properties of tomato leaves might be critical to verify.

Dehydration Stress Related Modifications in Wax Component Accumulation of Tomato Fruits

The observation of high variability in plant reaction to different environmental conditions was reported from several studies with particular attention to comparisons of plant performance under different osmotic stresses (Monforte *et al.*, 1997; Foolad *et al.*, 2002; Frankel *et al.*, 2003; Zinsou *et al.*, 2005). Previous investigations documented that the response to water deficit was associated with increased deposition of cuticular waxes and/or reduced transpiration rate of many plant species including wheat (*Triticum aestivum* L., *Triticum turgidum* L.; Uddin and Marshall, 1988), corn (*Zea mays* L.; Premachandra *et al.*, 1991), pea (*Pisum sativum* L.; Sánchez *et al.*, 2001), peanut (*Arachis hypogaea* L.; Samdur *et al.*, 2003), alfalfa (*Medicago truncatula* Gaertn.; Zhang *et al.*, 2005) and sesame (*Sesamum indicum* L.; Kim *et al.*, 2007). Therefore, signals of water deficiency might induce the enhanced wax production by up-regulating the expression of genes related to wax biosynthesis or transport processes, which benefit the water barrier efficiency of cuticular wax load. Based on these observations, MicroTom wild type plants were cultivated under four different watering regimes: high and low atmospheric humidity as well as high and low soil moisture. Limitations in water supply were determined to different degrees in mature green fruits of MicroTom wild type. Obviously, stress conditions evoked considerable selection pressure in mature green fruits because seeds were not matured at this early fruit developmental stage.

The water balance of MicroTom fruits under water limited conditions varied significantly in comparison to fruits of well-watered plants. Water limiting conditions at 40% humidity in combination with 'no watering' conditions showed the highest impairment of fruit water content, whereas exclusively low soil moisture ('no watering') or low atmospheric humidity (40%), respectively, evoked only moderate loss of water in tomato fruits. Thus, the dimension of water stringency clearly revealed the accumulation of water in mature green MicroTom fruits. Moreover, a decrease in fruit size at low atmospheric humidity (40%) may be based on alterations in cell division and/or cell expansion. This phenotypic change lowered the contact of the fruit surface with the dry atmosphere and,

particularly, led to a reduction of the transpirational surface area resulting in a limited dehydration.

In order to determine the variation of the cuticular wax coverage as consequence of dehydration stress, the wax quantity and wax composition were analyzed. As documented earlier (CHAPTER I), under well-watered conditions, at 80% atmospheric humidity and daily watering, cuticular waxes of mature green MicroTom fruits (category II) were dominated by triterpenoids and sterol derivatives (45% of total wax), with *n*-alkanes being the next-most abundant compound class (39% of total wax). In comparison, 'no watering' conditions caused only a weak increase in total wax amount on fruits. Hence, in contrast to the findings of Jaglo-Ottosen *et al.* (1998) and Kasuga *et al*. (1999) for different osmotic stresses such as drought, salt and freezing in *Arabidopsis*, the cuticular wax quantity of MicroTom fruits does not significantly increase due to dehydration stress.

However, Kim *et al.* (2007) demonstrated the functional impact of changes in wax quality. They observed variations in the barrier property of cuticular waxes mainly due to alterations of *n*-alkanes. Previously, Goodwin and Jenks (2005) suggested that an increase in polar components embedded in the cuticle and a shift in the chain length distribution to longer constituents could change the cuticles' permeability to water. Indeed, similar results were found for grape (*Vitis vinifera* L.; Radler; 1965), cotton (*Gossypium hirsutum* L.; Bondada *et al*. (1996), juniper (*Juniperus communis* L.; Dodd and Poveda, 2003), rose (*Rosa* x hybrida L.; Jenks *et al.* (2001) and tobacco (*Nicotiana glauca* L. Graham; Cameron *et al.* (2006). Nonetheless, in tomato plants the dehydration stress did not lead to alterations in the cuticular wax composition with particular regard to higher proportion of very-long-chain *n*-alkanes of mature green fruits. These results show clearly that water limitation in tomato plants provokes only minor variations in cuticular wax formation of mature green fruits with respect to the wax amount, but does not affect the wax composition.

Furthermore, it was shown that only one of the four experimental conditions significantly reduced the permeance for water of MicroTom fruits. High atmospheric humidity (80%) and 'no watering' conditions led to an improved water loss barrier property. Alterations in the wax biosynthesis leading to an increased stress resistance may explain these observations. Similarly, Schönherr (1976) mentioned that a deficit of water may cause shrinkage of the cuticle membrane and hence an increased resistance to the water loss through the cuticle resulting in a reduced water permeance. These observations possibly support the tendencies of an enhanced fruit wax accumulation under conditions of

80% atmospheric humidity and 'no watering', which might be a result of alterations in the wax production and consequential an increased stress resistance. In contrast, the permeance for water found in MicroTom fruits grown under low atmospheric humidity (40%) was even elevated in comparison to fruits under high atmospheric humidity (80%). A significant correlation between the total wax amount or the presence of single wax compounds and the permeance for water could not be shown. Likely reasons include an increase in water permeability evoked by an impaired structural integrity of the proper water barrier as a consequence of the severe stress. However, mature green MicroTom fruits cultivated under different level of atmospheric and soil humidity were shown to have contrasting facilities to compensate dehydration stress.

However, it is important to note that variations in wax biosynthesis do not always correlate inversely with transpirational water loss or positively with dehydration stress resistance. Water loss across the plant surface may be more complex than simple diffusion through the cuticle membrane (Oppenheimer, 1960; Lownds *et al.*, 1993; Ristic and Jenks, 2002; Goodwin and Jenks, 2005). The ability to alter cuticular waxes in response to dehydration stress provides only one mechanism of plants to limit transpirational water loss, improve water balance and manage growth in water limiting environments (Sánchez *et al.*, 2001; Goodwin and Jenks, 2005). Indeed, studies reported that variation in cuticular wax quantity alone had only minor or no correlation with transpiration and that other factors likely were more significant in controlling water loss rates, for example in *Sorghum bicolor* Moench. (Jenks *et al.*, 1994) and bahiagrass (*Paspalum notatum* Flugge; Tischler and Burson, 1995). Other factors were found to play a more important role for control of water loss. Plants exposed to dehydration stress respond with characteristic phenotypic features, such as reduced transpiration area, thickened epidermal cell wall and cuticle membrane, decreased cell size and intercellular spaces and adapted solute leakage rates through plasma membranes (Oppenheimer, 1960; Premachandra *et al.*, 1991; Ristic and Jenks, 2002; Moctezuma *et al.*, 2003).

Analyses of the transcriptional level of dehydration stress related fruit development exposed the highest gene expression signals for a wax synthase (58), a CER2 homologue (75), a non-specific lipid transfer protein (91), an 1-aminocyclopropane-1-carboxylate oxidase (111), an abscisic stress ripening protein (135) and a hypothetical protein (lipid transfer protein; 165). These findings are consistent with data on the developmental aspects of the wax biosynthesis (CHAPTER II). However, variation in the gene expression pattern

was generated by different levels of atmospheric humidity but not by different levels of soil moisture.

Despite the fact that dehydration stress avoidance and tolerance as well as wax accumulation are complex regulatory mechanisms that are under control of multiple genes, the microarray experiments allowed the identification of only few genes that are either up- or down-regulated in mature green fruits. Gene expression that indicates dehydration stress effects induced by these different experimental conditions were most likely an anionic peroxidase (100), an allene oxide cyclase (116) and an abscisic stress ripening protein (DS2 protein; 134). So far, the direct physiological impact of these transcripts remains unclear. Alterations in gene expression activity associated with wax biosynthesis were not identified in the fruit peel tissue due to low level signals and/or the slightness of variances between them.

Allene oxide cyclase, abscisic stress ripening protein (DS2 protein) and anionic peroxidase are known to be induced by different stress regimes such as water deficit or oxidative stress (Roberts and Kolattukudy, 1989; Lagrimini *et al*., 1990; Silhavy *et al*., 1995; Yamada *et al*., 2002; Stenzel *et al*., 2003; Dóczi *et al*., 2005). Members of the allene oxide cyclase family (AOC) were found to be involved in the production of 12-oxo-phytodienoic acid (OPDA), a precursor of jasmonic acid (Hause *et al*., 2000; Liechti *et al*., 2002). Therefore, the allene oxide cyclase is suggested to be significantly linked to stress induced signaling of mature green MicroTom fruits. An increase in the activity of the allene oxide cyclase could be a stress related stimulus in the regulatory pathway of jasmonic acid further cascading stress mediated reactions to confine damage *via* dehydration.

Water deficiency is known to harm cell membrane functioning, since altered membrane properties are associated with changes in membrane fluidity and permeability as well as cellular decompartmentation, generating membrane ion leakage and accelerating water loss (Hsiao, 1973; Bray, 1997). Moreover, interaction of membrane components with highly reactive oxidation products also leads to an enhanced oxidative index of membranes. In turn, resulting lipid hydroperoxides, exerting deleterious effects on membranes and proteins, can be metabolized into less damaging, physiologically active products possibly mediated by peroxidase activity (Holmberg and Bülow, 1998; Torres-Schumann *et al.*, 1992). Anionic peroxidases in plants are responsible for the removal of hydroperoxide and the oxidation of toxic reductants for example in response to environmental stresses (Gilad *et al.*, 1997; Çakir *et al.*, 2003; Carrari *et al.*, 2004). Thus, the observed amplification of

anionic peroxidase transcripts and loss of epidermal integrity in response to water deficit corroborates the hypothesis of an essential role in resistance against dehydration stress of mature green MicroTom fruits.

The drought stress protein (DS2), a member of the abscisic stress ripening protein family (ASR), is supposed to play a role in osmotic protection (Iusem *et al.*, 1993; Picton *et al.*, 1993; Rossi and Iusem, 1994; Amitai-Zeigerson *et al*., 1994, 1995; Gilad *et al.*, 1997; Faurobert *et al*., 2007). The gene expression intensity of allene oxide cyclase and anionic peroxidase was increased upon exposure the tomato plants to dehydration stress, abundance of the transcript level of drought stress protein (DS2) declined. Since the drought stress protein (DS2) is also associated in fruit ripening, this gene expression pattern might specify retardation of the fruit ripening process in response to dehydration. Previously reported by Greenway and Munns (1980), Boyer (1982) and Bohnert *et al*. (1995), water deficiency limits majorly the productivity of plants.

Thus, in MicroTom fruits several adaptations to environmental conditions were identified by transcriptional control of stress related activation and repression of gene expression: changes in hormone homeostasis, detoxification of reactive oxygen species and influences on the developmental control (Cushman and Bohnert, 2000; Xiong *et al.*, 2002; Zhu, 2002).

This study on mature green MicroTom wild type fruits displayed the complexity of responses to certain dehydration treatments and enhanced understanding of mechanisms in stress adaptation. Undoubtedly, MicroTom fruits improved their resistance to water limited condition during fruit development. Thereby, modifications in wax accumulation contributed to a decrease in permeance for water or obviating deleterious stress effects, respectively. One may speculate that a reduction in fruit size and the degree of metabolic variance due to the environmental influences generates stress resistance in MicroTom fruits. Nevertheless, although cuticular waxes certainly play an important role in plant water loss, it was shown that changes in the amount of cuticular waxes may not exclusively be responsible for limiting transpirational water loss. However, to investigate effective modifications in cuticular wax biosynthesis processes as consequence of water deficiency, the MicroTom fruit turned out to be not the ideally suited model system.

Induction of Wax Component Regeneration by Mechanical Stress of Tomato Fruits

The waxy cuticle cover acts as the first line of maintenance to support the water balance in the plant interior and to defense against external influences and, furthermore, adjustment

of an osmotic potential (Schönherr, 1976; Knoche *et al.*, 2000; Brennan and Weinbaum, 2001; Aharoni *et al.*, 2004). Following a direct impairment of the wax coverage on tomato fruits by mechanical removal leading to an increasing permeance for water, the induction of wax biosynthesis and regenerative wax accumulation is expected since provision of a waterproof protective coat is essential. Especially fruits of early developmental stages may be well adapted to this external stimulus. In mature green fruits (category II), the process of cuticular wax accumulation is not completed (CHAPTER I) and gene expression activity contributed to wax biosynthetic pathways is very high (CHAPTER II). Moreover, a succeeding fruit development is essential until the seeds as a means of reproduction have matured.

Cuticular wax of mature green tomato fruits is characterized by a compositional gradient within the wax load related to the way and the rate by which several wax compounds were synthesized and exuded through the fruit cuticle (von Wettstein-Knowles, 1974). Thus, a heterogeneous arrangement of cuticular waxes is monitored by sequential deposition of different compound classes and chain length distributions generating distinct epi- and intracuticular regions during tomato fruit development. The epicuticular wax layer of mature green MicroTom fruits consisted entirely of very-long-chain fatty acids and aliphatic derivatives in particular *n*-alkanes. In contrast, the intracuticular wax layer was dominated by triterpenoids and sterol derivatives that could not be detected in the epicuticular waxes. Probably, these compounds react with the functional groups of the cutin matrix rendering a complex biopolymer (Kolattukudy, 1984; Khan and Marron, 1988; Riederer and Schönherr, 1988).

A simple method for the selective removal of epicuticular wax without disturbing the fruit tissue provides the use of aqueous gum arabic (Jetter and Schäffer, 2001). Stripping off dried gum arabic from plant surfaces led to a 50% reduction of the total wax load, whereas exclusively the aliphatic wax constituents of the cuticular wax are degraded. The remaining intracuticular waxes comprised 44% aliphatic and 50% cyclic wax compounds. Similar distribution of aliphatic and cyclic compounds in leaf waxes were found for *Arabidopsis* (Jenks *et al*., 1995), laurel cherry (*Prunus laurocerasus* L.; Jetter and Schäffer, 2001) and *Nepenthes alata* Blanco (Riedel *et al*., 2003).

In mature green MicroTom fruits a sevenfold increase in the permeance for water provides a direct consequence of the removal of the cuticular wax coverage. Moreover, a rise in elasticity and susceptibility to fracturing due to the mechanical manipulation of the

cuticle indicate the role of waxes as a support for the tensile strength of the cuticle membrane on MicroTom fruits (Petracek and Bukovac, 1995; Buchholz, 2006).

After triggering regeneration properties by the reduction of the cuticular wax of mature green fruits, the 'original' wax amount and composition was restored within less than fifteen days. In accordance with these data, studies in *Eucalyptus spec.* L'Hérit. (Hallam, 1970; Wirthensohn and Sedgley, 1996; Neinhuis *et al*., 2001), common bean (*Phaseolus vulgaris* L.), field bean (*Vicia faba* L.), pea (*Pisum sativum* L.), rape (*Brassica napus* L.; Percy and Baker, 1987), laurel cherry (*Prunus laurocerasus* L.; Jetter and Schäffer, 2001), caper spurge (*Euphorbia lathyris* L.), common snowdrop (*Galanthus nivalis* L.) and *Ipheion uniflorum* Grah. (Koch *et al*., 2004) documented the reformation of epicuticular wax especially on young leaves. However, these earlier examinations could not clearly show that development-dependent alterations in the cuticular wax formation were not disturbed by the mechanical removal. Moreover, the present study on MicroTom fruits documented the regeneration of epicuticular wax on a fleshy berry fruits, for the first time.

The regenerated MicroTom fruit samples displayed a fourfold increased wax coverage in comparison to dewaxed fruits. The regenerated wax predominantly consists of *n*-alkanes homologous ranging from C_{25} to C_{35} as well as triterpenoids and sterol derivatives. Iso- and anteiso-alkanes, alkenes, aldehydes, primary alkanols, primary alkadienols, alkanoic acids and dihydroxyalkanoic acids were identified in distinctly minor concentrations. These results may reflect coordinated changes in cuticular wax load mainly caused by wax regeneration, but also effects of the fruit ripening process. Obviously, the composition of regenerated cuticular wax corresponds with the general trend for longer aliphatic homologues to accumulate in the later developmental stages (Jenks *et al.*, 1996b). Hence, fruits of MicroTom wild type showed an analogous chain length pattern detected during the transition from the mature green (category II) to the breaker fruit stage (category III) as demonstrated in CHAPTER I. The quantitative and qualitative changes in the wax composition, evoked as effects of restoration of the removed wax layer and developmental regulation of wax deposition, led also to an increase in the cuticular water loss barrier property. Given a fifteen days recovery period before analyzing, the permeance for water in the regenerated fruit samples ranged significantly at the same level as the permeability of the untreated control.

This regeneration event, which decisively influences the cuticular wax deposition and distribution and affects the water permeability, was further characterized by investigations on the biosynthesis activity on the transcriptional level. A multiplicity of gene expression

signals showed similarity to the expression patterns monitored in fruit category II and category III in the course of the fruit development process as described in CHAPTER II. However, water stress associated signals, probably generated by the increased permeance for water, were not detected in a comparison of the gum arabic treated sample and the untreated control. Nevertheless, an amplified biosynthesis activity promoted by the mechanical wax manipulation was revealed by transcriptional modifications in the signal intensities for a CER1 homologue (68) and several lipid transfer proteins (84, 86, 87, 91, 92).

The aldehyde decarbonylase CER1 is described to catalyze the conversion of long-chain aldehydes to alkanes, an important biosynthetic step for aliphatic wax compounds (Hannoufa *et al*., 1993; Lemieux *et al*., 1994; Jenks *et al*., 1995). In addition, lipid transfer proteins as well as non-specific lipid transfer proteins are putatively involved in the transport process of wax and cutin components across cell membranes and through the cuticle (Post-Beittenmiller, 1996). However, these results may display crucial aspects of cuticular wax accumulation providing targets for the mechanistic deposition of the aliphatic wax domain.

Apart from the chemical distinction between epi- and intracuticular wax constituents, a regenerative capacity in cuticular wax biosynthesis of MicroTom fruits was shown as response to mechanical stress in the early developmental stage of mature green fruits. This regeneration event was quantitatively and qualitatively analyzed in a functional context and correlated with data at the molecular level. The mature green tomato fruit compensated the mechanical impairment of the water loss barrier in rapid succession and blended in the 'normal' course of fruit development. Ultimately, MicroTom wild type fruits exposed a regular accumulation of waxes at the fruit surface, whereas stress indications were not detectable.

Aspects of Cuticular Wax Accumulation as Transpirational Water Loss Barrier of Tomato

Our understanding of the relationship between wax composition, function and the basic mechanisms of the biosynthesis inclines, though many aspects still need further elucidation. In order to analyze how genetic variance or diverse environmental conditions elicit an adaptive response on the level of cuticular wax formation, several major issues were examined in tomato and represented in this study.

A comprehensive analysis was aimed to differences in cuticular wax amount and chemical composition between MicroTom fruits and leaves and, particularly, to the effect of LeCER6 deficiency on wax deposition. Both genetic features organ specificity and *lecer6* mutation, respectively, generated tremendous variations in the wax composition and thereof deduced effects of the cuticle architecture and integrity. Based on different phenotypic stress symptoms in MicroTom leaves and MicroTom fruits, a disparate system of organs and physiological mechanisms became obvious. Nevertheless, the fundamental role that LeCER6 plays in VLCFA biosynthesis related to the cuticular wax production of tomato was underlined.

Tomato fruits were investigated in order to succeed in the identification of genes potentially involved modifications in wax adaptation as stress resistance. Strong evidence was found that the stress response in tomato fruits, namely the establishment of internal conditions that prevent dehydration, showed strong variations to different water limited conditions. Water deficiency stress was characterized as an important stress that triggers many changes in the tomato gene expression with strong impact on physiology and metabolism. Thus, a variety of water stress related modifications affected the whole organ. The cuticular wax load was only identified to regulate minor variations in the water loss barrier property of mature green MicroTom fruits exposed to water stress.

The experimental analysis of an environmental stress specific to the cuticular wax load of MicroTom fruits was designed by a mechanical removal of the epicuticular wax layer. The represented results permitted an insight in the wax biosynthesis associated with chemical wax composition and their impact on the cuticular water permeability. Hence, the induction of cuticular wax accumulation is suggested to have a substantial function in plant resistance to avoid and/or tolerate water stress under the inescapable constraint of a continuous water loss to the atmosphere.

CHAPTER IV

LeCer6 Gene: Characterization of a Tomato β-Ketoacyl-CoA Synthase Belonging to the Very-Long-Chain Condensing Enzymes from the Molecular Level to the Phenotype

RESULTS

LeCer6 encodes a β-ketoacyl-CoA synthase, which contributes to the production of very-long-chain condensing enzymes in the wax biosynthetic pathway of tomato plants. Its genotypic characterization was realized on the molecular level, including the verification of structural and regulatory elements of the nucleotide and the deduced amino acid sequence (i). A 'reverse genetic' approach of a *lecer6* mutant revealed phenotypic changes that correspond to a block of enzymatic functions involved in elongation of very-long-chain fatty acids. Pleiotropic effects caused by LeCER6 deficiency were consistent with this functional significance of LeCER6 (ii). Additionally, several aspects secondarily associated with LeCER6 mutation were documented in the model system tomato fruit in the context of fruit development (iii).

Isolation and Characterization of the *LeCer6* Nucleotide Sequence

The nucleotide sequence of the β-ketoacyl-CoA synthase gene *LeCer6* was partially known from the tentative consensus sequence TC172551 of the DFCI tomato gene database. Using a genome walk approach the genomic sequence was expanded in 5' orientation to a total sequence of 4068 bp completing the *LeCer6* gene. The aligned sequence of the transcriptional regulatory region resulted from genome walk PCR fragments screened with a *Stu*I DNA library (PCR product A; Figure 1) and a *Pvu*II DNA library (PCR product B) as templates. For the PCR fragment A the genome walk PCR was performed with gene specific primer prom1 and the nested walk PCR with gene specific primer prom2. In the same way the PCR fragment B was generated with the gene specific primer prom2 in the genome walk PCR and with gene specific primer prom3 in the nested walk PCR. A control PCR was carried out with gene specific primer prom4 and primer prom5, respectively.

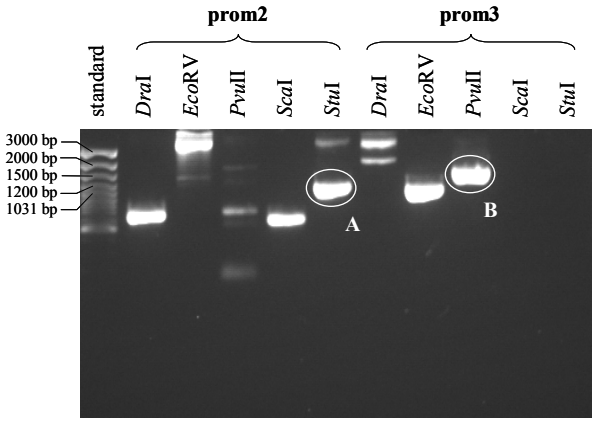

Figure 1. Photograph of nested walk PCR products of five different genomic DNA libraries *Dra*I, *Eco*RV, *Pvu*II, *Sca*I *and Stu*I separated by agarose gel electrophoresis and exposed to UV light. Gene specific primer prom2 or primer prom3 were used. Standard fragment sizes are given. PCR fragment A and PCR fragment B are marked with a circle.

The amplified fragments were aligned stepwise as indicated in Figure 2. The attained complete *LeCer6* gene sequence was illustrated at the translation start (ATG) and the translation stop codon (TAA) plus the intron. The *Ac/Ds* insertion element, which caused the deficiency of the *LeCer6* gene, was localized by Dr. Eyal Emmanuel.

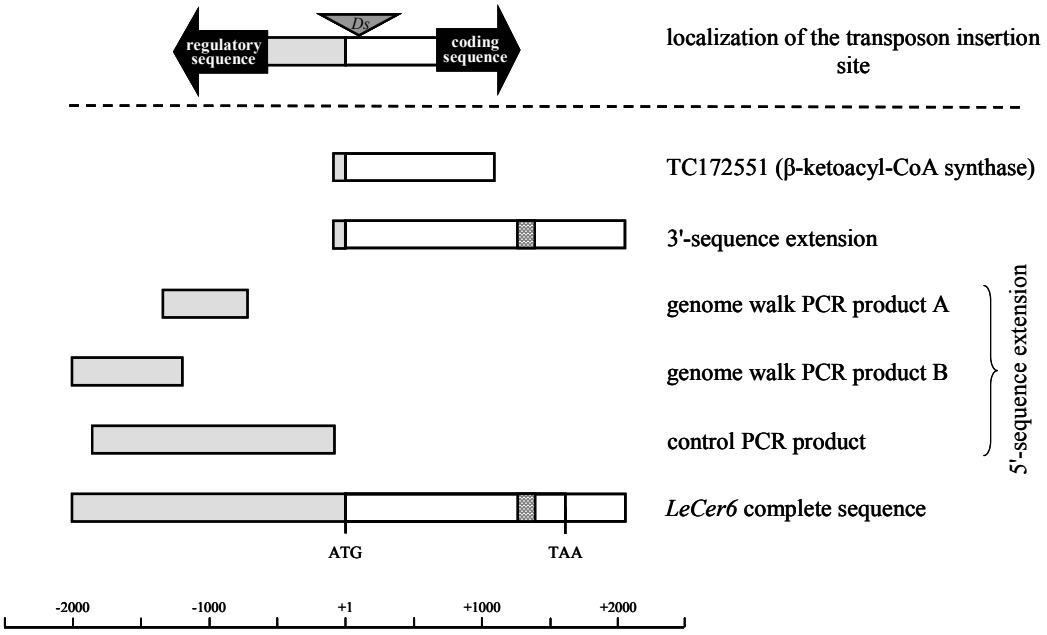

Figure 2. Schematic presentation of the 5'-sequence extension of the LeCER6 nucleotide sequence aligned by means of TC172551 (DFCI database; http://compbio.dfci.harvard.edu), 3'-sequence extension (Dr. Eyal Emmanuel), two genome walk fragments A and B as well as the amplified control PCR product. The position of the transposon insertion site, the ATG translation start codon, the TAA translation stop codon and the intron region (shaded) are given. The measuring rod points up the fragment sizes in bp.

The detailed study of the nucleotide sequence of the *LeCer6* gene revealed a protein encoding structure of 1617 bp beginning with an ATG translation start codon (position 1; Figure 3) and ending with a TAA translation stop codon (position 1617). Downstream of the ATG triplet this sequence contains a 126 bp intron at position 1271.

The approximately 2000 bp transcriptional regulatory region upstream of the ATG triplet includes three putative TATAAT boxes/enhancer elements at position -1830, -1561 and -1369. Furthermore, a number of probable regulatory elements were characterized in the *LeCer6* promoter region, such as light responsive elements: 4cl-CMA2b, box I, GATA-motif, I-box and CIRCADIAN element. Additionally, motifs for putative regulatory elements were found in the promoter region linked with defense and stress responsiveness: TCA/SARE (salicylic acid responsive element), LTR (low temperature responsive element), HSE (heat shock element), MBS (MYB binding site) and TC-rich repeats (in response to drought).

Within the gene sequence an ATG triplet terminates the transcriptional regulatory region at their 3' end that corresponds to the start codon of the LeCER6 translation. Analysis of the LeCER6 encoding region revealed an open reading frame of 1491 bp corresponding to a polypeptide of 496 amino acids with a predicted molecular mass of 55.8 kDa (http://www.expasy.ch/tools/peptide-mass.html).

Figure 3. Nucleotide sequence (small letter) and the deduced amino acid sequences (capital letter) of the β-ketoacyl-CoA synthase LeCER6 generated by using ExPASy (http://expasy.org/tools/#translate). Genomic sequences for the initiating translation start and stop codon are highlighted in bold letters on a black background. Putative TATAAT-boxes/enhancer are indicated by a black background. The intron nucleotide sequence is underlined (- - -). Numerous consensus sequences for putative regulatory elements 5´ to the coding region are illustrated: light responsive elements (light shadings) and stress and defense responsive elements (dark shadings; http://bioinformatics.psb.ugent.be/webtools/plantcare/html).

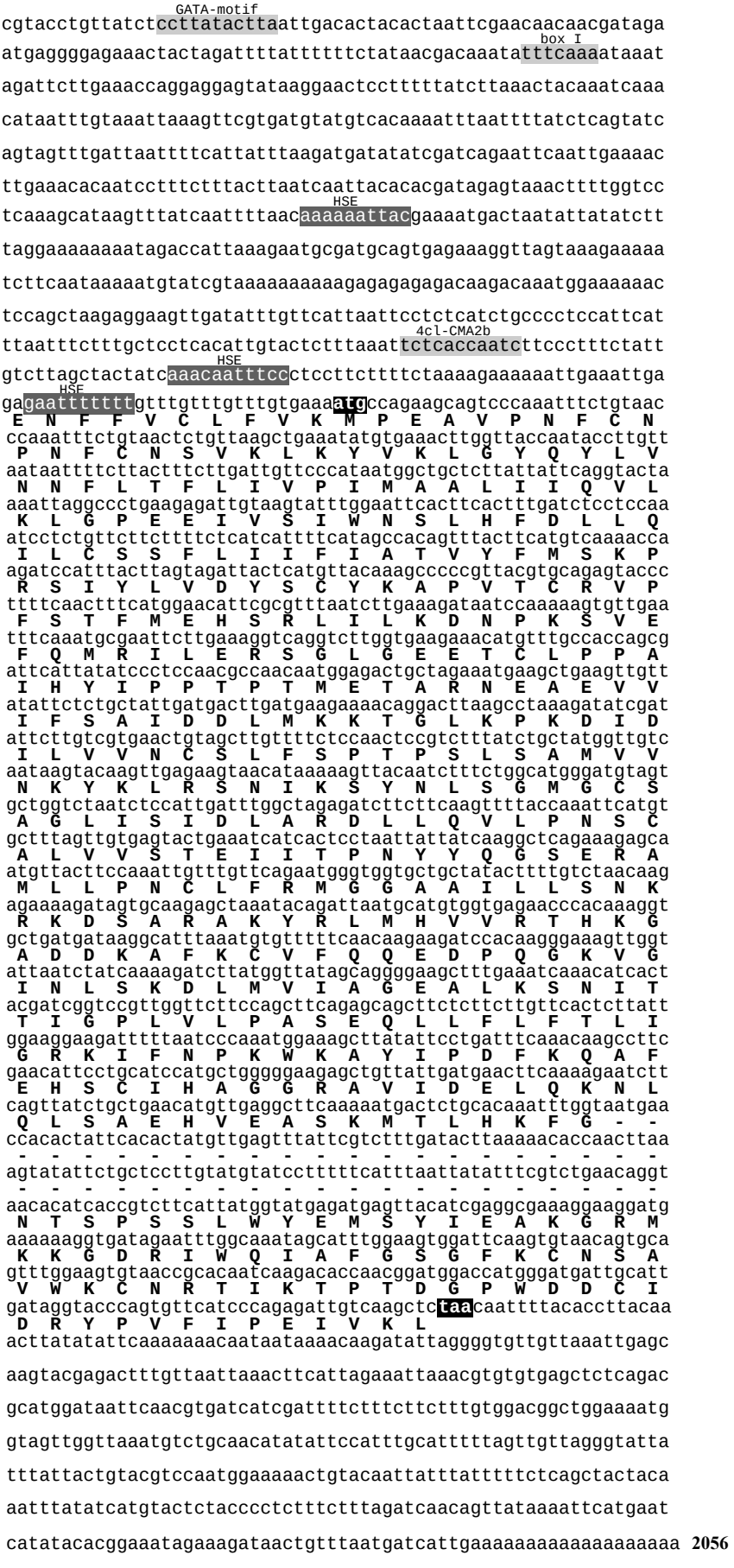

Figure 3. (continued)

Nucleotide Sequence Alignment of Different Tomato β-Ketoacyl-CoA Synthases

The *LeCer6* nucleotide sequence was comparatively analyzed with seven putative β-ketoacyl-CoA synthases from *Lycopersicon esculentum* Mill. and *AtCER6* and *AtCER60* from *Arabidopsis* as out-group. For the phylogenetic analysis of this gene family, the region downstream from the translational start codon in the frame of 510 bp to 750 bp was used. The tree view alignment displayed tomato β-ketoacyl-CoA synthases separated into distinct branches. As shown in Figure 4, except β-ketoacyl-CoA synthase EST326602, all annotated β-ketoacyl-CoA synthases are phylogenetically distant from *LeCer6*. The β-ketoacyl-CoA synthase TC172551 is identical with the *LeCer6* gene.

Further annotated tomato β-ketoacyl-CoA synthases TC180177, TC189513, TC188938, TC171376 and TC173761 could not be aligned with *LeCer6*, since sequence data were not available inside of the tested region.

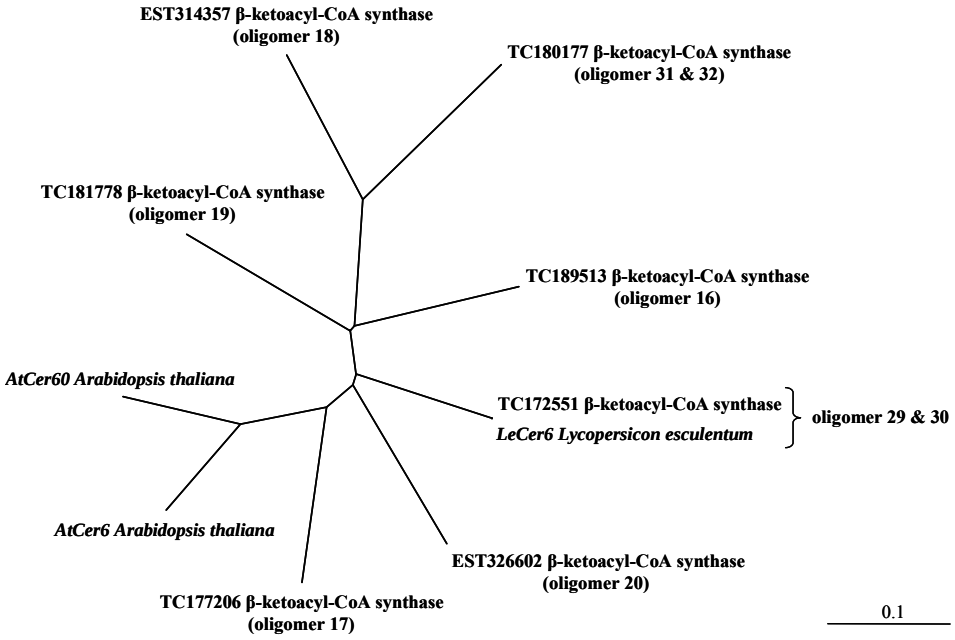

Figure 4. Phylogenetic relationship between seven different tomato β-ketoacyl-CoA synthases in comparison to *LeCer6* based on an alignment of nucleotide sequences constructed using ClustalW (http://www.ebi.ac.uk/Tools/clustalw/index.html) and displayed in TreeView 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). TC number, tentative annotation and internal oligomer number in the microarray analysis are given. *AtCer6* and *AtCer60* of *Arabidopsis thaliana* (L.) Heynh. (http://compbio.dfci.harvard.edu) were taken as out-group. The measuring rod points up the substitution of one base per 100 bases.

Amino Acid Sequence Alignment of β-Ketoacyl-CoA Synthases from Different Plant Species

The *in silico* predicted amino acid sequence of LeCER6 was screened by the BLAST database in order to assure distinction of LeCER6 to other proteins (http://www.ncbi.nlm.nih.gov/BLAST/). At the deduced amino acid sequence level LeCER6 of *Lycopersicon esculentum* is closely related to the condensing enzymes GhCER6 of *Gossypium hirsutum*, and to AtCER6 and AtCER60 of *Arabidopsis*. The generated alignment revealed amino acid homologies of 86% for LeCER6 and GhCER6 and homologies of 83% in comparison with AtCER6 and AtCER60 (Figure 5). The similarity of LeCER6 with amino acid sequences received from β-ketoacyl-CoA synthases of *Oryza sativa*, *Marchantia polymorpha* and *Physaria fendleri* ranged about 77%, 63% and 53%, respectively.

In the N-terminal position of the amino acid sequence of LeCER6 two hydrophobic domains were found corresponding to a putative transmembrane region. Using PSORT-II version 6.4 (http://psort.ims.u-tokyo.ac.jp/form.html), the LeCER6 protein was estimated to be localized in the plasma membrane with a certainty of 60% (Golgi body: 40%; chloroplast thylakoid membrane: 39%; endoplasmic reticulum membrane: 30%).

However, the catalytic domain of LeCER6 was characterized by the presence of a highly conserved cysteine (C^{263}) predicted on its homology to the active site residue cysteine FAE1 in *Arabidopsis* (Ghanevati and Jaworski, 2002). Furthermore, among all conserved histidine and asparagine residues only H^{430} and N^{463} aligned very well with the active site histidine and asparagine of both KAS III and chalcone synthase, as reported by Ghanevati and Jaworski (2001). Apart from these three residues essential for the condensing enzyme activity, a highly conserved phenylalanine residue (F^{312}) probably involved in substrate binding was determined in the LeCER6 reaction center (Ferrer *et al.*, 1999).

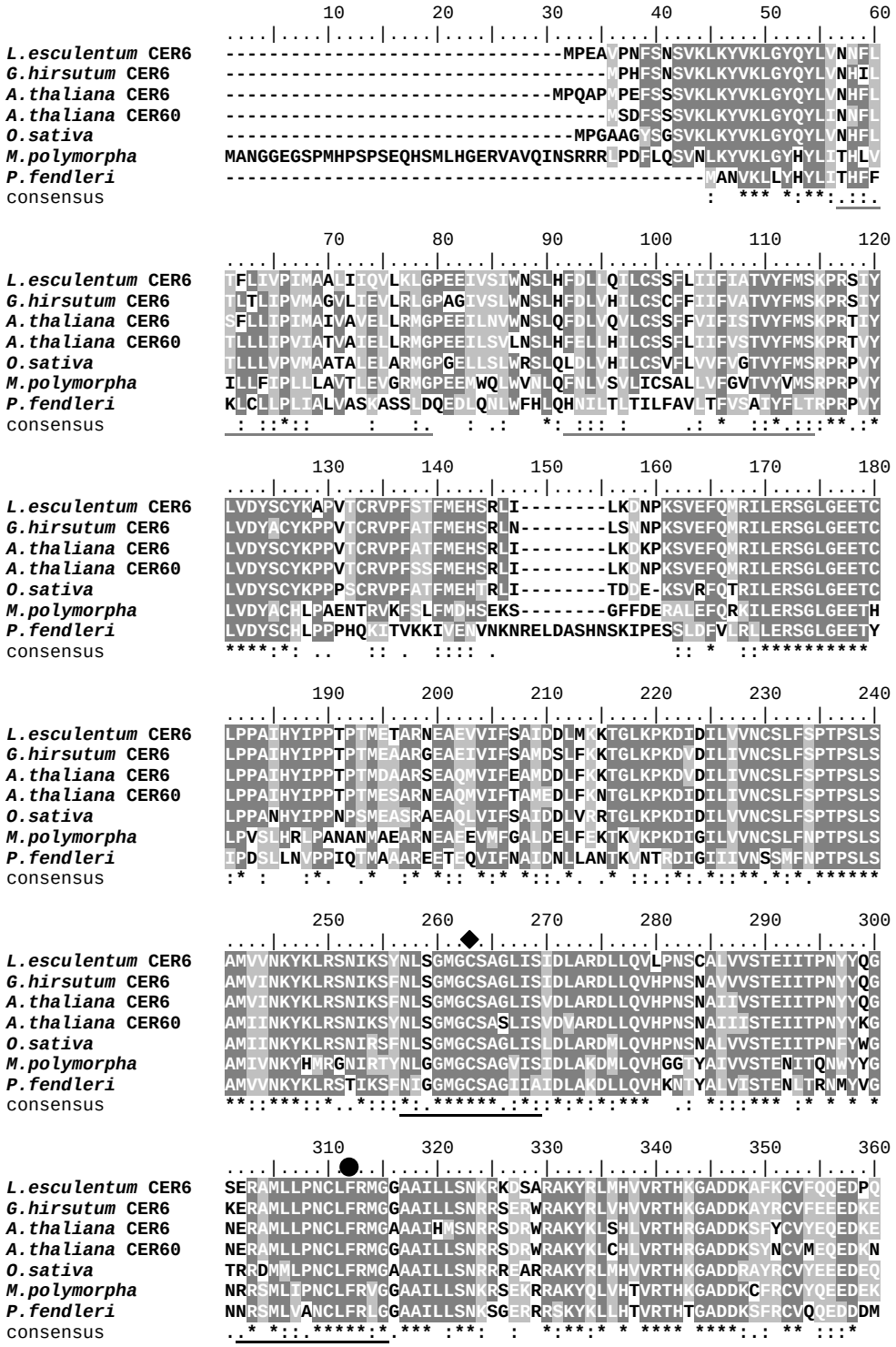

Figure 5. Amino acid sequence of *Lycopersicon esculentum* Mill. (Solanaceae) aligned with β-ketoacyl-CoA synthase sequences from *Gossypium hirsutum* L. (Malvaceae), *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae), *Oryza sativa* L. (Poaceae), *Marchantia polymorpha* L. (Marchantiaceae) and *Physaria fendleri* (A. Gray) O´Kane & Al-Shehbaz (Brassicaceae) generated by ClustalW multiple sequence alignment version 1.8 (http://www.ebi.ac.uk/clustalw/index.html). Consensus amino acid sequences are marked with ., : or *, as well as with light or dark shadings for similar or identical residues, respectively. Dashes are introduced to optimize the alignment. Amino acid sequences belonging to the two predicted transmembrane domains are indicated by gray lines (http://searchlauncher.bcm.tmc.edu/seq-search/struc-predict.html). Black lines specify highly conserved catalytic regions among all CER6 condensing enzymes. The position of the active site residues cysteine (C^{263}; quadrangle), phenylalanine (F^{312}; point), histidine (H^{430}; star) and asparagines (N^{463}; triangle) is illustrated.

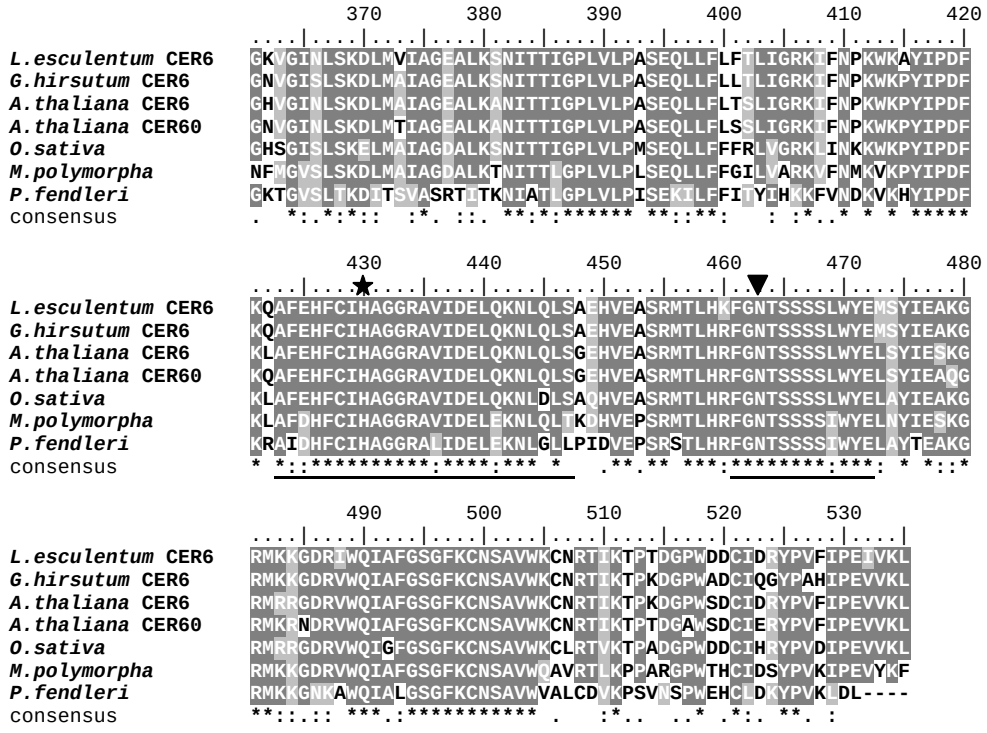

Figure 5. (continued)

Visual Differentiation of Tomato Wild Type and LeCER6-Deficient Plants

The MicroTom dwarf tomato plants were cultivated in a growth chamber under standardized conditions. The effects of an insertional mutation in the *LeCer6* gene in MicroTom plants led to a dumose growth form of the *lecer6* mutant with wrinkled leaves, visually distinguishable from the wild type (Figure 6).

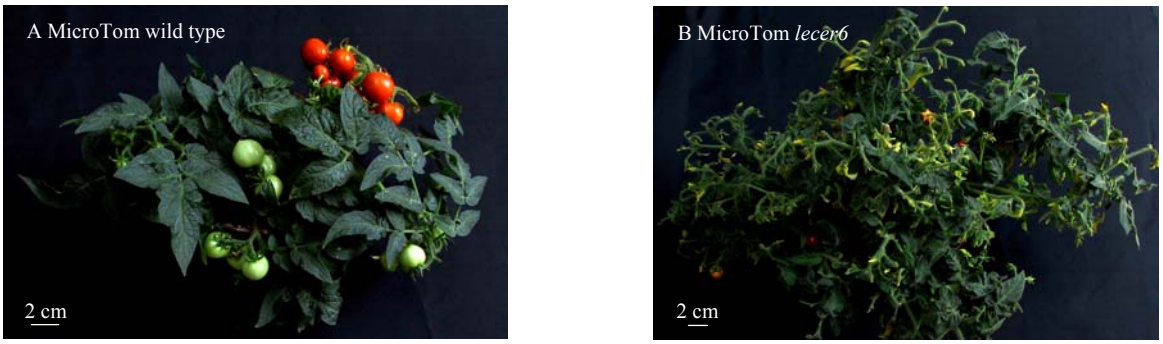

Figure 6. Photograph of plants of the miniaturized tomato cultivar MicroTom wild type (A) and MicroTom *lecer6* (B).

Moreover, a distinctly higher above-ground growth was found for *lecer6* plants when compared to wild type plants (Figure 8). Flowers of *lecer6* mutant were largely sterile and characterized by fused stamen and petals (Figure 7). A standard cultivation of both

MicroTom lines led to approximately 32 viable seeds per fruit for the wild type, whereas in *lecer6* mutant fruits less than one seed per fruit developed (Figure 9). In contrast to the wild type, seeds of MicroTom *lecer6* were only formed under conditions of a high humidity environment (> 90%; data not shown). The organ fusion phenotype of MicroTom *lecer6* was not reversible.

Figure 7. Photograph of MicroTom wild type (A) and fused MicroTom *lecer6* (B) flowers.

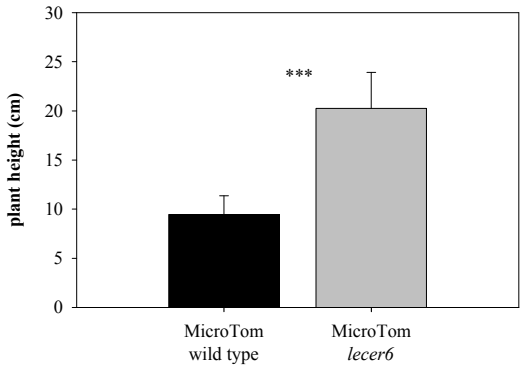

Figure 8. Plant height of MicroTom wild type and LeCER6-deficient MicroTom plants. Statistical differences between MicroTom wild type and MicroTom *lecer6* were analyzed with Mann-Whitney *U*-tests (n.s. = not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Data are shown as means ± SD ($n = 6$).

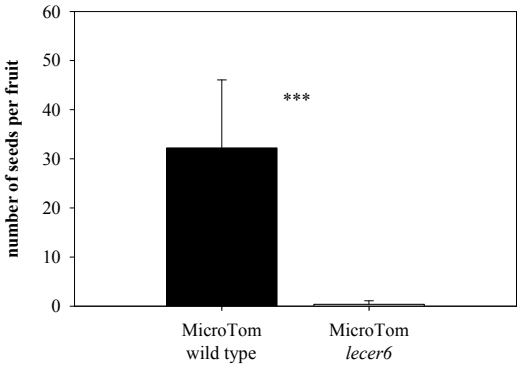

Figure 9. Number of seeds formed in MicroTom wild type and LeCER6-deficient MicroTom fruits by standard cultivation. Statistical differences between MicroTom wild type and MicroTom *lecer6* were analyzed with Mann-Whitney *U*-tests (n.s. = not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Data are shown as means ± SD ($n = 6$).

Development of Tomato Wild Type and LeCER6-Deficient Fruits

An insertional transposon mutagenesis evoked the LeCER6 deficiency of MicroTom. This loss-of-function mutation of LeCER6 affected several fruit parameters in the course of the development of fleshy berry tomato fruits.

Temporal Aspects of Fruit Development: Fruit ripening of MicroTom take about 80 days and is accompanied by a multiplication of size and changes of pigmentation (Figure

10). Both developmental parameters, fruit size and pigmentation, were used to arrange fruits of MicroTom wild type and MicroTom *lecer6* into seven categories. Fruit category I and category II are characterized by differently sized immature and mature green fruits of an age of 10 days and 20 days after flowering, respectively. The following period of 15 days of fruit ripening generated category II to category VI mainly differentiated on the basis of the respective fruit color. These categories were named early breaker (category III), breaker (category IV), orange (category V) and red ripe (category VI) tomato fruits. On day 45 after flowering MicroTom wild type fruits belong to category VI with a maximum fruit size and a fruit surface area of 10.21 ± 0.51 cm^2 (mean ± SD). Red overripe tomato fruits of category VII showed a shriveled fruit surface, diminished up to 15% after day 48 in comparison to the maximum surface area of category VI fruits, probably caused by manipulation by measurements and the progressive fruit softening.

Optical Aspects of Fruit Development: Visually changes in the fruit pigmentation were supported by alterations of the chlorophyll fluorescence of developing MicroTom wild type fruits (Figure 11). In both immature and mature green fruits the chlorophyll fluorescence, representing the photochemical efficiency of photosystem II (PSII), was at the highest levels of 0.65 ± 0.08 and 0.42 ± 0.05, respectively.

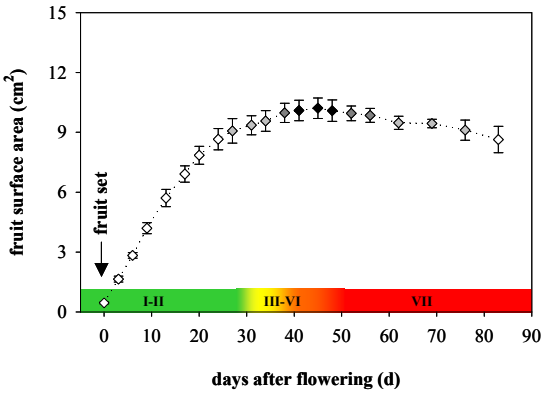

Figure 10. Fruit expansion of MicroTom wild type during the course of fruit development. The alteration of the fruit pigmentation and the resulting categorization is illustrated. Data are shown as means ± SD (n = 8 to 10).

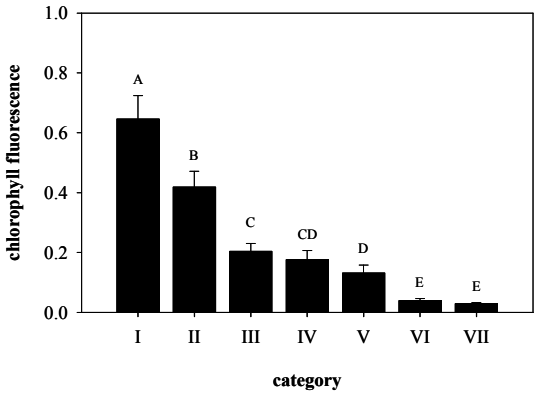

Figure 11. Chlorophyll fluorescence of MicroTom wild type fruits assigned into seven developmental categories. Chlorophyll fluorescence was compared by one-way ANOVA followed by HSD post-hoc test for unequal n: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SD (n = 20 to 25).

The bright-yellow, dark-yellow and orange fruits of category III to category V had a clearly reduced chlorophyll fluorescence. The PSII efficiency of these fruit categories were significantly 4.9-fold decreased compared to the PSII efficiency of fruits in category I. The chlorophyll fluorescence diminishment further continued during tomato fruit ripening. In fruit developmental categories VI and VII the chlorophyll fluorescence in red fruits drastically dropped down to 6% and 4% of the initial PSII efficiency.

Physical Aspects of Fruit Development: The surface areas of fruits of category I of both MicroTom lines were about the same size with areas of 0.54 ± 0.14 cm^2 (wild type) and 0.50 ± 0.11 cm^2 (*lecer6* mutant), respectively (Figure 12). The transition of category I to category II revealed a considerable increase of the fruit surface area of both MicroTom lines that was strongest expressed in the wild type fruits. Surface areas of wild type fruits of category II were 8.61 ± 1.24 cm^2 and 5.67 ± 1.04 cm^2 of fruits of *lecer6* mutant. Whereas the surface area of MicroTom *lecer6* fruits remained approximately constant at this level, fruits of the MicroTom wild type expanded their surface area up to 10.56 ± 1.50 cm^2. With respect to the fruit surface area the ratio between both MicroTom lines increased from 1:1 in category I to 1:2 in category VII.

However, the differences in fruit size between MicroTom wild type and MicroTom *lecer6* were more pronounced by the fruit volume (Figure 13).

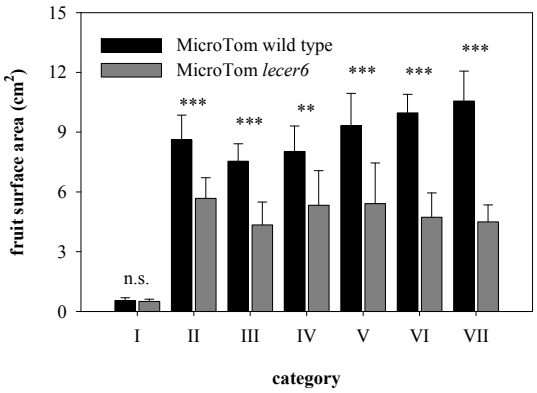

Figure 12. Surface area of MicroTom wild type and LeCER6-deficient MicroTom during fruit development. Statistical differences between MicroTom wild type and MicroTom *lecer6* were analyzed with Mann-Whitney *U*-tests (n.s. = not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Data are shown as means ± SD (n = 4 to 6).

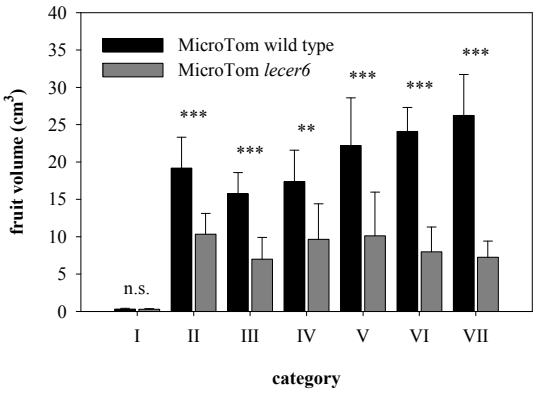

Figure 13. Volume of MicroTom wild type and LeCER6-deficient MicroTom during fruit development. Statistical differences between MicroTom wild type and MicroTom *lecer6* were analyzed with Mann-Whitney *U*-tests (n.s. = not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Data are shown as means ± SD (n = 4 to 6).

Whereas the fruit volumes were similar in category I, distinct differences between MicroTom wild type and *lecer6* mutant fruits started to become obvious in the early stage of category II. The fruit volume of MicroTom wild type increased from 0.31 ± 0.12 cm^3 in category I to 26.24 ± 5.49 cm^3 in category VII. However, the expansion of LeCER6-deficient fruits was smaller and ranged from 0.28 ± 0.09 cm^3 in category I up to 7.25 ± 2.14 cm^3 in category VII. Consequently, in category VII MicroTom wild type fruits reached a 3.6-fold higher volume than the mutant fruits. Hence, during the course of ripening the ratio of fruit surface area to fruit volume decreased from a factor of 1.8 in category I for both MicroTom lines to 0.4 for the wild type and to 0.6 for the *lecer6* mutant.

Similar to the fruit surface area and volume parameters, the fresh weight of MicroTom wild type and MicroTom *lecer6* fruits showed a parallel increase (Figure 14). The immature green fruits of category I were small and weighed only 0.04 ± 0.02 g, but increased to 2.50 ± 0.60 g for wild type and 1.35 ± 0.33 g for *lecer6* mutant fruits in category II. Wild type fruits of this developmental stage were 1.8-fold heavier than LeCER6-deficient fruits. Concerning the development of fruits in category III to category VII, the fresh weight of the *lecer6* mutant fruits stagnated at the level of category II, while the fresh weight of the wild type fruits further increased with an 1.5-fold enhancement within the transition of category IV to category VII. Therefore, the ratio of the fresh weights of both MicroTom lines reached 1:3 in category VII.

Freeze drying of tomato fruits led to a reduction of about 83% of the fresh weight throughout all developmental categories (Figure 15). Again, the characteristic shift of the fruit weight within the transition of category I and category II became obvious with an increase in fruit dry weight by a factor of 50.8 for the wild type and by 28.8 for the *lecer6* mutant. Thereby, weight differences between the MicroTom wild type and MicroTom *lecer6* fruits diminished. Ultimately, a significant 1:2 ratio of dry weight was only found in category VII.

The relationship between fruit dry weight and fruit volume was specified as 'fruit density'. In comparison, the ' fruit density' of category I of MicroTom wild type and LeCER6-deficient MicroTom fruits was calculated as 0.015 g cm^{-3} and 0.023 g cm^{-3}, respectively. With beginning of the early stage of category II, the 'fruit density' averaged 0.012 g cm^{-3} for the wild type and 0.019 g cm^{-3} for the *lecer6* mutant.

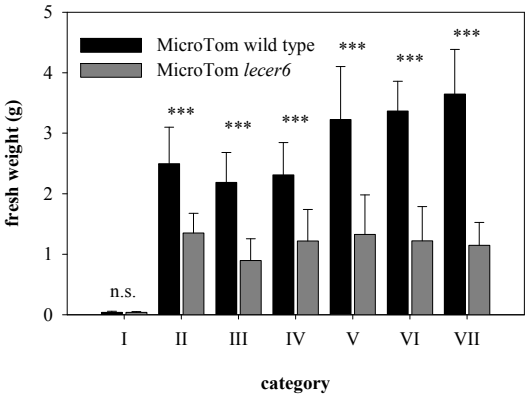

Figure 14. Fresh weight of MicroTom wild type and LeCER6-deficient MicroTom during fruit development. Statistical differences between MicroTom wild type and MicroTom *lecer6* were analyzed with Mann-Whitney *U*-tests (n.s. = not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Data are shown as means ± SD (n = 4 to 6).

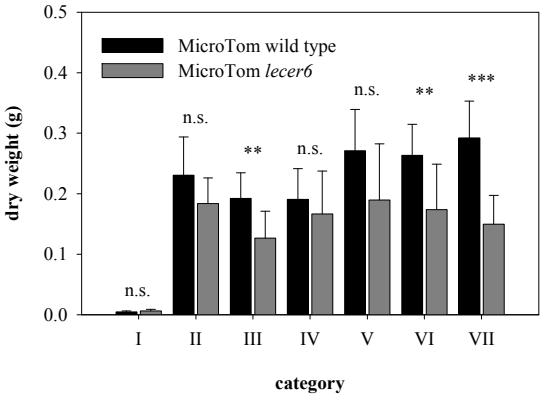

Figure 15. Dry weight of MicroTom wild type and LeCER6-deficient MicroTom during fruit development. Statistical differences between MicroTom wild type and MicroTom *lecer6* were analyzed with Mann-Whitney *U*-tests (n.s. = not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Data are shown as means ± SD (n = 4 to 6).

Metabolic Aspects of Fruit Development: For a further examination of MicroTom wild type and MicroTom *lecer6* fruits, the content of extractable protein and glucose was investigated after removal of the seeds and the surrounding locular tissue (gel). Immature green fruits of category I contained the highest protein amount: 26.11 ± 1.99 μg g^{-1} dry weight for the wild type and 30.49 ± 6.64 μg g^{-1} dry weight for the *lecer6* mutant, respectively (Figure 16). During the transition of category I to category II the MicroTom fruits increased in size and weight, whereas the protein content fell drastically. Between categories II and VII the protein content ranged at a reduced level between 8.98 ± 1.08 μg g^{-1} dry weight and 3.62 ± 0.85 μg g^{-1} dry weight. The protein content in wild type fruits was significantly increased by 24% in category II to category IV compared to the *lecer6* mutant fruit. In category VII, red overripe fruits contained 3.3-fold less protein for the wild type and 5.0-fold for the *lecer6* mutant as immature green fruits of the initial category I.

Furthermore, the content of the monosaccharide glucose was analyzed (Figure 17). For the wild type as well as for the LeCER6-deficient fruits the lowest glucose contents were detected in fruits of category I. However, in fruit category II the glucose content was about 2.2-fold increased to 0.83 ± 0.26 mmol g^{-1} dry weight in the wild type and 0.93 ± 0.37 mmol g^{-1} dry weight in *lecer6* mutant. The amount of glucose in wild type fruits remained almost constant at the level of mature green fruits in category II. In contrast, the glucose

level of LeCER6-deficient fruits increased continuously to a maximum of 1.59 ± 0.36 mmol g^{-1} dry weight in category VII. Ultimately, in the investigated fruit stage of category VII the glucose content resulted in a significant 1.7-fold difference between both MicroTom lines.

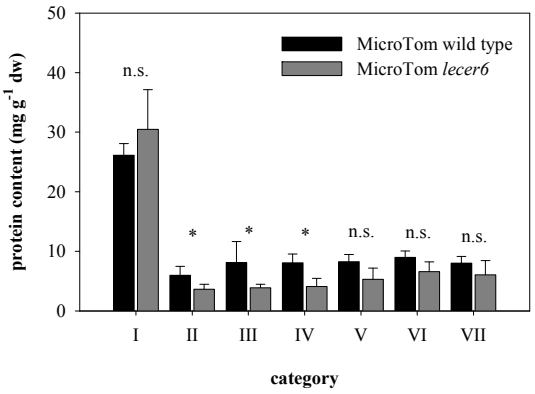

Figure 16. Protein content of MicroTom wild type and LeCER6-deficient MicroTom fruits without seeds during fruit development. Statistical differences between MicroTom wild type and MicroTom *lecer6* were analyzed with Mann-Whitney *U*-tests (n.s. = not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Data are shown as means ± SD ($n = 4$ to 6).

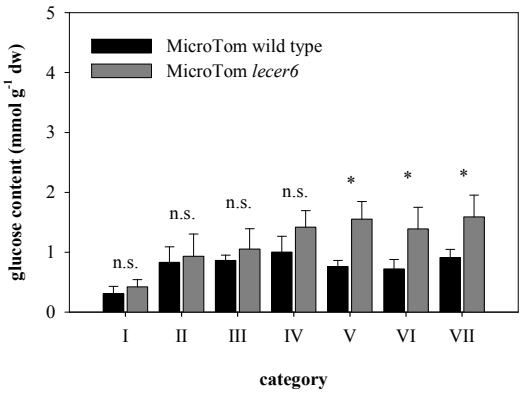

Figure 17. Glucose content of MicroTom wild type and LeCER6-deficient MicroTom fruits without seeds during fruit development. Statistical differences between MicroTom wild type and MicroTom *lecer6* were analyzed with Mann-Whitney *U*-tests (n.s. = not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Data are shown as means ± SD ($n = 4$ to 6).

Surface Aspects of Fruit Development: A micromorphological investigation of the MicroTom cuticular surface matrix was performed by scanning electron microscopy. Exemplary, the surface areas of category VI enzymatically isolated cuticles was imaged, which showed a laminar, crystal-free wax film of the fruit surface of MicroTom wild type and MicroTom *lecer6* (Figure 18). A visual comparison between the wax cover of these red ripe fruits of both MicroTom lines displayed more distinct contours of *lecer6* epidermal cells.

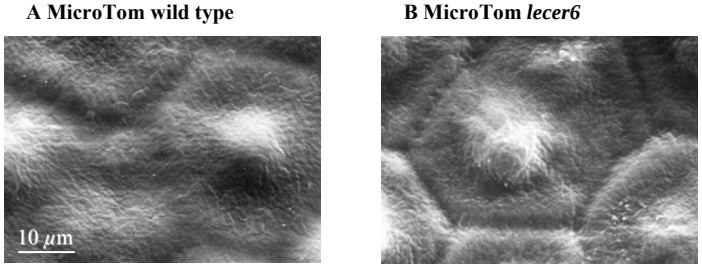

Figure 18. Scanning electron microscopic plan views of MicroTom wild type (A) and LeCER6-deficient MicroTom (B) fruit cuticle of category VI.

Enzymatically isolated MicroTom wild type and MicroTom *lecer6* cuticles of each category are represented in Figure 19.

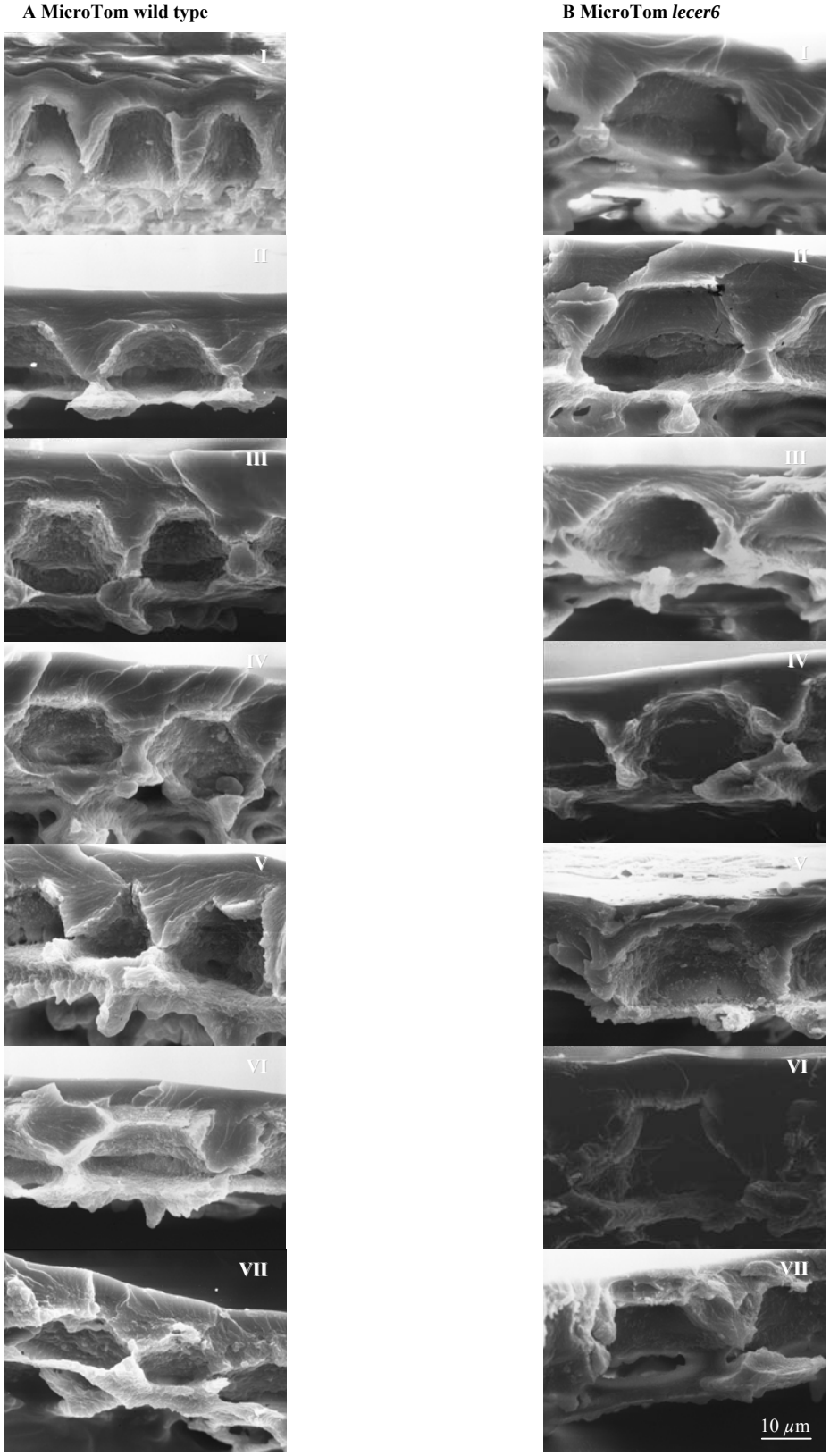

Figure 19. Scanning electron microscopic cross sections of MicroTom wild type (A) and LeCER6-deficient MicroTom (B) fruit cuticles of the seven developmental categories.

Cross sections show the cuticular membrane with increased cutin and wax layers of developing fruits. MicroTom wild type fruits of category I to category II are characterized by a cuticle that does not fully enclose whole epidermal cells. Following the developmental course, the complete epidermal cell layer and partially the subepidermal cell layer were embedded with this membrane with onset of developmental category III for the wild type and all categories of the *lecer6* mutant, respectively.

For further characterization of the outermost layer of the MicroTom fruits, the total number of epidermal cells forming the boundary layer of the fruit with its environment was determined. In category I the epidermal cell density of the outermost cuticle layer started with a mean level of 52 ± 8 cells cm^{-2} in the wild type and 71 ± 16 cells cm^{-2} in the *lecer6* mutant, respectively (Figure 20). Between category I and category II the number of cells increased considerably, leading to maximum values of approximately 877 cells cm^{-2} in mature green fruits in category II of both MicroTom lines. During the further fruit developmental process an increase of the epidermal cell density was not detectable. For the MicroTom wild type fruits this stagnation occurred at slightly elevated levels compared to the *lecer6* mutant. With regard to the whole fruit level, the epidermal cell number was calculated as 1183 ± 84 cells per fruit for the wild type and as 945 ± 231 cells per fruit for the *lecer6* mutant representing mean values for developmental category II to category VII.

A biotic specification of fruit surface features of MicroTom wild type and MicroTom *lecer6* was performed by a bioassay with fungus *Blumeria graminis* f. sp. *hordei*. The conidial morphogenesis of *Blumeria graminis* was analyzed on freshly peeled fruit tissue and enzymatically isolated fruit cuticle corresponding to developmental fruit category II and category VI (Figure 21). Freshly peeled fruit tissues of MicroTom wild type and MicroTom *lecer6* stimulated germination and appressorial differentiation of *Blumeria graminis* conidia. Thereby, the germination-inducing activity of mature green and red ripe fruits was higher for the MicroTom wild type (51%) than for the *lecer6* mutant (44%). Furthermore, wild type fruits showed a 6% or 7% higher percentage of conidia differentiation in comparison to the analogous developmental fruit category of LeCER6-deficient fruits. Within both MicroTom lines the appressorial differentiation of *Blumeria graminis* was slightly reduced on fresh peeled surfaces of red ripe fruits compared to mature green fruits.

An enzymatic isolation of fruit cuticles of MicroTom wild type and MicroTom *lecer6* resulted in a clear decrease in germination activity of *Blumeria graminis* in comparison to

the germination activity on freshly peeled fruit tissue. On none of four different surfaces the germination of *Blumeria graminis* conidia exceeded 36% by a rate of appressorial differentiation of < 1.5%. A similar pattern of germination and appressorial differentiation was observed on a glass slide as negative control. The conidial mortality rate on all fruit surfaces ranged between 38% and 62%, but tended to be elevated at enzymatically isolated cuticles.

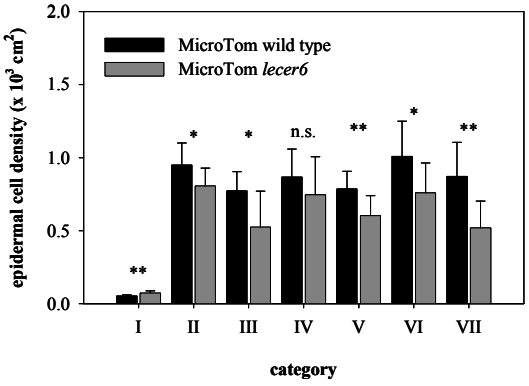

Figure 20. Epidermal cell density of MicroTom wild type and LeCER6-deficient MicroTom during fruit development. Statistical differences between MicroTom wild type and MicroTom *lecer6* were analyzed with Mann-Whitney *U*-tests (n.s. = not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Data are shown as means ± SD (n = 10 to 15).

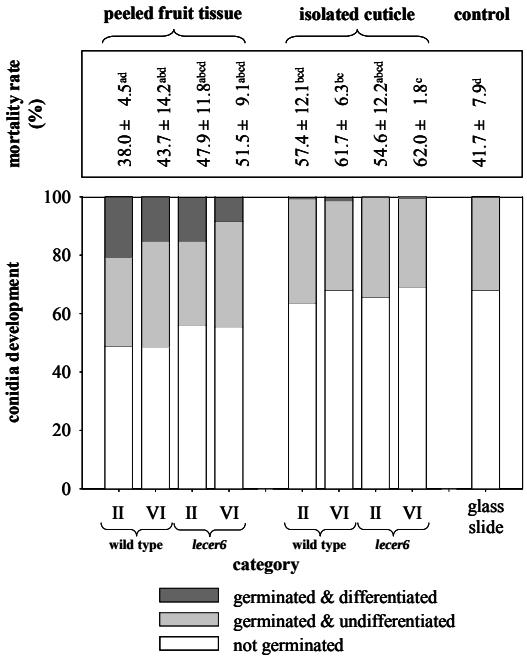

Figure 21. *Blumeria graminis* conidia development of different MicroTom wild type and LeCER6-deficient MicroTom fruit surfaces. The conidia development was compared by multi-way ANOVA followed by HSD post-hoc test for unequal n: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SD (n = 650 to 800 consisting of 6 independent experiments with > 100 conidia).

Relationship of Different Fruit Parameters of the Tomato Model System

In order to analyze possible correlations of developmental variation in fruit parameter values of MicroTom wild type and MicroTom *lecer6*, a Spearman's correlation of ranks was performed with fruit parameter data of all developmental categories of the two MicroTom lines (Table I). The additional data for water permeance, wax accumulation, water content and cuticle amount, discussed in CHAPTER I and CHAPTER II of this work, were also taken in consideration to this respect. Water content, surface area, volume, fresh weight, dry weight and epidermal cell density of the MicroTom fruits were found to

be highly associated during fruit development. Moreover, these parameters showed a positive but moderate correlation with cuticular wax accumulation of the MicroTom fruit. Furthermore, a negative correlation was detected for the same parameters in relation to the permeance for water. The negative correlation between the permeance for water and the total wax amount was not significant. None of these parameters were found to correlate with the amount of cuticle during the course of tomato fruit development. However, the amount of cuticle negatively correlates with protein content, but positively associates with glucose content.

Table I. Spearman's correlation of ranks was performed with different tomato fruit parameters of MicroTom wild type and MicroTom *lecer6*. Significant correlation coefficient values between permeance for water (m s^{-1}), wax accumulation (μg cm^{-2}), water content (%), surface area (cm^2), volume (cm^3), fresh weight (g), dry weight (g), epidermal cell density (cm^2), amount of cuticle (μg cm^{-2}), protein content (μg g^{-1} dw), glucose content (μmol g^{-1} dw) are marked in bold ($P < 0.05$).

parameter	wax accumulation	water content	surface area	volume	fresh weight	dry weight	epidermal cell density	amount of cuticle	protein content	glucose content
permeance for water	-0.48	**-0.86**	**-0.89**	**-0.89**	**-0.91**	**-0.88**	**-0.86**	0.12	-0.12	0.16
wax accumulation		0.46	**0.60**	**0.60**	**0.59**	**0.63**	0.38	0.31	-0.05	0.35
water content			**0.81**	**0.81**	**0.80**	**0.74**	**0.71**	-0.35	0.34	-0.28
surface area				**1.00**	**1.00**	**0.98**	**0.92**	-0.06	0.03	-0.14
volume					**1.00**	**0.98**	**0.92**	-0.06	0.03	-0.14
fresh weight						**0.98**	**0.93**	-0.06	0.05	-0.14
dry weight							**0.87**	-0.06	0.06	-0.13
epidermal cell density								-0.01	-0.07	-0.14
amount of cuticle									**-0.77**	**0.89**
protein content										**-0.74**

DISCUSSION

The present study focuses on the *LeCer6* molecular structure and describes direct and indirect changes of wax biosynthesis *via* the knock-out of this gene in the tomato plants. This 'reverse genetic' strategy was used to bridge the gap between *LeCer6* gene and its functional significance. Thereby, the analysis of the *lecer6* mutant in comparison to the MicroTom wild type provides a useful tool for the exploration of the temporal regulation of wax biosynthesis processes in tomato fruits. Differential gene expression led to coordinated biochemical and physiological changes, shown by extensive studies of both

the chemical (CHAPTER I) and the transcriptional levels (CHAPTER II) of ripening fruits. Strong effects of the deficiency in this single gene are demonstrated for cuticular wax coverage that is controlled by VLCFA biosynthesis, and also for several pleiotropic and secondary effects on tomato plants. Thus, multiple, often seemingly unrelated phenotypic changes could be directly or indirectly attributed to the *lecer6* mutation.

Detailed Genetic Make-up of β-Ketoacyl-CoA Synthase Gene *LeCer6* and Homologies of Its Gene Product a Very-Long-Chain Condensing Enzyme

With the above described experiments the *LeCer6* gene was identified at the molecular level and characterized to be identical with the expressed sequence tag (EST) TC172551, annotated as β-ketoacyl-CoA synthase (DFCI database).

A putative promoter fragment of 1.4 kb was identified upstream of the ATG translation start codon within the transcriptional regulatory region of *LeCer6* gene sequence. This promoter fragment included a multitude of transcriptional regulatory elements putatively involved in light sensitivity or acting on stress and defense responsiveness. Previously, the GATA-motif was specified as the common, conserved element for light regulated genes, whereas I-boxes were predicted to be functionally important in many light inducible promoters (Terzaghi and Cashmore, 1995; Hooker *et al.*, 2002). The CIRCADIAN element is related to the control of gene expression in plants by circadian rhythms. In addition, several motifs included in the *LeCer6* promoter fragment are known to be involved in transcriptional gene expression activation by salicylic acid. Salicylic acid is recognized as an endogenous signal that generates a significant impact on gene expression on plants associated with a variety of plant defense processes and mediated responses against stress conditions (Yalpani *et al*., 1994; Shirasu *et al*., 1997; Senaratna *et al*., 2000; Borsani *et al*., 2001; Singh *et al*., 2002). The presence of transcriptional regulatory elements in the coding region of *LeCer6*, which are also found in light- and stress-responsive genes, suggests that *LeCer6* gene expression may be influenced by these stimuli. Moreover, this regulatory sensitivity at the transcriptional level may trigger alterations in VLCFA biosynthesis by changing *LeCer6* gene expression and subsequent abundance of LeCER6 enzyme.

The *LeCer6* gene of *Lycopersicon esculentum* shows a strong homology to the *AtCer6* gene in *Arabidopsis*, which exhibits an 1.2 kb promoter fragment and a similar exon-intron organization in comparison to *LeCer6*. Based on a promoter analysis and experimental data, Hooker *et al.* (2002) reported that light as well as abscisic acid plays a pivotal role in the transcriptional regulation of the *AtCer6* gene. In contrast to the *Lecer6* gene sequence,

abscisic acid responsive elements (ABRE) instead of salicylic acid responsive elements were identified in the transcriptional regulatory region of *Atcer6*. ABRE elements are part of a number of ABA-regulated genes that are responsive to osmotic stress (Guiltinan *et al.*, 1990).

Analyses of the coding region of the *LeCer6* gene sequence revealed a 496 amino acid sequence, which encode a polypeptide with a predicted molecular mass of 56 kDa. The LeCER6 sequence of *Lycopersicon esculentum* is phylogenetically closely related to GhCER6 of *Gossypium hirsutum* and AtCER6 of *Arabidopsis* at the *in silico* deduced protein sequence level. The *GhCer6* and the *AtCer6* nucleotide sequence encode polypeptides with predicted molecular masses of 55 kDa or 56 kDa, respectively, that place these proteins in the size of LeCER6 (Millar *et al.*, 1999; Kunst and Samuels, 2003; Qin *et al.*, 2007). Deduced from the strong sequence identity to GhCER6, AtCER6 and AtCER60, LeCER6 was identified as β-ketoacyl-CoA synthase responsible for the condensation reaction in VLCFA biosynthesis. The LeCER6 enzyme showed also a similarity to other known β-ketoacyl-CoA synthases of *Oryza sativa*, *Marchantia polymorpha* and *Physalis fendleri*, but amino acid sequence identity was on a distinctly lower level in comparison to GhCER6, AtCER6 and AtCER60.

Sequence analysis of LeCER6 displayed two putative transmembrane-spanning domains close to the N-terminus, indicating that LeCER6 protein might be anchored in a membrane. Previously, Bessoule *et al.* (1989) noted the membrane-associated nature of fatty acid elongation enzymes in epidermal cells, the major site of VLCFA biosynthesis. Furthermore, the presence of two adjacent transmembrane domains is consistent with amino acid sequences of GhCER6, AtCER6 and AtCER60 (Millar *et al.*, 1999; Qin *et al.*, 2007). However, a C-terminal consensus motif comprehending two lysine (K) residues that might serve as a retention signal of endoplasmic reticulum proteins, as described by Jackson *et al.* (1990), were not detectable.

The region downstream of the transmembrane domains constitutes the globular, catalytic domain of LeCER6. Based on its identity to the specific structures of FAE1 of *Arabidopsis*, highly conserved cysteine (C^{263}), histidine (H^{430}) and asparagine (N^{463}) were predicted to be the catalytically critical residues in the active site architecture of LeCER6 (Ghanevati and Jaworski, 2001, 2002). The functional similarity and limited sequence identity of membrane-bound and soluble condensing enzymes strongly support the idea of a generally tripartite reaction mechanism (Ghanevati and Jaworski, 2002). During this enzymatic reaction, cysteine, histidine and asparagine catalyze the condensation reaction

in fatty acid elongation with the use of malonyl-CoA as donor and long-chain acyl-CoA as acceptor in order to generate VLCFA. The initial step involves the transfer of a long-chain acyl-CoA substrate onto the cysteine residue at the active site of the enzyme. The catalytic cysteine, a nucleophilic thiolate, serves as a covalent anchor that generates an acyl-CoA thioester intermediate (Lanz *et al.*, 1991; Lassner *et al.*, 1996). Obviously, in the following step the decarboxylation of the malonyl-CoA substrate produces a carbanion intermediate *via* catalysis of the active site histidine and asparagine residues. There is some evidence that hydrogen bonds stabilize the carbanion intermediate, which operates as the nucleophile for the condensation reaction (Davies *et al.*, 2000; Jez and Noel, 2000). The subsequent nucleophilic attack of the carbanion intermediate on the carbonyl carbon atom of the primed thioester intermediate leads to the release of a condensing β-ketoacyl-CoA product from the enzyme (Qiu *et al.*, 1999; Ferrer *et al.*, 1999). Apart from this putative catalytic triad, a phenylalanine residue (F^{233}) is proposed to promote the orientation of the substrates and assist the intermediate conversion at the active site (Ferrer *et al.*, 1999).

Closely connected to the functional importance of at least these protein residues participating directly in catalysis of LeCER6, the arrangement of catalytic residues also benefits the activity of the overall fatty acid elongation system. Because the active site constellation probably governs the structural features for substrate specificity of the enzyme (Huang *et al.*, 1998; Davies *et al.*, 2000). This is of particular importance, since the condensation reaction is proposed to be the rate-limiting step of the VLCFA elongation pathway (Millar and Kunst, 1997). Thereby, very-long-chain condensing enzymes control the functional uniqueness of the elongation systems (Shimakata and Stumpf, 1982; Stumpf, 1984; Trenkamp *et al*., 2004). Both factors mirror the immense role of the LeCER6 activity.

'Reverse genetic' analysis reported in this study reinforces a role for LeCER6 as a key enzyme in the generation of very-long-chain fatty acids > C_{31}. Similar to these findings, previous studies found the essential role of AtCER6 and GhCER6 for the elongation of acyl-CoA as substrates longer than C_{25}, respectively (Millar *et al.*, 1999; Qin *et al.*, 2007). Loss-of-function mutation of these genes displayed no analogous activity of this enzyme. Coherent with the requirements for the biosynthesis of a variety of VLCFA products in different plant tissues, the tomato genome contains a multigene family encoding β-ketoacyl-CoA synthases. Although LeCER6 provides a part of this β-ketoacyl-CoA synthase family, there was no evidence for any paralogous origins in the tomato genome

that might explain a redundancy of function. Hence, a deficiency in this enzyme function originated a block in the elongation of very-long-chain fatty acids.

So far, there was little knowledge about the effects of *Cer6* overexpression in the wild type background. Based on fatty acid profiles of transgenic *Arabidopsis* plants, Hooker *et al.*, 2002 reported that the overexpression of condensing enzyme AtCER6 under the control of epidermis-specific *AtCer6* promoter led to a significantly higher VLCFA deposition on the stem. Moreover, effective manipulation of surface VLCFA biosynthesis appears to require correctly timed gene expression (Millar *et al.*, 1999). In transgenic tomato plants, the *AtCer6* promoter was demonstrated to be very effective in directing epidermis-specific expression, which altered the cuticular characteristics by enhancing the epicuticular wax layer. Karaba (2007) demonstrated that tomato transformants revealed a reduced cuticular water loss and an improved drought resistance compared to the wild type.

Changes in Cell-Cell Communication: Pleiotropic Effects of LeCER6 Deficiency on the Epidermal Barrier Properties of Tomato Plants

On the biochemical level, LeCER6 deficiency affects the VLCFA biosynthesis of chain lengths > C_{31}, which modify the cuticular wax composition and, consequently, the transpirational barrier property of MicroTom fruit and leaf cuticles. However, additional phenotypic differences between MicroTom wild type and MicroTom *lecer6* plants were identified, for example a change in floral morphology that results from organ fusion of stamen and petals and the impairment of pollen grain hydration. Closer investigations of these apparently distinct processes are necessary to understand whether or not one common underlying mechanism controls these biochemical and morphological properties. Primarily, these findings support the idea that VLCFA biosynthesis catalyzed by LeCER6 contributes cuticular wax production, floral organ separation as well as pollen grain germination in tomato.

Floral Organ Fusion: From the mechanical point of view, the epidermis and its extracellular cuticle forms a multifunctional interface that acts as a selective barrier against environmental influences and, in particular, has consequences for cell-cell interactions (Lolle and Pruitt, 1999; Riederer and Schreiber, 2001). Thereby, the architecture of the epidermal cell layer influences biological interactions: blocking some reactions while permitting others (Sieber *et al.*, 2000). Cusick (1966) already noted the epidermal response of post-genital organ fusion achieved by a change in the sensitivity of the epidermis to

physical contact with other epidermal cells, as seen for MicroTom *lecer6*. This exceptional biological event, mainly occurring during floral ontogenesis, was further manifested in a large collection of *Arabidopsis* mutants (Koornneef *et al.*, 1989; Lolle *et al.*, 1998). Among others, studies on *CER10*, *DEADHEAD* (*DED*; Lolle *et al.*, 1998), *FIDDLEHEAD* (*FDH*, β-ketoacyl-CoA synthase; Yephremov *et al.*, 1999), *WAX2* (acyl-CoA reductase; Chen *et al.*, 2003) and *LACERATA* (*LCR*, cytochrome P_{450} monooxygenase; Wellesen *et al.*, 2001) genes also revealed a critical role for lipid biosynthesis. Although a number of these mutants are characterized at the molecular level, little is known about the mechanistic regulation of the organ fusion process so far. The diverse functions ascribed to these genes in lipid biosynthesis suggest that a change in the epidermal permeability that arises from perturbation of the structural integrity provides the only common denominator (Krolikowski *et al.*, 2003).

However, Siegel and Verbeke (1989) provided evidence that this epidermis-specific interaction is controlled by a reciprocal recognition event, mediated by small diffusible signaling molecules, which are exchanged between apposed cells. Consequently, alterations in epidermal barrier properties may permit co-segregation of a morphogenetic active factor that initiates a signal transduction cascade leading to epidermal cell adhesion and organ fusion.

In conclusion, LeCER6 plays a role in modifying the cuticular and therefore the epidermal barrier properties. Nevertheless, on the basis of existing examinations it is not clear how *lecer6* mutation can be reconciled with the organ fusion phenotype. Several possible scenarios are possible consequences of LeCER6 loss-of-function: VLCFA contribute to proper organ separation or VLCFA influence members of the signaling pathway participating in this process. Otherwise, VLCFA may be required for the optimal epidermal assembly that prevents the susceptibility of the epidermis for the organ fusion promoting factor. The nature of the signaling molecules for cell adhesion competence remains elusive, but ultimately the organ fusion phenotype of *lecer6* mutant may also indicates an indirect effect of an enhanced epidermal permeability, although no corroborating evidence supported this hypothesis (Lolle *et al.*, 1998).

However, despite the structurally abnormal flowers of MicroTom *lecer6* compared to MicroTom wild type plants, it can be concluded that the fused flowers of *lecer6* mutant were functionally intact. In light of self-compatibility of tomato plants female and male gametophytes were able to interact in self-pollination. Hence, it is important in this context

that fertilization is sterically not hindered by the floral imperfection of MicroTom *lecer6*, but eventually impaired in their rate when compared with MicroTom wild type plants.

Pollen-Stigma Communication: Following pollination, pollen-stigma interaction provides another important cell-cell communication between epidermal derivatives in fertile flowering plants (Gillaspy *et al.*, 1993). Mature pollen grains of angiosperms are highly desiccated and metabolically inert at time of dispersal. Only at the point of landing on the stigma, the pollen grain becomes hydrated by the stigmatic surface. This is necessary to develop osmotic pressure and to regain an activate metabolism as well as to initiate elongation of the pollen tube into the stigma (Cheung *et al*., 2000). The requirement for pollen hydration is a crucial regulatory barrier in fertilization, which allows specific pollen grain discrimination by stigmatic water release (Herrero and Dickinson, 1981). However, the molecular details of this pollen-stigma signaling are still unknown (Swanson *et al.*, 2004).

Particularly with regard to the pollen-stigma interaction, the presence of a pollen coat surrounding the pollen grain of species with 'dry-type' stigma (for example brassicaceous species) or the existence of exudates on stigmatic surface of species with 'wet-type' stigma (for example solanaceous species) was shown to be essential (Wolters-Arts *et al*., 2002; Bots *et al*., 2005). In plants with naturally 'dry' stigma, a lipid- and protein-rich, extracellular pollen coat is necessary for recognition of the pollen grain by the stigmatic surface. Fluid transfer from the stigma to the pollen grain depends on lipid-based channels formed by the pollen coat *via* direct physical contact with the stigmatic surface (Preuss *et al.*, 1993; Hülskamp *et al.*, 1995). Conversely, 'wet-type' stigma has a secretory zone found to produce complex lipid-rich exudates in a distinct surface secretion. The exudates matrix, which regulates the water flow from the stigmatic surface to the pollen grain, is arranged in a hydrophobic and a hydrophilic layer surrounding the surface of the secretory zone. At the binding side with the stigma surface, the pollen grain sinks through the exudates and thus comes into contact with a thin layer containing free water (Goldman *et al.*, 1994; Wolters-Arts *et al.*, 2002).

The LeCER6 deficiency resulted in sterility of the tomato plants. However, the sterile phenotype of *lecer6* mutant plants was reversed in a high humidity environment, where atmospheric water most likely bypassed the loss-of-function mutation. The fertility defect was only conditional, whereas the floral organ fusion was not averted. Moreover, it could be demonstrated that the competence for producing viable male pollen grains or female stigma cells was not affected by LeCER6 deficiency. Thus, seed maturation and

furthermore dispersal of *lecer6* mutant was possible without restriction. This observation strongly indicates that a reduction of VLCFA and its derivatives in MicroTom *lecer6* generated a disruption in stigmatic communication with the pollen grains at low humidity.

In *Arabidopsis*, characteristic features of *eceriferum* (*cer*) mutants include both, alterations of hydrophobic surface structures of aerial plant organs and the partial lack of fertility (Koornneef *et al.*, 1989; Post-Beittenmiller, 1996). Among these, AtCER6 plays an important role in biosynthesis of very-long-chain lipids (Preuss *et al.*, 1993). Very-long-chain lipids are required for the production of cuticular waxes and are also essential components in the assembly of the pollen coat, contribute pollen hydration and have an effect on migration of the coating onto the stigma surface in *Arabidopsis* (Zinkl *et al.*, 1999; Fiebig *et al.*, 2000). However, a mutation of AtCER6 substantially decreases all classes of very-long-chain lipids > C_{25} (Jenks *et al.*, 1995; Fiebig *et al.*, 2000). Exposed to a relatively dry environment, *atcer6* mutant plants are defective in pollen recognition and fail in pollen hydration by depleting these lipids, which are apparently functionally important signals to stimulate the release of water from the stigma to the pollen (Preuss *et al.*, 1993; Hülskamp *et al.*, 1995). Consequently, AtCER6 deficiency evokes a conditionally male sterility, which specifically affects pollen function. Deduced from outcomes in *Arabidopsis* and based on previous findings on solanaceous species, LeCER6 mutation might cause female sterility due to a stigmatic defect of tomato plants, though the possibility of a sterile pollen grain can not be ruled out. One can expect that pollen grains of *lecer6* mutant that reach the stigma are unable to communicate with the stigma, since contact itself is probably not sufficient to induce pollen hydration. Due to the lack of VLCFA and their derivatives, *lecer6* mutant pollen grains may remain desiccated at the stigma surface bearing an abnormal exudates matrix instead of germinating. Malfunction in fertilization and seedlessness are the consequences of these tomato plants.

To conclude these considerations, the adhesion of a compatible pollen grain triggers fluid mobilization of the 'dry'- or 'wet-type' stigma, revealing a highly sensitive and specific regulation system (Zinkl *et al.*, 2000). Although molecules that mediate in pollen-stigma recognition are not identified, the role of very-long-chain lipids in tomato and *Arabidopsis* evidently supports the idea that these compounds directly control the intercellular water flux, thereby directing the pollen tube growth. Hülskamp *et al.* (1995) proposed that the combination of specific lipid matrix properties and some other type of molecules biochemically determines the pollen-stigma-communication.

Above-Ground Plant Growth: The present study demonstrates the effects of LeCER6 condensing enzyme that contributes to the VLCFA elongation in cuticular wax biosynthesis of tomato in detail. It is clearly shown that LeCER6 activity affects epidermal barrier properties against uncontrolled passage of water or small water-soluble signaling molecules across a lipid interface. An orchestration of biosynthetic pathways and cell-cell interactions is necessary to mediate the fusion of male and female gametophytes in order to produce viable seeds. The complex of seed formation is controlled by an intricate interplay of genetic make-up, environmental influences and hormonal signals. In consequence, almost seedless plants are supposed to affect the pattern of endogenous hormone production and thus plant development (Gillaspy *et al.*, 1993). The nearly seedless plants of MicroTom *lecer6* had an increased formation of flowers, which resulted in a higher rate of fruit set and a highly elevated number of ripe fruits. Evidently, these attributes are associated with an augmented production of plant biomass. In contrast to MicroTom wild type plants of a small size of approximately 10 cm, LeCER6 deficient plants exhibited a bushier and twofold increased plant height (Scott and Harbaugh, 1989; Meissner *et al.*, 1997). However, the abnormal phenotype of MicroTom *lecer6* produces fruits with extraordinary small number of seeds or fruits even lacking seeds, although the mean total number of fruits per plant is enhanced. Consequently, the increase of plant growth combined with elevated numbers of fruits partially compensates the low quantity of seeds per fruit and furthermore provides seed dispersal. In accordance, Mazzucato *et al.* (1998, 1999) demonstrated that a shift in hormonal balance was strictly related with the production of seedless fruits, which boosted the mechanism of fruit set of parthenocarpic tomato lines.

Apart from a strictly hormone-associated altered plant development, changes in source-sink relationships within the plant might also affect the tomato plant size. A modified number of seed-bearing fruits as a potential sink may influence source demand. Accordingly, in MicroTom the balance between source and sink shifts towards seedless plants, which offers a transition of resources to an increased biomass accumulation (Gillaspy *et al.*, 1993). Despite significant effects on the hormonal and physiological status primarily originated from a disturbance of VLCFA biosynthesis in *lecer6* MicroTom plants, these variations are not directly linked with LeCER6 activity.

Variations between Tomato Wild Type and *lecer6* Mutant: Secondary Effects of LeCER6 Deficiency on Tomato Fruit Development

With their highly specific visual, physical, chemical and genetic characteristics fleshy berry tomato fruit can be regarded as functionally unique. In order to understand potential molecular, biochemical and physiological changes of the metabolic mechanisms, distinct fruit parameters were comparatively investigated for MicroTom wild type and MicroTom *lecer6* during fruit development.

Aspects of the Temporal Factor: MicroTom represents an excellent model system for studies on fruit development due to its relatively short life cycle, namely a production cycle of approximately a three month span (Meissner *et al*., 1997). The average number of days from sowing to anthesis valued at most 40 days, followed by additional 38 days to 42 days from date of fruit set to fruit color turning. Thus, red ripe MicroTom wild type fruits yielded approximately 75 days after sowing.

The course of fruit development differentiates into several phases (Giovannoni, 2004). During the transition from immature green (category I) to mature green (category II) stage the tomato fruits expanded and the epidermal of cell density increased. These green fruit stages are the longest period in fruit development that contributed to about 85% of the increase in fresh weight of the MicroTom fruits (Lemaire-Chamley *et al.*, 2005). In contrast to the green and photosynthetically active stages, the later changed in color from bright yellow (category III) to orange (category V) represents a short time frame only. Red fruit phases (category VI and VII) are the latest stages in fruit development, representing ripe and overripe fruits. These fully developed, bilocular fruits enclose mature seeds in a red fleshy, soft and thick pericarp surrounded by a gel (Gillaspy *et al.*, 1993).

Aspects of the Physical Appearance: Tomato fruit development is influenced by genetic determination, hormonal and physiological regulation as well as environmental conditions including plant cultivation. Grown under standardized conditions MicroTom wild type produced red fruits with a surface area and a fresh weight, which is in accordance with data documented by Meissner *et al.* (1997). In contrast to the MicroTom wild type, fully ripened red *lecer6* mutant fruits had a more than 50% reduced surface area and fresh weight. Corresponding to that effect, the volume of LeCER6-deficient fruits was distinctly diminished compared with the MicroTom wild type.

In particular, the final size and weight of the fruit reflects a genetically controlled number of cell divisions and rate of cell expansion occurring during fruit development (Coombe, 1976; Gillaspy *et al.*, 1993). In determining the rate of fruit expansion, the fruit

epidermis is of importance (Thompson *et al.*, 1998). A continuous rise in epidermal fruit cell density as well as the extension of cell volume indicated that both, cell division and cell enlargement take place in the green developmental stages (category I and II) of MicroTom wild type and MicroTom *lecer6*. After fully maturation the number of cells per fruit remained more or less constant. Nevertheless, the number of cells per fruit varied significantly between fruits of both MicroTom lines that contributed to the final size of wild type and *lecer6* mutant fruits. The number of epidermal cells, which enclosed the mutant fruit, was distinctly decreased in comparison to MicroTom wild type.

On the one hand, it is generally accepted that the number of enclosed seeds can hormonally control the rate of cell division in the surrounding tissue and thus fruit growth (Nitsch, 1970; Asahira and Hosoki, 1977; Falavigna *et al.*, 1978). Taking developing seeds as regulatory signal generator failed almost completely in fruits of *lecer6* mutant, which might lead to a reduced fruit size of MicroTom *lecer6*.

On the other hand, it can be concluded that MicroTom *lecer6* exhibited a reduced fruit size due to an increased water deficit, causing restricted metabolic capacity of the fruit. Fruit physical attributes, such as fresh weight, dry weight, surface area, volume, water content and epidermal cell density are highly associated with each other and varied significantly between MicroTom wild type and MicroTom *lecer6*. Moreover, these factors showed a negative correlation with the rate of transpirational water loss suggesting an association with water management of the tomato fruit. Similar observations were made in studies on pepper fruits (*Capsicum annuum* L.) by Lownds *et al.* (1994) and Maalekuu *et al.* (2005), although the rate of water loss and the surface area only weakly correlated.

Aspects of the Optical Feature: Color perception during fruit development depended on both, type and concentration of pigments contained in the fruit. Major pigment changes in tomato fruits refer to the degradation of chlorophyll and synthesis of carotenoids (Grierson, 1986). This deposition was associated with the transition from green to red as chloroplasts were transformed to chromoplasts during tomato fruit development. In addition to the known role of the pigments, for example in energy transfer and photoprotection, the structure of the tomato fruit also affected their visual appearance (Demmig-Adams and Adams, 1996).

The light scatter and absorption had an impact on this property, for example *via* color, opacity, translucency and gloss (Goodwin, 1976). Thus, the optical feature of tomato fruits can be regarded as a cumulative effect of color and structure. At the beginning of fruit development, the texture of green tomato fruits (category I and II) was compact with very

small intercellular spaces. Cells that contribute to most of the pericarp tissue were large and vacuolated and contained most of the chloroplasts, which give the developing fruits their green appearance. Cells in the inner layer as well as the outer layer were smaller and had fewer chloroplasts (Gillaspy *et al.*, 1993). The incident light was highly scattered and reflected, consequently, the tomato fruit appeared matt.

The efficiency of photosystem II in tomato fruits, measured by chlorophyll fluorescence (F_V/F_M), was at most detected at 0.65 ± 0.08 in category I, whereas the quantum yield of photosystem II in intact tomato leaves was calculated approximately of 0.80 (Grichko *et al.*, 2005). The decline of chlorophyll a and chlorophyll b valued about 75% up to the breaker stage of fruit development, when fruit color turned. These data correlated positively with the decrease of photosystem II efficiency during the transition from immature green stage (category I) to breaker stage (category IV) by 73% reported in the present study. The time course of degradation for gene expression profiles for components of the photosynthetic apparatus, the content of photosynthetic pigments and the photosynthetic activity showed a simultaneous decrease in tomato fruits (Alba *et al.*, 2004).

With beginning of the breaker stage, lycopene and β-carotene, which are the pigments largely responsible for the characteristic coloration of red ripe tomato fruit, started to accumulate. In red fleshy tomato fruits, the cells were swelled with fluid, more intercellular spaces emerged and fruits softened in comparison to green fruit stages causing an alteration of the fruit texture, which less distorts the path of incident light (Hetherington and MacDougall, 1992). Ultimately, more light was transmitted rather than scattered or reflected. However, red tomato fruits exhibited a glossy exterior.

Following this progression, photosystem II efficiency was only marginal detectable. Compared with green fruits, in fleshy fruits of category VII carotenoid concentration increased fivefold and actually dominated the pigment composition (Fraser *et al.*, 1994). In accordance with the developmental classification *via* chlorophyll fluorescence in the model system tomato fruit, the visual arrangement of MicroTom fruits into seven developmental categories could be verified for most fruits. Hence, five out of seven fruit categories could be significantly differentiated by a quantitative and non-invasive measurement.

Aspects of the Metabolic Character: Complex fruit developmental processes involve multiple metabolic changes, which may affect sensory quality of tomato fruits. In particular, contents of soluble sugars and amino acids are a very important fruit quality

determinant and may play an essential role in stress responses caused by osmotic imbalance. For example, sugars represent up to 60% of the total dry weight, which enhances sweetness and flavor intensity of red tomato fruits. Sucrose, glucose and fructose are the most abundant sugars found in fruits of cultivated tomato *Lycopersicon esculentum*, with characteristically high hexose accumulations (Yelle *et al.*, 1991).

As mentioned above, developing fruits have functional chloroplasts and contain photosynthetically active proteins (Piechulla *et al.*, 1986, 1987). During the photosynthetic active phase, immature green and mature green fruits contribute to the accumulation of glucose of tomato fruits. However, the fleshy berry fruit type of tomato follows a transition from a partially photosynthetic to a heterotrophic metabolism during development. Consequently, photosynthetic carbon dioxide fixation in leaves combined with a translocation of photo-assimilates in a sink-source relationship is essential for tomato fruits (Ho, 1988, 1996; Gillaspy *et al.*, 1993; Carrari and Fernie, 2006).

The increase in glucose correlates with the initiation of fruit expansion in the two MicroTom lines. While the glucose content increases in similar levels in MicroTom wild type and MicroTom *lecer6* fruits, the later ripening stages (category V to VII) *lecer6* mutant stored a significant higher level of glucose than the wild type. Ultimately, the glucose content of the fruit tissue was twofold higher in red ripe *lecer6* mutant fruits when compared with the wild type fruits. Based on the fact that the size of LeCER6-deficient fruits is less than half of those of MicroTom wild type, these findings suggest that the total glucose content per fruit corresponds with its physiological demands. In *lecer6* mutant fruits growth reduction may lead to an improved the fruit quality *via* glucose accumulation (Daie, 1996; Mazzucato *et al.*, 2003; Claussen, 2005). A decrease in water content in LeCER6-deficient fruits was obviously not accompanied by a changed flow of photoassimilates from source leaves to fruits, but may explain higher glucose content in red ripe mutant fruits compared to fruits of wild type plants.

Glucose can serve as immediate sources of energy and is not metabolically inert (Yancey, 2005). Based on a strong affinity for water, glucose might be implicated in osmotic stress responsiveness (Cushman and Bohnert, 2000). In general, the accumulation of osmotically active compounds, predominantly sugars, lowers the osmotic potential and probably improves the capacity to maintain the turgor pressure in pericarp cells changing from 0.14 to 0.03 MPa during tomato fruit development (Tyree and Jarvis, 1982; Grierson, 1986; Shackel *et al.*, 1991; Ruan *et al.*, 1996; Almeida and Huber, 1999; Guichard *et al.*, 2001; Claussen, 2005, Claussen *et al.*, 2006). The degree of metabolic variance between

MicroTom wild type and MicroTom *lecer6* may determine the plants adaptation to cuticular water loss stress (Gong *et al.*, 2005; Granier *et al.*, 2006; Bohnert *et al.*, 2006; Mittler, 2006; Yamaguchi-Shinozaki and Shinozaki, 2006).

The permeance for water of tomato fruits significantly controls the water balance of tomato fruits, and hence their osmotic potential. It can be hypothesized that the osmotic potential substantially differs between fruits of both MicroTom lines in response to limited water barrier property of *lecer6* mutant fruits. Higher concentrations of glucose in fruits of MicroTom *lecer6* may be responsible for an improved adaptation to water limiting conditions displaying a greater stress tolerance.

Furthermore, an interconnection between abscisic acid, jasmonic acid, ethylene and sugar metabolism under stress relevant conditions is already known from the literature. For example, glucose stimulates jasmonic acid responsiveness of lipoxygenases (LOX; Creelman and Mullet, 1995; Gazzarrini and McCourt, 2001; Çakir *et al.*, 2003; León and Sheen, 2003; Carrari *et al.*, 2004). In this case, the enhanced stress tolerance is not predicated on osmotic adaptation, but rather by other mechanisms such as protection from reactive oxygen and stabilization of macromolecular structures (Deguchi *et al.*, 2006).

The level of protein in fruit was used to assess the influence of LeCER6 deficiency on changes in protein biosynthesis and possibly in biosynthesis activity. During fruit development, numerous proteins act in a time-based delimitation: photosynthetically active proteins or proteins related to the wax biosynthesis are active in the earlier fruit stages, while the expression of other genes is not induced until tomato fruit ripens, for example proteins participating in ethylene biosynthesis (Gillaspy *et al.*, 1993). Induction and reduction events evoke tremendous changes in protein biosynthesis influencing the general protein level during fruit development.

The highest total protein content was found in immature green fruits, which are in the biosynthesis active phase in fruit development. In the course of fruit maturation, the total protein content decreased as consequence of fruit expansion of MicroTom wild type and MicroTom *lecer6*. Therefore, the total protein level per fruit varied only weakly within the fruit ripening process. Similar fluctuations were detected in the protein content of both MicroTom lines. Minor differences in protein content between MicroTom wild type and *lecer6* mutant fruits were probably based on the lower water balance in the mutant fruits. Even though a slightly increased level of protein content in MicroTom wild type fruits was detected, similar protein concentration per fruit of both MicroTom lines were generated.

A similar pattern, substantiating these results, was revealed by examining RNA content in MicroTom wild type and *lecer6* mutant fruits within this study (CHAPTER II). In this case, fruit tissues without enclosed seeds showed also a decline between wild type und LeCER6-deficient fruits. Accordingly, a balanced gradient in concentration of RNA and proteins may be based on different amounts of water contained in fruits of both MicroTom lines.

In conclusion, the content of soluble metabolites varied greatly in tomato fruits, mainly depending on the development stage and the tomato line. Thereby, alterations in biochemical demands and the physiological state of plants played decisive roles for water stress tolerance during fruit development. Obviously, protective mechanisms evolved, those for example affecting the transcriptional and translational machinery as well as physiological and metabolic processes to various degrees (Gong *et al.*, 2005).

Aspects of the Surface Property (Ultrastructural Architecture): Scanning electron microscopic analyses of enzymatically isolated MicroTom wild type and *lecer6* mutant fruits revealed a thin cuticular membrane, which enclosed incrementally the epidermis and extended into the subepidermal tissue. The thickening of the cuticle during tomato fruits ripening determines the overall biomechanical strength of the whole organ (Jones *et al.*, 1997; Matas *et al.*, 2004; Bargel and Neinhuis, 2005). The tensile forces are most probably highest in the outermost layer of the fleshy berry, which reinforce the significant contribution of the cuticle for mechanically stability particularly in soft, red ripe tomato fruits (Niklas, 1992). Hence, the material behavior of ripe tomato fruit cuticles was described as visco-elastic by Petracek and Bukovac (1995). Crucial in this context, the cuticular membrane does not only thicken during the fruit ripening process, but it also has to compensate growth of the epidermal cells and finally the entire fruit without impairing their physiological and mechanical integrity (Baker *et al.*, 1982; Edelmann *et al.*, 2005). This process is accompanied by an increase in biosynthesis activity and a significantly accumulation of the cuticular material, especially during the earlier fruit stages, which represent the fruit expansion phase of MicroTom wild type and MicroTom *lecer6*, as observed in the present study.

The cuticle of higher plants is a chemically heterogeneous membrane, basically consisting of mainly two components: the insoluble biopolymer cutin, which generates the framework of the cuticle, and the overlying, solvent-soluble wax fractions (Kolattukudy, 1980; Jeffree, 2006). Evidence is provided that the chemical composition of the fruit cuticle in relation to their developmental stage was subject to distinct dynamic changes of

both cutin and cuticular waxes (CHAPTER I). However, albeit substantial variations in the total cuticle amount between MicroTom wild type and *lecer6* mutant fruits, a visual discrimination of both MicroTom lines based on the structural cuticular characteristics was not possible.

A closer look at the tomato fruit cutin reveals a three-dimensional, high molecular weight polyester of various mostly dihydroxyhexadecanoic acids (Kolattukudy, 1980, 1996; Holloway, 1982; Holloway and Wattendorff, 1987; Walton, 1990; von Wettstein-Knowles, 1993). It forms a matrix with different texture depending on its chemical status as well as on its degree of density and cross-linking. For example, higher degrees of cross-linking were found after esterification of secondary hydroxyl groups of the cutin monomers in addition to the formation of elongated and orientated superstructures, which increased during the later stages of fruit development. Described as an effect of this interlacing, the topography of the outer surface of young fruits was modulated by soft and spaced wrinkles, whereas surfaces of ripened fruits appeared flatter (Benítez *et al.*, 2004). Although significant quantitative differences between the two MicroTom lines did not lead to modifications in the cuticular architecture, an almost double cuticle amount but a similar cutin monomer distribution might imply a higher molecular density of the mutant cuticle in comparison the wild type. Equivalent alterations in cuticle architecture were an integral element of the ripening program in both MicroTom lines. These observations were not unexpected, because as sufficiently described in the literature, there was no relationship detectable between structural appearance and permeability of the plant cuticle (Schönherr, 1982; Becker *et al.*, 1986; Lendzian and Kerstiens, 1991; Vega *et al.*, 1991; Schreiber and Riederer, 1996; Riederer and Schreiber, 2001).

Cuticular waxes, a general term for complex mixtures of homologous series of long chain aliphatic compounds with the addition of varying proportions of cyclic compounds, are associated with the cutin matrix and act as supporting filler of the cuticle necessary to maintain integrity and elasticity of the tomato fruit (Kolattukudy, 1980; Baker, 1982; Holloway, 1984; Walton, 1990; von Wettstein-Knowles, 1993, 1995; Riederer and Markstädter, 1996; Heredia, 2003). Based on experiments with dewaxed fruits, the biomechanical importance of the waxy fruit exterior to prevent cracking of tomato fruits was documented proceeding with fruit ripening in the two MicroTom lines (data not shown). Furthermore, it is supposed that very-long-chain aliphatic hydrocarbons of the cuticular wax, which were found for MicroTom wild type to a high degree, exhibit a closer molecular packing, forming a higher barrier to water loss within the cuticle, than cyclic

wax compounds dominating the *lecer6* mutant wax (Casado and Heredia, 2001; Riederer and Schreiber, 2001; Burghardt and Riederer, 2006). Nonetheless, the astomatous fruit surface of MicroTom wild type as well as MicroTom *lecer6* was enclosed with a crystal-free, epicuticular wax film. Thereby, the wrinkled waxy surface shown for red ripe MicroTom fruits (category VI) possibly imitate the cutin surface structure, which is documented by Benítez *et al.* (2004). In this case a direct comparison disclosed more diffuse epidermal contours of red ripe MicroTom wild type fruits than for MicroTom *lecer6* and lead to the notion of a higher epicuticular wax accumulation on wild type fruits. This agrees well with the quantitative variations within the epicuticular waxes yielding approximately 4 μg cm^{-2} for MicroTom wild type and less than 1 μg cm^{-2} for MicroTom *lecer6*, respectively (data not shown).

In conclusion, the cuticular membrane underwent an extensive increase in thickness and compositional changes during the development of fruits of both, MicroTom wild type and *lecer6* mutant, respectively. These modifications of the cuticle membrane caused directly, providing a biomechanical support, and indirectly, regulating water balance and physiological integrity of the tomato fruit (Saladié *et al.*, 2007).

Aspects of the Surface Property (Pathogenic Resistance): The chemical composition of the cuticle plays an important role in resistance against pathogenic invasion representing a potent source of defensive compounds, since the hydrophobic cuticular waxes guard the fruit surface from accumulation of atmospheric particles and prevent the formation of water films; both are capable of impairing germination of pathogen conidia spore (Holloway, 1970; Swain, 1977; Doss *et al.*, 1993; Knoll and Schreiber, 1998). LeCER6 is implicated in the control of the barrier property of the cuticular membrane of the MicroTom fruit. Therefore, the altered cuticle profile of LeCER6-deficient fruits might be linked with a modified pathogenic resistance of tomato fruits.

In a bioassay, the powdery mildew fungus *Blumeria graminis* was found to trigger pre-penetration processes on both freshly peeled skin and enzymatically isolated cuticular membrane of tomato fruits depending on the fruit developmental stage. Mature green (category II) and red ripe fruits (category VI) of MicroTom wild type and MicroTom *lecer6*, respectively, were in the focus of this investigation. During the primary infection of *Blumeria graminis*, plant cutin plays a central role favoring attachment and development of primary and appressorial germ tubes (Edwards, 2002). The conidium produces exudates containing cutinase to mediate tenacious anchorage on the plant surface as well as to enzymatically degrade the cuticle; both facilitate the initial penetration by

Blumeria graminis. Small amounts of cutin monomers released from cutin hydrolysis in turn stimulate appressorial differentiation of *Blumeria graminis* (Kunoh *et al.*, 1988).

Whereas cutin was detected in all tomato fruit cuticle samples, MicroTom *lecer6* possibly showed a higher degree of cutin cross-linking accompanied with an elevated firmness compared to the wild type. Thus, the germination activity of *Blumeria graminis* was similar on cuticles of mature green and red ripe fruits within both MicroTom lines. Observations revealed that the germ tube emergence on fruit peel tissues differed only between MicroTom wild type and *lecer6* mutant, therefore an influence of the cutin matrix on conidial morphogenesis of *Blumeria graminis* can not be ruled out.

MicroTom wild type and MicroTom *lecer6* fruit peel tissue was able to induce appressorial differentiation of *Blumeria graminis*. The results displayed highest appressorial differentiation-inducing activity for mature green wild type fruits. In contrast, fruit peel tissue of red ripe *lecer6* mutant fruit was more likely to have an inhibitory effect on appressorial differentiation. The stimulation of the appressorial differentiation of *Blumeria graminis* was described as specific to the cuticular wax layer (Tsuba *et al.*, 2002).

A series of examination on non-host plants published by Tsuba *et al.* (2002) resulted in approximately 15% appressorial differentiation of *Blumeria graminis* conidia on epidermal leaf strips of tomato. The datasets of tomato leaves and freshly peeled tomato fruit surfaces were consistent and lead to the conclusion that tomato in general hardly stimulate appressorial differentiation of *Blumeria graminis*. In the interpretation of Tsuba *et al.* (2002) the frequency of appressorial differentiation refers to the specific composition of cuticular waxes; a mixture of several very-long-chain aliphatic and cyclic compounds.

Various wax components are known to affect the frequency of appressorial differentiation in a dose-dependent manner. On the one hand, very-long-chain lipids ($> C_{25}$) enclosed in the cuticular wax promote the species-specific germination and differentiation of conidia (Podila *et al.*, 1993). Thereby, the aldehyde fraction of the cuticular waxes, in particular hexacosanal (C_{26}), and to a minor degree alkanols, precursors in aldehydes biosynthesis, was shown to stimulate appressorial differentiation of *Blumeria graminis* (Tsuba *et al.*, 2002). Nonetheless, it is still unclear whether or not hexacosanal alters the physical properties of the fruit surface, such as hydrophobicity and structure of the epicuticular wax, rather than acting as a chemical signal promoting the appressorial differentiation (Carver *et al.*, 1996; Tsuba *et al.*, 2002; Zabka *et al.*, 2008).

On the other hand, one can conclude that the inhibition of *Blumeria graminis* differentiation might refer to the toxicity of certain compound classes and/or the chain length distribution within the cuticular wax mixture. Fruit specific anti-fungal effects of cyclic compounds associated with the cuticular membrane, such as terpenoids and sterol derivatives, were pronounced by a multitude of studies (Singh and Dubey, 2001; Jain *et al.*, 2001; Smania *et al.*, 2003; Zinsou *et al.*, 2006). However, the character of these constituents in the pre-penetration processes is still controversially discussed (Kolattukudy *et al.*, 1995).

The present study suggested that appressorial differentiation was favored on mature green MicroTom fruits with lower quantities of surface waxes. The higher wax accumulation in combination with a putative higher cuticle cross-linking as observed in red MicroTom fruits might considerably contribute to a reduced differentiation rate of *Blumeria graminis*. In addition, mature green wild type fruits exhibited lower quantity of triterpenoids and sterol derivatives (inhibitory effect) and a higher proportion of very-long-chain aliphatic compounds (inducing effect) in the wax fraction as produced by the *lecer6* mutant fruit.

Appressorial differentiation was demonstrated almost exclusively on peeled fruit tissues. Enzymatically isolated cuticles exhibited a reduced germination activity and induced only minor appressorial activity of *Blumeria graminis* on MicroTom wild type and MicroTom *lecer6*, respectively. The pattern of conidial morphogenesis was similar to findings with conidia of *Blumeria graminis* inoculated on artificial surfaces. Conversely to the declined germination and differentiation rates, the mortality of *Blumeria graminis* conidia distinctly increased on enzymatically isolated fruit cuticles in comparison to freshly peeled fruit tissues. These results indicate a requirement for contact with the cuticle underlying fruit tissue, almost probably due to nutrient availability.

The bioassay focusing on the early steps of the infection process of *Blumeria graminis* revealed only minor variations between non-host MicroTom wild type and MicroTom *lecer6*. Nevertheless, the cuticular membrane of *lecer6* mutant fruits provided a less effective water loss barrier but showed a higher resistance against pathogenic invasion compared to the MicroTom wild type. Thus, the interaction between cuticle permeability and cell-cell signaling in mature green fruit peel tissue of MicroTom wild type might be necessary for appressorial differentiation *Blumeria graminis*.

In summary, the treatment of fruit tissues, the stage of fruit development and the compositional differences between both MicroTom lines affected variation in conidial

morphogenesis of *Blumeria graminis* to various degrees. The results lead to the conclusion that conidia germination and differentiation are two differently regulated processes in *Blumeria graminis* morphogenesis. An induction of germination does not unavoidably lead to stimulation of appressorial differentiation. Moreover, both events in conidial morphogenesis are not necessarily triggered by only a single factor, but rather a complex signaling machinery. A series of morphogenetic signals are almost certainly required for the successful infection of the contact surface.

Comprehensive Character of LeCER6 Deficiency in Tomato Plants

The 'reverse genetic' analysis of MicroTom mutant plants revealed several pleiotropic and secondary effects, which are based in the VLCFA biosynthesis by LeCER6 deficiency. An implicated interference in the water management and the hormonal balance of the plants led to alterations in the physiological status and further development of the tomato plants accompanied by a distorted appearance of the plant.

The highly complex process of development of MicroTom wild type and *lecer6* mutant fruits revealed a series of dynamic changes in pigmentation, firmness, soluble solid levels and cell wall metabolism. This compilation of datasets gained a first insight into the developmental time frame of regulatory networks that coordinates major alterations in fruits of the two MicroTom lines. Furthermore, this comparative approach of developing MicroTom fruits provides a suit of fruit-specific strategies that act additively or interactively to overcome LeCER6 deficiency. Nevertheless, these findings proceed as integral and regulated parts of the complex fruit ripening program either by masking cuticle disturbance, by improvement of water management or by minimizing deleterious effects.

CHAPTER V

Post-Harvest Formation of the Suberized Stem Scar Tissue during Storage of Tomato Fruits

RESULTS

By harvesting fleshy berry tomato fruits the contact between fruits and their parent plant, which are naturally associated in a source-sink relationship, is terminated. Thereby, a ragged wound is introduced to the tomato fruit tissue. As a consequence of wounding uncontrolled water loss and an associated ion leakage occurs from the interior of the fruit. In addition, plant pathogens from the exterior are able to penetrate the fruit tissue unrestrictedly. Therefore, the rapid 'wound healing' is essential for maintaining the osmotic balance of the fruit and for survival in a hostile environment containing a variety of pathogenic microorganisms. The objective of this research was to investigate post-harvest alterations at the stem scar tissue of tomato fruits in relation with its barrier function. The *de novo* biosynthetic processes producing a hydrophobic stem scar tissue were studied at the transcriptional and biochemical level in order to characterize compositional changes at the wound site of tomato fruits. The functional relevance of the wound response and the involvement of abscisic acid (ABA) as a putative regulator in the wound signaling cascade were examined in the course of fruit storage.

Functional Analysis of the Stem Scar Tissue during Storage of Tomato Fruits

The cuticular transpiration was routinely determined as a function of weight reduction resulting from the water loss over time. The stem scar tissue of intact tomato fruits was sealed with watertight paraffin wax in order to exclude the water leakage at the stem scar due to the wounding of the fruit by the harvesting process. Thus, without sealing the tomato fruits at the stem scar tissue with paraffin wax, transpiration plus additional evaporation processes were investigated simultaneously.

Both mature green (category II) and red ripe (category VI) tomato fruits of MicroTom wild type were harvested and studied with regard to their water loss barrier properties. In a period of fourteen days, mature green and red ripe fruits lost weight at the stem scar tissue,

regardless to paraffin wax treatment or non-treatment (Figure 1). MicroTom fruits of both ripening stages, treated with paraffin wax, reduced weight along a slope of a linear regression (r^2 = 0.999). During fourteen days of storage the mature green and red ripe fruits lost between 4% and 5% of the initial fruit weight.

The fruits without paraffin wax at the stem scar tissue lost considerably more weight in the same time, but predominantly in the first three days of storage. After three days, the weight decrease of the mature green tomato fruits, unlike the red ripe fruits, extended also along a slope of a linear regression (mature green: r^2 = 0.998, red ripe: r^2 = 0.984). However, the weight of the fully ripened red fruits decreased drastically mainly as a consequence of the high water loss rate after the third day of storage. At the last day of storage the weight of mature green fruits was decreased by 9% and the red ripe fruits lost 12% of their initial, non-stored weight.

At the beginning of storage the ratio of weight loss between untreated, mature green fruits and those fruits sealed with paraffin wax was 1:7. The discrepancy caused by the treatment diminished slowly and reached, ultimately, with beginning of day four a ratio of 1:2. Quite contrary to red ripe fruits, the weight loss of treated and untreated fruits differed by a factor of 1:9 at the initial measurement and after a period of fourteen days a significant difference of 1:3 still existed.

After enzymatic isolation with pectinase and cellulase the weight of the stem scar membrane was determined at the beginning and at the end of the fourteen days storage period (Figure 2). Non-stored, mature green and red ripe fruits differed only marginally with respect to their stem scar weight. In progress of the storage the weight of the stem scar membrane, isolated from fruits sealed with paraffin wax, did not increase significantly. However, significant differences in the stem scar weight were found in fruits of both ripening stages without paraffin wax treatment. The stem scar membrane of mature green fruits increased 3.3-fold in weight during the storage, whereas the stem scar weight of red ripe fruits rose by a factor of 4.3. The efficiency of mature green fruits to seal the wound was significantly higher to the performance of red ripe fruits. As an observation, the red ripe fruits were more seriously wounded than the mature green fruits due to the harvesting process.

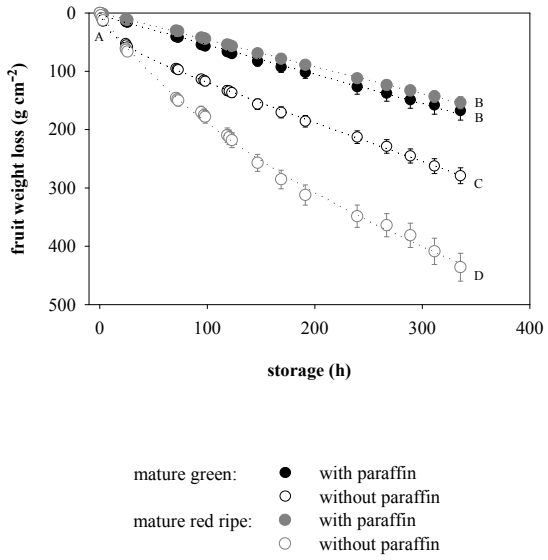

Figure 1. Weight loss of mature green and red ripe MicroTom wild type fruits during fourteen days of storage. The harvested, mature green and mature red ripe tomato fruits were stored with or without watertight paraffin wax at the stem scar tissue. Weight loss was compared by multi-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SE ($n = 10$).

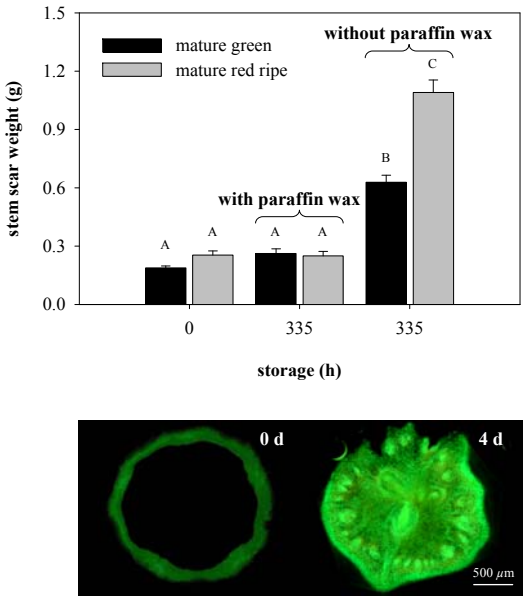

Figure 2. Weight of enzymatically isolated stem scar membrane of mature green and red ripe MicroTom wild type fruits at the beginning and at the end of fourteen days of storage. The harvested, different developed tomato fruits were stored with or without watertight paraffin wax at the stem scar tissue. Stem scar weight was compared by multi-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SE ($n = 6$ to 10). Auto-fluorescence microscopic images of enzymatically isolated stem scar membrane of non-stored and four days stored mature green MicroTom wild type fruits.

In consideration of the higher water loss barrier property, mature green fruits were harvested and microscopically investigated during four days of storage that was carried out with no paraffin wax sealing. In Figure 3 the accumulation of a novel tissue coupled with an increasing, intense green auto-fluorescence at the outermost layer of the stem scar tissue became distinctly visible.

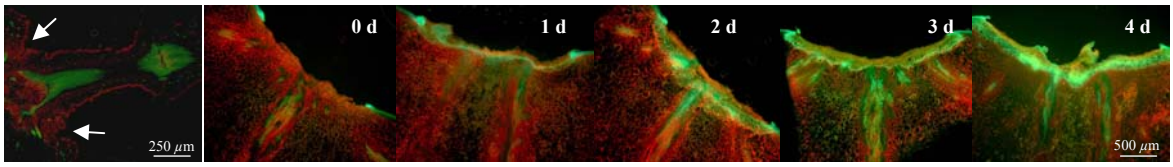

Figure 3. Auto-fluorescence microscopic images of longitudinal sections of mature green MicroTom wild type fruits. The cutting presented the stem scar tissue of freshly harvested and post-harvested stored tomato fruits without treating with paraffin wax. Arrows point at the interface between the fruit and the petiole (stem scar region).

This novel tissue completely covered the wound at the stem scar tissue generated upon fruit harvesting.

A more detailed view is presented by the data obtained for the water permeance of harvested mature green fruits over time with reference to their stem scar membrane weight (Figure 4). During the first day of storage the permeance for water of those mature green MicroTom fruits, which were not sealed with paraffin wax at the stem scar tissue, was reduced to 41% of the initial value, while at the second storage day the water permeance declined further by 11%. During the following four days of storage the water loss rate remained constant. The permeance for water amounted between 2.23 x 10^{-5} m s^{-1} and 2.39 x 10^{-5} m s^{-1}, respectively. These values corresponded to 22% of the water permeance of non-stored fruits directly after the harvesting process.

In comparison to the mature green fruits of MicroTom wild type *lecer6* mutant fruits, phenotypically characterized by a modified cuticular membrane, showed a similar pattern of water permeance reduction, however at a higher level. In this case the decrease of permeance for water progressed slower and not that strong as for the wild type. The permeance for water of MicroTom *lecer6* fruits was twofold higher compared to the wild type fruits at the beginning of the storage. At the same time, the permeance for water of wild type fruits significantly decreased by 79% of the initial value, whereas the value of the *lecer6* mutant fruits was reduced only by 66%. Nevertheless, for both MicroTom wild type and MicroTom *lecer6* the permeance for water declined more than twofold in the course of storage. Furthermore, it was shown that this alteration was prevented if the storage took place at 4°C instead of 25°C. For both MicroTom lines the permeance for water was not significantly changed after five days of storage at 4°C.

However, the cuticular permeance for water of mature green MicroTom fruits, which were covered with watertight paraffin wax at the stem scar tissue, showed only a 0.8-fold lower level in comparison to six days stored fruits without paraffin wax sealing. In turn, freshly harvested fruits exhibited a difference even by a factor of 5.9 for MicroTom wild type and by a factor of 4.4 for MicroTom *lecer6* in comparison to six days stored fruits.

The weight of the enzymatically isolated stem scar membrane was screened for both MicroTom lines in the same experiment as the permeance for water was detected (Figure 4). During the storage of tomato fruits the weight of the stem scar membrane increased threefold, whereas the stem scar weight of fruits stored at 4°C remained significantly constant when compared to the starting level.

A significant negative correlation between the permeance for water and the accumulation of the novel tissue at the fruit stem scar throughout the storage of MicroTom wild type and MicroTom *lecer6* fruits was found (wild type: R^2 = -0.79, *lecer6*: R^2 = -0.95; $P < 0.05$, Spearman's correlation of ranks).

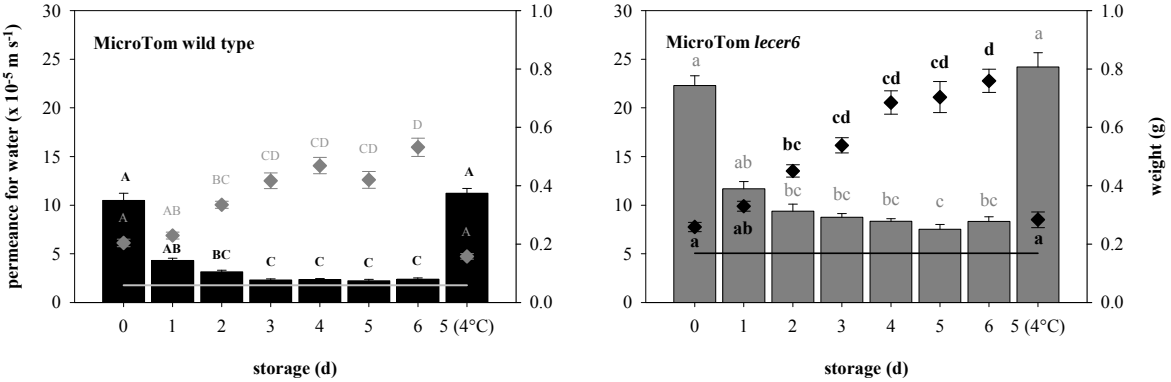

Figure 4. Harvested, green mature fruits of MicroTom wild type and MicroTom *lecer6* were stored without paraffin wax on the stem scar area at 25°C or 4°C. The permeance for water of these fruits during a period of six days is shown as bars. The weight of the enzymatically isolated stem scar membrane is given as diamonds. Permeance for water of non-stored mature green fruits which were sealed with paraffin wax is represented with a line. Permeance for water and the weight of the stem scar membrane of different stored fruits within each line were compared by one-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SE ($n = 20$).

For characterizing additional functional properties of the novel tissue at the fruit stem scar the infection rates of the pathogenic fungi *Cladosporium herbaceum*, *Alternaria alternata*, *Colletotrichum coccodes* and *Fusarium solani* were specified in a bioassay. A distinct entry of the fungi into the interior of the tomato fruit was indicated by a brownish discoloration along the vascular bundles as a sign for the fungal infection. A preliminary experiment on the filamentous fungus *Fusarium solani* showed that the highest infection success was found on freshly harvested, mature green fruits. In contrast, these fruits were least susceptible to *Cladosporium herbaceum* (Josef Gall, personal communication).

Fruits were harvested and at the stem scar area immediately inoculated with spores and actively growing mycelium of *Fusarium solani*. Seven days after inoculation 65% of the treated fruits were infected with the pathogen. The infection rate was significantly reduced to 38% in fruits that were stored for four days at 25°C after harvesting prior to the incubation with *Fusarium solani* (Figure 5).

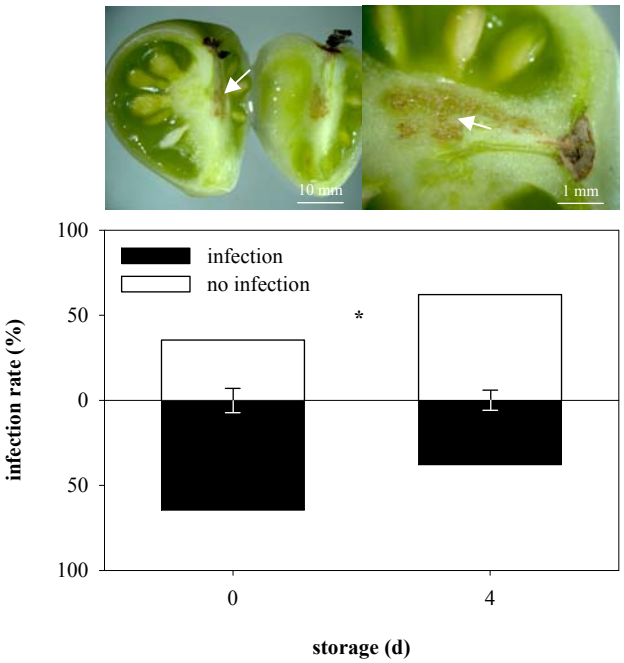

Figure 5. Mature green MicroTom wild type fruits infected with *Fusarium solani*. Binocular microscopic images of bisected mature green fruits infected with *Fusarium solani* without pre-storage. Fungal infection sites are marked with arrows. Infection rate of non-stored and four days stored mature green fruits was detected after an incubation with *Fusarium solani* at the stem scar tissue. The ratio between non-stored and stored fruits was tested with Mann-Whitney *U*-test (n.s. = not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Data are shown as means ± SE (n = 400 consisting of 4 independent experiments à 100 fruits).

Chemical Analysis of the Suberized Stem Scar Membrane of Tomato Fruits

In order to analyze the composition of the novel tissue formed during fruit storage, the stem scar was enzymatically isolated and subsequently monomerized (Figure 6). The stem scar membrane mainly consisted of saturated and unsaturated alkanols as well as alkanoic acids, ω-hydroxyalkanoic acids and α, ω-alkandioic acids with chain lengths of C_{16} to C_{30} as aliphatic compounds and ferulic acid as aromatic component that are characteristic for suberized tissues. While even-numbered, saturated alkanols, alkanoic acids, ω-hydroxyalkanoic acids and α, ω-alkandioic acids were found for all chain lengths corresponding mono- and diunsaturated components were only quantified for the chain length of C_{18}.

Aliphatics with chain lengths of C_{22} followed by C_{18} and C_{16} compounds were most of the stem scar membrane. Strikingly, with a proportion of 29% α, ω-alkandioic acids as the major compound class were found.

The compositions of the stem scar membrane and the suberized membrane of the wounded cuticle were investigated in direct comparison. Both isolated fruit membranes were similar regarding to their chemical compounds and the microscopic analyses showed in both cases strong green auto-fluorescence.

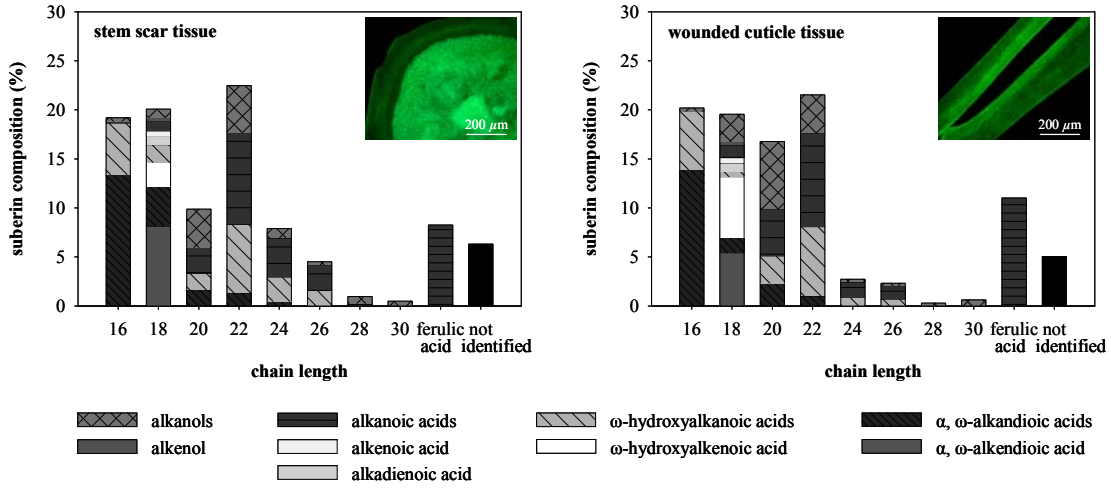

Figure 6. Relative suberin composition isolated from the stem scar tissue (A) and wounded cuticle tissue (B) of mature green MicroTom wild type fruits. Stem scar membrane was enzymatically isolated of fruits stored at 25°C for five days. The wounded cuticle tissue of fruits, injured with a scalpel and harvested after two days, was separated after an enzymatical digestion. Chain lengths are given for the aliphatic compositions. Data are shown as means (n = 1 or 3). Auto-fluorescence microscopic images of stem scar tissue and wounded cuticle membrane.

Transcriptional Changes at the Stem Scar Tissue during Storage of Tomato Fruits

In the course of storage the content of total RNA was extracted from the stem scar tissue for gene expression analyses. During the first two days the solvent-soluble RNA content increased significantly by a factor of 1.2 comparing to a level of 558.53 ± 97.40 μg g^{-1} fresh weight (mean ± SE) in non-stored fruits. During a period of four days the amount of total RNA decreased again and reached the initial level (Figure 7).

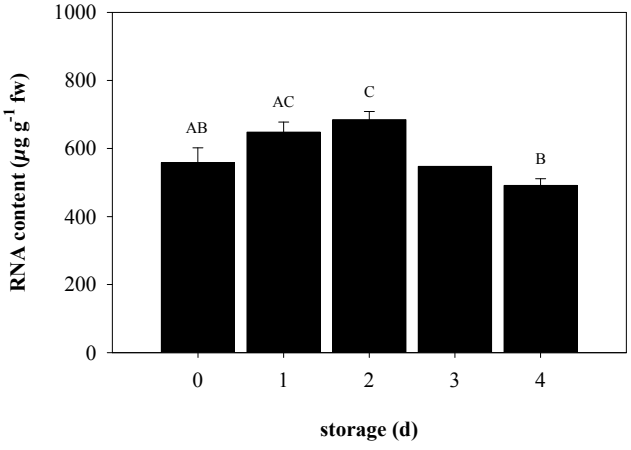

Figure 7. Content of solvent-soluble total RNA of non-stored and stored mature green MicroTom wild type fruits. Samples consisted exclusively of the stem scar tissue of the tomato fruits. RNA content was compared by one-way ANOVA followed by HSD post-hoc test for unequal n: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SE (n = 2 to 6).

For the microarray experiments equal amounts of total RNA were used to avoid any discrimination between the stem scar tissues of fruits stored for different times. An

overview of the distribution of the gene expression signals of each individual oligomer represented on the microarray is given in Figure 8. The expression signal intensities of the microarray experiments were normalized using the data of non-stored fruits as reference signals. The total expression signal intensities rose when comparing the microarray experiments of day one, two and four, respectively.

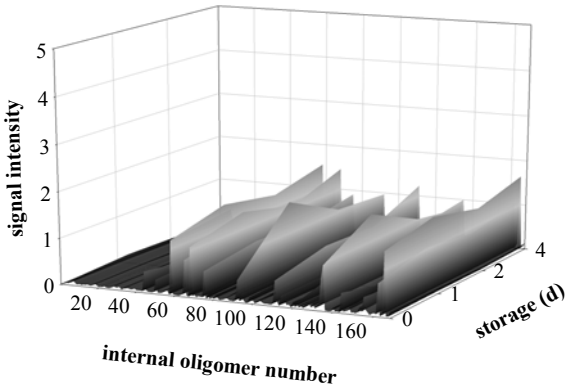

Figure 8. Differential microarray gene expression signals of 167 oligomers of stored and non-stored mature green MicroTom wild type fruits. Samples consisted exclusively of the stem scar tissue of the tomato fruits. Data are shown as means (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

Only few transcripts corresponding to the oligomers on the microarray were distinctly expressed in the stem scar tissue. On the basis of the highest microarray gene expression signals several oligomers were selected and shown in Table I.

Table I. Selection of the strongest microarray signal intensities of mature green MicroTom wild type fruits labeled with internal oligomer number during tomato fruit storage. Samples consisted exclusively of stem scar tissue. Data are shown as means ± SD (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

	oligomer	**fruit storage**			
		0 d	**1 d**	**2 d**	**4 d**
58	Wax synthase	1.2 ± 0.6	1.5 ± 0.6	1.3 ± 0.3	1.5 ± 0.6
65	CER1 homologue	0.8 ± 0.4	1.0 ± 0.5	0.9 ± 0.7	1.0 ± 0.8
68	CER1 homologue	1.1 ± 0.6	1.4 ± 0.8	1.1 ± 0.6	1.5 ± 0.9
92	Non-specific lipid transfer protein	0.5 ± 0.2	1.7 ± 0.3	1.1 ± 0.1	1.0 ± 0.2
111	1-aminocyclopropane-1-carboxylate oxidase	0.5 ± 0.3	0.8 ± 0.4	0.8 ± 0.2	1.2 ± 0.7
134	Abscisic stress ripening protein (DS2 protein)	0.7 ± 0.4	1.6 ± 0.6	1.0 ± 0.4	0.8 ± 0.5
135	Abscisic stress ripening protein	1.0 ± 0.5	1.1 ± 0.4	0.8 ± 0.3	1.0 ± 0.7
165	Hypothetical protein (lipid transfer protein)	1.4 ± 0.7	1.6 ± 0.9	1.4 ± 0.5	1.6 ± 0.6

Relevant oligomers in the stem scar tissue were a wax synthase (58), two *cer* homologues (65, 68), a non-specific lipid transfer protein (92), an 1-aminocyclopropane-1-carboxylate oxidase (111), two abscisic stress ripening proteins (DS2 protein 134, 135) and a hypothetical protein (165). In particular, the highest gene expression signals were detected in all microarray experiments for the hypothetical protein (165) and the wax synthase (58).

To determine an elevated or decreased gene expression at the stem scar tissue after one, two and four days of storage the ratio of the gene expression signals between non-stored and stored fruits were separately estimated for each microarray experiment. The intensity of gene expression signals was distinctly changed when calculating an expression ratio < 0.5 or > 2.0. A ratio < 0.5 indicated an outvalued expression in the stem scar tissue of non-stored fruits. In turn, a ratio > 2.0 specified an increased gene expression signal in the stored fruits compared with freshly harvested fruits (Figure 9).

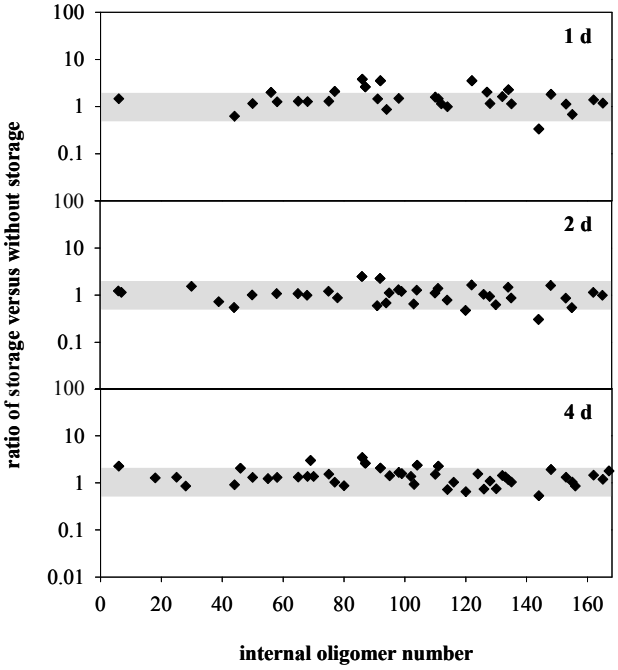

Figure 9. Ratio between the gene expression signals of stored and non-stored mature green MicroTom fruits corresponding to 167 oligomers. Samples consisted exclusively of the stem scar tissue of the tomato fruits. Data are shown as means (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots). A ratio ≤ 0.5 and ≥ 2.0 calculated between gene expression signal of non-stored and stored MicroTom fruits was illustrated with gray bars.

Only a small number of oligomers were found representing transcriptional modifications in the stem scar tissue. Differences in the signal levels were found between non-stored fruits and those which were stored for one day. Transcripts for the non-specific lipid transfer protein (92), the abscisic acid and environmental stress inducible protein (Dehydrin TAS14; 122) and the abscisic stress ripening protein (DS2 protein; 134) increased during the first day of storage (Figure 10). The incremental gene expression

signal averaged between the 2.3-fold and the 3.5-fold of the initial value. During the further course of storage, the transcript levels were distinctly reduced. At the fourth day of storage the gene expression signal of the abscisic stress ripening protein (DS2 protein; 134) was almost adjusted to the starting level. The difference in gene expression signals of non-stored and four days stored fruits for the non-specific lipid transfer protein (92) and the abscisic acid and environmental stress inducible protein (Dehydrin TAS14; 122) was only recorded at an intensity level of 2.0.

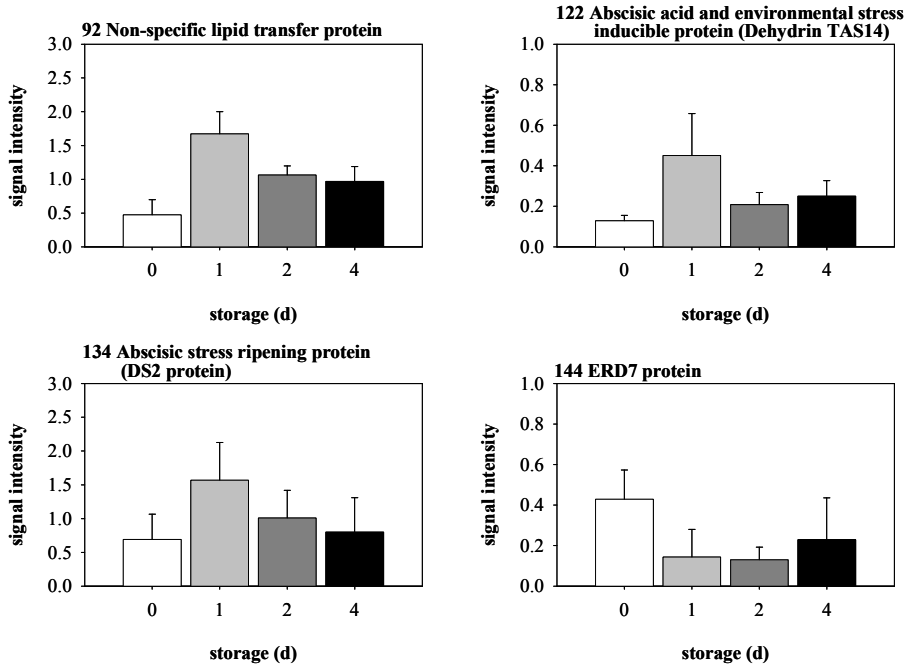

Figure 10. A detailed comparison of microarray gene expression signals of stored and non-stored mature green MicroTom wild type fruits. Samples consisted exclusively of the stem scar tissue of the tomato fruits. Data are shown as means ± SD (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

In contrast, the gene expression signal of the ERD7 protein (144) declined in comparison to the non-stored sample. This development started on the first day of storage and reached the maximum by a factor of 3.3 on the second day of storage. Four days after harvest the distinct transcriptional difference still existed with an alleviated intensity.

Findings of selected EST or gene sequences characterized in the microarray experiments were corroborated by using the more sensitive, semi-quantitative RT-PCR analysis (Figure 11).

Figure 11. (continued) additional control. The size of the amplified RT-PCR fragments is given. Microarray data are shown as means ± SD (n = 12 consisting of 2 microarray experiments with dye switch à 6 spots).

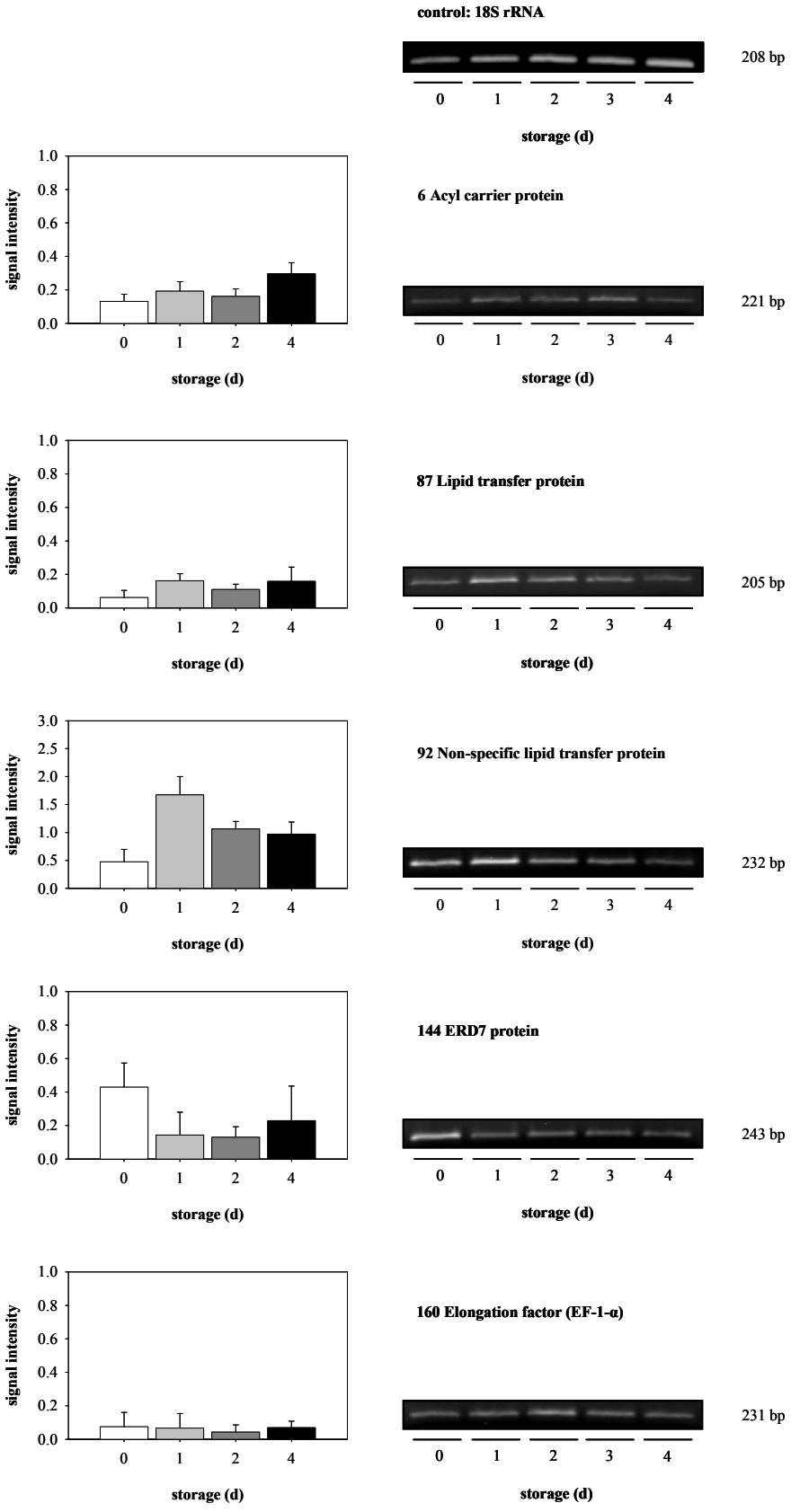

Figure 11. Comparison of gene expression signals for selective oligomers obtained from microarray experiments and of RT-PCR analysis. Samples consisted exclusively of the stem scar tissue of the of non-stored and stored mature green MicroTom fruits. The RT-PCR fragments of 18S rRNA were used as an

Additionally, the transcripts of the stem scar tissue of fruits stored for three days were specified to complete the expression pattern of the four days spanning period pointed out in the microarray experiments. As a control, RT-PCR analysis with 18S rRNA primers was performed. The transcript level of the elongation factor (EF-1-α, internal oligomer number 160) and the 18S rRNA (control) were constantly expressed during the fruit storage. In contrast, a slightly increased expression value was screened for the acyl carrier protein (6) and the lipid transfer protein (87) in stored fruits compared to freshly harvested, non-stored fruits. The signal intensity for the non-specific lipid transfer protein (92) increased especially within the first day of storage, whereas a reduced signal level in all stored fruits was found for the ERD7 protein (144) in comparison to the initial value of non-stored fruits.

Content of Endogenous Abscisic Acid in the Stem Scar Tissue during Storage of Tomato Fruits

The content of abscisic acid (ABA) in the stem scar tissue of mature green MicroTom wild type fruits was analyzed (Figure 12). The stem scar tissue of freshly harvested, non-stored fruits exhibited an ABA content of 6.71 ± 0.64 nmol g^{-1} fresh weight. Within the first day of storage the ABA analysis resulted in a significant increase of endogenous ABA at the stem scar tissue. After this 3.7-fold increase the ABA percentage was weakly alleviated and adjusted to a level of 18.02 ± 2.02 nmol g^{-1} fresh weight. Four days after harvest, the ABA content was still enhanced by a factor of 3.0 compared to non-stored fruits.

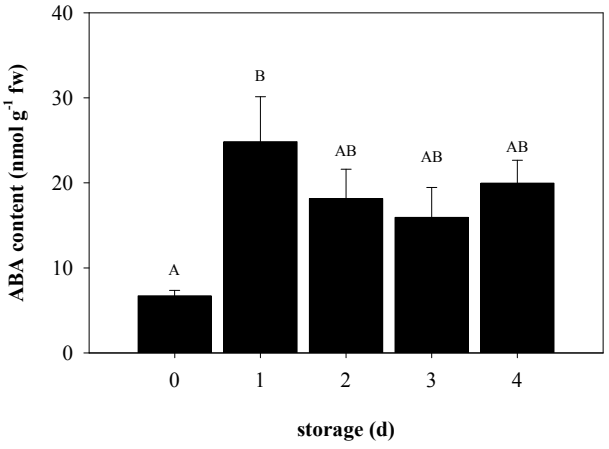

Figure 12. ABA content of mature green MicroTom wild type fruits during a period of four days. Samples consisted exclusively of the stem scar tissue of the tomato fruits. ABA content was compared by one-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SE ($n = 3$ to 8). The quantitative analysis was done by the workgroup of Prof. Wolfram Hartung.

To examine the effect of ABA on modifications at the stem scar tissue, mature green fruits of tomato mutant lines with defects in the ABA biosynthesis were additionally analyzed. The tomato lines *flacca*, *sitiens* and *notabilis* with the genetic background of Rheinlands Ruhm and Ailsa Craig were selected. For the phenotypic characterization the fruit size of the ABA mutants was determined. Irrespective of the mutation level, the surface area of *flacca*, *sitiens* as well as *notabilis* mutant fruits had significantly the same size as the corresponding wild type fruits of Rheinlands Ruhm and Ailsa Craig, respectively. Nevertheless, the fruit surface area of Rheinlands wild type, Rheinlands Ruhm *flacca* and Rheinlands Ruhm *sitiens* as well as Ailsa Craig wild type and Ailsa Craig *notabilis* were 2.9- and 4.8-fold, respectively, increased in comparison to MicroTom wild type fruits (Figure 13).

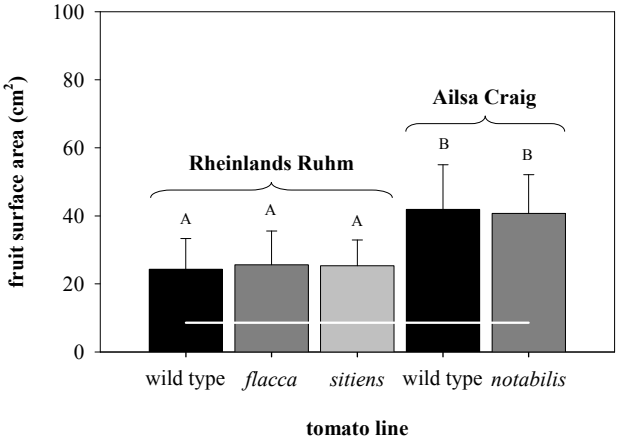

Figure 13. Fruit surface area of mature green Rheinlands Ruhm wild type, Rheinlands Ruhm *flacca*, Rheinlands Ruhm *sitiens*, Ailsa Craig wild type and Ailsa Craig *notabilis* fruits. For comparison the fruit surface area of mature green MicroTom wild type fruits is represented with a line. Fruit surface area was compared by one-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SD (n = 75 to 200).

Concerning the ABA content in the stem scar tissue, a similar pattern as seen for the MicroTom wild type was also found in wild type fruits of Rheinlands Ruhm and Ailsa Craig, however at a fourfold lower level (Figure 14).

Within the first two days of storage the ABA level distinctly increased by a factor of 3.7 for Rheinlands Ruhm wild type, while Ailsa Craig wild type exhibited an increase of 3.1 compared with freshly harvested fruits. Following the course of storage the ABA content was slightly reduced, but a 3.3- and 2.5-fold, respectively, elevated ABA content still remained. The ABA amount determined in the stem scar tissue of the ABA mutants *flacca*, *sitiens* and *notabilis* was not significantly enhanced within four days after harvest. During the four days of storage the ABA content of the stem scar tissue of Rheinlands Ruhm *flacca* and Ailsa Craig *notabilis* peaked only slightly though not significantly at the second day of storage.

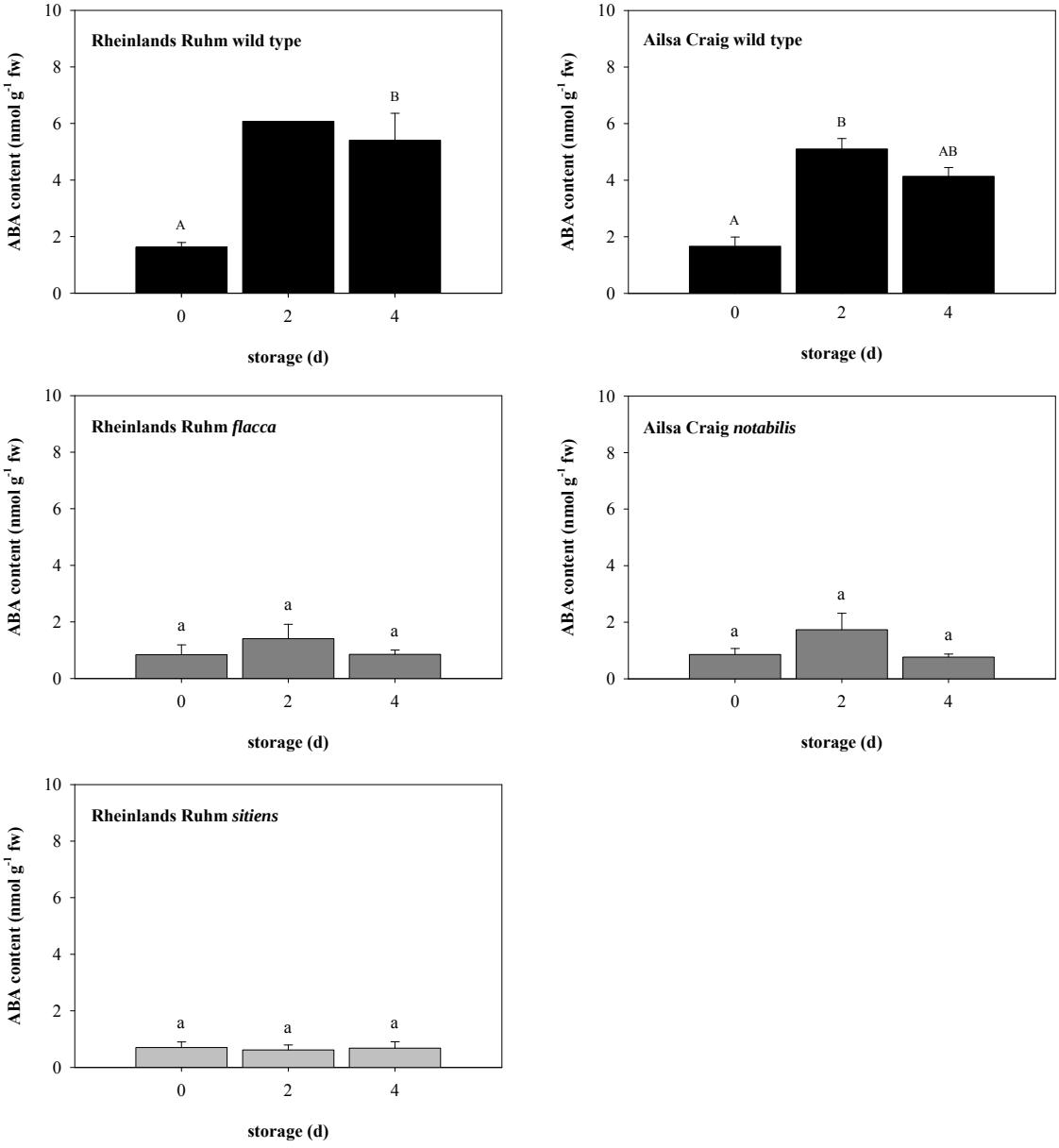

Figure 14. ABA content of Rheinlands Ruhm wild type, Rheinlands Ruhm *flacca*, Rheinlands Ruhm *sitiens*, Ailsa Craig wild type and Ailsa Craig *notabilis* mature green fruits during a period of four days. Samples consisted exclusively of the stem scar tissue of the tomato fruits. ABA content was compared by one-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SE (n = 2 to 4). The quantitative analysis was done by the workgroup of Prof. Dr. Wolfram Hartung.

During a period of six days changes of the water permeance and of the stem scar weight were studied of Rheinlands Ruhm wild type, Rheinlands Ruhm *flacca*, Rheinlands Ruhm *sitiens*, Ailsa Craig wild type and Ailsa Craig *notabilis* mature green tomato fruits. This storage process paralleled in both wild type lines (Figure 15). Freshly harvested tomato

fruits that were not sealed with paraffin wax at the stem scar tissue exhibited a water permeance of 6.00 ± 1.55 x 10^{-5} m s^{-1} for Rheinlands Ruhm wild type and 5.67 ± 1.10 x 10^{-5} m s^{-1} for Ailsa Craig wild type, respectively. This amount was only half of the initial permeance for water detected in freshly harvested MicroTom wild type fruits.

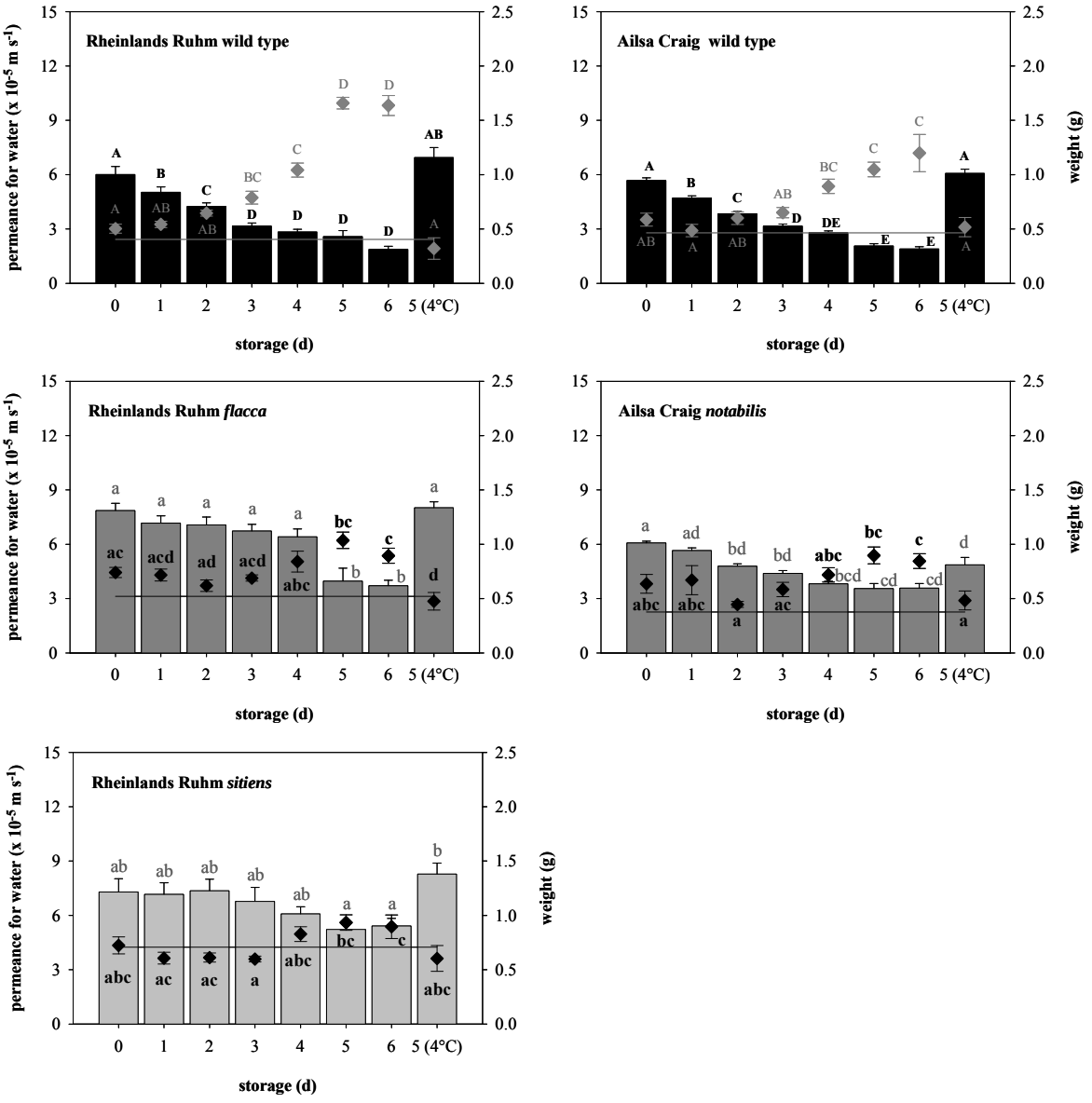

Figure 15. Harvested, green mature fruits of Rheinlands Ruhm wild type, Rheinlands Ruhm *flacca*, Rheinlands Ruhm *sitiens*, Ailsa Craig wild type and Ailsa Craig *notabilis* were stored without paraffin wax on the stem scar area at 25°C or 4°C. The permeance for water of these fruits during a period of six days is shown as bars. The weight of the enzymatically isolated stem scar membrane is given as diamonds. Permeance for water of non-stored mature green fruits which were sealed with paraffin wax is represented with a line. Permeance for water and the weight of the stem scar membrane of different stored fruits within each line were compared by one-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SE ($n = 6$ to 60).

The permeance for water of Rheinlands Ruhm wild type and Ailsa Craig wild type fruits fell continuously to a third of the initial value within five days of fruit storage, whereas the weight of the stem scar membrane increased by a factor between 2.0 and 3.3 in comparison of non-stored fruits at the same time. Similarly to the MicroTom lines this development was prevented through fruit storage at 4°C. A significant negative correlation between the permeance for water and the weight of the stem scar membrane throughout the storage of Rheinlands Ruhm and Ailsa Craig wild type fruits similar to the MicroTom fruits was found, as described above (Rheinlands Ruhm: R^2 = -0.96, Ailsa Craig: R^2 = -0.96; $P < 0.05$, Spearman's correlation of ranks).

The reduction of the permeance for water in all three ABA mutants was not to the same extent as for the corresponding wild type fruits. Directly after harvesting, non-stored fruits of Rheinlands Ruhm *flacca* and Rheinlands Ruhm *sitiens* showed a similar level as the wild type fruits. During a period six days the permeance for water decreased significantly to 47% for Rheinlands Ruhm *flacca* and to 74% for Rheinlands Ruhm *sitiens*. A similar alteration was mirrored for Ailsa Craig *notabilis* with a 59% reduction of the initial value before storage. A significant accumulation of the stem scar tissue became evident at the fifth day of storage. Finally the ratio between the stem scar weight of non-stored and six days stored fruits was only 1.4. The decline of the water permeance did not correlate with the increased weight of the isolated stem scar membrane.

Effects of Exogenous ABA Applications at the Stem Scar Tissue during Storage of Tomato Fruits

Application experiments were done with exogenously applied ABA, a plant growth regulator, tetcyclacis an inhibitor of ABA metabolism and cycloheximide an inhibitor of protein biosynthesis in eukaryotic cells. Different concentrations of ABA, tetcyclasis and cycloheximide solutions, and, additionally, deionized water as control were applied daily onto the stem scar tissue after harvest of mature green MicroTom wild type fruits. This experimental arrangement was studied in comparison to untreated fruits. After four days of storage the permeance for water was recorded. ABA and tetcyclasis solutions with a concentration range of 0.005 μM to 1000 μM had no significant consequences on the permeance for water in comparison to fruits without any application (data not shown).

Significant discrepancies were only found for fruits, which were treated with cycloheximide with a concentration of 10 μg ml^{-1}. After four days of storage the permeance for water of fruits was distinctly higher due to the cycloheximide treatment in

comparison with untreated fruits (Figure 16). Applied deionized water resulted only in insignificantly elevated water permeance compared with untreated fruits after four days of storage. The water permeance of fruits treated with cycloheximide still exhibited a proportion of 45% of the initial value of freshly harvested fruits, while the permeance for water of deionized water treated fruits amounted 29% and untreated fruits 23% compared with freshly harvested fruits. As an effect of the cycloheximide treatment the reduction of the water permeance was significantly retarded in the course of storage. A weight accumulation of the stem scar was not significantly detectable (data not shown).

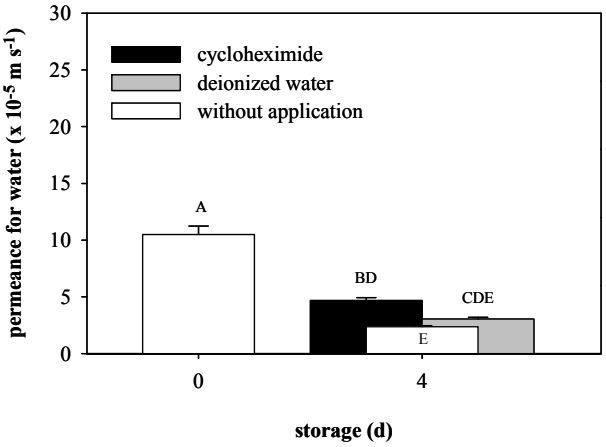

Figure 16. Permeance for water of harvested, mature green MicroTom wild type fruits in the course of storage with or without application at the stem scar tissue. A treatment with deionized water was screened as control. The permeance for water of different stored fruits was compared by one-way ANOVA followed by HSD post-hoc test for unequal *n*: significant differences are marked with different letters ($P < 0.05$). Data are shown as means ± SE ($n = 10$ to 27).

DISCUSSION

Fruits integrity of the horticultural crop tomato *Lycopersicon esculentum* Mill. is of prime economical importance. The attached fruit and its parent plant are connected in a source-sink relationship, which is ensured by a system of concentric vascular bundles radiating through the fruit pericarp. By harvesting of the fruit the vascular bundles are disrupted, the connection is disconnected and the tomato fruits incur wounds. Wounding that occurs during harvest or subsequent handling in principle imperils fruit homeostasis. Once detached, tomato fruits have no renewable source to compensate the uncontrolled water loss and, therefore, experience water stress (Nakano *et al*., 2003). Moreover, the tomato fruit is vulnerable to infections due to the harvesting process itself, during post-harvest storage or treatment for example by contaminated water in the grading and packaging line (Spotts and Cervantes, 1986; Sugar and Spotts, 1993). In order to protect the exposed fruit tissue, a rapid response to reduce water loss across the wound surface is

crucial, since a desiccated layer of dead or incompetent cells at the wound site is not able to control dehydration and pathogen infection of the tomato fruit (Lulai *et al.*, 2006).

Following fruit harvest, wound-induced biosynthesis generated a special novel tissue at the stem scar of the tomato fruit most notably protective against water loss. Hence, this 'wound healing' allows for seed maturation as well as fruit ripening processes and furthermore increases the chance for reproduction of tomato plants. In spite of the physiological importance in protection of the tomato fruit, there are many open questions in wound response of higher plants and the signaling pathways that effect or regulate this process. Hence, investigations on *de novo* biosynthetic activity at the stem scar tissue to improve biophysical properties and evaluation of the physiological importance of this hydrophobic barrier represent a high biotechnological potential to yield novel approaches for example in prevention of plant diseases and methods to enhance biological interactions associated with suberin biosynthesis.

Wounding of the Tomato Fruit Due to Harvesting Process

Tomato fruits are usually harvested by removing the fruit from the pedicel (Poole and McLeod, 1994). The mechanical process of harvest produces an irregular wound surface and injures the integrity of the tomato fruit. However, this wounding displays an abiotic stress that induces a variety of responses, since the ragged wound at the stem scar of the fruit affected a transpirational and/or evaporational water loss from the harvested fruit through the open vascular bundles and the surrounding fruit tissue. Subsequently, wound stress promotes modifications in many physiological processes, which often make the fruit more perishable and diminishes its shelf life. Otherwise, wounding also elicits several physiological responses in the fruit associated with 'wound healing' in order to survive. The 'wound healing' process and associated barrier properties are crucial for the fruit because the wound tissue might be a portal for uncontrolled water leakage and the unrestricted entry of pathogenic fungi invading the vascular system of tomato fruits.

This 'wound healing' of the fruit requires the rapid biosynthesis of an efficient barrier with a low permeability to gases and water and a high chemical resistance with elasticity to strengthen the integrity and extent the shelf life of the whole tomato fruit (Fortes and Nogueira, 1989; Grappin *et al.*, 2000; Morris *et al.*, 2004). In general, the epidermal cell wall and the cuticle of plants maintain the water balance and the stability of the fruit and, furthermore, protect the fruit tissue from a hostile environment (CHAPTER I and CHAPTER IV). Post-harvest alterations at the fruit stem scar evoke a novel tissue that is

visually different from the surrounding fruit tissue. The brownish discoloration of the wound site increases during the storage of harvested fruits. Ultimately, an apoplastic and brown discolored stem scar membrane is formed.

At the stem scar tissue of mature green tomato fruits highly induced gene expression activity was found for a wax synthase (58), *cer* homologues (65, 68), lipid transfer proteins (92, 165), an 1-aminocyclopropane-1-carboxylate oxidase (111) and abscisic stress ripening proteins (134, 135). Except for the non-specific lipid transfer protein (92), these transcripts were also most prominent in the intact fruit peel tissue of mature green fruits and, thus, probably represent a constitutively high level gene expression in of tomato fruits (CHAPTER II). Nonetheless, during fruit storage modified transcript amounts were determined for the non-specific lipid transfer protein (92), the abscisic acid and environmental stress inducible protein (Dehydrin TAS14; 122), the abscisic stress ripening protein (DS2 protein; 134) and the ERD7 protein (144) at the stem scar tissue.

Lipid transfer proteins (LTP), a protein family of small, basic enzymes, are functional characterized by their *in vitro* ability to catalyze the exchange of lipid molecules between natural and artificial membranes (Kader *et al.*, 1984; Kader, 1997; Trevino and O'Connell, 1998).Thus, Sterk *et al.* (1991) and Ma *et al.* (1995) discussed the role for plant LTP in the transport of cutin and wax monomers across the cell membrane to the sites of cuticle formation, where the lipophilic components may be released and esterified into the growing cutin matrix or assembled into the wax coverage. In general, LTP bind a broad spectrum of lipophilic molecules and, therefore, these LTP belong to the group of non-specific LTP (Kader, 1996). At the stem scar tissue the gene expression of a putative non-specific LTP was detected to be induced very rapidly in response to harvest of tomato fruits. According to the present model, LTP function as soluble carriers of lipophilic compounds, which lead to the suggestion that the non-specific LTP is required for the formation of the novel tissue at the stem scar of harvested tomato fruits (Mundy and Rogers, 1986; Sterk *et al.*, 1991; Pyee *et al.*, 1994; Ma *et al.*, 1995). Moreover, the increased level of non-specific LTP transcripts may indicate a post-harvest stimulation of transport activity of lipophilic compounds in order to produce a water loss barrier. Earlier studies demonstrated that the accumulation of LTP was stimulated by different osmotic stress stimuli or ABA treatment (Sossountzov *et al.*, 1991; Tsuboi *et al.*, 1992; Yamada, 1992; Torres-Schumann *et al.*, 1992; Thoma *et al.*, 1993; Molina and Garcia-Olmedo, 1993; Kader, 1996; Kawaguchi *et al.*, 2003). Previously, it was also reported that LTP gene expression in strawberry (*Fragaria ananassa* Duchesne) is stimulated by wounding

(Yubero-Serrano *et al.*, 2003). Taking into account that LTP possibly are responsible for the transfer of lipophilic molecules, the induction under a variety of stresses might be contributed to the adaptation to environmental changes or to the repair of stress-induced damage (Torres-Schuhmann *et al.*, 1992; Kader, 1997; Hollenbach *et al.*, 1997; Dunn *et al.*, 1998; Trevino and O'Connell, 1998; Sohal *et al.*, 1999). However, in spite of many studies on plant LTP, the *in vivo* function of these enzymes is still not fully understood.

Dehydrins form an important group of dehydration-induced enzymes representing a protein family that is structurally characterized by the presence of a conserved lysine rich-domain (Eze *et al.*, 1986; Close *et al.*, 1989; Godoy *et al.*, 1990; Roberton and Chandler, 1992; del Mar Parra *et al.*, 1996). The tomato dehydrin TAS14, an abscisic acid and environmental stress inducible protein, was shown to accumulate in response to different osmotic stresses or ABA treatment with distinct organ, tissue and cellular distribution (Godoy *et al.*, 1990, 1994; del Mar Parra *et al.*, 1996). The harvesting process of tomato fruits was also sufficient for induction of TAS14 gene expression at the stem scar tissue, clearly peaking already during the first day of fruit storage. Interestingly, Godoy *et al.*, 1994 described TAS14 gene expression to be not influenced by wounding in tomato seedlings (*Lycopersicon esculentum* Mill.). Deduced from these findings, it can be suggested that the TAS14 gene expression detected in the stem scar tissue of tomato fruits was promoted by the dehydration stress, which was evoked by the transient uncontrolled water loss at the ragged stem scar tissue of the fruit. The structural features and the biochemical properties of dehydrins including high hydrophilicity and thermostability may indicate a water-retaining capacity of dehydrins probably essential for the osmotic regulation and the maintenance of cell structure and integrity and, therefore, support the putative protective reactions of dehydrins under conditions of dehydration (Close, 1996, 1997; Wisniewski *et al.*, 1999; Koag *et al.*, 2003; Porat *et al.*, 2004). Strikingly, there is a relationship between the level of dehydrins and plant tolerance to dehydration (Allagulova *et al.*, 2003). Furthermore, the stimulation of dehydrin TAS14 is a prominent response to water limitation in tomato probably representing a general role in adapting plants to dehydration stress, although the function of dehydrin TAS14 remains to be elucidated.

The drought stress inducible DS2 proteins are members of the abscisic stress ripening protein family ASR termed corresponding to its stimulation by water deficit and regulation by fruit ripening (Silhavy *et al.*, 1995; Wang *et al.*, 1998). The rapid and strong induction of DS2 gene expression at the stem scar of tomato fruits is in agreement with results of Silhavy *et al.* (1995) found in desiccated leaves of wild potato (*Solanum chacoense* Bitter).

Several reports showed that DS2 accumulation is developmentally regulated as well as subjected to stress stimuli. In particular, the gene expression of DS2 is highly specific to dehydration but not inducible by wounding or ABA treatment (Silhavy *et al.*, 1995; Schneider *et al.*, 1997; Dóczi *et al.*, 2002, 2005). The latter feature distinguishes the DS2 protein from the water-stress-inducible dehydrin TAS14, described above (Godoy *et al.*, 1990). Previously, Çakir *et al.* (2003) and Dóczi *et al.* (2005) speculated that DS2 proteins may be involved in the signal transduction pathways by which plants translate exposure to dehydration stress into changes in gene expression, whereas DS2 acts in response to osmotic stress to survive the severe desiccation conditions.

Furthermore, the differential gene expression of the stem scar tissue included transcripts, which encode an early responsive to dehydration protein ERD7. Yet, the function of this senescence-related ERD7 protein is not determined, although changes in ERD7 gene expression in response to water stress and ABA treatment was documented (Seki *et al.*, 2002; Oono *et al.*, 2003; Vanderauwera *et al.*, 2007). In studies of Bray (2004) and Street *et al.* (2006) altered gene expression patterns in response to different water-deficit stresses of black and eastern cottonwood (*Populus trichocarpa* Hook., *Populus deltoides* (W.Bartram) Marshall) and *Arabidopsis* were documented. ERD7 was found to be commonly up-regulated by dehydration conditions. In addition, Oono *et al.* (2003) identified the induction of ERD7 gene expression in response to dehydration and characterized repressed ERD7 gene expression based on rehydration treatment. Thus, the ERD7 gene product may function in water stress tolerance or response to water limitation (Taji *et al.*, 1999). Unexpectedly, during storage of tomato fruits ERD7 gene expression decreased already in the stem scar tissue at the first day after fruit harvest. According to these results, the water stress determining a rapid rise of the ERD7 transcript level might be only transient. Therefore, the ERD7 transcript is probably related to the earlier alterations at the stem scar tissue of tomato fruits suggesting a regulatory role for the protein encoded by the ERD7 gene.

Accordingly, the products of these wound-inducible genes are classified to contribute to the biosynthesis of a lipophilic layer, to function in direct protection against environmental stresses and to regulate gene expression and signal transduction in stress reactions. Ultimately, these results are in agreement with several lines of evidence suggesting a general role for these genes in protecting against dehydration, although their function remains mostly hypothetical (Bray, 1997; Hasegawa *et al.*, 2000; Shinozaki and Yamaguchi-Shinozaki, 1997; Thomashow, 1999). A functional characterization of these

enzymes is important for elucidating the response to wounding and associated water stress at the molecular level to promote understanding of the molecular mechanisms that underlie the plant reaction to abiotic stress (Skriver and Mundy, 1990; Bray, 1993; Chandler and Robertson, 1994; Xiong *et al.*, 2002; Shinozaki *et al.*, 2003).

Fruit Stem Scar as a Special Tissue of Tomato Fruits

The apoplastic membrane of the fruit stem scar is shown to be visually different from the surrounding fruit tissue. Commonly, this browning is generated by the accumulation of phenolic compounds at or near the wound site, which follows wound-induced *de novo* biosynthetic activity (Bernards, 2002). The stem scar membrane is insoluble, but can be removed by enzymatic isolation. The monomerization of the removed stem scar membrane of tomato fruits released a complex mixture of saturated and unsaturated alkanoic acids, ω-hydroxyalkanoic acids and α, ω-alkandioic acids as major components together with alkanols. The main depolymerization product was α, ω-hexadecandioic acid. In addition, the monomerization of the stem scar membrane exhibited the presence of ferulic acid among the depolymerization products. According to these findings, the stem scar membrane of the tomato fruit consisted of characteristic suberin monomers. These even-numbered long-chain aliphatic constituents of tomato suberin with chain lengths varying mainly between C_{16} and C_{22} were similarly found for suberized cells forming a wound membrane after mechanical manipulation of tomato fruits. This wound membrane is unique and distinct in both the chemical composition and the subcellular location (Dean and Kolattukudy, 1976; Nawrath, 2002; Bernards *et al.*, 2004).

Suberin, a lipophilic biopolymer composed of an aliphatic and an aromatic domain, acts as a barrier between plants and its environment (Kolattukudy, 1977, 1980, 1981, 2001; Bernards and Lewis, 1998). The aromatic domain primarily consists of coumaric acid and ferulic acid. These monophenolic acids are presumably involved in covalently linking the aliphatic domain to the cell wall (Bernards and Lewis, 1998; Bernards and Razem, 2001; Bernards, 2002; Santos and Graça, 2006). The aliphatic domain of suberin is comprised of long-chain, aliphatic compounds belonging to different compound classes such as ω-hydroxyalkanoic acids and α, ω-alkandioic acids and in smaller quantities of alkanols and alkanoic acids (Kolattukudy *et al.*, 1975; Holloway, 1983; Graça and Pereira, 2000). The C_{16}, C_{18}, and C_{22} aliphatic compounds were the most prominent suberin monomers, which ultimately showed an overall chain length distribution ranging from C_{16} to C_{30} (Kolattukudy *et al.*, 1975; Kolattukudy, 1980; Holloway, 1983; Schreiber *et al.*, 1999;

Franke *et al.*, 2005). Most of the C_{18} suberin monomers have mid-chain substituents, such as an unsaturation or an epoxide group (Graça and Santos, 2007). Long-chain C_{16} and C_{22} suberin monomers consist only of saturated alkyl chains.

Nevertheless, the understanding how these suberin monomers are assembled at the macromolecular level remains largely unknown. A recently proposed model described the structure of suberin as a three-dimensional polyester biopolymer based on glycerol as a 'linker' between carboxylated aliphatic suberin monomers (Graça and Pereira, 2000; Bernards, 2002; Graça and Santos, 2007). This aliphatic polyester structure is closely associated with the polymeric aromatics of suberin (Kolattukudy, 2002; Bernards, 2002).

The occurrence of the complex biopolymer suberin in plants is highly variable and restricted to specialized tissues (Schreiber *et al.*, 1999; Groh *et al.*, 2002). Suberin deposition is localized for example in the cell wall of hypodermal, endodermal and peridermal cells in roots and in stems of *Arabidopsis* (Wilson and Peterson, 1983; Zimmermann *et al.*, 2000; Nawrath, 2002; Franke *et al.*, 2005). Furthermore, suberin is detected in the Casparian bands and is formed after mechanical or pathogenic damages in plants (Espelie and Kolattukudy, 1979; Agrios, 1997). Molecular evidence also suggests that abscission and dehiscence for example of falling leaves comprise cell-cell separation processes by the formation of suberized cell layers (Kolattukudy, 1981, 2001; Szymkowiak and Irish, 1999; Roberts *et al.*, 2000; Tabuchi *et al.*, 2000; Mao *et al.*, 2000; Franke *et al.*, 2005).

Strikingly, there is a common pattern in the monomeric composition of the aliphatic suberin domain from different dicotyledonous and monocotyledonous plant species, some variability exists only in the substituted suberin monomers. Graça and Pereira (2000) described the basic pattern of glycerol, ω-hydroxyalkanoic acids and α, ω-alkandioic acids as to be ubiquitous in suberin. The suberin monomers of the stem scar membrane and the wounded epidermis of the tomato fruit with high relative amounts of ω-hydroxyalkanoic acids and α, ω-alkandioic acids and a high proportion of C_{18} unsaturated aliphatic compounds are in agreement with the suberin composition from other plant sources for example onion (*Allium cepa* L.; Zeier and Schreiber, 1998), corn (*Zea mays* L.; Zeier *et al.*, 1999), rice (*Oryza sativa* L.; Schreiber *et al.*, 2005) and *Arabidopsis* (Franke *et al.*, 2005).

In addition to the occurrence and the chemical composition of suberin at the stem scar membrane of tomato fruits, the increasing degree of suberization of the stem scar membrane was microscopically documented *via* auto-fluorescence. The strong auto-fluorescence of the stem scar membrane that originated by the aromatic suberin domain

displayed ordered structures extending several cell layers from the wound (Spotts *et al.*, 1998). Thus, the alterations in the auto-fluorescence at the stem scar tissue visually reflected the thickening of cell walls by suberization. In the course of suberization, the proportion of suberin may form up to 50% of the total chemical composition of the cell walls (Pereira, 1988). Previously, Spotts *et al.* (1998) reported the histochemical analysis of suberin formation in wounded pear fruits (*Pyrus communis* L.) over time. These findings agree very well with the accumulation of suberin compounds at the tomato stem scar membrane produced within few days.

In a direct comparison the aliphatic compound classes of tomato fruits suberin and cutin are strikingly similar (CHAPTER I). Differences were detected concerning the chain length distribution, the saturation of C_{18} suberin monomers and the dominance of varying compound classes (Kolattukudy, 1981; Matzke and Riederer, 1991; Zeier *et al.*, 1999; Schreiber *et al.*, 1999; Franke *et al.*, 2005). Based on the ubiquity in all higher plants and the compositional similarity, the biosynthesis of suberin and cutin monomers may require analogous enzyme activities and most probably share common pathways, although no enzyme directly involved in cutin and/or suberin biosynthesis is characterized in detail yet (Franke *et al.*, 2005).

The biosynthesis of the aliphatic suberin domain is proposed to consist of two major biochemical pathways (Kolattukudy, 1980). The first step involves the elongation of fatty acids precursors producing long-chain fatty acids (Cassagne *et al.*, 1994; Barret *et al.*, 1998; Todd *et al.*, 1999). Bernards (2002) and Kunst *et al.* (2004) determined the pool of C_{16} and C_{18} carboxylic acids derived from plastidial fatty acid biosynthesis to supply the precursors of this pathway. A contribution of VLCFA pathways to the suberin biosynthesis may be excluded, since *lecer6*-deficient tomato fruits defective in a VLCFA elongase showed also suberization of the fruit stem scar as seen for the wild type fruits. The second step in suberin biosynthesis includes modifications of the long-chain fatty acids, for example by ω-oxidation, ω-hydroxylation and dehydrogenation, to generate the characteristic suberin monomers (Agrawal and Kolattukudy, 1978; Pinot *et al.*, 1993; Benveniste *et al.*, 1998; Le Bouquin *et al.*, 1999, 2001; Schuler and Werck-Reichhart, 2003; Duan and Schuler, 2005).

Furthermore, a role of wax accumulation in suberin formation is discussed by several authors, which are synthesized in epidermal cells of aerial organs (Soliday *et al.*, 1979; Kolattukudy, 1968; Espelie *et al.*, 1980; Nawrath, 2002; Jeffree, 2006; Lulai *et al.*, 2006, 2008). However, suberized tissues are deposited generally at the sites of protection,

including after harvest, whereby plant tissues are damaged and require the establishment of a shielding barrier as part of the 'wound healing' process (Kumar and Knowles, 2003; Aharoni *et al.*, 2004). An involvement of waxes incrusted or associated with the suberin biopolymer can be excluded for the formation of the stem scar tissue of tomato fruits, because neither very-long-chain aliphatic compounds nor triterpenoids and sterol derivatives as specific wax components of tomato fruits were detected at the stem scar tissue (data not shown). Only long-chain fatty acid (C_{16} to C_{26}) and derivatives were found probably encrusting the suberin polymer as matrix.

Impact of the Suberized Stem Scar Tissue on Tomato Fruits

The formation of an apoplastic suberin membrane is suggested to play a physiologically pivotal role as plant-environment interface acting wherever and whenever confinement or shielding of the plant surface against the surroundings is essential (Kolattukudy, 2001). Suberin restricts uncontrolled water loss, gas leakage and nutrient depletion as an inward barrier and represents an outward barrier that protects the tomato fruit against environmental aggressions like incorporation of toxic solutes, radiation and infection by pathogens (Schreiber *et al.*, 1999; Groh *et al.*, 2002; Franke and Schreiber, 2007). These features reflect the dual functional barrier character of the suberin biopolymer, primarily determined by its aliphatic domain that is able to co-align and form a hydrophobic layer (Schreiber *et al.*, 1999; Hose *et al.*, 2001; Franke *et al.*, 2005).

At the stem scar tissue of tomato fruits, suberization was shown to generate an effective hydrophobic barrier to suppress transpirational and/or evaporational water loss within three days after harvest. This barrier's property of the suberized stem scar was similar to the stem scar of those fruits, which were artificially sealed with paraffin wax. According to these findings, stress conditions evoked by uncontrolled water loss through the wound tissue are restricted within a few days. Hence, even mature green fruits have the ability to ripen post-harvestly. In addition to the regulatory barrier property at the stem scar tissue, mature green tomato fruits exhibited post-harvest ripening effects since the tomato fruits were no more attached to the parent plant. Such post-harvest ripening effects improved seed quality and thus germination rate of seeds (Cavero *et al.*, 1995). Moreover, post-harvest ripening conditions result in an increased accumulation of antioxidant components such as lycopene, β-carotene, ascorbic acid and phenolic compounds in tomato fruits (Giovanelli *et al.*, 1999).

However, the establishment of the barrier property at the stem scar tissue can be retarded by blocking biosynthetic activity, which is associated with suberin accumulation. Exposure to low temperature as well as cycloheximide treatment of harvested fruits generated a delay in formation of the water loss barrier property. Low temperature indirectly restricts biosynthetic pathways largely by deviation from the optimal reaction conditions and, therefore, effectively held the suberization process near to the minimum detected for freshly harvested fruits (Huner *et al.*, 1993). Lakshminarayana *et al.* (1987) gave also evidence for a drastic reduction of 'wound healing' in developing apple fruits, which were kept at low temperature when compared to fruits stored at room temperature. In contrast, application of cycloheximide directly interferes with biosynthetic activity. Cycloheximide produced by the bacterium *Streptomyces griseus* acts as an inhibitor of the peptidyl transferase activity of the 60S ribosome and, thus, inhibits the mechanism of the translation process (Morris, 1966; Müller *et al.*, 2007). Nevertheless, both treatments have the capacity to affect the *de novo* biosynthesis of the suberized stem scar tissue significantly.

In most cases, pathogens require a wound in the epidermis or stem to enter the susceptible fruit tissue and initiate infection. The wound evoked by fruit harvesting represents such a potential infection zone through which pathogens can spread into the interior of the tomato fruit. To study the relationship between 'wound healing' and decay symptoms caused by pathogens, tomato fruits were tested in two groups: freshly harvested and stored.

The fungal tomato pathogens *Cladosporium herbaceum*, *Alternaria alternata* and *Colletotrichum coccodes* causing anthracnose, black spot disease and canker were found to be less aggressive pathogens during storage of mature green tomato fruits (data not shown; Dillard and Cobb, 1998; Eshel *et al.*, 2002). In comparison, *Fusarium solani*, the causal agent of fruit rot, was found to be a more aggressive, fast growing plant pathogen (Rojas *et al.*, 1999). Nevertheless, the infection rate of *Fusarium solani* was considerably less efficient at the stem scar tissue of stored fruits than in freshly harvested fruits possibly triggered by inhibitory components of the suberized tissue (Poole and McLeod, 1991; Sharrock and Hallett, 1991). Post-harvest changes at the stem scar tissue of the fruit, such as the production of aromatic compounds associated with suberin or other secondary metabolites, may contribute to this resistance by affecting the susceptibility of the fruit (McLeod and Poole, 1994; Poole and McLeod, 1994; Kpémoua *et al.*, 1996). In turn, several studies specified explicitly the aliphatic suberin domain as the final barrier to

fungal infection (Lulai and Corsini, 1998; Lulai *et al.*, 2006; Thomas *et al.*, 2007). Regardless of the different views, the suberized stem scar tissue made the stem end of the fruit unsuitable as route of entry. The results of this analysis are comparable to those of Pennycook and Manning (1992) describing a reduced infection of wounded kiwifruits (*Actinidia deliciosa* (A.Chev.) C.F. Liang & A.R. Ferguson) inoculated with *Botrytis cinerea* with increasing fruit storage. In addition, the decay of pear fruits (*Pyrus communis* L.) was reduced by the formation of suberin compounds (Spotts *et al.*, 1998).

Consequently, the produced 'wound healing' tissue of the fruit was shown to provide both, a dehydration barrier and an interface between the outermost plant surface and their hostile environment. However, the examinations of this study focused on mature tomato fruits, but the developmental stage of tomato fruits at harvest could also be an important factor for determining the effect of wounding. Fruit ripening involves biochemical changes such as cell wall breakdown, membrane alteration and fruit softening, which may influence the 'wound healing' process. Recent work by Torres *et al.* (2003) revealed a closer relationship between these biochemical changes and susceptibility of apple fruits (*Malus domestica* Borkh.). The results indicated a lower resistance of ripe apple fruits to *Penicillium expansum* than unripe fruits. In particular, the barrier property of the stem scar tissue of red ripe fruits was characterized to be less capable to limit water loss in comparison to mature green fruits. Thus, the suberin deposition at the stem scar tissue of tomato fruits by harvest might be dependent on the fruit developmental stage. Differences in the stem scar tissues of mature green and red ripe tomato fruits, respectively, might be evoked by differences in the efficiency of polymerization during the suberization process or based on variable suberin composition (Prusky *et al.*, 1985; Lavy-Meir *et al.*, 1989). The weight of the stem scar tissue is approximately twofold higher in red fruits compared to mature green fruits, whereas the barrier property did not exceed the efficiency of mature green fruits. These contrasting rates of water loss and stem scar weights between fruits of both developmental stages might probably reinforce the hypothesis that preferentially mature green fruits exhibit a higher physiological potential for an efficient protection layer providing for seed and fruit ripening processes than red ripe fruits representing full developed fruits that enclose matured seeds. However, the discrepancy between the suberin amount and the reduction in water loss found in mature green and red ripe fruits and, additionally, findings of Hose *et al.* (2001) and Schreiber *et al.* (2005) imply that more than only quantity of the suberin biopolymer is important of a selective water barrier property.

Abscisic Acid as a Regulatory Molecule in Formation of the Stem Scar Tissue

Wounding produces a signal that migrates through the plant tissue and promotes gene expression resulting in the formation of a suberized tissue. A concomitantly wound-initiated dehydration stress also causes specific alterations in the gene expression pattern. The mechanisms by which plants translate exposure to stress into changes in gene expression remain still unclear, although a number of genes responding to wounding or dehydration are known (Silhavy *et al.*, 1995). Both effects on gene expression wounding and water loss stress might be commonly triggered by the regulatory molecule abscisic acid (ABA), which induces or represses various genes (Bray, 1993; Chandler and Robertson, 1994). The critical role of ABA includes a number of physiological processes both during normal development and in response to stress conditions (Leung and Giraudat, 1998; Marion-Poll and Leung, 2006). Thereby, ABA was extensively related to different kinds of osmotic changes and wounding, although its role in the response mechanism is not yet established. ABA is known as a growth regulator of plants that acts mainly to inhibit growth, promotes dormancy and particularly supports stress tolerance, whereby ABA stimulates adaptation of the osmotic potential and a decrease in metabolism in order to prevent water loss and further adverse conditions. In addition, evidence exists for the involvement of ABA in the initial response to mechanical wounding (Mansfield, 1987; Peña-Cortés *et al.*, 1989; Peña-Cortés and Willmitzer, 1995; Reymond *et al*., 2000).

The endogenous ABA content at the stem scar tissue of tomato fruits showed significant changes. Following the initial increase to a putative threshold a slight reduction occurred and, ultimately, adjusted to this elevated level up to the fourth day of fruit storage. A previous study using wounded potato tuber (*Solanum tuberosum* L.) as model system documented similar changes in the ABA content as found for the stem scar tissue of harvested tomato fruits exhibiting three different stages: a basal, a wound related and a 'wound healing' level of endogenous ABA content (Lulai *et al.*, 2008). Accordingly, Lulai *et al.* (2008) proposed an involvement of the early wound-induced peak in ABA accumulation into the formation of the aromatic domain during suberization. The later induced accumulation of the aliphatic domain of suberin during the 'wound healing' process is subjected to this prearrangement of aromatic compounds for assembly and further covalent arrangement of the aliphatic suberin components. Moreover, the immediate availability of a basal ABA level might be necessary for the rapid initiation and regulation of the biosynthesis of the suberin layer protecting plant tissues from dehydration (Lulai *et al.*, 2008).

As a consequence of the rise in endogenous ABA that occurs in wounded and dehydrated plant tissues, a pivotal role for ABA in regulating gene expression at the stem scar tissue after fruit harvest is suggested (Cottle and Kolattukudy, 1982; Skriver and Mundy, 1990; Silhavy *et al.*, 1995; Bray, 1997; Moons *et al.*, 1997; Fowler and Thomashow, 2002; Kreps *et al.*, 2002; Seki *et al.*, 2002, 2003; Gong *et al.*, 2005). In fact, ABA responsiveness is a common feature of most genes detected to be differentially expressed at the stem scar tissue of tomato fruits. The differential expression of ERD7, non-specific LTP and dehydrin TAS14 in response to dehydration stress and wounding are analyzed and discussed above.

The stimulation of LTP gene expression along with increased endogenous ABA levels was seen in tomato (*Lycopersicon esculentum* Mill.) and, recently, in strawberry (*Fragaria ananassa* Duchesne; Torres-Schumann *et al.*, 1992; Molina and Garcia-Olmedo, 1993; Cohen *et al.*, 1999; Yubero-Serrano *et al.*, 2003). Under conditions of cadmium exposure, Hollenbach *et al.* (1997) demonstrated a relationship between the transcriptional level of a non-specific LTP, increase in ABA content and deposition of lipophilic compounds on the surface of barley leaves (*Hordeum vulgare* L.). Moreover, Trewavas and Jones (1991) reported a weak correlation between endogenous ABA contents and the production of suberin. All these findings may support the hypothesis that non-specific LTP participate in the transfer of suberin monomers from the subcellular site of biosynthesis to the wounded plant tissue such as the stem scar tissue of tomato fruits.

The regulation of dehydrin gene expression occurs *via* ABA-dependent and ABA-independent pathways (Choi *et al.*, 1999; Giordani *et al.*, 1999; Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu, 2002). The tomato gene encoding dehydrin TAS14 was shown to be strongly induced by ABA treatment and, thus, probably representing also a component of the ABA controlled mechanism in stress response (Godoy *et al.*, 1990, 1994). Although dehydrin TAS14 is ABA-inducible, its gene regulation by stress is complex and may involve internal signals in addition to ABA and/or factors modulating the ABA signal (Godoy *et al.*, 1990).

The ERD7 gene expression was also reported to be responsive to ABA treatment (Vanderauwera *et al.*, 2007). Only for the DS2 protein no indication for a regulation by ABA at the transcriptional level was given (Dóczi *et al.*, 2005).

These findings together with the increased suberization and the reduced water loss stress corroborate the conclusion that ABA mediates stress signaling in the cascade of events that lead from the stress perception to the induction of specific gene expression and,

hence, influences the amount of the several transcripts at the stem scar tissue of tomato fruits.

Driven by a wounding signal, adaptations in biosynthetic processes develop and then turn into responses to stress reaction features. Several reports revealed genes that are induced by water deficit or wounding, but are unresponsive to exogenous ABA treatment, suggesting a possibility of the existence of both an ABA-dependent and an ABA-independent signal transduction cascades, respectively (Guerrero *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1992; del Mar Parra *et al.*, 1996; Bray, 1997; Wang *et al.*, 1998). This indicates complex regulatory mechanisms in the 'wound healing' process between stress signaling and gene expression activity in plants for example induced by jasmonic acid or ethylene representing plant regulatory molecules, which also contribute to plant defense (Reinbothe *et al.*, 1992; Blechert *et al.*, 1995; Peñia-Cortés *et al.*, 1995; Doares *et al.*, 1995; Conconi *et al.*, 1996; Wasternack *et al.*, 1998). Creelman and Mullet (1995) speculated that the production of higher levels of endogenous jasmonic acid stimulates only short-time adaptations during water stress conditions, whereas Nakano *et al.* (2003) showed induced ethylene production within few days modulated by water loss. So far, the presented data confirm that endogenous ABA acts as a key regulator in the signaling cascade in the stem scar tissue of tomato, but other regulatory mechanisms could also be involved (Peña-Cortés *et al.*, 1996; Wasternack and Parthier, 1997; Birkenmeier and Ryan, 1998).

To investigate a putative link between ABA levels and the formation of the stem scar tissue, different concentrations of ABA or tetcyclacis were exogenously applied to the stem scar tissue. Tetcyclacis, a growth retardant, influences ABA activity by inhibiting hydroxylation of ABA to 8'-hydroxymethyl-ABA and, thus, the oxidative pathway of ABA degradation (Rademacher *et al.*, 1987; Zeevaart *et al*., 1989; Daeter and Hartung, 1990; Betz *et al.*, 1993). Both treatments did not result in significant changes in the water loss rate probably because of minor absorption into the fruit interior (data not shown).

Therefore, a 'reverse genetic' tool of ABA mutant fruits was investigated to determine the influence of ABA in 'wound healing' processes at the stem scar tissue and the involvement in the resistance to water loss. Experiments were performed with tomato fruits of ABA-deficient mutants *notabilis*, *sitiens* and *flacca*. The corresponding wild type fruits Rheinlands Ruhm and Ailsa Craig were distinctly distinguishable from fruits of MicroTom wild type in relation to their fruit size but not referring to their initial water loss barrier property.

The mutant fruits *notabilis*, *sitiens* and *flacca* contained different levels of endogenous ABA *via* blockage of different steps in the ABA biosynthesis, which involve the oxidative cleavage of a 9-*cis*-epoxycarotenoid precursor to xanthoxin and a subsequent conversion of xanthoxin to ABA aldehyde (Taylor and Tarr, 1984; Linforth *et al.*, 1987; Marin and Marion-Poll, 1997; Burbidge *et al.*, 1999; Herde *et al.*, 1999; Taylor *et al.*, 2000). Measurements on ABA mutant fruits showed that the reduced levels of endogenous ABA biosynthesis inhibited the decrease in water loss and the accumulation of suberin monomers at the stem scar tissue with regard to wounding. The combined analysis of the water loss rate and the degree of suberization in wild type and mutant fruits revealed a close relationship with ABA content controlling these parameters (Lulai *et al.*, 2008). These data provide evidence for a positive correlation between the presence of a physiological ABA level and the elevated suberization of the stem scar tissue, which, in turn, led to the distinctly reduced dehydration of the tomato fruit.

Apart from the previous discussions, the question remains whether or not ABA increases in stem scar tissues, exposed in order to wounding to regulate the water deficit and/or is required for suberin accumulation. Findings of Birkenmeier and Ryan (1998) suggest that ABA is not a component of the wound-inducible signal transduction pathway, since ABA levels increase preferentially near the wound site. In agreement, Cohen *et al.* (1999) demonstrated that exclusively dehydration stress led to the accumulation of ABA beginning approximately four hours after a short lag time and reaching a maximum up to the second day. These findings are consistent with the relationship between dehydration stress and endogenous ABA accumulation in several plant tissues (Zeevaart and Creelman, 1988; Gomez *et al.*, 1988; Marion-Poll and Leung, 2006).

Contribution of Suberization to the Barrier Properties of Tomato Fruits

Stress conditions cause several important developmental and physiological alterations in plants, whereas stress-induced changes in gene expression provide the basis for the development of protective mechanisms responsible for adaptation and resistance to environmental restrictions (Skriver and Mundy, 1990; Bray, 1993; Chandler and Roberton, 1994; Shinozaki and Yamaguchi-Shinozaki, 1997; Xiong *et al.*, 2002; Shinozaki *et al.*, 2003). The regulation of the complex plant responses to stress depends on the extent of stress, the affected plant tissue and its developmental stage (Bray, 2002).

The tomato fruit is subjected to high stress conditions by the fruit harvest process. Wound stress induced *via* harvesting requires 'healing' biosynthetic processes directly after

wounding, which catalyze defense mechanisms in order to protect the fruit. Very rapidly after fruit harvest, a shielding layer including suberin deposition restricted the loss of gas, water and nutrients and avoided the entry of plant pathogens. Among the constitutive resistance mechanism of suberin, produced at wound sites, little is known about the macromolecular structure, the coordinated biosynthetic activities and the mediating signaling cascade in 'wound healing'. The current study indicate the stem scar tissue of tomato fruits as a model for suberized tissues due to the confirmation of suberin deposition in other plant species (Franke *et al.*, 2005).

The negative correlation between *de novo* suberin biosynthesis and its impact on water permeability was indicated and its supporting effect in pathogen defense during fruit storage was clarified. Investigations at the stem scar tissue clearly show a critical regulatory role for endogenous ABA in post-harvest stress signaling concerning the suberization and the development of resistance to water loss. Obviously, ABA profoundly influences the conversion of the stress signal into alterations in plant gene expression (Wang *et al.*, 1998). The determination of ABA as essential modulator for post-harvest alterations and the characterization of different wound-related gene expression patterns, make this study of major interest because of future post-harvest technologies controlling wound-related damage of the agriculturally important tomato fruit. In general, consequences of such stress-responsive signaling that facilitates wound response may have a wide influence on fruit ripening, but the analysis of the molecular aspects of signaling in response to fruit harvest is only at the beginning.

CONCLUDING REMARKS AND FUTURE PROSPECTS

For the first time it could be demonstrated that an intact plant model system, fruits of the horticultural crop tomato (*Lycopersicon esculentum* Mill.), offers the opportunity for an integrative functional approach that correlates cuticular wax biosynthesis, quantitative and qualitative wax deposition, and its function as a transpiration barrier. The focus was set on molecular, biochemical and physiological mechanisms of cuticular wax formation in the course of the fruit developmental process of MicroTom wild type and MicroTom *lecer6*, a wax mutant.

Cuticular Wax Architecture and Function: For intact tomato fruits the developmental and functional relationship between permeance for water and the qualitative composition of their cuticular waxes was shown. These results demonstrate the important role of cuticular wax constituents, mainly the accumulation of very-long-chain *n*-alkanes, which reduced the cuticular water permeance during the developmental course of tomato fruit ripening. Further studies on the compositional complexity of epi- and intracuticular waxes are necessary to shed some additional light on the distribution of the varying compounds within the cuticular wax load during fruit development and their proportion of the transpiration barrier of the tomato fruit. Moreover, the relative contribution of the polar and the apolar pathways for the water transport across the tomato fruit cuticle remains to be elucidated.

Spatial and Temporal Regulation of Wax Biosynthesis: The molecular dissection of developmental processes of tomato fruits with particular emphasis on genes involved in cuticular wax biosynthesis and lipid transfer process, were studied in microarray experiments. In combination with localization studies, for example RNA *in situ* hybridizations, the microarray data obtained in this thesis will help to substantiate the molecular and structural basis of the water barrier property in the course of fruit development. Future investigations should also include the protein level for revealing putative differences between the transcriptional and translational regulation of the underlying mechanisms of wax biosynthesis and wax accumulation. Thereby, establishing of even more sophisticated tools may lead to dissect metabolic pathways, for instance in water balancing at both, the spatial and the temporal levels, in order to allow comprehensive understanding of networks occurring within tomato fruit development.

Transgenic Characterization of* LeCER6:* Up to now, the β-ketoacyl-CoA synthase LeCER6 involved in very-long-chain fatty acid elongation associated with cuticular wax biosynthesis was functionally characterized by the knock-out mutation of LeCER6. The question remains what phenotype would mark transgenic tomato plants upon overexpression of the *LeCer6* gene. In this context, the cellular localization of the LeCER6 enzyme and enzyme substrate pools has to be analyzed as well. Heterologously expressed LeCER6 in yeast is discussed to facilitate the gain of detailed information on the substrate specificity of this membrane associated enzyme. Furthermore, constructs of the *LeCer6* promoter region fused to the β-glucuronidase (GUS) reporter gene could provide more insights into the regulation of *LeCer6* gene expression that is suggested to be responsive to light and different osmotic stresses based on a promoter element analysis.

Organ-Specific Character of* LeCER6*: In order to explore the integrative role of the β-ketoacyl-CoA synthase LeCER6 in tomato plants, in addition to the fruits and leaves, all parts of the plant need to be analyzed. In particular, experiments on the flowers remain to be established, since the function of the β-ketoacyl-CoA synthase LeCER6 participating in floral organ separation and pollen-stigma communication of tomato is still unclear.

Environmental Parameters: Several aspects of environmental influences or mechanical manipulation concerning the fruit wax layer were investigated and related to the overall water barrier property. For future research projects other potential effectors in wax biosynthesis have to be examined, such as light, circadian rhythm, temperature, salt and mycorrhization. Regulatory molecules like salicylic acid and abscisic acid, already discussed for the transcriptional gene regulation of *LeCer6* and *AtCer6* of *Arabidopsis thaliana* (L.) Heynh., have to be analyzed.

Fruit Stem Scar: The stem scar forms a special tissue of the tomato fruit. After the harvest, suberization of the fruit stem scar reduces water loss and pathogenic attacks. Although compositional biosynthetic pathways are still poorly understood, endogenous abscisic acid was specified as a signal molecule, which plays an important role in the suberization process. Further examinations have to clarify if wounding of the fruit tissue by the harvesting process additionally influences the endogenous level of jasmonic acid or ethylene. Moreover, the effect of exogenously vacuum infiltrated signaling molecules such as abscisic acid, jasmonic acid and corresponding antagonists should be investigated.

SUMMARY

Cuticular waxes cover all above-ground growing parts of plants. They provide the outermost contact zone between plants and their environment and play a pivotal role in limiting transpirational water loss across the plant surface. The complex mechanisms in cuticular wax biosynthesis conferring proper barrier function still remain to be elucidated.

The present study focuses on biosynthetic pathways in wax formation, cuticular wax accumulation and composition and its impact on the epidermal barrier property of the intact system of the astomatous tomato fruit (*Lycopersicon esculentum* Mill.). Fruits of all developmental stages of the wild type cultivar MicroTom and its *lecer6* mutant defective in a β-ketoacyl-CoA synthase involved in very-long-chain fatty acid elongation were analyzed. This 'reverse genetic' approach clarified the importance of the β-ketoacyl-CoA synthase LeCER6 for epidermal barrier property *in vivo* on the biochemical-analytical level, on the transcriptional level and, furthermore, on the physiological level comparatively between MicroTom wild type and MicroTom *lecer6*.

Surfaces of MicroTom wild type and MicroTom *lecer6* fruits showed similar patterns of quantitative wax accumulation, but differed considerably in the permeance for water. Qualitative analyses of the chemical composition of fruit cuticular waxes in the course of fruit development revealed the meaning of the β-ketoacyl-CoA synthase deficiency in the *lecer6* mutant. Fruits of this mutant exhibited a distinct decrease in the proportion of *n*-alkanes of chain lengths $> C_{28}$. Moreover, a concomitant increase in pentacyclic triterpenoids became discernible in the mature green fruit stage of the mutant. Since quantitative changes of the cutin matrix were not sufficient to affect transpiration barrier properties of the *lecer6* mutant presumably the shift in cuticular wax biosynthesis of the *lecer6* mutant is responsible for the observed increase of water permeance.

In order to investigate the molecular basis of wax formation, a microarray experiment was established that allows the simultaneous and comprehensive analysis of the timing and abundance of transcriptional changes in MicroTom wild type and MicroTom *lecer6*. This microarray consists of 167 oligonucleotides corresponding to EST and gene sequences of tomato potentially participating in wax biosynthesis, wax modification, transport processes and stress responsiveness. These parameters were correlated with the course of fruit development. This comparison of gene expression patterns showed a variety of differential

expressed transcripts encoding for example lipid transfer proteins and the dehydrin TAS14. On the basis of these findings, it can be proposed that diverse regulatory mechanisms like lipid transfer processes or osmotic stress response are affected by the LeCER6 deficiency, which is primarily accompanied by an impaired water barrier property of the fruit cuticle.

This present study correlates the continuous increase of *LeCer6* gene expression and the accumulation of very-long-chain *n*-alkanes within the cuticular waxes during the transition from the immature green to the early breaker fruit phase displaying a developmental regulation of the cuticular wax biosynthesis.

Organ-specific wax biosynthesis resulted in different cuticular wax pattern in tomato fruits and leaves. Moreover, in contrast to the fruits, LeCER6-deficient leaves showed a significantly reduced wax accumulation, mainly due to a decrease of *n*-alkanes with chain lengths > C_{30}, while the proportion of pentacyclic triterpenoids were not affected. Deduced from these biochemical-analytical data on tomato fruits and leaves LeCER6 was characterized as a key enzyme in VLCFA biosynthetic pathway responsible for cuticular wax accumulation.

In silico analysis of the LeCER6 sequence revealed the presence of two putative transmembrane domains in the N-terminal position. In addition, highly conserved configurations of catalytic residues in the active site of the enzyme were observed, which are probably essential to its overall structure and function in the fatty acid elongation process. High sequence homology of LeCER6 to the very-long-chain condensing enzymes GhCER6 of *Gossypium hirsutum* L. and AtCER6 of *Arabidopsis thaliana* (L.) Heynh. was found, which might be a good evidence for similar biochemical functions.

Apart from developmental regulation of the cuticular wax biosynthesis, environmental factors influenced the cuticular wax coverage of tomato fruits. Mechanical removal of epicuticular fruit wax evoked large-scale modifications of the quantitative and qualitative wax composition, such as a reduction of aliphatic wax components, and therewith affected the cuticular water permeability. A subsequent regeneration event was included in the regular wax biosynthesis process and led to the compensation of the detached wax amounts and increased the water barrier properties of the cuticular membrane again. In contrast, water-limited conditions had only minor impact on alterations in cuticular wax biosynthesis and, consequently, on the permeance for water of tomato fruits.

Floral organ fusion and conditional sterility, as observed in this study, are caused as pleiotropic effects in cell-cell signaling by the loss-of-function mutation in LeCER6. These

findings corroborated the functional impact of LeCER6 on the epidermal integrity and are consistent with the current knowledge on *eceriferum* mutants of *Arabidopsis*.

Investigations of phenotypic and biochemical characteristics of tomato fruits allowed a broader system-orientated perspective of the fruit development of MicroTom wild type and its *lecer6* mutant. These analyses highlight more precisely alterations in the fruit surface area, fresh and dry weight, epidermal cell density, photosynthetic activity or glucose content in the course of fruit development. The differences between MicroTom wild type and MicroTom *lecer6* characterize very well the large-scale consequences of the LeCER6 deficiency on the physiological status of tomato fruits. Moreover, the results clearly show a part of the genetic controlled network that governs tomato fruit metabolism and mediates extensive changes of the tomato fruit life cycle.

The analyses of the stem scar tissue of the tomato fruit revealed a complex set of responses caused by the harvesting process in detail. Throughout storage of the tomato fruits barrier properties were attributed to the suberized stem scar tissue in regard to water loss limitation and reduction of the fungal infection rate. Thereby the endogenous level of abscisic acid was found to be involved in the molecular signaling pathway that regulates the *de novo* formation of this tissue. For the first time, the chemical composition and physiological importance could be correlated with molecular changes at the transcriptional level during suberization of the stem scar of tomato fruits.

In conclusion, this work indicates a novel intact model system for an integrative functional approach for plant barrier properties that was successfully established and carefully studied. The results highlight correlations between wax biosynthesis, distribution of cuticular waxes, and its relevance on the transpirational water loss across the plant surface and, thus, promote the global understanding of plant cuticle biology.

ZUSAMMENFASSUNG

Kutikuläre Wachse bedecken alle oberirdischen Pflanzenteile und stellen somit die Kontaktzone zwischen Pflanzen und ihrer Umwelt dar. Zudem spielen sie eine entscheidende Rolle für den Schutz der Pflanzen vor unkontrolliertem Wasserverlust. Die komplexen Mechanismen der Wachsbiosynthese, die zur dieser Barrierefunktion beitragen, sind jedoch noch weitgehend unaufgeklärt.

Die vorliegende Arbeit untersucht Biosynthesewege von kutikulären Wachsen, ihre chemische Beschaffenheit sowie deren funktionelle Bedeutung als Transpirationsbarriere an dem intakten System der astomatären Tomatenfrucht (*Lycopersicon esculentum* Mill.). Untersuchungen wurden dabei an Früchten unterschiedlicher Entwicklungsstadien des Tomatenkultivars MicroTom Wildtyp und dessen *lecer6* Mutante durchgeführt. Die *lecer6* Mutante ist durch einen genetisch determinierten Defekt in der β-Ketoacyl-CoA Synthase LeCER6 unfähig zur Verlängerung von sehr langkettigen Fettsäuren. Durch diesen '*reverse genetic*' Ansatz wurde der Einfluss der β-Ketoacyl-CoA Synthase LeCER6 auf die Barrierefunktion der Epidermis zunächst *in vivo* auf der biochemisch-analytischen und physiologischen Ebene vergleichend zwischen MicroTom Wildtyp und MicroTom *lecer6* analysiert. Daran schlossen sich Untersuchungen auf transkriptioneller Ebene an.

Die den Früchten von MicroTom Wildtyp und der *lecer6* Mutante aufgelagerten Wachse unterscheiden sich quantitativ nur wenig, weisen hingegen deutliche Unterschiede in der qualitativen Zusammensetzung und den Wasserleitwerten auf. Die Analyse der chemischen Zusammensetzung der kutikulären Wachse zeigte im Verlauf der Fruchtentwicklung, dass die Defizienz in der β-Ketoacyl-CoA Synthase LeCER6 eine Abnahme des *n*-Alkananteils in den Wachsen ab einer Kettenlängen > C_{28} bewirkt, was bereits im Stadium der reifen grünen Früchte zu erkennen ist. Die in der *lecer6* Mutante vermehrt eingelagerten pentazyklischen Triterpenoide können die Transpirationsbarriereeigenschaft der aliphatischen *n*-Alkane nicht adäquat ersetzen. Ein möglicher Einfluss der ebenso untersuchten Kutinmatrix der Tomatenfrucht konnte ausgeschlossen werden.

Für eine umfangreiche Genexpressionsanalyse von MicroTom Wildtyp und MicroTom *lecer6* wurde ein *microarray* Experiment konzipiert, welches 167 Oligonukleotide umfasst entsprechend zu bekannten EST- und Gensequenzen der Tomate, die möglicherweise an

der Wachsbiosynthese, Wachsmodifikation, relevanten Transportprozessen oder Stressreaktionen beteiligt sind. Der Vergleich der Genexpression zwischen Wildtyp und der *lecer6* Mutante zeigte eine Vielzahl von differentiell expremierten Transkripten unter anderem Lipidtransferproteine und das Dehydrin TAS14. Anhand derer kann davon ausgegangen werden, dass der Verlust der LeCER6 Funktion unterschiedliche regulative Mechanismen beeinflusst, wie zum Beispiel Lipidtransportprozesse und Reaktionen des osmotischen Stresses, die mit einer Schwächung der kutikulären Transpirationsbarriere der Fruchtepidermis einhergehen.

Die vorliegende Studie belegt zudem erstmals einen Zusammenhang zwischen der Steigerung der *LeCer6* Genexpression, der nur geringfügig zeitverzögerten Anreicherung sehr langkettiger *n*-Alkane in den kutikulären Wachsen und der daraus resultierenden Barriereleistungsfähigkeit. Ebenso wird eine Regulation der kutikulären Wachsbiosynthese in Abhängigkeit von den jeweiligen Stadien der Fruchtentwicklung veranschaulicht.

Der organspezifische Vergleich der kutikulären Wachsbiosynthese zeigte, dass sich die Wachsmuster von Früchten und Blättern der Tomatenpflanzen deutlich voneinander unterscheiden. Die Wachsakkumulation auf der Blätterepidermis ist durch die LeCER6-Defizienz hauptsächlich im Anteil sehr langkettiger *n*-Alkane $> C_{30}$ signifikant herabgesenkt, während der Gehalt an pentazyklischen Triterpenoiden jedoch nicht, so wie in den Früchten der *lecer6* Mutante beobachtet, ansteigt. Aufgrund dieser Untersuchungen der Tomatenfrüchte und -blätter konnte LeCER6 als ein Schlüsselenzym für die Verlängerung sehr langkettiger Fettsäurederivate innerhalb der kutikulären Wachsbiosynthese funktionell charakterisiert werden.

Anhand von vergleichenden *in silico* Sequenzanalysen mit den Fettsäurenelongasen GhCER6 aus *Gossypium hirsutum* L. und AtCER6 aus *Arabidopsis thaliana* (L.) Heynh. konnten sowohl zwei mögliche transmembrane Proteindomänen im N-terminalen Bereich als auch hochkonservierte Bereiche im katalytischen Zentrum des LeCER6-Enzyms lokalisiert werden, die vermutlich zur funktionellen Struktur des Enzyms beitragen.

Neben der bereits angeführten entwicklungsabhängigen Regulation der Wachsbiosynthese beeinflussen auch Umweltstressoren die kutikuläre Wachsauflage der Tomatenfrüchte. Ein mechanisches Entfernen der epikutikulären Wachse führt zu einer beträchtlichen Reduktion der aliphatischen Wachsbestandteile, welche maßgeblich die Barriereeigenschaft der Kutikulamembran bestimmen. Die einsetzende Regeneration der manipulierten Wachsoberfläche führt zu einer vollständigen Kompensation der entfernten Wachskomponenten, so dass die Tomatenfrüchte in nur kurzer Zeit wieder eine dem

Reifestadium entsprechende normale Verteilung der kutikulären Wachse aufweisen. Im Gegensatz dazu führt Wassermangel nur zu sehr geringfügigen qualitativen und quantitativen Veränderungen der kutikulären Wachsschicht und folglich des Wasserleitwertes der Tomatenfrüchte.

Die hier dokumentierte Organfusion der Blüte und die eingeschränkte Sterilität der Tomatenpflanzen wurden als pleiotrope Effekte der *lecer6* Mutation auf die Zell-Zell-Kommunikation charakterisiert, was der funktionellen Bedeutung von LeCER6 für die Epidermisintegrität entspricht und mit Beobachtungen an *eceriferum* Mutanten in *Arabidopsis* übereinstimmt.

Die kombinierte Untersuchung phänotypischer und biochemischer Merkmale der Tomatenfrucht erlaubt eine breitere, systemorientierte Gegenüberstellung der Fruchtentwicklung von MicroTom Wildtyp und MicroTom *lecer6*. Dabei werden durch die Analysen von Größe, Frisch- und Trockengewicht, Dichte der Epidermiszellen, Photosyntheseaktivität und Glukosegehalt der Früchte die Unterschiede zwischen MicroTom Wildtyp und der *lecer6* Mutante deutlich aufgezeigt. Die LeCER6-Defizienz der Mutante führt dabei zu weitreichenden Veränderungen im physiologischen Status der Frucht. Diese Ergebnisse spiegeln somit einen Teil des physiologischen Netzwerkes wider, welches weitreichende sekundäre Veränderungen im Lebenszyklus der Tomatenfrucht vermittelt.

Das Stielnarbengewebe der Tomatenfrucht wird infolge der Verletzung durch den Ernteprozess gebildet. Basierend auf der *de novo* Suberinbiosynthese kann diesem Gewebe eine wichtige Barrierefunktion sowohl zur Einschränkung des unkontrollierten Wasserverlustes als auch zur Verringerung der Infektionsrate durch einen pilzlichen Erreger während der Lagerung von Tomatenfrüchten beigemessen werden. Eine Beteiligung der endogenen Abscisinsäure an dem der Bildung des suberinisierten Gewebes der Fruchtstielnarbe zugrunde liegendem, molekularen Signalweg konnte nachgewiesen werden.

Zusammenfassend dokumentiert diese Arbeit erstmalig detaillierte Studien im Hinblick auf pflanzliche Barriereeigenschaften an einem intakten Modellsystem. Die präsentierten Ergebnisse zu molekularen Untersuchungen der Wachsbiosynthese und qualitative and quantitative Analysen der Wachsakkumulation werden im Zusammenhang des Schutzes der Pflanzenoberfläche gegen Wasserverlust durch Transpiration diskutiert und bieten somit neue Erkenntnisse über die pflanzliche Kutikula.

MATERIALS AND METHODS

Plant Material

Fruits and leaves from tomato (*Lycopersicon esculentum* Mill.) cultivar MicroTom were investigated. Both wild type (Florensis, Stuttgart, Germany) and Ac/Ds transposon-tagged plants with a deficiency in a fatty acid β-ketoacyl-CoA synthase (LeCER6; Prof. Avraham A. Levy, Department of Plant Sciences, Weizmann Institute of Science, Rehovot, Israel) were analyzed. Identification and characterization of this knock-out insertional mutant was reported previously by Vogg *et al. (*2004). The tomato plants were cultivated in a growth chamber with 75% relative humidity, a 14-h photoperiod at 450 μmol photons m^{-2} s^{-1}, and a temperature regime of 22°C/18°C (day/night). Plants were grown in commercial pots with 510 ml capacity, irrigated daily and fertilized with 1‰ (w/v) Hakaphos Blau nutrient solution (Compo, Münster, Germany) on a weekly basis.

Fruits from tomato cultivar Rheinlands Ruhm and Ailsa Craig with the corresponding mutants *flacca*, *sitiens* and *notabilis*, limited in their abscisic acid (ABA) content, were additionally investigated (Tomato Genetics Resource Center, Department of Plant Sciences, University of California, Davis, CA, USA).

For analyzing the effects of atmospheric humidity and soil irrigation, plants were cultivated under modified conditions in a growth chamber with 40% or 80% relative humidity, respectively. Once a week the potted plants were substrate saturated watered. Two days after, only a group of plants was irrigated with a defined volume of water for the next 4 days on a daily basis. The weight of the potted plants was documented using a balance with a precision of 5 g (Wedo Karat, Münster, Germany).

Developmental Categorization of Tomato Fruits

MicroTom wild type and LeCER6-deficient fruits were classified on the basis of their size and pigmentation into comparable categories. Immature green fruits of developmental category 'fruit set' were harvested between 1 and 3 days after flowering and were only investigated in the microarray experiments. Immature green fruits of category I were collected between 7 and 10 days after flowering. This particular developmental stage was the earliest suitable for determination of fruit permeance for water and chloroform wax extraction. Category II fruits, representing mature green fruits, were harvested between 25

to 30 days after flowering. For categories III to VI, early breaker, breaker, orange, and red ripe pigmentation rather than fruit size was the major criterion of categorization. At about 40 days after flowering all fruits entered into category VII, named as red overripe.

Determination of Fruit and Leaf Surface Area

Fruit surface area in addition to fruit volume was calculated from the average of vertical and horizontal diameters by assuming a spherical shape of the fruit. Leaf surface area was estimated by means of a commercial image scanner. The pixel counts of a reference image generated by the scanner were measured and positively correlated with the amount per unit leaf surface area.

Determination of Fruit Fresh Weight, Dry Weight and Water Content

The fresh weight of tomato fruits was measured using a balance with a precision of 0.1 mg (Scaltec SBA 31, Heiligenstadt, Germany). Subsequently the tomato fruits were lyophilized at -55°C under vacuum. After 48 h in an freeze dryer (Christ Alpha 1-2 LD, Osterode am Harz, Germany) and an adaptation at room temperature in an exsiccator the fruit dry weight was recorded and the percentage of fruit weight loss, representing the total water content, calculated.

Enzymatic Isolation of Cutin and Suberin Fruit Tissues

Cuticular membranes of intact tomato fruits were isolated enzymatically with pectinase (Trenolin Super DF; Erbslöh, Geisenheim, Germany) and cellulase (Celluclast; Novo Nordisk AIS, Bagsvaerd, Denmark) in 20 mM citrate buffer (pH 3.0; Merck, Darmstadt, Germany), supplemented with 1 mM sodium azide (Sigma-Aldrich, St. Louis, MO, USA) according to Schönherr and Riederer (1986). The isolated cuticle tissues were initially washed with 10 mM borax buffer (disodium tetraborate decahydrate; Roth, Karlsruhe, Germany) at pH 9.18 and then washed with deionized water. The weight of the air-dried tissues was measured using a balance with a precision of 1 μg (Satorius MC 5, Göttingen, Germany).

To enforce tissue suberization, the outermost layer of the fruit was wounded with a scalpel. After 2 days the wounded fruits were harvested. Enzymatic isolation of the suberized tissue was performed as described above.

After harvest, tomato fruits were stored in darkness at a constant temperature of 25°C for several days. Cylindrical sections with a diameter of 4 mm for the MicroTom lines and

8 mm for the Rheinlands Ruhm and the Ailsa Craig lines, respectively, were cut from stem scar. These sections were similarly isolated.

Microscopic Analysis

Cylindrical stem scar sections with a diameter of 4 mm were sampled from tomato fruits, embedded in Tissue-Tek medium (Sakura Finetek, Zoeterwoude, Netherlands) and cooled down to -22°C. Frozen longitudinal sections of 30 μm thicknesses were cut with a Leica CM 1900 cryostat (Leica Microsystems, Nussloch, Germany).

These semi-thin fruit sections, enzymatically isolated stem scar membrane and wounded cuticular membrane were scanned with a Leica DMR HC microscope system (Leica Microsystems, Wetzlar, Germany) provided with an isolated fluorescent lamp (ebq 100 Leistungselektronik, Jena, Germany). Images were recorded using a Leica DC 50 digital camera (Leica Microsystems, Heerbrugg, Switzerland) combined with Leica IM 1000 image manager software version 1.2 (Imagic Bildverarbeitung, Glattbrugg, Switzerland).

Scanning Electron Microscopy

Enzymatically isolated fruit cuticles were mounted on an aluminum holder by means of conductive carbon double-sided adhesive tape (Plannet Plano, Wetzlar, Germany), 5 min sputter coated with gold palladium at 25 mA (Bal-Tec SCD 005, Balzers, Switzerland) and examined in a scanning electron microscope at 15 kV (Zeiss DSM 962, Oberkochen, Germany). The sputtering conditions, depositing approximately 20 nm of the alloy on the tissue samples, were optimized for the acceleration voltage used in the scanning electron microscope.

Determination of Epidermal Cell Density

Tomato fruit cuticles were enzymatically isolated and placed on a stage of a light microscope and imaged. Epidermal cell number was counted per unit surface area of the fruit cuticular membrane.

Chlorophyll Fluorescence Measurements with Pulse Amplitude Modulation

Photochemical efficiency of photosystem II (PSII) electron transport was measured as variable chlorophyll fluorescence of 30 min dark-adapted tomato fruits with a pulse amplitude modulation fluorometer (Walz PAM 2000, Effeltrich, Germany) at room

temperature. The fruit surface area was examined by using a fiber optics (Walz, Effeltrich, Germany).

The initial chlorophyll fluorescence (F_0) was excited following exposure modulated at 600 Hz and 650 nm. The maximum chlorophyll fluorescence (F_M) was elicited at 1000 Hz modulation frequency. The maximum fluorescence yield was obtained by submitting the fruit to a saturated light pulse of 0.8 s duration with an intensity of 8000 μmol m^{-2} s^{-1}. The maximal quantum yield of photosystem II was calculated as $F_V/F_M = (F_M - F_0)/F_M$, where F_V is the variable fluorescence.

Analyses of Protein and Glucose Concentrations

After removal of seeds frozen fruits were pulverized in a mixer mill (Retsch MM 301, Haan, Germany), extracted four times in 500 μl extraction buffer (pH 5.5; 200 mM Tris, Roth, Karlsruhe, Germany; 10 mM EDTA, Roth, Karlsruhe, Germany) on ice and centrifuged at 13000 rpm and 4°C for 10 min. Measurements of solvent-soluble protein and glucose concentration were conducted with 20 μl or 175 μl of each supernatant, respectively.

Determination of total protein was performed according to Bradford (1976), using 250 μl commercial chromogen reagent (Sigma-Aldrich, St. Louis, MO, USA) and 1.4 g l^{-1} bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as protein standard. After 5 min of shaking the absorbance at 595 nm was measured at room temperature on a Multiscan EX spectrophotometer (Thermo Labsystem, Vantaa, Finland). Protein concentration was calculated from obtained values using a cubic polynomial standard curve.

Glucose concentration was measured by addition of 50 μl freshly mixed color reagent, which contained 57 units ml^{-1} glucose oxidase (*Aspergillus niger*; Fluka, Buchs, Switzerland), 5.6 units ml^{-1} peroxidase (*Armoracia rusticana*; Fluka, Buchs, Switzerland), 2.8 mM 4-aminoantipyrine (Fluka, Buchs, Switzerland), 136 mM imidazol (Sigma-Aldrich, St. Louis, MO, USA) and 30.7 mM phenol (Roth, Karlsruhe, Germany) according to Trinder (1969) and Kunst *et al.* (1984). The glucose content was determined by measuring the absorbance at 492 nm on a Multiscan EX spectrophotometer (Thermo Labsystem, Vantaa, Finland) at room temperature for 45 min with a frequency of one measurement per minute and shaking in between. The rate of absorbance increase of *N*-(4-antipyryl)-*p*-benzoquinone imine as final chromogen was specified and analyzed for a linear range of enzyme activity. Maximum rate of color production was observed between 5 min and

15 min. By relating the sample maximum rate to those of the standard curve, the glucose concentration was obtained for each sample.

Analysis of Abscisic Acid Concentration

After harvest tomato fruits were stored in darkness at constant 25°C for several days. Exclusively stem scar tissue of these fruits was removed with a scalpel to a depth of approximately 2 mm and immediately frozen in liquid nitrogen. The frozen explants were homogenized in liquid nitrogen and two times extracted in 80% aqueous methanol (v/v; Roth, Karlsruhe, Germany) at -20°C over night.

According to Weiler (1986) the methanolic extracts were collected, passed through a C_{18} Sep-Pak cartridge and evaporated to the water phase. The aqueous residue was acidified to pH 3.0 and partitioned three times against ethyl acetate. The organic fractions were combined and evaporated to dryness, diluted in TBS buffer at pH 7.8 and subjected to enzyme-linked immunosorbent assay (ELISA). The immunoassay was performed using rabbit anti-mouse immunoglobulin, monoclonal anti-ABA antibody and alkaline phosphatase as a tracer. The antibody-sample ABA complex was colorimetrically assayed *via* phosphatase activity and colorless p-nitrophenyl phosphate as substrate. The released *p*-nitrophenol that gave a yellow color was spectrophotometrically assayed at a wavelength of 405 nm. ABA concentration was calculated using a linear regression from obtained values of ABA standards.

The extract purification and quantification of ABA was achieved by the workgroup of Prof. Wolfram Hartung (Department of Botany I: Molecular Plantphysiology and Biophysics, Julius-von-Sachs-Institute for Biosciences, University of Würzburg, Germany).

Characterization of Permeance for Water

Cuticular water permeance was determined for intact tomato fruits of different developmental stages. The attachment site of the pedicel (stem scar) was sealed with paraffin (Merck, Darmstadt, Germany). The amount of water transpired versus time (five to eight data points per individual fruit) was measured using a balance with a precision of 0.1 mg (Sartorius AC210S, Göttingen, Germany). Cuticular water flow rate (F, in g s^{-1}) of individual fruits was determined from the slope of a linear regression line fitted through the gravimetric data. Coefficient of determination (r^2) averaged 0.999. Flux (J, in g m^{-2} s^{-1}) was calculated by dividing F by total fruit surface area. Between measurements, the fruits

were stored at 25°C over dry silica gel (Applichem, Darmstadt, Germany). Under these conditions, the external water vapor concentration was essentially zero. The vapor phase-based driving force (Δc) for transpiration is therefore 23.07 g m^{-3}. For calculating water permeance (P, in m s^{-1}) based on water vapor concentration, J was divided by Δc.

In addition, the permeance for water of tomato fruits after different modifications of the cuticular membrane was analyzed: For quantitative removal of the epicuticular wax layer, a thin film of aqueous gum arabic (Roth, Karlsruhe, Germany) was applied to the fruit surface. After 1 h, the dried polymer, including the outermost epicuticular waxes, was mechanically removed. To extract the solvent-soluble epi- and intracuticular waxes, fruits were dipped in chloroform (Roth, Karlsruhe, Germany) at room temperature for 1 min (category I) or 2 min (categories II to VII). To avoid solvent penetration at the stem scar, only about 80% of each fruit surface was dipped. Alternatively, the tomato fruit was peeled off completely to investigate effects derived from the whole epidermal cell layer.

According to Bauer *et al.* (2004a), fruits were completely dipped in tert-butylmethylether:methanol (TBME:methanol, 9:1, v/v; Fluka, Buchs, Switzerland; Roth, Karlsruhe, Germany) by using an ultra sonic bath at room temperature for 5 min.

The permeance for water of stored fruits without paraffin wax sealing of the stem scar was studied for the stem scar experiments. Additionally, application experiments were done with abscisic acid, tetcyclasis (both obtained from Prof. Wolfram Hartung), cycloheximide (Sigma-Aldrich, St. Louis, MO, USA) or deionized water as negative control. Daily 50 μl of those solutions were applied exogenously at the stem scar tissue during storage in darkness at constant 25°C.

Fruit Wax Extraction for Gas Chromatographic Analysis

To extract total wax mixtures, tomato leaves and fruits of category I were immersed for 1 min and fruits of categories II to VII for 2 min in chloroform (Roth, Karlsruhe, Germany) at room temperature. Approximately 80% of the fruit surfaces were dipped to avoid solvent penetration *via* the stem scar. As an external standard, *n*-tetracosane (Sigma-Aldrich, Steinheim, Germany) was added to all extracts and the solvent was evaporated under a continuous flow of nitrogen. Lipids from internal tissues with chain lengths of C_{16} and C_{18} could not be detected in these extracts. Dipping efficiency was tested in a series of experiments with extraction times ranging from 5 s to 15 min (Figure 1).

Epi- and intracuticular waxes were separately detached with gum arabic and chloroform. For removal of the epicuticular wax layer a thin film of 1 g ml^{-1} aqueous gum arabic (Roth, Karlsruhe, Germany) was applied to the fruit surface. After 1 h the dried polymer including the outermost epicuticular waxes was mechanically removed. This procedure was repeated for several times before the fruit was dipped into chloroform for extracting the intracuticular waxes. The collected gum arabic fraction containing the epicuticular waxes was dissolved in chloroform:deionized water (1:1). Gum arabic bound epicuticular wax compounds were extracted from this two-phase-system three times with chloroform. The combined organic phases were dried over sodium sulfate (anhydrous; Applichem, Darmstadt, Germany). All extracts were filtered and the organic solvent was evaporated under a continuous flow of nitrogen.

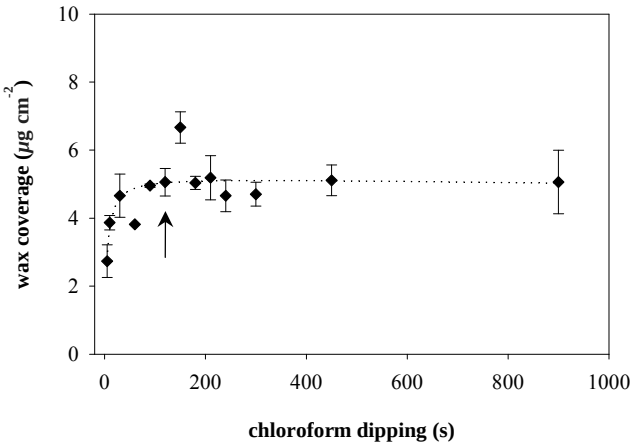

Figure 1. Gravimetrically determination of fruit wax coverage. Total waxes of MicroTom wild type fruits of category VI were extracted with chloroform in a time series of 5 s to 15 min. The time point of 2 min within the chloroform extraction curve was marked with an arrow. Regression is represented as dotted line. Data are shown as means ± SD (n = 3).

Fruit Cutin and Suberin Extraction for Gas Chromatographic Analysis

The total wax was removed by immersing enzymatically isolated cutin and suberin membranes in chloroform (Roth, Karlsruhe, Germany) at room temperature. The wax-free membranes were transesterified with BF_3-methanol (approximately 1.3 M boron trifluoride in methanol; Fluka, Steinheim, Germany) at 70°C over night to release methyl esters of cutin and suberin acid monomers as well as phenolics. Sodium chloride-saturated aqueous solution (Applichem, Darmstadt, Germany), chloroform, and, as an external standard, *n*-dotriacontane (Sigma-Aldrich, St. Louis, MO, USA) were added to all reaction mixtures. From this two-phase-system, the depolymerized transmethylated components were extracted three times with chloroform. The combined organic phases were dried over sodium sulfate (anhydrous; Applichem, Darmstadt, Germany) and filtered. The organic solvent was evaporated under a continuous flow of nitrogen.

Gas Chromatographic Analysis of Fruit Wax, Cutin and Suberin

Prior to gas chromatographic analysis, hydroxyl-containing wax, cutin and suberin compounds were transformed into the corresponding trimethylsilyl derivatives using *N,O*-bis-trimethylsilyl-trifluoroacetamide (Macherey-Nagel, Düren, Germany) in pyridine (Merck, Darmstadt, Germany). The qualitative composition of wax was identified with temperature-controlled capillary gas chromatography (6890N; Agilent Technologies, Santa Clara, CA, USA) and on-column injection (30 m DB-1, 320 μm i.d., d_f = 1 μm; J&W Scientific, Folsom, CA, USA) with helium carrier gas inlet pressure programmed at 50 kPa for 5 min, 3 kPa min^{-1} to 150 kPa, and at 150 kPa for 30 min using a mass spectrometric detector (70 eV, *m*/*z* 50-750, 5973N; Agilent Technologies, Santa Clara, CA, USA). Cutin and suberin composition were analyzed at 50 kPa for 41 min, 10 kPa min^{-1} to 150 kPa, and at 150 kPa for 40 min.

Separation of the wax mixtures was achieved using an initial temperature of 50°C for 2 min, raised by 40°C min^{-1} to 200°C, held at 200°C for 2 min, and then raised by 3°C min^{-1} to 320°C and held at 320°C for 30 min. Accordingly, separation of cutin and suberin mixtures was performed using a temperature program of 50°C for 1 min, raised by 10°C min^{-1} to 150°C, held at 150°C for 2 min, and then raised by 3°C min^{-1} to 320°C and held at 320°C for 30 min. Quantitative composition of the mixtures was studied using a capillary gas chromatography (5890 II; Hewlett Packard, Avondale, PA, USA) and flame ionization detection under the same gas chromatographic conditions as above but with hydrogen as carrier gas. Single compounds were quantified against the external standard. Compounds with a proportion < 0.01% of the total wax, cutin or suberin composition were summarized as 'not identified'.

RNA Isolation

According to the developmental stage of the fruits, samples were composed of 5 to 15 whole fruits with seeds being removed (category 'fruit set' to II). The locular tissue (gel) that surrounded the seeds and fills the locular cavity were detached. Otherwise, samples contained exclusively the fruit peel, which was removed with a scalpel to a depth of approximately 1 mm to 2 mm (category II to VII). After sampling point the plant material was immediately frozen in liquid nitrogen and stored at -80°C until use.

Total RNA was isolated using the RNeasy plant mini kit (Qiagen, Hilden, Germany) complying with the manufacturer’s instructions including optional washing steps and a treatment with RNase-free DNase (Qiagen, Hilden, Germany).

The isolated total RNA was precipitated with 2.5 volumes of 100% ethanol (Applichem, Darmstadt, Germany) and 0.1 volume of 3 M sodium acetate (pH 6.5; Applichem, Darmstadt, Germany) at -20°C over night, washed with 70% ice cold ethanol (Applichem, Darmstadt, Germany) and finally resuspended in diethyl pyrocarbonate (Roth, Karlsruhe, Germany) treated, deionized water. For cDNA synthesis total RNA was quantified by measuring UV absorbance (Biophotometer; Eppendorf, Hamburg, Germany). RNA quality was checked by gel electrophoresis.

Alternatively total RNA was isolated from fully developed tomato leaves. The stem scar samples of stored fruits, which were used for isolation of total RNA, were composed of exclusively the stem scar tissue, which was removed with a scalpel to a depth of approximately 2 mm.

Microarray Design and Construction

The tomato wax oligonucleotide microarray was established using oligomer probes specific to 167 tomato gene sequences and expressed sequence tags (EST) involved in a range of wax biosynthesis and fruit development related processes designed by MWG Biotech (50 bp oligomers; Ebersberg, Germany) or selected from the database of DFCI tomato gene index release 11.0 (70 bp oligomers; http://compbio.dfci.harvard.edu; Table I). The oligomer name, representing a putative functional assignment, based on sequence homology according to BLAST search results (http://compbio.dfci.harvard.edu). In addition, probes for non-plant genes as positive control (*Saccharomyces cerevisiae* GAS1) and as an external standard (*Coleoptera* luciferase) were included in the microarray. Oligomers were diluted in 3x SSC (pH 7.0; 450 mM sodium chloride, Roth, Karlsruhe, Germany; 45 mM sodium citrate, Applichem, Darmstadt, Germany) and 1.5 M betaine (Sigma-Aldrich, Steinheim, Germany) and were spotted in duplicates (2 grids à 3 spots per oligomer) on epoxy-coated Nexterion HiSens glass slides (Peqlab, Erlangen, Germany) using an OmniGrid Microarrayer (Protedyne, Windsor, CT, USA). After spotting, the oligomers were attached covalently to the epoxy-functionalized glass slides by baking.

For blocking, the spotted microarray slides were equilibrated with 0.1% triton x-100 (v/v; Sigma, Taufkirchen, Germany), sequentially treated with 1 mM hydrochloric acid (Merck, Darmstadt, Germany) and with 100 mM potassium chloride (Applichem, Darmstadt, Germany), washed in deionized water and immersed in blocking solution consisting of 100 mM Tris (pH 9.0; Roth, Karlsruhe, Germany), 50 mM ethanolamine

(Sigma-Aldrich, Steinheim, Germany) and 0.1% SDS (w/v; Applichem, Darmstadt, Germany). Blocked slides were washed sequentially in deionized water and dried by centrifugation at 1500 rpm for 2 min.

Table I. Internal oligomer number, oligomer name and *Lycopersicon esculentum* gene index (LeGI identifier) of 167 oligomer sequences with a chain length of 50 bp or 70 bp corresponding to tomato genes and EST designed by MWG Biotech or DFCI.

number	oligomer name (putative protein function/annotation)	LeGI identifier	oligomer sequence (3´-5´ oligonucleotide sequence)
1	Acetyl-CoA carboxylase	TC155719	TATCGAAGATTGGTGATTCGACATTGGATTTACATGCCCTACCACAAGGT
2	Acetyl-CoA carboxylase	AM087200	GTAATTGAACAAACATTGAATAAAACAGTACCCGAAGGTTCACAAGCAGC
3	Biotin carboxylase carrier protein	TC161595	ATATCTGCTCAGAAGATTGGTTCACTATTACTCTTGTTGAAATCTATGAT
4	Biotin carboxylase carrier protein	TC163903	CCTTTTTCAGTTTGACATATTTATGTGAGGTTTGTACCTCGATAACAATC
5	Biotin carboxylase	TC156241	AGTGAATATAGTTTAAGAACATTACTCTCATTGGAACTTGAAGTGAGTGC
6	Acyl carrier protein	TC154599	GAGAGGAACTCTCATTCATGCTTGTTTGTTTCATTTTCTGTAAGACTGAT
7	Acyl carrier protein	TC162699	CGTGAGCTGTCTTGTTTTCGCCGAATCCTGCAATGAAATCTAAAGATATA
8	Malonyl-CoA:ACP transacylase	TC155340	TTGAAGAGCTATGCTCGCGGTGCTTGCTTGTTAAACGATGGGATTAAACG
9	β-ketoacyl-ACP synthase	TC155174	TAGCAGCCAATACATAATTCATCTCACATCTGCTTGGAAATGAGAAATAT
10	β-ketoacyl-ACP synthase	TC155699	AGGCTTTCATTGGCAGAATCCCCTTTTCGTCTCTCTGGTTTTCTTCTGTA
11	β-ketoacyl-ACP synthase	TC158629	ATTATACTTACTTAACTAAATTGTTTCTCTTACACATAGCAGCAAATGAC
12	β-ketoacyl-ACP synthase	TC163946	CATACCCCAATTCACCAATGTAAAGCCATTGAAACCCAAAAGAACCCCTT
13	β-ketoacyl-ACP synthase	TC165966	TTCTGCATCTACTGTTTCTGCTCCAAAAAGAGAGAAAGACCCAAAGAAAA
14	β-ketoacyl-ACP reductase	TC154326	GTTTCTATGTTAAAGCTGAGTAAACACTATTTGACGCACTTCCTTTGGTC
15	β-ketoacyl-CoA synthase	TC163172	TTGAAAGAAATACCCTGTTCTTGAATCTTGCATCATTTTATTTTGTTTGT
16	β-ketoacyl-CoA synthase	TC166171	GTTAGAAAAAAATCTTGAATTGAGTGAATGGCATATGGAGCCATCAAGAA
17	β-ketoacyl-CoA synthase	TC155622	CAGATATCCTGTTGATATTCCAGATGTTGTTAGGCTCTAATTATCTTTAA
18	β-ketoacyl-CoA synthase	TC166800	ACAAGTCCAATGATCGAACCCGGTCTAAATATGAGCTGGTCCACACCGTT
19	β-ketoacyl-CoA synthase	TC166849	TTGAGAGGTCTGGCCTTGGTGAATCAACTTATCTCCCTGAAGCTGTGCTG
20	β-ketoacyl-CoA synthase	AW648148	GCTGCATACAGTGAGAACACACAAAGGATCTAATGACAAATCTTATAATT
21	β-ketoacyl-CoA synthase (*Fiddlehead*)	TC164538	CAAATTGCTTTTTTCGAATGGGTTGCTCCGCCCTCCTCCTCTCCAATCGT
22	β-ketoacyl-CoA reductase	TC154540	AATTGGGTAATGCTAGTTACTGTAATGCAACTAGAAGCTTGATTCTGTTC
23	β-ketoacyl-CoA reductase	TC156747	TCTCATCCTCTTTTCTCCATCTATGCTGCAACTAAAGCCATAAGAACTGG
24	Enoyl-ACP reductase	TC154761	GCCCAGCTTTCACGGGTCTCGACATCCCAAAAGATAATAAGAGCTAGTGC
25	Acyl-CoA reductase	TC168406	AAAGGACTTATTCAAAGATCTAAGAGAAAAATGTGGACCAAATTTTACTA
26	Elongation of fatty acids protein	TC165530	TTTGTATCCATTGCTTATCTCTATTGCTTATCTACTTTGGTAGTTTTTAA
27	Long-chain acyl-CoA synthetase	TC154510	ACGAAATCTTTTCTGAGATAAATAATTTTAACCTCGACAGCTTATATCCG
28	Long-chain acyl-CoA synthetase	TC156228	ACAGTTTAGTGAGTGAATGTAGCCAAAAAGGTGAAAGGTTGAAATATCAT
29	Very-long-chain fatty acid condensing enzyme	TC154916	CGGTCCGTTGGTTCTTACAGCTTCAGAGCAGCTTCTCTTCTTGTTCACTC
30	Very-long-chain fatty acid condensing enzyme (*LeCer6*)	TC154916	TGTGTTTTTCAACAAGAAGATCCACAAGGGAAAGTTGGTATTAATCTATC
31	Very-long-chain fatty acid condensing enzyme	TC164957	CCTATTTTATTGTAGTTATTTTCCTGATAAACATCTATTCATGACTGAGC
32	Very-long-chain fatty acid condensing enzyme	TC164957	TAGTGAGTTCGATCTTGAGCCGTCTCGGATGACTTTGCACCGCTTTGGAA
33	Acyl-ACP thioesterase	TC154590	CGTCACAATCCTTTGTGAATCCTCCAACTTGTGCTCTTTGATCTTTATAT
34	Acyl-ACP thioesterase	TC164326	GGACTTTGAAATAATAGGTGTCGAACTGAATGGGAGAAAGAAGTCTGCTA
35	Acyl transferase	TC166117	GGTCCCGGTTACGGCTACGTCATCTTCTGATGCTGCGCCTGTGAAAATGG
36	Carboxyl transferase	TC157552	TTAAGGCACAGGATGCTAAAGTTCCGAAAACTTGGTGGCTTCCAGGAGGG
37	Dihydrolipoamide S-acetyl transferase	TC159737	ATTATTGAGAATCCAGATAGCTTGACAATGTAGATGGAGAGCTAAGATTA
38	Dihydrolipoamide dehydrogenase	TC166025	AATGAATTTGGCGTAGAAACAATTTTGTTTTCTAGTTTATTAGCGTTTAA

Table I. (continued)

number	oligomer name (putative protein function/annotation)	LeGI identifier	oligomer sequence (3´-5´ oligonucleotide sequence)
39	Hydroxymyristoyl-ACP dehydratase	TC163900	ATGAATGCGAATTCCTGTATCTATGATAAGGTCCCTTTCTCTTTGAGTGC
40	Stearoyl-ACP desaturase	TC163909	TGACAACCTATTCGAACACTTCTCTGCCGTAGCTCAACGCCTTGGTGTAT
41	Stearoyl-ACP desaturase	TC164870	GTTTTTGATTCTCTGCGCGATTGGGCTGCACAGAATCTCTTGGTGAACCT
42	Stearoyl-ACP desaturase	TC167423	GCCGAAGAGACAGGCATGGTGATCTCCTCACAAGTATCTTTACCTTTCCG
43	ω-3 fatty acid desaturase	TC162310	AGTTGGTTGTATTAAGCACTACTAGTTGAGCACAAAGTAAAGTTAGAAGA
44	ω-6 fatty acid desaturase	TC155495	TTTATAATGATTGCATATTCAGAATCAAAGAGGGAGGAAGACGAAAATGG
45	ω-6 fatty acid desaturase	TC156147	TTGATTTTCAATATCTCAGGCAAAAAATATGACCCGTTTTGCATGTCATT
46	ω-6 fatty acid desaturase	TC163281	GAAGAAAAGAAGAATGATATTGCAAAAAGAGTTCCATCTTCAAAGCCCCC
47	δ-9 desaturase	TC165278	ATTGGAGAAGCAGGGTTCTATCATTCATTCGTGATACTTATGTGATCCAT
48	δ-9 desaturase	BG629606	GTTTGTTGTGCCAACCTTCTCCAAATGCAAGCAATGCCACCCCTCGTGCC
49	Aldehyde oxidase	TC163875	GAATTCGGACTCCTCAAGGAGATACCCTTGGTACAGTCCTGTTTCCATTG
50	Fatty acid hydroxylase	TC161119	CCTCACGGCGAAAATGAAAGGTGGTTTCCATGTGACAATTGAGGAAAGGG
51	Fatty acid hydroxylase	TC157396	TGCAACTGCCATTCTCCTACTCCCACTATGGAGTGTAATTAAATTGTTAG
52	Fatty acid hydroxylase	TC163531	TTTGTACCTTGTACCAGTTTGGCAATTGGAAAAATCCTGTTGATTCATGC
53	Fatty acid hydroxylase	TC165333	ATTTATAAGGGTTGTATGCTATCGATTATGAGAATCTTAGTAGTTCAGTT
54	Fatty acid ω-hydroxylase	TC154668	CAAGTTGAAAAAACAAGTTTATTGTATGTGTTCTTAGGTTATTTGCTTTT
55	Fatty acid ω-hydroxylase	TC155232	ATCCCTACGCAATGGGGAGAGCAGAGGAACTATGGGGAAGTGATTGGAAC
56	Fatty acid ω-hydroxylase	TC161085	AAACATTGGCTCCCGGCTTGCCCGATAATACGTTTGCATCGGCTTTTTGA
57	Wax synthase	TC156999	CTTCAGCTAGAGCCACAGTTCAATGAACCGTATTTATCAACTTCACTACA
58	Wax synthase	TC159852	TTGTCAGAAGTTGTCGGGGAGGAATGTGTGCCCTACGTGTTGCCGTGCTG
59	Wax synthase	TC166314	GGTTCTTATTCATGTACCATAGAGTATGTTGTAAAACATTCATCAAATTG
60	Wax synthase	AW615959	GTTGAGACCACAAAAAAGGCTACAAATTCAACTTTCAATCTTGTTACAAA
61	WIN1 transkriptionsfaktor (ERF)	TC165186	TCAAGCCACTTGAAGATGATCACATTGAACAAATGATTGAAGAATTGTTG
62	WIN1 transkriptionsfaktor (ERF)	AI776626	TGAAGAGCCAAACTAGAGGAAATAAAGATCATTTCAAATGTGACGGACGC
63	WIN1 transkriptionsfaktor (ERF)	BG642554	CCGGAGCCCGGCCTGATTCTAGCTGGGTCATGACGGTTGAATTTGGCAAT
64	CER1 homologue	TC154074	TATGTAACACCTTTCTTTGTTTCTTTAAGAATTTCCTATGTTGTTTTCCG
65	CER1 homologue	TC154074	ACACATGTGATCTGGCATTGATGTGGTGGCTTATGAGTTAAAGAAAAAGTTCACTGTTGTTCAACTTCAC
66	CER1 homologue	TC162208	TAAGATGTAATATGAACCAAACTGGAGCATATTGAAGGATGATTTCTATA
67	CER1 homologue	TC162208	TGATCATGGTTTTCGCCCGTTAACATTAGTGGCTACTTCTACGGGATCATCCAGAACTAGAAGTATAGAT
68	CER1 homologue	TC167714	GTGGCCTTTTGCCTTTGTGGTGGTGCTTACTATGTGGCTGAAGTCTAAGA
69	CER1 homologue	TC154678	TAATTCCTAGCAACTCAATGGCCTGTACTATATGTAATAAATAACTTTTG
70	CER1 homologue	TC164385	AAAAGAGGATTGGCTTGGAGTTCACTAATTGATTCAATTCAAATAGAGAA
71	CER1 homologue	TC164385	TTCATGATGTGTCCATCCTTCTAATGAATGAACCACCCCTCCTGCATGGCATGCATGGACTACCCCTCTC
72	CER1 homologue	TC164385	GATTACACCAAGAGAACAAAGTTATGCACCTAAAGGAACTCATTTTCACC
73	CER1 homologue	TC167446	TGAAACCTTTGTATAATTCATCATAGTGATACCATTTGTCTCATATTCAT
74	CER1 homologue	BE354679	GGCGTTCTACATTGCATACCTGAAGTTCTCAGAGACTCATCACTTGCCTC
75	CER2 homologue	TC162943	AGTATTTGTTTCATAGTTTATTTTGGTTTAATTGTGGTGTTGGTTTGTTA
76	CER3 homologue	AW429151	ACTTGTCTGCTATATTCGTAATAATATCCAATTTGTCTACTATCTTTGTA
77	CER10 homologue	TC154549	TCATGATTGTTGCTGCTAGCATTATGACTAACTGGGCTCTTGGAAAGCATCGTCGTCTGAAGAAGCTCTT
78	CER10 homologue	TC155449	ACTGGTGTTTCCACTACTTCAAGCGGATCATGGAGACCTTCTTTGTGCACCGTTTCAGCCACGCCACTTC
79	CER10 homologue	TC155449	AGTTCCCACTTCTTTGTGATGGCTTCAAGAACCATCTTTGTTTAATCTAA
80	Male sterility protein	TC169786	CAAATTATATTTCAAAGGAATTGTTAAATCGACATCATGAAGTTGAAGAT
81	β-amyrin synthase	TC160438	GGTGTTTGCTTCACATATGGTTCCTGGTTTGCTCTTGGAAGGCTTGTTGC
82	Lupeol synthase	TC167994	GGCTGTGAAAATTAGATTGAGAAAAAGTATAAGTTATTACTCAACAATTC
83	Lipid transfer protein	TC159152	TTCTCCCATATACAATTTCAGAGCCACTTTATTATTTCTTGCATAAAAGC
84	Lipid transfer protein	TC159469	AAGATTAGCCCTAAAGTTGATTGCTCAAAGGTCAGATAAAAGGATGAATT

Table I. (continued)

number	oligomer name (putative protein function/annotation)	LeGI identifier	oligomer sequence (3´-5´ oligonucleotide sequence)
85	Lipid transfer protein	TC160076	GTATACTCTGCCTCCTGTTGGGCATACCACTACTTGTTAGTGCAATGGAT
86	Lipid transfer protein	TC161801	CATGAGGAGAATTAAGAATAAGATAGATAGCATTGATCTTGCTTATGGAT
87	Lipid transfer protein	TC161801	CATCTACCTTGGCTCCTTGTCTCCCTTATCTAATGAATCGCGGTCCTCTC
88	Lipid transfer protein	TC162870	CTTGTTTGGGACTGAGACGCATGTAGTTGTTACATTTGTTGTTGTATTCT
89	Lipid transfer protein	TC163287	TCATATAATATCCAACCCATTTGGTCCTTTGGAGACTTGATCTTATTTGT
90	Lipid transfer protein	TC169166	TCAGTTAAATCAGCTCGTGATTCACCGGGAATAATGCTTCGCAATGGTGT
91	Non-specific lipid transfer protein	TC162479	ATCTTTTCTCAAATTTTTGTAACAAATGGAAATGTCTAGCAAAATTGCAT
92	Non-specific lipid transfer protein	TC162505	TCTTTTTTCGTTTTGTCTTTTGGGGGTCTTGTATCGAAACTTCACTATCG
93	Non-specific lipid transfer protein	TC164001	ATTTGGTACAAGTGCAATTATCATGAGGAGGAGAAGAATAAGCTATGGTC
94	Tfm5 protein	TC162134	ATTCGTCAACCAGTTGATATTTGTACTCAAAAGGCTTACTACTACTATAT
95	ABC transporter	TC155151	GAACCGTCTTTTATAGAGAAAGGGCTGCTGGGATGTATTCAGCATTACCATATGCTATGGCACAGGTAAT
96	ABC transporter	TC165234	ATTGCCTAATGATCTTCCTAAGCCATTTTGGAAGTACCCAATGTATTACATTGCATTTCATAAATATGCA
97	ABC transporter	TC169292	GACTCCCCACGACATCCCAAAGCCTTTCTGGCGTTACCCTATGTCTTACCTCAGTTTCCATTTTGGGCAC
98	Anionic peroxidase	TC154576	AACGTTTTCCTTGGAGATTTTGCTGCTGCCATGATCAAGATGGGAGACTTGCCCCCCTCCGCCGGCGCTC
99	Anionic peroxidase	TC154576	AATGTTACTGTTTTTCTTGAAGATTTTGCTGCTGCTATGATCAAAATGGGGAATTTACCTCCCTCCGCTG
100	Anionic peroxidase	TC154577	TGTTTTCATCTTTTTCAGTATTGATGCAATAATATATATGTCCCTATGTTCGCAGTGTTTTTAATCCCCC
101	Caffeic acid O-methyltransferase	TC155422	CACAAGTCATGAATGTAACTGTGAGTGGTATGAACGTAGTTTTGTTTTAA
102	4-coumarate-CoA ligase	TC162879	TCCAGAGGCCACTACGGGAACAATAGACAAAGAAAGGTGGTTACATACGG
103	Phenylalanine ammonia-lyase	TC153588	ATCAGCGATTTTTACTGAAGTGATGAACGGAAAGCCCGAATTCACTGACT
104	Phenylalanine ammonia-lyase	TC153686	ACTTAAATGAAAAGTGACAGTGTGCTTATGTCTATTCAATTCTTCTGTTC
105	Ethylene receptor	TC156201	AACATTAGAAACTTTATTGCCTTGGCTTGACACAACAATAGATTATTATG
106	Ethylene inducible protein kinase	TC156632	TCTCAGAATATTACAAACACATCCATTTTCTCCATCCTCTTTCTTACTGT
107	1-aminocyclopropane-1-carboxylate synthase	TC154562	GCTTAACGTCTCGCCTGGATCTTCGTTTGAATGTCAAGAGCCAGGGTGGTTCCGAGTTTGTTTTGCAAAT
108	1-aminocyclopropane-1-carboxylate synthase	TC162669	CTCCTCATTCACCTATGTCTTCTCCTATGGTCCAAGCAAGGAATTAATCG
109	1-aminocyclopropane-1-carboxylate oxidase	TC154595	CTGTGCATCACGCTCTTGTACTTCCCGTTGGTAATCACCTCAAGCTGGTCACCAAGGTTAACCACAATAG
110	1-aminocyclopropane-1-carboxylate oxidase	TC154940	TGATGCATTGATCTATCCAGCACCAGCTCTAGTTGATAAAGAGGCAGAGGAACATAACAAACAAGTTTAC
111	1-aminocyclopropane-1-carboxylate oxidase	TC154595	AAGTGCATGGAACAGAGGTTTAAGGAACTAGTGGCAAGTAAGGGACTTGAGGCTGTTCAAGCTGAGGTTA
112	1-aminocyclopropane-1-carboxylate oxidase	TC154814	GGGGTATATTGTAAAGTTTCCTCTTTTACTTTAATGAAATCTATGCTTAA
113	Cytokinin synthase	AW622109	AGGCATTTGTCCACAACAGTAACTCATATAATTTTAGTACAAGGTACGAT
114	Cytokinin oxidase	TC164231	ACATTAACTGTAGCGGCGAGAGGCAACGGTCATTCCATTAACGGCCAAGC
115	Allene oxide synthase	TC165425	CTGGTCGAACTGTTCCTCCGATATGATTCGTTTGAGATTGAAGTCGGTGC
116	Allene oxide cyclase	TC155191	GGGGTCGTTCTGAAATAATCAATAACTACGTGCTCTATAATTAAAAAAAA
117	Chalcone flavanone isomerase	TC154241	ATGCAATAGTGTGCAATAAGGTCCCACTTTAAGTCTGTTAATTATGTAAA
118	Naringenin 2-oxoglutarate 3-dioxygenase	TC159713	CGAAAACTATTATTTTATGTAACTAGTAATCTGGTGTGATTGTGTATGTT
119	Naringenin-chalcone synthase	TC161468	CATTTTGGTCTCAGAAATACCCAAACTTGGTAAAGAGGCTGCCCTTATGG
120	Dehydrin	TC153563	AAGGGAAAAGGTTTGATACCTTAACTTTGTCATTTAGAATTGATATATTT
121	Dehydrin	TC161916	CCAACTCTGTTATATGAAAACGTATTGTTCAAAACATATCGAGTTATTAT
122	Abscisic acid and environmental stress inducible protein (Dehydrin TAS14)	TC163263	CCGTGTTTAATGATGTTTTGGAAATAATATAACTTGTGCTATATTTCTTT
123	Lipoxygenase	TC154671	CTTTCAGGACTTAGTGATAAAAAAAGTAGCCTTTTTAACATCGTACCAAG
124	Lipoxygenase	TC154788	ACATGTAAAATGTAAGAAAGCTGGAGTTTGAATGAATCTTCAAATAAAAT
125	Lipoxygenase	TC162124	GCCACTATTGAGGAAAATTAACATGTCAGCAAACACAAAAGCCTATGCCT
126	Chaperonin	TC153554	CCTGGTTGGAGTAGCAGCCTTCAGGGTTCGGCACTGTTAATTAACATGCAATCCAATGTGTAATTGCAGC
127	Hsp20.0 protein	TC162620	TCTTCGAATCGGCTCTGTTTCTCACCTAATGGCGTAGTTGATGTACTTGC
128	DnaJ protein	TC158095	TTTCCCCGAGTCATTAACAGCAGAGCAGTGCAAGAACCTTGAGGCTGTGCTGCCTCCAAAACCCAAATTG
129	Salt and low temperature response protein	TC163235	TATGGATTGGTAATTTATTGCCTTTTGTTCTAAGGTGTTAAATTTCTCGT
130	PHAP2A protein (APETALA2)	TC156099	GATTCCGATGCCGGTGCGGCGAGTTGTTTTGCGGTGAACATCGATACTCCGATCGTCATGATTGTAGCTA

Table I. (continued)

number	oligomer name (putative protein function/annotation)	LeGI identifier	oligomer sequence (3´-5´ oligonucleotide sequence)
131	TDR6 protein (APETALA3)	TC154953	GCTTTTCGCCTACAACCATTGCACCCCAATCTTCAAAACGAAGGAGGATTTGGTTCTCGTGATCTACGTC
132	MADS-box transcription factor MADS-RIN	TC164766	CTGGATGGATGCTTTGAATTTGGAGTATATGGAGAGAAGAAATCCTCTTAGTTATACAAGTTATTTATTT
133	MADS-box JOINTLESS protein (LeMADS)	TC156874	CTCAGGCTGAAGAGATCAAAAGCAAGGTGTGGCTATTTTTGTATGTTATT
134	Abscisic stress ripening protein (DS2 protein)	TC154684	GAAAACAAGTTATGGTGGGGGAGATGAAAACAAGTATGGTGAGAAAACTA
135	Abscisic stress ripening protein	TC162125	ACCAATCTCGTGTGATGTAAAGAAGGATGTATTGTGATTTCCAAAATGAT
136	Phytoene synthase	TC153853	CTCCTACAAAAATGCCTCTCTTCAAAGATAAAGCATGAAATGAAGATATA
137	Lycopene beta cyclase	TC157834	TAAATATGATCAACAATTTGTTACAGGATAAAGAATGAATCCGAGTAATT
138	CPD photolyase	TC160981	CAGGGAGAAGCAATAGACACAATTCCCAACTTTCTTAAAGAATGTGGAGC
139	Photolyase/blue light photoreceptor	TC161789	AAAGAATTTGTCGCCATTTCTAGCAAGTCGCTCTACAATCCTTCTTATGC
140	9-*cis*-epoxycarotenoid dioxygenase	TC154656	AGGAAGAAGACGATGGTTATATTTTAGCTTTCGTTCACGATGAGAAAGAA
141	ζ-carotene desaturase	TC163067	AGAACTTTGTCACACATAAGCTTTTAATAATGTTAACCTTAAGTCAGACC
142	Acid beta-fructofuranosidase (acid invertase)	TC161907	CCACTAGTAGAGCAACTGAAACCTACATGATCTGCTTCAATTATTCCTTG
143	Polygalacturonase	TC166124	AAGCTTTATACTTGCCAAAATGAGTCCCTTAGCAATTTTCTTCCTCTTTT
144	ERD7 protein	TC155774	TGTATTGGCTTTATTACTCTAGTGTGGTTGCTTGAAAATTAGAAATGGAA
145	Senescence associated protein	TC153712	GTGATTAAATCTAGTGTACAACTTGCACATGTTTTGTTCTTGCTCCTCTA
146	Senescence associated protein	TC155638	GGTTCTATTTGTATTTCATCTGCACATATTATTTCTACCATCTTATCTTT
147	Wound inducible carboxypeptidase	TC155683	AGAGTGGCTAGAGGGCAACAATAATATAAATGAATAATGAAGACTGAATT
148	Wound induced protein	TC162831	CAAGGTTGCCAGAGCCAATGCAGAGGTGGACCAACTCCAACTCCGACTCC
149	N-hydroxycinnamoyl-CoA:tyramine N-hydroxycinnamoyl transferase	TC161982	GTTTAATATTGTAAAAGCAGAACGCTCATTGTATTTGTAATTTGAAAAAT
150	Glutathion S-transferase	TC153757	TGATTTTGTGTGGCAATAAAATGGAGATAATGGATTTAGGATGAGTTTTT
151	Pathogenesis related leaf protein	TC155130	ATTACATGTCTAGGAATTAAAATGATAAGTGGATCGGATTGATATCCTAT
152	Pathogenesis related protein	TC162553	TACTTTAATTTGTCTTACACATGATGTTCAAAATTCTCACACAAGAGTGC
153	α-tubulin	TC153680	ATTTTAGGGTTTCGAATTTTTCAGTTTCTGTATCGAAAATGAGGGAGTGC
154	α-tubulin	TC153680	GCATGTTCCTAGAGCTGTCTTTGTAGATCTTGAGCCCACTGTCATTGATG
155	Ubiquitin	TC153696	ATGGCTGTGTGCTTTGTTGTTTTATTTCATGACTTTTAGTGTTCTTCGTT
156	Ubiquitin	TC153696	TTGCAGACTATAACATCCAGAAAGAGTCTACTCTTCATCTTGTCCTGAGG
157	Histone H4	TC154669	TAGATATCAGTTTCTAGTGATGACATTGGTTCGACTTTTAGCTGCTGATT
158	Actin	TC160487	GGATGCTGAGAAGAGTTACGAACTTCCAGATGGGCAAATAGTCACTATTG
159	Actin	TC161925	TCCCTCTATTGTCCACAGGAAGTGCTTCTAATTTTTCCAAGATTGACAATGTTGGTGAAAGGAAAAGACT
160	Elongation factor (EF-1-α)	TC161869	ATGCGGCACAGCCTCGTGCCCGCCAGTTAACATCTCTCTTCACTTTTCTCAGTATCACTTCTCAACAGAA
161	Phosphoglycerate kinase (PKG)	TC161873	CAATCTTGCTGTTTGCATCAGGAGCAAATTTATCTGCAATCACAACATCAGGAGAGAAGGAGCTTCCTTT
162	Glyceraldehyde-3-phosphate dehydrogenase	TC161943	CGAATGGGGTTACAGTTCCCGTGTGATTGATTTGATCTGCCATATGGCTAAGGCTTGATTGATGCTGCTG
163	Protein kinase	TC153603	ATTTGTTCATTAATTCCTCTCATCTGCCCCTCCATTCATTTAATTTCTTT
164	Ribosomal protein L37a	TC161876	ATCTCAATTAGACATTAGTATAAAACTGTGTTTTTCAGACAAGTATTTAC
165	Hypothetical protein (lipid transfer protein)	TC163886	ATTGTGGTGCAAGGTGTGCAGTTTCTTGGGTTTGAAGTGGGTTACGATTC
166	Zinc finger (C3HC4-type RING finger) family protein	TC164745	TTACATGAAACCCCCCTTCATAATCTTCATAGACATTGCTGGACAGTTAA
167	Molybdenum cofactor sulfurase (FLACCA)	TC165810	TACTGCTAAGATATGAGAATAATACGAAAACCGAGTCAGATACATGGATT

Preparation of Fluorescent Dye Labeled cDNA Samples

Aliquots of 20 μg total RNA were spiked with *in vitro* synthesized 18 ng or 2 ng standard yeast mRNA, respectively, and 5 ng of luciferase control RNA (Promega, Mannheim, Germany) and reversely transcribed using 200 units of Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany) in the presence of 0.5 μg oligo(dT)$_{12\text{-}18}$ primer and 4.5 μg random nonamers. In addition to the RNA template and the annealing

primer, each reaction contained 500 μM dATP, 500 μM dGTP, 500 μM dTTP and 200 μM dCTP, 1 mM dithiothreitol, 40 units RNase inhibitor (RNase out; Invitrogen, Karlsruhe, Germany) and dCTP-coupled 1.25 nM Cyanine 3 or Cyanine 5 (Amersham Biosciences, Uppsala, Sweden) in the provided first-strand buffer with a final volume of 40 μl. After incubation at room temperature for 10 min and at 50°C for 90 min another 200 units of Superscript III reverse transcriptase were added and the incubation was continued at 50°C for 90 min. The cDNA synthesis was stopped by heating the sample at 70°C for 15 min. Subsequently 100 units RNase A (Fermentas, St. Leon-Rot, Germany) was added to all reactions followed by an incubation at 37°C for 45 min.

Cyanine dye labeled cDNA was purified using G-50 columns (Amersham Biosciences, Uppsala, Sweden) according to manufacturer's instructions. Each cDNA sample was concentrated to a final volume of 10 μl in a speedvac centrifuge (Holbrook Savant SC 110, NY, USA) evaporating water under vacuum.

Microarray Hybridization and Data Acquisition

Both Cyanine 3 and Cyanine 5 labeled cDNA samples were pooled and then mixed with hybridization solution containing 3x SSC (pH 7.0; 450 mM sodium chloride, Roth, Karlsruhe, Germany; 45 mM sodium citrate, Applichem, Darmstadt, Germany) and 0.1% SDS (w/v; Applichem, Darmstadt, Germany). After incubation at 95°C for 2 min, the probes were immediately chilled on ice, then applied to the blocked microarray surface and finally covered by a glass slide. Hybridization was performed at 42°C for 18 h in a hybridization chamber (MWG Biotech, Martinsried, Germany). Post-hybridization washes were done sequentially at room temperature in 2x SSC with 0.2% (w/v) SDS for 10 min, in 2x SSC for 15 min and in 0.2x SSC for 15 min. Slides were dried by centrifugation at 1500 rpm for 2 min. In order to protect the fluorescence signals against photo bleaching the slides were briefly immersed in DyeSaver2 solution (Implen, Munich, Germany).

Microarray slides were scanned using a ScanArray 4000 (Packard Bioscience, Meriden, CT, USA) adjusted to 532 nm for Cyanine 3 and to 635 nm for Cyanine 5 (Figure 2). Fluorescence data were processed of total pixel intensities within a fixed area obtained for each spot using the ArrayVision 8.0 image analysis software (Imaging Research, St. Catharines, Canada). Hybridization signals were quantified by subtracting the individual background values from the corresponding spot intensity in order to correct for non-specific fluorescence. Normalization of the two samples per hybridization was performed using the mean hybridization signal of the spiked luciferase control RNA to correct the

expression ratio for scanner channel specific effects. Signal intensities below 0.1x of the background signal, calculated from the hybridization signals of the spotting buffer only, were filtered out as non-hybridization signal.

Each of the microarray experiments was performed in duplicate with Cyanine 3 and Cyanine 5 dye-swap replicates of the cDNA labeling. In all hybridization experiments cDNA of wild type samples compared to cDNA of mutant samples were probed according to the respective developmental stage. Fluorescence signals were inspected visually and non-homogeneous and aberrant spots were flagged.

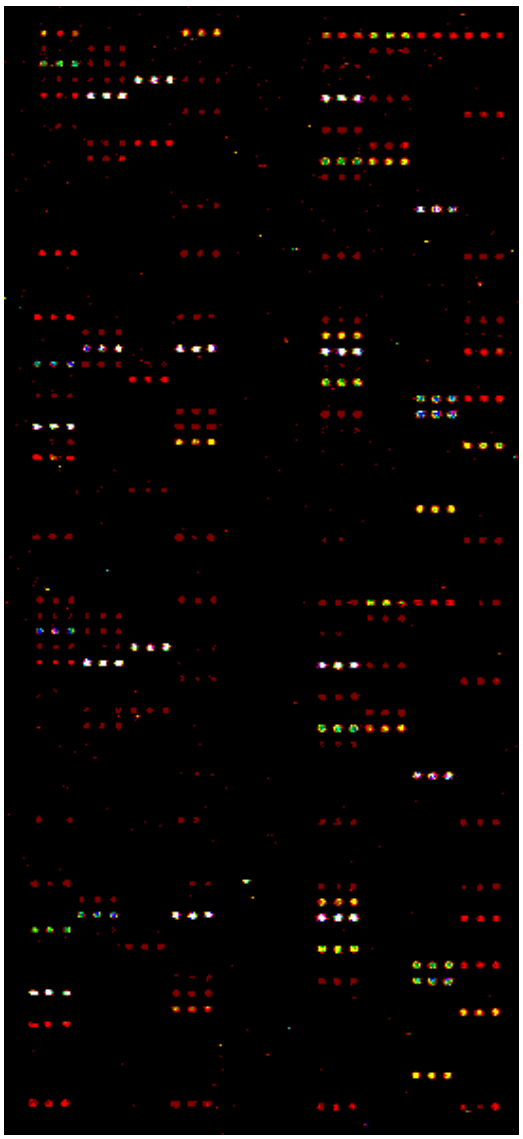

Figure 2. Tomato wax microarray slide. A comprehensive system of gene expression analysis involved in several aspects of wax biosynthesis in tomato using an oligonucleotide microarray consisting of 167 50mers and 70mers.

Semi-Quantitative Reverse Transcription PCR Analysis

For reverse transcription (RT) PCR experiments 2 μg DNase treated total RNA were denatured and reversely transcribed by using 200 units Superscript III reverse transcriptase

and 0.5 μg oligo(dT)$_{12-18}$ primer for cDNA synthesis with a final volume of 20 μl as described by the supplier (Invitrogen, Karlsruhe, Germany). Subsequently 1 μl cDNA was used to perform a PCR using 0.5 units Supratherm DNA polymerase (Genecraft, Lüdingshausen, Germany) according to the manufacturer's instructions. Specific primers were created with LightCycler Probe Design software version 1.0 (Idaho Technologies, Salt Lake City, UT, USA) to amplify tomato cDNA (Table II). The reaction conditions for PCR included a denaturing step of 2 min at 95°C, followed by 25 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 78°C, ending with a final elongation step of 10 min at 72°C (Mastercycler gradient; Eppendorf, Hamburg, Germany). For control RT-PCR experiments, 18S rRNA primers were applied.

Table II. Internal oligomer number and oligomer name of six primer pair sequences corresponding to tomato genes and EST created with LightCycler Probe Design software. The size of the resulting amplified RT-PCR fragments is specified.

number	oligomer name (putative protein function/annotation)	5´-3´ oligonucleotide sequence		amplicon size
		forward primer sequence	reverse primer sequence	
6	Acyl carrier protein	TGAGTTGTAGTAGCTTGGT	ACCAAAGGATGTCCTTAAAT	221 bp
30	Very-long-chain fatty acid condensing enzyme (*LeCer6*)	GTCGTGAACTGTAGCTTG	CACCACCCATTCTGAACAA	271 bp
66	CER1 homologue	TTCAGGTTGCCACGTTAG	CGTTCCAGCCTTCCAATG	351 bp
87	Lipid transfer protein	TCTCAGAAACTGTTGTGATG	CTGAACTTTAGAGCAGTC	205 bp
92	Non-specific lipid transfer protein	TTATCTTCAAGGCCGTGG	ACTTTACTGAACCGTGG	232 bp
144	ERD7 protein	AATGTGATGTCAACGACC	CCGGATCTACTTCTGCT	243 bp
160	Elongation factor (EF-1-α)	CAAGAAGGACCCAACTG	CGCCTATCAAGTACCCA	231 bp
control	18S rRNA	ATTCCTAGTAAGCGCGAGTCAT	GTTCACCTACGGAAACCTTGTT	208 bp

DNA Extraction

Plant material composed of tomato wild type leaves was frozen in liquid nitrogen and stored at -80°C until use. Genomic DNA was isolated by thawing pulverized leave tissues at 65°C in extraction buffer containing of 2% cetyltrimethyl ammonium bromide (CTAB; w/v; Roth, Karlsruhe, Germany), 1.42 mM sodium chloride (Roth, Karlsruhe, Germany), 0.02 M EDTA (Roth, Karlsruhe, Germany), 0.1 M Tris (Roth, Karlsruhe, Germany), 2% polyvinyl pyrrolidone (PVP40; w/v; Sigma-Aldrich, Steinheim, Germany), 5 mM ascorbic acid (Merck, Darmstadt, Germany), 4 mM diethyldithiocarbamic acid (Roth, Karlsruhe, Germany). After 1 min vortexing, 70 μg RNase A (Fermentas, St. Leon-Rot, Germany) were added to all reactions and the incubation continued at 65°C for 5 min. The DNA was then extracted with a chloroform:isoamylalcohol phase system (25:1; Roth, Karlsruhe, Germany; Applichem, Darmstadt, Germany) and precipitated with 0.7 volumes cold

isopropanol (Merck, Darmstadt, Germany) on ice for 60 min, washed with 70% ice-cold ethanol (Applichem, Darmstadt, Germany) and finally resuspended in deionized water. For DNA library generation genomic DNA was quantified using UV absorbance measurements (Biophotometer; Eppendorf, Hamburg, Germany) and qualified by agarose gelelectrophoresis.

Conception of DNA Libraries and Genome Walk PCR

The universal GenomeWalker universal kit (Clontech Laboraties, Saint-Germain-en-Laye, France) was used for the genome walk according to the manufacturer's instructions. The walk was performed with aliquots of 2.5 mg genomic DNA and five different restriction enzymes *Dra*I, *Eco*RV, *Pvu*II, *Sca*I and *Stu*I, respectively. GenomeWalker adaptors were ligated to blunt-end genomic DNA of each library construction (Table III).

Table III. Oligonucleotide sequences of GenomeWalker adaptor, adaptor primers (Clontech Laboraties) and gene specific primers corresponding to tomato β-ketoacyl-CoA synthase *LeCer6* gene created with LightCycler Probe Design software. The size of the resulting amplified fragments for control PCR is specified.

sequence name	5´-3´ oligonucleotide sequence	amplicon size
PCR		
adaptor primer	GTAATACGACTCACTATAGGGC	
gene specific primer prom1	CAGCTAAGAGGAAGTTGATATTTGTTCA	
gene specific primer prom2	ATTCCTCTCATCTGCCCCTCCATTCAT	
nested PCR		
adaptor primer	ACTATAGGGCACGCGTGGT	
gene specific primer prom2	ATTCCTCTCATCTGCCCCTCCATTCAT	
gene specific primer prom3	TGTGAAAATGCCAGAAGCAGTCCCAA	
control PCR		
forward gene specific primer prom4	GTCATTATCAAAACCTTGAC	
reverse gene specific primer prom5	GTCTTAGCTACTATCAAACA	1781 bp
GenomeWalker adaptor	GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT	

PCR and nested PCR were performed using two-step cycle parameters. The PCR condition included 7 cycles of 25 s at 94°C and 3 min at 72°C as well as 32 cycles of 25 s at 94°C and 3 min at 67°C, ending with a final elongation step of 7 min at 67°C (Mastercycler gradient; Eppendorf, Hamburg, Germany). The nested PCR was programmed at 5 cycles of 25 s at 94°C and 3 min at 72°C and additionally 20 cycles of 25 s at 94°C and 3 min at 67°C, ending with a final elongation step of 7 min at 67°C.

The reaction conditions for control PCR included a denaturing step of 2 min at 94°C, followed by 40 cycles of 60 s at 94°C, 45 s at 55.2°C and 120 s at 72°C, ending with final elongation step of 10 min at 72°C.

Agarose Gel Electrophoresis

Aliquots of 25 μl cDNA amplicons spiked with 5 μl 6x loading buffer (Peqlab, Erlangen, Germany) were separated by electrophoresis on a 0.9% (w/v) agarose (low EEO; Applichem, Darmstadt, Germany) Tris-acetate-EDTA (pH 8.0; 40 mM Tris-acetate, 1 mM EDTA, Roth, Karlsruhe, Germany) gel with 140 μM ethidium bromide (Applichem, Darmstadt, Germany) at 75 V for 120 min. An aliquot of 100 bp^+ standard DNA ladder (Fermentas, St. Leon-Rot, Germany) was used for estimation the size of the PCR fragments. In order to visualize and to photograph the PCR products the agarose gels were exposed to UV light in a GelDoc EQ station in combination with Quantity One 1-D analysis software (Biorad, Munich, Germany).

PCR products of semi-quantitative RT-PCR were separated by electrophoresis on a 2% (w/v) agarose gel for 90 min. An aliquot of peqGold ultra low range standard (Peqlab, Erlangen, Germany) was used for estimation the size of the PCR fragments.

Gel Elution and Sequence Analysis

PCR products of the expected amplicon size were purified from the agarose gel using an E.Z.N.A. gel extraction kit (Peqlab, Erlangen, Germany) complying with the manufacturer's instructions including optional washing steps, and sequenced.

Conidia Development of *Blumeria graminis*

Blumeria graminis f. sp. *hordei* (isolate CC1; Dr. Timothy L. W. Carver, Institute of Grassland and Environmental Research, Aberystwyth, UK) was propagated on its host barley (*Hordeum vulgare* L.; Nordic Gene Bank, Alnarp, Sweden) until distinct white powdery pustules appeared. One day before conidia were required for examination, barley leaves bearing sporulating colonies were shaken to remove older conidia, thus only freshly emerged conidia were available for subsequent bioassay.

Freshly peeled cuticle layer and enzymatically isolated cuticles of tomato fruits were fixed on glass slides at the base of a settling tower. Empty glass slides served as negative control. Conidia from infected barley leaves were blown into the tower using pressurized air to ensure an even distribution. For 24 h the samples were incubated in a moisture

chamber with a 16-h photoperiod at 400 μmol photons m^{-2} s^{-1} and a temperature regime of 24°C/18°C (day/night). One hundred intact and individual conidia were analyzed by light microscopy on each fruit surface. Germinating conidia with single or multiple germ tubes or differentiation into appressoria were determined. In addition, the quality of fungal conidia was verified by detecting the mortality rate of conidia apparently damaged or desiccated within the inoculation procedure.

Fruit Infection with Plant Pathogenic Fungi

The pathogenic fungi *Cladosporium herbaceum*, *Alternaria alternata*, *Colletotrichum coccodes* and *Fusarium solani* (Dr. Günther Bahnweg, GSF-National Research Center for Environment and Health, Institute of Biochemical Plant Pathology, Neuherberg, Germany) were maintained in darkness at 25°C on a V8 agar medium (pH 7.2; 167 ml l^{-1} V8 Grünfink vegetable juice, Dohrn & Timm, Diedersdorf, Germany; 30 mM calcium carbonate, Merck, Darmstadt, Germany; 15 g l^{-1} agar agar, Roth, Karlsruhe, Germany). Blocks of 7 days old V8 agar cultures with an extent of 15 mm^2 were applied to the stem scar area of freshly harvested or 4 days stored tomato fruits. The inoculated fruits were incubated in a moisture chamber at room temperature in darkness. After 7 days the fruits were bisected and visually distinguished into infected and non-infected fruits.

With *Fusarium solani* infected tissues was visualized with a Leica MZ 16 stereoscopic binocular microscope provided with a halogen lamp (Leica Microsystems, Wetzlar, Germany).

Statistical Analyses

Data within tomato lines were tested by one-way analyses of variance (ANOVA) followed by Tukey's honestly significant difference mean-separation test for unequal *n* or Kruskal-Wallis ANOVA and subsequent multiple comparisons of ranks. One-way ANOVA was only used when homogeneity of variances was given. Accordingly, comparisons between tomato lines within fruit-developmental categories were tested with Student's *t*-test or Mann-Whitney *U*-test. Spearman's correlation of ranks was used to estimate the correlation coefficient (R^2) of data belonging to developmental categories or of days within storage ($P < 0.05$). Statistical analyses were performed with STATISTICA 7.1 (StatSoft, Tulsa, OK, USA).

LITERATURE CITED

Aarts MGM, Keijzer CJ, Stiekema WJ, Pereira A (1995) Molecular characterization of the *CER1* gene of *Arabidopsis* involved in epicuticular wax biosynthesis and pollen fertility. Plant Cell **7**: 2115-2127

Abe I, Rohmer M, Prestwich GD (1993) Enzymatic cyclization of squalene and oxidosqualene to sterols and triterpenes. Chem Rev **93**: 2189-2206

Abel S, Nguyen MD, Chow W, Theologis A (1995) *ASC4*, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in *Arabidopsis thaliana*: structural characterization, expression in *Eschericha coli*, and expression characteristics in response to auxin. J Biol Chem **270**: 19093-19099

Abeles FB, Morgan PW, Saltveit ME (1992) Ethylene in plant biology. Academic Press, San Diego, CA, pp 113-114

Adams-Phillips L, Barry C, Giovannoni J (2004a) Signal transduction systems regulating fruit ripening. Trends Plant Sci **9**: 331-338

Adams-Phillips L, Barry C, Kannan P, Leclercq J, Bouzayen M, Giovannoni J (2004b) Evidence that CTR1-mediated ethylene signal transduction in tomato is encoded by a multigene family whose members display distinct regulatory features. Plant Mol Biol **54**: 387-404

Agrawal VP, Kolattukudy PE (1978) Purification and characterization of a wound-induced ω-hydroxyfatty acid:NADP oxidoreductase from potato tuber disks (*Solanum tuberosum* L.). Arch Biochem Biophys **191**: 452-465

Agrawal VP, Stumpf PK (1985) Characterization and solubilization of an acyl chain elongation system in microsomes of leek epidermal cells. Arch Biochem Biophys **240**: 154-165

Agrios GN (1997) Plant pathology. Academic Press, San Diego, CA, pp 46-52

Aharoni A, Dixit S, Jetter R, Thoenes E, van Arkel G, Pereira A (2004) The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in *Arabidopsis*. Plant Cell **16**: 2463-2480

Alba R, Cordonnier-Pratt MM, Pratt LH (2000) Fruit-localized phytochromes regulate lycopene accumulation independently of ethylene production in tomato. Plant Physiol **123**: 363-370

Alba R, Fei Z, Payton P, Liu Y, Moore SL, Debbie P, Cohn J, D´Ascenzo M, Gordon JS, Rose JKC, Martin G, Tanksley SD, Bouzayen M, Jahn MM, Giovannoni J (2004) ESTs, cDNA microarrays, and gene expression profiling: tools for dissecting plant physiology and development. Plant J **39**: 697-714

Alba R, Payton P, Fei Z, McQuinn R, Debbie P, Martin GB, Tanksley SD, Giovannoni JJ (2005) Transcriptome and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development. Plant Cell **17**: 2954-2965

Alexander L, Grierson D (2002) Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. J Exp Bot **53**: 2039-2055

Allagulova CR, Gimalov FR, Shakirova FM, Vakhitov VA (2003) The plant dehydrins: structure and putative functions. Biochemistry **68**: 945-951

Almeida DPF, Huber DJ (1999) Apoplastic pH and inorganic ion levels in tomato fruit: a potential means for regulation of cell wall metabolism during ripening. Physiol Plantarum **105**: 506-512

Amitai-Zeigerson H, Scolnik PA, Bar-Zvi D (1994) Genomic nucleotide sequence of tomato *Asr2,* a second member of the stress/ripening-induced *Asr1* gene family. Plant Physiol **106**: 1699-1700

Amitai-Zeigerson H, Scolnik PA, Bar-Zvi D (1995) Tomato *Asr1* mRNA and protein are transiently expressed following salt stress, osmotic stress and treatment with abscisic acid. Plant Sci **110**: 205-213

Andersen CL, Jensen JL, Ørntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res **64**: 5245-5250

Andersson MX, Hamberg M, Kourtchenko O, Brunnström Å, McPhail KL, Gerwick WH, Göbel C, Feussner I, Ellerström M (2006) Oxylipin profiling of the hypersensitive response in *Arabidopsis thaliana*: formation of a novel oxo-phytodienoic acid-containing galactolipid, Arabidopside E. J Biol Chem **281**: 31528-31537

Andrews J, Adams SR, Burton KS, Evered CE (2002) Subcellular localization of peroxidase in tomato fruit skin and the possible implications for the regulation of fruit growth. J Exp Bot **53**: 2185-2191

Anterola AM, Lewis NG (2002) Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. Phytochemistry **61**: 221-294

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55**: 373-399

Arora R, Wisniewski ME (1994) Cold acclimation in genetically related (sibling) deciduous and evergreen peach (*Prunus persica* [L.] Batsch): a 60-kilodalton bark protein in cold-acclimated tissues of peach is heat stable and related to the dehydrin family of proteins. Plant Physiol **105**: 95-101

Asahira T, Hosoki T (1977) *In vitro* studies of controlling tomato puffiness by growth regulators. Sci Hortic **7**: 319-328

Auldridge ME, McCarty DR, Klee HJ (2006) Plant carotenoid cleavage oxygenases and their apocarotenoid products. Curr Opin Plant Biol **9**: 315-321

Ayub R, Guis M, Amor MB, Gillot L, Roustan JP, Latché A, Bouzayen M, Pech JP (1996) Expression of ACC oxidase antisense gene inhibits ripening of cantaloupe melon fruits. Nat Biotechnol **14**: 862-866

Bafor M, Smith MA, Jonsson L, Stobart K, Stymne S (1993) Biosynthesis of vernoleate (*cis*-12-epoxyoctadeca-*cis*-9-enoate) in microsomal preparations from developing endosperm of *Euphorbia lagascae*. Arch Biochem Biophys **303**: 145-151

Baker EA, Bukovac MJ, Hunt GM (1982) Composition of tomato fruit cuticle as related to fruit growth and development. *In* DF Cutler, KL Alvin, CE Price, *eds*, The Plant Cuticle. Academic Press, London, pp 33-44

Bakker JC (1991) Leaf conductance of four glasshouse vegetable crops as affected by air humidity. Agr Forest Meteorol **55**: 23-36

Bargel H, Barthlott W, Koch K, Schreiber L, Neinhuis C (2004) Plant cuticles: multifunctional interfaces between plant and environment. *In* AR Hemsley, I Poole, *eds*, The Evolution Of Plant Physiology. Elsevier Academic Press, London, pp 171-194

Bargel H, Neinhuis C (2005) Tomato (*Lycopersicon esculentum* Mill.) fruit growth and ripening as related to the biomechanical properties of fruit skin and isolated cuticle. J Exp Bot **56**: 1049-1060

Barret P, Delourme R, Renard M, Domergue F, Lessire R, Delseny M, Roscoe TJ (1998) A rapeseed *FAE1* gene is linked to the E1 locus associated with variation in the content of erucic acid. Theor Appl Genet **96**: 177-186

Barry CS, Blume B, Bouzayen M, Cooper W, Hamilton AJ, Grierson D (1996) Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. Plant J **9**: 525-535

Barry CS, Llop-Tous MI, Grierson D (2000) The Regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. Plant Physiol **123**: 979-986

Bartley GE, Scolnik PA (1995) Plant carotenoids: pigments for photoprotection, visual attraction, and human health. Plant Cell **7**: 1027-1038

Bartley GE, Scolnik PA, Giuliano G (1994) Molecular biology of carotenoid biosynthesis in plants. Annu Rev Plant Phys **45**: 287-301

Bartley GE, Viitanen PV, Bacot KO, Scolnik PA (1992) A tomato gene expressed during fruit ripening encodes an enzyme of the carotenoid biosynthesis pathway. J Biol Chem **267**: 5036-5039

Bartley GE, Viitanen PV, Pecker I, Chamovitz D, Hirschberg J, Scolnik PA (1991) Molecular cloning and expression in photosynthetic bacteria of a soybean cDNA coding for phytoene desaturase, an enzyme of the carotenoid biosynthesis pathway. P Natl Acad Sci USA **88**: 6532-6536

Bauer S (2002) Die Zusammensetzung der Oberflächenwachse von Tomaten, Paprika und Auberginen. PhD Thesis. University of Münster, Münster, Germany

Bauer S, Schulte E, Thier HP (2004a) Composition of the surface wax from tomatoes: identification of the components by GC/MS. Eur Food Res Technol **219**: 223-228

Bauer S, Schulte E, Thier HP (2004b) Composition of the surface wax from tomatoes: quantification of the components at the red ripe stage and during ripening. Eur Food Res Technol **219**: 487-491

Beaudoin N, Serizet C, Gosti F, Giraudat J (2000) Interaction between abscisic acid and ethylene signaling cascades. Plant Cell **12**: 1103-1115

Becker M, Kerstiens G, Schönherr J (1986) Water permeability of plant cuticles: permeance, diffusion and partition coefficients. Trees-Struct Funct **1**: 54-60

Becraft PW (1999) Development of the leaf epidermis. Curr Top Dev Biol **45**: 1-40

Bengtson C, Larsson S, Liljenberg C (1979) Water stress, epicuticular wax and cuticular transpiration rate. *In* LA Appelqvist, C Liljenberg, *eds*, Advances In The Biochemistry And Physiology Of Plant Lipids. Elsevier Biomedical Press, Amsterdam, pp 269-274

Benítez JJ, Matas AJ, Heredia A (2004) Molecular characterization of the plant biopolyester cutin by AFM and spectroscopic techniques. J Struct Biol **147**: 179-184

Benveniste I, Tijet N, Adas F, Philipps G, Salaün JP, Durst F (1998) *CYP86A1* from *Arabidopsis thaliana* encodes a cytochrome P450-dependent fatty acid omega-hydroxylase. Biochem Bioph Res Co **243**: 688-693

Bernards MA (2002) Demystifying suberin. Can J Bot **80**: 227-240

Bernards MA, Lewis NG (1998) The macromolecular aromatic domain in suberized tissue: a changing paradigm. Phytochemistry **47**: 91-933

Bernards MA, Razem FA (2001) The poly(phenolic) domain of potato suberin: a non-lignin cell wall bio-polymer. Phytochemistry **57**: 1115-1122

Bernards MA, Summerhurst DK, Razem FA (2004) Oxidases, peroxidases and hydrogen peroxide: the suberin connection. Phytochem Rev **3**: 113-126

Bernhard WR, Thoma S, Botella J, Somerville CR (1991) Isolation of a cDNA clone for spinach lipid transfer protein and evidence that the protein is synthesized by the secretory pathway. Plant Physiol **95**: 164-170

Bessoule JJ, Lessire R, Cassagne C (1989) Partial purification of the acyl-CoA elongase of *Allium porrum* leaves. Arch Biochem Biophys **268**: 475-484

Betz C, Ullrich CI, Hartung W (1993) Tetcyclacis and abscisic acid-sensitive reduction of extracellular ferricyanide by mesophyll cells of *Valerianella locusta* and *Lemna gibba*. J Exp Bot **44**: 35-39

Birkenmeier GF, Ryan CA (1998) Wound signaling in tomato plants: evidence that ABA is

not a primary signal for defense gene activation. Plant Physiol **117**: 687-693

Blechert S, Brodschelm W, Holder S, Kammerer L, Kutchan TM, Mueller MJ, Xia ZQ, Zenk MH (1995) The octadecanoic pathway: signal molecules for the regulation of secondary pathways. P Natl Acad Sci USA **92**: 4099-4105

Blée E, Schuber F (1990) Efficient epoxidation of unsaturated fatty acids by a hydroperoxide-dependent oxygenase. J Biol Chem **265**: 12887-12894

Blée E, Schuber F (1993) Biosynthesis of cutin monomers: involvement of a lipoxygenase peroxygenase pathway. Plant J **4**: 113-123

Bleecker AB, Esch JJ, Hall AE, Rodríguez FI, Binder BM (1998) The ethylene-receptor family from *Arabidopsis*: structure and function. Philos T Roy Soc **353**: 1405-1412

Bleecker AB, Kende H (2000) Ethylene: a gaseous signal molecule in plants. Annu Rev Cell Dev Bi **16**: 1-40

Bohnert HJ, Gong Q, Li P, Ma S (2006) Unraveling abiotic stress tolerance mechanisms: getting genomics going. Curr Opin Plant Biol **9**:180-188

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to environmental stresses. Plant Cell **7**: 1099-1111

Bondada BR, Oosterhuis DM, Murphy JB, Kim KS (1996) Effect of water stress on the epicuticular wax composition and ultrastructure of cotton *(Gossypium hirsutum* L.) leaf, bract, and boll. Environ Exp Bot **36**: 61-69

Booker J, Auldridge M, Wills S, McCarty D, Klee H, Leyser O (2004) MAX3/CCD7 is a carotenoid cleavage dioxygenase required for the synthesis of a novel plant signaling molecule. Curr Biol **14**: 1232-1238

Borsani O, Valpuesta V, Botella MA (2001) Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in *Arabidopsis* seedlings. Plant Physiol **126**: 1024-1030

Botella MA, Quesada MA, Kononowicz AK, Bressan RA, Pliego F, Hasegawa PM, Valpuesta V (1994) Characterization and *in situ* localization of a salt-induced tomato peroxidase mRNA. Plant Mol Biol **25**: 105-114

Bots M, Feron R, Uehlein N, Weterings K, Kaldenhoff R, Mariani T (2005) PIP1 and PIP2 aquaporins are differentially expressed during tobacco anther and stigma development. J Exp Bot **56**: 113-121

Bourgis F, Kader JC (1997) Lipid-transfer proteins: tools for manipulating membrane lipids. Physiol Plantarum **100**: 78-84

Bovy A, de Vos R, Kemper M, Schijlen E, Pertejo MA, Muir S, Collins G, Robinson S, Verhoeyen M, Hughes S, Santos-Buelga C, van Tunen A (2002) High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes *LC* and *C1*. Plant Cell **14**: 2509-2526

Bovy A, Schijlen E, Hall RD (2007) Metabolic engineering of flavonoids in tomato (*Solanum lycopersicum*): the potential for metabolomics. Metabolomics **3**: 399-412

Bowsher CG, Ferrie BJM, Ghosh S, Todd J, Thompson JE, Rothstein SJ (1992) Purification and partial characterization of a membrane-associated lipoxygenase in tomato fruit. Plant Physiol **100**: 1802-1807

Boyer JS (1982) Plant productivity and environment. Science **218**: 443-448

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72**: 248-254

Brady CJ (1987) Fruit ripening. Annu Rev Plant Phys **38**: 155-178

Bramley P, Teulieres C, Blain I, Bird C, Schuch W (1992) Biochemical characterization of transgenic tomato plants in which carotenoid synthesis has been inhibited through the expression of antisense RNA to pTOM5. Plant J **2**: 343-349

Brash AR (1999) Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. J Biol Chem **274**: 23679-23682

Bray EA (1993) Update on water deficit: molecular responses to water deficit. Plant Physiol **103**: 1035-1040

Bray EA (1997) Plant responses to water deficit. Trends Plant Sci **2**: 48-54

Bray EA (2002) Classification of genes differentially expressed during water-deficit stress in *Arabidopsis thaliana*: an analysis using microarray and differential expression data. Ann Bot **89**: 803-811

Bray EA (2004) Genes commonly regulated by water-deficit stress in *Arabidopsis thaliana*. J Exp Bot **55**: 2331-2341

Brennan EB, Weinbaum SA (2001) Performance of adult psyllids in no-choice experiments on juvenile and adult leaves of *Eucalyptus globules.* Entomol Exp Appl **100**: 179-185

Brett C, Waldron K (1990) Cell wall structure and the skeletal functions of the wall. *In* C Brett, K Waldron, *eds*, Physiology And Biochemistry Of Plant Cell Walls. Unwin Hyman, London, pp 4-57

Browse J, Somerville C (1991) Glycerolipid synthesis: biochemistry and regulation. Annu Rev Plant Phys **42**: 467-506

Brunner AM, Yakovlev IA, Strauss SH (2004) Validating internal controls for quantitative plant gene expression studies. BMC Plant Biol **4**: 14-20

Buchholz A (2006) Characterization of the diffusion of non-electrolytes across plant cuticles: properties of the lipophilic pathway. J Exp Bot **57**: 2501-2513

Buchholz A, Baur P, Schönherr J (1998) Differences among plant species in cuticular permeabilities and solute mobilities are not caused by differential selectivities. Planta **206**: 322-328

Burbidge A, Grieve TM, Jackson A, Thompson A, McCarty DR, Taylor IB (1999) Characterization of the ABA-deficient tomato mutant *notabilis* and its relationship with maize *Vp14*. Plant J **17**: 427-431

Burghardt M, Riederer M (2003) Ecophysiological relevance of cuticular transpiration of deciduous and evergreen plants

in relation to stomatal closure and leaf water potential. J Exp Bot **54**: 1941-1949

Burghardt M, Riederer M (2006) Cuticular transpiration. *In* M Riederer, C Müller, *eds*, Biology Of The Plant Cuticle. Blackwell Scientific Publishers, Oxford, pp 291-308

Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol **29**: 23-29

Cahoon EB, Marillia EF, Stecca KL, Hall SE, Taylor DC, Kinney AJ (2000) Production of fatty acid components of meadowfoam oil in somatic soybean embryos. Plant Physiol **124**: 243-251

Çakir B, Agasse A, Gaillard C, Saumonneau A, Delrot S, Atanassova R (2003) A grape ASR protein involved in sugar and abscisic acid signalling. Plant Cell **15**: 2165-2180

Cameron KD, Teece MA, Smart LB (2006) Increased accumulation of cuticular wax and expression of lipid transfer protein in response to periodic drying events in leaves of tree tobacco. Plant Physiol **140**: 176-183

Carrari F, Fernie AR (2006) Metabolic regulation underlying tomato fruit development. J Exp Bot **5**: 1883-1897

Carrari F, Fernie AR, Iusem ND (2004) Heard it through the grapevine? ABA and sugar cross-talk: the ASR story. Trends Plant Sci **9**: 57-59

Carver TLW, Zhang L, Zeyen RJ, Robbins MP (1996) Phenolic biosynthesis inhibitors suppress adult plant resistance to *Erysiphe graminis* in oat at 20°C and 10°C. Physiol Mol Plant P **49**: 121-141

Casado CG, Heredia A (1999) Structure and dynamics of reconstituted cuticular waxes of grape berry cuticle (*Vitis vinifera* L.). J Exp Bot **50**: 175-182

Casado CG, Heredia A (2001) Specific heat determination of plant barrier lipophilic components: biological implications. Biochim Biophys Acta **1511**: 291-296

Cassagne C, Lessire R (1978) Biosynthesis of saturated very long chain fatty acids by purified membrane fractions from leek epidermal cells. Arch Biochem Biophys **191**: 146-152

Cassagne C, Lessire R, Bessoule JJ, Moreau P, Creach A, Schneider F, Sturbois B (1994) Biosynthesis of very long chain fatty acids in higher plants. Prog Lipid Res **33**: 55-69

Cavero J, Ortega RG, Zaragoza C (1995) Influence of fruit ripeness at the time of seed extraction on pepper (*Capsicum annuum*) seed germination. Sci Hortic **60**: 345-352

Cerff R (1982) Separation and purification of NAD- and NADP-linked glyceraldehyde-3-phosphate dehydrogenases from higher plants. *In* M Edelman, RB Hallick, NH Chua, *eds*, Methods In Chloroplast Molecular Biology. Elsevier Biomedical Press, Amsterdam, pp 683-694

Chandler PM, Robertson M (1994) Gene expression regulated by abscisic acid and its relation to stress tolerance. Annu Rev Plant Phys **45**: 113-141

Chang C, Stadler R (2001) Ethylene hormone receptor action in *Arabidopsis*. Bioessays **23**: 619-627

Cheesbrough TM, Kolattukudy PE (1984) Alkane biosynthesis by decarbonylation of aldehydes catalyzed by a particulate preparation from *Pisum sativum*. P Natl Acad Sci USA **81**: 6613-6617

Chen G, Hackett R, Walker D, Taylor A, Lin Z, Grierson D (2004) Identification of a specific isoform of tomato lipoxygenase (TomLoxC) involved in the generation of fatty acid-derived flavor compounds. Plant Physiol **136**: 2641-2651

Chen X, Goodwin SM, Boroff VL, Liu X, Jenks MA (2003) Cloning and characterization of the *WAX2* gene of *Arabidopsis* involved in cuticle membrane and wax production. Plant Cell **15**: 1170-1185

Cheng CS, Samuel D, Liu YJ, Shyu JC, Lai SM, Lin KF, Lyu PC (2004) Binding mechanism of nonspecific lipid transfer proteins and their role in plant defense. Biochemistry **43**: 13628-13636

Cheung AY, McNellis T, Piekos B (1993) Maintenance of chloroplast components during chromoplast differentiation in the tomato mutant green flesh. Plant Physiol **101**: 1223-1229

Cheung AY, Wu HM, Di Stilio V, Glaven R, Chen C, Wong E, Ogdahl J, Estavillo A (2000) Pollen-pistil interactions in *Nicotiana tabacum*. Ann Bot **85**: 29-37

Choi DW, Zhu B, Close TJ (1999) The barley (*Hordeum vulgare* L.) dehydrin multigene family: sequences, allele types, chromosome assignments, and expression characteristics of 11 *Dhn* genes of cv. Dicktoo. Theor Appl Genet **98:** 1234-1247

Clark KL, Larsen PB, Wang XX, Chang C (1998) Association of the *Arabidopsis* CTR1 Raf-like kinase with the ETR1 and ERS ethylene receptors. P Natl Acad Sci USA **95**: 5401-5406

Claussen W (2005) Proline as a measure of stress in tomato plants. Plant Sci **168**: 241-248

Claussen W, Brückner B, Krumbein A, Lenz F (2006) Long-term response of tomato plants to changing nutrient concentration in the root environment: the role of proline as an indicator of sensory fruit quality. Plant Sci **171**: 323-331

Close TJ (1996) Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. Physiol Plantarum **97**: 795-803

Close TJ (1997) Dehydrins: a commonalty in the response of plants to dehydration and low temperature. Physiol Plantarum **100**: 291-296

Close TJ, Kortt AA, Chandler PM (1989) A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. Plant Mol Biol **13**: 95-108

Cohen A, Moses MS, Plant ÁL, Bray EA (1999) Multiple mechanisms control the expression of abscisic acid (ABA)-requiring genes in tomato plants exposed to soil water deficit. Plant Cell Environ **22**: 989-998

Coker JS, Davies E (2003) Selection of candidate housekeeping controls in tomato plants using EST data. Biotechniques **35**: 740-748

Conconi A, Smerdon MJ, Howe GA, Ryan CA (1996) The octadecanoic signalling pathway in plants mediate a response to ultraviolet radiation. Nature **383**: 826-829

Coombe BG (1976) The development of fleshy fruits. Annu Rev Plant Phys **27**: 507-528

Costaglioli P, Joubès J, Garcia C, Stef M, Arveilerc B, Lessire R, Garbaya B (2005) Profiling candidate genes involved in wax biosynthesis in *Arabidopsis thaliana* by microarray analysis. Biochim Biophys Acta **1734**: 247-258

Cottle W, Kolattukudy PE (1982) Abscisic acid stimulation of suberization: induction of enzymes and deposition of polymeric components and associated waxes in tissue cultures of potato tuber. Plant Physiol **70**: 775-780

Creelman RA, Mullet JE (1995) Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. P Natl Acad Sci USA **92**: 4114-4119

Crozier A, Kamiya Y, Bishop G, Yokota T (2000) Biosynthesis of hormones and elicitor molecules. *In* BB Buchanan, W Gruissem, RL Jones, *eds*, Biochemistry And Molecular Biology Of Plants. American Society of Plant Physiologists, Rockville, MD, pp 850-929

Cunningham FX, Gantt E (1998) Genes and enzymes of carotenoid biosynthesis in plants. Annu Rev Plant Phys **49**: 557-583

Cushman JC, Bohnert HJ (2000) Genomic approaches to plant stress tolerance. Curr Opin Plant Biol **3**: 117-124

Cusick F (1966) On phylogenetic and ontogenetic fusions. *In* EG Cutter, *ed*, Trends In Plant Morphogenesis. Longmans, Green and Co., London, pp 170-183

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. Plant Physiol **139**: 5-17

D'Auria JC (2006) Acyltransferases in plants: a good time to be BAHD. Curr Opin Plant Biol **9**: 331-340

Daeter W, Hartung W (1990) Compartmentation and transport of abscisic acid in mesophyll cells of intact leaves of *Valerianella locusta*. J Plant Physiol **136**: 306-312

Daie J (1996) Metabolic adjustments, assimilate partitioning, and alterations in source-sink relations in drought-stressed plants. *In* E Zamski, A Schaffer, *eds*, Photoassimilate Distribution In Plants And Crops: Source-Sink Relationships. Marcel Dekker, New York, NY, pp 407-420

Dan Y, Yan H, Munyikwa T, Dong J, Zhang Y, Armstrong CL (2006) MicroTom: a high-throughput model transformation system for functional genomics. Plant Cell Rep **25**: 432-441

Danyluk J, Perron A, Houde M, Limin A, Fowler B, Benhamou N, Sarhan F (1998) Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. Plant Cell **10**: 623-638

Davies C, Heath RJ, White SW, Rock CO (2000) The 1.8 Å crystal structure and active-site architecture of β-ketoacyl-acyl carrier protein synthase III (FabH) from *Escherichia coli*. Structure **8**: 185-195

Dawson JH (1988) Probing structure-function relations in heme-containing oxygenases and peroxidases. Science **240**: 433-439

de Bruxelles G, Roberts MR (2001) Signals regulating multiple responses to wounding and herbivores. Crit Rev Plant Sci **20**: 487-521

Dean BB, Kolattukudy PE (1976) Synthesis of suberin during wound-healing in jade leaves, tomato fruit, and bean pods. Plant Physiol **58**: 411-416

Deguchi M, Bennett AB, Yamaki S, Yamada K, Kanahama K, Kanayama Y (2006) An engineered sorbitol cycle alters sugar composition, not growth, in transformed tobacco. Plant Cell Environ **29**: 1980-1988

Deikman J (1997) Molecular mechanisms of ethylene regulation of gene transcription. Physiol Plantarum **100**: 561-566

del Mar Parra M, del Pozo O, Luna R, Godoy JA, Pintor-Toro JA (1996) Structure of the dehydrin *tasl4* gene of tomato and its developmental and environmental regulation in transgenic tobacco. Plant Mol Biol **32**: 453-460

DellaPenna D, Lincoln JE, Fischer RL, Bennett AB (1989) Transcriptional analysis of polygalacturonase and other ripening associated genes in Rutgers, *rin*, *nor*, and *Nr* tomato fruit. Plant Physiol **90**: 1372-1377

DellaPenna D, Pogson BJ (2006) Vitamin synthesis in plants: tocopherols and carotenoids. Annu Rev Plant Biol **57**: 711-38

Demmig-Adams B, Adams WW (1996) The role of xanthophyll cycle carotenoids in the protection of photosynthesis. Trends Plant Sci **1**: 21-26

Demmig-Adams B, Adams WW (2000) Harvesting sunlight safely. Nature **403**: 371-374

Dillard HR, Cobb AC (1998) Survival of *Colletotrichum coccodes* in infected tomato tissue and in soil. Plant Dis **82**: 235-238

Dixon RA, Dey PM, Lamb CJ (1983) Phytoalexins: molecular biology and enzymology. Adv Enzymol RAMB **55**: 1-135

do Rêgo ER, Finger FL, Casali VWD, Cardoso AA (1999) Inheritance of fruit color and pigment changes in a yellow tomato (*Lycopersicon esculentum* Mill.) mutant. Genet Mol Biol **22**: 101-104

Doares SH, Syrovets T, Weiler EW, Ryan CA (1995) Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. P Natl Acad Sci USA **92**: 4095-4098

Dóczi R, Csanaki C, Bánfalvi Z (2002) Expression and promoter activity of the desiccation-specific *Solanum tuberosum* gene, *StDS2*. Plant Cell Environ **25**: 1197-1203

Dóczi R, Kondrák M, Kovács G, Beczner F, Bánfalvi Z (2005) Conservation of the drought-inducible *DS2* genes and divergences from their *ASR* paralogues in solanaceous species. Plant Physiol Bioch **43**: 269-276

Dodd RS, Poveda MM (2003) Environmental gradients and population divergence contribute to variation in cuticular wax composition in *Juniperus communis*. Biochem Syst Ecol **31**: 1257-1270

Doss RP, Potier SW, Chastagner GA, Christian JK (1993) Adhesion of nongerminated *Botrytis cinerea* conidia to several substrata. Appl Environ Microb **59**: 1786-1791

Droillard M, Rouet-Mayer MA, Bureau JM, Lauriere C (1993) Membrane-associated and

soluble lipoxygenase isoforms in tomato pericarp: characterization and involvement in membrane alterations. Plant Physiol **103**: 1211.1219

Duan H, Schuler MA (2005) Differential expression and evolution of the *Arabidopsis* CYP86A subfamily. Plant Physiol **137**: 1067-1081

Dunn MA, Hughes MA, Zhang L, Pearce RS, Quigley AS, Jack PL (1991) Nucleotide sequence and molecular analysis of the low temperature induced cereal gene, *BLT4*. Mol Gen Genet **229**: 389-394

Dunn MA, White AJ, Vural S, Hughes MA (1998) Identification of promoter elements in a low-temperature-responsive gene (*blt4.9*) from barley (*Hordeum vulgare* L.). Plant Mol Biol **38**: 551-564

Durbin ML, McCaig B, Clegg MT (2000) Molecular evolution of the chalcone synthase multigene family in the morning glory genome. Plant Mol Biol **42**: 79-92

Dure L (1993) Structural motifs in lea proteins. *In* TJ Close, EA Bray, *eds*, Plant Responses To Cellular Dehydration During Environmental Stress. American Society of Plant Physiologists, Rockville, MD, pp 91-104

Ealing PM (1994) Lipoxygenase activity in ripening tomato fruit pericarp tissue. Phytochemistry **34**: 547-552

Eckey-Kaltenbach H, Ernst D, Heller W, Sandermann H (1994) Biochemical plant responses to ozone: cross-induction of defensive pathways in parsley (*Petroselinum crispum* L.) plants. Plant Physiol **104**: 67-74

Edelmann HG, Neinhuis C, Bargel H (2005) Influence of hydration and temperature on the rheological properties of plant cuticles and their impact on plant organ integrity. J Plant Growth Regul **24**: 116-126

Edwards D, Abbott GD, Raven JA (1996) Cuticles of early land plants: a palaeoecophsiological evaluation. *In* G Kerstiens, *ed*, Plant Cuticles: An Integrated Functional Approach. Bios Scientific Publishers, Oxford, pp 1-32

Efrati A, Eyal Y, Paran I (2005) Molecular mapping of the chlorophyll retainer (cl) mutation in pepper (*Capsicum* spp.) and screening for candidate genes using tomato ESTs homologous to structural genes of the chlorophyll catabolism pathway. Genome **48**: 347-351

Eigenbrode SD, Espelie KE (1995) Effects of plant epicuticular lipids on insect herbivores. Annu Rev Entomol **40**: 171-194

Emmanuel E (2001) A comprehensive system for functional genomics in tomato. Master Thesis. Weizmann Institute of Science, Rehovot, Israel

Emmanuel E, Levy AA (2002) Tomato mutants as tools for functional genomics. Curr Opin Plant Biol **5**: 112-117

Eshel D, Miyara I, Ailing T, Dinoor A, Prusky D (2002) pH regulates endoglucanase expression and virulence of *Alternaria alternata* in persimmon fruit. Mol Plant Microbe In **15**: 774-779

Espelie KE, Davis RW, Kolattukudy PE (1980) Composition, ultrastructure and function of the cutin- and suberin-containing layers in the leaf, fruit peel, juice-sac and inner seed coat of grapefruit (*Citrus paradisi* Macfed.). Planta **149**: 498-511

Espelie KE, Franceschi VR, Kolattukudy PE (1986) Immunocytochemical localization and time course of appearance of an anionic peroxidase associated with suberization in wound-healing potato tuber tissue. Plant Physiol **81**: 487-492

Espelie KE, Kolattukudy PE (1979) Composition of the aliphatic components of 'suberin' from the bundle sheaths of *Zea mays* leaves [maize]. Plant Physiol Bioch **15**: 225-230

Eze JMO, Mayak S, Thompson JE, Dumbroff EB (1986) Senescence in cut carnation flowers: temporal and physiological relationship among water status, ethylene, abscisic acid and membrane permeability. Physiol Plantarum **68**: 323-328

Falavigna A, Badino M, Soressi GP (1978) Potential of the monomendelian factor *pat* in the tomato breeding for industry. Genet Agrar **32**: 159-160

Faurobert M, Mihr C, Bertin N, Pawlowski T, Negroni L, Sommerer N, Causse M (2007) Major proteome variations associated with cherry tomato pericarp development and ripening. Plant Physiol **143**: 1327-1346

Fehling E, Lessire R, Cassagne C, Mukherjee KD (1992) Solubilization and partial purication of constituents of acyl-CoA elongase from *Lunaria annua*. Biochim Biophys Acta **1126**: 88-94

Fehling E, Mukherjee KD (1991) Acyl-CoA elongase from a higher plant (*Lunaria annua*): metabolic intermediates of very-long-chain acyl-CoA products and substrate specificity. Biochim Biophys Acta **1082**: 239-246

Fei Z, Tang X, Alba RM, White JA, Ronning CM, Martin GB, Tanksley SD, Giovannoni JJ (2004) Comprehensive EST analysis of tomato and comparative genomics of fruit ripening. Plant J **40**: 47-59

Feinbaum RL, Ausubel FM (1988) Transcriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. Mol Cell Biol **8**: 1985-1992

Fernie AR, Trethewey RN, Krotzky AJ, Willmitzer L (2004) Metabolite profiling: from diagnostics to systems biology. Nat Rev Mol Cell Biol **5**: 763-769

Ferrer I, Dezotti M, Durán N (1991) Decolorization of kraft effluent by free and immobilized lignin peroxidases and horseradisch peroxidase. Biotechnol Lett **13**: 577-582

Ferrer JL, Jez JM, Bowman ME, Dixon RA and Noel JP (1999) Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. Nat Struct Biol **6**: 775-784

Ferrie BI, Beaudoin N, Burkhart W, Bowsher CC, Rothstein SJ (1994) The cloning of two tomato lipoxygenase genes and their differential expression during fruit ripening. Plant Physiol **106**: 109-118

Feussner I, Wasternack C (2002) The lipoxygenase pathway. Annu Rev Plant Biol **53**: 275-97

Fiebig A, Mayfield JA, Miley NL, Chau S, Fischer RL, Preuss D (2000) Alterations in *CER6*, a gene identical to *CUT1*, differentially

affect long-chain lipid content on the surface of pollen and stems. Plant Cell **12**: 2001-2008

Finkelstein RR, Gampala SSL, Rock CD (2002) Abscisic acid signalling in seeds and seedlings. Plant Cell **14**: 15-45

Finkelstein RR, Gibson SI (2001) ABA and sugar interactions regulating development: cross-talk or voices in a crowd? Curr Opin Plant Biol **5**: 26-32

Fleming AJ, Mandel T, Hofmann S, Sterk P, Devries SC, Kuhlemeier C (1992) Expression pattern of a tobacco lipid transfer protein gene within the shoot apex. Plant J **2**: 855-862

Flores FB, Martinez-Madrid MC, Sanchez-Hidalgo FJ, Romojaro F (2001) Differential rind and pulp ripening of transgenic antisense ACC oxidase melon. Plant Physiol Bioch **39**: 37-43

Fluhr R, Mattoo AK (1996) Ethylene: biosynthesis and perception. Crit Rev Plant Sci **15**: 479-523

Foolad MR, Zhang LP, Khan AA, Niño-Liu D, Lin GY (2002) Identification of QTLs for early blight (*Alternaria solani*) resistance in tomato using backcross populations of a *Lycopersicon esculentum* x *L. hirsutum* cross. Theor Appl Genet **104**: 945-958

Fortes MA, Nogueira MT (1989) The poisson effect in cork. Mater Sci Eng **122**: 227-232

Fowler S, Thomashow MF (2002) *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell **14**: 1675-1690

Franke R, Briesen I, Wojciechowski T, Faust A, Yephremov A, Nawrath C, Schreiber L (2005) Apoplastic polyesters in *Arabidopsis* surface tissues: a typical suberin and a particular cutin. Phytochemistry **66**: 2643-2658

Franke R, Schreiber L (2007) Suberin: a biopolyester forming apoplastic plant interfaces. Curr Opin Plant Biol **10**: 252-259

Frankel N, Carrari F, Hasson E, Iusem ND (2006) Evolutionary history of the *Asr* gene family. Gene **378**: 74-83

Frankel N, Hasson E, Iusem ND, Rossi MS (2003) Adaptive evolution of the water stress-induced gene *Asr2* in *Lycopersicon* species dwelling in arid habitats. Mol Biol Evol **20**: 1955-1962

Fraser PD, Bramley PM (2004) The biosynthesis and nutritional uses of carotenoids. Prog Lipid Res **43**: 228-226

Fraser PD, Enfissi EMA, Goodfellow M, Eguchi T, Bramley PM (2007) Metabolite profiling of plant carotenoids using the matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Plant J **49**: 552-564

Fraser PD, Hedden P, Cooke DT, Bird CR, Schuch W, Bramley PM (1995) The effect of reduced activity of phytoene synthase on isoprenoid levels in tomato pericarp during fruit development and ripening. Planta **196**: 321-326

Fraser PD, Kiano JW, Truesdale MR, Schuch W, Bramley PM (1999) Phytoene synthase-2 enzyme activity in tomato does not contribute to carotenoid synthesis in ripening fruit. Plant Mol Biol **40**: 687-698

Fraser PD, Schuch W, Bramley PM (2000) Phytoene synthase from tomato (*Lycopersicon esculentum*) chloroplasts: partial purification and biochemical properties. Planta **211**: 361-369

Fraser PD, Truesdale MR, Bird CR, Schuch W, Bramley PM (1994) Carotenoid biosynthesis during tomato fruit development: evidence for tissue-specific gene expression. Plant Physiol **105**: 405-413

Fray RG, Grierson D (1993) Identification and genetic analysis of normal and mutant phytoene synthase genes of tomato by sequencing, complementation and co-suppression. Plant Mol Biol **22**: 589-602

Fray RG, Wallace A, Fraser PD, Valero D, Hedden P, Bramley PM, Grierson D (1995) Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. Plant J **8**: 693-701

Fry SC (1986) Cross-linking of matrix polymers in the growing cell walls of angiosperms. Ann Rev Plant Physiol **37**: 165-186

Galpaz N, Ronen G, Khalfa Z, Zamir D, Hirschberg J (2006) A chromoplast-specific carotenoid biosynthesis pathway is revealed by cloning of the tomato white-flower locus. Plant Cell **18**: 1947-1960

Gamble RL, Coonfield ML, Schaller GE (1998) Histidine kinase activity of the ETR1 ethylene receptor from *Arabidopsis*. P Natl Acad Sci USA **95**: 7825-7829

Gao Z, Chen YF, Randlett MD, Zhao XC, Findell JL, Kieber JJ, Schaller GE (2003) Localization of the Raf-like kinase CTR1 to the endoplasmic reticulum of *Arabidopsis* through participation in ethylene receptor signaling complexes. J Biol Chem **278**: 34725-34732

Gazzarrini S, McCourt P (2001) Genetic interactions between ABA, ethylene and sugar signaling pathways. Curr Opin Plant Biol **4**: 387-391

Gazzarrini S, McCourt P (2003) Cross-talk in plant hormone signalling: what *Arabidopsis* mutants are telling us. Ann Bot **91**: 605-612

Geyer U, Schönherr J (1990) The effect of the environment on the permeability and composition of *Citrus* leaf cuticles: water permeability of isolated cuticular membranes. Planta **180**: 147-153

Ghanevati M, Jaworski JG (2001) Active-site residues of a plant membrane-bound fatty acid elongase β-ketoacyl-CoA synthase, FAE1 KCS. Biochim Biophys Acta **1530**: 77-85

Ghanevati M, Jaworski JG (2002) Engineering and mechanistic studies of the *Arabidopsis* FAE1 β-ketoacyl-CoA synthase, FAE1 KCS. Eur J Biochem **269**: 3531-3539

Ghassemian M, Nambara E, Cutler S, Kawaide H, Kamiya Y, McCourt P (2000) Regulation of abscisic acid signaling by the ethylene response pathway in *Arabidopsis*. Plant Cell **12**: 1117-1126

Gilad A, Amitai-Zeigerson H, Scolnik PA, Bar-Zvi D (1997) *ASR1*, a tomato water stress-regulated gene: genomic organization, developmental regulation and DNA-binding activity. Acta Hortic **447**: 441-453

Gillaspy G, Ben-David H, Gruissem W (1993) Fruits: a developmental perspective. Plant Cell **5**: 1439-1451

Giordani T, Natali L, D'Ercole A, Pugliesi C, Fambrini M, Verzieri P, Vitagliano C, Cavallini A (1999) Expression of a dehydrin gene during embryo development and drought stress in ABA-deficient mutants of sunflower (*Helianthus annuus* L.). Plant Mol Biol **39**: 739-748

Giorio G, Stigliani AL, D'Ambrosio C (2008) Phytoene synthase genes in tomato (*Solanum lycopersicum* L.): new data on the structures, the deduced amino acid sequences and the expression patterns. FEBS J **275**: 527-535

Giovanelli G, Lavelli V, Peri C, Nobili S (1999) Variation in antioxidant components of tomato during vine and post-harvest ripening. J Sci Food Agr **79**: 1583-1588

Giovannoni JJ (2001) Molecular biology of fruit maturation and ripening. Annu Rev Plant Phys **52**: 725-749

Giovannoni JJ (2004) Genetic regulation of fruit development and ripening. Plant Cell **16**: 170-180

Giovannoni JJ (2007) Fruit ripening mutants yield insights into ripening control. Curr Opin Plant Biol **10**: 283-289

Giovinazzo G, D'Amico L, Paradiso A, Bollini R, Sparsoli F, DeGara L (2005) Antioxidant metabolite profiles in tomato fruit constitutively expressing the grapevine stilbene synthase gene. Plant Biotechnol J **3**: 57-69

Giuliano G, Bartley GE, Scolnik PA (1993) Regulation of carotenoid biosynthesis during tomato development. Plant Cell **5**: 379-387

Godoy JA, Lunar R, Torres-Schumann S, Moreno J, Rodrigo RM, Pintor-Toro JA (1994) Expression, tissue distribution and subcellular localization of dehydrin TAS14 in salt-stressed tomato plants. Plant Mol Biol **26**: 1921-1934

Godoy JA, Pardo JM, Pintor-Toro JA (1990) A tomato cDNA inducible by salt stress and abscisic acid: nucleotide sequence and expression pattern. Plant Mol Biol **15**: 695-705

Goidin D, Mamessier A, Staquet MJ, Schmitt D, Berthier-Vergnes O (2001) Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and β-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. Anal Biochem **295**: 17-21

Goldman MHS, Goldberg RB, Mariani C (1994) Female sterile tobacco plants are produced by stigma-specific cell ablation. EMBO J **13**: 2976-2984

Gómez J, Sánchez-Martínez D, Stiefel V, Rigau J, Puigdomènech P, Pagès M (1988) A gene induced by the plant hormone abscisic acid in response to water stress encodes a glycine-rich protein. Nature **334**: 262-264

Gong Q, Li P, Ma S, Rupassara SI, Bohnert HJ (2005) Salinity stress adaptation competence in the extremophile *Thellungiella halophila* in comparison with its relative *Arabidopsis thaliana*. Plant J **44**: 826-839

Goodwin SM, Jenks MA (2005) Plant cuticle function as a barrier to water loss. *In* MA Jenks, PM Hasegawa, *eds*, Plant Abiotic Stress. Blackwell Scientific Publishers, Oxford, pp 1-36

Goodwin TW (1976) Distribution of carotenoids. *In* TW Goodwin, *ed*, Chemistry And Biochemistry Of Plant Pigments. Academic Press, London, pp 225-261

Goodwin TW (1980) The biochemistry of the carotenoids: biosynthesis of carotenoids. Chapman and Hall, London, pp 33-76

Gordon DC, Percy KE, Riding RT (1998) Effects of UV-B radiation on epicuticular wax production and chemical composition of four *Picea* species. New Phytol **138**: 441-449

Gowri G, Paiva NL, Dixon RA (1991) Stress responses in alfalfa (*Medicago sativa* L.): sequence analysis of phenylalanine ammonia-lyase (PAL) cDNA clones and appearance of PAL transcripts in elicitor-treated cell cultures and developing plants. Plant Mol Biol **17**: 415-429

Graça J, Pereira H (2000) Suberin structure in potato periderm: glycerol, long-chain monomers, and glyceryl and feruloyl dimers. J Agr Food Chem **4**8: 5476-5483

Graça J, Santos S (2006) Linear aliphatic dimeric esters from cork suberin. Biomacromolecules **7**: 2003-2010

Graça J, Santos S (2007) Suberin: a biopolyester of plants' skin. Macromol Biosci **7**: 128-135

Granier C, Aguirrezabal L, Chenu K, Cookson SJ, Dauzat M, Hamard P, Thioux JJ, Rolland G, Bouchier-Combaud S, Lebaudy A, Muller B, Simonneau T, Tardieu F (2006) PHENOPSIS, an automated platform for reproducible phenotyping of plant responses to soil water deficit in *Arabidopsis thaliana* permitted the identification of an accession with low sensitivity to soil water deficit. New Phytol **169**: 623-635

Grappin P, Bouinot D, Sotta B, Miginiac E, Jullien M (2000) Control of seed dormancy in *Nicotiana plumbaginifolia*: post-imbibition abscisic acid synthesis imposes dormancy maintenance. Planta **210**: 279-285

Gray JE, Picton S, Giovannoni JJ, Grierson D (1994) The use of transgenic and naturally-occurring mutants to understand and manipulate tomato fruit ripening. Plant Cell Environ **17**: 557-571

Greenway H, Munns R (1980) Mechanisms of salt tolerance in nonhalophytes. Annu Rev Plant Phys **31**: 149-190

Grichko VP, Glick BR, Grishko VI, Pauls KP (2005) Evaluation of tomato plants with constitutive, root-specific, and stress-induced ACC deaminase gene expression. Russ J Plant Physl **52**: 359-364

Grierson D (1986) Molecular biology of fruit ripening. Oxf Surv Plant Mol Cell Biol **3**: 363-383

Grierson D, Fray R (1994) Control of ripening in transgenic tomatoes. Euphytica **79**: 251-263

Grierson D, Maunders MJ, Slater A, Ray J, Bird CR, Schuh W, Holdsworth MJ, Tucker GA, Knapp JE (1986) Gene expression during tomato ripening. Philos T Roy Soc **314**: 399-410

Griffiths A, Barry C, Alpuche-Soli AG, Grierson D (1999) Ethylene and developmental signals regulate expression of lipoxygenase genes during tomato fruit ripening. J Exp Bot **50**: 793-798

Grisebach H (1965) Biosynthesis of flavonoids. *In* TW Goodwin, *ed*, Chemistry And Biochemistry Of Plant Pigments. Academic Press, New York, NY, pp 279-308

Groh B, Hübner C, Lendzian KJ (2002) Water and oxygen permeance of phellems isolated from trees: the role of waxes and lenticels. Planta **215**: 794-801

Guerrero FD, Jones JT, Mullet JE (1990) Turgor-responsive gene transcription and RNA levels increase rapidly when pea shoots are wilted: sequence and expression of three inducible genes. Plant Mol Biol **15**: 11-26

Guhling O, Kinzler C, Dreyer M, Bringmann G, Jetter R (2005) Surface composition of myrmecophilic plants: cuticular wax and glandular trichomes on leaves of *Macaranga tanarius*. J Chem Ecol **31**: 2323-2341

Guichard S, Bertin N, Leonardi C, Gary C (2001) Tomato fruit quality in relation to water and carbon fluxes. Agronomie **21**: 385-392

Guiltinan MJ, Marcotte WR, Quatrano RS (1990) A plant leucine zipper protein that recognizes an abscisic acid response element. Science **250**: 267-271

Haas K (1974) Untersuchungen zum chemischen Aufbau der Cuticula während der Organogenese von Blättern und Früchten sowie zur Cuticulartranspiration. PhD Thesis. University of Hohenheim, Hohenheim, Germany

Hahlbrock K (1981) Flavonoids. *In* EE Conn, *ed*, The Biochemistry Of Plants: Secondary Plant Products. Academic Press, New York, NY, pp 425-456

Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Phys **40**: 347-369

Hallam D (1970) Growth and regeneration of waxes on the leaves of *Eucalyptus.* Planta **93**: 257-268

Hamberg M, Hamberg G (1990) Hydroperoxide-dependent epoxidation of unsaturated fatty acids in the broad bean (*Vicia faba* L.). Arch Biochem Biophys **283**: 409-416

Hamilton AJ, Lycett GW, Grierson D (1990) Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. Nature **346**: 284-287

Hamilton RJ (1995) Analysis of waxes. *In* RJ Hamilton, *ed*, Waxes: Chemistry, Molecular Biology And Functions. The Oily Press, Dundee, pp 311-342

Han J, Lühs W, Sonntag K, Zähringer U, Borchardt DS, Wolter FP, Heinz E, Frentzen M (2001) Functional characterization of β-ketoacyl-CoA synthase genes from *Brassica napus* L. Plant Mol Biol **46**: 229-239

Hannoufa A, McNevin J, Lemieux B (1993) Epicuticular waxes of *eceriferum* mutants of *Arabidopsis thaliana*. Phytochemistry **33**: 851-855

Hansen JD, Pyee J, Xia Y, Wen TJ, Robertson DS, Kolattukudy PE, Nikolau BJ, Schnable PS (1997) The *glossy7* locus of maize and an epidermis-specific cDNA from *KIeínía odora* define a class of receptor-like proteins required for the normal accumulation of cuticular waxes. Plant Physiol **113**: 1091-1100

Harker M, Hirschberg J (1998) Molecular biology of carotenoid biosynthetic organisms. Method Enzymol **297**: 244-263

Harwood CE (1980) Frost resistance of subalpine *Eucalyptus* species: experiments using a radiation frost room. Aust J Bot **28**: 587-599

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular response to high salinity. Annu Rev Plant Phys **51**: 463-99

Haslam E (1998) Practical polyphenolics: from structure to molecular recognition and physiological action. University Press, Cambridge

Hatfield RD, Ralph J, Grabber JH (1999) Cell wall cross-linking by ferulates and diferulates in grasses. J Sci Food Agr **79**: 403-407

Hauke V, Schreiber L (1998) Ontogenetic and seasonal development of wax composition and cuticular transpiration of ivy (*Hedera helix* L.) sun and shade leaves. Planta **207**: 67-75

Hause B, Stenzel I, Miersch O, Maucher H, Kramell R, Ziegler J, Wasternack C (2000) Tissue-specific oxylipin signature of tomato flowers: allene oxide cyclase is highly expressed in distinct flower organs and vascular bundles. Plant J **24**: 113-126

Hayashi H, Huang P, Kirakosyan A, Inoue K, Hiraoka N, Ikeshiro Y, Kushiro T, Shibuya M, Ebizuka Y (2001) Cloning and characterization of a cDNA encoding β-amyrin synthase involved in glycyrrhizin and soyasaponin biosyntheses in licorice. Biol Pharm Bull **24**: 912-916

Heitz T, Bergey DR, Ryan CA (1997) A gene encoding a chloroplast-targeted lipoxygenase in tomato leaves is transiently induced by wounding, systemin, and methyl jasmonate. Plant Physiol **114**: 1085-1093

Hemm MR, Rider SD, Ogas J, Murry DJ, Chapple C (2004) Light induces phenylpropanoid metabolism in *Arabidopsis* roots. Plant J **38**: 765-778

Herde O, Atzorn R, Fisahn J, Wasternack C, Willmitzer L, Peña-Cortés H (1996) Localized wounding by heat initiates the accumulation of proteinase inhibitor II in abscisic acid-deficient plants by triggering jasmonic acid biosynthesis. Plant Physiol **112**: 853-860

Herde O, Cortés HP, Wasternack C, Willmitzer L, Fisahn J (1999) Electric signaling and *Pin2* gene expression on different abiotic stimuli depend on a distinct threshold level of endogenous abscisic acid in several abscisic acid-deficient tomato mutants. Plant Physiol **119**: 213-218

Heredia A (2003) Biophysical and biochemical characteristics of cutin, a plant barrier biopolymer. Biochim Biophys Acta **1620**: 1-7

Herrero M, Dickinson HG (1981) Pollen tube development in *Petunia hybrida* following compatible and incompatible intraspecific matings. J Cell Sci **47**: 365-383

Hetherington MJ, MacDougall DB (1992) Optical properties and appearance characteristics of

tomato fruit (*Lycopersicon esculentum*). J Sci Food Agr **59**: 537-543

Hille J, Koornneef M, Ramanna MS, Zabel P (1989) Tomato: a crop species amenable to improvement by cellular and molecular methods. Euphytica **42**: 1-23

Himmelbach A, Yang Y, Grill E (2003) Relay and control of abscisic acid signalling. Curr Opin Plant Biol **6**: 470-479

Hirschberg J (2001) Carotenoid biosynthesis in flowering plants. Curr Opin Plant Biol **4**: 210-218

Ho LC (1988) Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. Annu Rev Plant Phys **39**: 355-78

Ho LC (1996) The mechanism of assimilate partitioning and carbohydrate compartmentation in fruit in relation to the quality and yield of tomato. J Exp Bot **47**: 1239-1243

Hoeberichts FA, Linus HW, Woltering EJ (2002) Ethylene perception is required for the expression of tomato ripening-related genes and associated physiological changes even at advanced stages of ripening. Postharvest Biol Tec **26**: 125-133

Hoh F, Pons JL, Gautier MF, de Lamotte F, Dumas C (2005) Structure of a liganded type 2 nonspecific lipid-transfer protein from wheat and the molecular basis of lipid binding. Acta Crystallogr **61**: 397-406

Hollenbach B, Schreiber l, Hartung W, Dietz KJ (1997) Cadmium leads to stimulated expression of the lipid transfer protein genes in barley: implications for the involvement of lipid transfer proteins in wax assembly. Planta **203**: 9-19

Holloway PJ (1970) Surface factors affecting the wetting of leaves. Pestic Sci **1**: 156-163

Holloway PJ (1982) The chemical constitution of plant cutins. *In* DF Cutler, KL Alvin, CE Price, *eds*, The Plant Cuticle. Academic Press, London, pp 45-85

Holloway PJ (1983) Some variations in the composition of suberin from the cork layers of higher plants. Phytochemistry **22**: 495-502

Holloway PJ (1984) Surface lipids of plants and animals. *In* HK Mangold, G Zweig, J Sherma, *eds*, Handbook Of Chromatography: Lipids. CRC Press, Boca Raton, FL, pp 347-380

Holloway PJ, Wattendorff J (1987) Cutinized and suberized cell walls. *In* KC Vaughn, *ed*, Handbook Of Plant Cytochemistry. CRC Press, Boca Raton, FL, pp 1-35

Holmberg N, Bülow L (1998) Improving stress tolerance in plants by gene transfer. Trends Plant Sci **3**: 61-66

Holroyd GH, Hetherington AM, Gray JE (2002) A role for the cuticular waxes in the environmental control of stomatal development. New Phytol **153**: 433-439

Holton TA, Cornish EC (1995) Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell **7**: 1071-1083

Hong JH, Cowan AK, Lee SK (2004) Glucose inhibits ACC oxidase activity and ethylene biosynthesis in ripening tomato fruit. Plant Growth Regul **43**: 81-87

Hooker TS, Millar AA, Kunst L (2002) Significance of the expression of the CER6 condensing enzyme for cuticular wax production in *Arabidopsis*. Plant Physiol **129**: 1568-1580

Hörtensteiner S (2006) Chlorophyll degradation during senescence. Annu Rev Plant Biol **57**: 55-77

Hose E, Clarkson DT, Steudle E, Schreiber L, Hartung W (2001) The exodermis: a variable apoplastic barrier. J Exp Bot **52**: 2245-2264

Hsiao TC (1973) Plant responses to water stress. Annu Rev Plant Phys **24**: 519-570

Hua J, Meyerowitz EM (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell **94**: 261-271

Huang W, Jia J, Edwards P, Dehesh K, Schneider G, Lindqvist Y (1998) Crystal structure of β-ketoacyl-acyl carrier protein synthase II from *E. coli* reveals the molecular architecture of condensing enzymes. EMBO J **17**: 1183-1191

Huang Y, Li H, Hutchison CE, Laskey J, Kieber JJ (2003) Biochemical and functional analysis of CTR1, a protein kinase that negatively regulates ethylene signaling in *Arabidopsis*. Plant J **33**: 221-233

Hülskamp M, Schneitz K, Pruitt RE (1995) Genetic evidence for a long-range activity that directs pollen tube guidance in *Arabidopsis*. Plant Cell **7**: 57-64

Huner NPA, Oquist G, Hurry VM, Krol M, Falk S, Griffith M (1993) Photosynthesis, photoinhibition and low temperature acclimation tolerant plants in cold. Photosynth Res **37**: 19-39

Hunt GM, Baker EA (1980) Phenolic constituents of tomato fruit cuticles. Phytochemistry **19**: 1415-1419

Ibrahim RK, Varin L (1993) Flavonoid enzymology. Meth Plant Biochem **9**: 99-131

Ichimura K, Shinozaki K, Tena G, Sheen J, Henry Y, Champion A, Kreis M, Zhang S, Hirt H, Wilson C, Heberle-Bors E, Ellis BE, Morris PC, Innes RW, Ecker JR, Scheel D, Klessig DF, Machida Y, Mundy J, Ohashi Y, Walker JC (2002) Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends Plant Sci **7**: 301-308

Iskandar HM, Simpson RS, Casu RE, Bonnett GD, Maclean DJ, Manners JM (2004) Comparison of reference genes for quantitative real-time polymerase chain reaction analysis of gene expression in sugarcane. Plant Mol Biol Rep **22**: 325-337

Iusem ND, Bartholomew DM, Hitz WD, Scolnik PA (1993) Tomato *(Lycopersicon esculentum)* transcript induced water deficit and ripening. Plant Physiol **102**: 1353-1354

Jackson MR, Nilsson T, Peterson PA (1990) Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO J **9**: 3153-3162

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. Science **280**: 104-106

Jain M, Nijhawan A, Tyagi AK, Khurana JP (2006) Validation of housekeeping genes as internal control for studying gene expression in

rice by quantitative real-time PCR. Biochem Bioph Res Co **345**: 646-651

Jain SC, Singh B, Jain R (2001) Antimicrobial activity of triterpenoids from *Heliotropium ellipticum*. Fitoterapia **72**: 666-668

James DW, Lim E, Keller J, Plooy I, Ralston E, Dooner HK (1995) Directed tagging of the *Arabidopsis FATTY ACID ELONGATION1* (*FAE1*) gene with the maize transposon *Activator*. Plant Cell **7**: 309-319

Jandet P, Douillet-Breuil AC, Bessis R, Debord S, Sbaghi M, Adrian M (2002) Phytoalexins from the vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism. J Agr Food Chem **50**: 2731-2741

Jefferson PG (1994) Genetic variation for epicuticular wax production in altai wildrye populations that differ in glaucousness. Crop Sci **34**: 367-371

Jeffree CE (1996) Structure and ontogeny of plant cuticles. *In* G Kerstiens, *ed*, Plant Cuticles: An Integrated Functional Approach. Bios Scientific Publishers, Oxford, pp 33-82

Jeffree CE (2006) The fine structure of the plant cuticle. *In* M Riederer, C Müller, *eds*, Biology Of The Plant Cuticle. Blackwell Scientific Publishers, Oxford, pp 11-125

Jenks MA, Andersen L, Teusinka RS, Williams MH (2001) Leaf cuticular waxes of potted rose cultivars as affected by plant development, drought and paclobutrazol treatments. Physiol Plantarum **112**: 62-70

Jenks MA, Ashworth EN (1999) Plant epicuticular waxes: function, production, and genetics. *In* J Janick, *ed*, Horticultural Reviews. John Wiley and Sons, New York, NY, pp 1-68

Jenks MA, Eigenbrode SD, Lemieux B (2002) Cuticular waxes of *Arabidopsis*. *In* CR Somerville, EM Meyerowitz, *eds*, The *Arabidopsis* Book. American Society of Plant Biologists, Rockville, MD

Jenks MA, Joly RJ, Peters PJ, Rich PJ, Axtell JD, Ashworth EN (1994) Chemically induced cuticle mutation affecting epidermal conductance to water vapor and disease susceptibility in *Sorghum bicolor* (L.) Moench. Plant Physiol **105**: 1239-1245

Jenks MA, Rashotte AM, Tuttle HA, Feldmann KA (1996a) Mutants in *Arabidopsis thaliana* altered in epicuticular wax and leaf morphology. Plant Physiol **110**: 377-385

Jenks MA, Tuttle HA, Eigenbrode SD, Feldmann KA (1995) Leaf epicuticular waxes of the *eceriferum* mutants in *Arabidopsis*. Plant Physiol **108**: 369-377

Jenks MA, Tuttle HA, Feldmann KA (1996b) Changes in epicuticular waxes on wild type and *eceriferum* mutants in *Arabidopsis* during development. Phytochemistry **42**: 29-34

Jetter R, Kunst L, Samuels AL (2006) Composition of plant cuticular waxes. *In* M Riederer, C Müller, *eds*, Biology Of The Plant Cuticle. Blackwell Scientific Publishers, Oxford, pp 145-181

Jetter R, Schäffer S (2001) Chemical composition of the *Prunus laurocerasus* leaf surface: dynamic changes of the epicuticular wax film during leaf development. Plant Physiol **126**: 1725-1737

Jetter R, Schäffer S, Riederer M (2000) Leaf cuticular waxes are arranged in chemically and mechanically distinct layers: evidence from *Prunus laurocerasus* L. Plant Cell Environ **23**: 619-628

Jez JM, Noel JP (2000) Mechanism of Chalcone Synthase. pK_a of the catalytic cysteine and the role of the conserved histidine in a plant polyketide synthase. J Biol Chem **275**: 39640-39646

Jiang Y, Fu J (2000) Ethylene regulation of fruit ripening: molecular aspects. Plant Growth Regul **30**: 193-200

Jiang Y, Huang B (2002) Protein alterations in tall fescue in response to drought stress and abscisic acid. Crop Sci **42:** 202-207

Johnson PR, Ecker JR (1998) The ethylene gas signal transduction pathway: a molecular perspective. Annu Rev Genet **32**: 227-254

Jones DH (1984) Phenylalanine ammonia-lyase: regulation of its induction, and its role in plant development. Phytochemistry **23**: 1349-1359

Jones L, Seymour GB, Knox JP (1997) Localization of pectic galactan in tomato cell walls using a monoclonal antibody specific to (1→4)-β-D-galactan. Plant Physiol **113**: 1405-1412

Jouili H, Bouazizi H, Rossignol M, Borderies G, Jamet E, El Ferjani E (2008) Partial purification and characterization of a copper-induced anionic peroxidase of sunflower roots. Plant Physiol Biochemistry **46**: 760-767

Jung KH, Han MJ, Lee DY, Lee YS, Schreiber L, Franke R, Faust A, Yephremov A, Saedler H, Kim YW, Hwang I, An G (2006) *Wax-deficient anther1* is involved in cuticle and wax production in rice anther walls and is required for pollen development. Plant Cell **18**: 3015-3032

Kader JC (1996) Lipid-transfer proteins in plants. Annu Rev Plant Phys **47**: 627-54

Kader JC (1997) Lipid-transfer proteins: a puzzling family of plant proteins. Trends Plant Sci **2**: 66-70

Kader JC, Julienne M, Vergnolle C (1984) Purification and characterization of a spinach-leaf protein capable of transferring phospholipids from liposomes to mitochondria or chloroplasts. Eur J Biochem **139**: 411-416

Kahn RA, Le Bouquin R, Pinot F, Benveniste I, Durst F (2001) A conservative amino acid substitution alters the regiospecificity of CYP94A2, a fatty acid hydroxylase from the plant *Vicia sativa*. Arch Biochem Biophys **391**: 180-187

Kangasjärvi J, Talvinen J, Utriainen M, Karjalainen R (1994) Plant defense systems induced by ozone. Plant Cell Environ **17**: 783-794

Karaba A (2007) Improvement of water use efficiency in rice and tomato using *Arabidopsis* wax biosynthetic genes and transcription factors. PhD Thesis, Wageningen University, Wageningen, Netherlands

Karakurt Y, Huber DJ (2004) Ethylene-induced gene expression, enzyme activities, and water soaking in immature and ripe watermelon

(*Citrullus lanatus*) fruit. J Plant Physiol **16**1: 381-388

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol **17**: 287-291

Kaulen H, Schell J, Kreuzaler F (1986) Light-induced expression of the chimeric chalcone synthase-NPT II gene in tobacco cells. EMBO J **5**: 1-8

Kausch KD, Handa AK (1997) Molecular cloning of a ripening-specific lipoxygenase and its expression during wild-type and mutant tomato fruit development. Plant Physiol **113**: 10411050

Kawaguchi R, Williams AJ, Bray EA, Bailey-Serres J (2003) Water-deficit-induced translational control in *Nicotiana tabacum*. Plant Cell Environ **26**: 221-229

Kende H (1993) Ethylene biosynthesis. Annu Rev Plant Phys **44**: 283-307

Kerstiens G (1996) Cuticular water permeability and its physiological significance. J Exp Bot **47**: 1813-1832

Kerstiens G (2006) Water transport in plant cuticles: an update. J Exp Bot **57**: 2493-2499

Kerstiens G, Schreiber L, Lendzian KJ (2006) Quantification of cuticular permeability in genetically modified plants. J Exp Bot **57**: 2547-2552

Khan W, Prithiviraj B, Smith DL (2004) Chitosan and chitin oligomers increase phenylalanine ammonia-lyase and tyrosine ammonia-lyase activities in soybean leaves. J Plant Physiol **160**: 859-63

Khan ZH, Marron K (1988) Replacement of benzene and carbon tetrachloride with less hazardous eluants in chromatography for the determination of saturates content of hydrocarbon oils. J Liq Chromatogr **11**: 1605-1613.

Kieber JJ, Rothenberg M, Roman G, Feldman KA, Ecker JR (1993) CTR1, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the Raf family of protein kinases. Cell **72**: 427-441

Kim BR, Nam HY, Kim SU, Kim SI, Chang YJ (2003) Normalization of reverse transcription quantitative-PCR with housekeeping genes in rice. Biotechnol Lett **25**: 1869-1872

Kim KS, Park SH, Jenks MA (2007) Changes in leaf cuticular waxes of sesame (*Sesamum indicum* L.) plants exposed to water deficit. J Plant Physiol **164**: 1134-1143

Kiss E, Balagh A, Tisza V, Koncz T, Heszky L (2007) Ethylene biosynthetic and signalling genes in strawberry fruit: isolation and characterization of ACC-synthase, -oxidase and CTR1. *In* A Ramina, C Chang, J Giovannoni, H Klee, P Perata, E Woltering, *eds*, Advances In Plant Ethylene Research, Springer-Verlag, Berlin, pp 41-43

Klee HJ (1993) Ripening physiology of fruit from transgenic tomato (*Lycopersicon esculentum*) plants with reduced ethylene synthesis. Plant Physiol **102**: 911-916

Klotz KL, Liu TTY, Liu L, Lagrimini LM (1998) Expression of the tobacco anionic peroxidase gene is tissue-specific and developmentally regulated. Plant Mol Biol **36**: 509-520

Knoche M, Peschel S, Hinz M, Bukovac MJ (2000) Studies on water transport through the sweet cherry fruit surface: characterizing conductance of the cuticular membrane using pericarp segments. Planta **212**: 127-135

Knoll D, Schreiber L (1998) Influence of epiphytic micro-organisms on leaf wettability: wetting of the upper leaf surface of *Juglans regia* and of model surfaces in relation to colonization by micro-organisms. New Phytol **140**: 271-282

Koag MC, Fenton RD, Wilkens S, Close TJ (2003) The binding of maize DHN1 to lipid vesicles: gain of structure and lipid specificity. Plant Physiol **131**: 309-316

Koch K, Neinhuis C, Ensikat HJ, Barthlott W (2004) Self assembly of epicuticular waxes on living plant surfaces imaged by atomic force microscopy (AFM). J Exp Bot **55**: 711-718

Koes RE, Quattrocchio F, Mol JNM (1994) The flavonoid biosynthetic pathway in plants: function and evolution. Bioessays **16**: 123-132

Kolattukudy PE (1967) Mechanisms of synthesis of waxy esters in broccoli (*Brassica oleracea*). Biochemistry **6**: 2705-2717

Kolattukudy PE (1968) Further evidence for an elongation-decarboxylation mechanism in the biosynthesis of paraffins in leaves. Plant Physiol **43**: 375-383

Kolattukudy PE (1977) Lipid polymers and associated phenols, their chemistry, biosynthesis, and role in pathogenesis. *In* FA Loewus, VC Runeckles, *eds*, The Structure, Biosynthesis And Degradation Of Wood. Plenum Press, New York, NY, pp 185-246

Kolattukudy PE (1980) Biopolyester membranes of plants: cutin and suberin. Science **208**: 990-1000

Kolattukudy PE (1981) Structure, biosynthesis, and biodegradation of cutin and suberin. Annu Rev Plant Phys **32**: 539-567

Kolattukudy PE (1984) Biochemistry and function of cutin and suberin. Can J Bot **62**: 2918-2933

Kolattukudy PE (1996) Biosynthetic pathways of cutin and waxes and their sensitivity to environmental stresses. *In* G Kerstiens, *ed*, Plant Cuticles: An Integrated Functional Approach. Bios Scientific Publishers, Oxford, pp 83-108

Kolattukudy PE (2001) Polyesters in higher plants. *In* W Babel, A Steinbüchel, *eds*, Biopolyesters. Springer-Verlag, Berlin, pp 1-49

Kolattukudy PE (2002) Suberin from plants. *In* Y Doi, A Steinbüchel, *eds*, Polyesters. Wiley-VCH, Weinheim, pp 41-73

Kolattukudy PE, Espelie KE (1985) Biosynthesis of cutin, suberin and associated waxes. *In* T Higuchi, *ed*, Biosynthesis And Biodegradation Of Wood Components. Academic Press, New York, NY, pp 161-207

Kolattukudy PE, Kronman K, Poulose AJ (1975) Determination of structure and composition of suberin from the roots of carrot, parsnip, rutabaga, turnip, red beet, and sweet potato by combined gas-liquid chromatography and mass spectrometry. Plant Physiol **55**: 567-573

Kolattukudy PE, Rogers LM, Li D, Hwang CS, Flaishman MA (1995) Surface signaling in

pathogenesis. P Natl Acad Sci USA **92**: 4080-4087

Koornneef M (1986) Genetic aspecs of abscisic acid. *In* AD Blonstein, PJ King, *eds*, A Genetic Approach To Plant Biochemistry, Springer-Verlag, Berlin, pp 35-54

Koornneef M, Hanhart CJ, Thiel F (1989) A genetic and phenotypic description of *eceriferum (cer)* mutants in *Arabidopsis thaliana.* J Hered **80**: 118-122

Kpémoua K, Boher B, Nicole M, Calatayud PA, Geiger JP (1996) Cytochemistry of defense responses in cassava infected by *Xanthomonas campestris* pv. *manihotis*. Can J Microbiol **42**: 1131-1143

Kreps JA, Wu Y, Chang HS, Zhu T, Wang X, Harper JF (2002) Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. Plant Physiol **130**: 2129-2141

Krolikowski KA, Victor JL, Nussbaum-Wagler T, Lolle SJ, Pruitt RE (2003) Isolation and characterization of the *Arabidopsis* organ fusion gene *HOTHEAD*. Plant J **35**: 501-511

Kumar GNM, Knowles NR (2003) Wound-induced superoxide production and PAL activity decline with potato tuber age and wound healing ability. Physiol Plantarum **117**: 108-117

Kunoh H, Yamaoka N, Yoshioka H, Nicholson RL (1988) Preparation of the infection court by *Erysiphe graminis*. Contact-mediated changes in morphology of the conidium surface. Exp Mycol **12**: 325-335

Kunst A, Draeger B, Ziegenhorn J (1984) D-glucose: colorimetric methods with glucose oxidase and peroxidase. *In* HU Bergmeyer, J Bergmeyer, M Graßl, *eds*, Metabolites: Carbohydrates. Verlag Chemie, Weinheim, pp 178-185

Kunst L, Jetter R, Samuels AL (2006) Biosynthesis and transport of plant cuticular waxes. *In* M Riederer, C Müller, *eds*, Biology Of The Plant Cuticle. Blackwell Scientific Publishers, Oxford, pp 182-215

Kunst L, Samuels AL (2003) Biosynthesis and secretion of plant cuticular wax. Prog Lipid Res **42**: 51-80

Kunst L, Samuels AL, Jetter R (2004) The plant cuticle: formation and structure of epidermal surfaces. *In* DJ Murphy, *ed*, Plant Lipids: Biology, Utilisation And Manipulation. Blackwell Scientific Publishers, Oxford, pp 270-302

Kurata T, Kawabata-Awai C, Sakuradani E, Shimizu S, Okada K, Wada T (2003) The *YORE-YORE* gene regulates multiple aspects of epidermal cell differentiation in *Arabidopsis*. Plant J **36**: 55-66

Kushiro T, Shibuya M, Masudab K, Ebizuka Y (2000) A novel multifunctional triterpene synthase from *Arabidopsis thaliana*. Tetrahedron Letters **41**: 7705-7710

Lagrimini LM, Bradford S, Rothstein S (1990) Peroxidase-induced wilting in transgenic tobacco plants. Plant Cell **2**: 7-18

Lagrimini LM, Gingas V, Finger F, Rothstein S, Liu TTY (1997) Characterization of antisense transformed plants deficient in the tobacco anionic peroxidase. Plant Physiol **114**: 1187-1196

Lagrimini LM, Rothstein S (1987) Tissue specificity of tobacco peroxidase isozymes and their induction by wounding and tobacco mosaic virus infection. Plant Physiol **84**: 438-442

Lai C, Kunst L, Jetter R (2007) Composition of alkyl esters in the cuticular wax on inflorescence stems of *Arabidopsis thaliana cer* mutants. Plant J **50**: 189-196

Lakshminarayana S, Sommer NF, Polito V, Fortlage RJ (1987) Development of resistance to infection by *Botrytis cinerea* and *Penicillium expansum* in wounds of mature apple fruits. Phytopathology **77**: 1674-1678

Lång V, Robertson M, Chandler PM (1998) Allelic variation in the dehydrin gene family of 'Himalaya' barley (*Hordeum vulgare* L.). Theor Appl Genet **96**: 1193-1199

Lanz T, Tropf S, Marner FJ, Schröder J, Schröder G (1991) The role of cysteines in polyketide synthases. Site-directed mutagenesis of resveratrol and chalcone synthases, two key enzymes in different plant-specific pathways. J Biol Chem **266**: 9971-9976

Lardizabal KD, Metz JG, Sakamoto T, Hutton WC, Pollard MR, Lassner MW (2000) Purification of a jojoba embryo wax synthase, cloning of its cDNA, and production of high levels of wax in seeds of transgenic *Arabidopsis*. Plant Physiol **122**: 645-655

Lassner MW, Lardizabal K, Metz JG (1996) A jojoba β-ketoacyl-CoA synthase cDNA complements the canola fatty acid elongation mutation in transgenic plants. Plant Cell **8**: 281-292

Lavy-Meir G, Barkai-Golan R, Kopeliovitch E (1989) Resistance of tomato ripening mutants and their hybrids to *Botrytis cinerea*. Plant Dis **73**: 976-978

Lawlor DW, Cornic G (2002) Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell Environ **25**: 275-294

Le Bouquin R, Pinot F, Benveniste I, Salaün JP, Durst F (1999) Cloning and functional characterization of CYP94A2, a medium chain fatty acid hydroxylase from *Vicia sativa*. Biochem Bioph Res Co **261**: 156-162

Le Bouquin R, Skrabs M, Kahn R, Benveniste I, Salaün JP, Schreiber L, Durst F, Pinot F (2001) Cyp94A5, a new cytochrome P450 from *Nicotiana tabacum* is able to catalyze the oxidation of fatty acids to the omega-alcohol and to the corresponding diacid. Eur J Biochem **268**: 3083-3090

Leclercq J, Adams-Phillips LC, Zegzouti H, Jones B, Latché A, Giovannoni JJ, Pech JC, Bouzayen M (2002) *LeCTR1*, a tomato *CTR1*-like gene, demonstrates ethylene signaling ability in *Arabidopsis* and novel expression patterns in tomato. Plant Physiol **130**: 1132-1142

Lee PD, Sladek R, Greenwood CMT, Hudson TJ (2001) Control genes and variability: absence of ubiquitous reference transcripts in diverse mammalian expression studies. Genome Res **12**: 292-297

Lemaire-Chamley M, Petit J, Garcia V, Just D, Baldet P, Germain V, Fagard M, Mouassite M, Cheniclet C, Rothan C (2005) Changes in transcriptional profiles are associated with early fruit tissue specialization in tomato. Plant Physiol **139**: 750-769

Lemieux B (1996) Molecular genetics of epicuticular wax biosynthesis. Trends Plant Sci **1**: 312-318

Lemieux B, Koornneef M, Feldmann KA (1994) Epicuticular wax and *eceriferum* mutants. *In* EM Meyerowitz, CR Somerville, *eds*, *Arabidopsis.* Cold Spring Harbor Laboratory Press, New York, NY, pp 1031-1047

Lendzian KJ, Kerstiens G (1991) Sorption and transport of gases and vapors in plant cuticles. Rev Environ Contam T **121**: 65-128

León P, Sheen J (2003) Sugar and hormone connections. Trends in Plant Sci 8: 110-116

Lessire R, Bessoule JJ, Cassagne C (1985). Solubilization of C_{18}-CoA and C_{20}-CoA elongases from *Allium porrum* L. epidermal cell microsomes. FEBS Lett **187**: 314-320

Lessire R, Chevalier S, Lucet-Levannier K, Lellouche JP, Mioskowski C, Cassagne C (1999) Study of the 3-hydroxy eicosanoyl-coenzyme A dehydratase and (*E*)-2,3 enoyl-coenzyme A reductase involved in acyl-coenzyme A elongation in etiolated leek seedlings. Plant Physiol **119**: 1009-1015

Leung J, Giraudat J (1998) Abscisic acid signal transduction. Annu Rev Plant Phys **49**: 199-222

Li F, Murillo C, Wurtzel ET (2007) Maize γ9 encodes a product essential for 15-*cis*-ζ-carotene isomerization. Plant Physiol **144**: 1181-1189

Li F, Wu X, Lam P, Bird D, Zheng H, Samuels L, Jetter R, Kunst L (2008) Identification of the wax ester synthase/acyl-coenzyme A:diacylglycerol acyltransferase WSD1 required for stem wax ester biosynthesis in *Arabidopsis*. Plant Physiol **148**: 97-107

Liavonchanka A, Feussner I (2006) Lipoxygenases: occurrence, functions and catalysis. J Plant Physiol **163**: 348-357

Liechti R, Farmer EE (2002) The jasmonate pathway. Science **296**: 1649-1650

Lin Z, Hackett RM, Payton P, Grierson D (1998) A tomato sequence *tCTR2*, encoding an *Arabidopsis CTR1* homologue. Plant Physiol **117**: 1126

Lincoln JE, Cordes S, Read E, Fischer RL (1987) Regulation of gene expression ethylene during *Lycopersicon esculentum* (tomato) fruit development. P Natl Acad Sci USA **84**: 2793-2797

Lindgren LO, Stålberg KG, Höglund AS (2003) Seed-specific overexpression of an endogenous *Arabidopsis* phytoene synthase gene results in delayed germination and increased levels of carotenoids, chlorophyll, and abscisic acid. Plant Physiol **132**: 779-785

Linforth RST, Bowman WR, Griffin DA, Marples BA, Taylor IB (1987) 2-*trans*-ABA alcohol accumulation in the wilty tomato mutants *flacca* and *sitiens*. Plant Cell Environ **10**: 599-606

Liu Y, Hoffman NE, Yang SF (1985) Promotion by ethylene of the capability to convert 1-aminocyclopropane-1-carboxylic acid to ethylene in preclimacteric tomato and cantaloupe fruits. Plant Physiol **77**: 407-411

Liu Y, Schiff M, Dinesh-Kumar SP (2002) Virus-induced gene silencing in tomato. Plant J **31**: 777-786

Lois LM, Rodríguez-Concepción M, Gallego F, Campos N, Boronat A (2000) Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase. Plant J **22**: 503-513

Lolle SJ, Hsu W, Pruitt RE (1998) Genetic analysis of organ fusion in *Arabidopsis thaliana*. Genetics **149**: 607-619

Lolle SJ, Pruitt RE (1999) Epidermal cell interactions: a case for local talk. Trends Plant Sci **4**: 1360-1385

López-Casado G, Matas AJ, Domínguez E, Cuartero J, Heredia A (2007) Biomechanics of isolated tomato (*Solanum lycopersicum* L.) fruit cuticles: the role of the cutin matrix and polysaccharides. J Exp Bot **58**: 3875-3883

Lownds NK, Banaras M, Bosland PW (1993) Relationships between postharvest water loss and physical properties of pepper fruit (*Capsicum annuum* L.). HortScience **28**: 1182-1184

Lownds NK, Banaras M, Bosland PW (1994) Postharvest water loss and storage quality of nine pepper (*Capsicum*) cultivars. HortScience **29**: 191-193

Luckwill LC (1943) The genus *Lycopersicon*: a historical, biological, and taxonomic survey of the wild and cultivated tomatoes. University Press, Aberdeen

Lulai EC, Corsini DL (1998) Differential deposition of suberin phenolic and aliphatic domains and their roles in resistance to infection during potato tuber (*Solanum tuberosum* L.) wound-healing. Physiol Mol Plant P **53**: 209-222

Lulai EC, Suttle JC, Pederson SM (2008) Regulatory involvement of abscisic acid in potato tuber wound-healing. J Exp Bot **59**: 1175-1186

Lulai EC, Weiland JJ, Suttle JC, Sabba RP, Bussan AJ (2006). Pink eye is an unusual periderm disorder characterized by aberrant suberization: a cytological analysis. Am J Potato Res **83**: 409-421

Luque P, Bruque S, Heredia A (1995) Water permeability of isolated cuticular membranes: a structural analysis. Arch Biochem Biophys **317**: 417-422

Ma DP, Tan H, Si Y, Creech RG, Jenkins JN (1995) Differential expression of a lipid transfer protein gene in cotton fiber. Biochim Biophys Acta **1257**: 81-84

Maalekuu K, Elkind Y, Leikin-Frenkel A, Lurie S, Fallik E (2006) The relationship between water loss, lipid content, membrane integrity and LOX activity in ripe pepper fruit after storage. Postharvest Biol Tec **42**: 248-255

Maalekuu K, Tuvia-Alkalai S, Shalom Y, Fallik E, Elkind Y, Jenks MA, Goodwin MS (2005) Characterization of physiological and biochemical factors associated with postharvest water loss in ripe pepper fruit during storage. J Am Soc Hortic Sci **130:** 735-741

Maccarrone M, Veldink GA, Agrò AF, Vliegenthart JFG (1995) Modulation of soybean lipoxygenase expression and membrane oxidation by water deficit. FEBS Lett **371**: 223-226

Manavella PA, Arce AL, Dezar CA, Bitton F, Renou JP, Crespi M, Chan RL (2006) Cross-talk between ethylene and drought signaling pathways is mediated by the sunflower Hahb-4 transcription factor. Plant J **48**: 125-137

Mansfield TA (1987) Hormones as regulators of water balance. *In* RD Davies, *ed*, Plant Hormones And Their Role In Plant Growth And Development. Martinus Nijhoff Publishers, Dordrecht, pp 411-430

Mao L, Begum D, Chuang HW, Budiman MA, Szymkowiak EJ, Irish EE, Wing RA (2000) *JOINTLESS* is a MADS-box gene controlling tomato flower abscission zone development. Nature **406**: 910-913

Marga F, Pesacreta TC, Hasenstein KH (2001) Biochemical analysis of elastic and rigid cuticles of *Cirsium horridulum*. Planta **213**: 841-848

Marin E, Marion-Poll A (1997) Tomato *flacca* mutant is impaired in ABA aldehyde oxidase and xanthine dehydrogenase activities. Plant Physiol Bioch **35**: 369-372

Marion-Poll A, Leung J (2006) Abscisic acid synthesis, metabolism and signal transduction. *In* P Hedden, TG Thomas, *eds*, Plant Hormone Signaling. Blackwell Scientific Publishers, Oxford, pp 1-35

Maskin L, Gudesblat GE, Moreno JE, Carrari FO, Frankel N, Sambade A, Rossi M, Iusem ND (2001) Differential expression of the members of the *Asr* gene family in tomato (*Lycopersicon esculentum*). Plant Sci **16**1: 739-746

Matas AJ, Cuartero J, Heredia A (2004) Phase transitions in the biopolyester cutin isolated from tomato fruit cuticles. Thermochim Acta **409:** 165-168

Matzke K, Riederer M (1991) A comparative study into the chemical constitution of cutins and suberins from *Picea abies* (L.) Karst., *Quercus robur* L., and *Fagus sylvatica* L. Planta **185**: 233-245

Mayhew M, Hartl FU (1996) Molecular chaperone proteins. *In* F Neidhardt, *ed*, *Escherchia coli* And *Salmonella typhimurium*: Cellular And Molecular Biology. American Society for Microbiology, Washington, DC, pp 922-937

Mazzucato A, Olimpieri I, Ciampolini F, Cresti M, Soressi GP (2003) A defective pollen-pistil interaction contributes to hamper seed set in the *parthenocarpic fruit* tomato mutant. Sex Plant Reprod 16: 157-164

Mazzucato A, Taddei AR, Soressi GP (1998) The *parthenocarpic fruit* (*pat*) mutant of tomato (*Lycopersicon esculentum* Mill.) sets seedless fruits and has aberrant anther and ovule development. Development **125**: 107-114

Mazzucato A, Testa G, Biancari T, Soressi GP (1999) Effect of gibberellic acid treatments, environmental conditions, and genetic background on the expression of the *parthenocarpic fruit* mutation in tomato. Protoplasma **208**: 18-25

McLeod LC, Poole PR (1994) Changes in enzymatic activities after harvest and in early stages of *Botrytis cinerea* infection of kiwifruit. J Sci Food Agr **64**: 95-106

McMurchie EJ, McGlasson WB, Eaks IL (1972) Treatment of fruit with propylene gives information about the biogenesis of ethylene. Nature **237**: 235-236

McNevin JP, Woodward W, Hannoufa A, Feldmann KA, Lemieux B (1993) Isolation and characterization of *eceriferum* (*CER*) mutants induced by T-DNA insertions in *Arabidopsis thaliana*. Genome **36**: 610-618

Meissner R, Chaque V, Zhu Q, Emmanuel E, Elkind Y, Levy AA (2000) A high throughput system for transposon tagging and promoter trapping in tomato. Plant J **22**: 265-274

Meissner R, Jacobson Y, Melamed S, Levyatuv S, Shalev G, Ashri A, Elkind Y, Levy A (1997) A new model system for tomato genetics. Plant J **12**: 1465-1472

Metz JG, Pollard MR, Anderson L, Hayes TR, Lassner MW (2000) Purification of a jojoba embryo fatty acyl-coenzyme A reductase and expression of its cDNA in high erucic acid rapeseed. Plant Physiol **122**: 635-644

Mietkiewska E, Giblin EM, Wang S, Barton DL, Dirpaul J, Brost JM, Katavic V, Taylor DC (2004) Seed-specific heterologous expression of a nasturtium *FAE* gene in *Arabidopsis* results in a dramatic increase in the proportion of erucic acid. Plant Physiol **136**: 2665-2675

Millar AA, Clemens S, Zachgo S, Giblin EM, Taylor DC, Kunst L (1999) *CUT1*, an *Arabidopsis* gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. Plant Cell **11**: 825-838

Millar AA, Kunst L (1997) Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. Plant J **12**: 121-131

Millar AA, Wrischer M, Kunst L (1998) Accumulation of very-long-chain fatty acids in membrane glycerolipids is associated with dramatic alterations in plant morphology. Plant Cell **11**: 1889-1902

Mintz-Oron S, Mandel T, Rogachev I, Feldberg L, Lotan O, Yativ M, Wang Z, Jetter R, Venger I, Adato A, Aharoni A (2008) Gene expression and metabolism in tomato fruit surface tissues. Plant Physiol **147**: 823-851

Mittler R (2006) Abiotic stress, the field environment and stress combination. Trends Plant Sci **11**: 15-19

Moctezuma E, Smith DL, Gross KC (2003) Antisense suppression of a β-galactosidase gene (*TBG6*) in tomato increases fruit cracking. J Exp Bot **54**: 2025-2033

Mohan R, Vijayan P, Kolattukudy PE (1993) Developmental and tissue-specific expression of a tomato anionic peroxidase (*tap1*) gene by a minimal promoter, with wound and pathogen induction by an additional 5'-flanking region. Plant Mol Biol **22**: 475-490

Moire L, Schmutz A, Buchala A, Yan B, Stark RE, Ryser U (1999) Glycerol is a suberin

monomer: new experimental evidence for an old hypothesis. Plant Physiol **119**: 1137-1146

Mol JNM, Schram AW, de Vlaming P, Gerats AGM, Kreuzaler F, Hahlbrock K, Reif HJ, Veltkamp E (1983) Regulation of flavonoid gene expression in *Petunia hybrida*: description and partial characterization of a conditional mutant in chaleone synthase gene expression. Mol Gen Genet **192**: 424-429

Molina A, Garcia-Olmedo F (1993) Developmental and pathogen-induced expression of three barley genes encoding lipid transfer proteins. Plant J **4**: 983-991

Monforte AJ, Asíns MJ, Carbonell EA (1997) Salt tolerance in *Lycopersicon* species: genotype-by-salinity interaction in quantitative trait loci detection: constitutive and response QTLs. Theor Appl Genet **95**: 706-713

Moon H, Chowrira G, Rowland O, Blacklock BJ, Smith MA, Kunst L (2005) A root-specific condensing enzyme from *Lesquerella fendleri* that elongates very-long-chain saturated fatty acids. Plant Mol Biol **56**: 917-927

Moons A, Prinsen E, Bauw G, van Montagu M (1997) Antagonistic effects of abscisic acid and jasmonates on salt stress-inducible transcripts in rice roots. Plant Cell **9**: 2243-2259

Moore S, Payton P, Wright M, Tanksley S, Giovannoni J (2005) Utilization of tomato microarrays for comparative gene expression analysis in the Solanaceae. J Exp Bot **56**: 2885-2895

Morgan PW, Drew MC (1997) Ethylene and plant responses to stress. Physiol Plantarum **100**: 620-630

Morita M, Shibuya M, Kushiro T, Masuda K, Ebizuka Y (2000) Molecular cloning and functional expression of triterpene synthases from pea (*Pisum sativum*): new a-amyrin-producing enzyme is a multifunctional triterpene synthase. Eur J Biochem **267**: 3453-3460

Morris I (1966) The effect of cycloheximide (actidione) on protein and nucleic acid synthesis by Chlorella. J Exp Bot **18**: 54-64

Morris WL, Ducreus L, Griffiths DW, Stewart D, Davies HV, Taylor MA (2004) Carotenogenesis during tuber development and storage in potato. J Exp Bot **55**: 975-982

Muir SR, Collins GJ, Robinson S, Hughes S, Bovy A, de Vos CHR, van Tunen AJ, Verhoeyen ME (2001) Overexpression of petunia chalcone isomerase in tomato results in fruit containing increased levels of flavonols. Nat Biotechnol **19**: 470-474

Müller WJ, Albertyn J, Smit MS (2007) Cycloheximide resistance in the Lipomycetaceae revisited. Can J Microbiol **53**: 509-513

Murata J, Roepke J, Gordon H, de Luca V (2008) The leaf epidermome of *Catharanthus roseus* reveals its biochemical specialization. Plant Cell **20**: 524-542

Nakano R, Ogura E, Kubo Y, Inaba A (2003) Ethylene biosynthesis in detached young persimmon fruit is initiated in calyx and modulated by water loss from the fruit. Plant Physiol **131**: 276-286

Nakatsuka A, Murachi S, Okunishi H, Shiomi S, Nakano R, Kubo Y, Inaba A (1998) Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, of 1-aminocyclopropane-1-carboxylateoxidase, and ethylene receptor genes in tomato fruit during development and ripening. Plant Physiol **118**: 1295-1305

Nawrath C (2002) The biopolymers cutin and suberin. *In* CR Somerville, EM Meyerowitz, *eds*, The *Arabidopsis* Book. American Society of Plant Biologists, Rockville, MD

Nawrath C (2006) Unraveling the complex network of cuticular structure and function. Curr Opin Plant Biol **9**: 281-287

Negruk V, Yang P, Subramanian M, McNevin JP, Lemieux B (1996) Molecular cloning and characterization of the *CER2* gene of *Arabidopsis thaliana*. Plant J **9**: 137-145

Neinhuis C, Koch K, Barthlott W (2001) Movement and regeneration of epicuticular waxes through plant cuticles. Planta **213**: 427-434

Nie X, Singh RP, Tai GCC (2002) Molecular characterization and expression analysis of 1-aminocyclopropane-1-carboxylate oxidase homologs from potato under abiotic and biotic stresses. Genome **45**: 905-913

Niklas KJ (1992) Plant biomechanics: an engineering approach to plant form and function. University Press, Chicago, IL

Nitsch JP (1970) Hormonal factors in growth and development. *In* AC Hulme, *ed*, Biochemistry Of Fruits And Their Products. Academic Press, London, pp 427-472

Niyogi KK (1999) Photoprotection revisited: genetic and molecular approaches. Annu Rev Plant Phys **50**: 333-359

Novikova GV, Moshkov IE, Smith AR, Hall MA (2000) The effect of ethylene on MAPKinase-like activity in *Arabidopsis thaliana*. FEBS Lett **474**: 29-32

Nylander M, Svensson J, Palva ET, Welin BV (2001) Stress-induced accumulation and tissue-specific localization of dehydrins in *Arabidopsis thaliana*. Plant Mol Biol **45**: 263-279

O'Neill SD, Tong Y, Sporlein B, Forkmann G, Yoder YI (1990) Molecular genetic analysis of chalcone synthase in *Lycopersicon esculentum* and an anthocyanin-deficient mutant. Mol Gen Genet **224**: 279-288

Oeller PW, Wong LM, Taylor LP, Pike DA, Theologis A (1991) Reversible inhibition of tomato fruit senescence by antisense RNA. Science **254**: 437-439

Ohlrogge JB, Jaworski JG, Post-Beittenmiller D (1993) *De novo* fatty acid biosynthesis. *In* TS Moore, *ed*, Lipid Metabolism In Plants. CRC Press, Boca Raton, FL, pp 3-33

Olsen KM, Lea US, Slimestad R, Verheul M, Lillo C (2008) Differential expression of four *Arabidopsis PAL* genes: *PAL1* and *PAL2* have functional specialization in abiotic environmental-triggered flavonoid synthesis. Journal of Plant Physiol **165**: 1491-1499

Olson DC, White JA, Edelman L, Harkins RN, Kende H (1991) Differential expression of two genes for 1-aminocyclopropane-1-carboxylic acid synthase in tomato fruits. P Natl Acad Sci USA **88**: 5340-5344

Oono Y, Seki M, Nanjo T, Narusaka M, Fujita M, Satoh R, Satou M, Sakurai T, Ishida J, Akiyama K, Iida K, Maruyama K, Satoh S, Yamaguchi-Shinozaki K, Shinozaki K (2003) Monitoring expression profiles of *Arabidopsis* gene expression during rehydration process after dehydration using ca. 7000 full-length cDNA microarray. Plant J **34**: 868-887

Oppenheimer HR (1960) Adaptation to drought: xerophytism. *In* Plant-Water Relationships In Arid And Semi-Arid Conditions. Reviews of Research: Arid Zone Research, pp 105-138

Parr AJ, Bolwell GP (2000) Phenols in the plant and in man: the potential for possible nutritional enhancment of the diet by modifying the phenols content or profile. J Sci Food Agr **80**: 985-1012

Pecker I, Chamovitz D, Linden H, Sandmann G, Hirschberg J (1992) A single polypeptide catalyzing the conversion of phytoene to C-carotene is transcriptionally regulated during tomato fruit ripening (carotenoid biosynthesis/phytoene desaturase/pahtids). P Natl Acad Sci USA **89**: 4962-4966

Pelletier MK, Burbulis IE, Winkel-Shirley B (1999) Disruption of specific flavonoid genes enhances the accumulation of flavonoid enzymes and end-products in *Arabidopsis* seedlings. Plant Mol Biol **40**: 45-54

Peña-Cortés H, Prat S, Atzorn R, Wasternack C, Willmitzer L (1996) Abscisic acid-deficient plants do not accumulate proteinase inhibitor II following systemin treatment. Planta **198**: 447-451

Peña-Cortés H, Sanchez-Serrano JJ, Mertens R, Willmitzer L, Prat S (1989) Abscisic acid involved in the wound-induced expression proteinase inhibitor II gene in potato and tomato. P Natl Acad Sci USA **86:** 9851-9855

Peña-Cortés H, Willmitzer L (1995) The role of hormones in gene activation in response to wounding. *In* PJ Davis, *ed*, Plant Hormones: Physiology, Biochemistry And Molecular Biology. Kluwer Academic Publishers, Dordrecht, pp 395-414

Pennycook SR, Manning MA (1992) Picking wound curing to reduce *Botrytis* storage rot of kiwifruit. New Zeal J Crop Hort **20**: 357-360

Percy KE, Baker EA (1987) Effects of simulated acid rain on production, morphology and composition of epicuticular wax and on cellular membrane development. New Phytol **107**: 577-589

Pereira H (1988) Chemical composition and variability of cork from *Quercus suber* L. Wood Sci Technol **22**: 211-218

Petracek PD, Bukovac MJ (1995) Rheological properties of enzymatically isolated tomato fruit cuticle. Plant Physiol **109**: 675-679

Pfündel E, Agati G, Cerovic ZG (2006) Optical properties of plant surfaces. *In* M Riederer, C Müller, *eds*, Biology Of The Plant Cuticle. Blackwell Scientific Publishers, Oxford, pp 215-248

Picton S, Barton SL, Bouzayen M, Hamilton AJ, Grierson D (1993) Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene. Plant J **3**: 469-481

Piechulla B, Gruissem W (1987) Diurnal mRNA fluctuations of nuclear and plastid genes in developing tomato fruits. EMBO J **6**: 3593-3599

Piechulla B, Pichersky E, Cashmore AR I, Gruissem W (1986) Expression of nuclear and plastid genes for photosynthesis-specific proteins during tomato fruit development and ripening. Plant Mol Biol 7: 367-376

Pighin JA, Zheng H, Balakshin LJ, Goodman IP, Western TL, Jetter R, Kunst L, Samuels AL (2004) Plant cuticular lipid export requires an ABC transporter. Science **306**: 702-704

Pinot F, Bosch H, Alayrac C, Mioskowski C, Vendais A, Durst F, Salaün JP (1993) ω-hydroxylation of oleic acid in *Vicia sativa* microsomes: inhibition by substrate analogs and inactivation by terminal acetylenes. Plant Physiol **102**: 1313-1318

Pinot F, Salaün JP, Bosch H, Lesot A, Mioskowski C, Durst F (1992) ω-hydroxylation of Z9-octadecenoic, Z9,10-epoxystearic and 9,10-dihydroxystearic acids by microsomal cytochrome P450 systems from *Vicia sativa*. Biochem Bioph Res Co **184**: 183-193

Podila GK, Rogers LM, Kolattukudy PE (1993) Chemical signals from avocado surface wax trigger germination and appressorium formation in *Colletotrichum gloeosporioides*. Plant Physiol **103**: 267-272

Pollard M, Beisson F, Li Y, Ohlrogge JB (2008) Building lipid barriers: biosynthesis of cutin and suberin. Trends Plant Sci **13**: 236-246

Pons JL, de Lamotte F, Gautier MF, Delsuc MA (2003) Refined solution structure of a liganded type 2 wheat nonspecific lipid transfer protein. J Biol Chem **278**: 14249-14256

Poole PR, McLeod LC (1991) Inhibition of *Botrytis cinerea* infection in kiwifruit. Acta hortic **297**: 599-603

Poole PR, McLeod LC (1994) Development of resistance to picking wound entry *Botrytis cinema* storage rots in kiwifruit. New Zeal J Crop Hort **22**: 387-392

Popp C, Burghardt M, Friedmann A, Riederer M (2005) Characterization of hydrophilic and lipophilic pathways of *Hedera helix* L. cuticular membranes: permeation of water and uncharged organic compounds. J Exp Bot **56**: 2797-2806

Porat R, Pasentsis K, Rozentzvieg D, Gerasopoulos D, Falara V, Samach A, Lurie S, Kanellis AK (2004) Isolation of a dehydrin cDNA from orange and grapefruit citrus fruit that is specifically induced by the combination of heat followed by chilling temperatures. Physiol Plantarum **120**: 256-264

Porta H, Rocha-Sosa M (2002) Plant lipoxygenases: physiological and molecular features. Plant Physiol **130**: 15-21

Porta H, Rueda-Benitez P, Campos F, Colmenero-Flores JM, Colorado JM, Carmona MJ, Covarrubias AA, Rocha-Sosa M (1999) Analysis of lipoxygenase mRNA accumulation in the common bean (*Phaseolus vulgaris* L.) during development and under stress conditions. Plant Cell Physiol **40**: 850-858

Post-Beittenmiller D (1996) Biochemistry and molecular biology of wax production in plants. Annu Rev Plant Phys **47**: 405-430

Premachandra GS, Saneoka H, Kanaya M, Ogata S (1991) Cell membrane stability and leaf surface wax content as affected by increasing water deficits in maize. J Exp Bot **42**: 167-171

Preuss D, Lemieux B, Yen G, Davis RW (1993) A conditional sterile mutation eliminates surface components from *Arabidopsis* pollen and disrupts cell signalling during fertilization. Gene Dev **7**: 974-985

Pruitt RE, Vielle-Calzada JP, Ploense SE, Grossniklaus U, Lolle SJ (2000) *FIDDLEHEAD*, a gene required to suppress epidermal cell interactions in *Arabidopsis*, encodes a putative lipid biosynthetic enzyme. P Natl Acad Sci USA **97**: 1311-1316

Prusky D, Kobiler I, Jacoby B, Sims JJ, Midland SL (1985) Inhibitors of avocado lipoxygenase: their possible relationship with the latency of *Colletotrichum gloeosporioides*. Physiol Plant Pathol **27**: 269-279

Pyee J, Kolattukudy PE (1995) The gene for the major cuticular wax-associated protein and three homologous genes from broccoli (*Brassica oleracea*) and their expression patterns. Plant J **7**: 49-59

Pyee J, Yu H, Kolattukudy PE (1994) Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (*Brassica oleracea*) leaves. Arch Biochem Biophys **311**: 460-468

Qin YM, Pujol FM, Hu CY, Feng JX, Kastaniotis AJ, Hiltunen JK, Zhu YX (2007) Genetic and biochemical studies in yeast reveal that the cotton fibre-specific *GhCER6* gene functions in fatty acid elongation. J Exp Bot **58**: 473-481

Qiu X, Janson CA, Konstantinidis AK, Nwagwu S, Silverman C, Smith WW, Khandekar S, Lonsdale J, Abdel-Meguid SS (1999) Crystal structure of β-ketoacyl-acyl carrier protein synthase: a key condensing enzyme in bacterial fatty acid biosynthesis. J Biol Chem **274**: 36465-36471

Quiroga M, Guerrero C, Botella MA, Barceló A, Amaya I, Medina MI, Alonso FJ, de Forchetti SM, Tigier H, Valpuesta V (2000) A tomato peroxidase involved in the synthesis of lignin and suberin. Plant Physiol **122**: 1119-1127

Rademacher W, Maisch R, Liessegang J, Jung J (1987) Water consumption and yield formation in crop plants under the influence of synthetic analogues of abscisic acid. *In* AF Hawkins, AD Steadand, NJ Pinfield, *eds*, Plant Growth Regulators For Agricultural and Amenity Use. BCPC Publications, Thornton Heath, pp 53-66

Radler F (1965) Reduction of loss of moisture by the cuticle wax components of grapes. Nature **207**: 1002-1003

Radonić A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A (2004) Guideline to reference gene selection for quantitative real-time PCR. Biochem Bioph Res Co **313**: 856-862

Raffaele S, Vailleau F, Léger A, Joubès J, Miersch O, Huard C, Blée E, Mongrand S, Domergue F, Robya D (2008) A MYB transcription factor regulates very-long-chain fatty acid biosynthesis for activation of the hypersensitive cell death response in *Arabidopsis*. Plant Cell **20**: 752-767

Ramos LJ, Narayanan KR, McMillan RT (1992) Association of stomatal frequency and morphology in *Lycopersicon* species with resistance to *Xanthomonas campestris* pv. *vesicatoria*. Plant Pathol **41**: 157-164

Rashotte AM, Jenks MA, Ross AS, Feldmann KA (2004) Novel *eceriferum* mutants in *Arabidopsis thaliana*. Planta **219**: 5-13

Rasori A, Bertolasi B, Furini A, Bonghi C, Tonutti P, Ramina A (2003) A functional analysis of peach ACC oxidase promoters in transgenic tomato and in ripening peach fruit. Plant Sci **165**: 523-530

Raven JA (1977) The evolution of vascular land plants in relation to supracellular transport processes. Adv Bot Res **5**: 153-219

Ray J, Moureau P, Bird C, Bird A, Grierson D, Maunders M, Truesdale M, Bramley P, Schuch W (1992) Cloning and characterization of a gene involved in phytoene synthesis from tomato. Plant Mol Biol **19**: 401-404

Raz V, Ecker JR (1999) Regulation of differential growth in the apical hook of *Arabidopsis*. Development **126**: 3661-3668

Reina JJ, Heredia A (2001) Plant cutin biosynthesis: the involvement of a new acyltransferase. Trends Plant Sci **6**: 296

Reinbotbe S, Reinbothe C, Lehmann J, Parthier B (1992) Differential accumulation of methyl jasmonate-induced mRNAs in response to abscisic acid and desiccation in barley (*Hordeum vulgare*). Physiol Plantarum **86**: 49-56

Renan M, François J, Marion D, Axelos MAV, Douliez JP (2003) Study of the interaction between end-capped telechelic polymers and the wheat lipid transfer protein LTP1, in solution and at the air/water interface. Colloid Surface **32**: 213-221

Reymond P, Weber H, Damond M, Farmer EE (2000) Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. Plant Cell **12**: 707-719

Reynhardt EC, Riederer M (1994) Structures and molecular dynamics of plant waxes: cuticular waxes from leaves of *Fagus sylvatica* L. and *Hordeum vulgare* L. Eur Biophys J **23**: 59-70

Rick CM, Yoder JI (1988) Classical and molecular genetics of tomato: highlights and perspectives. Annu Rev Genet **22**: 281-300

Riedel M, Eichner A, Jetter R (2003) Slippery surfaces of carnivorous plants: composition of epicuticular wax crystals in *Nepenthes alata* Blanco pitchers. Planta **218**: 87-97

Riederer M, Friedmann A (2006) Transport of lipophilic non-electrolytes across the cuticle. *In* M Riederer, Müller, *eds*, Biology Of The Plant Cuticle. Blackwell Scientific Publishers, Oxford, pp 250-279

Riederer M, Markstädter C (1996) Cuticular waxes: a critical assessment of current knowledge. *In* G Kerstiens, *ed*, Plant Cuticles: An Integrated Functional Approach. Bios Scientific Publishers, Oxford, pp 189-200

Riederer M, Schneider G (1990) The effect of the environment on the permeability and composition of *Citrus* leaf cuticles: composition of soluble cuticular lipids and correlation with transport properties. Planta **180**: 154-165

Riederer M, Schönherr J (1988) Development of plant cuticles: fine structure and cutin composition of *Clivia miniata* Reg. leaves. Planta **174**: 127-138

Riederer M, Schreiber L (1995) Waxes: the transport barriers of plant cuticles. *In* RJ Hamilton, *ed*, Waxes: Chemistry, Molecular Biology And Functions. The Oily Press, Dundee, pp 130-156

Riederer M, Schreiber L (2001) Protecting against water loss: analysis of the barrier properties of plant cuticles. J Exp Bot **52**: 2023-2032

Ristic Z, Jenks MA (2002) Leaf cuticle and water loss in maize lines differing in dehydration avoidance. J Plant Physiol **159**: 645-651

Roberton M, Chandler PM (1992) Pea dehydrins: identification, characterisation and expression. Plant Mol Biol **19**: 1031-1044

Roberts E, Kolattukudy PE (1989) Molecular cloning, nucleotide sequence, and abscisic acid induction of a suberization-associated highly anionic peroxidase. Mol Gen Genet **217**: 223-232

Roberts E, Kutchan T, Kolattukudy PE (1988) Cloning and sequencing of cDNA for a highly anionic peroxidase from potato and the induction of its mRNA in suberizing potato tubers and tomato fruits. Plant Mol Biol **11**: 15-26

Roberts JA, Whitelaw CA, Gonzalez-Carranza ZH, McManus MT (2000) Cell separation processes in plants: models, mechanisms and manipulation. Ann Bot **86**: 223-235

Robertson M, Chandler PM (1994) A dehydrin cognate protein from pea (*Pisum sativum* L.) with an atypical pattern of expression. Plant Mol Biol **26**: 805-816

Rodríguez-Concepción M, Querol J, Lois LM, Imperial S, Boronat A (2003) Bioinformatic and molecular analysis of hydroxymethylbutenyl diphosphate synthase (GCPE) gene expression during carotenoid accumulation in ripening tomato fruit. Planta **217**: 476-482

Rogiers SY, Kumar GNM, Knowles NR (1998) Maturation and ripening of fruit of *Amelanchier alnifolia* Nutt. are accompanied by increasing oxidative stress. Ann Bot **81**: 203-211

Rojas MG, Morales-Ramos JA, Harrington TC (1999) Association between *Hypothenemus hampei* (Coleoptera: Scolytidae) and *Fusarium solani* (Moniliales: Tuberculariaceae). Ann Entomol Soc Am **92**: 98-100

Rolland F, Moore B, Sheen J (2002) Sugar sensing and signalling in plants. Plant Cell 185-205

Rom S, Gilad A, Kalifa Y, Konrad Z, Karpasas MK, Goldgur Y, Bar-Zvi D (2006) Mapping the DNA- and zinc-binding domains of ASR1 (abscisic acid stress ripening), an abiotic-stress regulated plant specific protein. Biochimie **88**: 621-628

Ronen G, Cohen M, Zamir D, Hirschberg J (1999) Regulation of carotenoid biosynthesis during tomato fruit development: expression of the gene for lycopene epsilon-cyclase is down-regulated during ripening and is elevated in the mutant Delta. Plant J **17**: 341-351

Rose JKC, Bashir S, Giovannoni JJ, Jahn MM, Saravanan RS (2004) Tackling the plant proteome: practical approaches, hurdles and experimental tools. Plant J **39**: 715-733

Rossi M, Carrari F, Cabrera-Ponce JL, Vázquez-Rovere C, Herrera-Estrella L, Gudesblat G, Iusem ND (1998) Analysis of an abscisic acid (ABA)-responsive gene promoter belonging to the *Asr* gene family from tomato in homologous and heterologous systems. Mol Gen Genet **258**: 1-8

Rossi M, Iusem ND (1994) Tomato (*Lycopersicon esculentum*) genomic clone homologous to a gene encoding an abscisic acid-induced protein. Plant Physiol **104**: 1073-1074

Rottmann WH, Peter GF, Oeller PW, Keller JA, Shen NF, Nagy BP, Taylor LP, Campbell AD, Theologis A (1991) 1-aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. J Mol Biol **222**: 937-961

Rowland O, Zheng H, Hepworth SR, Lam P, Jetter R, Kunst L (2006) *CER4* encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in cuticular wax production in *Arabidopsis*. Plant Physiol **142**: 866-877

Roy BA, Stanton ML, Eppley SM (1999) Effects of environmental stress on leaf hair density and consequences for selection. J Evolution Biol **12**: 1089-1103

Ruan YL, Patrick JW, Brady CJ (1996) The composition of apoplast fluid recovered from intact developing tomato fruit. Aust J Plant Physiol **23**: 9-13

Ruperti B, Bonghi C, Rasori A, Ramina A, Tonutti P (2001) Characterization and expression of two members of the peach 1-aminocyclopropane-1-carboxylate oxidase gene family. Physiol Plantarum **111**: 336-344

Saladié M, Matas AJ, Isaacson T, Jenks MA, Goodwin SM, Niklas KJ, Xiaolin R, Labavitch JM, Shackel KA, Fernie AR, Lytovchenko A, O'Neill MA, Watkins CB, Rose JKC (2007) A reevaluation of the key factors that influence tomato fruit softening and integrity. Plant Physiol **144**: 1012-1028

Samdur MY, Manivel P, Jain VK, Chikani BM, Gor HK, Desai S, Misra JB (2003) Genotypic differences and water-deficit induced enhancement in epicuticular wax load in peanut. Crop Sci **43**: 1294-1299

Samuel D, Liu YJ, Cheng CS, Lyu PC (2002) Solution structure of plant nonspecific lipid transfer protein-2 from rice (*Oryza sativa*). J Biol Chem **277**: 35267-35273

Samuels L, Kunst L, Jetter R (2008) Sealing plant surfaces: cuticular wax formation by epidermal cells. Annu Rev Plant Biol **59**: 683-707

Sánchez FJ, Manzanares M, de Andrés EF, Tenorio JL, Ayerbe L (2001) Residual transpiration rate, epicuticular wax load and leaf colour of pea plants in drought conditions: influence on harvest index and canopy temperature. Eur J Agron **15**: 57-70

Santos S, Graça J (2006) Glycerol-ω-hydroxyacid-ferulic acid oligomers in cork suberin structure. Holzforschung **60**: 171-177

Šantrůček J (1991) Patterns of statistical distribution of stomatal resistance over the leaf

surface of tobacco plants cultivated *in vitro*. Photosynthetica **25**: 527-536

Schijlen E, de Vos CHR, Jonker H, van den Broeck H, Molthoff J, van Tunen A, Martens S, Bovy A (2006) Pathway engineering for healthy phytochemicals leading to the production of novel flavonoids in tomato fruit. Plant Biotechnol J **4**: 433-444

Schijlen EGWM, de Vos CHR, Martens S, Jonker HH, Rosin FM, Molthoff JW, Tikunov YM, Angenent GC, van Tunen AJ, Bovy AG (2007) RNA interference silencing of chalcone synthase, the first step in the flavonoid biosynthesis pathway, leads to parthenocarpic tomato fruits. Plant Physiol **144**: 1520-1530

Schmidt HW, Merida T, Schönherr J (1981) Water permeability and fine structure of cuticular membranes isolated enzymatically from leaves of *Clivia miniata* Reg. Z Pflanzenphysiol **105**: 41-51

Schneider A, Salamini F, Gebhardt C (1997) Expression patterns and promoter activity of the cold-regulated gene *ci21A* of potato. Plant Physiol **113**: 335-345

Schneider F, Lessire R, Bessoule JJ, Juguelin H, Cassagne C (1993) Effect of cerulenin on the synthesis of very-long-chain fatty acids in microsomes from leek seedlings. Biochim Biophys Acta **1152**: 243-252

Schnurr J, Shockey J, Browse J (2004) The acyl-CoA synthetase encoded by *LACS2* is essential for normal cuticle development in *Arabidopsis*. Plant Cell **16**: 629-642

Schofield A, Paliyath G (2005) Modulation of carotenoid biosynthesis during tomato fruit ripening through phytochrome regulation of phytoene synthase activity. Plant Physiol Biochemistry **43**: 1052-1060

Schönherr J (1976) Water permeability of isolated cuticular membranes: the effect of cuticular waxes on diffusion of water. Planta **131**: 159-164

Schönherr J (1982) Resistance of plant surfaces to water loss: transport properties of cutin, suberin and associated lipids. *In* OL Lange, PS Nobel, CB Osmond, H Ziegler, *eds*, Physiological Plant Ecology. Springer-Verlag, Berlin, pp 153-179

Schönherr J (2000) Calcium chloride penetrates plant cuticles *via* aqueous pores. Planta **212**: 112-118

Schönherr J, Baur P (1996) Effects of temperature, surfactants and other adjuvants on rates of uptake of organic compounds. *In* G Kerstiens, *ed*, Plant Cuticles: An Integrated Functional Approach. Bios Scientific Publishers, Oxford, pp 134-154

Schönherr J, Lendzian K (1981) A simple and inexpensive method of measuring water permeability of isolated plant cuticular membranes. Z Pflanzenphysiol **102**: 321-327

Schönherr J, Riederer M (1986) Plant cuticles sorb lipophilic compounds during enzymatic isolation. Plant Cell Environ **9**: 459-466

Schönherr J, Riederer M (1989) Foliar penetration and accumulation of organic chemicals in plant cuticles. Rev Environ Contam T **108**: 1-70

Schöppner A, Kindl H (1979) Stilbene synthase (pinosylvine synthase) and its induction by ultraviolet light. FEBS Lett **108**: 349-352

Schreiber L (2006) Characterisation of polar paths of transport in plant cuticles. *In* M Riederer, C Müller, *eds*, Biology Of The Plant Cuticle. Blackwell Scientific Publishers, Oxford, pp 280-291

Schreiber L, Franke R, Hartmann KD, Ranathunge K, Steudle E (2005) The chemical composition of suberin in apoplastic barriers affects radial hydraulic conductivity differently in the roots of rice (*Oryza sativa* L. cv. IR64) and corn (*Zea mays* L. cv. Helix). J Exp Bot **56**: 1427-1436

Schreiber L, Hartmann K, Skrabs M, Zeier J (1999) Apoplastic barriers in roots: chemical composition of endodermal and hypodermal cell walls. J Exp Bot **50**: 1267-1280

Schreiber L, Riederer M (1996) Ecophysiology of cuticular transpiration: comparative investigation of cuticular water permeability of plant species from different habitats. Oecologia **107**: 426-432

Schuler MA, Werck-Reichhart D (2003) Functional genomics of P450s. Annu Rev Plant Biol **54**: 629-667

Schwab W, Davidovich-Rikanati R, Lewinsohn E (2008) Harnessing plant biomass for biofuels and biomaterials: biosynthesis of plant-derived flavor compounds. Plant J **54**: 712-732

Scott JW, Harbaugh BK (1989) 'Micro-Gold' miniature dwarf tomato. University Press, Gainesville, FL

Seki M, Kamei A, Yamaguchi-Shinozaki, Shinozaki K (2003) Molecular responses to drought, salinity and frost: common and different paths for plant protection. Curr Opin Biotech **14**: 194-199

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002) Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J **31**: 279-292

Senaratna T, Touchell D, Bunn E, Dixon K (2000) Acetyl salicylic acid (aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. Plant Growth Regul **30**: 157-161

Seo M, Koshiba T (2002) Complex regulation of ABA biosynthesis in plants. Trends Plant Sci **7**: 41-48

Shackel KA, Greve C, Labavitch JM, Ahmadi H (1991) Cell turgor changes associated with ripening in tomato pericarp tissue. Plant Physiol **97**: 814-816

Sharrock KR, Hallet IC (1991) Physiology of *Botrytis* infection in kiwifruit. Acta hortic **297**: 551-558

Shepherd T, Griffiths DW (2006) The effects of stress on plant cuticular waxes. New Phytol **171**: 469-499

Shepherd T, Griffiths DW (2006) The effects of stress on plant cuticular Waxes. New Phytol **171**: 469-499

Shih MC, Heinrich P, Goodman HM (1991) Cloning and chromosomal mapping of nuclear genes encoding chloroplast and cytosolic

glyceraldehyde-3-phosphate-dehydrogenase from *Arabidopsis thaliana*. Gene **104**: 133-138

Shimakata T, Stumpf PK (1982) Isolation and function of spinach leaf β-ketoacyl-[acyl-carrier-protein] synthases. P Natl Acad Sci USA **79**: 5808-5812

Shinozaki K, Yamaguchi- Shinozaki K (1997) Gene expression and signal transduction in water-stress response. Plant Physiol **115**: 327-334

Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol **6**: 410-417

Shirasu K, Nakajima H, Rajasekhar VK, Dixon RA, Lamb C (1997) Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. Plant Cell **9**: 261-270

Shirley BW (1996) Flavonoid biosynthesis: 'new' functions for an 'old' pathway. Trends Plant Sci **1**: 377-382

Sieber P, Schorderet M, Ryser U, Buchala A, Kolattukudy P, Métraux JP, Nawrath C (2000) Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell **12**: 721-737

Siedow JN (1991) Plant lipoxygenase: structure and function. Annu Rev Plant Phys **42**: 145-188

Siegel BA, Verbeke JA (1989) Diffusible factors essential for epidermal cell redifferentiation in *Catharanthus roseus*. Science **244**: 580-582

Silhavy D, Hutvágner G, Barta E, Bánfalvi Z (1995) Isolation and characterization of a water-stress-inducible cDNA clone from *Solanum chacoense*. Plant Mol Biol **27**: 587-595

Simkin AJ, Schwartz SH, Auldridge M, Taylor MG, Klee HJ (2004) The tomato *carotenoid cleavage dioxygenase 1* genes contribute to the formation of the flavor volatiles β-ionone, pseudoionone, and geranylacetone. Plant J **40**: 882-892

Singh B, Dubey MM (2001) Estimation of triterpenoids from *Heliotropium marifolium* Koen. ex Retz. *in vivo* and *in vitro*: antimicrobial screening. Phytother Res **15**: 231-234

Singh KB, Foley RC, Oñate-Sánchez L (2002) Transcription factors in plant defense and stress responses. Curr Opin Plant Biol **5**: 430-436

Skirycz A, Jozefczuk S, Stobiecki M, Muth D, Zanor MI, Witt I, Mueller-Roeber B (2007) Transcription factor AtDOF4;2 affects phenylpropanoid metabolism in *Arabidopsis thaliana*. New Phytol **175**: 425-438

Skriver K, Mundy J (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell **2**: 503-512

Slabas AR, Fawcett T (1992). The biochemistry and molecular biology of plant lipid biosynthesis. Plant Mol Biol **19**: 169-191

Smania EFA, Delle Monache F, Smania A, Yunes RA, Cuneo RS (2003) Antifungal activity of sterols and triterpenes isolated from *Ganoderma annulare*. Fitoterapia **74**: 375-377

Sohal AK, Pallas JA, Jenkins GI (1999) The promoter of a *Brassica napus* lipid transfer protein gene is active in a range of tissues and stimulated by light and viral infection in transgenic *Arabidopsis*. Plant Mol Biol **41**: 75-87

Soliday CL, Dean BB, Kolattukudy PE (1978) Suberization: inhibition by washing and stimulation by abscisic acid in potato disks and tissue culture. Plant Physiol **61**: 170-174

Soliday CL, Kolattukudy PE, Davis RW (1979) Chemical and ultrastructural evidence that waxes associated with the suberin polymer constitute the major diffusion barrier to water vapor in potato tuber (*Solanum tuberosum* L.). Planta **146**: 607-614

Somerville C, Browse J, Jaworski JG, Ohlrogge JB (2000) Lipids. *In* BB Buchanan, W Gruissem, RL Jones, *eds*, Biochemistry And Molecular Biology Of Plants. American Society of Plant Physiologists, Rockville, MD, pp 456-527

Song J, Wang Z (2008) Molecular cloning, expression and characterization of a phenylalanine ammonia-lyase gene (*SmPAL1*) from *Salvia miltiorrhiza*. Mol Biol Rep

Sossountzov L, Ruiz-Avila L, Vignols F, Jolliot A, Arondel V, Tchang F, Grosbois M, Guerbette F, Miginiac E, Delseny M, Puigdomenèch P, Kader JC (1991) Spatial and temporal expression of a maize lipid transfer protein gene. Plant Cell **3**: 923-933

Spotts RA, Cervantes LA (1986) Populations, pathogenicity, and benomyl resistance of *Botrytis* spp., *Penicillium* spp., and *Mucor piriformis* in packinghouses. Plant Dis **70**: 106-108

Spotts RA, Sanderson PG, Lennox CL, Sugar D, Cervantes LA (1998) Wounding, wound healing and staining of mature pear fruit. Postharvest Biol Tec **13**: 27-36

Stafford HA (1990) Flavonoid metabolism. CRC Press, Boca Raton, FL, pp 123-146

Steinbrenner J, Linden H (2001) Regulation of two carotenoid biosynthesis genes coding for phytoene synthase and carotenoid hydroxylase during stress-induced astaxanthin formation in the green alga *Haematococcus pluvialis*. Plant Physiol **125**: 810-817

Stenzel I, Hause B, Miersch O, Kurz T, Maucher H, Weichert H, Ziegler J, Feussner I, Wasternack C (2003) Jasmonate biosynthesis and the allene oxide cyclase family of *Arabidopsis thaliana*. Plant Mol Biol **51**: 895-911

Sterk P, Booij H, Schellekens GA, van Kammen A, de Vries SC (1991) Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell **3**: 907-921

Stewart AJ, Bozonnet S, Mullen W, Jenkins GI, Lean MEJ, Crozier A (2000) Occurrence of flavonols in tomatoes and tomato-based products. J Agr Food Chem **48**: 2663-2669

St-Pierre B, Laflamme P, Alarco AM, Luca VD (1998) The terminal O-acetyltransferase involved in vindoline biosynthesis defines a new class of proteins responsible for coenzyme A-dependent acyl transfer. Plant J **14**: 703-713

Street NR, Skogström O, Sjödin A, Tucker J, Rodríguez-Acosta M, Nilsson P, Jansson S, Taylor G (2006) The genetics and genomics of the drought response in *Populus*. Plant J **48**: 321-341

Stumpf PK (1980). The biosynthesis of saturated and unsaturated fatty acids. *In* PK Stumpf, EE Conn, *eds*, The Biochemistry Of Plants. Academic Press, New York, NY, pp 177-204

Stumpf PK (1984) Plant lipid biosynthesis in 1959 and 1984. J Lipid Res **25**: 1508-1510

Stumpf PK, Pollard MR (1983) Pathways of fatty acid biosynthesis in higher plants with particular reference to developing rapeseed. *In* JK Kramer, F Sauer, WJ Pigden, *eds*, High And Low Erucic Acid Rapeseed Oils. Academic Press, New York, NY, pp 131-141

Sturaro M, Hartings H, Schmelzer E, Velasco R, Salamini F, Motto M (2005) Cloning and characterization of *GLOSSY1*, a maize gene involved in cuticle membrane and wax production. Plant Physiol **138**: 478-489

Sugar D, Spotts RA (1993) The importance of wounds in infection of pear fruit by *Phialophora malorum* and the role of hydrostatic pressure in spore penetration of wounds. Phytopathology **83**: 1083-1086

Suh MC, Samuels AL, Jetter R, Kunst L, Pollard M, Ohlrogge J, Beisson F (2005) Cuticular lipid composition, surface structure, and gene expression in *Arabidopsis* stem epidermis. Plant Physiol **139**: 1649-1665

Suzuki H, Achnine L, Xu R, Matsuda SPT, Dixon RA (2002) A genomics approach to the early stages of triterpene saponin biosynthesis in *Medicago truncatula*. Plant J **32**: 1033-1048

Suzuki T, Higgins PJ, Crawford DR (2000) Control selection for RNA quantitation. Biotechniques **29**: 332-337

Swain T (1977) Secondary compounds as protective agents. Annu Rev Plant Phys **28:** 479-501

Swanson R, Edlund AF, Preuss D (2004) Species specificity in pollen-pistil interactions. Annu Rev Genet **38**: 793-818

Szafranek BM, Synak EE (2006) Cuticular waxes from potato (*Solanum tuberosum*) leaves. Phytochemistry **67**: 80-90

Szymkowiak EJ, Irish EE (1999) Interactions between *jointless* and wild-type tomato tissues during development of the pedicel abscission zone and the inflorescence meristem. Plant Cell **11**: 159-175

Tabuchi T, Ito S, Arai N (2000) Structural changes in the abscission zones of pedicels at different ripening stages of tomato. J Jpn Soc Hortic Sci **69**: 705-707

Taji T, Seki M, Yamaguchi-Shinozaki K, Kamada H, Giraudat J, Shinozaki K (1999) Mapping of 25 drought-inducible genes, *RD* and *ERD*, in *Arabidopsis thaliana*. Plant Cell Physiol **40**: 119-123

Takeo T, Tsushida T (1980) Changes in lipoxygenase activity in relation to lipid degeneration in plucked tea shoots. Phytochemistry **19**: 2521-2522

Tanaka H, Machida Y (2006) The cuticle and cellular interactions. *In* M Riederer, C Müller, *eds*, Biology Of The Plant Cuticle. Blackwell Scientific Publishers, Oxford, pp 312-334

Tanaka Y, Tsuda S, Kusumi T (1998) Metabolic engineering to modify flower color. Plant Cell Physiol **39**: 1119-1126

Taylor IB, Burbidge A, Thompson AJ (2000) Control of abscisic acid synthesis. J Exp Bot **51**: 1563-1574

Taylor IB, Tarr AR (1984) Phenotypic interactions between abscisic acid deficient tomato mutants. Theor Appl Genet **68**: 115-119

Terzaghi WB, Cashmore AR (1995) Light-regulated transcription. Annu Rev Plant Phys **46**: 445-474

Thelander M, Narita JO, Gruissem W (1986) Plastid differentiation and pigment biosynthesis during tomato fruit ripening. Curr Top Plant Biochem Physiol **5**: 128-141

Thellin O, Zorzi W, Lakaye B, de Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E (1999) Housekeeping genes as internal standards: use and limits. J Biotechnol **75**: 291-295

Theologis A (1993) One rotten apple spoils the whole bushel: the role of ethylene in fruit ripening. Cell **70**: 181-184

Thoma S, Hecht U, Kippers A, BotellaJ, De Vries S, Somerville C (1994) Tissue-specific expression of a gene encoding a cell wall-localized lipid transfer protein from *Arabidopsis*. Plant Physiol **105**: 35-45

Thoma S, Kaneko Y, Somerville C (1993) A non-specific lipid transfer protein from *Arabidopsis* is a cell wall protein. Plant J **3**: 427-436

Thomas DA, Barber HN (1974) Studies on leaf characteristics of a cline of *Eucalyptus urnigera* from Mount Wellington, Tasmania: water repellency and the freezing of leaves. Aust J Bot **22**: 501-512

Thomas R, Fang X, Ranathunge K, Anderson TA, Peterson CA, Bernards MA (2007) Soybean root suberin: anatomical distribution, chemical composition, and relationship to partial resistance to *Phytophthora sojae*. Plant Physiol 144: 299-311

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Phys **50**: 571-599

Thompson AJ, Jackson AC, Symonds RC, Mulholland BJ, Dadswell AR, Blake PS, Burbidhge A, Taylor IB (2000) Ectopic expression of a tomato 9-*cis*-epoxycarotenoid dioxygenase gene causes over-production of abscisic acid. Plant J **23**: 363-374

Thompson DS, Davies WJ, Ho LC (1998) Regulation of tomato fruit growth by epidermal cell wall enzymes. Plant Cell Environ **21**: 589-599

Tijet N, Helvig C, Pinot F, Le Bouquin R, Lesot A, Durst F, Salaün JP, Benveniste I (1998) Functional expression in yeast and characterization of a clofibrate-inducible plant cytochrome P450 (CYP94A1) involved in cutin monomers synthesis. Biochem J **332**: 583-589

Tischler CR, Burson BL (1995) Evaluating different bahiagrass cytotypes for heat tolerance and leaf epicuticular wax content. Euphytica **84**: 229-235

Todd J, Post-Beittenmiller D, Jaworski JG (1999) *KCS1* encodes a fatty acid elongase 3-ketoacyl-CoA synthase affecting wax biosynthesis in *Arabidopsis thaliana*. Plant J **17**: 119-130

Todd JF, Paliyath G, Thompson JE (1990) Characteristics of a membrane-associated lipoxygenase in tomato fruit. Plant Physiol **94**: 1225-1232

Torres R, Valentines MC, Usall J, Viñas I, Larrigaudiere C (2003) Possible involvement of hydrogen peroxide in the development of resistance mechanisms in 'Golden Delicious' apple fruit. Postharvest Biol Tec **27**: 235-242

Torres-Schumann S, Godoy JA, Pintor-Toro JA (1992) A probable lipid transfer protein gene is induced by NaCl in stems of tomato plants. Plant Mol Biol **18**: 749-757

Trenkamp S, Martin W, Tietjen K (2004) Specific and differential inhibition of very-long-chain fatty acid elongases from *Arabidopsis thaliana* by different herbicides. P Natl Acad Sci USA **101**: 11903-11908

Treviño MB, O´Connell MA (1998) Three drought-responsive members of the nonspecific lipid-transfer protein gene family in *Lycopersicon pennellii* show different developmental patterns of expression. Plant Physiol **116**: 1461-1468

Trewavas AJ, Jones HG (1991) An assessment of the role of ABA in plant development. *In* WJ Davies, HG Jones, *eds*, Abscisic Acid: Physiology And Biochemistry. Bios Scientific Publishers, Oxford, pp 169-188

Trinder P (1969) Determination of blood glucose using 4-amino phenazone as oxygen acceptor. J Clin Pathol **22**: 158-161

Tsuba M, Katagiri C, Takeuchi Y, Takada Y, Yamaoka N (2002) Chemical factors of the leaf surface involved in the morphogenesis of *Blumeria graminis.* Physiol Mol Plant P **60**: 51-57

Tsuboi S, Osafune T, Tsugek IR, Nishimura M, Yamada M (1992) Nonspecific lipid transfer protein in castor bean cotyledon cells: subcellular localization and a possible role in lipid metabolism. J Biochem **111**: 500-508

Tuomainen J, Pellinen R, Roy S, Kiiskinen M, Eloranta T, Karjalainen R, Kangasjärvi J (1996) Ozone affects birch (*Betula pendula* Roth) phenylpropanoid, polyamine and active oxygen detoxifying pathways at biochemical and gene expression level. J Plant Physiol **148**: 179-188

Tyree MT, Jarvis PG (1982) Water in tissues and cells. *In* PS Nobel, CB Osmond, H Ziegler, *eds*, Encyclopedia Of Plant Physiology. Springer-Verlag, Berlin, pp 37-77

Uddin MN, Marshall DR (1988) Variation in epicuticular wax content in wheat. Euphytica **38**: 3-9

van der Hoeven R, Ronning C, Giovannoni J, Martin G, Tanksley S (2002) Deductions about the number, organization, and evolution of genes in the tomato genome based on analysis of a large expressed sequence tag collection and selective genomic sequencing. Plant Cell **14**: 1441-1456

Vanderauwera S, de Block M, van de Steene N, van de Cotte B, Metzlaff M, van Breusegem F (2007) Silencing of poly(ADP-ribose) polymerase in plants alters abiotic stress signal transduction. P Natl Acad Sci USA **104**: 15150-15155

Vega A, Luza J, Espina S, Lizana LA (1991) Characterization of the epidermis of three blueberry (*Vaccinium corymbosum* L.) cultivars and water loss at different storage temperatures. Proc Interamer Soc Tropic Hort **35**: 263-274

Verhoeyen ME, Bovy A, Collins G, Muir S, Robinson S, de Vos CHR, Colliver S (2002) Increasing antioxidant levels in tomatoes through modification of the flavonoid biosynthetic pathway. J Exp Bot **53**: 2099-2106

Vioque J, Kolattukudy PE (1997) Resolution and purification of an aldehyde-generating and an alcohol-generating fatty acyl-CoA reductase from pea leaves (*Pisum sativum* L.). Arch Biochem Biophys **340**: 64-72

Vogg G, Fischer S, Leide J, Emmanuel E, Jetter R, Levy AA, Riederer M (2004) Tomato fruit cuticular waxes and their effects on transpiration barrier properties: functional characterization of a mutant deficient in a very-long-chain fatty acid β-ketoacyl-CoA synthase. J Exp Bot **55**: 1401-1410

von Wettstein-Knowles P (1974) Ultrastructure and origin of epicuticular wax tubes. J Ultra Mol Struct R **46**: 483-498

von Wettstein-Knowles P (1982) Biosynthesis of epicuticular lipids as analyzed with the aid of gene mutations in barley. *In* JFGM Wintermans, PJC Kuiper, *eds*, Biochemistry And Metabolism Of Plant Lipids. Elsevier Biomedical Press, Amsterdam, pp 69-78

von Wettstein-Knowles P (1993) Waxes, cutin and suberin. *In* TS Moore, *ed*, Lipid Metabolism In Plants. CRC Press, Boca Raton, FL, pp 127-166

von Wettstein-Knowles P (1995) Biosynthesis and genetics of waxes. *In* RJ Hamilton, *ed*, Waxes: Chemistry, Molecular Biology And Functions. The Oily Press, Dundee, pp 91-130

von Wettstein-Knowles P, Avato P, Mikkelsen JD (1979) Light promotes synthesis of the very long chain fatty acyl chains in maize wax. *In* D Mazliak, P Bennveniste, C Costes, R Douce, *eds*, Biogenesis And Function Of Plant Lipids. Elsevier Biomedical Press, Amsterdam, pp 271-274

Wallace G, Fry SC (1999) Action of diverse peroxidases and laccases on six cell wall-related phenolic compounds. Phytochemistry **52**: 769-773

Walton TJ (1990) Waxes, cutin and suberin. *In* JL Harwood, J Boyer, *eds*, Lipids, Membranes And Aspects Of Photobiology. Academic Press, London, pp 105-158

Wang CS, Liau YE, Huang JC, Wu TD, Su CC, Lin CH (1998) Characterization of a desiccation-related protein in lily pollen during development and stress. Plant Cell Physiol **39**: 1307-1314

Wang H, Cutler AJ (1995) Promoters from *kin1* and *cor6.6*, two *Arabidopsis thaliana* low-temperature- and ABA-inducible genes, direct strong beta-glucuronidase expression in guard cells, pollen, and young developing seeds. Plant Mol Biol **28**: 619-634

Wang KLC, Li H, Ecker JR (2002) Ethylene biosynthesis and signaling networks. Plant Cell 131-151

Wang ZY, Hopkins A, Mian R (2001) Forage and turf grass biotechnology. Crit Rev Plant Sci **20**: 573- 619

Wasson AP, Pellerone FI, Mathesius U (2006) Silencing the flavonoids pathway in *Medicago truncatula* inhibits root nodule formation and prevents auxin transport regulation by Rhizobia. Plant Cell **18**: 1617-1629

Wasternack C, Miersch O, Kramell R, Hause B, Ward J, Beale M, Boland W, Parthier B, Feussner I (1998) Jasmonic acid: biosynthesis, signal transduction, gene expression. Fett-Lipid **100**: 139-146

Wasternack C, Panthier B (1997) Jasmonate-signalled plant gene expression. Trends Plant Sci **2**: 302-307

Weiler EW (1986) Plant hormone immunoassay based on monoclonal and polyclonal antibodies. *In* HF Linskens, JF Jackson, *eds*, Modern Methods Of Plant Analysis. Springer-Verlag, Berlin, pp 1-17

Wellesen K, Durst F, Pinot F, Benveniste I, Nettesheim K, Wisman E, Steiner-Lange S, Saedler H, Yephremov A (2001) Functional analysis of the *LACERATA* gene of *Arabidopsis* provides evidence for different roles of fatty acid ω-hydroxylation in development. P Natl Acad Sci USA **98**: 9694-9699

Welling A, Moritz T, Palva ET, Junttila O (2002) Independent activation of cold acclimation by low temperature and short photoperiod in hybrid aspen. Plant Physiol **129**: 1633-1641

Welsch R, Wüst F, Bär C, Al-Babili S, Beyer P (2008) A third phytoene synthase is devoted to abiotic stress-induced abscisic acid formation in rice and defines functional diversification of phytoene synthase genes. Plant Physiol **147**: 367-380

Wijmans JG, Baker RW (1995) The solution-diffusion model: a review. J Membrane Sci **107**: 1-21

Wilson CA, Peterson CA (1983) Chemical composition of the epidermal, hypodermal, endodermal and intervening cortical cell walls of various plant roots. Ann Bot **51**: 759-769

Winkel-Shirley B (2001) Flavonoid biosynthesis: a colourful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol **126**: 485-493

Wirthensohn MG, Sedgley M (1996) Epicuticular wax structure and regeneration on developing juvenile *Eucalyptus* leaves. Aust J Bot **44**: 691-704

Wirtz KWA, Gadella TWJ (1990) Properties and modes of action of specific and non-specific phospholipid transfer proteins. Experientia **46**: 592-599

Wisniewski M, Webb R, Balsamo R, Close TJ, Yu XM, Griffith M (1999) Purification, immunolocalization, cryoprotective, and antifreeze activity of PCA60: a dehydrin from peach (*Prunus persica*). Physiol Plantarum **105**: 600-608

Wolters-Arts M, van der Weerd L, van Aelst AC, van der Weerd J, van As H, Mariani C (2002) Water-conducting properties of lipids during pollen hydration. Plant Cell Environ **25:** 513-519

Wu XY, Moreau RA, Stumpf PK (1981) Studies of biosynthesis of waxes by developing jojoba seed: biosynthesis of wax esters from acyl-CoA and long-chain alcohols. Lipids **6**: 897-902

Xia Y, Nikolau BJ, Schnable PS (1996) Cloning and characterization of *CER2*, an *Arabidopsis* gene that affects cuticular wax accumulation. Plant Cell **8**: 1291-1304

Xia Y, Nikolau BJ, Schnable PS (1997) Developmental and hormonal regulation of the *Arabidopsis CER2* gene that codes for a nuclear-localized protein required for the normal accumulation of cuticular waxes. Plant Physiol **115**: 925-937

Xiong L, Schumaker KS, Zhu JK (2002) Cell signaling during cold, drought, and salt stress. Plant Cell **14**: 165-184

Xu R, Fazio GC, Matsuda SPT (2004) On the origins of triterpenoid skeletal diversity. Phytochemistry **65**: 261-291

Xu XJ, Dietrich CR, Delledonne M, Xia YJ, Wen TJ, Robertson DS, Nikolau BJ, Schnable PS (1997) Sequence analysis of the cloned *glossy8* gene of maize suggests that it may code for a β-ketoacyl reductase required for the biosynthesis of cuticular waxes. Plant Physiol **115**: 501-510

Yalpani N, Enyedi AJ, León J, Raskin I (1994) Ultraviolet light and ozone stimulate accumulation of salicylic acid, pathogenesis-related proteins and virus resistance in tobacco. Planta **193**: 372-376

Yamada A, Saitoh T, Mimura T, Ozeki Y (2002) Expression of mangrove allene oxide cyclase enhances salt tolerance in *Escherichia coli*, yeast, and tobacco cells. Plant Cell Physiol **43**: 903-910

Yamada M (1992) Lipid transfer proteins in plants and microorganisms. Plant Cell Physiol **33**: 1-6

Yamaguchi-Shinozaki K, Koizumi M, Urao S, Shinozaki K (1992) Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in *Arabidopsis thaliana*: sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. Plant Cell Physiol **33**: 217-224

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol **57**: 781-803

Yamamoto N, Tsugane T, Watanabe M, Yano K, Maeda F, Kuwata C, Torki M, Ban Y, Nishimura S, Shibata D (2005) Expressed sequence tags from the laboratory-grown miniature tomato (*Lycopersicon esculentum*) cultivar MicroTom and mining for single nucleotide polymorphisms and insertions/deletions in tomato cultivars. Gene **356**: 127-134

Yancey PH (2005) Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J Exp Biol **208**: 2819-2830

Yang SF, Hoffman NE (1984) Ethylene biosynthesis and its regulation in higher plants. Annu Rev Plant Phys **35**: 155-189

Yeats TH, Rose JKC (2008) The biochemistry and biology of extracellular plant lipid-transfer proteins (LTPs). Protein Sci **17**: 191-198

Yelle S, Chetelat RT, Dorais M, DeVerna JW, Bennett AB (1991) Sink metabolism in tomato fruit: genetic and biochemical analysis of sucrose accumulation. Plant Physiol **95**: 1026-1035

Yephremov A, Wisman E, Huijser P, Huijser C, Wellesen K, Saedler H (1999) Characterization of the *FIDDLEHEAD* gene of *Arabidopsis* reveals a link between adhesion response and cell differentiation in the epidermis. Plant Cell **11**: 2187-2201

Yubero-Serrano EM, Moyano E, Medina-Escobar N, Muñoz-Blanco J, Caballero JL (2003) Identification of a strawberry gene encoding a non-specific lipid transfer protein that responds to ABA, wounding and cold stress. J Exp Bot **54**: 1865-1877

Zabka V, Stangl M, Bringmann G, Vogg G, Riederer M, Hildebrandt U (2008) Host surface properties affect prepenetration processes in the barley powdery mildew fungus. New Phytol **177**: 251-263

Zarembinski T, Theologis A (1994) Ethylene biosynthesis and action: a case of conservation. Plant Mol Biol **26**: 1579-1597

Zeevaart JAD, Creelman RA (1988) Metabolism and physiology of abscisic acid. Annu Rev Plant Phys **39**: 439-73

Zeevaart JAD, Heath TG, Gage DA (1989) Evidence for a universal pathway of abscisic acid biosynthesis in higher plants from ^{18}O incorporation patterns. Plant Physiol **91**: 1594-1601

Zegzouti H, Jones B, Frasse P, Marty C, Maître B, Latché A, Pech JC, Bouzayen M (1999) Ethylene-regulated gene expression in tomato fruit: characterization of novel ethylene-response and ripening-related genes isolated by differential-display. Plant J **18**: 589-600

Zeier J, Ruel K, Ryser U, Schreiber L (1999) Chemical analysis and immunolocalisation of lignin and suberin in endodermal and hypodermal/rhizodermal cell walls of developing maize (*Zea mays* L.) primary roots. Planta **209**: 1-12

Zeier J, Schreiber L (1998) Comparative investigation of primary and tertiary endodermal cell walls isolated from the roots of five monocotyledoneous species: chemical composition in relation to fine structure. Planta **206**: 349-361

Zhang JY, Broeckling CD, Blancaflor EB, Sledge MK, Sumner LW, Wang ZY (2005) Overexpression of *WXP1*, a putative *Medicago truncatula* AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*). Plant J **42**: 689-707

Zhu HL, Zhu BZ, Fu DQ, Xie YH, Hao YL, Luo Y B (2005) Role of ethylene in the biosynthetic pathways of aroma volatiles in ripening fruit. Russ J Plant Physl **52**: 691-695

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol **53**: 247-273

Zimmermann HM, Hartmann K, Schreiber L, Steudle E (2000) Chemical composition of apoplastic transport barriers in relation to radial hydraulic conductivity of corn roots (*Zea mays* L.). Planta **210**: 302-311

Zinkl GM, Preuss D (2000) Dissecting *Arabidopsis* pollen-stigma interactions reveals novel mechanisms that confer mating specificity. Ann Bot **85**: 15-21

Zinkl GM, Zwiebel BI, Grier DG, Preuss D (1999) Pollen-stigma adhesion in *Arabidopsis*: a species-specific interaction mediated by lipophilic molecules in the pollen exine. Development **126**: 5431-5440

Zinsou V, Wydra K, Ahohuendo B, Hau B (2005) Genotype x environment interactions in symptom development and yield of cassava genotypes with artificial and natural cassava bacterial blight infections. Eur J Plant Pathol **111**: 217-233

Zinsou V, Wydra K, Ahohuendo B, Schreiber L (2006) Leaf waxes of cassava (*Manihot esculenta* Crantz) in relation to ecozone and resistance to *Xanthomonas* blight. Euphytica **149**: 189-198

ABBREVIATIONS

Δc	vapor phase-based driving force
ABA	abscisic acid
ABC	ATP-binding cassette
ABRE	abscisic acid responsive element
Ac	*Activation*
ACC	1-aminocyclopropane-1-carboxylate
ACO	ACC oxidase
ACP	acyl carrier protein
ACS	ACC synthase
ANOVA	analysis of variance
AOC	allene oxide cyclase
AS	β-amyrin synthase
ASR	abscisic stress ripening protein
BF_3	boron trifluoride
BLAST	basic local alignment search tool
Borax	disodium tetraborate decahydrate
bp	base pair
cDNA	complementary DNA
cer	*eceriferum*
CHS	naringenin-chalcone synthase
CM	cuticular membrane
CoA	coenzyme A
CPC	cyclobutane pyrimidine dimer
CTAB	cetyltrimethyl ammonium bromide
CTR	constitutive triple response
dATP	deoxyadenosine triphosphate
DCM	dewaxed cutin matrix
dCTP	deoxycytidine triphosphate
ded	*deadhead*
DFCI	Dana-Faber Cancer Institute
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
Ds	*Dissociation*
DS	drought stress protein
dTTP	deoxythymidine triphosphate
ECR	2,3-enoyl-CoA reductase
ECW	epicuticular waxes
EDTA	ethylenediaminetetraacetic acid
EEO	electroendosmosis
EF	elongation factor
EFE	ethylene forming enzyme
ELISA	enzyme-linked immunosorbent assay
ERD	early response to drought protein
ERF	ethylene response factor
EST	expressed sequence tag
ETR	ethylene receptor
F	water flow rate
F_0	initial chlorophyll fluorescence
FA	fatty acid
FAE	fatty acid elongation/elongase
FAS	fatty acid synthesis/synthase
fdh	*fiddlehead*
F_M	maximum chlorophyll fluorescence
F_V	variable chlorophyll fluorescence
fw	fresh weight
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAS	growth arrest-specific protein
GGPP	geranylgeranyl diphosphate
gl	glossy
GUS	β-glucuronidase
H_2O_2	hydrogen peroxide
HCD	β-hydroxyacyl-CoA dehydrogenase
HSD	Tukey's honestly significant difference mean-separation test
HSE	heat shock element
HSP	heat shock protein
i.d.	internal diameter
ICW	intracuticular waxes
J	flux
KAS	β-ketoacyl-acyl carrier protein synthase
KCR	β-ketoacyl-CoA reductase
KCS	β-ketoacyl-CoA synthase
lcr	*lacerata*
LeGI	*Lycopersicon esculentum* gene index
LOX	lipoxygenase
LTP	lipid transfer protein
LTR	low temperature responsive element
m*/*z	mass-to-charge ratio
MAPKKK	mitogen-activated protein kinase kinase kinase
MBS	MYB binding site
n.s.	not significant
OPDA	12-oxo-phytodienoic acid
P	permeance for water
PAL	phenylalanine ammonia-lyase
PAM	pulse amplitude modulation fluorometer
PCR	polymerase chain reaction
PKG	phosphoglycerate kinase
PPDP	pre-phytoene diphosphate
PSII	photosystem II
PSORT	prediction of protein localization sites
PSY	phytoene synthase
PVP	polyvinyl pyrrolidone
r	resistance
R^2	coefficient of correlation
r^2	coefficient of determination
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
RT	reverse transcription
SAM	*S*-adenosyl-L-methionine
SD	standard deviation
SDS	sodium dodecyl sulfate
SE	standard error
SSC	saline-sodium citrate buffer
TAS	tomato ABA and salt induced protein
TBME	tert-butylmethylether
TBS	Tris-buffered saline
TC	tentative consensus sequence
TCA/SARE	salicylic acid responsive element
TDR	tapetum degeneration retardation protein
Tris	tris(hydroxymethyl)aminomethane
UV	ultra violet
v/v	volume per volume
VLCFA	very-long-chain fatty acid
w/v	weight per volume
WIN	wax inducer
WS	wax synthase

PUBLICATIONS

Gerd Vogg, Stephanie Fischer, Jana Leide, Eyal Emmanuel, Reinhard Jetter, Avarham A. Levy, Markus Riederer (2004) Tomato fruit cuticular waxes and their effects on transpiration barrier properties: functional characterization of a mutant deficient in a very-long-chain fatty acid β-ketoacyl-CoA synthase. Journal of Experimental Botany **55**: 1401-1410

Jana Leide, Ulrich Hildebrandt, Kerstin Reussing, Markus Riederer, Gerd Vogg (2007) The developmental pattern of tomato fruit wax accumulation and its impact on cuticular transpiration barrier properties: effects of a deficiency in a β-ketoacyl-coenzyme A synthase (LeCER6). Plant Physiology **144**: 1667-1679

Jana Leide, Ulrich Hildebrandt, Wolfram Hartung, Markus Riederer, Gerd Vogg (2008) Post-harvest formation of a suberized stem scar tissue during storage of tomato fruits. in preparation

PRESENTATIONS AT CONFERENCES

Jana Leide, Josef Gall, Markus Riederer, Gerd Vogg (2004) The function of a wound-induced suberized tissue in tomato fruits during post-harvest storage. Deutsche Botanikertagung in Braunschweig

Jana Leide, Kerstin Reußing, Markus Riederer, Gerd Vogg (2005) Changes in the cuticular waxes during tomato fruit development and their importance as a transpiration barrier. International Botanical Congress in Vienna

CURRICULUM VITAE

Jana Leide
Pilziggrundstrasse 73
97076 Würzburg

geboren am 09. Juni 1976 in Grevesmühlen
Staatsangehörigkeit deutsch
Familienstand ledig

Schul- und Hochschulausbildung

seit 2003 Julius Maximilians Universität Würzburg
Wissenschaftliche Mitarbeiterin am Lehrstuhl für Ökophysiologie und Vegetationsökologie im Rahmen des DFG geförderten Projektes „Biosynthese und Funktion kutikulärer Wachse der Tomate“ und des SFB 567 „Mechanismen der interspezifischen Interaktion von Organismen“

2002-2003 Ernst Moritz Arndt Universität Greifswald
Diplomarbeit am Lehrstuhl für Botanik und Landschaftsökologie zum Thema „Untersuchungen zur Dynamik von Cytokininoxidase, Cytokininoxidase-mRNA und Cytokininen bei der Seneszenz von Poaceenblättern“

2001 Martin Luther Universität Halle/Wittenberg
Molekularbiologisches Methodenpraktikum am Institut für Pflanzenphysiologie

1996-2002 Ernst Moritz Arndt Universität Greifswald
Studium der Biologie mit den Haupt- und Nebenfächern Botanik, Angewandte Mikrobiologie/Biotechnologie und Genetik

1995-1996 häusliche Pflegeperson Schwerin

1991-1995 Juri Gagarin Gymnasium Schwerin
Abitur mit den Leistungsfächern Biologie und Französisch, weitere Prüfungsfächer Geschichte, Deutsch und Mathematik

1983-1991 Bertolt Brecht Oberschule Schwerin

Würzburg, im November 2008

DANKSAGUNG

Mein besonderer Dank gilt Herrn Prof. Markus Riederer für die engagierte Betreuung und interessante Aufgabenstellung, die weit genug gefasst war, mir die Freiheit für die Ausgestaltung dieser Arbeit zu geben. Meinen Betreuern Dr. Gerd Vogg und Dr. Ulrich Hildebrandt möchte ich für die beständige und vielfältige Unterstützung in allen Belangen meiner Arbeit danken. Für die Finanzierung dieses Projektes danke ich der DFG (VO 934-1-3) und dem SFB 567 „Mechanismen der interspezifischen Interaktion von Organismen“.

Den Mitarbeitern des Lehrstuhles für Botanik II sei ebenfalls für die stete Diskussionsbereitschaft, das aufgeschlossene Interesse meiner Arbeit gegenüber und die freundliche Unterstützung herzlich gedankt. Allen voran möchte ich mich an dieser Stelle bei Dr. Christian Popp, Dr. Kerstin Reifenrath, Dr. Nora Travers-Martin und Dr. Vanessa Zabka nicht nur für die zahlreichen Ratschläge und ihre zu jeglicher Zeit gewährte, große Hilfsbereitschaft sondern auch für die gemeinsame Zeit bedanken, die uns Freunde werden ließ.

Dank gebührt im Besonderen Lena Reinhardt für die sehr gute Zusammenarbeit, ihren großen Fleiß bei der Durchführung auch kniffliger Experimente und vor allem dafür, dass sie die Augen für die notwendigen Details stets offen hielt. Jutta Winkler-Steinbeck danke ich für die Geduld bei der Anzucht und Pflege der Versuchpflanzen. Meinen Dank auch an Kerstin Reußing, Mirjam Schelter und Josef Gall, die durch ihre praktische Arbeit dieses Projekt vorangetrieben haben.

Nicht versäumen möchte ich, mich bei Monika Noak, Wilma Kreßmann, Michaela Jäger, Julia Blass-Warmuth, Natascha Sieling, Olga Frank und Torsten Volkmar zu bedanken, die nicht nur für eine sehr herzliche Arbeitsatmosphäre im Institut sorgten, sondern mir auch jederzeit Hilfe jeglicher Art zuteil werden ließen.

Ein ganz herzlicher Dank für die gewährte Zusammenarbeit in fachspezifischen Belangen geht an Prof. G. Krohne (Elektronenmikroskopie), Prof. T. Roitsch (Pharmazeutische Biologie), Prof. W. Hartung (Botanik I), Dr. S. Kneitz (Virologie und Immunbiologie), Prof. M. Frosch (Hygiene und Mikrobiologie), Prof. M. Sendtner (Klinische Neurobiologie) sowie Prof. A. Levy und Dr. E. Emmanuel (Weizmann Institute of Science, Rehovot, Israel).

Mein herzlicher Dank gilt meiner Familie, die mich offenen Herzens meinen Weg gehen lässt und so auf ihre Weise durch ihre grenzenlose und uneingeschränkte Unterstützung, Geduld und Liebe zum Gelingen dieser Arbeit beigetragen hat.

ERKLÄRUNG

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die hier angegebenen Quellen und Hilfsmittel verwendet habe.

Ferner erkläre ich, dass die Arbeit in gleicher oder ähnlicher Form noch zu keinem anderen Prüfungsverfahren vorgelegt wurde. Ich erkläre, dass ich bisher keine akademischen Grade erworben oder zu erwerben versucht habe.

Würzburg, im November 2008