The Plasticity of Barley (*Hordeum vulgare*) Leaf Wax Characteristics and their Effects on Early Events in the Powdery Mildew Fungus (*Blumeria graminis* f.sp. *hordei*): Interactive Adaptations at the Physiological and the Molecular Level

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

vorgelegt von
Vanessa Zabka
aus Wuppertal

Würzburg 2007

Eingereicht am: .....

Mitglieder der Promotionskommission:

Vorsitzender: Prof. Dr. Martin Müller

Erstgutachter: Prof. Dr. Markus Riederer

Zweitgutachter: Prof. Dr. Werner Kaiser

Tag des Promotionskolloquiums: .....

Doktorurkunde ausgehändigt am: .....

Meinen Eltern

CONTENTS

INTRODUCTION

The cuticle is a multifunctional structure that covers all plant organs and thereby provides numerous functions of essential importance for aboveground plants (Kerstiens, 1996). Since it provides the first contact zone between plant surfaces and their environment, it incorporates several protective functions, e.g. maintaining the structural integrity of plant tissues, regulating the intensity of harmful radiation or preventing invasion by various microbes (Riederer & Müller, 2006).

The primary physiological function of plant cuticles is to protect the tissue against a relatively dry atmosphere, and thus to prevent desiccation by minimizing non-stomatal water loss (Riederer & Schreiber, 2001; Kerstiens, 2006). The cuticular membrane is composed of a polymer matrix (cutin) and associated solvent–soluble lipids (cuticular waxes), which can be divided into two spatially distinct layers, the epicuticular waxes coating the surface, and the intracuticular waxes embedded in the cutin matrix. Typical compositions of cuticular waxes comprise several long-chain aliphatic compounds, e.g. primary and secondary alcohols, aldehydes, esters, ketones or alkanes, while others may be dominated by cyclic components like, triterpenes (Baker, 1982; Jeffree, 1986; Barthlott, 1990).

For some species, it has been shown that the epi- and intracuticular wax portions display different chemical compositions. Sheer gradients between the smooth epicuticular wax film and intracuticular wax layers were detected for leaves of *Prunus laurocerasus* (Jetter *et al.*, 2000), with aliphatic compounds exclusively occurring in mechanically harvested epicuticular wax layers, while the triterpenoids ursolic and oleanolic acid represent almost two-thirds of the intracuticular wax. Riedel *et al.* (2003) observed gradients of aldehydes and countergradients of alcohols and fatty acids between intra- and epicuticular waxes of *Nepenthes alata* pitchers.

The epicuticular waxes may be present as a thin film upon the cutin matrix, or they may appear as shaped like microscopic aggregates (epicuticular wax crystals) protruding from this film (Barthlott *et al.*, 1998; Jetter & Schäffer, 2001). The shape and density of single wax crystals determines the structure of

epicuticular waxes. The morphology of such crystals depends on the plant's species-specific chemistry (Baker, 1982) and can be determined by a predominating wax compound (Jetter & Riederer, 1994; 1995). However, the amount and chemical composition of surface waxes is not only species- and organ-specific, but may also vary due to plant growth and development. Rhee *et al.* (1998) reported that the amount and orientation of the leaf wax crystals differ between leaf regions of variable age in expanding leek leaves (*Allium porrum* L.). For Japanese cedar *(Cryptomeria japonica*), Sase *et al.* (1998) reported a positive correlation between wax amounts and leaf age during the growing phase. The wax on upper surfaces of *Prunus laurocerasus* leaves was found to change very drastically in the total amount, and the relative composition of cuticular waxes during organ development varied (Jetter & Schäffer, 2001). The authors suggest that different compound classes dominating the wax composition in distinct developmental stages are correlated with specific functions. Moreover, differences in wax amount and composition may also occur within different tissues of one plant organ. Gniwotta *et al.* (2005) showed differences in total wax amounts and in the chemical composition of adaxial and abaxial leaf surfaces of *Pisum sativum*.

The variable regulation of the cuticular wax production in tissues and organs, in dependence of the developmental stage, demands sophisticated control mechanisms for the expression of an intricate biosynthetic pathway (Post-Beittenmiller, 1996). The complex mixtures of long-chain hydrocarbons, aldehydes, alcohols, acids and esters are almost entirely derived from fatty acids. *De novo* biosynthesis of C_{16}/ C_{18} fatty acids in plants is operated by three different types of fatty acid synthase complexes (FAS), localized in the plastid stroma (Kunst *et al.*, 2005). Via a cycle of four reactions, an acyl chain, attached to an acyl carrier protein (ACP), is extended by two carbons per cycle: a condensation of C_2 moieties originating from malonyl-ACP to acyl-ACP, followed by the reduction of β-ketoacyl-ACP, the dehydration of β-hydroxyacyl-ACP, and the reduction of trans-Δ^2-enoyl-ACP (Ohlrogge *et al.*, 1993; Ohlrogge & Browse, 1995). The three different types of FAS complexes differ in their condensing enzymes, which have strict acyl chain length specificities: KASIII initiates fatty acid biosynthesis with acetyl-CoA as a substrate (C_2-C_4), KASI extends the chain to C_{16}, and KASII completes the chain elongation to C_{18}. The involved reductases

and the dehydratase have no apparent acyl chain length specificity and are shared by all three types of plastidial FAS complexes.

The further extension of the C_{16} and C_{18} fatty acids to very long chain fatty acid (VLCFA) chains used for the production of cuticular wax components is catalyzed by fatty acid elongases (FAE, von Wettstein-Knowles, 1982). Since these multi-enzyme complexes are bound to the endoplasmic reticulum (ER; Xu *et al.*, 2002; Kunst & Samuels, 2003; Zheng *et al.*, 2005), the saturated C_{16} and C_{18} acyl groups first have to get hydrolyzed from the ACP by an acyl-ACP thioesterase, then exported from the plastid by an unknown mechanism, and esterified to CoA to reach the fatty acid elongation sites at the ER. Two classes of acyl-ACP thioesterases have been described in plants, i. e. FATA and FATB, which show different preferences for saturated and non-saturated fatty acids. Similar to the processes in the FAS, elongation of long-chain fatty acids (C_{16}, C_{18}) to VLCFA in the FAE involves four consecutive enzymatic reactions, but in this case the 2-carbon donor for FAE is malonyl-CoA, generated by the multifunctional extraplastidial acetyl-CoA carboxylase (ACCase; Kunst *et al.*, 2005). The activities of β-ketoacyl-CoA synthases and β-ketoacyl-CoA reductases are involved in this elongation processes. The chain lengths of aliphatic wax components are typically in the range of 20–34 carbons; thus multiple elongation cycles are needed to extend the acylchain to the final length.

Following elongation, VLCFAs are modified by various alternative pathways to form aliphatic wax components (e.g. primary alcohols, esters, *n*-alkanes). Investigations in *Brassica oleracea* leaves have led to the suggestion that primary alcohol production is a two-step process carried out by two separate enzymes – an NADH-dependent acyl-CoA reductase required for a reduction of fatty acids to aldehydes, and an NADPH-dependent aldehyde reductase required for a further reduction of aldehydes to primary alcohols (Kolattukudy, 1971). The primary alcohols generated in the epidermal cells may be further used for the synthesis of wax esters. Biochemical studies of wax ester formation in *B. oleracea* leaves (Kolattukudy, 1967a) and jojoba (*Simmondsia chinensis*) seeds (Wu *et al.*, 1981) suggested that this modification reaction is catalyzed by a membrane-bound acyltransferase (wax synthase). For alkane biosynthesis Kolattukudy (1966, 1967b) proposed an elongation–decarboxylation reaction in which C_{16} fatty acids are elongated by the addition of C_2 units and are decarboxylated after reaching the

appropriate length (C_{30}–C_{32}). In the following, the generated alkanes can be hydroxylated to secondary alcohols, which can give rise to a ketone by oxidation.

However, very little is known about the cellular localization of the enzymes catalyzing the corresponding reactions, and about how the resulting wax precursors and products are channeled between the biosynthetic enzymes and the cuticle. Based on current knowledge, there are at least two hypothetical mechanisms which could manage the transport wax components from the ER to the plasma membrane (PM): 1. direct transfer to the PM via sites of close apposition of ER domains with the protoplasmic face of the PM, or 2. vesicular transport from the ER to the Golgi and finally to the PM (exocytosis, Kunst & Samuels, 2003). It was also suggested that special proteins might serve to solubilize wax compounds in the cytoplasm and shuttle them across aqueous compartments on their way to the cuticle. However, a number of alternative hypotheses for this aspect of cuticle formation have been put forward, also based on analogies with other intracellular lipid transport processes (Kunst & Samuels, 2003).

Once at the cell surface, wax components could be pulled out of the bilayer by ABC-transporters and either transferred directly through the cell wall or carried by non-specific lipid transfer proteins (LTPs) to the cuticle. The identification of an *Arabidopsis thaliana* ATP binding cassette (ABC) transporter, CER5 gave evidence that this transporter might be involved in wax export (Pighin *et al.*, 2004). This was suggested from the analysis of the *cer5* mutant in which wax components are reduced on the cuticle surface and accumulate inside the cells instead. Previously, plant ABC-transporters have been characterized primarily in heavy metal detoxification and secondary metabolite transport (Rea *et al.*, 1998; Jasinski *et al.*, 2001; 2003). They are supposed to have the ability to pump a wide variety of substrates. In mammals and bacteria, the functionality of ABC-transporters as lipid transporters in outer membrane lipid A export could already be verified (Pohl *et al.*, 2005).

Wax transport through the cell wall has usually been attributed to LTPs (Sterk *et al.*, 1991; Moreau *et al.*, 1998). Non-specific LTPs (nsLTPs) of plants are part of a larger superfamily of small, basic proteins that move phospholipids between lipid bilayers in vitro (Kader, 1996; Arondel *et al.*, 2000; Rogers & Bankaitis, 2000). A few representatives of the protein family have been localized in

the epidermal cell wall (Thoma *et al.*, 1993) as well as in the cuticle (Pyee *et al.*, 1994). LTPs have been grouped into two classes, nsLTP1 (molecular weight, 9 kDa) and nsLTP2 (7 kDa) (Kader, 1996). Emerging data describing their properties makes them an interesting candidate for transport of cutin and wax monomers through the cell wall, although there is no direct experimental evidence confirming LTP participation in these processes.

Within the hydrophobic environment of the cuticle, wax molecules are supposed to self-arrange into intracuticular layers and epicuticular films, which finally leads to crystalline three-dimensional micro- and nanostructures that emerge from the underlying wax film (Koch *et al.*, 2004). Since plant cuticular waxes play pivotal physiological and ecological roles, it might be advantageous to adapt their composition and properties to fluctuating environmental conditions. Such dynamic changes may occur on several levels, including individual compounds, compound classes, or entire wax mixtures, and affect their relative percentages, or absolute amounts (Riederer & Müller, 2006).

Some studies investigated the influence of distinct abiotic factors (e.g. radiation, ozone, temperature, light, drought and humidity) on wax characteristics. The occurrence of the wax modification often depends on the type, length and intensity of the respective treatment, and on the combinations of species and treatments (Whitecross & Armstrong, 1972; Ashraf & Mehmood, 1991; Gordon *et al.*, 1998). Extensive studies have been carried out, to test the effects of light, temperature and air humidity during growth of leaves of *B. oleracea* (Baker, 1974). Both, enhanced temperature and decreased relative humidity, caused an increase in total wax amounts, and a shift in proportional quantities from alkanes and ketones to aldehydes and primary alcohols. These results are of great interest, as they suggest a feedback mechanism of increasing wax accumulation under transpiration stress. Furthermore, changes in wax amounts are also reported for several species subjected to distinct treatments (Whitecross & Armstrong, 1972; Steinmüller & Tevini, 1985; Günthard-Georg & Keller, 1987; Ashraf & Mehmood, 1991; Dixon *et al.*, 1997; Gordon *et al.*, 1998; Jenks *et al.*, 2001).

Apparently, the basic characteristics of wax production are associated with a certain variation range, which represents an acclimatization tool to the particular plant's exposure. Adaptations to specific exposure conditions also include dynamic effects on the chemical composition, and in more detail the chain length

distribution of several component classes. The primary physiological function of the cuticle is linked to the chain length distribution in the homologous series of the wax constituents, since the chain length distribution of aliphatics influences the size and geometry of crystalline domains in the wax, and consequently limits the water flow across the cuticle (Riederer & Schreiber, 1995). Giese (1975) evaluated the wax composition of barley leaves in light and dark and found that these different light conditions can strongly influence the chain length composition of wax classes. Effects on chain length distribution were shown for different species and treatments (Steinmüller & Tevini, 1985; Shepherd *et al.*, 1995; Dixon *et al.*, 1997).

The regeneration of epicuticular lipids on surfaces of living plants is a highly dynamic process, reflecting the importance of a steadily present continuous outer leaf coverage. There is a negative correlation between water repellency of microstructure surfaces and the contamination with particles (Barthlott & Neinhuis, 1997). Seasonal changes in leaf surface contamination of beech (*Fagus sylvatica* L.), oak (*Quercus robur* L.) and gingko (*Ginkgo biloba* L.) are closely related to leaf micromorphology and wettability (Neinhuis & Barthlott, 1998). It was concluded that the specific wax structure and chemistry influences the efficiency of self cleaning and is therefore important for the plant's survival in strong air pollution

Plant surfaces are also of ecological importance, as they essentially represent the first zone of contact with a variety of organisms, as herbivores, pathogens, and fungi (Riederer & Müller, 2006). It has been suggested that plant surfaces may contain signals that influence germination of biotrophic fungi already in early infection stages stipulating host specificity of plant pathogens, such as powdery mildews (Carver *et al.*, 1990; Tsuba *et al.*, 2002).

Powdery mildew, caused by the obligately biotrophic pathogen *Blumeria graminis* Speer f. sp. *hordei* Marchal (*Bgh*), is one of the most destructive foliar diseases of barley (*Hordeum vulgare* L.), and may cause up to 40% yield loss in temperate regions (Jørgensen, 1988). The asexual conidia of *Bgh* germinate and develop reasonably synchronously, proceeding through a highly ordered morphogenetic sequence (Green *et al.*, 2002). After initial contact with the host surface, *Bgh* asexual conidia form a primary germ tube (pgt), which attaches to the leaf surface and forms a short peg that penetrates the cuticle (Edwards, 2002). Subsequently, an appressorial germ tube (agt) elongates and differentiates

a lobed, apical appressorium (app) with a penetration peg, that breaches both host cuticle and cell wall, to finally establish a haustorium within an epidermal cell. These processes are completed 24h after inoculation. The central structure of the intracellular formed haustorium, the haustorial complex, exhibits an important function, as it operates the nutrient acquisition from the host plant to feed the fungus (Bushnell *et al.*, 1987). Moreover, the haustorial complex may also be involved in recognition of the host via molecular signalling (Heath & Skalamera, 1997). The successful establishment of the haustorium is followed by the formation of additional superficial hyphae. Secondary appressoria are formed, which in turn establish secondary haustoria in other epidermal cells. About 3–4 days after primary infection, conidiophores are formed on the leaf surface and the newly–formed conidia can be wind–dispersed to initiate new infection cycles.

The leaf surface provides the first barrier that fungi must overcome in order to gain access to the leaf, but it also provides chemical and physical cues that are necessary for the development of infection structures for many fungal pathogens (Walters, 2006). A specific role of plant surfaces in providing signals that influence germination and stipulation of the host specificity of biotrophic pathogens, such as powdery mildews, has been suggested by several authors (Carver *et al.*, 1990; Hedge & Kollattukudy, 1997; Iwamoto *et al.*, 2002; Tsuba *et al.*, 2002, Gniwotta *et al.*, 2005). Specifically, the early events of the infection process, such as fungal germination and differentiation, might be triggered by surface characteristics. The chemical composition and the physical structure of the cuticular wax layer may comprise factors affecting *Bgh* germination and appressorial differentiation. Hence, there is a strong interest to understand the series of events leading to successful appressorium formation, and to identify potential surface effectors triggering *Bgh* pre–penetration events.

It is important to ask which features of the host surface affect the infection process, and to what extent alterations of such parameters may diminish the fungal attack. On some non–host plant surfaces and artificial surfaces, the typical developmental sequence of *Bgh* pre–penetration processes is altered or impeded (Carver & Ingerson, 1987; Kobayashi *et al.*, 1991). On host leaves and on some largely hydrophilic surfaces, such as water agar or certain cellulose membranes, conidia were described to germinate regularly and differentiate into the agt stage

(Yang & Ellingboe, 1972; Carver & Ingerson, 1987; Kobayashi *et al.*, 1991; Kinane *et al.*, 2000; Green *et al.*, 2002). However, they do form multiple short germ tubes (sgt), and a very low percentage of agts on hydrophilic glass slides.

In order to analyze the effects of different surfaces on germination and appressorium formation of *Bgh*, Yang and Ellingboe (1972) applied different eceriferum (*cer*) barley mutants that were affected in physical and chemical properties of their cuticular wax layer. The authors found a high proportion of malformed appressoria on both, *cer*–mutant leaves and artificial substrates, including reconstructed wax layers. Due to the distinct micro–morphology of the *cer*–mutant leaf surfaces, they concluded that the distribution of wax crystals may be a determining factor for the formation of mature appressoria. However, after mechanical removal of the leaf epicuticular wax layer, a completely normal *Bgh* germling development was observed, and the physical structure of epicuticular waxes was found to be of little importance (Carver & Thomas, 1990). Instead, the fungal pathogen may recognize signal substances from the cuticle, or from the underlying cellulose cell wall. In line with this, Francis *et al.* (1996) suggested cutin monomers to be involved in triggering agt development, whereas Nicholson *et al.* (1993) demonstrated that agt formation requires a conversion of the conidium surface from a hydrophobic to a hydrophilic state. More recently, Tsuba *et al.* (2002) identified hexacosanal (C_{26}–aldehyde), a chemical constituent of the epicuticular wax layer, to strongly induce app formation of *Bgh* germlings. However, it remained unclear whether the C_{26}-aldehyde hexacosanal alters the physical surface properties of the host, e.g., by modifying the surface's hydrophobicity, rather than acting as chemical signal involved in app formation (Tsuba *et al.*, 2002). Further support for the idea of wax derived signals is given by Gniwotta *et al.* (2005). They demonstrated that different germination and differentiation rates of pea mildew (*Erysiphe pisi*) on adaxial and abaxial leaf surfaces of pea (*Pisum sativum*) can be attributed to the ultra–structural morphology of epicuticular wax crystals and to the chemical composition of epicuticular waxes. In conclusion, the chemical composition of epicuticular wax crystals, rather than their surface structure, carry the surface cues that stimulate early fungal development (Gniwotta *et al.*, 2005). Nevertheless, integrating historical data on potential physical and chemical surface signals of pre–

penetration processes is hardly possible, since earlier studies featured different cultivars and inconsistent experimental procedures.

MOTIVATION

The present study was conducted in order to clarify those contact cues that are important for early barley/ *Bgh* interaction, and in order to evaluate and integrate different surface factors that were assumed to affect *Bgh* pre–penetration processes, by using a single barley cultivar (cv Bonus), and a defined *Bgh* isolate. Although total leaf wax analyses for several accessions of the genus *Hordeum* had been reported previously (von Wettstein–Knowles, 1971; Baum *et al.*, 1989), there has been no data up to date on surface hydrophobicity and possible compositional and/ or structural differences in epi– and intracuticular waxes of ad– and abaxial barley leaf surfaces. Finally, the question arose whether *Hordeum* may adapt its leaf surface parameters to variable environmental conditions, with potential effects on the conidial development of its species specific pathogen (*Bgh*).

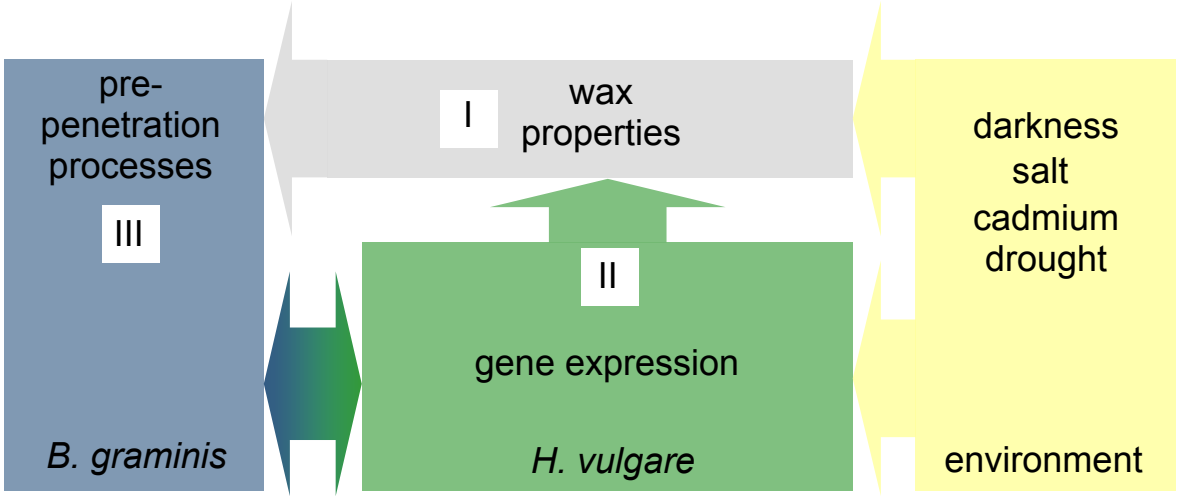


Figure 1: Illustration of investigated aspects in this study. Barley (*Hordeum vulgare)* surface wax properties (***chapter I****: characterization of different leaf wax parameters*) depend on molecular processes of wax biogenesis (***chapter II****: gene expression studies investigating different aspects of wax biogenesis*), whereas both provide important prerequisites for determination of distinct host surface cues which trigger development of powdery mildew (*Blumeria graminis*) conidia (***chapter III****: impact of different surface features on conidial development*). Impacts of abiotic environmental factors and molecular interactions between host and pathogen are additionally considered to explore different mechanistic levels of the species specific interaction.

Therefore, we investigated the impact of different abiotic habitat factors (drought-, cadmium-, salinity-stress and darkness) on distinct physical and chemical parameters of barley wild-type leaf surface waxes (phenotypic approach, Fig. 1).

Moreover, we also tested whether a biotic stressor, powdery mildew infection, affects local and systemic alterations in wax characteristics, with regard to its potential to modify the developmental success of following secondary infections. In order to obtain a high diversity of different natural leaf surfaces for bioassays with fungal conidia, a detailed characterization of various eceriferum (*cer*-) mutants leaf wax features was added (genotypic approach) and compared to the wild-type. Leaf surfaces resulting from both approaches should serve as important prerequisite to combine the different modifications in surface waxes with conidial germination and differentiation success on natural surfaces. Additionally, we studied growth responses of powdery mildew on artificial substrates to reveal the chemical and physical surface wax factors determining the ability of conidia to successfully develop on barley leaves during first steps of the barley/ *Bgh* interaction.

In order to clarify the plant's mechanisms to regulate wax accumulation and composition, we established a barley wax-microarray which allowed us to correlate molecular events of wax biogenesis with observed analytical wax data. Gene expression in different stages of wax biogenesis supporting *de novo* fatty acid synthesis (C_{16}/C_{18}), their elongation to very long chain fatty acids (VLCFAs), and modification to wax precursors, were considered. To set the transcriptional events into a physiological context, the transcriptional activity in intracellular lipid-transport, and the export of wax components, should be verified. In addition, the expression of several stress inducible genes and putative housekeeping genes should be screened. The regulatory impact of variable abiotic treatments (darkness-, cadmium-, NaCl- and drought-treatment) on gene expression was combined with observed wax modifications of respective treatments. Moreover, we investigated potential inferences of biotic stress, namely fungal infection and development, with proceeding wax biogenesis, reflecting molecular aspects of barley/ *Bgh* interaction events. With the broad range of different aspects investigated in this study we attempted to improve our current understanding of both, the highly coordinated mechanisms of the early steps of barley/ powdery mildew interaction on plant surfaces, and the different levels of reciprocal adaptations within this highly specific system.

CHAPTER I presents a detailed characterization of barley surface wax parameters (amount, portions of epi- and intracuticular waxes, chemistry and hydrophobicity) of native wild-type, stressed wild-type and of a selection of *cer*-mutants, which were most distinctly impaired in their wax characteristics in comparison to the wild-type.

Gene expression studies investigating different aspects of wax biogenesis are presented in **CHAPTER II**. In order to demonstrate the benefit of the established barley wax-micorarray, two experimental setups are described: 1. The "stress-microarray" correlates the molecular responses within barley leaves during cadmium-, salt- and drought- stress as well as deficiency of light (darkness-treatment) with the distinct modifications in wax characteristics enforced by the respective treatments. 2. To test the impact of biotic stress the second experiment examined the potential effects of powdery mildew (*Blumeria graminis* f. sp. *hordei*) infection on transcriptional events of wax biogenesis and was therefore described as "*Bgh*-microarray".

CHAPTER III presents various bioassays with *Bgh* conidia on natural barley leaf surfaces (characterized in the first chapter) and on artificial surfaces, that further examine single chemical and physical wax and leaf surface factors, to evaluate the roles of potential surface signals governing germination and structure differentiation of *Bgh* conidia.

CHAPTER I:
Characterization of Different Leaf Wax Parameters

RESULTS

The detailed characterization of cuticular waxes covering barley leaf surfaces is an important prerequisite to investigating potential recognition cues in the barley/ powdery-mildew system. Thus, the total amount of leaf cuticular waxes, the proportion of epicuticular and intracuticular waxes, and the respective chemical composition of both fractions, were determined by GC-MS analysis. As wax amount and chemical composition largely determine the specific micro-morphology of wax crystals, the crystal structures of barley surface waxes were examined by SEM, while contact angle measurements were used to define surface hydrophobicity.

Four abiotic treatments and one biotic treatment, *i.e. Blumeria graminis* f.sp. *hordei* (*Bgh*)- infection, were applied, to test the impact of environmental effects on surface wax characteristics (phenotypic approach). In comparison, features of surface waxes of several *eceriferum*- (*cer*-) mutants were determined (genotypic approach). At last, the modified chemical and physical wax parameters observed within the corresponding phenotypic and genotypic approach were tested in assays with *Bgh* conidia, to investigate their potential effect in influencing the fungal pre-penetration processes (chapter III).

1. Characterization of Barley Wild-Type Leaf Waxes

Amount- On average, the total cuticular wax coverage of 14d old leaves of barley wild-type resulted in 14μg cm^{-2}.

Chemical composition- Barley wild-type leaf waxes were composed of several distinct aliphatic compound classes (Fig. 1). Primary alkanols with chain lengths between C_{22} to C_{30} formed the most prominent portion of total cuticular wax (10.8μg cm^{-2}). With an absolute amount of 9.8μg cm^{-2}, hexacosanol (C_{26}) not only dominated within fraction of primary alkanols (91% of alkanols), but also made up 69% of total wax, whereas hexacosanal (C_{26}-aldehyde) formed the second main compound with 0.6μg cm^{-2} leading to 4% of total wax. Esters with even-numbered carbon chain lengths (C_{38} to C_{52}) contributed 1.6μg cm^{-2}. Main components of this fraction were given for carbon chain lengths C_{46} and C_{48}.

Long-chain fatty acids (C_{24} & C_{26}) and *n*-alkanes (*n*-C_{27}) were present in trace amounts. Adaxial and abaxial cuticular waxes were analyzed separately, but no distinct differences in wax quantity or quality were detected (data not shown, for details see Stangl, 2005).

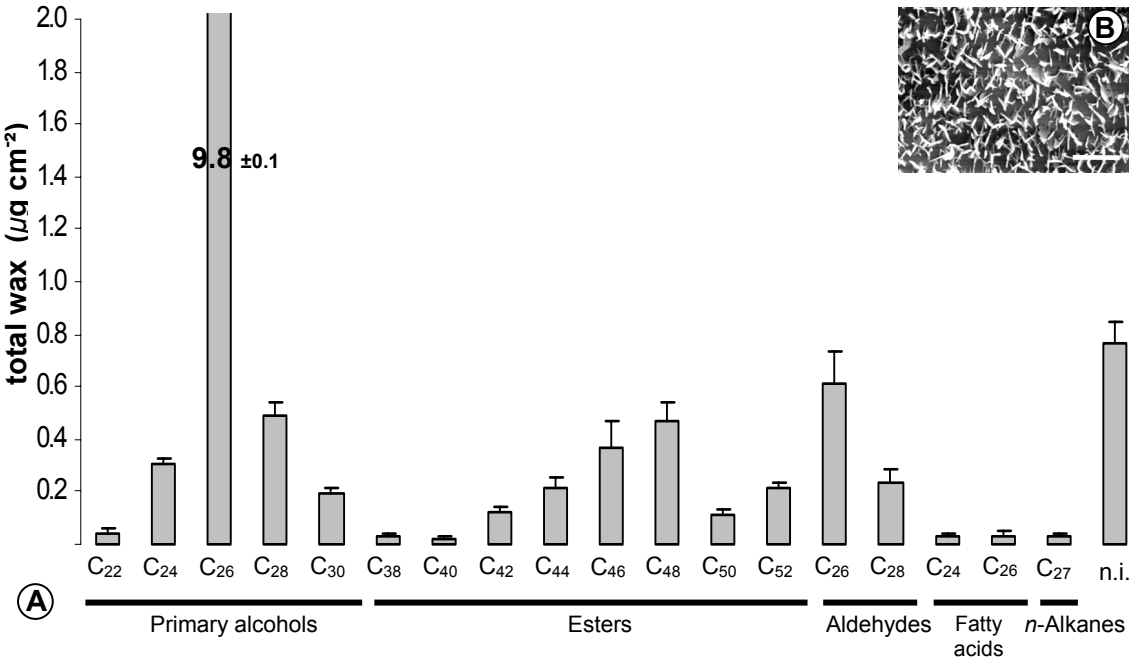


Figure 1: A-Cuticular wax composition of barley wild-type (cv Bonus). Chain length distribution within wax compound classes given in absolute amounts (mean ±SD, n=5). B-SEM picture of epicuticular wax crystal structure of adaxial wild type leaf surface. Bar 2µm.

Wax crystal morphology- The adaxial epicuticular wax layer of wild-type leaves was composed of densely packed wax crystal platelets, vertically protruding 1-1.5µm from the leaf surface. A dense and relatively thick network, in which the smooth surface of the cuticle itself was rarely visible, was established through these platelets.

Hydrophobicity- The determination of hydrophobicity on this surfaces revealed average contact angles of 140° ±3 (mean ±SD, n=20).

2. Modifications in Wild-Type Leaf Waxes Due to Different Environmental Stresses

In order to test the impact of environmental factors on wax characteristics, four abiotic treatments, *i.e.* darkness, NaCl-, cadmium- and drought-stress, as well as one biotic treatment, *i.e.* infection with *B. graminis*, were applied. Effects on total wax amount, chemical composition, wax crystal morphology, and surface hydrophobicity, were described in comparison to native wild-type leaves.

2.1 Alterations in Wax Amount and Composition Due to Different Abiotic Stresses

Darkness-treatment- Total wax amount of plants grown in darkness with 10 ±0.3 μg cm^{-2} was significantly reduced (mean ±SD, n=5; Student´s *t*-test; Fig. 2) to about 31% compared to control plants with 14.3 ±3.0 μg cm^{-2}. As noticed by visual inspection, the leaf firmness of etiolated leaves was slightly reduced compared to natively grown leaf blades.

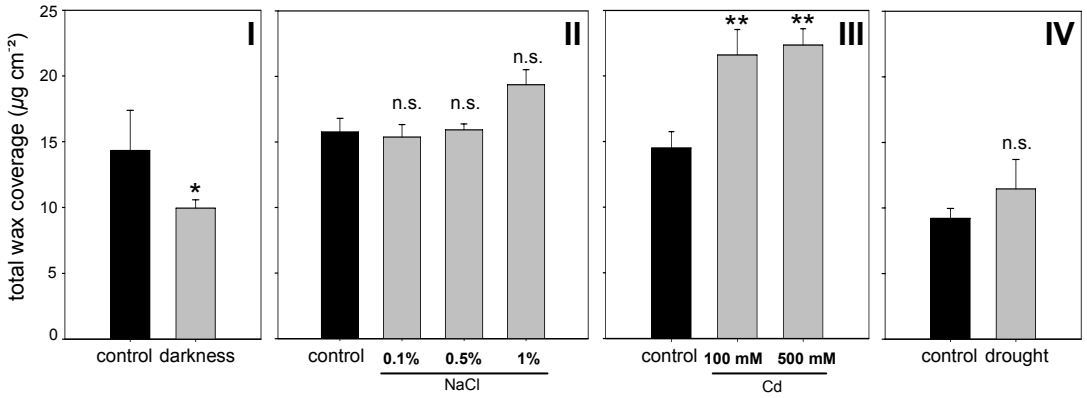


Figure 2: Modifications of total leaf wax amount in response to different treatments: I- darkness, 14d old etiolated plants, II- plants grown on hydroponics of different NaCl concentrations, III- plants treated with 100 mM and 500 mM cadmium (Cd) solution, and IV- 21 d old plants set under drought stress (for details see materials and methods 2.0). Given are means ±SD of n=5 replications. Statistical differences (p≤0.05) were tested with Student´s *t*-test.

The proportions of all compound classes diverged significantly in etiolated plants compared to waxes of native wild-type (Fig. 3). In etiolated 14d old barley leaves a significant increase of 5% of primary alcohol fraction was revealed, which was mainly ascribed to an amplification of docosanol (C_{22}) and tetracosanol (C_{24}) within this compound class. The effect was accompanied by a 2% reduction of the ester fraction, which attributed in detail to a decrease of carbon chain lengths C_{46} and C_{48}. Compared to controls, fraction of fatty acids significantly increased, mainly due to C_{24} and C_{26} carbon skeletons. Compared to non-treated plants, little amounts of aldehydes and *n*-alkanes emerged from the surface waxes of etiolated plants. Similar to the wax composition of non-treated plants, the left over of 0.3% of aldehydes in etiolated leaves was dominated by hexacosanal, while octacosanal was not detectable.

NaCl-treatment- Increasing salt concentrations of 0.1%-1% NaCl in hydroponic solution did not significantly affect the wax amount (Student´s *t*-test, n=5, Fig. 2). 1% NaCl tended to slightly increase the wax overlay to 19.4± 1.2 μg cm^{-2} compared to 15.8μg cm^{-2} in control plants (mean ±SD). As observed visually,

plant growth was impeded upon NaCl-treatment, an effect that increased in parallel with rising salt-concentrations.

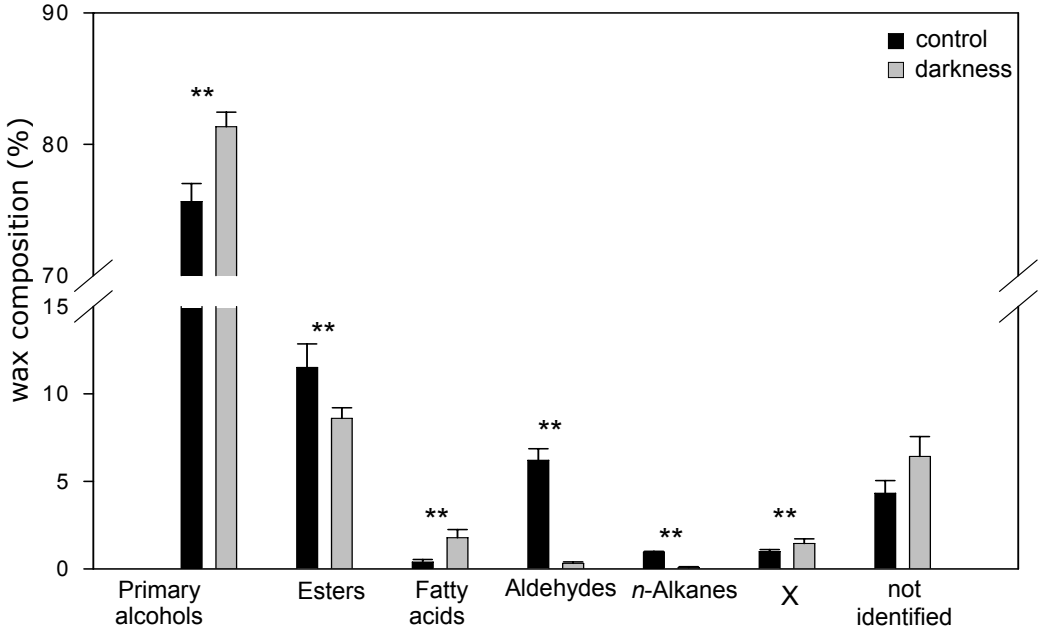


Figure 3: Leaf wax composition of 14d-old etiolated, in darkness grown plants, given are means ±SD. Significant differences ($p \leq 0.05$) were tested in a Student´s t-test (n=5) and are marked with stars. X: a series of homologous aliphatics of an unidentified compound class with corresponding diagnostic ion masses: m/z 268/281- 505/520, m/z 268/281- 533/548, and m/z 282/295- 575/590. Fraction of *not identified* was not statistically tested, proportions of aldehydes and X were Welch-corrected.

Cadmium-treatment- Total wax amount of cadmium stressed plants was significantly increased up to 154% compared to controls (Student´s *t*-test, n=5, Fig. 2). Maximum cuticular wax coverage of 22.4± 1.3μg cm^{-2} was achieved by a treatment with a 500mM cadmium solution. As visually observed, increasing heavy metal concentrations reduced the leaf blade area, as well as their flexibility, resulting in a relatively firm and rigid appearance of the plant individuals.

Drought-treatment- drought-stress did not significantly affect the wax amount (Fig. 2), a slight increase to 11.4 ±1.0 μg cm^{-2} appeared, when compared to 9.2 ±0.3 μg cm^{-2} in corresponding controls. Reduced values in total wax coverage of control plants within the drought-treatment, compared to controls of all other treatments, may be caused by cultivation of single plant germlings in smaller plastic pots.

The chemical composition of cuticular waxes was not affected by NaCl-, cadmium- and drought-stress. Likewise, for all different abiotic treatments, the allocation of epi- and intracuticular wax portions remained unaltered (data not shown).

2.2 Wax Crystal Structure Due to Different Abiotic Stresses

Epicuticular wax crystal shape was not affected by the different abiotic stress treatments. Size and arrangement of surface wax plates were similar across the differently treated test plants and the control (Fig. 4 A I-IV). Wettability of adaxial leaf waxes was also not strikingly affected by the different treatments, since all contact angle values were in similar range. However, a weak alteration of surface hydrophobicity on NaCl treated plants appeared.

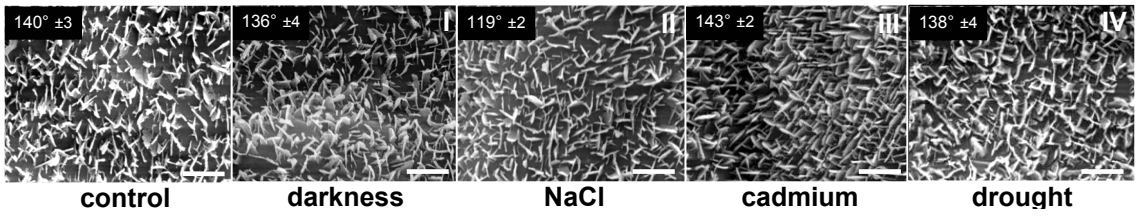


Figure 4: SEMs of barley adaxial leaf surfaces. Wax crystal structure of wild type adaxial leaf surfaces: I- grown in darkness, II- grown on hydroponics with 1% NaCl concentration, III- treated with 500 mM cadmium solution and IV- set under drought stress, control- untreated wild-type (for treatment details see materials and methods 2.0). Contact angles are given as means ±SD of n=20 independent measurements. Bars = 2μm.

2.3. Biotic Stress Due to Powdery Mildew (*Bgh*)-infection: Wax Characteristics of Local and Systemic Tissues

Local Tissue- After 6d inoculation with *Bgh* conidia, the amount of total waxes remained unaltered between infected and control leaves (infected leaves: 11.1 ±3.7μg cm^{-2}, controls: 11.0 ±1.9μg cm^{-2}, mean ±SD, n=5). Moreover, the chemical composition of surface waxes was comparable in the treatments and control plants (data not shown). Since leaf surfaces were densely covered with powdery mildew pustules, determination of the hydrophobicity by contact angle measurement was abandoned.

Systemic effects- In order to investigate systemic effects of powdery mildew on leaf wax features, the secondary leaves were infected exclusively, whereas wax analysis was carried out for the non-infected fourth leaves. The wax analysis of the later exhibited a wax amount of 7.6 ±0.3μg cm^{-2} (mean ±SD, n=5), which was similar to controls with 7.0 ±1.2μg cm^{-2} (data not shown). A slight but not significant increase in wax amount was detectable after 10d of inoculation with 15.3 ±2.4μg cm^{-2} compared to 11.7 ±0.9μg cm^{-2} of controls. This trend remained after further incubation with about 18.5 ±1.0μg cm^{-2} leaf wax coverage on fourth leaves of systemically infected plants, compared to 16.2 ±1.1μg cm^{-2} for non-

infected controls, after 15d conidia infection. Chemical wax composition of the analyzed fourth leaves of systemically infected plants remained unaltered upon *Bgh*-infection. Contact angle measurement showed comparable degrees of surface hydrophobicity for systemically infected plant tissue, and control (137° ±4; mean ±SD, n=20). A visual inspection of systemically infected plants did not reveal differences in size, leaf length, and leaf area, between the infected second, and later emerged fourth leaves.

3. *Cer*-Mutants´ Wax Characteristics

Different leaf surface properties of 18 barley *cer* mutants (lines), known to be impaired in their leaf wax properties, were characterized. The characterization pointed to a selection of five *cer*-mutants, which were most distinctly modified in the chemistry and/ or morphology of their epicuticular waxes. The focus was set on *cer*-yj.667, *cer*-yp.949, *cer*-zd.67, *cer*-zh.54, and *cer*-j.59. Analytical data for these five *cer* mutants were compared to wild type wax characteristics. For wax amount, chemical composition, and epicuticular wax crystal structure of the remaining 13 *cer*-mutants see appendix (Fig. 1).

Figure 5: Phenotype of 14d old barley wild-type cv "Bonus" and a selection of five *cer*- wax mutants.

The phenotype of 14d old *cer*-mutant plants was not different from that of wild-type (Fig. 5). A closer visual inspection in comparison to the other *cer*-mutants and the wild-type, revealed an impaired leaf firmness, and slight variations in the leaf blade color of mutant *cer*-yp.949.

3.1 *Cer*-Mutants´ Total Leaf Wax Coverage and Composition

The selected wax mutants *cer*-yp.949, *cer*-zd.67, *cer*-zh.54, and *cer*-j.59 exhibited a significantly reduced wax coverage compared to wild-type. The *cer*-mutant zh.54 showed the lowest amount with 2.3 ±0.7*µ*g cm^{-2} (mean ±SD, n=5). In case of *cer*-yj.667 (13.0 ±3.0*µ*g cm^{-2}), no distinct differences concerning wax amount were detected in comparison to the wild-type (Tab. I).

Table I. Compound class composition (%) and total wax coverage (*µ*g cm^{-2}) of entire barley leaves from wild type (cv Bonus) and from five of its *cer*-mutants. Significant differences (p≤0.05) between all leaf surfaces within total wax coverage and compound classes (rows) were tested with a one way ANOVA, followed by a Tukey HSD post hoc test. Data with significant differences to all assays within one compound class are styled fat, differences within wax coverage are marked with letters (n.d. = not detected). A series of homologous aliphatics of an unidentified compound class is responsible for the relatively high percentage of unidentified compounds in the leaf wax of cer-j.59. Corresponding diagnostic ion masses are: m/z 268/281- 505/520, m/z 268/281- 533/548 and m/z 282/295- 575/590.

	Wild-type		*cer*-yj.667		*cer*-yp.949		*cer*-zd.67		*cer*-zh.54		*cer*-j.59	
Wax coverage	14.30^{a}	±3.00	13.00^{a}	±3.00	4.30^{b}	±1.40	3.70^{b}	±0.30	2.30^{b}	±0.70	4.50^{b}	±1.00
Compound Class												
Primary alcohols	75.76	±1.37	73.40	±1.88	**45.08**	±4.54	70.99	±2.26	69.55	±1.94	**38.18**	±4.43
Esters	11.52	±1.35	10.27	±0.58	15.75	±1.74	17.10	±0.72	**20.30**	±0.90	**38.18**	±4.10
Aldehydes	6.20	±0.66	6.03	±0.44	**29.81**	±0.97	3.11	±0.61	1.60^{b}	±1.59	2.89^{b}	±1.51
Fatty acids	0.39	±0.15	**4.19**	±0.54	1.35	±0.49	2.03	±0.21	0.89	±0.23	0.55	±0.20
n-Alkanes	0.95	±0.06	0.06	±0.01	0.20	±0.07	**n.d.**		0.32	±0.04	0.79	±0.21
Unidentified	5.32	±0.74	6.05	±0.50	7.81	±1.66	6.77	±1.74	7.34	±1.32	19.41	±7.02

n=5, mean± SD

The leaf wax composition varied largely among some of the mutants. Wax mutants *cer*-yp.949 and *cer*-j.59 exhibited a significantly modified wax composition. In comparison to the wild-type, the proportion of primary alkanols in the wax of *cer*-yp.949 was significantly reduced to nearly 50%, while the proportion of aldehydes showed a five-fold increase. Focusing on compound chain lengths in detail, 91% of primary alkanols in *cer*-yp.949 were composed of hexacosanol, while 89% of the aldehydes consisted of hexacosanal, the corresponding C_{26}-aldehyde. In general, the primary alkanol fraction was dominated by hexacosanol in both, wild-type and selected mutants, except for *cer*-j.59. This mutant exhibited a modified primary alkanol fraction, predominantly composed of tetracosanol (C_{24}) and hexacosanol (C_{26}) with similar portions of 15% and 17%, respectively. The proportion of primary alkanols in *cer*-j.59 was distinctly

reduced to 50% of the wild type value, whereas esters showed a nearly four-fold increase.

In contrast to the significantly modified wax composition in *cer*-yp.949 and *cer*-j.59, the other mutants exhibited only moderate variations in wax composition. Nonetheless, they were selected for analyses because of the significant differences in wax characteristics, as total wax amount in *cer*-zd.67 or modified wax crystal morphology in *cer*-yj.667 and *cer*-zh.54 (Fig. 6). However, *cer*-yj.667 exhibited a significant increase in the proportion of fatty acids by one order of magnitude, while the proportion of esters was slightly increased by about 30% in *cer*-zd.67. Concerning other components, the wax composition of *cer*-zd.67 and *cer*-yj.667 was similar to the wild-type. In *cer*-zh.54 the ester fraction was increased two-fold increased compared to the wild-type, while the proportion of primary alkanols was slightly reduced. Aldehydes decreased to a minimum of 25% of the proportion in the wild-type. In general, the proportion of *n*-alkanes was below 1% of total leaf cuticular wax in both, wild-type and *cer*-mutants.

3.2 *Cer*-Mutants´ Epi- and Intracuticular Waxes

About 25% of the adaxial cuticular waxes of Bonus wild type leaves were found to be epicuticular, while the rest was embedded in the cuticular matrix (Tab. II). The leaves of *cer*-yj.667, *cer*-zd.67 and *cer*-zh.54 exhibited similar ratios of epi- and intracuticular waxes. Absolute amounts of the epicuticular wax fractions of *cer*-yj.667 and *cer-yp.*949 did not significantly differ from the wild-type, whereas *cer*-zh.54 exhibited slightly reduced amounts, and *cer*-zd.67 showed a significant four-fold reduction of epicuticular waxes. In contrast to all other lines, *cer*-j.59 exhibited a significantly increased portion of epicuticular waxes. Compared with to the other mutants and the wild-type, the extractable intracuticular wax fraction of *cer*-yp.949 was significantly reduced, and the ratio of epi- to intracuticular wax was almost inverted on *cer*-yp.949 and *cer*-j.59. However, the analysis of the chemical wax composition did not reveal any significant qualitative differences between epi- and intracuticular wax layers in all lines assayed (data not shown).

The selective extraction of epi- and intracuticular waxes led to total wax contents different from those of total wax extractions. These deviations resulted from different methods: for selective extractions of adaxial leaf waxes, the abaxial leaf surface was covered with water based *gum arabic* to protect the wax crystals

from being solved, afterwards the solvent was applied. With this procedure wax crystals positioned at the leaf edges were extracted as well. In consequence, the values obtained for the adaxial wax amount increased. Hence, the sum of epicuticular and intracuticular wax portions slightly deviates from the total wax coverage data presented in table I.

Table II. Amount of cuticular adaxial leaf wax coverage of barley wild-type (cv Bonus) and five *cer*-mutants. Significant differences ($p \leq 0.05$) between corresponding portions of wax coverage (epicuticular, intracuticular, total and ratio of epi/intra) were tested in one way ANOVA, followed by Tukey HSD post hoc test. Letters mark differences between every approach within one portion (rows).

	Wild-type	*cer*-yj.667	*cer*-yp.949	*cer*-zd.67	*cer*-zh.54	*cer*-j.59
Adaxial leaf wax coverage (μg cm^{-2})						
Epicuticular wax	4.23 ^{a} ±0.46	3.33 ^{ab} ±0.28	3.93 ^{a} ±0.17	1.15 ^{c} ±0.16	2.55 ^{b} ±0.4	6.04 ^{d} ±0.66
Intracuticular wax	13.11 ^{ab} ±1.06	13.97 ^{bc} ±1.76	1.62 ^{d} ±0.51	3.66 ^{cd} ±0.89	5.72 ^{b} ±0.63	4.01 ^{bc} ±0.90
Total wax load	16.72 ^{a} ±3.03	17.07 ^{a} ±1.56	5.93 ^{b} ±0.83	6.21 ^{b} ±1.07	5.9 ^{b} ±0.89	7.01 ^{b} ±0.39
Ratio epi/ intra	0.32 ^{a}	0.24 ^{ab}	2.43 ^{c}	0.31 ^{a}	0.45 ^{a}	1.51 ^{bc}

n=5, mean± SD

3.3 *Cer*-Mutants´ Epicuticular Wax Crystal Structure and Surface Hydrophobicity

Alterations detected in the chemical composition of barley wild-type and *cer*-mutant cuticular waxes were clearly reflected in the differing morphology of epicuticular wax crystals (Fig. 6).

Unlike the wild-type, *cer*-yj.667 exhibited lengthwise joined platelets, which protruded much further (2-2.5μm) from the leaf surface (Fig. 6B). In contrast, wax crystals of *cer*-yp.949 were more horizontally oriented and appeared to be embedded in an amorphous, crust-like mass (Fig. 6C). The epicuticular wax crystals of *cer*-zd.67 were smaller and less abundant than the platelets on wild type leaves, whereas *cer*-zh.54 and *cer*-j.59 exhibited single, irregularly scattered wax bodies (Fig. 6E & F). In the case of *cer*-zh.54, the wax crystals were more fragile than those of *cer*-j.59, which appeared more compact. Concerning wax crystal morphology, no distinct differences were detected between adaxial and abaxial leaf surfaces within plant individuals of both, barley wild-type and investigated *cer*-mutants.

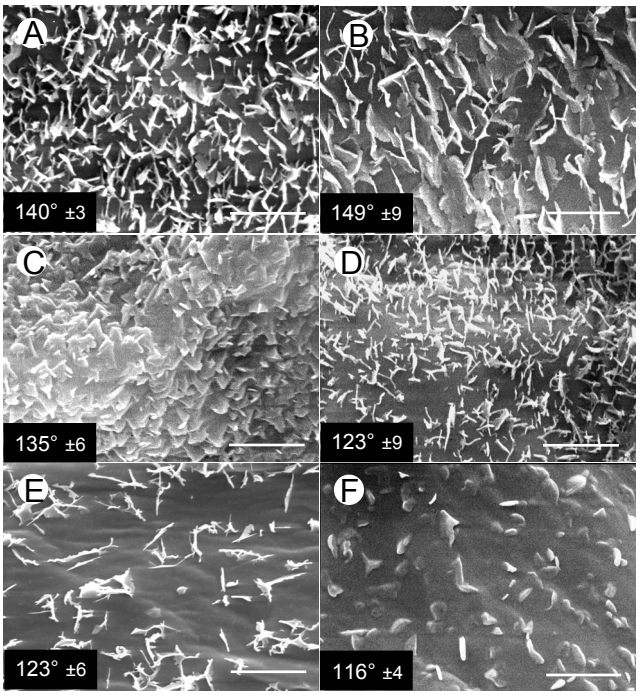


Figure 6: SEMs of barley adaxial leaf surfaces. Wax crystal structure of wild-type cv Bonus (A) and *cer*-mutants *cer*-yj.667 (B), *cer*-yp.949 (C), *cer*-zd.67 (D), *cer*-zh.54 (E) and *cer*-j.59 (F). Contact angles are given as means ±SD, n = 20. Bars = 2μm

The determination of contact angles as a measure of surface hydrophobicity revealed differences between wild-type cv Bonus and its selected *cer* mutants (Fig. 6). While the adaxial leaf surfaces of wild-type and *cer*-yp.949 exhibited comparable values of 140° and 135°, respectively (means, n = 20, Fig. 6), *cer*-zd.67 and *cer*-zh.54 showed a slightly decreased average contact angle of 123°. The most prominent surface hydrophobicity, with a contact angle of 149°, was obtained with *cer*-yj.667 leaves. In contrast, the most hydrophilic leaf surface of this assay was exhibited by *cer*-j.59 with a contact angle of 116°.

DISCUSSION

1. Barley Wild-Type Leaf Wax Characteristics

Total wax coverage and chemical composition of wild-type barley (cv Bonus) leaves were largely in accordance with data found in previous studies (von Wettstein-Knowles, 1971; Giese, 1976). “Bonus” leaf cuticular wax was dominated, as in other barley cultivars, by the primary alcohol 1-hexacosanol (C_{26}-alcohol), which comprised about 69% of the total extractable cuticular waxes, while the primary seedling leaf wax of the “Bonus” wild-type was comprised of 73% primary alcohols altogether (Giese, 1976). Typically, additional compounds, like β-diketones and hydroxy-β-diketones, were found in the internode and spike waxes of barley. As expected, such compounds were absent in barley wild-type leaf waxes.

2. Modifications in Wild Type Leaf Waxes Due to Different Environmental Stresses

2.1 Etiolation Reduces the Cuticle Wax Load, Changes the Relative Composition and Shifts the Major Components to Shorter Chain-Lengths

As reported in several studies examining diverse plant systems, environmental factors may change the amount and/ or composition of cuticular waxes (Shepherd & Griffiths, 2006). Such alterations in surface waxes provide an acclimatization to the particular environmental conditions and therefore maintain the protective functionality of the cuticle with its embedded cuticular waxes.

The various abiotic stress factors investigated in this study induced different modifications of barley surface waxes. The significantly reduced cuticular wax amounts of plants grown in darkness, and their modified chemical composition, *i.e.* aldehyde amount and chain lengths’ variations of the major components within the alcohol fraction, were comparable to Giese (1975). Based on these observations, it was suggested that enzymes carrying out a given reaction in wax biosynthesis may select specific chain lengths as substrates, and that this functionality might be differentially sensitive to light. Similar to this study, and in accordance with our observed data, von Wettstein-Knowles *et al.* (1980) reports an almost entire disappearance of the aldehyde fraction in maize, due to growth in darkness. These

observations led to the suggestion, that the formation of aldehydes could be light stimulated. One might further expect that a light stimulus regulates ratios of aldehyde versus alcohol contents, by operating the activity of fatty acyl reductase, which in turn supports the modification step from aldehydes to primary alcohols (Kollatukudy, 1971).

From investigations of surface waxes of *Arabidopsis thaliana* mutants, *cer1* and *cer22*, which, compared to wild type plants, exhibited increases in aldehyde levels and dramatic reductions in the accumulation of alkanes, secondary alcohols, and ketones, a block in the conversion of aldehydes to alkanes was suggested (Hannoufa *et al.*, 1993; Lemieux *et al.,* 1994; Jenks *et al.*, 1995; Rashotte *et al.*, 2004). Based on this biochemical phenotype, it was proposed that *Cer1* may encode an aldehyde decarbonylase (Hannoufa *et al.* 1993, Mc Nevin *et al.* 1993). Concurrently, a lack of light stimulus in etiolated test plants may reduce the effectiveness of the aldehyde decarbonylase pathway-step, which could result in a greater flux of carbon towards primary alcohols, thus inducing elevated concentrations of this compound class.

According to Shepherd *et al.* (1995), who investigated different *Brassica* species exhibiting variations in ester chain-length related to variations in alcohol chain length, the overall effects of environmental variation on the distribution of the wax esters might be dependent on the formation of the precursor acid and alcohol moieties, and the esterification process itself.

Referring to our study, the reduced ester amount in etiolated test plants might be attributable to a decreased flow of fatty acids and primary alcohols into the esterification process, which in turn might explain the accumulation of precursor compounds of respective chain lengths. However, it remains to be discussed in how far these chemical modifications upon etiolation alter the physiological properties of the cuticle. Since the chain length distribution of aliphatics influences the size and geometry of crystalline domains in the wax, the water flow across the cuticle might be affected (Riederer & Schreiber, 1995).

Properties of the epicuticular wax layer underlie a permanent modification, which depends on the developmental stage of the plant under prevailing environmental factors (Baker, 1974). In general, the availability of light is an important factor for cell activity, as it delivers energy from the photosynthetic machinery. Based on investigations in cuticular wax deposition of growing barley

leaves, Richardson *et al.* (2005) assumed light to be the most likely candidate to affect wax deposition. Although they did not survey differences in photosynthetically active radiation between enclosed and emerged leaf portions, they proposed light to be influencing wax deposition through changes in photon flux density. In conclusion, it might be suggested that a lack of light might have enforced retardation of wax deposit-processes, thus explaining the lower wax amount in etiolated leaves, which, as the case may be, could have been compensated by extended developmental time.

2.2 Cadmium-Exposition Highly Increases the Leaf Wax Amount

Among the four investigated abiotic stresses, cadmium exposure enforced the most striking increase in wax accumulation. This elevated wax amount was similar to Hollenbach *et al.* (1997), who reported the same effect for cadmium exposed barley seedlings. It might be possible that the increased amounts of cuticular waxes in cadmium-stressed plants rather result from decreased cell surface areas than from increased cuticle wax load. Khudsar *et al.* (2001) report a reduction of stomata and stomatal pore dimensions, as well as a decrease of trichome length and density upon cadmium treatment in *Cajanus cajan* (Khudsar *et al.*, 2001). This indicates that reduced leaf areas might be the result of decreased single cell surface areas. In this case, an unaltered wax amount per smaller epidermal cell would have led to increased wax amounts per area. However, the cuticle wax load coating the cell surfaces was increased upon cadmium exposition.

It has been postulated that the mobility of water or solutes in plant cuticular waxes will be decisively determined by the volume fraction of wax present in a crystalline state, and by the geometry of the crystallites (Riederer & Schreiber, 1995). Thus, it might be possible, that cadmium exposed plants maintain the protective functionality of the cuticle upon cadmium stress, by increasing the cuticular wax load. Nevertheless, the increased wax overlay did not strikingly affect surface hydrophobicity, since contact angle measurements of cadmium exposed leaves exhibited equivalent values to native leaves.

As also detected in our experiments, growth of the cadmium-stressed plant individuals, as well as their leaf blade area, and firmness, seemed to be affected. This was congruent with other studies, which described cadmium exposure to

retard plant biomass and development in barley, depending on the distinctive tolerance of the cultivar (Wu *et al.* ,2004; Gou *et al.*, 2007). Generally, metal stress in plants appears to excessively disturb the cell metabolism in multiple ways, altering the activity and quantity of the key enzymes of various metabolic pathways. This may cause changed ultra-structure of the cell organelles, reduced chlorophyll content, and even inhibited plant growth and respiration (Khudsar *et al.*, 2001; Yamamoto *et al.*, 2002). Therefore, abnormal growth might be a consequence of induced disorder in nutrient metabolism due to highly concentrated heavy metals in plant tissues (Guo *et al.*, 2007).

2.3 Drought- and Salinity-Stress Do Not Affect the Leaf Wax Characteristics

Several studies investigating a range of different plant species often describe drought-stress as enforcing elevated amounts of cuticular waxes (Ashraf & Mehmood, 1990; Premachandra *et al.*, 1991; Xu et al, 1995; Bondada *et al.*, 1996; Dixon *et al.*, 1997; Jenks *et al.*, 2001). However, among the drought experiment in barley investigated in this study, no significant increase in total wax amount in stressed plants appeared, even though plants were kept under drought stress for a 21d period. Nevertheless, a slight 20% increase of surface wax load was revealed, which corresponded to Jenks *et al.* (2001), who reported a 9-15% enhancement of leaf surface waxes in water stressed rose cultivars. However, it remains unsolved, if this slight increase in surface wax load sufficiently strengthens the cuticle barrier upon drought-stress to sustain the protective function of the cuticle against transpirational water loss (Riederer & Schreiber, 2001; Kerstiens, 2006).

Similar to studies of Fricke and Peters (2002) and Fricke *et al.* (2006), who investigated effects of salt exposure to barley, we found no significant amplification of wax coverage for salt-stressed plants. Moreover, in accordance to our observations, the authors report changes in leaf growth upon NaCl-treatment (Fricke *et al*., 2006). It was concluded, that salt-stress changes the water potential gradient between leaf xylem and peripheral elongating cells, which in turn reduces the leaf elongation velocity. Thus, it might be suggested, that the reduced leaf area observed in our experiments was derived from decreased cell elongation. However, similar to our study, the authors reported that, even high levels of salinity did not obviously affect wax deposition, and a short term-exposure to salt-stress

did not affect wax density. This lack of wax modification due to salt-stress could refer to an evolved adaptation to habitat conditions, dealing with water deficiency and associated high concentrations of mineral nutriments, since barley is known to be a major crop well growing in arid and semiarid regions (Mass,1984; Francois & Mass, 1999). To cope with such common habitat stresses, barley might have evolved adaptive mechanisms different from those influencing the surface wax characteristics.

2.4 Abiotic Stress Does Not Alter the Epicuticular Wax Crystal Structure

The surface waxes often form complex crystalline microstructures, that originate from self-assembly processes (Bargel *et al.*, 2006; Koch *et al.*, 2006). The relationship between wax morphology and chemistry was reviewed by Baker (1982). Jetter and Riederer (1994, 1995) found that the shape of wax crystals is largely determined by the predominating wax compound. Based on observations of the leaf and leaf-sheath waxes of cereals, Lundqvist *et al.* (1968) proposed a crystallization in the shape of plates as a result of the primary alcohol fraction.

However, in our study, as already described for untreated wild-type, the changed chemical composition of etiolated plants was unalterably dominated by primary alcohols. Thus, the epicuticular wax crystal structure exhibited the typical platelet form. As expected, the wax microstructure and associated leaf surface wettability were not affected. According to literature, low leaf wettability was assumed to correlate with high density of epicuticular crystalline waxes (Holloway, 1970; Beattie & Marcell, 2002). However, the cuticular wax reduction in etiolated leaves did apparently not suffice enough to influence density of epicuticuler wax crystal bodies. Although cadmium-treatment exhibited elevated amounts of surface waxes, this was also not distinctly reflected in the density of epicuticular wax crystals.

Since crystal morphology and density of wax platelets was also not affected by salt- and drought-stress, the weak alteration of surface hydrophobicity on NaCl treated plants might be explained by traces of salt crystals, that had probably contaminated the leaf surfaces upon growth in hydroponics, thus influencing the contact angle measurements.

2.5 Biotic Stress Due to *Bgh*-Infection: Wax Characteristics Remain Unaffected

Neither 6d locally *Bgh* infected leaf tissue, nor later emerged leaves of systemically infected test plants exhibited alterations in wax load and composition. This was contradictory to the study of Uchiyama *et al.* (1989), who reported a 12% elevated aldehyde portion, accompanied by an 8% increase of wax esters, due to local *Bgh*-infection. Simultaneously, the primary alcohol fraction was 20% increased.

However, the relative composition of the barley control plants applied in this study, distinctly deviates from that of wild-type cv Bonus investigated in our experiments. It remains unsolved, in how far cultivar dependent variations, or the utilization of different solvents, might lead to such differences. Nevertheless, the highly sensitive GC-MS detection methods, used in our analytical approach, did not exhibit changes in chemical composition that might be attributed to powdery mildew infection.

3. Modifications of *Cer*-Mutants´ Wax Characteristics

3.1 Alterations in *Cer*-Mutants´ Total Leaf Wax Coverage and Composition

Providing a basis for studies of fungal pre-penetration processes (chapter III), the discussion of analytical wax data is restricted to a selection of five screened *cer*-mutants most distinctly impaired in chemical composition, epicuticular crystal structure, and surface hydrophobicity of their leaf waxes, in comparison to the wild-type.

The barley mutant line *cer*-j.59, that exhibited distinctly reduced leaf wax coverages (von Wettstein-Knowles, 1971; Giese, 1976), has been selected primarily because of its elevated cuticular wax ester portion (Tab. I). In comparison to Bonus wild type, this specific increase was attributed to the drastically decreased amount of 1-hexacosanol in *cer*-j.59 wax (von Wettstein-Knowles, 1974; Giese, 1976). When compared to the wax composition data of primary leaves (Giese, 1976), this distinct decrease in primary alcohols was even more pronounced in the secondary leaves of *cer*-j.59, resulting in equal amounts of primary alcohols and esters in the cuticular wax of 14d old plants examined in the present study (Tab. I). In accordance with von Wettstein-Knowles (1971), the leaf

wax coverage of *cer*-zd.67 exhibited a distinctly reduced total amount, yet no substantial differences in the proportions of the lipid classes composing this wax were recognized. To the best of our knowledge, with respect to *cer*-zh.54, *cer*-yp.949 and *cer*-yj.667, no data on the chemical composition of the respective leaf waxes is available at present. The mutant line *cer*-zh.54 has been selected because of its approximately doubled relative portion of esters (Tab. I). In contrast, *cer*-yp.949 and *cer*-j.59 exhibited a massive decrease of primary alcohols and, unlike *cer*-j.59, a distinct increase in the proportion of aldehydes. The aldehyde fraction of the barley leaf waxes was of particular interest, since the presence of aldehydes (C_{26} and C_{30}) was found to have a high *Bgh* differentiation-stimulating activity (Tsuba *et al.* 2002). The phenotypical description of *cer*-yj.667 (Lundqvist & Franckowiak, 1997) proposed absence of wax coating on the blades on the upper three leaves of the plant. However, our results demonstrated that wax amounts on the second leaf of 14d old plants of *cer*-yj.667 were in fact comparable with Bonus wild-type. This obvious difference might be due to sampling at different time points of plant development.

3.2 Alterations in *Cer*-Mutants´ Epi- and Intracuticular Wax Portions

With the mechanical removal of epicuticular waxes by applying glue-like aqueous *gum arabic*, separate analyses of epi- and intracuticular waxes became possible (Jetter & Schäffer, 2001). This useful sampling strategy has yielded direct information on the surface composition of diverse plant species: the epi-cuticular wax film on *Prunus laurocerasus* leaves was found to be dominated by aliphatic acetates, alcohols, and alkanes, depending on leaf developmental stages, while the intracuticular wax fraction was mainly characterized by triterpenoids (Jetter *et al.*, 2000; Jetter & Schäffer, 2001). Significant differences in the epi- and intracuticular wax compositions have been reported for leaves of *Prunus laurocerasus* (Jetter *et al.*, 2000; Jetter & Schäffer, 2001), *Nepenthes alata* (Riedel *et al.*, 2003) and tomato fruits (Vogg *et al.*, 2004; Leide *et al.*, 2007).

Our analyses of barley leaf waxes showed a similar composition of epi- and intracuticular wax layers, irrespective of their adaxial or abaxial origin (data not shown). With respect to the significantly changed distribution of epi- and intracuticular wax portions in *cer*-yp.949 and *cer*-j.59, one may assume that such variations in surface wax distribution of barley *cer* mutants are based on modified

processes involved in wax transport and arrangement of wax components within the cuticle layer.

3.3 Alterations in *Cer*-Mutants´ Wax Crystal Structure and Surface Hydrophobicity

Among the structural characterization of several *cer*-mutants, Lundqvist *et al.* (1968), and von Wettstein-Knowles (1974), described the wax structures on the leaves of the *cer*-j.59 mutant as thin plates pressed to the cuticle with few large irregularly shaped bodies. Our results correspond to this description of the extensively modified chemical composition of *cer*-j.59 wax. The almost plate-less outward appearance of the *cer*-j.59 leaf surface (Fig. 1F) may point to the distinctly reduced portion of primary alcohols, which are considered to be responsible for the plate-like occurrence of epicuticular wax crystal bodies. This goes in line with the largely altered surface structure of *cer*-yp.949. Again, the widely missing vertical wax plates (Fig. 1C) lead to the assumption that the content of primary alcohols is reduced in the *cer*-yp.949 leaf surface wax. This was confirmed by our chemical analyses. Nevertheless, *cer*-yp.949 exhibited a wax-crystal micro-morphology distinctly different from that of *cer*-j.59. One may speculate that this difference refers to the increased aldehyde fraction present in the cuticular leaf wax of *cer*-yp.949.

However, during re-crystallization of plant leaf waxes, the underlying substrate appears to be an important determinant of wax crystal morphology (Koch *et al.*, 2006). Hence, the cuticle structure itself may influence the formation of wax crystal structures as well. In contrast to *cer*-yp.949 or *cer*-j.59, the wax mutant *cer*-zd.67 was described to possess leaf areas covered with almost the same density of similar sized rods, and simple plates, as the wild-type, though wax crystals more often formed tiny rods, and more angular, rather than lobed, plates, on the upper epidermis of this mutant (von Wettstein-Knowles, 1971). However, due to our observations, *cer*-zd.67 simply exhibited a reduced wax crystal density, in combination with distinctly smaller wax plates. Such differences could be explained by different conditions during cultivation, and distinct leaf developmental stages, assayed in both studies. Nonetheless, *cer*-zd.67 leaves were covered with a wax coat morphologically different from that of “Bonus” wild type. From the enlarged wax plates formed on leaves of *cer*-yj.667, one might infer that even

small changes concerning the chemical composition of cuticular waxes, as the observed substantial increase in fatty acids, could result in considerable modifications of size and shape of wax crystal bodies.

Leaf surfaces exhibiting a strongly pronounced three-dimensional wax crystal structure, as *cer*-yj.667 and Bonus wild-type, tend to result in increased hydrophobicity levels. Correspondingly, Holloway (1970) and Beattie and Marcell (2002) reported a correlation of pronounced water repellency and high density of crystalline epicuticular waxes.

CHAPTER II:

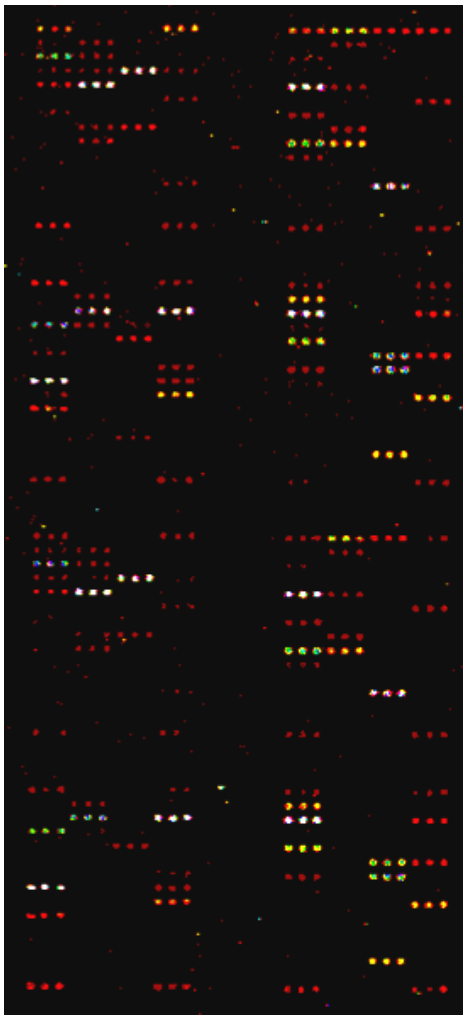
Gene Expression Studies Investigating Different Aspects of Wax Biogenesis

RESULTS

In order to confirm potential environmental factors, which induce wax biosynthesis associated processes, this chapter presents a correlation of transcriptional modifications in response to different external stressors, with alterations in the surface wax characteristics due to these respective stresses. Furthermore, the interference of powdery mildew infection with several aspects of leaf wax biogenesis demonstrates the molecular level of the host/ pathogen interaction.

1. The Barley Wax-Microarray

In this study, a barley wax-microarray has been successfully established, (Fig. 1) in order to investigate the transcriptional activity of several aspects of wax biosynthesis and transport of wax associated components.



The design of the barley wax-microarray took advantage of barley ESTs and gene sequences putatively functioning within processes of fatty acid biosynthesis, fatty acid elongation and modification of very long chain fatty acids (VLCFAs), as well as lipid transfer proteins (LTPs) and ABC-transporters, which probably support the lipid trafficking between cell compartments and transport of wax components for cuticle formation. Additionally, supposed housekeeping genes and putatively stress regulated genes were integrated, resulting in a total number of 254 oligo-nucleotide sequences.

Figure 1: The barley wax-microarray: an oligo-nucleotide microarray consisting of 254 unique 70mers, focusing several aspects of wax biosynthesis in barley leaves.

To demonstrate the benefit of the wax-micorarray, two experimental setups were carried out: while the first approach screens the molecular responses of barley leaves to salt-, cadmium-, and drought- stress, as well as deficiency of light (darkness-treatment), henceforth termed “stress-microarray”, the second experiment examined the potential molecular effects of powdery mildew (*Blumeria graminis* f.sp. *hordei*) infection on barley, and was therefore described as “*Bgh*-microarray”.

For both approaches, the 254 target genes were arranged into putative functional categories (I-V, Tab. I) involving subordinated groups in order to create sections of issues which can be more comprehensively compared with the detected modifications of surface waxes presented and discussed in chapter I, and to exemplify time dependent expression trends upon processing *Bgh*-infection.

Table I: Arrangement of 254 investigated oligo-nucleotide sequences in putative functional categories focused in the barley wax-microarray. No.= absolute number of genes included in the respective group, Signal: relative signal intensity of the respective group as proportion of the signal intensity of total spots representing the initial molecular state of non-treated leaves (16 experiments including dye switch with a series of 6 spots per oligo-nucleotide).

Category	Putative function	No.	Signal (%)
I	Fatty acid synthesis/ Elongation/ Modification	**∑ 116**	**43**
II	*a* Lipid transfer proteins	51	19
	b Carrier/ Transferases	13	7
	c ABC-transporters	19	9
		∑ 83	**35**
III	Stress	**39**	**16**
IV	Others	**7**	**1**
V	Housekeeping	**9**	**5**
		∑ 254	**100**

The major part of the barley wax-microarray targeted 199 genes involved in processes that potentially operate fatty acid biosynthesis, fatty acid modification, and transport of wax components (category I&II, Tab. I). Genes in category I probably encode condensing enzymes of the plastidial fatty acid synthesis (FAS)-complex, and enzymes known to take part in the fatty acid elongation (FAE)-complex, e.g. β-ketoacyl CoA-reductases or -synthases. Moreover, such genes which are supposed to modify VLCFAs resulting in certain component classes,

e.g. wax synthases generating wax esters or decarbonylases which support the modification of aldehydes to *n*-alkanes, were involved in category I. For component transport, the functionality of ABC-transporters, channel specific carriers, and lipid transfer proteins in intracellular lipid-transport, as well as in processes of cutin and wax formation, are discussed. Thus, potential ABC-transporter encoding genes, several carrier or transferases, and genes of different classes of lipid transfer proteins, constitute category II. In sum, category I & II comprise approximately 78% of the total signal intensity of the barley wax-microarray.

Additionally, several putatively stress regulated genes, like PR-proteins, or several classes of dehydrins, and genes involved in plant growth and development (e.g. encoding senescence associated proteins), were included in category III. A selection of supposed housekeeping genes (e.g. actin, ubiquitin) was additionally integrated and summarized as category V.

The barley wax-microarray exhibited a pronounced sensitivity to a high range of signal intensities, which was reflected in several orders of magnitude within the numerical dataset. For our experimental setting, the amounts of isolated RNA for the stress-microrarray was not sufficient to apply 20*µ*g for cDNA synthesis, as it was done in the *Bgh*-microarray. Therefore, the stress-microarray was carried out with 8*µ*g RNA for each sample. According to this 2.5-fold reduced amount of applied RNA, signal intensities for the stress-microarray were about one-third weaker compared to those of the *Bgh*-microarray. Furthermore, signal intensities of print puffers (lowest level used as reference for non-expressed signals) differed within both experiments. Consequently, the minimum level for expressed signals within the stress-microarray was defined as >0.1 and expression levels passing the high threshold value of >1 were regarded as strong signals (for the *Bgh*-microarray >0.5 and >2.5, respectively).

At first, the differences between relative signal intensities of treatment minus controls, is presented in functional categories for both- “stress”- and “*Bgh*”-microarray. On the one hand, this procedure allows the demonstration of the highly different modifications between the expression patterns of the four abiotic treatments within the stress-microarray, on the other hand, the impact of the time-dependent development of the *Bgh*-infection can be verified. Subsequently, single differentially expressed genes are introduced to correlate certain molecular

aspects of wax biosynthesis and wax arrangement with observed analytical wax data (presented and discussed in chapter I).

2. Stress-Microarray: Transcriptional Events of Wax Biogenesis in Barley Leaves in Response to Different Abiotic Stress-Treatments

Compared to native controls, all stress treatments led to a reduction of signal intensities (Tab. II), which resulted in an increased proportion of signals <0.1. The major part of the modifications were found for signal intensities between 0.1-1, while the proportion of strong signals >1 was hardly affected by the different abiotic treatments.

Table I: Distribution of signal intensities (nVol) within the stress-microarray.

signal intensity	control	**darkness**	control	**NaCl**	control	**cadmium**	control	**drought**
<0.1	90	**187**	174	**181**	23	**126**	19	**174**
0.1-1	142	**49**	58	**52**	210	**110**	219	**59**
>1	22	**19**	22	**22**	21	**19**	26	**22**

2.1 Trends of Gene Expression in Functional Categories in Response to Different Abiotic Stresses

The transcriptional profiles, which are based on the created functional categories, differed distinctly between the four abiotic treatments (Fig. 2).

Darkness-treatment: in etiolated leaves, the proportion of category I and category II signals was distinctly increased. Signal intensities of categories III-V were decreased due to this treatment, which was pronounced for putatively stress regulated genes (III), and selected housekeeping genes (V).

NaCl-treatment: the group signal intensities were weakly modified by salinity-stress. A slight increase revealed for group of ABC-transporters (IIc), while signals of stress regulated genes (III) and involved housekeeping genes (V) were weakened.

Cadmium-treatment: cadmium exposure led to an expression pattern comparable to that of the NaCl treatment, with the exception of the distinct

decrease in signals of FA-synthesis (I), and a minimum increase in housekeeping genes (V).

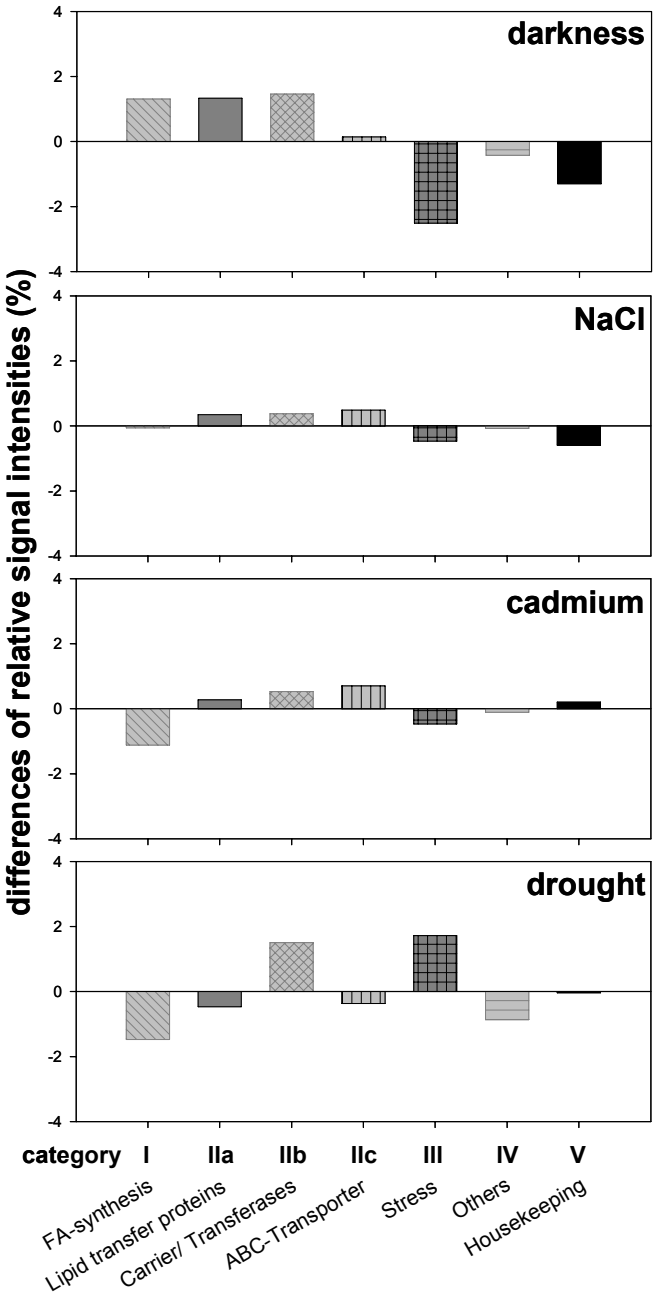


Figure 2: Differences of relative signal intensities (treatment - control) per category between plants of different abiotic treatments (darkness, NaCl, cadmium and drought, see materials and methods 2.0)) in the stress-microarray. Total pixel intensity was defined 100% and the series of signals for each oligo-nucleotide was used to calculate relative expression. Deviations from zero indicate trends of expression modifications. Positive values imply up regulation, negative values indicate down-regulation due to the respective treatment.

Drought-treatment: in drought stressed plants, signal intensities of targeted sequences involved in category I and category IV were distinctly down-regulated. The three groups screening lipid-transport in category II responded inhomogenously. Groups of lipid-transfer proteins (IIa) and ABC-transporters (IIc)

slightly decreased, while signal intensities of carrier/ transferases (IIb) increased. Most prominent up-regulation was given for group of putative stress regulated genes (III). Signal intensities of screened sequences involved in processes of housekeeping (V) were hardly affected by this treatment.

Within the treatments, darkness and NaCl differences in category IIb were strikingly affected by the high signal intensity of hydroxylcinnamoyl transferase (161), which dominated 77% of the group´s signal intensity.

2.2 Impact of Different Abiotic Stresses on the Gene Expression Pattern

Darkness-treatment: in etiolated leaves, the average signal ratios tended to shift to that of native controls, given that most of the calculated ratios were below 1 (Fig. 3). A total number of 118 sequences was differentially expressed in this treatment (ratio <0.5 and >1.5). A more than 1.5-fold up-regulation was given for probable β-keto-acyl reductase (oligomer number 4), putative *fiddlehead*-like protein (5), acyl-CoA thioesterase (34), *glossy1* homolog (60), omega 3 fatty acid desaturase (94), similar to probable non-specific lipid transfer protein (115) and histon (203), while the most striking down-regulation <0.3 revealed for fatty acyl-CoA reductase (12), acyl-CoA binding protein (22), CUT1 (43), fatty acyl-CoA reductase (51) and elongation factor 1-alpha (199).

NaCl-treatment: salinity-stress led to different expression of only 31 genes. This low number included the homologous to *glossy1* protein (69), very long chain fatty acid condensing enzyme (97), lipid transfer protein (108), probable phospholipid transfer protein (114), non-specific lipid transfer protein 4.3 (120), phospholipid transfer protein precursor (154), lipid transfer-like protein (157), basic pathogenesis related protein 5 (211), dehydrin 4 (213), and an APETALA2-like protein (229), which were accumulated 1.5-fold within salinity stress. A few sequences showed a distinct down-regulation <0.3-fold - an ABC-transporter (1), β-keto-acyl reductase (25), and fatty acyl-CoA reductase (49).

Cadmium-treatment: the cadmium exposition had the weakest effect on signal ratios. 27 genes were differentially expressed, among them the most prominent up-regulation was given for the non-specific lipid transfer protein 4.2 (117, 1.5-fold), and the strongest down-regulation was revealed for a non-specific lipid transfer protein 4.1 (119, ratio <0.4).

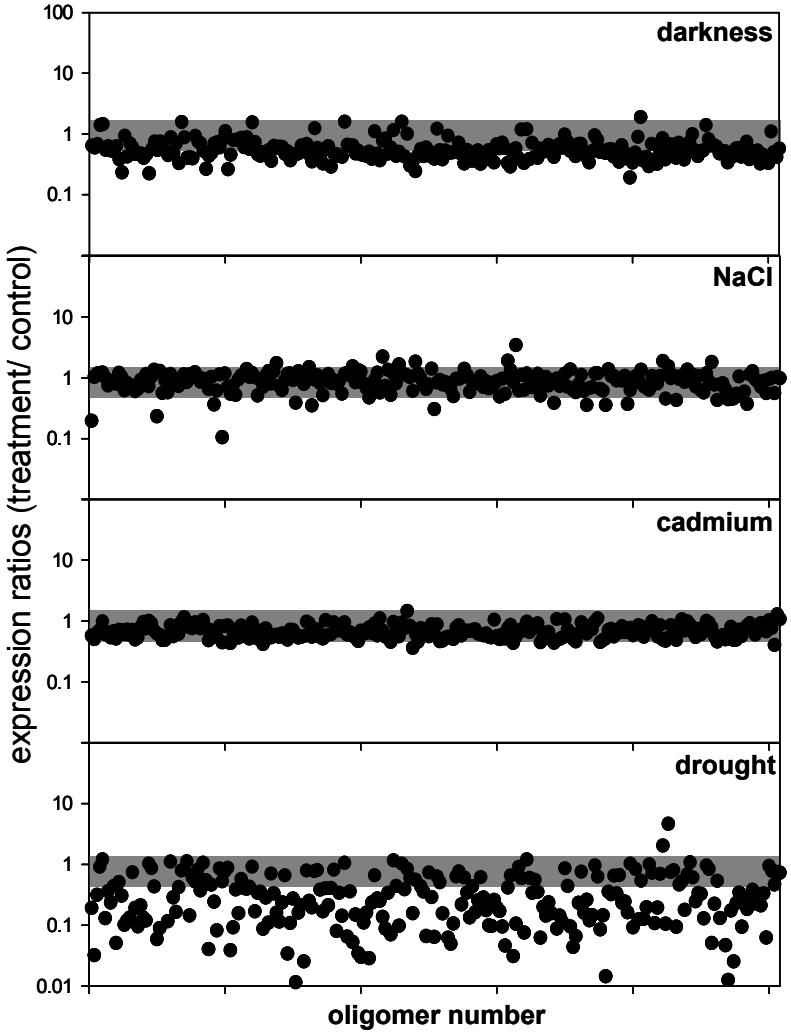


Figure 3: Ratios of signal intensities (treatment/ control) due to different abiotic stresses. Darkness: plants grown in darkness for 14 days, NaCl: plants grown on nutrient solution with 1% NaCl, cadmium: 14d old cadmium stressed plants (500 mM), drought: 21d old drought stressed plants. Values passing the high threshold 1.5 indicate up-regulation while a decrease below the threshold of 0.5 implies down-regulation of expression due to abiotic stresses. Values not strikingly differing through treatment remain within grey background bars. Given are means of (n=12) normalized and background corrected signal values (nVol). Oligomer number: internal oligo-nucleotide number (see appendix table III).

Drought-treatment: drought-stress enforced the most striking modifications within the expression pattern, 182 of the screened genes (72% of total signal) were differentially expressed, while the predominant number of these genes was down-regulated (Fig. 3). Merely two transcripts, namely the basic pathogenesis related protein 5 (211) and dehydrin 4 (213) accumulated 1.4-fold compared to controls. A dramatic down-regulation to <0.02 was observed for acyl carrier protein I chloroplast precursor (2), β-keto-acyl-CoA synthase (52), similar to *CER1* (73), *CER1*-like protein (79), putative FAE1 (99), very long chain fatty acid condensing enzyme (100), probable non-specific lipid transfer protein precursor (156), PDR-like ABC-transporter (190), steroid 5-alpha reductase (235) and probable amylase/ protease inhibitor (237).

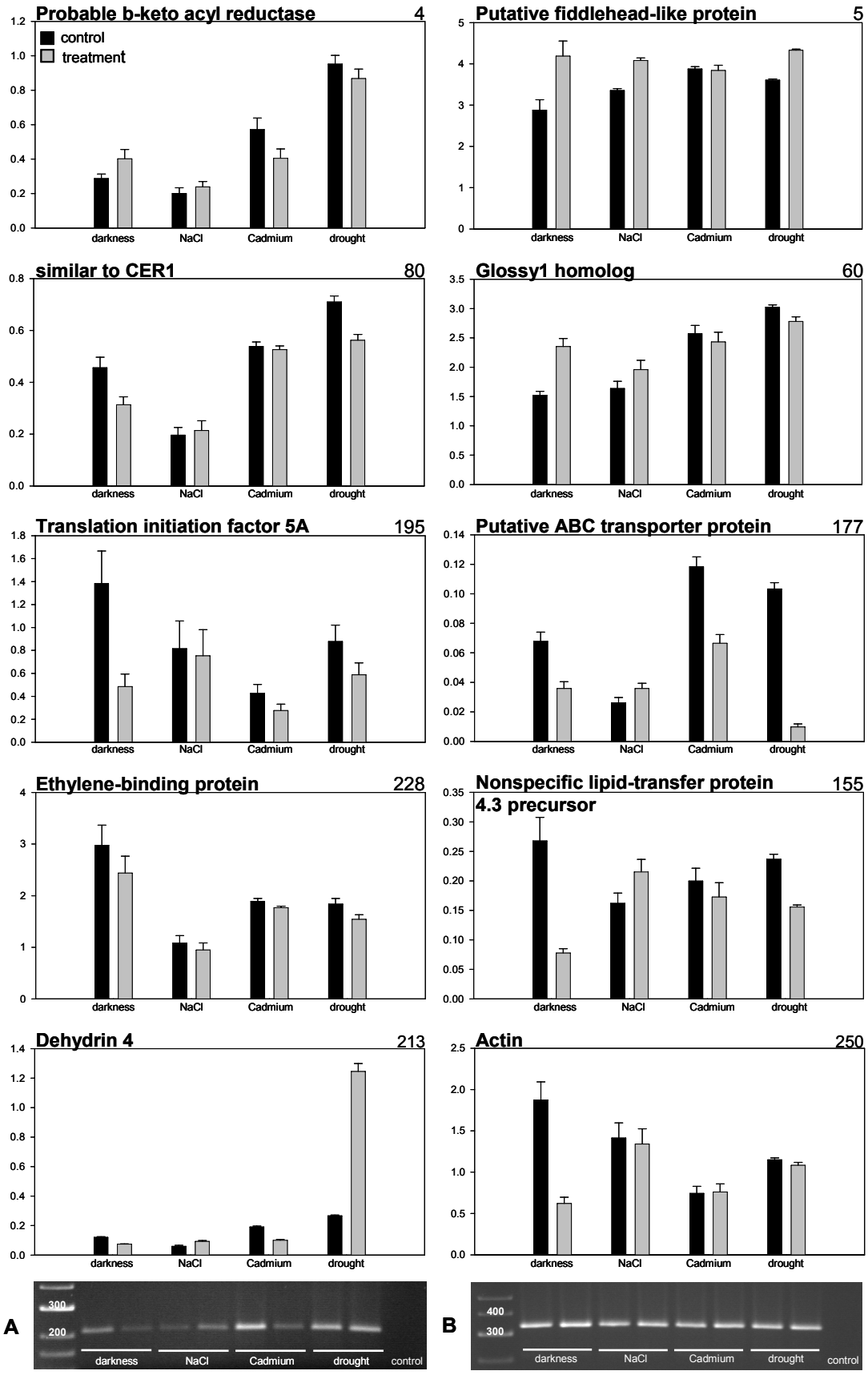

Probable b-keto acyl reductase 4
control
treatment
Putative fiddlehead-like protein 5
similar to CER1 80
Glossy1 homolog 60
Translation initiation factor 5A 195
Putative ABC transporter protein 177
Ethylene-binding protein 228
Nonspecific lipid-transfer protein 155
4.3 precursor
Dehydrin 4 213
Actin 250
darkness
NaCl
Cadmium
drought
A
300
200
control
B
400
300
18 S rRNA
C

Figure 4: Expression levels of a selection of barley genes, which were differentially regulated in response to four abiotic stresses (grey bars): darkness: plants grown in darkness for 14 days, NaCl: plants grown on nutrient solution with 1% NaCl, cadmium: cadmium stressed plants (500 mM), drought: 21d old drought stressed plants, compared to native controls (black bars). Given are means ±SE of (n=12) normalized and background corrected signal values (nVol). Top right number represents the number of the internal register (see appendix table III). A-C independent semiquantitative confirmation of the microarray data by RT-PCR. Agarose gel with PCR-products of dehydrin 4 (A), actin (B) and 18S rRNA as control of respective treatments (C).

2.3 A Selection of Genes Differentially Expressed in Response to Different Abiotic Stresses

In order to demonstrate a variety of highlights within the transcriptional modifications, in response to the different abiotic stresses, a selection of 10 genes is presented below. For ratios of the top 20 differentially expressed genes due to the respective stress-treatments see appendix table I.

Darkness-treatment: in etiolated leaves, a total of 118 genes were differentially expressed. 5 of those- integrated in category I- were up-regulated including probable β-keto-acyl reductase (oligomer number 4), putative *fiddlehead*-like protein (5), and *glossy1* homolog (60, Fig. 4). In comparison to the other abiotic stress treatments, the darkness treatment led to a most striking up-regulation of the genes 1.4-, 1.5-, and even 1.6-fold in mentioned order. 113 genes of various expression levels were down-regulated through etiolation.
Among these genes, similar to *cer1* (80), translation initiation factor 5A (195), ethylene-binding protein (228), putative ABC-transporter protein (177), non-specific lipid transfer protein 4.3 precursor (155), dehydrin 4 (213), and actin (250, Fig. 4) were most prominent. Signals of ethylene-binding protein (228) and similar to *cer1* (80) were weakly affected by the treatment (0.8- 0.7-fold), while signal of dehydrin 4 (213) was down-regulated 0.6-fold.

NaCl-treatment: in the NaCl treatment, signal intensities were hardly affected. Slight variations (1-1.2-fold) due to salt-stress were revealed for actin (250), similar to *cer1* (80), probable β-keto-acyl reductase (4), putative *fiddlehead*-like protein (5) and *glossy1* homolog (60). Stronger effects (1.3-1.5-fold) appeared for non-specific lipid transfer protein 4.3 precursor (155), putative ABC-transporter protein (177) and dehydrin 4 (213, Fig. 4). 21 sequences were down-regulated to ratios of <0.5, affecting genes involved in several groups of the wax microarray, e.g. translation initiation factor 5A (195) and ethylene-binding protein (228).

Cadmium-treatment: a total number of 27 sequences exhibited reduced signal intensities during cadmium exposition, affected were genes operating

diverse aspects of wax biogenesis. Striking down-regulation (0.5- 0.6-fold) was found for dehydrin 4 (213), putative ABC-transporter protein (177), translation initiation factor 5A (195), and probable β-keto-acyl reductase (4). In comparison to controls, none of the modified signals was increased in the cadmium-treatment.

Drought-treatment: out of a total of 183 differentially regulated genes, two were clearly up-regulated (>1.5) in the drought treatment, namely pathogenesis related protein 5 (211) and dehydrin 4 (213, Fig. 4). Signal of dehydrin 4 culminated in a 4.7-fold increase in stressed plants. While screened probable β-keto-acyl reductase (4), *glossy1* homolog (60), ethylene-binding protein (228), and actin (250), were almost unaffected, the signal decrease of translation initiation factor 5A (195), and non-specific lipid transfer protein 4.3 precursor (155), was decreased to 0.7-fold upon drought-stress. Signal of similar to *cer1* (80) was 0.8-fold decreased and the most striking down-regulation (0.1-fold) was revealed for putative ABC-transporter protein (177).

3. *Bgh*-Microarray: Transcriptional Events of Wax Biogenesis in Barley Leaves During Powdery Mildew Infection

3.1 The Transcriptional Profile of the *Bgh*-Microarray

The expression pattern of the different time points within the *Bgh*-microarray showed different signal intensities ranging over several orders of magnitude (Fig. 5). During the time course of the *Bgh*-infection, single ESTs attained different signal intensities. High signal intensities (>10) of both, infected and control tissue, were obtained from several genes grouped in category I: putative *fiddlehead*-like protein (oligomer number 5), β-keto-acyl-CoA synthase (23), β-keto-acyl-ACP synthetase I (30), omega 3 fatty acid desaturase (94), and transport associated genes like type 2 non-specific lipid transfer protein (112), hydroxycinnamoyl transferase (161), PDR-like ABC-transporter (186), translation initiation factor 5A (195). Also, indicators for stress responses (category V), e.g. dehydrin 11 (215), were expressed on a high level.

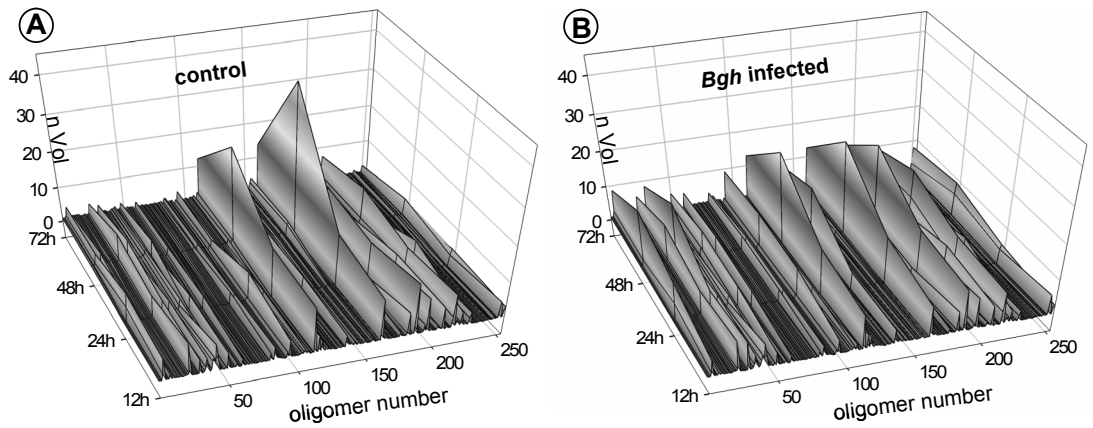


Figure 5: Expression analysis of 254 barley genes screened in the barley wax-microarray due to *B. graminis* infection of different inoculation intervals (12h-72h). A- expression pattern of non-infected control plants, B- expression pattern of *Bgh* infected leaf tissue. Given are means of (n=12) normalized and background corrected signal values (nVol). Oligomer number: internal oligo-nucleotide number (see appendix table III).

Modification of signal intensities through *Bgh*-treatment occurred in both directions, up and down, for several sequences embedded in various categories. The total expression level of untreated control plants (Fig. 5A) also varied between the different time points of tissue harvesting. Within the experimental progress, total expression trended to be intensified among controls. The major part of the signals exhibited intensities <0.5. Rather those signals were affected upon *Bgh*-infection, than the signals of intensities in the range of 0.5-2.5.

3.2 Trends of Gene Expression Due to *Bgh*-Infection in Functional Categories

To gain a general impression to which extent infection with powdery mildew affects molecular events in the *Bgh*-microarray, the relative differences in functional categories of treatment minus control signals were considered (Fig. 6). During the first 12h-24h interval, some of the regulation trends were reversed, while they were enhanced during the 24h-48h interval of the infection.

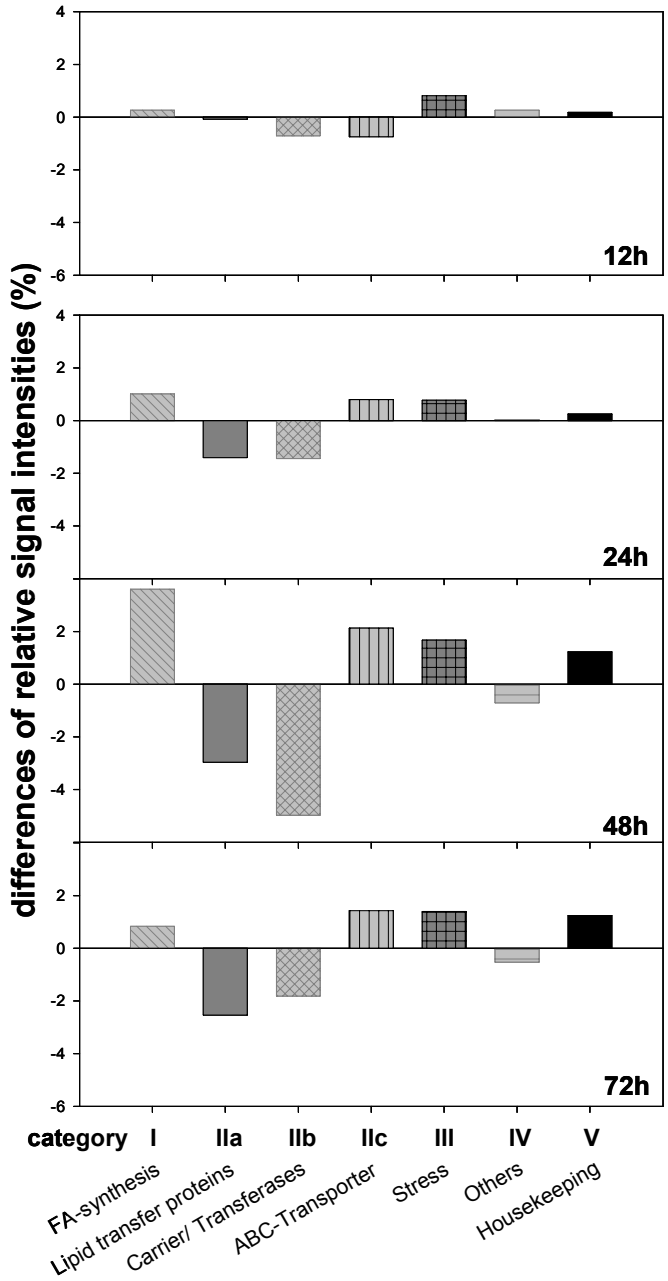


Figure 6: Calculated differences of relative signal intensities (*Bgh*-infection - control) per category after different incubation periods (12h-72h) in the *Bgh*-microarray. Total pixel intensity was defined 100% and the series of signals for each oligo-nucleotide was used to calculate relative expression. Deviations from zero indicate trends of expression modifications. Positive values imply up-regulation, and negative values indicate down-regulation due to fungal infection.Since the first 12h of *Bgh*-incubation occurred within a full light period, the 12h dataset was separated in the graphic.

Already after 12h, weak alterations in group signals due to *Bgh*-infection were detected. Slight up-regulation of relative signals occurred for carrier/ transferases (category IIb), and ABC-transporters (IIc), while signals of indicators for stress responses (III) increased. It is important to notice, that the first 12h of *Bgh*-infection occured under light conditions, whereas the following daily screenings include a full 16h/ 8h light/ dark period.

After 24h, category I (fatty acid biosynthesis /elongation/ modification) was up-regulated, and signals of category IIa (lipid-transfer proteins) distinctly decreased. The decrease of category IIb (carrier/ transferases) expression level became more accentuated, while category IIc (ABC-transporters) was reversed, in comparison to 12h post inoculation. The level of putatively stress responsive genes (III) remained.

After 48h incubation time, the transcriptional profiling showed strongly accentuated differences between native and *Bgh*-infected leaf tissue. Group signal differences within category I-III achieved maximum levels. In addition, category IV slightly decreased, while category of putative housekeeping genes (V) was distinctly up-regulated.

With further incubation (72h), the regulation trends remained more or less unchanged, in general the described effects were solely weakened.

3.3 Impact of *Bgh*-Infection on the Expression Pattern

12h Bgh-infection: 12h incubation with powdery mildew led to the differential expression of a number of 53 genes, most of which tended to be up-regulated trough the infection (ratio >1.5, Fig. 7). Transcripts of fatty acyl-CoA reductase (oligomer number 49), acyl-CoA reductase-like protein (54), weakly similar to CER1-like protein (75), ATPase 11 (189), and a dehydrin (210), accumulated more than two-fold in infected plants.

24h Bgh-infection: the time course of the *Bgh*-microarray highlighted 90 differentially expressed genes after 24h of inoculation (Fig. 7). In comparison to controls, signal intensities of several genes in category I & II were more than three-fold increased due to *Bgh* inoculation including acyl carrier protein I chloroplast precursor (2), β-ketoacyl-ACP synthase (20), β-ketoacyl synthase (52), and non-specific lipid transfer protein 1 (130).

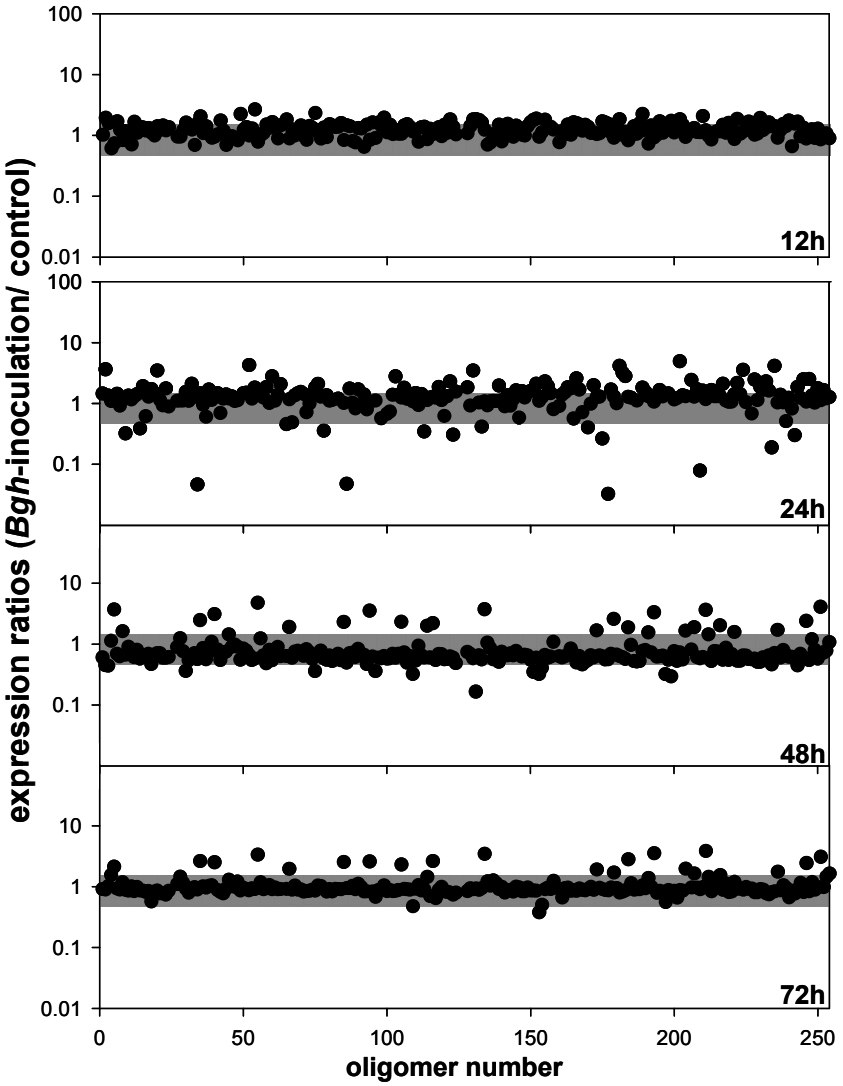


Figure 7: Signal ratio of *B. graminis* (*Bgh*) inoculated plants to native control plants of corresponding time interval (12h-72h) per internal oligo-nucleotide number (oligomer number, see appendix table III). Since the first 12h of *Bgh*-incubation occurred within a full light period, the 12h dataset was separated in the graphic. Values passing the high threshold of 1.5 indicate up-regulation while a decrease below the threshold of 0.5 implies down-regulation of expression through fungal infection. Values not strikingly differing among *Bgh* treatment remain within grey background bars. Given are means of (n=12) normalized and background corrected signal values (nVol).

A striking down-regulation to a ratio of < 0.1 was given for acyl-CoA thioesterase (34), putative fatty acid elongase (86), and putative ABC-transporter protein (177). Among categories III-V signals of cytosolic HSP90 (202), and senescence associated protein (224), were also reinforced, in contrast signals of pathogenesis related protein 10 (209) were dramatically down-regulated.

48h Bgh-infection: after 48h of *Bgh*-inoculation, the former spreading of signal ratios was restricted, as 43 differentially expressed genes were detectable after that infection period. Prominent candidates more than three-fold up-regulated were putative *fiddlehead*-like protein (5), 3-ketoacyl-CoA reductase (40), cytosolic acetyl-CoA carboxylase (55), omega 3 fatty acid desaturase (94), and a 7 kDa lipid

transfer protein (134). A down-regulation <0.3 occurred for a lipid transfer protein-like sequence (131), and elongation factor 1-alpha (199). In contrast to these genes, which are potentially operating in fatty acid biosynthesis and lipid-transport (category I & II), basic pathogenesis related protein 5 (211), as one indicator for stress responses (category V), and ubiquitin-protein ligase E3- alpha-like (251), were more than two-fold increased.

72h Bgh-infection: out of a total of 20 differentially expressed genes 17 were distinctly up-regulated after 72h *Bgh*-infection. Within these candidates, all accumulated transcripts of time point 48h occurred again. Additionally, a non-specific lipid transfer protein 4.1 (116), and a putative ATP dependent transmembrane transporter, (184) were detected, which were both based on a doubled expression level, due to powdery mildew infection. In contrast, a selection of lipid transfer proteins was distinctly reduced <0.5-fold: lipid transfer protein (109), 7 kDa lipid transfer protein (153), and phospholipid transfer protein precursor (154).

3.4 A Selection of Genes Differentially Expressed in Response to *Bgh*-Infection

In order to demonstrate the variety of molecular aspects of host/ fungus interaction, a selection of 10 genes, which were differentially expressed in response to *Bgh*-infection, is presented below. For ratios of the top 20 differentially expressed genes, depending on the different *Bgh*-incubation periods, see appendix table II.

Category I: out of 116 ESTs involved in category (I) (fatty acid synthesis/ elongation/ modification), 25 candidates exhibited strong signal intensities >10. Among these, 24 signals were up-regulated, including putative *fiddlehead*-like protein (oligomer number 5), translation initiation factor 5A (195), putative wax synthase protein (83), and *glossy1* homolog (60, Fig. 8). 0.5-fold down-regulation occurred for signal intensities of short chain alcohol dehydrogenase (93), after 48h of infection, and was weakened further with progressing incubation time (72h). The expression patterns and signal intensities of putative *fiddlehead*-like protein (5,) and translation initiation factor 5A (195), were nearly similar: in infected tissue the signals increased until 48h *Bgh*-infection, with further incubation (72h) this effect was weakened for putative *fiddlehead*-like protein, while it was further increased for translation initiation factor 5A.

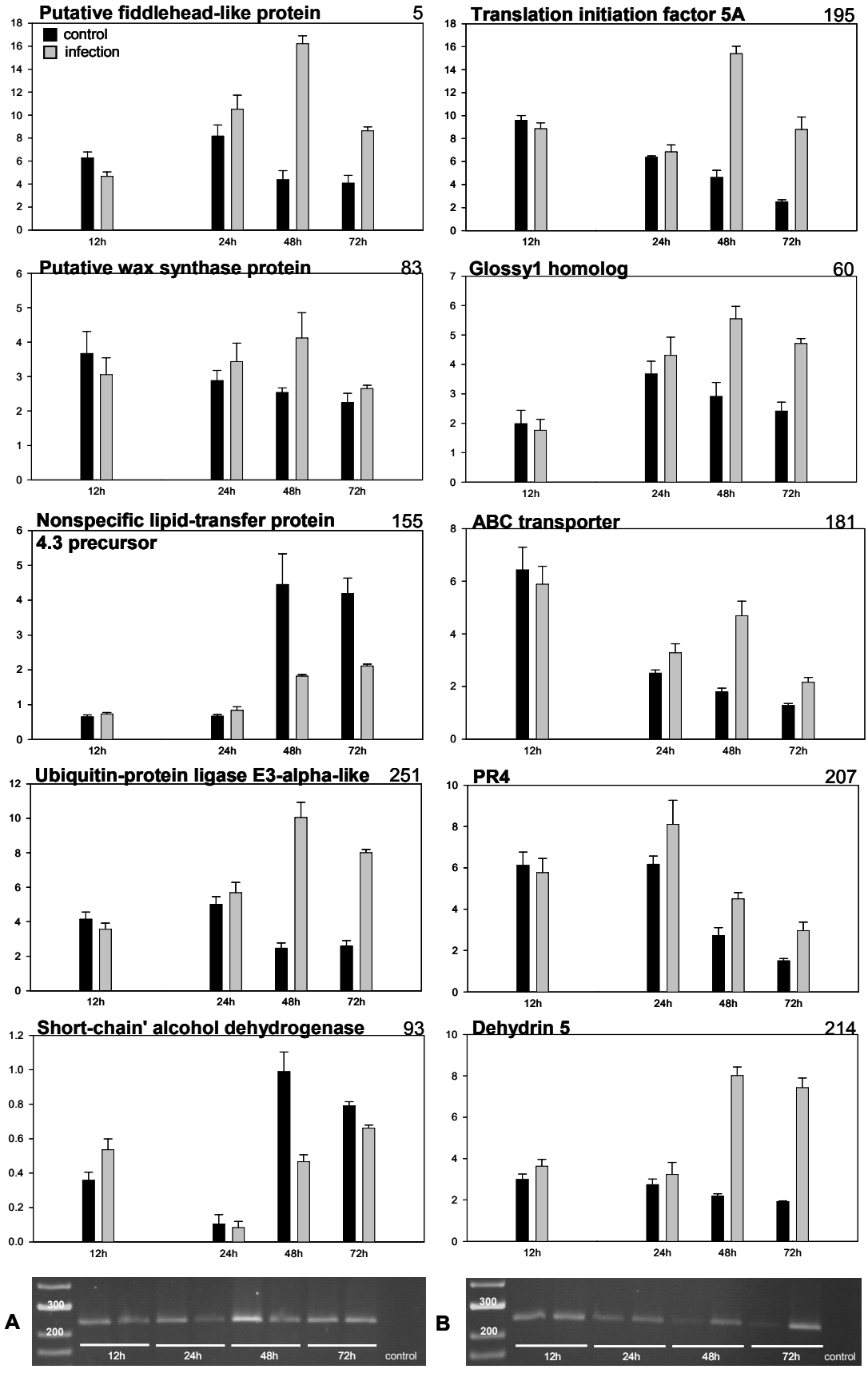

Putative fiddlehead-like protein 5
control
infection
Translation initiation factor 5A 195
Putative wax synthase protein 83
Glossy1 homolog 60
Nonspecific lipid-transfer protein 155
4.3 precursor
ABC transporter 181
Ubiquitin-protein ligase E3-alpha-like 251
PR4 207
Short-chain' alcohol dehydrogenase 93
Dehydrin 5 214
12h
24h
48h
72h
A
B
C
300
200
control
18 S rRNA

Figure 8: Expression levels of a selection of barley genes which were differentially regulated in response to *B. graminis* infection (grey bars), within time periods between 12h and 72h compared to native controls (black bars). Since the first 12h of *Bgh*-incubation occurred within a full light period, the 12h dataset was separated in the graphic. Given are means ± SE of (n=12) normalized and background corrected signal values (nVol). Top right number represents number of internal register (see appendix table III). A-C -independent semiquantitative confirmation of the microarray data by RT-PCR. Agarose gel with PCR-products of short-chain alcohol dehydrogenase (A), dehydrin 5 (B), and 18S rRNA controls (C) upon respective periods of infection (12h-72h).

Generally, these two types of expression profiles similarly occurred for various screened ESTs of different intensity levels included in several categories (oligomer numbers 60, 83, 181, 207, 214 & 251 Fig. 8). In each case, differences between infected tissue and controls culminated at 48h *Bgh*-inoculation. In control plants, the expression level of some genes continuously decreased between 24-48h of the experiment (black bars oligomer numbers 5, 60, 181, 195 & 207 Fig. 8).

Category II: out of the 83 ESTs associated with lipid-transport, 24 sequences exhibited a strong signal intensity (>10). 17 of these candidates were lipid transfer proteins (II), which responded inhomogeneously to *Bgh*-infection. Five of these showed a distinct up-regulation, while the non-specific lipid transfer protein 4.3 precursor (155, Fig. 8) was distinctly down-regulated through treatment, culminating at 48h in an only 0.4-fold intensity. Among group carrier/ transferases (IIb), eight ESTs of various signal intensities were differentially expressed, seven of these were down-regulated with the most striking suppression at 48h. Hydroxcinnamoyl transferase (161) was included in this group, which exhibited the overall strongest microarray signal. Among the 19 investigated ABC-transporters (IIc), four high expressed signals (>10) were detectable, three of these candidates were dramatically up-regulated in response to *Bgh*-infection. After 48h experimental time, a 2.6-fold accumulation of an ABC-transporter was observed (181, Fig. 8), which was decreased to 1.7-fold of the control level with further incubation (72h).

Other categories: out of a total of 26 selected stress induced genes, 12 were differentially expressed in response to *Bgh*-infection. Five of these were up-regulated, e.g. the pathogenesis related protein 4 (207, Fig. 8), which was increased to almost two-fold after 72h of inoculation. In comparison, dehydrin 5 (214) responded delayed. Here, a pronounced increase of signal intensity occurred at 48h post inoculation, culminating in a 3.9-fold up-regulation after 72h. After 48h of infection ubiquitin-protein ligase E3-alpha-like (251, Fig. 8) was also

dramatically increased to 4.1-fold of control level, which was slightly decreased to 3.1-fold after 72h.

DISCUSSION

In order to understand the molecular basis of several plant regulatory mechanisms, microarray analyses offer a useful tool for creating gene expression profiles (Alba *et al.*, 2004). In recent years, several microarrays have been established, which contain defined subsets of sequences of target genes involved in certain processes of physiological, developmental, or metabolic aspects. Although these “theme microarrays” are based on a limited number of sequences, they may sufficiently support specific research applications. Especially for comparisons of modifications in transcript accumulation in response to different environmental stresses and/or time-course analyses, microarrays provide a common research method.

The development of the barley wax-microarray allows investigations of transcriptional processes, exclusively targeting wax biogenesis and lipid transport in response to different abiotic stresses, and the infection with powdery mildew as a biotic stressor, for the first. The complex dataset of this study offers various possibilities of effect evaluation, which are, on the one hand, discussed in relation to observed wax modifications regarding amount and chemical composition, as described and discussed in chapter I, and, on the other hand, in correlation to the surface derived processes, which influence the development of powdery mildew conidia (chapter III).

1. Stress-Microarray: Transcriptional Events of Wax Biogenesis in Barley Leaves in Response to Different Abiotic Stress-Treatments

1.1.1 Darkness I- Differential Expression in Fatty Acid Biosynthesis, Elongation and Modification Correlates with Alterations in the Chemical Composition of Surface Waxes

As already described in chapter I, the darkness-treatment led to a 30% decrease of the total leaf wax amount of etiolated plants, and to a distinct modification of the relative chemical composition. While the proportions of primary alcohols and esters were significantly increased, aldehydes and *n*-alkanes almost entirely disappeared due to etiolation. These changes in chemical composition

might have resulted from altered gene expression within the modification process of very long chain fatty acids (VLCFAs).

Little is known about the putative genes involved in this processes: a decrease of *n*-alkane fraction detected in *Arabidopsis* mutants has already been associated with the *cer1* sequence previously, encoding for an aldehyde decarbonylase (Hannoufa *et al.*,1993; Mc Nevin *et al.*, 1993). This gene is essential for alkane biosynthesis, as it was found to regulate the decarbonylation of fatty aldehydes to alkanes (Lemieux *et al.*, 1994). Investigations in *Brassica oleracea* leaves showed that primary alcohol production is a two-step process carried out by two separate enzymes – an NADH-dependent acyl-CoA reductase is required for a reduction of fatty acids to aldehydes, and an NADPH-dependent aldehyde reductase is required for a further reduction of aldehydes to primary alcohols (Kolattukudy, 1971). The primary alcohols generated in the epidermal cells may be further used for the synthesis of wax esters. Consequently, aldehydes may, on the one hand, serve as precursors for the synthesis of primary alcohols, and, on the other hand, may act as intermediates for the production of *n*-alkanes. This might imply an interrelation between the proportions of aldehydes and *n*-alkanes, and the increased amounts of primary alcohols in the surface wax of etiolated plants. This shift in the chemical wax composition correlates with a decrease in the expression of a gene similar to *cer1* (oligomer number 80), detected in the barley wax-microarray. Given that the lower expression of *cer1* during etiolation might have reduced the availability for decarbonylases, modification of aldehydes to *n*-alkanes could have been weakened, finally resulting in reduced amounts of *n*-alkanes in the surface wax. In consequence, higher amounts of aldehydes could be available as precursors for the synthesis of primary alcohols, increasing the respective amounts in the surface waxes. As synthesis of esters follows primary alcohol synthesis, one might further suggest, that the increase in ester fraction follows the enhanced production of primary alcohols.

The detailed analysis of the relative amounts of single components within fractions of primary alcohols and esters further exhibited alterations in chain length distribution in response to etiolation. The carbon chain lengths of the major homologues were shifted to two-carbon shorter chain lengths. There might be a connection between aldehyde formation and the determination of carbon chain

length. Investigations in maize plants with a mutant allele of the glossy*5* locus showed that the resulting ezyme not only supports the generation of aldehydes, but also plays an important role in determining the relative amounts of C_{16} and C_{18} fatty acid precursors with distinct effects on the chain lengths of resulting wax products (Bianchi *et al.* 1978). Thus, modifications in chain length distribution might be explained as results of alterations within the elongation of the C_{16}/ C_{18} precursors to VLCFA.

Although indicating an impairment of fatty acid elongation, a strong expressed gene encoding a *fiddlehead*-like protein (5), a named probable β-ketoacyl reductase (4), and a glossy*1* homolog sequence (60), were distinctly up-regulated in the expression profile of plants grown in darkness. As deducted from previous biochemical analyses in *Arabidopsis*, the *fiddlehead* genes encode for proteins, that are probably involved in the synthesis of long-chain lipids, found in the cuticle, and show similarity to a large class of genes encoding proteins related to β-ketoacyl-CoA synthases (Pruitt *et al.* 2000; Efremova *et al.*, 2004). These epidermis-specific enzymes are known to function within fatty acyl elongase complex (FAE), which operates extension of fatty acid precursors via four enzymatic steps (Kunst & Samuels, 2003). β-ketoacyl-CoA reductases are also known to work within the FAE, supporting the successive two carbon elongation of C_{16}/ C_{18} fatty acyl-CoA precursors (Kunst *et al.*, 2005). Moreover, GLOSSY*1* was also assumed to be required for an elongation step in cuticular wax biosynthesis in maize (Sturaro *et al.* 2005).

In summary, the differential expression of genes taking part within steps of fatty acid elongation, and their modification within the expression pattern, might be connected with the shift of chain lengths and the altered relative wax composition observed in the surface wax analysis of plants grown in darkness.

1.1.2 Darkness II- Modifications of Gene Expression within Processes of Component Transport

Our observation of 30% reduced wax amount in etiolated leaves might suggest that wax biogenesis is inducible by light, and was therefore not activated in etiolated leaf tissue. Surprisingly, in these tissues held in darkness, the expression analysis revealed a distinct over-expression of genes involved in fatty acid biosynthesis/ elongation, and modification (category I), which are responsible

for the production of wax precursors. The availability of wax precursors drives the formation of surface waxes and the transport of the components to the outer cell surface. This aspect was reflected by the expression of genes putatively supporting lipid-transport (category II), for which increases in gene expression were detected for screened LTPs (IIa) and category of carrier/ transferases (IIb).

Plant lipid transfer proteins (LTPs) were already attributed to play several roles involved in regulation of intracellular fatty acid pools, membrane biogenesis, and even defence reactions against phytopathogens (Kader, 1996). Mainly based on the localization of LTPs' in the cell wall, they were proposed to be involved in the formation of protective barriers, such as the cuticle, and associated wax assembly (Sterk *et al.*, 1991; Thoma *et al.*, 1994; Hollenbach *et al.*, 1997). Particularly in young leaves with active cutin deposition, LTPs are mainly concentrated in the surface wax (Pyee *et al.*, 1994).

The increased LTPs expression in etiolated leaves we found in our study, might reflect amplified activities in the export machinery of wax components, and even in the formation of the cuticle, indicating that these processes were still progressing. The dramatically high expression level of a single gene involved in category IIb (carrier/ transferases) namely cinnamoyl transferase (161), points to a further idea: if cinnamoyl transferases promote the accumulation of cell-wall-bound phenolic amines that are involved in cell wall strengthening (Clarke,1982), the assembly of cell wall structures is also impaired upon growth in darkness.

These observations of component transport processes may lead to the conclusion that the reduced cuticle wax load is the result of a non-completed stadium of wax deposition. Speculating further, the intensified gene expression in category I (fatty acid synthesis/ elongation and modification, described above) may reflect the intensified requirement for building material necessary for progressing wax deposition, as well as components for cuticle- and even cell wall- formation.

1.1.3 Darkness III- Light as Inductive Factor for Wax Formation

The correlation between the analytical wax data and the gene expression study indicates a retardation of several processes of wax biogenesis. Since gene expression depends on the availability of energy resulting from the photosynthetic machinery, the lack of light may have led to a retardation of wax biogenesis processes in etiolated plants. Furthermore, light also serves as a stimulus for the

differentiation of chloroplasts, observable in the pale-yellow leaf blade color of etiolated plants. The plastids serve as important cellular compartments, as the FAS complex that operats a *de novo* synthesis of C_{16}/ C_{18} fatty acids is localized in the plastid stroma (Ohlrogge *et al.*, 1993; Ohlrogge & Browse, 1995). Moreover, some investigations in plastid transcription demonstrate that different light conditions may affect the activity of certain chloroplast promoters in barley (Christopher *et al.*, 1997; Kim *et al.*, 1999; Thum *et al.*, 2001). Therefore, not only a decreased availability of fully differentiated chloroplasts, but also a diminished induction of the gene expression within plastidial fatty acid biosynthesis in darkness, may impede the production of wax precursors.

The expression analysis showed a down-regulation within the category of housekeeping genes, supporting the idea of process retardation. A general restriction of intracellular transcriptional activity can be seen in the strongly reduced expression of ubiquitin (253) and actin (250), to nearly one-third of control level among etiolation. However, since plant size and leaf areas of etiolated plants were similar to those of plants grown under natural light conditions, a retardation of the general developmental process of the plants grown in darkness was not obvious.

Although the production of wax is transcriptionally controlled, the transcriptional activators that up-regulate the expression of wax biosynthetic genes, during development on the one hand, and in response to the environment on the other hand, have not yet been identified. However, two studies report the isolation and characterization of an *Arabidopsis thaliana* transcriptional regulator of the APETALA/ ethylene-responsive-element binding protein (AP2/ EREBP) family. An over-expression was described to dramatically enhance wax accumulation in *A. thaliana* leaves and stems (Aharoni *et al.*, 2004; Broun *et al.*, 2004). Ethylene-responsive-element binding factor (ERF) proteins are a subfamily of the AP2/ EREBP transcription factor family, that is unique to plants (Singh *et al.*, 2002). Concluding from their transcriptional properties, most ERFs were identified as activators of the transcriptional machinery, although some may repress transcription (Fujimoto *et al.*, 2000). Our expression analysis pointed to one ERF, namely ethylene-binding protein (228), which was down-regulated upon etiolation. In addition wax array data showed a distinct down-regulation of translation initiation factor 5A (195) upon treatment of darkness. As described in several

studies investigating different eukaryotic organisms' function of translation, initiation factor 5A is potentially involved in cell proliferation and processes of senescence (Park *et al.*, 1993; Kang & Hershey ,1994; Thompson *et al.*, 2004). However, it remains unclear, if the differential expression of these named transcription factors affects the screened molecular processes of wax biogenesis in etiolated plants, finally resulting in modifications of wax amount, and changes within the chemical composition.

Nevertheless, summarizing several aspects of the transcriptional profiling of the darkness-treatment, demonstrates that the differential expression of single genes affects the elongation and modification of long chain lipids and components transport, which can be correlated with the observed modifications in the surface waxes. The progressing transcription of genes involved in components-transport might indicate ongoing wax deposition and/ or cuticle formation, thereby explaining decreasing surface wax amounts. Furthermore, one can expect that the lack of light weakens the intracellular energy level usually fed by the photosynthetic machinery, leading to the retardation of several processes involved in wax biogenesis among plants grown in darkness.

1.2 Drought- and Salinity-Stress- Adaptations on the Transcriptional Level

The major function of the cuticle layer is the protection against transpirational water loss (Riederer & Schreiber, 2001; Kerstiens, 2006). Providing an adaptation to drought-stress, an increase in wax overlay can be a possible strategy to strengthen this protective barrier. However, the analyzed wax parameters of drought-stressed barley did neither show alterations in wax amount, nor modifications of the chemical composition. Nevertheless, the expression analysis exhibited a high percentage of differentially expressed genes (70%) in this treatment, affecting several aspects of wax biogenesis. The major part of these genes was dramatically down-regulated, which included candidates of category I targeting fatty acid biosynthesis/ elongation and modification as well as some of the genes associated with lipid-transport represented in category II. Among these, a high number of target LTPs and ABC-transporters was down-regulated. The expression level of category carrier/ transferases (IIb) was dominated by the high signal intensity of cinnamoyl transferase (161). This slight increase in cinnamoyl transferase expression shifted the entire group expression

value to a positive level, although the expression of the major part of the genes involved in this category was dramatically decreased in the drought-treatment. For potato tissue, among other functions, cinnamoyl transferase was shown to be involved in the promotion of the accumulation of cell-wall-bound phenolic amines, which are involved in cell wall strengthening (Clarke, 1982). One might suggest that drought stress highly influences the fluidity of membranes, and even possibly affects the stability of the cell wall.

Dhn4 (213) is a putatively stress regulated gene, and one of the two candidates that were distinctly up-regulated in drought-treatments. The increase in its expression might be important in this respect. Dehydrins are one of the classes of proteins that are synthesized in plants in response to several stresses. Under water stress conditions, they might act as stabilizers of membranes or proteins (Close, 1997). Thus, the transcriptional increase of cinnamoyl transferase (161) and *dhn4* (213) may reflect a progressing regeneration of the plants´ protection barrier. Nevertheless, the strong trend of down-regulation of the transcriptional profile of drought-stressed plants did obviously not affect amount and composition of surface waxes.

The comparison of the transcriptional responses between a shock-like dehydration treatment, and the gradual imposition of drought, showed that transcriptional modifications within the expression pattern highly depend on the type of drought-stress (Talamè *et al.*, 2006). Moreover, the authors found that the majority of transcripts were regulated by only one of the drought treatments, with 57% of the differentially expressed transcripts exclusively affected in the dehydration shock. Therefore, it was suggested that shock-like treatments reveal the capacity of a plant to respond to a severe and acute change in the environment. Consequently, a slow decrease in soil moisture would allow more appropriate measures of potential transcript and protein acclimations providing long-term protection. The experimental setup applied in our study combined gradual drought-stress, enforced by three-times repeated dehydration periods, which were interrupted by rehydration. Since the tissue for expression analysis was harvested at the end of a dehydration period, it is possible that screened modifications of gene expression were composed of a mix of short- and long term responses. Nevertheless, strong modifications in the expression pattern revealed by drought-stress, did surprisingly not affect the amount and composition of

surface waxes. Since the drought-experiment was started with 4 days old barley seedlings placed into water saturated soil, a relatively low but steadily increasing drought-stress during the first days of this treatment was caused. It is possible that initial conditions were not sufficient to produce drought-stress effective enough to impair the production of the cuticle wax load.

Similar to drought-stress, NaCl-treatment did also not alter the wax characteristics of barley leaf surface waxes, but the number of genes responding to this treatment decreased distinctly. In accordance with our results, Ozturk *et al.* (2002) observed effects of drought- and salinity-stress on largely different sets of transcripts in barley, while the percentage of differentially regulated sequences was higher in drought- than in salt-stress. However, several microarray studies investigating *Arabidopsis* gene expression, in response to several abiotic stress inducers, reported that many genes may equally respond to a diversity of stress factors (Seki *et al.* 2001; 2002a; 2002b; Shinozaki *et al.*, 2003; Kilian *et al.*, 2007). For example, more than half of the drought-inducible genes were also induced by high salinity, and/ or ABA treatments, indicating the existence of significant crosstalk among the drought, high-salinity, and ABA responses. Walia *et al.* (2007) conducted studies screening the expression profile after a short term exposure to salinity-stress, and concluded an overlap of genes regulated by salinity stress and ABA application. Similar to these studies, Walia *et al.* (2006) reported, that various functional categories of genes respond to salinity-stress in barley, involving transporters and membrane proteins, cell-wall related proteins, transcription factors, and common abiotic and biotic stress-related genes. However, in contrast to drought-stress, the expression pattern was hardly modified by salinity-stress, but, for both treatments in common, the surface wax characteristics were not affected.

According to Shinozaki *et al.* (2003), plants may respond and adapt to environmental stresses through various biochemical and physiological processes, thereby acquiring stress tolerance. It is most likely that a complex machinery of transcriptional, as well as posttranscriptional, mechanisms is involved in the regulation (Ramagopal, 1987). Since barley is rated as moderately tolerant forage crop (Mass, 1984; Francois & Mass, 1999), and is found in drought and saline environments, it needs to be adapted to frequent environmental stresses. The particular alterations of gene expression under drought- and salt-stress probably

reflect the different adjustments to such common habitat stresses, whereas the modification of surface waxes is obviously not essential for these adaptations.

1.3 Cadmium-Stress - Differential Expression in Processes of Component Transport Correlates with Increased Amount of Surface Waxes

Cadmium exposition led to a significant 1.5-fold increase of the total leaf wax amount, while the chemical composition remained unaffected. Moreover, as noticed by visual inspection, leaf areas of cadmium stressed plants were distinctly reduced, accompanied by a pallid leaf blade color and an altered leaf blade firmness.

Generally, metal stress in plants appears to excessively disturb the cell metabolism in multiple ways, altering the activity and quantity of the key enzymes of various metabolic pathways. This may cause changes in the ultra-structure of the cell organelles, reduced chlorophyll content and even inhibited plant growth and respiration (Khudsar *et al.*, 2001; Yamamoto *et al.*, 2002). Comparable results were also reported in several other studies investigating cadmium stress (Aery & Rana, 2003; Wu *et al.*, 2004; Guo *et al.*, 2007). Moreover, it was shown, that the cadmium content in exposed plants accumulates with increased cadmium quantities applied to the soil (Aery & Rana, 2003). Abnormal growth might be a consequence of induced disorder in nutrient metabolism by high concentrations of heavy metals in plant tissues (Guo *et al.*, 2007). Thus, the phenotype of the cadmium stressed barley plants in this study might reflect the complexity and the extent of enforced imposition by this heavy metal.

Compared to the visible external impairment of the phenotypes, the molecular modifications in the expression pattern were rather weak in cadmium exposed plants. A distinct down-regulation was detected in processes of fatty acid biosynthesis/ elongation and modification (category I). This reduction was rather based on weak expression decreases of several genes than on a pronounced down-regulation of single candidates, which might have given clues for a certain interpretational direction.

However, Hollenbach *et al.* (1997) demonstrated that the expression of a barley LTP gene was stimulated in the presence of cadmium. This observed increase in the LTPs mRNA level was accompanied by an increase in the amount of several wax components, and by a correlative increase in the size and density

of the epicuticular wax plates. In conclusion, the authors suggest that the over-expressed LTP is involved in the transport of lipophilic components to the wax of barley leaves. The described increased wax overlay was congruent with our analytical observations. However, the expression regulation of screened LTPs in our stress-microarray was highly inhomogenous, depending on the distinct type of LTPs. Therefore, a connection of intensified LTP expression with increased wax amounts could not be verified.

The increased expression of ABC-transporters might be connected to the plant´s heavy metal stress management. ABC-transporters have been characterized primarily in heavy metal detoxification, and secondary metabolite transport in *Arabidopsis* (Rea *et al.*, 1998; Jasinski *et al.*, 2001). Such ABC-transporters are supposed to have the ability to pump a wide variety of substrates. Especially their described function as transporters of heavy metal chelates from the cytosol to the cell vacuole is important for the plant, in order to tolerate heavy metal stress. On the other hand, a CER5 ABC-transporter identified in *A. thaliana* was supposed to be involved in wax export (Pighin *et al.*, 2004).This suggestion comes from the analysis of the *cer5* mutant, in which wax components are reduced on the cuticle surface, and accumulated inside the cells instead. Therefore, in the same way, the alterations in ABC-transporter expression may take part in the export of wax components, which in turn might be related to the increased wax amounts in cadmium-stressed plants. However, since the chemical composition remained unaltered upon cadmium-treatment, the trend of expression decrease among genes taking part in fatty acid synthesis/ elongation and modification appears, to a certain extent, contradictory.

2. *Bgh*-Microarray: Transcriptional Events of Wax Biogenesis in Barley Leaves During Powdery Mildew Infection

2.1 The Alterations in the Expression Pattern Follow a Time-Dependent Development Correlating with Distinct Stages of Fungal Infection

The changes in the gene expression levels within the *Bgh*-microarray follow a time-dependent development, which correlates with progressing pathogen infection. First molecular effects were measurable after 12h *Bgh*-infection. The

increase of the number of differentially expressed genes to a total of 53 candidates correlates with the time point of fungal invasion. Usually, the conidia form appressoria to penetrate into the host epidermis in this stage of infection. Since the formation of appressoria represents the first step in the switch from surface to invasive fungal growth (Schulze-Lefert & Panstruga, 2003), the differential expression of host genes indicates first steps of host/ pathogen interaction at the molecular level.

Ongoing incubation exhibits an amplified number of differentially expressed gene candidates highlighting in 90 regulated genes after 24h of *Bgh*-inoculation. After this incubation time, fungal pre-penetration processes are completely finished: fungal haustoria have already been formed within the epidermal cells, which fundamentally direct the plants intracellular activity for nutrient acquisition and for growth of superficial hyphae, followed by further secondary infection structures (Green *et al.*, 2002; Schulze-Lefert & Panstruga, 2003). Modifications within the expression pattern of *Bgh*-microarray mirror this rearrangement of host cell resources by the fungus, to redirect the host´s metabolic flow to its own benefit (Schulze-Lefert & Panstruga, 2003). Moreover, a comparison between the 12h and 24h infection interval indicates additional effects derived from the different light conditions. While the first 12h of *Bgh*-infection occurred in a full light period, the plant material had been exposed to 14h/ 8h/ 2h light/ dark/ light intervals after 24h of fungal infection. Thus, the leaf tissue harvested after 12h of *Bgh*-infection, might have been altered in its energy level resulting from the photosynthetic activity, that in turn potentially affected the molecular responses during fungal infection.

Since modification of gene expression was highly complex within the first 24h, it was difficult to recognize certain regulatory trends. Further incubation (48h) addressed distinct genes, for which differential expression was further accentuated with progressive fungal development (72h). Obviously, the 72h observation period of our experiment completely records the early transcriptional events of *Bgh*-treatment, since a decrease to only 20 differentially expressed sequences was counted at the end of the experiment.

2.2 Activation of the Plants Defense Machinery

The formation of intracellular fungal structures requires successful penetration of the host cell wall. During this complex process, the intruder is exposed to cell wall-associated defense responses (Schulze-Lefert & Panstruga, 2003). This might result in the deposition of callose and other constituents in the inner surface of the epidermal cell wall directly underlying the penetration peg and appressorium (Zeyen *et al.*, 2002). These so called papillae are deposited, regardless of a plant´s potential resistance, or susceptibility to penetration by powdery mildew fungus.

In our study, changes in the barley expression profile were already detectable after 12h of *Bgh*-inoculation, with the distinctly enhanced expression of single genes. A nearly two-fold accumulation of HSP90 (202) pointed to an activation of cell wall associated defense responses. Heat shock proteins (HSPs) act as molecular chaperones, stabilizing the target proteins during processing and transport. The high accumulation of an HSP90 transcript in response to *Bgh*-infection might be explained with an enhanced requirement for this protein, since the secretory protein traffic is increased in the endoplasmic reticulum during formation of papillae (Walther-Larsen *et al.*, 1993). Thus, increased HSP90 transcripts in our expression analysis might reflect the plants' unspecific initial effort to prevent the pathogen attack. This goes in line with two wound induced transcripts (219 & 225), distinctly accumulated within 12-24h of infection, which additionally indicate a recognition of the fungal invasion by the host cells.

This process was accompanied by several differentially responding PR-proteins. Expression level of for PR1a (206) was distinctly down-regulated within the experiment, and expression for PR5 and PR10 highlighted already within first 24h of *Bgh*-incubation, while the accumulation of PR4 was accentuated with advanced fungal infection (48-72h). Different classes of PR proteins are known from investigations in tobacco (Cutt & Klessig, 1992). Some of these proteins display antifungal activity (Hejgaard *et al.*, 1992; Alexander *et al.*, 1993; Muradov *et al.*, 1993). The specific regulation, and the time point of PR response, may vary depending on the corresponding types of PR proteins. For PR4-like barley leaf and grain proteins, Hejgaard *et al.* (1992) demonstrated a growth inhibition of *Trichoderma harzianum* spores. After 7 days of inoculation, the protein contents still revealed a 5-fold increase of this protein. For mRNA of PR1, a continuous

accumulation within an earlier time frame of 72h of *Bgh*-infection in barley was reported (Mouradov *et al.* 1994). In this scenario, high transcript amounts between 48-72h were discussed to be the result of the cellular hypersensitive reaction which occurs at this time interval.

Bgh-infection primarily attacks the epidermal cell layer. Since in our study, entire leaf tissue was applied for gene expression analysis, the detected molecular modifications refer to a mix of epidermal and mesophyll tissue derived responses. The fungus is able to suppress defense gene activation in physical proximity to haustoria in the epidermal cells, whereas signals triggering defense induction must spread systemically to subtending mesophyll tissue (Gregersen *et al.*, 1997). Obviously, the highly different responses of the screened PR-proteins are a mix of local and systemic reactions of pathogen defense. However, the differential expression of several PR proteins due to *Bgh*-infection did obviously display the plants effort to fend off the fungal invasion.

After 48h of incubation, the expression analysis confirmed a conspicuous expression regulation of the screened dehydrins. The different types of dehydrins responded highly diverse, as *Dhn4*, *Dhn8* and *Dhn12* exhibited down-regulation, while *Dhn5*, *Dhn11* and an unspecific dehydrin (213) were distinctly accumulated, in response to *Bgh*-infection. Differential regulation of *Dhn* genes is known as a common response to dehydration (Close ,1996; Close, 1997; Grossi *et al.*,1995). These genes were found to act as stabilizers of membranes or proteins under water-stress conditions (Close, 1997; Egerton-Walburton *et al.*, 1997; Danyluk *et al.*, 1998).

In this stage of infection, the epidermal tissue of infected leaves was strongly damaged through the penetration by fungal appressoria and secondary hyphae. In this case, water could have been exuded through the damaged cuticular surface structures, causing dehydration-stress, and, finally, leading to differential expression of the screened dehydrins. In conclusion, different stepwise phases of plant defense are reflected in the gene expression profiles. Early responses included differential expression of PR-proteins, indicating the initial events of fungal invasion, whereas a subsequent regulation of dehydrins could display a progression of the cuticular damage in later infection stages.

2.3 *Bgh*-Infection Affects Fatty Acid Elongation and Modification

Gene expression of category I (fatty acid synthesis/ elongation and modification) exhibited a striking up-regulation during powdery mildew infection. This involved several genes, suggested to take part in processes of long chain lipids modification, like glossy*1* homolog (60), a gene similar to *cer1* (80), and the wax synthase (83), which were both strikingly amplified.

These genes are supposed to take part in the production of several wax component classes: Glossy*1* of *Zea mays* shares high sequence similarities to the *wax2* gene in *Arabidopsis* (Sturaro *et al.*, 2005), which may function as an aldehyde-generating acyl-CoA reductase (Chen *et al.*, 2003, Kurata *et al.* 2003). *Cer1* in Arabidopsis was supposed to function as an aldehyde-decarbonylase (Aarts *et al.*, 1995), putatively supporting *n*-alkane biosynthesis. Biochemical studies in *B. oleracea* leaves (Kolattukudy, 1967a) and *Simmondsia. chinensis* seeds (Wu *et al.*,1981) suggested that the formation of wax esters involves the transfer of an acyl chain from fatty acyl-CoA to a fatty alcohol, catalyzed by a membrane-bound acyltransferase (wax synthase). Therefore, the named wax synthase is supposed to function within the formation of alkyl-esters (Lardizabal *et al.*, 2000). In conclusion, the differential expression affects various steps within aldehydes-, *n*-alkanes-, and ester- formation.

Moreover, *Bgh*-infection obviously alters the expression level of putative *fiddlehead*-like protein (5), which was most distinctly 3.7-fold up-regulated after 48h of *Bgh*-inoculation. The *fiddlehead* genes were supposed to be impaired in steps of fatty acid elongation in *Arabidopsis* (Pruitt *et al.*, 2000), and showed a high similarity to a large class of genes encoding proteins related to β-ketoacyl-CoA synthases (Pruitt *et al.*, 2000; Efremova *et al.*, 2004). Our expression analysis revealed four further genes putatively operating fatty acid elongation (oligomer numbers 4, 30, 35 & 51), which were increased upon *Bgh*-infection.

In conclusion, *Bgh*-infection modifies the gene expression in processes of fatty acid elongation, and affects various steps of aldehyde-, *n*-alkane-, and ester-formation. However, these molecular modifications do not cause alterations in amount and chemical composition of surface waxes. Supposedly, the transcriptional events did not sufficiently alter those enzyme levels pushing the respective modification and elongation processes, since the wax-microarray reconfirmed the transcriptional events of a small part of genes within the barley

genome. Moreover, wax formation not only depends on the availability of wax components, but also on transport mechanisms operating lipid-trafficking between cell compartments, and the export of wax precursors to the outer cell surface. Thus, the transcripitional activity of screened transporters needs to be considered as well.

2.4 *Bgh*-Infection Changes Gene Expression within Processes of Component Transport

Bgh-infection led to the differential regulation of gene transcription within the three groups targeting components-transport. While group expression of screened LTPs and carrier/ transferases was continuously suppressed, signals of ABC-transporters increased with progressing infection. The group signal increase of carrier/ transferases was dominated by the high signal of a single gene econding a hydroxyl-cinnamoyl-transferase (oligomer number 161), but it hardly responded to *Bgh*-infection. Therefore, we focused on the general trends of LTPs and ABC-transporters.

The decrease of LTPs expression was contradictory to the study of Molina & García-Olmedo (1993), who reported a type dependent increase in LTPs transcription to 3- 9-fold after 18h *Bgh*-infection. However, the functionality of LTPs involves several aspects: the wax transport trough the cell wall has usually been attributed to LTPs (Sterk *et al.*, 1991; Moreau *et al.*, 1998). Additionally, they were supposed to transport the acyl monomers needed for the synthesis of cutin (Hendriks *et al.*, 1994). For several LTP-like proteins purified from barley leaves, and for an LTP isolated from maize leaves, growth inhibition of a bacterial pathogen and fungus was demonstrated (Molina *et al.*, 1993).

The utility of ABC-transporters implies similar aspects: localized in the plasma membrane, as well as in intracellular membranes, ABC-transporters play an important role for intracellular mechanisms of lipid trafficking (Moreau *et al.*, 1998). Known from investigations in different mammal and prokaryote systems, the proteins mediate transbilayer lipid-transport in various membranes of cells and organelles (Pohl *et al.*, 2005). Secondly, ABC-transporter can take part in cutin biosynthesis, which in general seems to be stimulated by pathogen infection (Kader, 1996). Investigations in *cer5* mutants in *Arabidopsis* led to an identification of the *cer5* gene, which encodes an ABC-transporter, probably localized in the

plasma membrane of the epidermal cells (Pighin *et al.*, 2004). Moreover, the authors proposed the ABC-transporter to function in the export machinery of plant waxes, which might include the transport of a variety of substrates. Finally, ABC transporters may also support aspects in pathogen-defense: an ABC-transporter subclass, namely GS-X pumps (glutathione-conjugate or multispecific organic anion Mg^{2+}-ATPases), is considered to participate in the transport of exogenous amphipathic anions and glutathionated compounds from the cytosol into the vacuole (Rea *et al.*, 1998). In pathogen-related processes, this functionality becomes especially important, since the vacuolar storage of antimicrobial compounds in the healthy cells, surrounding the hypersensitive lesion, might be facilitated by ABC-transporters (Li *et al.*, 1997). Consequently, ABC-transporters may support a variety of processes affecting intracellular lipid-trafficking, the transport of wax components to the outer cell surface, cuticle strengthening, and pathogen defense, which goes in line with several described functionalities of LTPs.

Biotrophic fungi suppress both, the induction of plant defense responses on physical proximity to infection sites, and the induction of specific host genes for the establishment of biotrophy (Schulze-Lefert & Panstruga, 2003). To establish a compatible interaction, the fungus either has to camouflage against recognition, suppress activation of defense, or counter-defend activated defense by detoxification or sequestration of potentially harmful compounds.

Our study shows that the antimicrobial function of LTPs, and the role of ABC-transporters in supporting the vacuolar sequestration of antifungal compounds, is important in managing the plant´s pathogen defense. In contrast, the functionality of ABC-transporters within intracellular lipid trafficking might also be useful for the enlargement of intracellular fungal structures. In this case, the increased activity of ABC-transporters could serve as an intracellular consumption of fatty acids used for membrane biogenesis. Invasive growth of biotrophs after cell wall penetration leads to invagination of the plasma membrane, and creates an interface between host and fungus, that consists of the haustorial membrane, an extra-haustorial matrix, and the host plasma membrane, following the contours of the haustorial membrane (Heath & Skalamera, 1997). This extrahaustorial membrane expansion during the development of the haustorial complex requires membrane synthesis. It was suggested that the new membrane is probably

derived from the plant, but its formation may also involve the dissolution of plant cell wall components by secreted fungal enzymes (Green *et al.*, 2002).

It is not possible to allocate specific modifications in the expression pattern to confirm the control of the haustorial structures. The molecular interrelations between the operations, which are either advantageous for the plant, or useful for the invasive fungus, may lead to a variety of antagonistic and/ or synergistic working processes. These plethora of responses might be reflected in the described differential regulation of screened genes taking part in transport mechanisms. Since the amount and composition of surface waxes remained unaltered by *Bgh*-infection, one might speculate that the ABC-transporters are more likely involved in intracellular lipid-trafficking, than in processes of wax export. Thus, the increased activity of ABC-transporters may not only support the sequestration of antifungal substances, but also the intracellular consumption of fatty acids for membrane biogenesis during the establishment of secondary infection structures, and the generation of new conidia.

2.5 Transcriptional Regulators during *Bgh*-Infection

The regulation of the temporal expression levels of specific stress inducible genes is an important part of the plant stress response. Transcription factors play an important role in orchestrating the cross-talk between multiple signaling pathways. Ethylene-responsive-element binding factor (ERF) proteins are a subfamily of the APETALA2 (AP2)/ ethylene-responsive-element binding protein (EREBP) transcription factor family that is unique to plants (Singh *et al.*, 2002). The RNA levels of ERFs are regulated by cold, drought, pathogen infection, wounding, or treatment with ethylene.

One out of five ERFs- ethylene-binding protein (228) screened in the barley wax-microarray was, strikingly, 2.5-fold increased within 48h-72h of *Bgh*-incubation. The accumulation of this gene transcript might imply enhancement of the plant´s pathogen defense, since constitutive expression of ERF1 in *Arabidopsis* was shown to confer resistance to several necrotrophic fungi (Berrocal-Lobo *et al.*, 2002). A sole ERF gene can have a major impact on a complex plant stress response (Jaglo-Ottosen *et al.*, 1998), therefore the named ethylene-binding protein may play an important role in regulating the interrelative molecular processes during *Bgh*-infection.

Moreover, in our study, the expression of three screened AP2 related genes (oligomer numbers 232, 233 & 237) was weakened due to *Bgh*-infection, accompanied by increased expression levels of ubiquitin-protein ligase E3 alpha-like (251). Regulators of the AP2 family showed homologies to WAX2 in Arabidopsis which has been proposed to be involved in both cutin and wax production (Riederer & Müller 2006; Chen *et al.*, 2003). The detected transcript accumulation of ubiquitin-protein ligase E3 alpha-like (251) also hints at the regulatory mechanisms in plant cuticular wax production, since E3 alpha like is related to Cer3 in Arabidopsis, a gene known to be involved in wax biosynthesis (Hannoufa *et al.* 1996).

Although these genes were differentially regulated in response to *Bgh*-infection, modifications of barley surface waxes were disclosed neither locally, nor systemically. However, these genes may exhibit additional functionalities. The differential regulation of the AP2- related genes due to *Bgh*-infection, and a decrease in expression of translation initiation factor 5A (195), might point to senescence related processes. A microarray expression approach in *Arabidopsis* transcription factors showed that genes expressed in response to different stresses overlap with those expressed during processes of senescence (Chen *et al.*, 2002). For AP2 related genes in maize, a participation in the control of vegetative and reproductive lateral organ identity was reported (Moose & Sisco, 2007), probably reflecting the activity level of meristems as general pointer for plant development. As described in several studies investigating different eukaryotic organisms, the function of the translation initiation factor 5A is potentially involved in cell proliferation and processes of senescence (Thompson *et al.*, 2004; Kang and Hershey ,1994; Park *et al.*, 1993).

In conclusion, these results might indicate modifications in the expression pattern, which are connected to senescence related processes. The proceeding pustule formation of powdery mildew obviously leads to the yellowing of the locally infected material, probably reflecting an acceleration of senescence within the tissue. Hence, *Bgh*-infection might have stressed the plant´s metabolism, probably resulting in outrunning aging of leaf tissue.

3. Abiotic- versus Biotic-Stress Responses

Comparing the transcriptional modifications between the investigated abiotic stresses, the major group effects turn out to be rather different, while differential expression of single sequences was partly similar. At first sight, this might reflect common strategies in the plants management of different environmental stresses. For example, the involvement of specific plasma membrane ion transporting pumps, carriers, and channels, in sequestration of ions and toxic metals from the cytosol into the vacuole, seems to be comparably effective during salinity- and metal-stress. Similarly, the activity of ion transporters, which shuttle ions between various cellular compartments, in the attempt to maintain ionic homeostasis, is also important in freezing, drought, and salt-stress (Langridge, 2006). Especially the functionality of ABC-transporters seems to reflect an internode in abiotic and biotic stress- related responses. Their ability to transport several components supports different aspects in cell detoxification and secondary metabolite transport, intracellular lipid-channeling, export of both waxes, and cutin components, and at last pathogen defense related mechanisms (Rea *et al.*, 1998, Jasinski *et al.*, 2001; Pighin *et al.*, 2004).

However, it is known that a highly complex cross-talk is involved in several stress-related mechanisms (Langridge, 2006; Knight & Knight 2001), in which central mediators often seem to play an important role in balancing the signaling pathways. Abscisic acid (ABA) might function as such a regulator, as its accumulation can be detected in response to several abiotic stresses, like freezing, drought, and salinity (Langridge, 2006; Knight & Knight, 2001). A connection of the functionality of ABA with processes of cuticular wax production was reported in Hollenbach *et al.* (1997). In this study, an increase of leaf ABA contents due to cadmium treatment in barley correlated with elevated levels of LTP transcripts, which was suggested to result in increased surface wax amounts. In conclusion, several regulatory mechanisms of stress-management may also affect the formation of surface waxes.

Within the cross-talk in signaling pathways, transcription factors display potential internodes which additionally aggravate the complexity of plant stress responses, in operating possible modifications that result in inhibitory and/ or amplifying effects (Knight & Knight, 2001). It needs to be considered that an accumulation of certain transcripts does not generally have a positive stimulating

effect on a defined process, since an inhibitory function is also possible. Furthermore, it has to be kept in mind that posttranscriptional down-stream processes in managing the plants stress response take place. There is a huge range of possible modifications, on several interactive levels, which do not always finally lead to modifications in surface waxes.

Among the investigated abiotic and biotic stressors, the correlation of the chemical modifications of surface waxes upon darkness-treatment, and the increases in wax amount upon cadmium-exposition, with the distinct modifications in the expression of wax biosynthesis-related genes, might somehow reflect the inductivity of wax biogenesis. Although powdery mildew infection, in representing a biotic stressor, seemed not to affect the wax characteristics, molecular modifications were detected, which included several aspects important in production and transport of wax components.

CHAPTER III:
Impact of Different Surface Features on Conidial Development

RESULTS

Germination and differentiation of *Blumeria graminis* (*Bgh*) conidia were studied in assays with native wild-type barley leaf surfaces, compared to treated wild-type resulting from the different abiotic stressors (darkness, salt-, cadmium- and drought-stress), and the characterized *cer*-mutants. Additionally, conidial development on several artificial surfaces covered with wax extracts or single wax components, on hydrophobic foils, isolated leaf cuticles, and cellulose membranes, were recorded, to investigate the effect of leaf surface alterations on pre-penetration processes of *Bgh* conidia.

1. Assays with *Bgh* Conidia on Leaf Tissue

1.1 Conidial Development on Stressed Wild-Type Leaf Surfaces

About 85% of the conidia deposited onto the adaxial surface of native barley wild-type leaves appeared undamaged and vital. About 95% of them germinated, and finally formed differentiated appressorial germ tubes (agts) and appressoria (apps, Fig. 1).

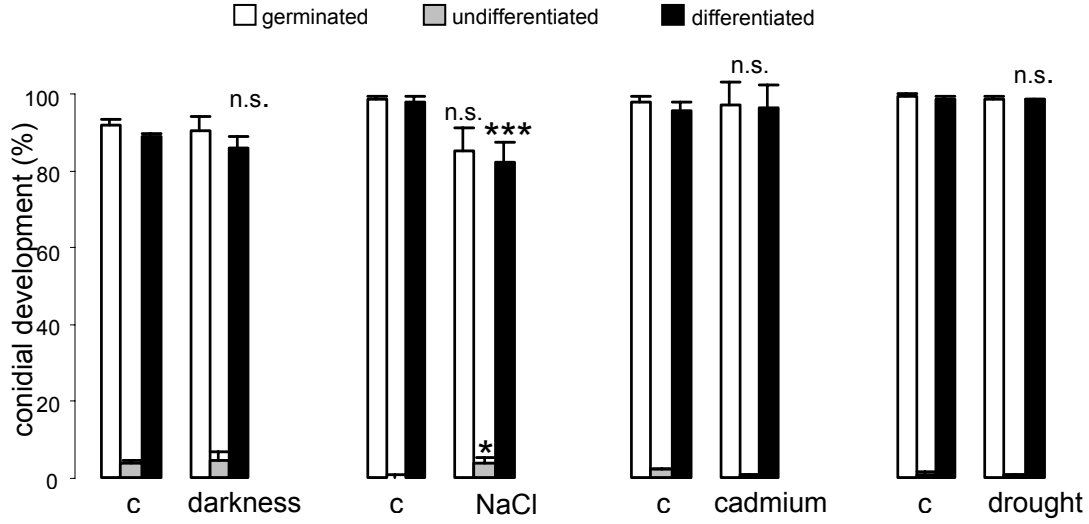


Figure 1: Development of *B. graminis* conidia on wild type leaf surfaces differently treated. Growth of plants: 14d in darkness (etiolation), 14d on hydroponic solution with 1% NaCl (NaCl), upon 28d cadmium exposition (500 mM cadmium), upon 21d drought stress (drought, for details see materials and methods 10.4), c: control. Germinated: percentage of germinated conidia; undifferentiated: percentage of conidia with undifferentiated germ tubes only; differentiated: percentage of conidia that differentiated by formation of an appressorium. Given are means ±SE of n=5 replications, 100 conidia each. Statistical differences ($p \leq 0.05$) were tested in Student´s *t*-test.

On leaf surfaces of etiolated, cadmium- and drought-stressed plants, the conidial germination and differentiation frequency remained unaltered in comparison to controls. 98-100% of the germinated conidia distinctly differentiated agts and apps. On leaf surfaces of 1% NaCl-exposed plants, the percentage of conidia, which had formed differentiated infection structures, was significantly reduced, to 82% in comparison to controls (Student´s *t*-test).

1.2 Conidial Development on Modified *Cer*-Mutants´ Leaf Surfaces

In the following, developmental frequencies of fungal conidia on leaf surfaces of a selection of five *cer*-mutants are presented, which were most distinctly impaired in surface wax characteristics. The *cer*-zd.67 mutant was selected as a representative for reduced wax amounts, while *cer*-yj.667 and *cer*-zh.54 showed distinct variations in wax crystal structure. The most striking differences in wax chemistry occurred in *cer*-yp.949 und *cer*-j.59, with regard to the proportion of aldehydes and esters, respectively. For results concerning conidia on leaf surfaces of additional 13 *cer*-mutant candidates analyzed in the screening program see appendix figure 2.

Rates of app formation were comparable to values obtained on wild type leaves on corresponding leaf surfaces of *cer*-zd.667, *cer*-zh.54, and *cer*-j.59, while leaves of *cer*-zd.67 showed a slightly reduced formation of appressoria (Fig. 2, I). In comparison to wild-type, development on leaves of *cer*-yp.949 led to a significantly reduced proportion of conidia that had formed apps after 24h. This decrease was not due to a developmental delay, as the proportion of conidia with fully differentiated apps did not change even after a prolonged incubation time of 48h (data not shown).

Neither germination, nor app formation on wild-type and *cer*-mutant leaves, were affected after mechanical removal of the epicuticular waxes, with the exception of *cer*-yj.667, where a distinct decrease of app formation (73% ±4) was observed (Fig. 2, II).

After chloroform extraction of total leaf cuticular waxes, the average rate of app formation significantly decreased on most leaf surfaces assayed. About 95% of conidia formed apps on untreated wild-type leaves. By de-waxing wild-type leaf surfaces, this rate was significantly reduced to 68% ($p \leq 0.001$, repeated measures ANOVA).

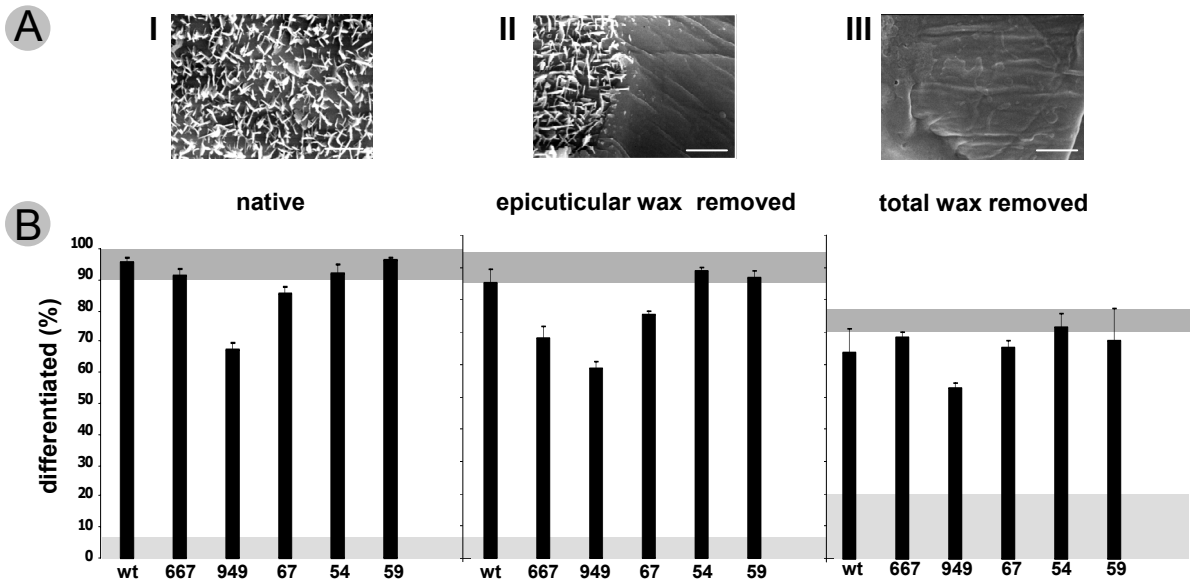


Figure 2. Development of *B. graminis* f.sp. *hordei* condia on different adaxial leaf surfaces. A: SEM pictures of adaxial wild-type leaf surfaces I. native, II. after removal of epicuticular waxes [a clear borderline is visible between the treated leaf area (right) and the adjacent untreated area (left)], III. after total wax extraction with chloroform. Bars = 2 μm. B: Percentages of conidia with differentiated germ tubes (appressoria) on barley adaxial leaf surfaces of wild-type (wt) and *cer*-mutants: *cer*-yj.667 (667), *cer*-yp.949 (949), *cer*-zd.67 (67), *cer*-zh.54 (54) and *cer*-j.59 (59) on leaves subjected to the different treatments. Given are means ±SE of n=5 replications, 100 conidia each. The upper grey horizontal bars display the overall average of appressorium formation (95% confidence interval), including data from leaves of 18 *cer*-mutants plus Bonus wild-type subjected to the following treatments: native 92%, epicuticular wax removed 90%, total wax removed 73%. Grey background basis depicts the average value of non-germinated conidia within each treatment. I. = 6% II = 5% III. = 20%.

After removal of total cuticular waxes, the initial proportion of conidia with fully differentiated apps was also distinctly reduced in *cer*-zd.667, *cer*-zh.54, and *cer*-j.59, whereas *cer*-yp.949 exhibited only a slight decrease of app formation under this treatment (Fig. 2 ,III). In contrast to all other lines, app formation on *cer*-yp.949 was significantly different from wild-type on native ($p \leq 0.025$) and *gum arabic* treated leaves ($p \leq 0.048$). However, these differences were no longer statistically significant after chloroform extraction. In comparison to the wild-type, and all other *cer* mutants involved, conidial differentiation on *cer*-yp.949 leaves was not significantly affected by any of the different wax removal treatments.

2. Assays with *Bgh* Conidia on Different Artificial Surfaces

2.1 Conidial Development on Wax Coated Glass Slides

In order to analyze the impact of barley cuticular waxes on the pre-penetration processes of powdery mildew, excluding the influence of living tissue, glass slides covered with extracted barley wax were inoculated with *Bgh*

conidia. After 24h of incubation, rates of germination and differentiation (agt & app formation) were recorded. For these experiments, the waxes of barley wild-type and of the *cer*-mutant yp.949 were selected. With respect to the chemical composition of leaf waxes, *cer*-yp.949 exhibited the most prominent differences of the selected *cer*-mutants, when compared to the wild-type wax. In contrast to the situation on untreated wild-type leaves, the germination rates of *Bgh* conidia on glass slides reached only about 34%, while the rate of app formation was reduced to a minimum of 1% (Fig. 3).

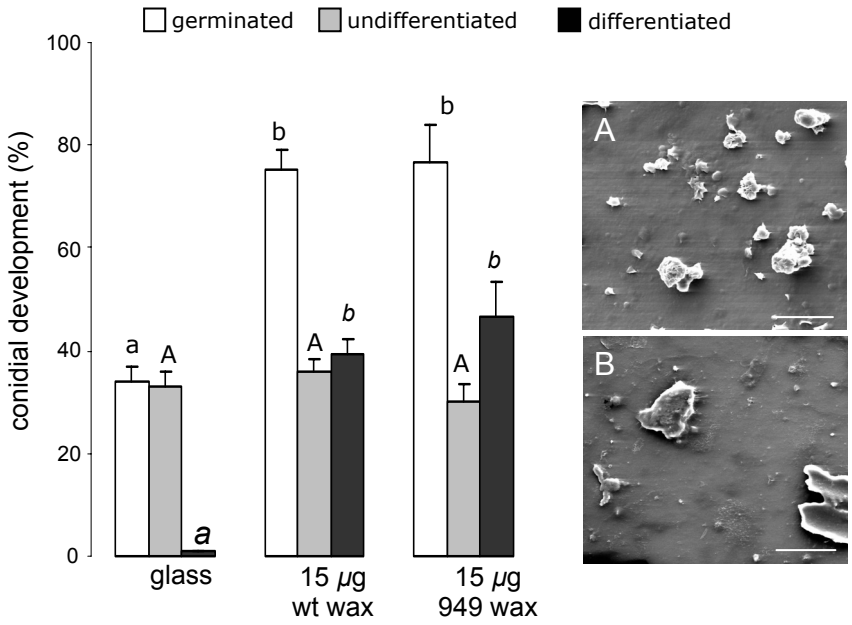


Figure 3. Development of *B. graminis* conidia on wax covered glass slides. glass: Histobond® glass slide; wt wax and 949 wax: glass slide covered wild-type wax respectively *cer*-yp.949 wax; germinated: percentage of germinated conidia; undifferentiated: percentage of conidia with undifferentiated germ tubes only; differentiated: percentage of conidia that differentiated by formation of an appressorial germ tube or an appressorium. Given are means±SE. Significant differences ($p \leq 0.05$) in conidial development of each category (germinated, undifferentiated and differentiated) were tested in one-way ANOVA, followed by Tukey HSD post hoc test with n=5 experiments with 100 conidia each. Statistical differences are marked by letter types: germination in small letters, formation of undifferentiated germ tubes in capital letters and appressorium formation in italics.
A & B: SEM pictures of a glass slide surface covered with 15µg wild type wax (A) and mutant *cer*-yp.949 wax (B).

On the other hand, germination rates on wax-sprayed glass slides were significantly increased, as now more than 75% of conidia developed germ tubes, irrespective of whether wild type or *cer*-yp.949 wax had been applied. Both waxes resulted in comparable germination and differentiation rates, which were significantly different from those on glass surfaces. Coverage with *cer*-yp.949 wax produced the highest rates of differentiation observed in this experiment (47%). In detail, 51% of these differentiated conidia distinctly formed a hooked app, while 49% generated a swollen appressorial germ tube (agt). The proportion of

differentiated apps was lower on wild type wax coverage, as here, no more than 36% of the differentiated conidia distinctly formed apps, while 64% formed agts (black bars, Fig. 3).

Different amounts of wild-type or *cer*-yp.949 wax sprayed onto glass-slides, demonstrated a positive correlation between increasing wax amount, germination rate, and a successful differentiation (data not shown). In both cases, highest rates were achieved on surfaces with wax amounts equivalent to wild type leaves (15 μg cm^{-2}). Irrespective of their wax loads, the glass slide surfaces did not exhibit the typical wax crystal structures known from native leaves (Fig. 3, A & B).

2.1.1 Conidial Development on Surfaces with Different Compounds and Hydrophobicity Levels

The two major compounds of the alkanol and aldehyde fraction of barley leaf cuticular wax, hexacosanol and hexacosanal, respectively, were described, to exert different effects on germination and differentiation of *Bgh* conidia (Tsuba *et al.*, 2002). To study these effects in more detail, conidiospore germination was analyzed in the presence of different amounts of these two compounds. For such analyses, the different chemical properties of hexacosanol and hexacosanal, resulting in different surface hydrophobicitiy characteristics, were taken into consideration (Fig. 4).

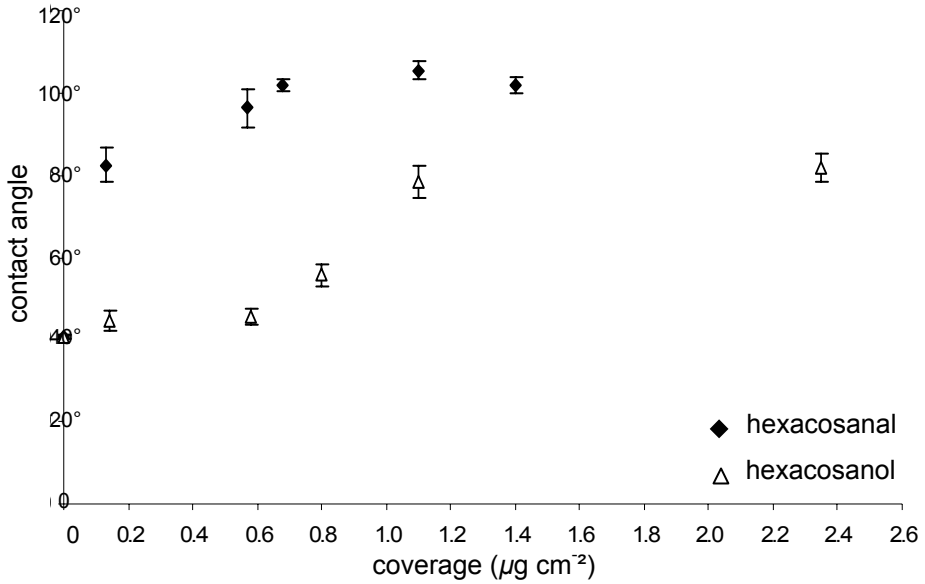


Figure 4. Contact angles of Histobond® glass slides coated with varying amounts of hexacosanol (C_{26}-alkohol) or hexacosanal (C_{26}-aldehyde). Given are means of 20 replications ±SD.

A glass slide covered with only 0.15μg cm^{-2} hexacosanal inherently exhibited a contact angle of 83°, while the same amount of hexacosanol resulted

in only 45°. Non-treated glass slides had a contact angle of approximately 40°. Increasing amounts of hexacosanal resulted in a maximum contact angle of 105° already formed on slides covered by 0.6μg cm^{-2}. With hexacosanol, the highest contact angle (82°) was obtained from glass slides covered with 1.1μg cm^{-2}. Increasing amounts of both substances did not result in any further increase of their maximum contact angles.

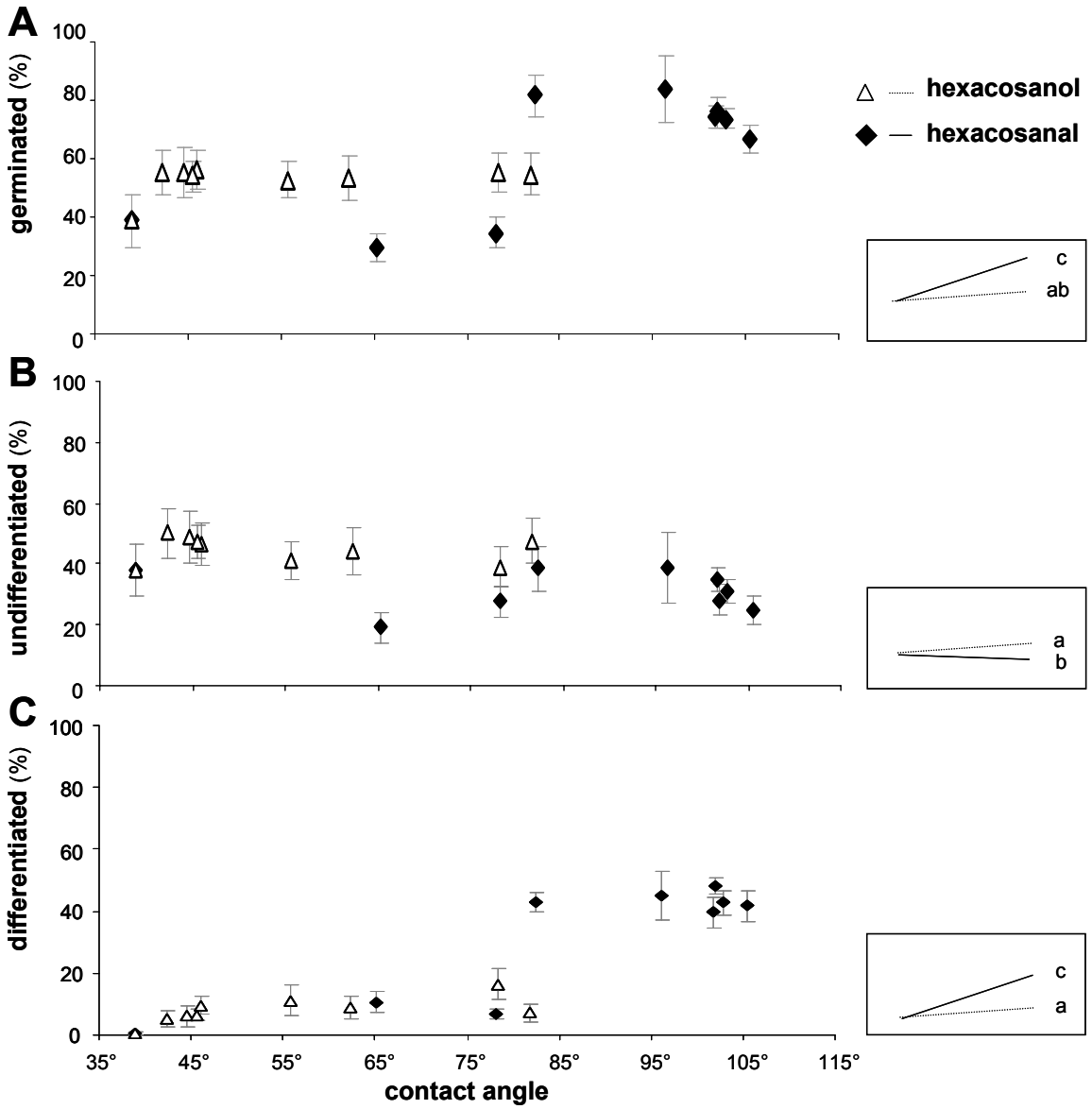


Figure 5. *B. graminis* conidia development on glass slides coated with varying amounts of hexacosanol or hexacosanal with resulting contact angles. A: percentage of conidia which germinated; B: percentage of conidia which formed undifferentiated germ tubes only; C: percentage of differentiated conidia with an appressorial germ tube or appressorium formed. Given are means ±SE. Boxes: Regression analysis. Regression lines were calculated for every data set consisting of hexacosanol and hexacosanal coated surfaces in combination with the corresponding stage of fungal development (A-C). Significant differences ($p \leq 0.05$) between slopes of every data set were tested by one-way ANOVA, followed by Tukey HSD post hoc test with n=5 experiments, 100 conidia each. Significantly different slopes within and between the three stages of fungal development (A-C), depending on surface chemistry and hydrophobicity, are indicated by different letters.

On hexacosanol covered glass slides (Fig. 5), conidia of *Bgh* exhibited maximum germination rates of merely 55%, and differentiation rates of approximately 16%, irrespective of different amounts and hydrophobicity values. In contrast, hexacosanal sprayed slides with contact angles below 80° showed a distinctly impaired fungal germination, as only about 32% (±3) of conidia formed a germination tube (Fig. 5A).

However, contact angles above 95° resulted in significantly higher average germination rates ($p = 0.0034$, Kruskal Wallis ANOVA) of 75% (±6), while the proportion of spores persisting in the undifferentiated germ tube stage slightly decreased depending on the amount of sprayed hexacosanal and the consequent surface hydrophobicity (Fig. 5B). Hexacosanal sprayed slides with contact angles above 75° led to a significant increase in the proportion of fully differentiated spores ($p = 0.001$, Kruskal Wallis ANOVA) to 48% at most (Fig. 5C).

In consideration of the ratio of app/ agt among the portion of differentiated conidia, the significant effect of hexacosanal compared to the non-inductive hexacosanol was even more pronounced, since it was 52% apps/ 48% agts for the aldehyde, contrasting 19% apps /81% agts for the alcohol. The regression lines additionally underline these effects on germination and differentiation of *Bgh* conidia.

2.1.1.1 Hydrophobicity as a Surface Cue

As the hydrophobicity of wax covered surfaces apparently influences germination and differentiation properties of *Bgh* conidia, different surfaces without wax coverage and contact angles ranging from 28° to 111° were tested for their effect on pre-penetration processes of *Bgh* (Fig. 6). The majority of fungal conidia (62%) did not germinate on thoroughly cleansed, fat- free glass slides with contact angles of about 28°. Furthermore, none of the germinated spores formed an app (Fig. 6).

A glass slide surface with a slightly increased contact angle of 39° did not affect spore germination rates, whereas about 1% of the conidia now formed an app. An ETFE foil with a comparably high contact angle of 94° resulted in significantly increased germination (59%) and slightly increased differentiation rates (3%).

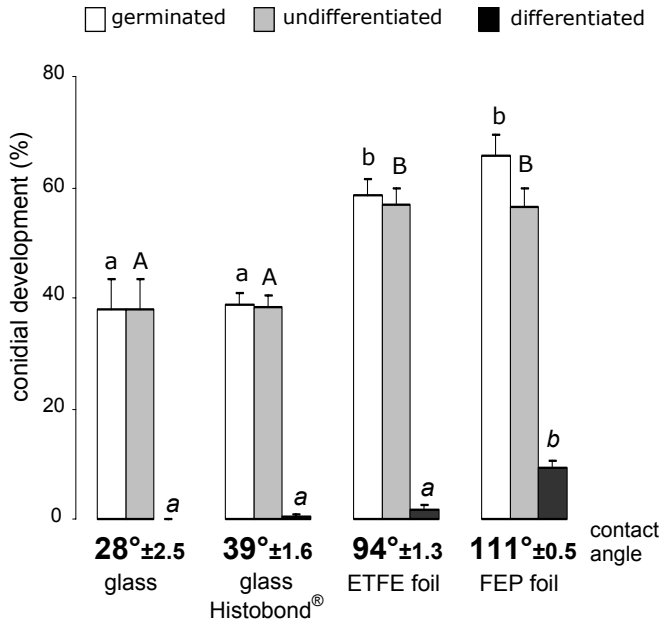


Figure 6. Development of *Bgh* conidia on planar artificial surfaces with distinct contact angles 28° - thoroughly cleaned glass slide, 39° - Histobond® glass slide, 94° - NOWOFLON® ET-6235 J (ETFE) and 111° - NOWOFLON® FEP (FEP) foils. Given are means ±SE. Significant differences ($p \leq 0.05$) in conidial development were tested by one-way ANOVA, followed by Tukey HSD post hoc test (n=5, 100 conidia each). The format of letters marks differences between rates of germination in small letters, formation of undifferentiated germ tubes in capital letters, and appressorium formation in italics.

The most striking results were obtained by employing a different, more hydrophobic ETFE-foil surface with a contact angle of 111°. Consequently, the germination rate was further increased to 66%, and the rate of app formation was significantly elevated to 10%.

2.2 Conidial Development on Isolated Leaf Cuticles

Development of *Bgh*-conidia on isolated barley leaf cuticular membranes resulted in reduced germination rates when compared with wax-sprayed glass slides (Fig. 7). About 59% of *Bgh* conidia germinated on isolated wild-type cuticles, whereas only 48% germinated on *cer*-yp.949 cuticular membranes.

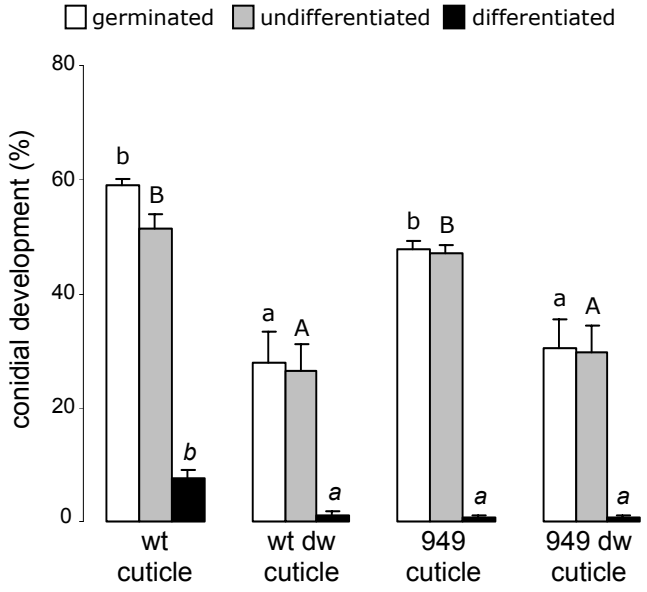


Figure 7. Development of *Bgh* conidia on enzymatically isolated barley leaf cuticles of wild-type (wt cuticle) and *cer*-yp.949. (949 cuticle): dw: dewaxed. Given are means ±SE. Significant differences ($p \leq 0.05$) in conidia development of each category (germinated, undifferentiated and differentiated) were tested in the one-way ANOVA, followed by Tukey HSD post hoc test with n=5 experiments, 100 conidia each. Statistical differences are marked by letter types.

On untreated wild type cuticular membranes, a significantly higher proportion (8%) of the conidia formed apps (one-way ANOVA), whereas on *cer*-yp.949 cuticles, only 1% of conidia differentiated into the app stage. After total wax extraction of wild type cuticles, the proportion of conidia forming apps was significantly reduced to almost 1%, whereas differentiation rates on de-waxed *cer*-yp.949 cuticles remained unaltered.

2.3 Conidial Development on Cellulose Membranes

Properties of living leaf tissue may have an impact on *Bgh* pre-penetration processes. Therefore, in order to create an artificial system that to some extent mimics these characteristics, development of *Bgh* conidia was screened on cellulose membranes applied onto glass slides (Fig. 8). On air dried cellulose dialyse membranes, a minimum of 4% of conidia had germinated, but no appressorium formation was detected. On cellulose membranes moistened with water, germination rates significantly increased to 35% (one-way ANOVA), and even a small percentage of differentiated apps could be detected (2%).

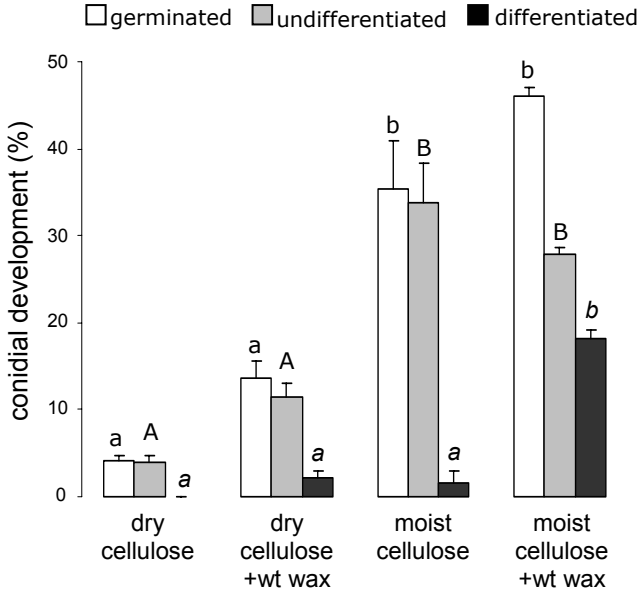


Figure 8: Development of *Bgh* conidia on cellulose membranes (for details see materials and methods 8.0) Wt wax: barley wild type wax. Given are means ±SE. Significant differences ($p \leq 0.05$) in conidiospore development were tested by one-way ANOVA, followed by Tukey HSD post hoc test (n=5, 100 conidia each). The format of letters marks differences between rates of germination in small letters, formation of undifferentiated germ tubes in capital letters, and appressorium formation in italics.

After the dry membranes were covered with wild type wax, rates of germination slightly increased to about 14%, accompanied by a slightly elevated percentage of apps (2%). Maximum germination and differentiation rates were achieved, when cellulose membranes were moist, and additionally covered with barley wild type wax. In this case, 46% of the conidia formed undifferentiated germ tubes, and 18% reached the app stage, which was highly significant when compared to wax

covered dry membranes. However, the conidial germination and differentiation frequency on the moistened and wax covered cellulose membranes did not achieve the level of those on wax covered glass slides (Fig. 3).

2.4 Conidial Survival Rates on Different Surfaces

In contrast to assays on living leaf tissue artificial surfaces apparently affected conidial success of survival. Therefore, rates of damaged and shrunken conidia were recorded in addition (Fig. 9, for details see materials and methods 8.0).

Irrespective, if wax of wild-type or of *cer*-mutant yp.949 was applied, a wax coverage on glass slides significantly improved conidial survival (one-way ANOVA), from 59% on plain glass, to almost 80% on wax coverage, a level fairly comparable to wild type leaves.

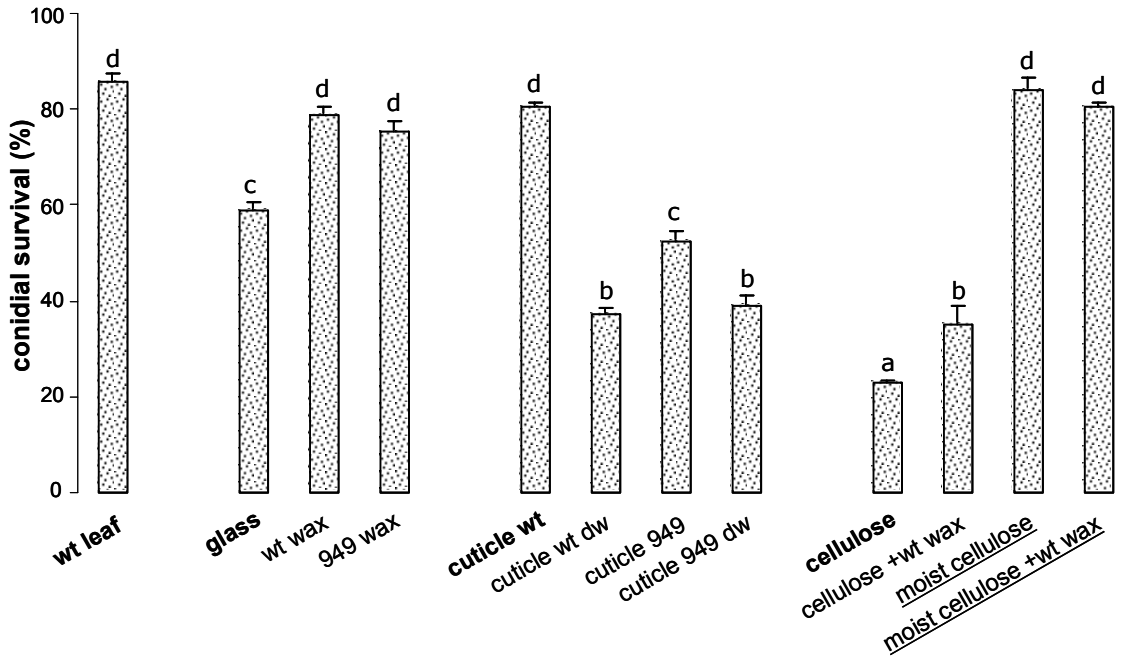


Figure 9: Rate of conidial survival on native wild type leaves and different artificial surfaces. Glass: Histobond® glass slide, wt: wild type, 949: mutant *cer*-yp.949, dw: dewaxed. Moist surfaces are underlined. Given are means ±SE. Letters mark significant differences ($p \leq 0.05$) in conidia survival, which were tested by one-way ANOVA, followed by Tukey HSD post hoc test (n=5, 100 conidia each).

On dry isolated wild type cuticles, survival rates were similar to those on natural leaf surfaces, and wax covered glass slides (ca. 80%). Whereas conidial survival on dry isolated *cer*-yp.949 cuticles was significantly reduced to 52%. After the removal of total cuticular waxes on both types of isolated cuticles, the percentage of surviving conidia significantly decreased to less than 40% in both

cases. This effect was more pronounced for wild type cuticles, for which less than one-half of applied conidia proved to be healthy after wax extraction.

On dry cellulose membranes, a minimum of 23% of conidia survived, whereas moistening of cellulose membranes, increased this proportion to a maximum survival rate of 84%. In each case, independent of an applied wax coverage, moist cellulose membranes exhibited identical survival rates to native wild type leaves.

DISCUSSION

1. Assays with *Bgh* Conidia on Leaf Tissue

1.1 Impact of Stressed Wild-Type Leaf Surfaces on Conidial Development

Our results demonstrate that even drastic differences in the amounts of cuticular waxes present on barley leaves do not significantly affect germination and differentiation properties of *Bgh* conidia. Neither the 1.5-fold increased wax load of cadmium exposed plant tissue, nor the distinctly reduced wax amounts of etiolated leaves, affected germination and differentiation of the conidia. Similarly, the modified composition of surface waxes due to etiolation had no effect on conidial development.

Surprisingly, the leaf surfaces of NaCl treated plants led to a significantly decreased proportion of conidia, which had formed appressoria (apps), although cuticular wax load and its chemical composition remained obviously unaltered under this stress-treatment. These results might be attributed to other surface derived factors, which are probably not directly connected with the investigated wax characteristics. One explanation could be that the plant´s leaf surfaces contained residues of salt crystals, resulting from the cultivation in hydroponic solution. The contact with these crystals, already during the conidial pgt-stage, might have caused osmolytic effects, which finally interfered with the successful formation of appressoria.

1.2 Impact on Modified *Cer*-Mutants´ Leaf Wax Characteristics on Conidial Development

Bgh conidia exhibited distinct responses when exposed to leaf surfaces of the selected *cer* mutants. Previous studies reported that *Bgh* conidia formed an increased proportion of malformed apps on flag or seedling leaves of various *cer* mutants of barley, suggesting that germlings failed to recognize and to respond to the leaf surface waxes on these mutants (Yang & Ellingboe, 1972; Rubiales *et al.*, 2001). However, in our hands, *Bgh* conidia did not exhibit distinct features of abnormal development on detached leaf surfaces of wild-type (Bonus) and selected *cer* mutants after 24h inoculation.

Rubiales *et al.* (2001) observed a significantly reduced percentage of *Bgh* germlings that produced an app (70%) on leaves of *cer*-zd.67. In the present study, by applying the same mutant, only a slight decrease in the proportion of apps was discernible. Compared to all other native leaf surfaces, inoculation of native *cer*-yp.949 leaves resulted in a significantly reduced proportion of apps in a similar range as reported for *cer*-zd.67 and *cer*-u.69 (Rubiales *et al.*, 2001). While Yang and Ellingboe (1972) considered that it might be the physical form, rather than the chemical composition, of epicuticular wax, to which *Bgh* responds by forming an app, Carver and Thomas (1990) showed that *Bgh* development was very similar on intact leaves and leaves from which the epicuticular waxes had been removed. Consequently, Rubiales *et al.* (2001) inferred that any effect of surface wax is most likely to be mediated by wax chemistry.

1.2.1 The "Wax-Effect"

In line with Carver and Thomas (1990), the removal of epicuticular waxes did not markedly affect the formation of apps on Bonus wild-type and on most *cer*-mutant leaves. This was not surprising, as the employed lines did not exhibit qualitative differences concerning epi- and intracuticular wax composition. These results underline that the physical structure of the epicuticular waxes plays at least a minor role in conidial recognition processes leading to app formation (Rubiales *et al.*, 2001). However, *cer*-yj.667 showed a distinct, although not significant, reduction in the proportion of differentiated conidia already after *gum arabic* treatment.

In most of the applied barley lines, the removal of total cuticular waxes by chloroform dipping resulted in a distinct decrease of the percentage of differentiated germlings forming apps by approximately 20% (Fig. 2, III). Hence, this "wax-effect" apparently accounts for at least 20% of the signals responsible for *Bgh* conidia differentiation on the leaf surface. Moreover, the "wax-effect" is reflected by decreasing germination rates in a similar order of magnitude. Thus, removing epi- and intracuticular waxes from the barley leaf surface clearly affects germination and app formation of *Bgh* conidia. However, there is a slight possibility that chloroform extraction releases compounds potentially inhibiting pre-penetration processes of *Bgh*. Likewise, the chemical removal of the barley

coleoptile cuticle by immersion in diethyl ether led to an increased frequency of premature appressorial tip collapse (Iwamoto *et al.*, 2002; 2007).

Cer-yj.667 and *cer*-yp.949 did not exhibit any further reduction of app formation on their leaf surfaces upon chloroform dipping, whereas Yang and Ellingboe (1972) found an abnormal germling development on *cer*-zj.78, known to contain an elevated portion of aldehydes (von Wettstein-Knowles, 1971). Similarly, *cer*-yp.949 leaf wax exhibited a drastically increased aldehyde fraction (mainly hexacosanal; Tab. I). Hence, the reduced portion of conidia forming apps on *cer*-yp.949 might be related to the modified chemical composition of leaf cuticular waxes in this mutant line. Surprisingly, neither removal of the epicuticular, nor of the intracuticular waxes, significantly affected the rates of app formation on *cer*-yp.949 leaves. Therefore, it seems unlikely that in this case the chemical composition of the mutant leaf wax influences *Bgh* germling differentiation. This is even more surprising as Tsuba *et al.* (2002) demonstrated hexacosanal to be active in inducing app formation *in vitro*.

2. Assays with *Bgh* Conidia on Different Artificial Surfaces

2.1 Impact of the Wax Coating on Conidial Development

To further substantiate the observed “wax-effect” concerning germination and app formation, glass slides were covered with isolated barley wild-type (cv Bonus) or *cer*-yp.949 leaf cuticular waxes. The reduced rates of *Bgh* germination and app formation on untreated control glass slides were perfectly in line with data presented by Yang and Ellingboe (1972). However, on wax treated glass slides, germination as well as app formation was greatly facilitated within a similar range by wild type and *cer*-yp.949 wax. This further supports the suggestion that it is not the distinctly modified chemical composition of the *cer*-yp.949 mutant wax that is responsible for the observed reduced app phenotype on detached leaves. On the contrary, *cer*-yp.949 wax covered glass slides resulted in even slightly improved rates of app formation when compared to wild type wax covered slides.

Our results demonstrated a verification of the observed “wax-effect” in an *in vitro* test system, which substantiated the importance of cuticular waxes in the *Bgh* pre-penetration processes. Nevertheless, the degree of germination and differentiation success on artificial surfaces did not achieve the rates observed on natural leaf surfaces. Since haustorium formation was proposed to be retarded by

light (Edwards, 1993; Carver *et al.*, 1994), the progression of conidial development might possibly depend on the light conditions during the gradual morphogenesis within the pre-penetration processes. Probably, conidial development on artificial surfaces can be optimized, resulting in differentiation rates comparable to those on leaf blades, by performing the inoculation procedure in the absence of light.

2.1.1 Impact of Substrate Chemistry and Hydrophobicity on Conidial Development

The hydrophobic nature of the epicuticular wax surface is its most important physicochemical property. It depends on surface roughness and the nature of the chemical groups exposed on the surface (Holloway, 1969). Previous studies analyzing the effect of surface waxes on pre-penetration processes of *Bgh* have not considered the distinct hydrophobic characteristics of the diverse wax constituents as possible surface cues. Hence, it remained unclear whether the C_{26}-aldehyde hexacosanal, with the potential to strongly induce app differentiation, alters the physical surface properties of the host, e.g., by changing the surface hydrophobicity, rather than acting as a chemical signal involved in app formation (Tsuba *et al.*, 2002).

By covering glass slides with distinct amounts of chemically synthesized hexacosanol or hexacosanal, which are the most prominent representatives of their substance classes in barley leaf wax, artificial surfaces of different hydrophobicity were produced. The results of the subsequent bioassays showed that the maximum contact angle, obtained on hexacosanol covered glass slides (82°, ≥1.2 μg cm^{-2} hexacosanol), was not sufficient to induce app formation, while hexacosanal induced germination and app formation only after the coverage gave rise to a threshold contact angle of approximately 80°. Nevertheless, both compounds exerted different effects on the germination rate of *Bgh* conidia. Despite increasing amounts of hexacosanol, the germination rates on such glass slides remained unaffected. Likewise, hexacosanal treatment below contact angles of 80° did not affect germination rates, though resulting in values comparable to those on untreated glass slides. Obviously, the presence of hexacosanol initially affects the germination rate, which is already increased about 15% by a faint hexacosanol overlay. Nevertheless, germination rates could not be increased further by applying additional hexacosanol. Strikingly, the average rate

of germlings that formed undifferentiated germ tubes only was significantly reduced on hexacosanal treated slides, irrespective of surface contact angles.

This further supports the idea that, below contact angles of 80°, neither germination, nor app formation is stimulated by hexacosanal. To boost the subsequent app stage of development, contact angles above 80° appear to be essential. Such contact angles could not be achieved with hexacosanol alone. According to Holloway (1969), the primary alcohol fractions are among the most wettable constituents of different plant leaf waxes, and form the major component of barley wax. Hence, on the natural barley leaf surface, the hexacosanal portion present in the epicuticular wax layer might in fact increase surface hydrophobicity, potentially affecting app formation of *Bgh* conidia.

Surprisingly, neither germination, nor formation of appressoria, was inhibited by hexacosanal in a dose-dependent manner above of 0.5 μg cm^{-2}, as described by Tsuba *et al.* (2002). In contrast, on glass slides covered with extracted *cer*-yp.949 wax, with a distinctly enhanced proportion of hexacosanal, *Bgh* conidia germination and differentiation were even slightly increased, as compared to slides covered with wax extracted from wild-type leaves. Likewise, this effect might be attributable to a simultaneously increased contact angle (117°±2) on the *cer*-yp.949 wax surface, in comparison with the applied wild-type wax (110°±1). However, triacontanal, the corresponding C_{30}-aldehyde, also exhibits an inductive effect on app differentiation, even though to a lesser degree (Tsuba *et al.*, 2002). This, and the differentiation-inducing effect of the C_{28}-aldehyde octacosanal on germlings of the wheat stem rust fungus *Puccinia graminis* f.sp. *tritici* (Reisige *et al.*, 2006), might support the idea of aldehydes being cuticular wax-derived inductive factors.

Nevertheless, the importance of the surface hydrophobicity in the *Bgh* experimental system is underlined by the fact that even wax-free artificial surfaces, like ETFE foils (Fig. 6), can induce considerable germination rates, comparable to those obtained on hexacosanal covered artificial surfaces. On the other hand, it needs to be determined if surface micro-structures resulting in contact angles ≥112° might further imply a possible differentiation inducing capacity.

2.2 Impact of the Cutin Matrix on Conidial Development: Wild-Type Versus *Cer*-Mutant yp.949

When compared to plain glass surfaces, germination and differentiation rates on isolated barley cuticles (wild type and *cer*-yp.949) were slightly increased. This might have been expected, as in accordance to Francis *et al.* (1996), cutin monomers were described to induce the formation of differentiated apps. Nevertheless, the germination and app formation rates observed are not in accordance with results of earlier studies (Yang & Ellingboe, 1972; Kobayashi *et al.*, 1991), reporting comparable rates of germination and app formation on isolated barley cuticles and detached leaves. These differences might be explicable by distinct experimental procedures, particularly, as fully dried cuticular membranes fixed on glass slides were applied in our study, while in other studies, isolated cuticles positioned on water agar were used.

Compared to wax sprayed glass slides, germination and differentiation rates were reduced on both types of isolated cuticles, wild type and cer-yp.949. The values on *cer*-yp.949 did not attain those on wild-type cuticles. Even the removal of intracuticular waxes did not alter the developmental frequency of the conidia on isolated cuticles of *cer*-yp.949, indicating that the observed “wax-effect”, was again, similar to assays on living leaf tissue, not verifiable in this *cer* mutant. Nevertheless, chloroform-extracted (“dewaxed”) isolated wild type leaf cuticles revealed a distinct “wax-effect” with a decrease of germination and app formation rates, resulting in rates comparable to those obtained on non-treated glass slides.

Similar to the germination- and differentiation-success, the survival-rates of fungal conidia on wax coated isolated *cer*-yp.949 cuticles were significantly reduced, in comparison to those on wild-type cuticles. After the removal of cuticular waxes in both lines, the survival rates were not only significantly reduced, but also reached a similar level (almost 40%).

Summarizing the various results of conidial development obtained on leaf surfaces, extracted waxes and isolated cuticles of *cer*-yp.949, different suggestions arise: isolated waxes of wild-type and *cer*-yp.949 were highly inductive to germination, differentiation, and even survival of *Bgh* conidia. On both types of dewaxed cuticles, low but similar germination rates and weak differentiation success were observed, accompanied by similar survival rates (Fig. 7 & 9). A linkage of the specific wax coverage to both types of cuticles led to

significantly higher germination rates of the conidia. In contrast, formation of differentiated germ tubes, as well as conidial survival rates, achieved higher levels on wild-type than on cuticles of mutant *cer*-yp.949. The results suggest that the construction of the cutin matrix, with embedded intracuticular wax components, may contain key information for conidial development.

The analysis of cutin monomer composition of isolated wild-type and *cer*-yp.949 in comparison, did not reveal differences in the composition between the cuticles of both lines (data not shown). Probably, a different construction of the cuticle network itself in the mutant *cer*-yp.949 could explain the different germination and differentiation rates of *Bgh* conidia. Although there is extensive information on the cutin monomer composition of several plant species, its polymeric architecture is largely unknown (Stark *et al.*, 2000; Tian, 2005). For the methodical determination of the chemical composition, the polymer's three dimensional network has to be degraded, to produce soluble products that can be identified. However, since the properties of the substratum may also substantially influence the arrangement of the wax components (Koch *et al.*, 2006), the suggested modifications in the *cer*-yp.949 cuticle might be reflected in its altered epicuticular wax crystal morphology (chapter I, Fig. 6C, p.22).

2.3 Impact of Surface Moisture on Conidial Development

Previous studies reported considerable rates of germling differentiation also on largely hydrophilic surfaces, such as water agar or cellulose membranes (Yang & Ellingboe, 1972; Carver & Ingerson, 1987; Kobayashi *et al.*, 1991; Kinane *et al.*, 2000; Green *et al.*, 2002). As even those surfaces can be inductive, other factors than hydrophobicity or wax chemical composition appear to be involved in determining the rates of differentiation achieved on natural host surfaces. The exact nature of such signals is largely unknown, although indications exist that cutin monomers (Francis *et al.*, 1996), or cellulose breakdown products (Carver *et al.*, 1996), might play an additional role in conidial surface recognition.

To substantiate additional effects, i. e. the impact of water availability, assays on dry and moist cellulose membranes were applied. The tissue moisture was shown to highly influence the conidial survival rates. The survival rates recorded on dry cellulose were dramatically increased when the matrix was moistened by underlying watery agar. Cellulose membranes show a pronounced

capability to soak water. Hence, the reduced rates on the dry membranes might have been the result of the high suction of the material, which possibly may have led to a desiccation of conidia. This effect seems to be balanced by the applied surface moisture.

The recorded survival rates on artificial surfaces attained those of native leaf blades, whether conidia had been applied on wax alone, whether wax had been embedded in the wild type cuticle, or whether sufficient humidity was given by the substrate surface (Fig. 9). However, tissue humidity does not only seem to be an important factor in influencing the conidial survival success, but also in stimulating conidial germination rates (Fig. 8). Referring to this, Carver *et al.* (1983) reported that unattached *Bgh* germlings grew well in humid conditions, but almost always stopped growing and shrivelled before differentiating an app when exposed to arid conditions. Thus, the authors suggested an increased attachment of the primary germ tubes (pgts) to the host surface, to reflect the conidial ability to respond to arid conditions. They supposed the pgt to play an important role in water uptake, thereby highly influencing the success of the following steps of the pre-penetration processes. Therefore, already the pgt´s contact with the dry cellulose membrane in our experiments might have reduced the conidial survival rates and their differentiation success, as they could not make water accessible.

However, the applied wild type wax coverage had obviously weakened this negative effect. In case of sufficiently available water, an additional wax coverage on moist cellulose membranes did not improve the conidial survival anymore (Fig. 9), but again the wax coverage significantly stimulated germination and differentiation of the conidia (Fig. 8). This resulted in a similar increase in app formation of approximately 20%, as has already been shown earlier for native leaf blades (“wax-effect”). Thus, humidity did rather support conidial survival and emergence of undifferentiated germ tubes, than stimulate the formation of differentiated apps. Furthermore, there seems to be a valuable difference between atmospheric humidity and surface moisture. The relative atmospheric humidity was similar in all experiments, but in contrast to the artificial surfaces, the natural leaf surfaces may, for the spores, serve advantageous water resources, which are not available on artificial substratum. In the proximity of the stomata, the water content may be locally increased by stomatal transpiration. Moreover, the conidia probably gain access to the leaf tissue moisture through the direct contact with the

surface. Kunoh *et al.* (1988) showed, that ungerminated conidia release a liquid exudate upon contact with a substratum (extracellular matrix), that contains hydrolytic enzymes, including esterases (Nicholson *et al.*, 1988), which are capable of degrading the surface of the leaf cuticle (Kunoh *et al.*, 1990). By direct contact between the conidial extracellular matrix and the leaf surface, the conidium may benefit from the water contents of the epidermal tissue.

In conclusion, the availability of water seems to be necessary for conidial survival, and therefore, serves as important prerequisite for supporting successful progression of conidial development. In addition, the inductive capacity of barley surface waxes on conidial differentiation could be underlined by an artificial system that is based on a moist cellulose matrix.

3. Resuming Several Leaf Surface Parameters in Affecting Conidial Development

This study demonstrates clearly that the availability of barley cuticular surface waxes is highly important for providing initial cues promoting *Bgh* pre-penetration processes ("wax-effect"). Exclusively focusing on the characteristics of the leaf waxes, the results further indicate synergistic effects of hydrophobicity and chemical composition (Fig. 10) to be jointly responsible for triggering the ordered morphogenetic sequence of spore germination and differentiation.

Surface hydrophobicity, which depends on the chemical nature and the micro-morphology of the surface constituents, obviously comprises a substantial portion of the "wax-effect". This could further be substantiated with single wax components, which, based on their respective chemical nature, also influence the surface hydrophobicity.

In comparison to wax coated glass slides, the differentiation rates on wax containing isolated cuticles and wax covered cellulose membranes were diminished (Fig. 10). This might indicate, that the embedding of waxes into an underlying substratum somehow impairs the accessibility of surface derived factors which are important for the successful conidial differentiation. The three-dimensional construction of the cutin polymer matrix may interfere with the reported stimulating effect of cutin monomers (Francis *et al.*, 1996). An alteration

in the cutin polymer construction of *cer*-yp.949 probably explains the diminished conidial developmental rates on leaf surfaces and cuticles of this mutant.

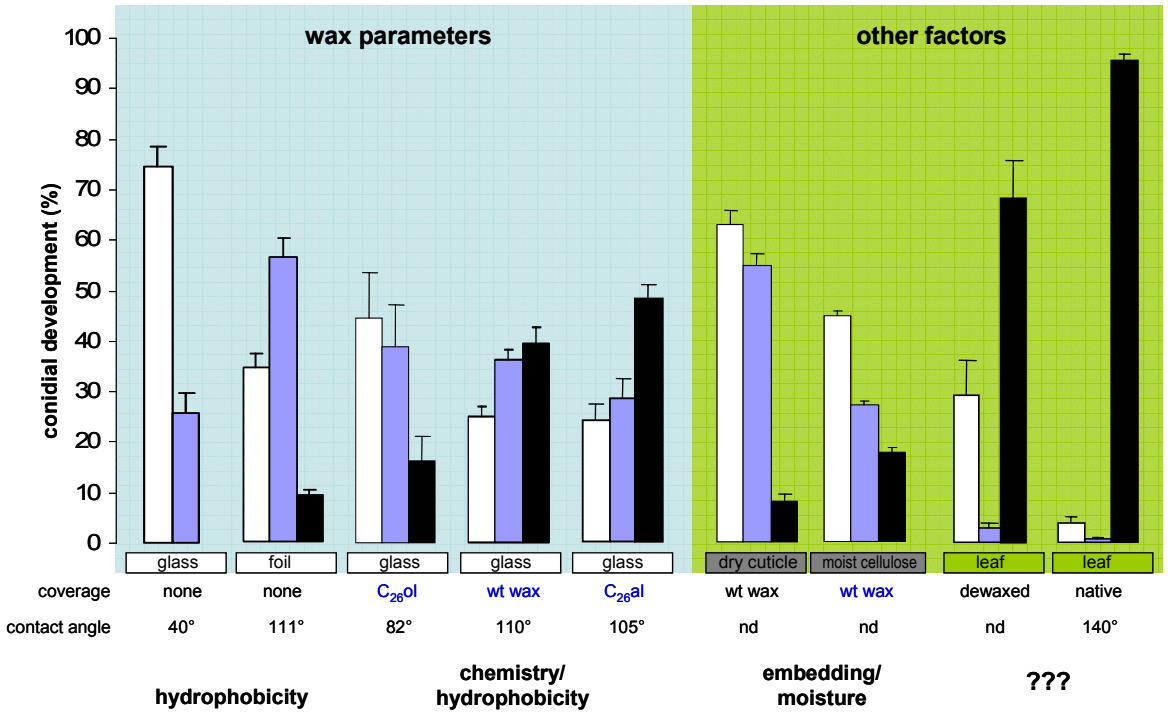


Figure 10: Illustration of observed proportional effects of distinct barley wax characteristics and tissue derived factors on *B. graminis* conidial development: white bars- germinated conidia, blue bars- conidia which had formed undifferentiated germ tubes only, black bars- conidia which had formed differentiated germ tubes and appressoria. The blue text marks applied coverages, nd- not determined. Given are means ±SE (n=5, 100 conidia each).

Moreover, the natural leaf tissue provides further factors that might potentially influence the fungal pre-penetration processes. Our experiments indicate that the surface moisture directly supports the survival of fungal conidia contacting a substratum and thereby rules the successful formation of undifferentiated germ tubes. Finally, the availability of other tissue derived parameters, like glucose, cellulose break-down products, or other factors, might play an additive role in the development of the fungal germlings, which shall be the subject of other studies. Nevertheless, our investigations display a high impact of distinctive leaf wax parameters on the progress of the conidial developmental sequence, ultimately leading to *Bgh* appressorial differentiation on barley epidermal surfaces.

CONCLUSIONS AND PERSPECTIVES

CHAPTER I demonstrates the plasticity of leaf waxes in response to environmental stresses in barley plants. Variations in the amount and the chemical composition of cuticular leaf waxes reflect the plants capability to maintain the protective function of the cuticle (e.g. against transpirational water loss) under extreme conditions. Quantitative changes were rather revealed in the reduction, or increase, of the total cuticular wax load, than in shifts of the ratio of the epi-/ intracuticular wax portions. Qualitative variations were restricted to modifications in relative amounts of several chemical compound classes, which are characteristic for barley leaves, and to alterations in chain length distribution of certain fractions. Entirely new wax components did not arise.

The respective type of abiotic stress determined the degree of changes in surface wax characteristics. In response to some common habitat stresses, barley may have evolved alternative strategies of adaptations, which do not affect the leaf wax properties, but do influence other regulatory levels e.g. processes of gene expression presented in chapter III. *Bgh*-infection, here presented as an example for a biotic stressor, evoked neither locally nor systemically derived changes in wax characteristics.

The modifications of wild type wax, in response to environmental stress, did not attain the degree of the alterations in *cer*-mutants leaf surface waxes. For each of the screened mutants, variations in wax quantities were expressed as a reduction of total wax amounts. At least for some special mutants, extraordinary reductions resulting in a minimum part of the wild-type wax load were observed (*cer*-zd.67, *cer*-zh.54). In addition, the ratios of epi-/ intracuticular wax fractions changed drastically. Dramatic changes in chemical composition, which especially affected quantities of aldehydes and esters, highly influenced the morphology of epicuticular wax crystals, resulting in distinct decreases of surface hydrophobicity.

It remains unclear, whether these observed differences in *cer*-mutants wax features may be compensated in case of exposure to a combination of abiotic stresses. On the one hand, this may possibly result in a shift, back to the wild type wax phenotype, on the other hand, the mutant specific wax modifications, may be intensified. However, further unknown alterations in surface wax characteristics

could appear, which might hint towards the type of mutation within the respective wax mutants.

CHAPTER II presents the establishment of the barley wax microarray, which offered the possibility to correlate analytical observations with certain transcriptional events of wax biogenesis. Changes in gene expression patterns in response to different environmental stresses were highly divers. However, also common trends occurred, which indicates significant cross-talk between several modes of transcriptional control. Since the differential expression in processes of elongation and modification of fatty acids in the darkness-treatment highly correlated with the changes in wax composition, light can be considered an important factor in determining the induction of wax biogenesis. Several of the detected gene responses were attributable to the respective stress related defense, rather than to the progression of wax biosynthesis. Some of the observed effects were difficult to evidently correlate to certain aspects, since they pointed to different sets of regulatory mechanisms. Various transcriptional modifications were revealed through *Bgh*-infection, which indicates several levels of molecular interaction between the pathogen and its host. The fungal invasion through the plants' cuticle into the host epidermis was reflected by the induction of several stress regulated genes. Furthermore, the rapid progression of the fungal infection correlated with a time-dependent development of numerous transcriptional events. Expression profiles were useful for the detection of speedy transcriptional modifications during initial events of powdery mildew infection, which offered complementary aspects of the host/ pathogen interaction at the molecular level. Although powdery mildew infection did not obviously affect the surface wax characteristics, the impact of the haustorial functionality on the rearrangement of the host plants' metabolic activity affected several aspects important for the production and transport of wax components.

For future studies, further benefits from the barley wax-microarray might be seen in investigations of the transcriptional responses of the prominent selection of *cer*-mutant candidates. It would be of highest interest to explore whether transcriptional processes affecting wax biogenesis in these mutants differ from those in the wild-type. Thereby, the modifications in *cer*-mutants surface waxes can be expected to correlate with differences in the expression of distinct gene

candidates, probably further indicating the type of mutation in respective wax-mutants. Transcriptional modifications due to *Bgh*-infection should especially be screened for the mutant *cer*-yp.949. Thereby, differences in the distinct molecular responses in comparison to the wild-type can be expected to correlate with the reduced conidial developmental rates on this mutants leaf surfaces described in chapter III. Since barley is commonly exposed to the attack of a variety of pathogens, the plants' molecular responses to a different biotrophic fungus, e.g. the rust fungus (*Puccinia graminis*), may reveal variations in molecular responses to different biotic stressors.

CHAPTER III offers detailed observation of fungal conidia development in dependence of several leaf surface parameters, with new insights into the mechanisms of surface recognition in the barley/ powdery mildew system. For the first time, the importance of leaf surface waxes for succeeding fungal pre-penetration processes were clearly demonstrated and validated. With regard to the wax characteristics, our results indicate synergistic effects of surface hydrophobicity and chemical composition of cuticular waxes, providing initial cues promoting *Bgh* pre–penetration processes. Furthermore, wax amounts and the shape of epicuticular wax crystals serve as factors of minor importance during early interactive events. Nevertheless, with regard to a further substantiation of the effects of surface hydrophobicity, future investigations should examine the indirect impact of microstructured superhydrophobic surfaces on conidial differentiation rates.

In reference to the conidial germination and differentiation success on leaf surfaces and isolated cuticles of *cer*-yp.949, the embedding of waxes into the cutin polymer matrix obviously bears information for spore development. It would be advantageous to explore further methods to clarify the three-dimensional architecture of the cuticle network in order to verify the constructional differences between this mutant and the wild-type. As one example for a tissue derived factor, the surface moisture obviously strongly supports the conidial survival and germination. Ultimately, the interfering effects of glucose or other nutrients with observed effects of barley surface waxes on the developing fungal conidia remain to be tested.

In conclusion, early events of the powdery mildew infection obviously remain unaffected by the stress state of the host plant during extreme abiotic environmental conditions. The adaptation of the highly ordered conidial morphogenesis to constant, species unspecific, leaf wax parameters, might be advantageous for the infection- and survival- success of the fungal conidia. Finally, conidial germination and differentiation reveal species independent processes, while species specific adaptations of interaction events are mirrored solely on the molecular level.

MATERIALS AND METHODS

1.0 Pathogen and Plant Material

Hordeum vulgare (L. cv. Bonus) was grown in plastic pots (Ø 9cm). The plants were kept in growth chambers, under 300 to 400 µmol photons m^{-2} s^{-1} light intensity in day /night cycle 16h/ 8h (24°C/ 18°C), and 70% relative humidity.

Initially, a collection of 18 barley *cer*–mutants in Bonus background (*cer*-yb.200, *cer*-yj.667, *cer*-yp.949, *cer*-ze.81, *cer*-zd.67, *cer*-yf.652, *cer*-za.126, *cer*-zh.54, *cer*-ye.792, *cer*-j.59, *cer*-zk.85, *cer*-ys.680, *cer*-zu.69, *cer*-zj.78, *cer*-p.57, *cer*-yp.955, *cer*-zv.1246, *cer*-zv.1795), obtained from the Nordic Gene Bank (Alnarp, Sweden), and Udda Lundqvist (Svalöv, Sweden), was analyzed with respect to surface morphology and wax chemistry, and subjected to *Bgh* bioassays. The barley wax mutants were grown under the same conditions as the Bonus wild type.

Blumeria graminis f.sp. *hordei*, (*Bgh*, isolate CC1, originally obtained from Tim Carver, Aberystwyth, U.K.) was propagated on its host barley (cv Bonus), until distinct white powdery pustules appeared. One day before conidia were required for experimentation, leaves bearing sporulating colonies were shaken to remove older conidia, so that freshly emerged conidia were available for subsequent bioassays.

2.0 Different Abiotic Stress-Treatments

To test whether abiotic factors may potentially lead to a modification of surface waxes, barley wild type plants (cv Bonus) were subjected to four different treatments (I-IV). In treatment I, plants were covered with cardboard boxes during growth, to ascertain absence of light (darkness). Similar to control plants, this setup was kept in growth chambers with equivalent conditions as described above. For wax analysis, harvested 14d old secondary leaves were scanned by means of a commercial image scanner. To determine the exact leaf surface area, the pixel counts of the leaf images were measured, and correlated with a reference area.

In the second approach, 3d old barley germlings were grown up on filter paper moistened with tap water, until an 3-4 cm primary leave had emerged, and were then transferred to Hoagland solution (0.5x), Tab.#), with different proportions of Sodium (NaCl, Hersteller, Land, w/v; 0.5%, 1% and 2%), to impose distinct levels

of salt stress (treatment II). During experimental time of 14d, a constant aeration within the hydroponics was provided, and the nutrient solutions were replaced daily. Control plants were grown on Hoagland solution exclusively, without addition of NaCl.

To examine the effect of cadmium-stress, a treatment (III) with different concentrations of cadmiumchloride solutions ($CdCl_3$, Hersteller, Land, 100µM and 500µM) was applied. Beforehand, barley seeds were kept in a moist mixture of 90% Lecaton (fleur ami GmbH, Tönisvorst, Germany), and 10% soil for 2d, until germlings were approximately 3-4 cm long. Subsequently, 40ml of the $CdCl_3$ solutions were poured on daily for one week, and afterwards every second day for further 2 weeks. Control plants were watered with tap water, for all assays a fertilization with Hoagland solution (0.5x) was added (40ml) once weekly. 9d and 16d old secondary leaves were harvested (n=5) for wax analysis, and leaf surface area was determined.

To test the effect of drought-stress (treatment IV), single potted (pots Ø 5cm) 4d old plants grown-up in growth chambers (see conditions above) were watered until the soil was saturated. Afterwards, pot weight was determined, and treated plants were kept in growth chambers without further watering, until weight loss of pots achieved approximately 50% of continuously watered control plants (Fig. 1). The procedure was repeated twice, and plant material for wax analysis was finally harvested on day 21 of treatment. Plant tissue samples for RNA isolation of native and treated (I-IV) wild type leaves were cut off simultaneously, and immediately frozen to -80°C until use.

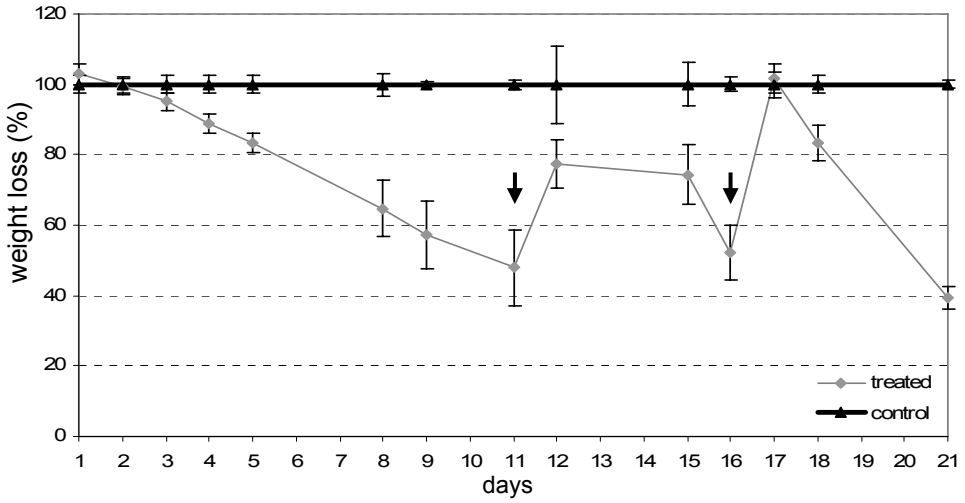


Figure 1: Weight loss of plant pots during drought stress (treatment IV). Arrows mark first and second rewatering, mean ±SD, control plants n=20, treated plants n=30

3.1 Analysis of Locally *Bgh* Infected Tissue

To investigate the local effect of *B. graminis* infection on wax composition and accumulation, 14d old plants were incubated for 6d with freshly emerged conidia, as described above. Afterwards, secondary infected leaves of test plants, and non-infected leaves of controls, were harvested, their leaf area was determined, and collected leaves were freeze dried at -55°C under vacuum. After 24h in the freeze dryer (Christ Alpha 1-2 LD, Osterode am Harz, Germany), and adaptation at room temperature in an exsiccator, wax analysis was carried out.

To examine the effect of powdery mildew infection on molecular events of wax biosynthesis, 14d old native wild type plants were inoculated with *B. graminis* onidia, and after different incubation periods (12 h-72h), in growth chambers with equivalent conditions, the second leaf was harvested for molecular investigations, while material of non-treated control plants was simultaneously collected.

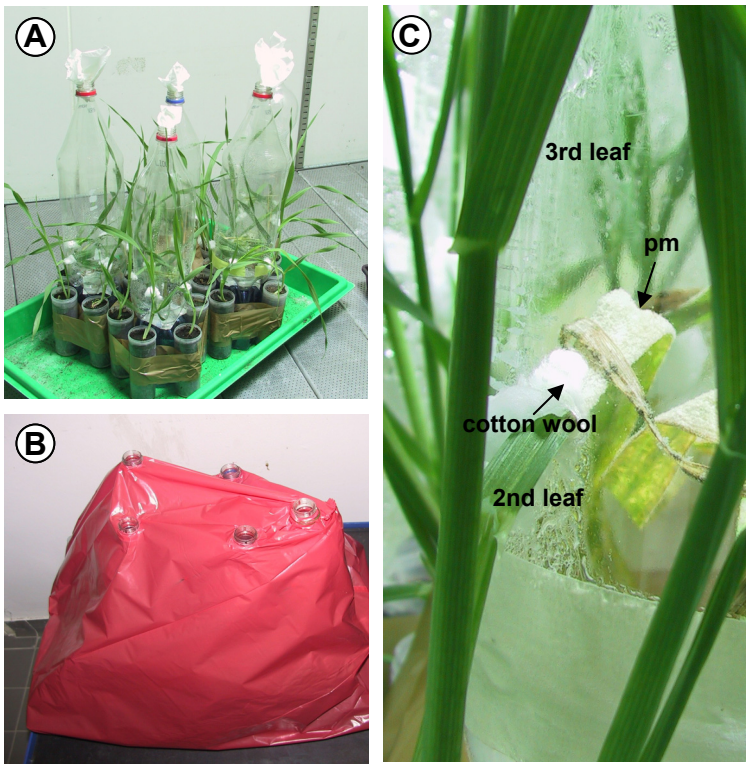


Figure 2: Experimental setup for inoculation of exclusively one barley leaf with *B. graminis* (*Bgh*) conidia. A: Single grown 14d old barley plants were fixed to plastic bottles (1.5l) with their second leaf inside the bottle lumen. B: While the plant body was covered with plastic foil, *Bgh* conidia were blown through the bottleneck to exclusively infect the secondary leaf. C: 5d after inoculation, powdery mildew pustules (pm) on the leaf area inside the bottle were distinctly visible, while the leaf area outside remained uninfected and healthy.

3.2 Systemic *Bgh* Infection

To systemically infect barley plants, exclusively the secondary leaves of 14d old single grown plants were inoculated with *B. graminis* conidia, using plastic bottles as an settling tower (Fig. 2). After an incubation time of 5d, 10d and 15d in growth chambers, emerged non-infected fourth leaf was harvested for wax analysis (n=5). Secondary leaves of control plants were inserted into bottles as well, without conidial inoculation.

4.1 Sampling of Cuticular Waxes

Total leaf extracts were obtained by dipping entire leaves (apart from the cut edge) for 2min into 20mL chloroform (>99%; Roth, Karlsruhe, Germany). *N*-tetracosane (Sigma–Aldrich, Steinheim, Germany) was added to all extracts as an internal standard. The solvent was removed under a continuous flow of nitrogen. In order to sample adaxial and abaxial cuticular leaf waxes separately, the respective opposite leaf surface was covered with a 50% aqueous solution of *gum arabic* (1:1, w/v, Roth, Karlsruhe, Germany), using a paint brush. Subsequently, the leaves were glued upside down onto the bottom of a glass Petri dish, and the waxes of the leaf surface of interest were rinsed off for 2min with 20mL chloroform. Prior to use, *gum arabic* was extracted exhaustively with hot chloroform, to remove impurities.

For the selective mechanical removal, and collection of epicuticular waxes, *gum arabic* was applied, and dried until it had formed a stable polymer film, in which epicuticular wax crystals were embedded. The polymer film was stripped off and the procedure repeated once. After the second application, epicuticular wax crystals were removed completely (chapter III, Fig. 2A-III). For the analysis of chemical composition, the dried *gum arabic* film was collected in a vial containing a water/chloroform mixture (1:2, v/v). After vigorous agitation and phase separation, the organic solution was removed, and the procedure was repeated once, after adding 2mL chloroform to the aqueous phase. Finally, the obtained organic phases were pooled and stored until analyzed. After *gum arabic* treatment, leaves were physically intact. The subsequent selective extraction of adaxial and abaxial intracuticular waxes was performed as described above.

4.2 Chemical Analysis

Prior to gas chromatography (GC) analysis, hydroxyl–containing compounds in all samples were transformed into the corresponding trimethylsilyl derivatives, by reaction with bis-N,O-trimethylsilyltrifluoroacetamide (Macherey–Nagel, Düren, Germany) in pyridine (30min at 70°C). The quantitative compositions of the mixtures were studied by using capillary GC (5890 Hewlett Packard Series II; Agilent Technologies, Santa Clara, USA), and flame ionization detector, under the same conditions as qualitative analysis (6890N, Agilent Technologies, Santa Clara USA), with mass spectrometric detection (*m/z* 50–750; MSD 5973, Agilent Technologies, Santa Clara, USA). GC was carried out with on–column injection (30m x 0.32mm inner diameter, DB–1, d_f= 0.1μm, J&W Scientific, Agilent Technologies, Santa Clara USA). Oven temperature was programmed for 2min at 50°C, 40°C min^{-1} to 200°C, 2min at 200°C, 3°C min^{-1} to 320°C, 30min at 320°C, and He carrier gas inlet pressures programmed 5min at 50kPa, 3kPa min^{-1} to 150kPa, and 30min at 150kPa. Wax components were identified by comparison of their mass spectra with those of authentic standards and literature data. For quantification of individual compounds, GC was used under conditions as described above, but with H_2 (5min at 5kPa, 3kPa min^{-1} to 50kPa, and 30min at 50kPa) as the carrier gas and a flame ionization detector (HP ChemStation software package).

5.0 Characterization of Wax Crystal Structures and Surface Hydrophobicity

Air-dried (four weeks, 25°C) pieces of leaf samples were mounted on aluminium holders, sputter coated with gold palladium (Bal–Tec SCD005 sputter coater; 25 mA, 300 s, Balzers, Switzerland), and examined by scanning electron microscopy (SEM; Zeiss DSM 962, 15kV, Zeiss, Oberkochen, Germany). Sputtering conditions, depositing approximately 20nm of the alloy on the tissue samples, were optimized for the acceleration voltage used in the SEM.

To measure surface hydrophobicity, contact angles of sessile 1µl droplets of distilled water were determined (Young-Laplace fitting, contact angle system OCA 15, software system SCA20; Dataphysics Instruments, Filderstadt, Germany). A total of 20 independent measurements per surface sample were performed.

6.0 Coating of Glass Slides

Different dilutions of chloroform extracted cuticular plant waxes were sprayed on glass slides (Histobond, Marienfeld, Germany), using a chromatography sprayer. The wax-coated glass slides were stored at room temperature for a minimum of 14d, to ensure complete evaporation of solvent traces. To determine the applied wax amount per glass surface area, the glass slide wax coverage was washed off thoroughly with chloroform. Subsequently, absolute wax amounts were measured by GC analysis.

Hexacosanal was synthesized, starting from the corresponding 1-alkanol, according to the protocol developed by Corey and Suggs (1975). A suspension of PCC (pyridiniumchlorochromate, 31mg, 3.3mmol, 98%: Fluka, Steinheim, Germany) and Celite (1g, Merck, Darmstadt, Germany) in CH_2Cl_2 (99.5%; Roth, Karlsruhe, Germany, 5mL) was cooled to 0°C, and 1-hexacosanol (50mg, 3.0mmol, 99%; Sigma, Steinheim, Germany) in CH_2Cl_2 (10mL) was added. After continuous stirring over night at room temperature, the formed brown slurry was filtered over a silica gel column (Ø 2.5cm). The synthesized aldehyde was further eluted and fractionated with petroleumether/ diethylether 9:1 (400mL). The aldehyde fractions obtained were pooled, and the purity was checked by NMR (data not shown). Pure hexacosanol and hexacosanal were dissolved in chloroform, and then applied to glass slides as described above.

7.0 Isolation of Barley Leaf Cuticles

Leaf cuticles were isolated from 14d old leaf pieces (8cm) that were fixed with parafilm (Pechiney Plastic Packaging, Chicago, Il., USA) on glass slides. The enzymatic isolation was performed according to Schönherr and Riederer (1986). Subsequently, the isolated cuticular membranes were incubated in distilled water, which was replaced ten times, once per day. The fully dried membranes were then prepared for use in bioassays. For total wax extraction, the isolated and dry cuticles were washed with chloroform (>99%; Roth, Karlsruhe, Germany) for 2min, and then stored at room temperature for a minimum of 14d, to ensure complete evaporation of solvent traces.

8.0 Studies of Fungal Pre–Penetration Processes

Detached 14d old secondary leaves of barley wild type, and of the wax mutants, with their adaxial surface upwards, and glass slides with different surface coatings, were fixed at the base of a settling tower. Conidia from infected barley leaves were instantly blown into the tower, using pressurized air to ensure their even distribution (100-150 conidia/cm²). After inoculation, artificial surfaces and leaves of the different treatments (*gum arabic* and/or chloroform) were kept in a sealed box on a moist filter paper applied underneath (relative humidity 90%). The samples were incubated for 24h in a growth chamber with a light intensity of 300 to 400µmol photons m^{-2} s^{-1} in a 16/8h day/night cycle (24/18°C).

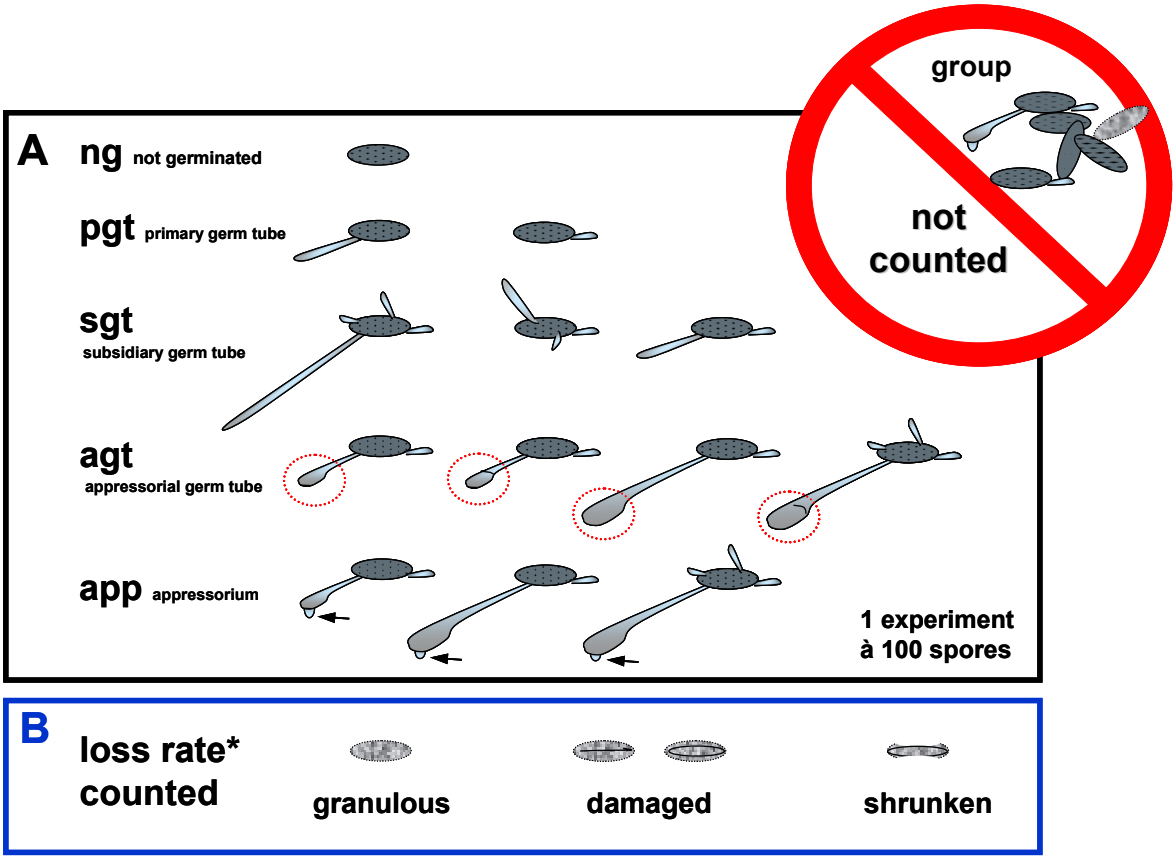


Figure 3: Categories of fungal development used in studies of fungal pre-penetration processes. Primary and subsidiary germ tubes were assessed to non-differentiated germ tubes, while swollen appressorial germ tubes and the successful formation of hooked appressoria were valued as differentiated germ tubes. Survival rates were calculated from 100- loss rate (%).

For microscopy, inoculated leaves were treated as described by Lyngkjær & Carver (1999), to avoid displacement of ungerminated conidia. Briefly, the leaves were placed, with their inoculated surface upwards, onto Whatman 3MM paper moistened with ethanol:acetic acid (3:1, v/v), until bleached and transferred to filter paper moistened with lactoglycerol (lactic acid:glycerol:water [1:1:1, v/v/v]) for 3h.

Finally, fungal structures were stained for 30min, by carefully pipetting a few droplets of trypanblue (Merck, Darmstadt, Germany) (0.05% [w/v]), acetic acid:glycerol:water [1:1:1, v/v/v]) onto the inoculated surface.100 intact individual spores were analyzed on each surface by light microscopy (Leica DMR with Leica IM1000 software package, Wetzlar, Germany), to determine whether they had germinated (visible single or multiple germ tubes), and, if so, whether swollen appressorial germ tubes or hooked appressoria had differentiated. In addition, the quality of fungal conidia was verified by detecting the loss rate of those apparently damaged or desiccated during the inoculation procedure. Only single, well separated conidia were counted, to eliminate the possibility of inhibition due to crowding. Five independent replications were examined in each trial. Two hydrophobic foils (Nowofol, Siegsdorf, Germany) with different contact angles were applied to the assays: NOWOFLON® ET-6235 J made of a copolymer of ethylene, and tetrafluorethylene (ETFE), and NOWOFLON® FEP, made of a copolymer from tetrafluorethylene and hexafluorpropylene (FEP).

Cellulose membranes (Zellu Trans Roth V Series, Roth, Karlsruhe, Germany; dry width 2.5cm, thickness 22µm, MWCO nominal 10.000) were 7d incubated in deionized and autoclaved water, and depending on respective experiments, afterwards sprayed with extracted wax. Dried wax-sprayed, or wet pieces of the membranes were applied on cleaned glass slides, or on 2% agar, to test the effect of surface humidity on conidial development, respectively.

9.0 Statistical Analysis

The basis for statistical analysis in chapter I was the sampling of n=5 independent experiments in each case. For assays with *B. graminis*, n=1 is based on the examination of 100 conidia. In the case of a significant homogeneity of variance (Levene´s test), data were transformed. Statistical differences ($P \leq 0.05$) within datasets were tested by one-way ANOVA followed by a Tukey HSD post hoc test (chapter III: Tab. I & II, Fig.3, 5-9). With respect to different surface treatments, depending on the according *cer* mutant, or wild-type leaf surfaces, the dataset of Fig.2 was tested in a repeated measures ANOVA, followed by Tukey HSD post hoc test.

10.0 Microarray Analysis

10.1 RNA Isolation

Plant material was immediately frozen in liquid nitrogen and stored at -80°C until use. Samples were composed of a mixture of about 40-50 secondary leaves of different plant individuals of respective experiments.

Total RNA was isolated using peqGOLD RNAPure reagent (peqLab), following the manufactor´s instructions. The isolated total RNA was precipitated with 2.5 volumes of 100% ethanol (Applichem, Darmstadt, Germany) and 0.1 volume of 3M sodium acetate (Applichem, Darmstadt, Germany) at -20°C over night, washed two times with 70% ethanol (Applichem, Darmstadt, Germany), and finally resuspended in diethyl pyrocarbonate (Roth, Karlsruhe, Germany) treated water. Total RNA for semiquantitative confirmation with RT-PCR was isolated, using the RNeasy mini kit (Qiagen, Hilden, Germany), complying with the manufacturer's instructions, including all optional washing steps, and a treatment with RNase-free DNase (Qiagen, Hilden, Germany). For cDNA synthesis, total RNA was quantified by measuring UV absorbance (Biophotometer; Eppendorf, Hamburg, Germany). RNA quality was checked by gel electrophoresis.

10.2 Microarray Design and Construction

The barley wax oligonucleotid-microarray was established using oligomer probes specific to 254 barley gene sequences, and ESTs (expressed sequence tags) involved in a range of wax biosynthesis related processes, designed by Operon (70 bp oligomers; Köln, Germany), or selected from the database of DFCI barley gene index release 10.0 (http://compbio.dfci.harvard.edu/), and designed by Operon. In addition, probes for non-plant genes as positive control (*Saccharomyces cerevisiae* GAS1), and as an internal standard (*Coleoptera* luciferase), were included in the microarray. Oligomers were diluted in 3x SSC (pH 7.0; 450mM sodium chloride, Roth, Karlsruhe, Germany; 45mM sodium citrate, Applichem, Darmstadt, Germany), and 1.5M 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 1mM hydrochloric acid (Merck, Darmstadt, Germany), and with 100mM potassium chloride (Applichem, Darmstadt, Germany), washed in deionized water, and immersed in blocking solution, consisting of 100mM Tris (pH 9.0; Roth, Karlsruhe, Germany), 50mM 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 2min.

10.3 Preparation of Fluorescent Labeled cDNA Samples

For the *Bgh*-microarray, aliquots of 20*µ*g of total RNA were spiked with *in vitro* synthesized 18ng and 2ng standard yeast mRNA, respectively, and 5ng of luciferase control RNA (Promega, Mannheim, Germany), and reverse transcribed using 200 units of Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany) in the presence of 0.5*µ*g oligo$(dT)_{12\text{-}18}$ primer, and 4.5*µ*g random nonamers. Amounts of isolated RNA for the stress-micorarray were not sufficient to apply 20*µ*g for cDNA synthesis, like it was done in the *Bgh*-microarray, therefore the stress-microarray was carried out with 8µg RNA for each sample. Each reaction contained, in addition to the RNA template, and the annealing primer, 500*µ*M dATP, 500*µ*M dGTP, 500*µ*M dTTP and 200*µ*M dCTP, 1mM dithiothreitol, 40 units RNase inhibitor (RNase out; Invitrogen, Karlsruhe, Germany), and dCTP-coupled 1.25nM Cyanine 3 or Cyanine 5 (Amersham Biosciences, Uppsala, Sweden), in the provided first-strand buffer, with a final volume of 40*µ*l. After incubation at room temperature for 10min, and at 50°C for 90min, another 200 units of Superscript III reverse transcriptase were added, and the incubation was continued at 50°C for 90min. The cDNA synthesis was stopped by heating at 70°C for 15min. Subsequently, 100 units RNase A (Fermentas, St. Leon-Rot, Germany) was added to all reactions followed by an incubation at 37°C for 45min.

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*µ*l in a Savant Speedvac SC 110 (Holbrook, NY, USA), evaporating the water under vacuum.

10.4 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; 450mM sodium chloride, Roth, Karlsruhe, Germany; 45mM sodium citrate, Applichem, Darmstadt, Germany) and 0.1% SDS (w/v; Applichem, Darmstadt, Germany). After incubation at 95°C for 2min, 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 18h 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 10min, in 2x SSC for 15min, and in 0.2x SSC for 15min. Slides were dried by centrifugation at 1500rpm for 2min. In order to protect the fluorescence signals against photo bleaching, the slides were briefly immersed in DyeSaver2 (Implen, Munich, Germany).

Microarray slides were scanned, using a ScanArray 4000 (Packard Bioscience, Meriden, CT, USA), adjusted to 532nm for Cyanine 3 and to 635nm for Cyanine 5. Fluorescence data were processed of total pixel intensities within a fixed area obtained for each spot, using 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, 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. Signals falling 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 switch of the cDNA labeling. In all hybridization experiments, cDNA of native barley wild type compared to cDNA of treated plants (abiotic stress and *B. graminis* infection) was probed according to the respective experiment. Fluorescence signals were inspected visually, and non-homogeneous and aberrant spots were flagged. Total pixel intensity was defined 100%, and the series of signals for each oligo-nucleotide was used to calculate relative expression.

10.5 Semi-Quantitative Reverse Transcription PCR Analysis

For the reverse transcription (RT) PCR experiments, 2µg of DNase treated total RNA was denatured and reversely transcribed using 200 units Superscript III reverse transcriptase and 0.5µg oligo(dT) 12-18 primer for cDNA synthesis, with a final volume of 20µl, as described by the supplier (Invitrogen, Karlsruhe, Germany).

Subsequently, 1µl 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; Tab. I), to amplify barley cDNA. The reaction conditions for PCR included a denaturing step of 2min at 95°C, followed by 30 cycles of 15s at 95°C, 30s at 60°C, and 30s at 78°C, and a final elongation step of 10min at 72°C (Mastercycler gradient; Eppendorf, Hamburg, Germany). For control RT-PCR 18S rRNA primers were applied.

Table I: Internal oligomer number and putative function of five primer pair sequences corresponding to barley genes and ESTs created with LightCycler Probe Design software. The size of the resulting amplified RT-PCR fragments was specified.

Oligo No.	Putative function	TC No.	Primer Sequence (5´-3´)		PCR product size (bp)
93	**Short-chain alcohol dehydrogenase**	TC132567	forward reverse	TGGATAGAGGGATGGGT CGTTTCTCGGACGAATG	235
213	**Dehydrin 4**	TC139232	forward reverse	CATGGACAAGATCAAGGAG ACCTAGATACATTCGCCC	207
214	**Dehydrin 5**	TC139233	forward reverse	GAGAAGAAGAGCCTCATGG TGAAGTAAAGGAAATCTGCAC	248
250	**Actin**	TC131547	forward reverse	GTCCATCCTAGCCTCAC CAAGCCAACCCAAGTTT	335
control	**18S rRNA**	AY552749	forward reverse	GACGATCAGATACCGTCC AGTCAAATTAAGCCGCAG	198

10.6 Agarose Gel Electrophoresis

Aliquots of 25*µ*l of the cDNA amplicons spiked with 5*µ*l 6x loading buffer (Peqlab, Erlangen, Germany) were separated by electrophoresis on a 2% (w/v) agarose (low EEO; Applichem, Darmstadt, Germany), Tris-acetate-EDTA (pH 8.0; 40mM Tris-acetate, 1mM EDTA, Roth, Karlsruhe, Germany) gel, with 140*µ*M

ethidium bromide (Applichem, Darmstadt, Germany), at 75V for 90min. 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).

APPENDIX

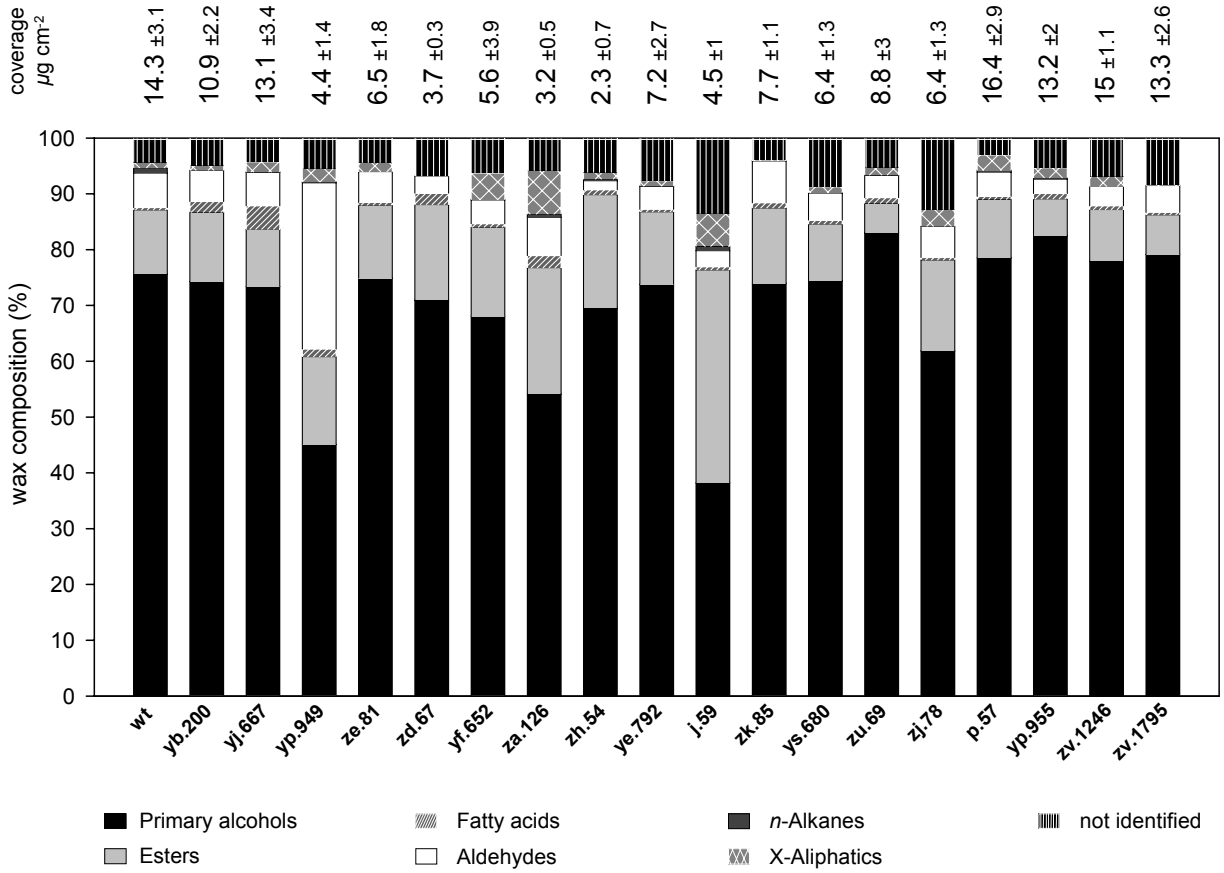


Figure 1: Total wax amount and relative chemical composition of barley wild type and 18 *cer*-mutant leaves screened in this study. For details concerning the leaf surface characteristics of *cer*-yj.667, *cer*-yp.949, *cer*-zd.67, *cer*-zh.54 and *cer*-j.59 view chapter I, 1.3. X-aliphatics: a series of homologous aliphatics of an unidentified compound class with corresponding diagnostic ion masses: m/z 268/281- 505/520, m/z 268/281- 533/548 and m/z 282/295- 575/590.

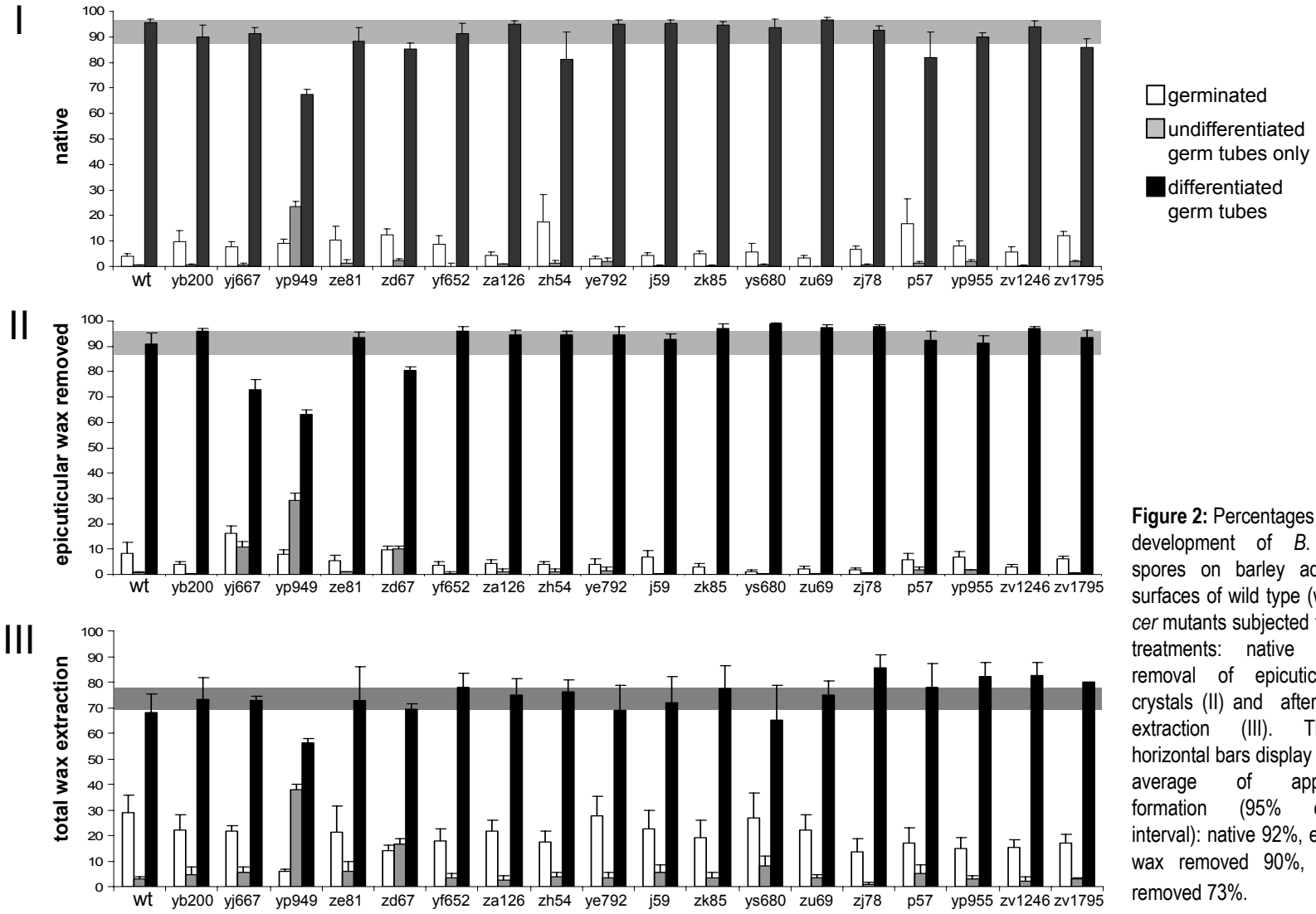


Figure 2: Percentages of conidial development of *B. graminis* spores on barley adaxial leaf surfaces of wild type (wt) and 18 *cer* mutants subjected to different treatments: native (I), after removal of epicuticular wax crystals (II) and after total wax extraction (III). The grey horizontal bars display the overall average of appressorium formation (95% confidence interval): native 92%, epicuticular wax removed 90%, total wax removed 73%.

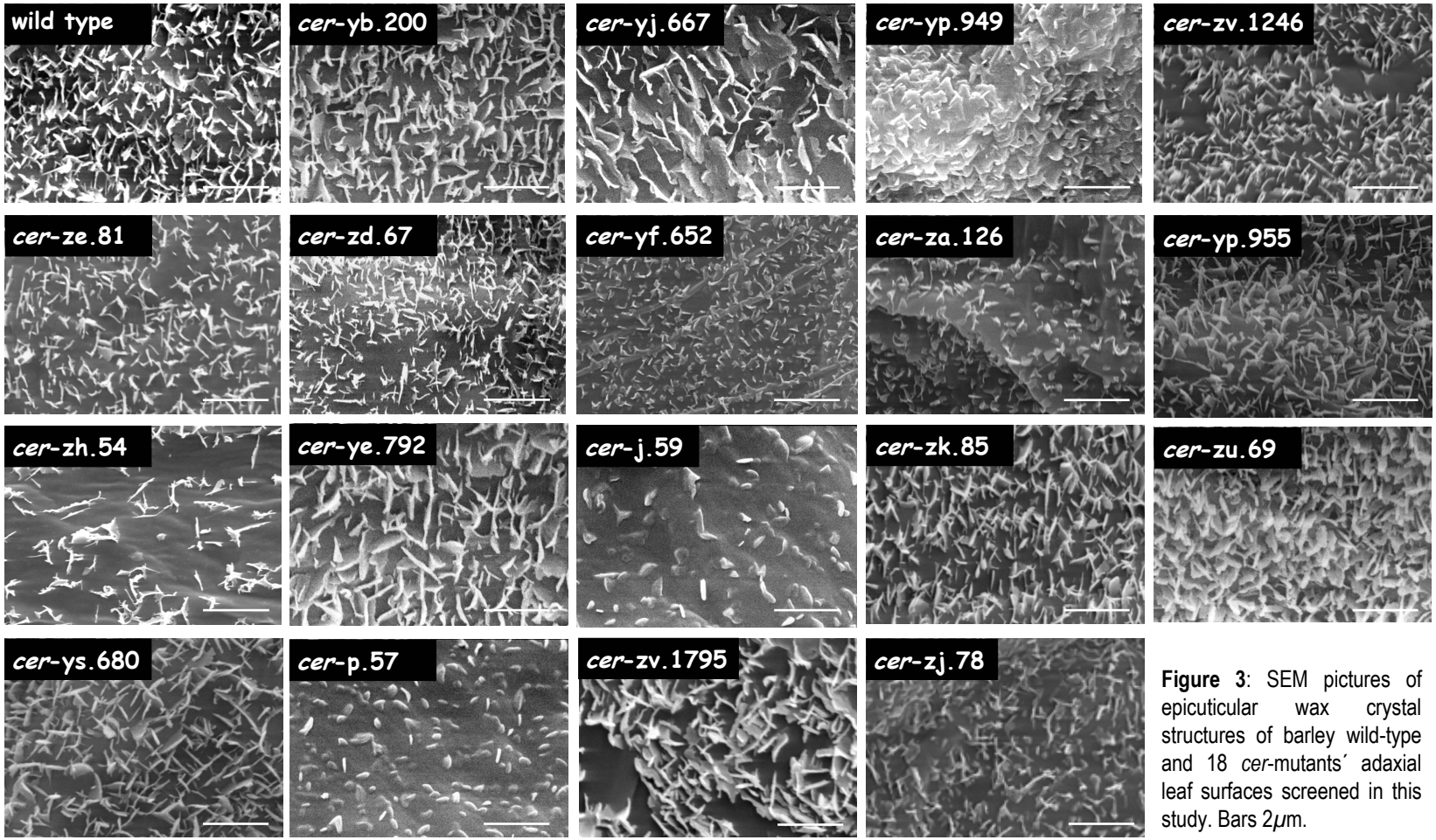


Figure 3: SEM pictures of epicuticular wax crystal structures of barley wild-type and 18 *cer*-mutants´ adaxial leaf surfaces screened in this study. Bars 2μm.

Table I: Ratios (treatment/ control) of the top 20 differentially expressed sequences in barley leaves within four different abiotic treatments (darkness, NaCl, cadmium & drought) in the stress-microarray. Strong signal intensities (nVol>1) are marked with grey background. Oligo No.: internal oligomer-number according to table III)

Oligo No.	Putative Function	darkness	NaCl	cadmium	drought
4	Probable β-keto acyl reductase	1.4	1.2	0.7	0.9
5	Putative *fiddlehead*-like protein	1.5	1.2	1.0	1.2
60	Glossy1 homologue	1.5	1.2	0.9	0.9
69	Homologueue to Glossy1 protein	0.6	1.7	0.6	0.2
80	Similar to CER1	0.7	1.1	1.0	0.8
83	Putative wax synthase protein	1.2	0.9	0.7	0.8
93	Short-chain alcohol dehydrogenase	0.4	0.5	0.7	0.1
112	Type 2 non specific lipid transfer protein	1.2	1.2	1.0	1.2
115	Similar to probable nonspecific lipid-transfer protein	1.6	1.2	0.9	1.0
155	Nonspecific lipid-transfer protein 4.3 precursor	0.3	1.3	0.9	0.7
161	Hydroxycinnamoyl transferase	1.2	1.2	1.0	1.2
177	Putative ABC transporter protein	0.5	1.4	0.6	0.1
181	ABC transporter	0.7	1.1	1.0	0.8
195	Translation initiation factor 5A	0.4	0.9	0.6	0.7
207	PR4pre	0.7	1.1	1.0	0.7
213	Dehydrin 4	0.6	1.5	0.5	4.7
214	Dehydrin 5	0.7	1.0	0.9	0.8
228	Ethylene-binding protein	0.8	0.9	0.9	0.8
250	Actin	0.3	0.9	1.0	0.9
251	Ubiquitin-protein ligase E3-alpha-like	1.1	1.0	0.8	0.8

Table II: Ratios (treatment/ control) of the top 20 differentially expressed sequences in barley leaves upon different infection periods with *B. graminis* conidia in the *Bgh*-microarray. Strong signal intensities (nVol>1.5) are marked with grey background. Oligo No.: internal oligomer-number, according to table III).

Oligo No.	Putative Function	12h	24h	48h	72h
4	Probable β-keto acyl reductase	0.6	1.1	1.1	1.5
5	Putative *fiddlehead*-like protein	0.7	1.3	3.7	2.1
30	β-ketoacyl-ACP synthetase I	2.0	1.5	2.5	2.6
60	*Glossy1* homologueue	0.9	1.2	1.9	1.9
80	Similar to CER1	0.8	1.0	2.3	2.5
83	Putative wax synthase protein	0.8	1.2	1.6	1.2
93	Short-chain alcohol dehydrogenase	1.5	0.8	0.5	0.8
94	Omega-3 fatty acid desaturase	0.8	1.1	3.5	2.6
105	FAE1	1.1	1.3	2.3	2.3
155	Nonspecific lipid-transfer protein 4.3 precursor	1.1	1.3	0.4	0.5
161	Hydroxycinnamoyl transferase	0.8	0.9	0.6	0.9
175	Putative ABC transporter family protein	1.0	1.3	1.7	1.9
181	ABC transporter	0.9	1.3	2.6	1.7
195	Translation initiation factor 5A	0.9	1.1	3.3	3.5
207	PR4pre	0.9	1.3	1.6	2.0
213	Dehydrin 4	2.1	1.6	0.6	1.0
214	Dehydrin 5	1.2	1.2	3.7	3.9
228	Ethylene-binding protein	0.9	1.1	2.4	2.4
250	Actin	1.3	1.3	1.2	1.2
251	Ubiquitin-protein ligase E3-alpha-like	0.9	1.1	4.1	3.1

Table III: Internal oligomer-numbers, tentative consensus sequence (TC), putative functionary names and nucleotide sequences of 254 70mer oligo-nucleotides corresponding to barley ESTs and gene sequences (expressed sequence tags) involved in a range of wax biogenesis related processes selected from the database of DFCI barley gene index release 10.0 (http://compbio.dfci.harvard.edu/).

No	TC	Functionary Name	Sequence
1	AV835375	ABC-transporter	GCGGGAGACATAGTGTATGAAAACTGTGCACTCTCGAGCTCTACGATTGATCGGATCTCCAAAGAGATCG
2	BF064504	Acyl-carrier-protein I chloroplast precursor	GAAGCAACTGGCTAGTTTTCTCTCCCCTTTTGGGGAAACTTGGTACACCTGTTACAGAGGAGTCGAAATT
3	BF258264	Very-long-chain fatty acid condensing enzyme CUT1	ATCGTACCATCCAATCACCGACAGATGGGCCTTGGGAGGATTGCATCCATAGGTATCCCGGTCATATCCC
4	BG343361	Probable β-ketoacyl reductase	GAGACCATCTCCAAGACCCGCGCCGTGCAGACCAAGACCGTCGTGTTCGACCTCTCGCTAGTTGCCACTC
5	BG417975	Putative fiddlehead-like protein	CACCTGTTGGGGCGGCCGTCGTGTGATGGCTATCAATGTCTGCTGTTGGCGGGTAGCTGTCCACGCTGTC
6	BG418575	β-ketoacyl-ACP synthase	AATCAAGTAACAACTACCTTTTATTCATTCAAAGGCCTGCAGATACTCAGGTAAGTAACCCACAACAGGT
7	BI955463	Ketoacyl reductase homologue	GTACGCCGTCGTTACCGGGGCTTCCGATGGACTAAGAAAAGAATACGCCATTCAGCTTGCCCAAAAAGGA
8	BI959952	Putative 3-ketoacyl-carrier-protein reductase	AAGGGTTCAGTTATCAACATTTCGTCTGTTGCTGGCCTCAACCGTGGCAATCTGCCTGGCTCCATTGGAT
9	BM442555	β-ketoacyl-CoA synthase	AGCTCTTCGCCAAGACCGGCGTGTGGCCCAAGGACATCGGCGTGCTCGTCGTCAACTGCAGCCTCTTCAA
10	BM817350	Similar to β-ketoacyl-CoA synthase	CCGATCTTGATGGGTGAGACCCTAAAGGTGGGCACAAATCTAGATATCGAATTCGAGTTAAATTTAATCA
11	BU991543	Acyl-CoA synthetase-like protein	GTGCTCCCAGATGGTTCAAAGGGGATTGTGAAGATTAAAGGACTGCCAGTAATGCAGGGATATTATAAGA
12	BU995180	Fattyacyl-CoA reductase	TTCGGGCTTCTAAGGGAGAAGCACGGGGACGGGTTTCAATCTTTCATCGGAGGGAAGATCGTCCCGTTGG
13	CB860427	Fatty acyl-CoA reductase	TGACCCAAAGACCCTCGACTGGGACGACTACTTCTCATGACACCAGGGATGTGGACCTTGAAGAAGTAGT
14	CB867644	Probable β-ketoacyl-CoA synthase	ACGACGCCCTCGAAGTTCCAGCGCAAGGATCCTCGAAGCGCTCGGGGGCTCAACGAAGGAAGACCTACTG
15	CB870423	Probable β-ketoacyl reductase	ATCTCCAAGGCCGTCTCGGAAACCCACGGCGTGCTGGCCAAGACCGTGCAGTTCGATTTCTCCCACGTCT
16	CB870444	Fatty acyl-CoA reductase	CCAAACCAACTCGCTCCAACTTACTACTACGTTACTTGAACGGACCTAAGTCATGGTGTAGGTAGATCCA
17	CB872347	Fatty acyl-CoA reductase	TTGTCTTGGTCACGGTCAGTCGGAGCCGCTCCAAGTTGATGTCGTCGAAGCATCCTTTGAATAAGGCGAA
18	CV059159	β-ketoacyl synthase (fiddlehead)	GGGCCTTTAATATCCCGGGACTTAAAGCGCGGCGTTCAAACACTTTGTGTTGACAGCGGCCAACCGGAAA
19	NP482509	Acetyl-CoA carboxylase	CAGATGACGGATTCAAGCCTACCGGTGGAAAAGTAAAGGAGATCAGTTTTAAAAGCAAGCCAAATGTTTG
20	TC131378	B-ketoacyl-ACP synthase	TTGCGCCACTAGTAACTTCTGCATTCTGAGTGCAGCTAATCATATCATGAGGGGGGAAACTGATTTGATG
21	TC131517	Fiddlehead-like protein	ACGTACCATAATGCGCAAGTGTATGTGCGCGCTCTTGTGTGAAACTTATGTCAACTTTGATTATCTACGT
22	TC131586	Acyl-CoA-binding protein	ATTCGAAGTACTAGTTGTTGTCAAGCTTATCATCGTTGTCGTGGATGTGTAATTACGTTGAACAAGCCCT
23	TC132557	β-ketoacyl-CoA synthase	GTCGGGTATTAATTTTAGATGATTCCATTTAGGTGGTACAGAGGGGTGTGATGTCGTGCTGTTCACTATT
24	TC132614	Acyl-CoA synthetase	CTGAAACTGCCATTTTACCAACGACCGATTAGCAGCAAGAAACATCCCCCGGTTCAGTCTTAAGATACTC
25	TC132825	B-ketoacyl reductase	CGACTAACTCCGCGTGTAGACTCTCTATAGAATACATCATACTCATTGCTGCGCTACCTATACCAGTAAT
26	TC134151	Putative fatty acyl-CoA reductase	CGTCCCAGCGGTCCTAATGTACGGCCGCAAAAATAAGGGAAGCGTATAGAAATGAGTGGAATCTTCCAAT
27	TC134290	long-chain acyl-CoA synthetase	TGATGGTTCTCTTACTCTGACTGATACCCTGAGATGACATAGGACAGTGCTCTTATGTCGGCACATACTA
28	TC134459	Fatty acyl-CoA reductase	CCTCTAGCTTCCCAGAGAAACCCGGCCACCATCTTCCACATTTTCAGGTTTGTCGATTCTCGGCCCTTAT
29	TC135123	β-ketoacyl reductase	ACTCTGCTCTCAATCTGTGGCGTCTCCGGGTTGGTATCCGGAAGAGAAACGAGATGAGAGCCCTGATCGG
30	TC135910	β-ketoacyl-ACP synthetase I	TGCGGCGGGTGGGCTAGAAGCTATTGCCACTATCAAGTCCATAACTACAGGATGGGTGCATCCTACCATT
31	TC136114	Acyl-CoA synthetase	GCCGCCATACTCCCAAAGTGCACAGCCCACATCAAAGCCATTGTTAGTTTTGGAGGTGTGCCAATTGAGC
32	TC137519	Fatty acyl-CoA reductase	TGGGGGTGAGGCACATGTGCGACTTCGCCCGTAAATGCCCCAACCTCGAGGTGCTCATGCACGTCTCCAC
33	TC140205	Acyl-CoA thioesterase	ACGAGGCTGCCAAATCCCAATCGCTTTTAAACTCGAGATATTATTGTCCGCCTTCCGGCGCGCAGCTGCT
34	TC140206	Acyl-CoA thioesterase	CAAGAGGCGTTGATTAGGAGAGCCAAGACACCTGGGCAAACCCCAAGGCCGAAGCTTTGAACCGTGCAGA
35	TC141024	Long -chain acyl-CoA synthetase	AAATAAACAGTCAATCAAGGAACCAAAACTAATCTTTCCAATGAACAACAGCCTGGGACAGTGCTTGGCG
36	TC142132	Probable β-ketoacyl-CoA synthase	CAGAGTGGTGCTGAGAAGGTCAGTCAATGAGATATGCCTCTTGTGTTGTTGTACTATGTACATTTGTTGT
37	TC142665	Putative β-ketoacyl-CoA synthase	CGTCTTAATGGTAGCTCCGGGTGTGGCACCCACCTCCCGCTTACCGCAACCCTTATACTCCTATATATCG
38	TC143043	β-ketoacyl reductase	CTGCGCTTGTTTGTGCATACTTTGTGTAGTACTGTGTGTGATTTGACATGGATCTGTGGACCGCAGTTCG
39	TC143425	Fatty acyl-CoA reductase	CATACCGGGTGTCCTCAAGTATGTGCTCAAGTAAAACTGCAGCAATTTGTATCCACATATATGCACCTTA
40	TC143649	3-ketoacyl-CoA reductase	GCTCCATGCCTTCTAAGGATATAAGGGTGTGGCGCGATCAGGTATGTTGATGGAAGAACAATGGAGCTGT
41	TC144718	Acetyl-CoA carboxylase	CTGCCGAGCGTGTAGTCCCTCAAGCAGGACAGGTGTGGTTCCCAGATTCAGCCTCAAAAACAGGCCAGGC
42	TC145117	Very-long-chain fatty acid condensing enzyme CUT1	GATCGTCGAGTTGTTGTTTGGACCTGTAGGGCGATTCGTGGAGCGGAGAATAAGCTCGGAACCTTCATCT
43	TC145432	Very-long-chain fatty acid condensing enzyme CUT1	GGACATCGACATCCTCGTCGTCAACTGCAGCCTCTTCGCCCCGACGCCGTCTTTCGCCGACATGATCGTC
44	TC146045	Putative 3-ketoacyl-CoA reductase	TGGCAAACAAGTCTAATCAGTCTCTCCCGCATCATTCGATGGTGTGCATGTACACATATCTTCCTTGTAT
45	TC147874	3-oxoacyl-[acyl-carrier-protein] reductase	ATGGTGGGATGGTAATGTGAAACCTTTGAGCAAGCTTTGATGGAAGGTTGACATTTGCCCTGCCTAGCGG
46	TC147878	β-ketoacyl-ACP synthase	AGAGAGCTGGCACATTAGCACAGGCTTTGCCGCAGACGAATCGAACCATTCCCCGTTCCATCCTTCGATC
47	TC148481	Long-chain acyl-CoA synthetase 6	ATTTACTCGTCCTTTATAAGTACAAAATAACCACGCCCAACACCAGTCCAGACTGTTGACAGGTGAAAGG
48	TC148482	Long-chain acyl-CoA synthetase 7	CTGTCAACAGTCTGGACTGGTGTTGGGCGTGGTTATTTTGTACTTATAAAGGACGAGTAAATGTGCCCGC
49	TC149068	Fatty acyl-CoA reductase	CTCTTCATGGTGCTCAAGTACCGTGTCCCCCTTGAGCTCCTTCGCCTGCTCTCCATCCTTTGTTGCGGCC
50	TC149091	Cytosolic acetyl-CoA carboxylase	TCTATGGTGGACTGGGTATCAAGGGAAGCGCAGACGACACGGAGCAAATTTTACTACTTGCGTGGCGAGC

Table III (continued)

No	TC	Functionary Name	Sequence
51	TC150629	Fatty acyl-CoA reductase	TGCTACTCCTAGCCTTCTAGTGGCAGGGCTCTGCTTCTCTCTCCCCCTCTCTCTCTCTTCAGATCTCG
52	TC151468	β-ketoacyl-CoA synthase	TGTACCTGCTCAACTACAGCTGCCACCTTCCGGACGCTGACCGTCAGTGCAACCTGGAGGTGTGCGAGTA
53	TC151638	Long-chain acyl-CoA synthetase	AATACCTAAAGGCGCACGTGTTGGTCTGTATTTTATAAATAGACCTGAGTGGACCATAGTTGACAATGCT
54	TC151728	Acyl-CoA reductase-like protein	CTCCATGTGTCTACCGCATTTGCAGCCGGCGACAGGGAAGGGCTGATCATGGAGAGGCCTTTCAAGAAGG
55	TC152335	Cytosolic acetyl-CoA carboxylase	TTCAGTTGCTCTGTGACAAGCATGGCAACGTGGCAGCACTGCACAGTCGAGACTGTAGTGTTCAAAGAAG
56	TC153410	Ketoacyl synthase	AGAAGCCAGAAACTTCTTCGATCGATCAGCAACAGTCGGGCGGACCAAGAGTCTGTTCGATCCGGCCGGT
57	AJ432405	CER3	TTGAGGAACAATGCCCAAAGGTTTGGATAATGATCATGTGATGTGCATATTTTCTGGAACTGTCGTCTAG
58	AV836642	Putative CER1	ACCTGCGCATTGACCGGAACCGCCTCCATACTCACGTTCGAGATGTATGTGATCTACATTGACTTCATGA
59	BF064697	CER10/ ECR; fatty acid elongase	CCTGAATGACCCTCAAATAAAAATCGGTTCTGCTTCCACGTCCTTTTCCCAATCTCAAAACCTTCTTACT
60	BG309170	Glossy1 homologue	CCAGATCGACGCCGAGTGGGACTGGGATAACATGGTGATCATGCAGACGCTGCTGGGGGCCATGGCGATC
61	BQ661120	CER1	AGGGTTATCCGAGGGTCTAGAGGCTATAGAGGCAATCCCAACCCTAAGATGATAAGTTGATCGCAAGTTG
62	BQ762923	Glossy1 protein homologue	ACCGAGCACCTCCTTTTTTTCATCCTCGCGGCCCGTCTCAATGTTTTTCCTCGTTGACTGACACCGCCGT
63	CB859878	Putative CER1	GGATGGGCAAGCTCAGCACCGAGACATGGGTCGTCCCACGATACTCCTCCCAGTACACGTCGAAGAAGGA
64	CB860229	Similar to glossy1 protein	CTCTCCTCCTGGCCATGGGCGAGCCTGGGCATATACAAGGCACGCACCGAGCACCTCCTTTTTTTCATCC
65	CB879522	Similar to glossy1 protein	GGGACTGGGACAACTTCCTGATACTGCAGATACTGATGGCGTCCACGGCGCTCTACGCCTTCCCGTCGCT
66	CD055162	CER3 ubiquitin-protein ligase	TGAAGCTTATCGCTGAACCGGCTTTTAAGTATGAGTTTGCTAAAGTTTTCATACAGTACTACCCTGTTGC
67	TC131363	Glyceraldehyde-3-phosphate dehydrogenase	GGCTCCTGGCTTTATAAAACCGGCCGCTTAAACCCTCCCCACCTCTCCATATCCGCTTCCAGACACTTCC
68	TC133120	Similar to CER3 protein	GTTCTTCTAGAAAGATATGTCAAGATGCATTGCCCTGATTGTGGTTCAGTGCCTGATGAGCCAGCTTTAT
69	TC133901	Homologue to Glossy1 protein	ATCGCCCCGTGCACTTCCGTCTAGCTACGGCCCAAGTCCGGCCCAAGACAAAACCTTCTACTCTATTTCT
70	TC134470	Similar to CER1	GATCCCGATGATCGCCTGCGCTCTGACAGGAACTGCTTCCATCATGACGTTTGAGCTCTACATGCTCTAC
71	TC134545	CER2	GGCTTGGCTGCGAGTCACTGCCATGATCTTCAGATGGCTATGACATAATTAAACATCCTATGTGGGAGAA
72	TC136116	Glossy1 homologue	CATCGCCTTCTGCCTCATGGTGCTCCAGGTGTTGTGCTCCAAGACCTTCACCGTCAGCTTCTACTGCCTC
73	TC136317	Similar to CER1	AGTACTAGTTTGATCCACCAAGGTTACTTGGTTAGCGTACCATTACTGCATTATTCGTCTCCTATGGTAT
74	TC139881	Glossy1 homologue	AACACATGCTTGTACAGTGGCTCGACACAATTGCCGTCCACGGAGACGGTTGCATCACTGTTGAGGAATG
75	TC141010	Weakly similar to CER1-like protein	TCCCTCTCACGAAAGCTTGACTGAGAAATGTTGCCTCCGTCCGATGCTAAGATGGTACTACCGAGAATTG
76	TC142242	CER1-like protein	CTCTGCTACCAGTAGTAATCCTGTCGCAAGAAATAATCCTGTCGCGTTACACCGTGGTATATAGTGCTGC
77	TC150380	CER1	TCCACACTTTATATGGGAAATGGCTTCCGCCCTTCGTATTTGTGATGTATAATCGGTTTAACATTGGACT
78	TC151042	CER3 protein	AGCAACATCCACTGATTAGATATATATCATGTTTCCCAAGTGGACGCTGTGCTGGCAATGCAGATGACAA
79	TC152410	CER1-like protein	ACCTCATGTACACTCCCTCGTAAGTGTAACATTTGAACAGCAAGCTTACGATATAGTTAAGATGTAACCC
80	TC152700	Similar to CER1	GAGTTCCTCTACTACTGGTTCCACCGCGCGCTGCACCACCATTTCCTCTACACCCGCTACCACTCGCACC
81	TC153353	Similar to CER1-like protein	ATCCCTGGGTCGCCGGTTTACCACACCCGTAGGGGGGATTAATAAAGTGGGCTGGGGTAACCTAACCTAA
82	BI955074	Putative fatty acid elongase	ACCCACTACGACGCGCTCGACGAGATGGACGCCTTTTTCGACGACGCCGTGCGCGGGGTCTTCTACAAGA
83	BI959726	Putative wax synthase protein	TTTTTTATGGCTGTGACTAGAGATTGAACGTGTGCCGTACGATAAGAGGGTGTGACGAGGGAATACTTGA
84	BM370045	Long-chain-fatty-acid-CoA ligase 1	TGAGGGGCATTGAGATTATTAACGGTGTTGTGCTTTCTGACGACGAGTGGACCCCGCAAAATGGTTTCAT
85	BQ463834	Putative fatty acid elongase	CTCCGAGTCCTGCGCCCCCAACTGGTACAACGGCACCGACAAGTCCATGATGCTCGGCAACTGCCTCTTC
86	BQ468862	Putative fatty acid elongase	TTCACCGTCCGCCTCCTGGCGCGCAAGCTGATGCGCCGGAAGCTCAAGTTCGAGGGCCCAAAGATCAACT
87	BQ656917	Putative fatty acid elongase	TTGATTTACACGGTAGTATAAGAAGAATTGCCCTTCATCAGTATATATAGTAAGCCCGGCCTGCAGCGAT
88	BU981032	Putative fatty acid elongase	AGAGCAGAGTAATGGCGTCGCGCATGGTAGACTGCGGTTCCGGCCATCTGCGTCCATGCCAAATTGCCAA
89	CA015158	Putative fatty acid elongase	TCCATCGACCTCGCCAGGACCTGCTGCAGGTGCACCCAAACTCGTACGCGCTGGTGATCAGCATGGAGAA
90	TC132371	Very-long-chain fatty acid condensing enzyme CUT1	GTCACACAAGGAGGGGATCGGTTCATTCACAAGGAGGTTGATTACACGTATATTTATATTGGATATTGGA
91	TC132372	Very-long-chain fatty acid condensing enzyme	CTGCGTCATGCTCGTGTTTCATTAAATAATTTTCCGTTTACCCCATTAACTGTTTGTTATCGCCCAAAGG
92	TC132373	Very-long-chain fatty acid condensing enzyme CUT1	TTCACCACCTTCATGGAGCACATCAAGCTCATCTCCAACAACGACAAGAGCCTCCGCTTCCAGACCCGCA
93	TC132567	Short-chain alcohol dehydrogenase	TATGTGCGACATGACCAGTGGGAAGGCAAGCACGCCTTGTACCGTCACCTAGTCAGTGAGTTTCAAGTAT
94	TC132736	Omega-3 fatty acid desaturase	CAAACCGACCCGAGCCTGAGTGGCGACAACTGGGCTGGAACCGACAAGCAGAAGTGATGAGAGACTGGAA
95	TC132789	Omega-6 fatty acid desaturase	ACAGGCGCAATTAAACGGCACAGTTCACTGTAGCTATCCTCGATGGATAGAGATCCTTTGCCATGACATA
96	TC133232	Short-chain type dehydrogenase	CCCTGCCATGCCATTAGCCATTACGGTGTGAACGAAATAATGCGTCTGTGTGTTGCAGAAAACCAGACAA
97	TC134382	Very-long-chain fatty acid condensing enzyme	GGCACAAAGGGCTTAATTTCTTCCGGTCATTCATAAACGTATAAATCGTGGTCCTCGATCGCGAGCGTAC
98	TC135102	Fatty acid elongase	TGGAGAGCGTCGTTTCATCGTCATGATAGACTCGTCAAGGTCGAGTACCAAACAGAGGGTTTTCTAGAAA
99	TC136696	Putative FAE1	AGATGCTTCTCCTAAGTACGCTGATGTATTTTCCTTAATTCCAGTCACGTGAGCAAACATTCCAGTCGTC
100	TC137802	Very-long-chain fatty acid condensing enzyme	GCTTAACAATCTCATGCCGGTTACATGATGACGAAAAAATAAGACTGATAGGCCTTTAGTGAGCGAATCG
101	TC140056	Short chain alcohol dehydrogenase-like	CACTTACAGAAGGACTTTTGGCTAACAAGGAATTTGAGGCCTCCGTTGTGAGTCGAACACCACTTGGGCG
102	TC141477	FAE1	AAGGTGGTGCCCATCGACGAGACCTCTTACCAAGTCCCAATAATCTAGCTCTAGCTCTAGCTTGCTAGCT
103	TC142465	Short-chain dehydrogenase (SDR) family protein	CACACAGTTCCAGCCCGTATCAGTTGTAATGTTCAGTCCAAGTGTAAAGTCCTCCTGGTTATTATGAAAT

Table III (continued)

No	TC	Functionary Name	Sequence
104	TC145423	Putative fatty acid elongase	TTGAACACTGCTGTGGGCCGCGGGAGATGTGTTCTTTTGTACGTAATTTAAATTTTCGGTGTCAATGTTA
105	TC151992	FAE1	GCGAGCTCACACGAACCGGCGGTGTGGAGAACCGTGGTAGCTAGGGGTACGGTTCCATTCACGAGGTACG
106	TC152883	Wax synthase-like protein	GGTTAGAGGCGTCGCTAAGCGTCTCATCGACCGTCTATTTGGGGCTGGTTCAAATTAGGGGTAAAAGAGT
107	X93171	Oxalate oxidase-like protein	ACACATTGGGTGATTACACGGACGACACGTGCGTAAATTAAGGGCATGATTGAGTTCCTAGATATGCATC
108	BG344956	Lipid transfer protein	TGAGCCTCCCACTAAGGGCCTGTCTATGAAAGGGCGGGGCTTAACTAATAAAATAACCCACGGACAATAC
109	BG415903	Lipid transfer protein	ACCCTAAACTTGTGCGGTATTCCCGTACCGCACTTGCTAGCCGCCTATCCTATCAAGGGCTCCAGGCACC
110	BI950000	Similar to lipid transfer protein 7a2b	CGGTGTCAGCGTGCCCTTTCCGATCAGCATGTCCACCGACTGCAACAAGGTGCACTAGCACGGATCAGAT
111	BM816471	Nonspecific lipid transfer protein 4.3	AAGCCCTCTTGAGGGGGCCTATAAAGCGCCTATGAGGGTTTTTCACGCCCCTCTATACTGCTCTACCGCC
112	BQ759090	Type 2 non specific lipid transfer protein	GCAGGCATGGCGATGGCGATGGCGATGGCGATGAGGATTTGTGCACGGAGGCCGTGATGATGGTGATGGT
113	BQ767884	Probable lipid transfer protein	ACGCCACTGCTGCTAGTCGATCAGTCTGTTTCTCCCGTCCAGGTTTCAGGGGGAGGCGCCATGAAGTACT
114	BU983160	Probable phospholipid transfer protein	CATGACAGATGAGGGACTATATCTATATGTAGCTAAGAACCTAGCTGAGTGCGTGATGCGTCAATAAAAT
115	CV053917	Probable nonspecific lipid transfer protein	AGAATATCCTAGGCGGATACTAAACCGAGCGAAGAGTGCTGTGTGAACCTGCGTGCACATGGGGGGTGCT
116	TC131296	Nonspecific lipid transfer protein 4.1	TCATCCATGTATTTTATTTCACATAAGAGAATCTCCAACGACCGTGCTATATAAGCGCCGCACCGTAAAA
117	TC131297	Nonspecific lipid transfer protein 4.2	GAAGGGACTGGAAGACCCTAGGTGGCCCAACTGGATAACCTAGAAGGCAAGCCCTTTGACAGGTAGGGAG
118	TC131302	Lipid transfer protein	GGGTGCCCGCCTATAGCGCCCTCCGTCGCCGCTGAGTATGCTGAGTTCACGAATACATATGAATAAACGC
119	TC131305	Nonspecific lipid transfer protein 4.1	GCCTTTTTTTTCATCTCCTGCGGTCATTTTTTCTCTGCCTTGAGCCCCTTTTTTTTCTATGCCCGCGGCA
120	TC131306	Nonspecific lipid transfer protein 4.3	GCAGGAATTCGGCACGAGAGCTAATCTAGCTATCTCATCATCTCCGGCTTAGCTCACCACCACTACTATT
121	TC131308	Nonspecific lipid-transfer protein 4.1	GATCAACGAATCTTAAAACAATCCACCGAAGCCCTTATGGCGTAAGGGACGCTGACCCCCCACATGGAGG
122	TC131846	Lipid transfer protein-like protein	TCTGATCCATCCATCCATGCATAATCGTTCTACTATTTTGCATGTCATACACACGCACCATATGTATGTT
123	TC131847	Lipid transfer protein-like protein	CGTGCATGCATGCTTGATAAGCTCGTGATTGATCATCAGGGTCATTGTATCCCGTTATGGTATCTAAATG
124	TC131848	Lipid transfer protein-like protein	TCGATTTACTCGTAGCTATTGCATGTCATACGCGCGCACTGTTACAGTTCATATGTCAGGCGTTTGCATG
125	TC131849	Lipid transfer protein-like protein	ACGAGGCTAGCTCAGCTACGAGCCAGTACACTCTAGGTAGCTACTAGCTTCATAGCAATGGCGCCCTCCA
126	TC131850	Lipid transfer protein-like protein	ATGGATTTACTCGTAGCTATATATTGCATGTCATACGCGCGCACCATATGTCAGGACTTTGCATGGGCTC
127	TC131851	Lipid transfer protein-like protein	CCCCGACAACTTCACCTGCCCCATGTGATCCATCTATCCATCCATTTACTCGTAGCTATTGCATGTCATA
128	TC131852	Lipid transfer protein-like protein	CAGGTTGTGTTTTTTCCCATGTGTGTTCAGGTTGCGTTAACTCGATCCGTGATGTGCCAGCTTGTATATT
129	TC132846	Nonspecific lipid transfer protein	GTCAGTGTTTCCCAACACAATCTAGCTACCCTGACGATGTATGTGGAGTTGTGGACCGATGTGTATCTAA
130	TC133181	Nonspecific lipid-transfer protein 1	GGCCTATTTAAGCTCCTACATTCCCCGGCATAGACAACGTACCTATAGCCATTTGATCGCTACCACCGCT
131	TC134057	Lipid transfer protein-like	ATACTTATCATCGTCAGAGTGCGTTAGTCTATGCGGATATAGTGATATTGACACACCCCTATACATTCAT
132	TC134650	Probable nonspecific lipid-transfer protein	CTTATTTTAATTTATAATAAAACTTCTCTTTGGGGGTGCCTCGCTAATCCATTTCCCCTTTTGGGGGGGG
133	TC135221	Lipid transfer protein-like protein	AGCACCACTAAAGAGAGATCGATATACACAGCCCATACGTACGTGCACTTCCACTGCCGGACTTGCACCG
134	TC137537	7 kDa Lipid transfer protein	TGCTAGGCGCCATGCTATGGCTCAGCTAGCTATACATCCGTCCACGACAGATGCGAGAGACTATATATAT
135	TC138142	Similar to Nonspecific lipid transfer protein	AGCGTTGCACGTACACTACGTACATTTGGCGATGTGATGGCCGTTCGAGTGCCTGCCGAGTATGTGAGAT
136	TC139050	Lipid transfer protein	CTCAAGCCAGCGCCACTCACACCACGCTCGTCCACTCAAGCTAGCTAGCGCCGTAGTTCACTTCAGTCCA
137	TC139051	Lipid transfer protein	CAAGCCAGCGCCACTCACATCCACGCTCGTCCACTCAAGCTAGCTAGCGCCGTAGTTCACTTCAGTCCAG
138	TC139179	Nonspecific lipid transfer protein 4.1	GTGCCGAATTCGGCACGAGGAGCAAATCTAGCCATCTCATCATCTCCAGCTGAGCTCGGCTGCGCTACTA
139	TC139180	Nonspecific lipid transfer protein 4.2	CGCGTCCGCCCACGCGTCCGCTTATTAGCAAATCTAGAGCTCACCACCACCACTATTGCAAACTCCTTAG
140	TC139226	Weakly similar to Lipid transfer protein	CGTAACGTACTAAGTACGTACGAACGAGCTCCTCCCATGGCTACTGGTACGACGTCCGTCGAACAGCTCC
141	TC139294	Similar to Nonspecific lipid transfer protein	TCTCTCAGCCTTCACTGACGCACCGACTAAGCCGACAAAGCAAGCACACTAGCTAGTTTCCACCATGGCA
142	TC139295	Nonspecific lipid transfer protein	TCTATTTGTATCACCCGTTCGCCCGGCGAACACGCACGGTCTATGATTATACCCCACGTAGAGCGGACAA
143	TC139296	Similar to Nonspecific lipid transfer protein	TAGCTGCTGTTCACTTTAGATGCGCTTTTGGAAATTTTGCATTGCTACAGAACACCAACCAGACGGACAG
144	TC139633	Glycolipid transfer protein-like	ATGTAACTGAGAATGCAGAGGATGCAGATATGTGCTGGTCTTGTCGTTCCATTTATAAACTGCTGTAAAA
145	TC140013	Probable nonspecific lipid transfer protein	GGGGCCCCGTTCCTTTTTTGCCCTTTGTGGTTCTTTTTCAATTTTCCGGCCCGGGTTTTTAGCCTTTTTT
146	TC140033	Putative lipid transfer protein	CGTTCTGCTGTTCGCACGCACCCCGCAGATTATACGGTAGTACTCCTTCAATACATACGCGTATTATTTC
147	TC140034	Lipid transfer protein precursor	TTCTGGCATCACGACCGTGTTCCGCACGCCGTAGAGTCGCGTACCCAATTTATATCGCATTGCAGTTGAC
148	TC140061	Lipid transfer protein-like	ACCTGGGTACGCAAACGCCATGTGTTGGTGCCAGATGCTTAACCACGCAGAGCTATGTTGATTGCTACTT
149	TC141534	Nonspecific lipid-transfer protein 3 precursor	AGCCCAGAGCACCGCTGACAAGCAAGCGGCGTGCAAGTGCATCAAGAGCCTCGCTACCAGCATCAAGGGC
150	TC146251	Nonspecific lipid transfer protein	GTCCGCATCTCGCTCGCTTGCCGGTCTCTGCTCCAGCATTTCAATCGCCAACACCGGCAAGGAAAGCAAG
151	TC146675	Lipid transfer protein 7a2b	GTGTGTACCTAGCTACTACTATATAGCAGTATGATGGAGCATCAGACATGTGTGCGCTATACCGATGTTT
152	TC146738	Similar Lipid transfer protein	TTGATAATAACATGGCACATTTCGTAATTTTGGAACCTAATGGCAAGAACCAGAAATACTTGGCGAAGGC
153	TC146891	7 kDa Lipid transfer protein	CTTCCCAGCATATCATATGTGGCTCAGCCTCAGCTATAGGTATGTAGGGTACCTCCGTCCATGACAGATG
154	TC147023	Phospholipid transfer protein precursor	TGTTGTACTTTGTACCATGTGCACTTTTGATATATGGCTATGCCTGCTCGCGTCATTATTTAGTATACGT
155	TC147024	Nonspecific lipid transfer protein 4.3 precursor	ATATATTATCTCCATGTGGGAGAGAAAGAGAGAGAGAGAGAGAGAGAAGTACGTACGTTGAGCCCGCT
156	TC147454	Probable nonspecific lipid-transfer protein precursor	TGATCTATGGCGGCTAGCTTGCTTCCTGGCTAGCAGCTGCTGTAATGAAATTTGTGTTGCAACTTTTTTT

Table III (continued)

No	TC	Functionary Name	Sequence
157	TC148609	Lipid transfer like protein	AACTGGCCGGATTTGCTATTTCAATAACAGTTACGAGCGCAGTAGGTGTGCTCGCCGTTCGTTTTCTGCT
158	TC150142	Nonspecific lipid transfer protein, complete	AGTCACTACTCACTACTACCAGCCGATCTACATTTCTTCGTCAGAGTTCCAGAGACGCCATGAAGTACTT
159	BQ767780	3-ketoacyl-acyl carrier protein reductase	ATGTAAAGGGCACATACCTCGTGACGAGGGCCATGCTGCCCCTCCTTTTGTTAGGGGGCGAAAAGACCAT
160	TC132098	Acyl-carrier protein II, chloroplast precursor	CCAGAACTCTTATCTGGCTAACGTTCGTTGGGAGACGTTTTGGTTCTTAGGTGAGTTCAAACGGTGGCAG
161	TC132937	Hydroxycinnamoyl transferase	GAACCGCCAAGCAAAAGCAAGCAAAGGGCGGAGGGGACAGGCGAGATCGAGATGAAGGTGGAGGTGGTGG
162	TC139306	Acyl-carrier protein III, chloroplast precursor	GACCATCTAAGTTCCTAGTTTTCGTGCGGCTGTGGTGCATGTTGATGTACGCATCTTTTGTGCCCAAAAA
163	TC139889	Homologueue to Acyl-carrier protein-like protein	ACCACCCCATGGCCGGCTGAGTGCCCGCCCTTCCGATTGTTGACCGCGCCAGGGTGAGGATTTTTCTTGT
164	TC140670	Hydroxyanthranilate hydroxycinnamoyltransferase3	GCTTCAGGTTGCGCCGAACGTTTTTCTGGGGGGTATATATATGCCTGGAGTTTCAGATTAACACATACTG
165	TC143966	3-Oxo/ 3-Ketoacyl carrier protein synthase	CGGAGGATTGCCTAACTTGTGGGCGTTGACTCGTAGGCCTGTAAATTTTTACAGTACGAGAATTATCGCT
166	TC147044	Hydroxyanthranilate hydroxycinnamoyltransferase3	GCAGCACCGGAGTAATTGTAGGATGCAGCAATGATTCTTTGATTACACACGTAGTTCCTGCACGTTTTCC
167	TC147267	Acyl carrier protein I, chloroplast precursor	TGTTGCTGCGGGTTAATTACCCAAAGGCTTCATAAACAAGCTAGGACCATGTCCTAAGTTCGAATTCATA
168	TC147905	3-Oxoacyl-[acyl-carrier-protein] synthase I (KASI)	TTTCAGGAAGCTCCGAGTTATTTTCGATGTGTTTTCTTACGGGAAGCAATTGTCGATTTGACTGCCAGCA
169	TC150849	Acyl-[acyl-carrier protein] thioesterase	TGGAAGAGCTACCAGCAAGTGGGTCATGATGAACCAAAATGGGCGCAGACTTCAAAGAGTCAGTGACGAA
170	TC152918	Similar to 3-oxoacyl-[acyl-carrier-protein]	ATCACAACTCAGACATTTGAAAGTACCGATTATTACCTGACATCTCTATCGTTAACTCTACCGCCTTTAC
171	TC153209	Oleoyl-acyl-carrier protein thioesterase	TAAACACCTCATCCCTGACTTCGTCACTGACTCTTTGAAGTCTGCGCGTATTTTGGTTCCTCGAGGATTC
172	AU252305	Fatty acyl-CoA reductase	
173	AV835407	Putative CER1	GACGGACGACCTGTCACCACGAGCTACATGAAGCAGATTTCCTCTTACGTGATGCAAGACGATCAGCTGT
174	BE437893	Putative ABC-transporter family protein	AAGAACACGGGTATAGTTTATGGCTTGCTTTATGGGTTTACATAGACATACGAGACGCTATGGCCCTGTT
175	BI777732	Putative ABC-transporter family protein	GACATACGTGGCATCGGAGGGGACGTGGTGAAAATGGATGGGCAACAGCCAAGCTTCCTCACGCAATCCT
176	BI947024	Putative ABC-transporter	AAGCACAGGGTGCACCGTCATCTTCACAATGTATCAAAGCAGCACGGAGGTTTTTGGACTTTTCGATCGG
177	BQ465940	Putative ABC-transporter protein	TATGATCGCATACTTTGAGGGAATAGGCGTCAATAATATACAAGATGGGCATAACCCCGCAACTTGGATG
178	BU991506	PDR-like ABC-transporter	TGTCAACGAAGTTCTTGAAACAATTGAGTTGGACGAAATTAGAGATTCTTTGGTCGGAATACCTGGGGTA
179	CA013652	PDR-like ABC-transporter	CCCTGATGTAGACTCAGAAGCAAGACAAATGTTTGTGGAACAAGTCATGGGACTCGTCGAGCTGACATCA
180	TC131529	PDR-like ABC transporter (PDR4)	GTTTTGTTCATGGGGGTGCAGAACTCAGCCTCAGTTCAGCCAGTTGTAGCTGTTGAGCGCACAGTCTTTT
181	TC132458	ABC-transporter	GCGATGCAGGGTGACACATAATTGGACGTTCACGGTTTGTTGCTGTCTTGGAAGGGCAGCAGGATAGGTA
182	TC135334	Putative ABC-transporter	TTATCCTCCTGGGCAGAGGAAGTTTGCTCTACTTTGGGAAGACAGCTGAAGCCATGCCCTACTTTTCATC
183	TC140581	PDR-like ABC-transporter	CTCCAGCTTCTTCACCAGCTCTAGCTGAGTAACTGCTAATTTACTCGTCGGAGAATGTAAACTTGACCAG
184	TC141271	Putative ATP-dependent transmembrane transporter	CTATTACTTGTGTATGCGTTTCCACTTGTTAGTTTTGCTTGCAGGTCTGCAGCTTCGCGAGGTTCATAAA
185	TC141870	PDR-like ABC-transporter	GTGGCGTTGACCCCGTCCTCGATGCTCGCAAACATACTGATATCTTTTGTACTTCCTCTTTGGAACCTGT
186	TC143431	PDR-like ABC-transporter (PDR4)	GTGGCGGCCACGACAGTAAAGAGAGAATAAGCATAATGTAGGGGAAGAGTAGAAGGGGGTGGTGCTGGTT
187	TC144475	ABC-transporter-like protein	CACCAGTGCCTTCATGGTGGGGAAAGTGCTGCAGCGCCATCAGCCAAAACGGCAGCGTCGTGGTCATGGC
188	TC149628	PDR-like ABC-transporter	TTGGCCAGGTTGTCATTGAACTCCCATACACACTCGCTCAGGCTACCGTATACGGAGTCATAGTTTACTC
189	TC150699	Potential phospholipid-transporting ATPase 11	ATCTCGTCAATGGTATGCTACTTCTTATATAAGAACATCACATTCGGCGTGACCCTGTTCCTGTACGAGT
190	TC153166	PDR-like ABC-transporter	TTTCAGTCTTTCATGTAGCAACGATGCTCAGGGACTGAAATCCTTTGAAAGACCAAACCCATCTGGTGCT
191	AJ475492	AP2/ EREBP transcription factor	ACAACAGCTGCCGGCGTGAAGGCCAGAGCCGCAAGGGCCGGCAAGTGTACCTCGGTGGCTATGATAAGGA
192	BG343755	Putative AP2 domain transcriptional regulator	CCAAGATGCGTGAAGTGCAGAAGCTCTGGAAGGACAGAAGGAAGATACGAAGGTTCTGATAAAAGGATAT
193	BU988090	Similar to AP2/EREBP transcription factor	TAGATATGTTTGACTTCTGATTGGTTACTGATGGTTTCTTGCAATGGTGTTCCAATATGGCGGTGCTTCA
194	BU993521	AP2/ EREBP transcription factor	GGGGCTTAGTGGTACATACAAGATAGAAGAACTGGTCGCAGCCTGTCAGTATTTGCTGCTGTATGATTCT
195	TC131406	Translation initiation factor 5A	TTTGGAATTGGAACTGAGTGGTGGCTGGAACTTAGTTTCCGGTTTGCTCATCCGCCCTATCTCTATGAAT
196	TC131837	AP2 domain transcription factor EREBP	ATCCAACGTCCGTCCGCCGCTGAAAACACCCTTCCCAGTCCAAAGCCTTTCCCGCCACCTAAAGCACCCG
197	TC139591	DRE binding factor 2	AAAAATCCCTAGATGGCTGCAGCTATAGATCTGTCCGGGGAGGATCTGGTGAGAGCACTCGAGCCTTTTA
198	TC143745	AP2/ EREBP transcription factor	CAACTTGATGTGTTATTTCTCCTTCATGGCTGTTATTCCTGGCGTCGTCTCCCTCCAGTGTACCTCGGTG
199	Z50789	Elongation factor 1-alpha	CTCGCTTTGCGACCCCTCCGTTCGTTCTTCTTCTTCGAGTTTGTTATCAGCCATGGGTAAGGAGAAGACT
200	TC132081	17kDaHSP	GTCTAGAGAGAAATCTCTGTATTGTGTTTCTTTCCCCAGTCGTTTCGGTACTTATGTAATCGCCAACAGG
201	AF043093	Dehydrin 8 gene	AGATATCACATGGTTGTTGCGGTTAATTACTGACCTCGTGTGTGATCGTCTGTACAGTCTAGTGACGAGG
202	TC138670	cytHSP90	TTGGGCTTTAAGCCTGGAGAACCCAAAAACCTTGGGACCCAAGAATCCCCCCATTGCTAAAACTTGGCCT
203	TC138881	Histon	TACCATGCTCTGTTCAATGCAATCAGTTCGATTCTGAATCCTGACGAAATGCTTCAGTGCTGAATCGTGT
204	TC146844	HSP20	TGAGTCTCTCGTGTCTGTGTTTCCAGTCCAGTTTGTTCGTCAGTCTGTACTATGTACTCCATCAGCAACT
205	L36093	Peroxidase	CCGGCAGCTCGCTAGCACGATACGTGAATGAATAAGGCGAACCAGTGGCCAATATAAACACCAGCACTTG
206	TC150640	PR1a	CGTGTAGTGCCCGCACACCTTCCCCCCTGCACAGGTGTTGGACCCGTAGTCGTAATCCTTCTTCTCGTCC
207	TC131682	PR4pre	CGACTGGGACACCGTCTTCGCCAAGATCGACACCAACGGGATCGGGTTCCAGCAGGGCCACCTCAACGTC
208	TC132008	PR1a Pathogenesis-related protein (Hv-1a) precursor	TATACATAGTCCAGTAGTCCCTCGTTCCTCACGTGCATGGGATTCATGCACGCTCGTATCTATAATTAGC
209	TC132822	Pathogenesis-related protein 10	ACATTCATGCACCGAGCACTTAGATACTATGCTCAGCAGCGCTAGTGTGCAGCGAGCCAGTGACCGACGG

Table III (continued)

No	TC	Functionary Name	Sequence
210	TC132980	Dehydrin	ACCGTGTGATAGATGTTGCAGTACTGTGACATGGCCTGGTTCTAAGTAGTTTGTCTGGTCGGTTTGCACT
211	TC133008	Basic pathogenesis-related protein PR5	CTGCTTATGATCGAGGATCATTTGTATGTGCTATGTCATATATGCATGCATCACTAGTGCCTGTATGTGG
212	TC138692	Wound-induced basic protein	TGTCTCTCGACCTCTGCCTGTTCTTCACCTGCACTCTTTAGCCTTGTATGCTTATCTTTTTGGCGTGACA
213	TC139232	Dehydrin 4	GGGCGAATGTATCTAGGTTCAAGTTTACGTGAACAACACTGTGTTAAGAGTGTTTTCGTCTGTTTACACC
214	TC139233	Dehydrin 5	AATAAGATGGCGAAGTTCCACCGTATGCACATTCACCTAGCTCACTTTCGTTGGTGGAGCGAATGTATCT
215	TC139234	Dehydrin 11	ACGAGTTAGATTTGGGGTAGTTCGAGTTGGTGTGTGGCTAATGTCCGGTGCTACCGTAAGTGTAGTTCCT
216	TC140295	18,9 kDa ABA-induced protein Stress responsive	AGAAATGCAACAAGCCCTGGAGTTAGTGCTGCTGGCAGCTACGTATTTGCATTTCGTACTTGTGTGTATT
217	TC140762	Cytochrome P450	TGCATGCTCCACGTACAGTTCGTGCCATATGGGGAAGGTCCGATGGACGAAGTTGGAAATGGCAAAAAAA
218	TC142835	Dehydrin 12	AGTTTGGGACCGGTGCAGAGGATAGATGATCCAGCAGCAAACTCAACAGAGGTGATTGGATGGAGTACCG
219	TC143368	Wound-induced protein	ACAAGGAGCACAAGTCAGTTTCCACTCTCCAGCAGCGCAGCAAGAATAAGAAGTATAGGAGAAGCGATGG
220	TC146488	Putative cytochrome P450	AGAAAGAAAATGGACGCGTATGGGCACCAGCGTGGATGCATGATCGATCTATACAGGTTGGCTTGACGCT
221	TC146605	Peptidylprolyl isomerase (cyclophilin)	GACAGGGGCAAGACACGACACGACTTCTTATTTAGACCGACGACACGACAGGGGACACATGGCACGACGA
222	TC148223	Universal stress protein USP1-like protein	TAGGCATCGTCCTGCAGCTAGGACGGCAAGCAAACCACAGCAGACCGAGCCAAGAAACCTACTCTCATCA
223	TC148899	Phospholipid hydroperoxide glutathione peroxidase	TATCAGATGGCACGAGCTTACCTTTGATAGTTTTCGAGCGTGGTTTCCTGAGGTTATTTCGGTGATCGAT
224	TC150945	Senescence-associated protein	CACGAGGGAACAAGATCTCCAAGGAGGCCTTCCTCGACATGACCGAGAGCACCGGCTGCTTCAACGACGA
225	TC147009	Winduced	GTCCATACTTGCTTGTTTCGGTGGAGTGATCTTTTATACTCCAGTAATTTCGCTATAAATTGGCAGGCTT
226	CA592528	Putative senescence-associated protein	AACATCACCACGCTGGGGCCACTGGTGCTGCCCCTCAGCGAGCAGCTCAAGTTCCTCAAGTCGCTCATGA
227	CB859433	Sutative steroid reductase DET2	TACACTTCTCCTCAGCCAGGGTGACACAAGTTTTCAGAGGCTAGTAGATGTACGGGATGACGGCCCTGCG
228	TC130819	Ethylene-binding protein	AAATGGTGCACAAGACATCCCTGCTTTTGACAATGAGGATGGGGCAAGCAGTGGGGATCTCTGGAGCCTC
229	TC132897	APETAL2-like protein	TCCCATAATTCTCGCTGTCAGATACTAACCTCAGCTCATCTCACCATCTGAGATGGATTCATACCATTGT
230	TC133736	AP2 domain-containing protein	CAGCGCTGCGTTTGCCTTTACTAAATTTAGGAGTCGGGCCATGTACAAATTGGGGTTAGCCCTGTGTTCT
231	TC134961	Putative ethylene response factor	TGCCCGTCCTCCTCCCTGTCAGAGAGAGAATCTCGCTCGCTCTGCATTCCGCAGAGAAACATCATGGTAC
232	TC136332	Putative ethylene response factor	AGGTGTTGGGCCGCTATTATGTACTTCATTTTAGGCATTTCATTACACAGGCCCCCCGTCGAAGATACTT
233	TC136836	Senescence-associated protein	GTATATAATCTCCCCACCGCACTTGTGATGTTTTACACTGCTGTGGGGAGAAAAGAATCATGTAATCTTC
234	TC137165	APETALA2-like protein	CATCGCCGGCCTTCAGTTAACTTTTGATTCCCCTGAGTCGTCAACCACAATGGTCTCCTCTCAGCCAATG
235	TC139592	Steroid 5-alpha reductase	GAAAGCCATCATTCGATTTCACGCAAACATTCCTACTAATCGTGCCCTGTACCAGCTGTCAAACCTCAAT
236	TC142284	Steroid 5-alpha-reductase-like protein	CCTATATCTTGTAGTGAGCTAGTTAATTGGTTGTCTGCCAGATTGTCTATTCACAACCAGTCTCATGCTT
237	TC146564	Probable amylase/ protease inhibitor	CTTGTCTCTCTGTTTCCTCACGTTCGCGTCTCATATAATAATTTACTTATGTGCTCTAGGATCGTAGTAC
238	TC147452	Abscisic acid and stress inducible gene	TACTCCTTCGTCACGCTCGTCGAGATGGTCCTCGAGTCCCTCATCTACTGGATACCGATCTGGTACGAGC
239	AY483150	Putative cellulose synthase catalytic subunit (CesA1)	ACAGTGATCGACTGACAACCCGTGGAGCCAGAGAAAATTTATGTTTGGGGTTGCGAATTACTACGTTTGA
240	TC131841	Similar to At5g64750	CAACATCTCCTCGCGTTTTGTCGCTACAAACGCCTTTACCCTGTAATGTAATCATCAGACACATACCTTT
241	TC141549	Calcium-dependent protein kinase	CCAAATCGCTTCTTGTACAGTGGTCCATGCTACTGTTCTGTGCTACGCAGGATGATGCTATTTTCCCTGT
242	TC143520	Ovule development protein aintegumenta (ANT)-like	GATATTAGCAACTACCGTGAAGATGGTTTGAAGAAACTAGAAGCCTCATCGGAGATAGCGAACCTGGAGG
243	TC146764	Endoplasmin homologue precursor (GRP94 homologue)	GTGATGGTTTTGAGGGACGTCCATTTTTACCGCGCGGAAGACGATTAATCATCGATCGTGATACGGGGGT
244	TC150994	Probable ATP-dependent permease precursor	TTCGATCAAGACCATTGAGGACTACTTCTCAACTCTTGGAATTCATGTCCCCGACCGTGTAAACCCGCCA
245	TC151223	Synaptic glycoprotein SC2	GGATTGGATCGGGAGTAGTCTTGGCTTACTACGTTTATCACCCTGCTTCATACACCCAACTTGAGTCAGA
246	TC131417	Actin	CTGTATTGCCGGATTAATTTGTTGGATCAAGATTGCGTACCTTGTAGACTCGTAATATGTTCAGTGGGCT
247	TC131375	a-Tubulin	CCTTGGCCTCTGTCTCCATTTTCCTGTTTCAATATGTGTTACTATCTGGTTATCTATGCCTTAGCCTCCT
248	AY220735	Ubiquitin-conjugating enzyme (UBC)	CATGATAGTCCCTATGCTGGAGGTGTTTTCTTGGTGAATATCCATTTCCCCCCGGACTACCCGTTCAAGC
249	TC131375	Tubulin alpha-2 chain	GGTTATCTATGCCTTGGCCTCTGTCTCCATTTTCCTGTTTCAATATGTGTTACTATCTGGTTATCTATGC
250	TC131547	Actin	TACGTGGCGGCTCCATGGTGCAAGTGCAAGTACACTATCTATGTTTGTGCATTGTCAGTGTGTTTGTGGG
251	TC151701	Ubiquitin-protein ligase E3-alpha-like	TTCACCACCTTCATGGAGCACATCAAGCTCATCTCCAACAACGACAAGAGCCTCCGCTTCCAGACCCGCA
252	TC152952	Ubiquitin-protein ligase E3-alpha-like protein	AATTGCCTTGCTTACCGCTGCCGGACCTGCGAGAGTGACTCCACATGCGCAATTTGCGTTCCATGCTTCC
253	TC138996	Ubiquitin	AGCGCCTCATCTTCGCTGGCAAGCAGCTTGAGGACGGCAGGACCCTTGCTGACTACAACATCCAGAAGGA
254	X04133	mRNA for Ubiquitin polyprecursor fragment	CCGGACCAGCAGCGCCTCATCTTTGCTGGCAAGCAGCTCGAGGATGGCCGCACCCTGGCTGACTATAACA

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. *The Plant Cell* **7:** 2115-2127.

Aery NC, Rana DK. 2003. Growth and cadmium uptake in barley under cadmium stress. *Journal of Environmental Biology* **24(2):** 117-123.

Aharoni A, Dixit S, Jetter R. et al. 2004. The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in *Arabidopsis*. *The Plant Cell* **16:** 2463–2480.

Alba R, Fei Z, Payton P *et al.* 2004. ESTs, cDNA microarrays, and gene expression profiling: tools for dissecting plant physiology and development. *The Plant Journal* **39:** 697-714.

Alexander D, Goodman RM, Gut-Rella M *et al.* 1993. Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. *Proceedings of the National Academy of Sciences* **90:** 7327-7331.

Arondel V, Vergnolle C, Cantrel C, Kader J-C. 2000. Lipid transfer proteins are encoded by a small multigene family in *Arabidopsis* thaliana. *Plant Science* **157:** 1–12.

Ashraf M, Mehmood S. 1990. Response to four *Brassicacea* species to drought stress. *Environmental and Experimental Botany* **30(1):** 93-100

Baker EA. 1974. The influence of environment on leaf wax development in *Brassica oleracea* var. Gemifera. *New Phytologist* **73:** 955-966

Baker EA. 1982. Chemistry and morphology of plant cuticular waxes. In: *The plant cuticle* (eds Cutler DF, Alvin KL, Price CE), Academic Press, New York pp.139-166.

Bargel H, Koch K, Cerman Z, Neinhuis C. 2006. Structure-function relationships of the plant cuticle and cuticular waxes - a smart material? *Functional Plant Biology* **33:** 893-910.

Barthlott W. 1990. Scanning electron microscopy of the epidermal surface in plants. In: *Scanning Electron Microscopy in Taxonomy and Functional Morphology* (ed. Claugher D), Clarendon Press, Oxford, pp. 69–94.

Barthlott W, Neinhuis C. 1997. Purity of the sacred lotus, or escape from contamination in biological surfaces. *Planta* **202:** 1-8.

Barthlott W, Neinhuis C, Cutler D *et al.* 1998. Classification and terminology of plant epicuticular waxes. *Botanical Journal of the Linnean Society* **126:** 237–260.

Baum BR, Tulloch AP, Bailey LG. 1989. Epicuticular waxes of the genus *Hordeum*: a survey of their chemical composition and ultrastructure. *Canadian Journal of Botany* **67:** 3219–3226.

Beattie GA, Marcell LM. 2002. Effect of alterations in cuticular wax biosynthesis on the physicochemical properties and topography of maize leaf surfaces. *Plant Cell & Environment* **25:** 1–16.

Berrocal-Lobo M, Molina A, Solano R. 2002. Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in *Arabidopsis* confers resistance to several necrotrophic fungi. *The Plant Journal* **29(1):** 23-32.

Bianchi G, Avato P, Salamini F. 1978. Glossy mutants of maize. VIII. Accumulation of fatty aldehydes in surface waxes of gl5 maize seedlings. *Biochmical Genetics* **16(9/10):** 1015-1021.

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. *Environmental and Experimental Botany* **36(1):** 61-69.

Broun P, Poindexter P, Osborne E, Jiang C-Z, Riechmann JL. 2004. WIN1, a transcriptional activator of epidermal wax accumulation in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the USA* **101:** 4706–4711.

Bushnell WR, Mendgen K, Liu Z. 1987. Accumulation of potentiometric and other dyes in haustoria of *Erysiphe graminis* in living host cells. *Physiology and Molecular Plant Pathology* **31:** 237-250.

Carver TLW, Bushnell WR. 1983. The probable role of primary germ tubes in water uptake before infection by *Erysiphe graminis*. *Physiological Plant Pathology* **23:** 229-240.

Carver TLW, Ingerson SM. 1987. Responses of *Erysiphe graminis* germlings to contact with artificial and host surfaces. *Physiology and Molecular Plant Pathology* **30:** 359–372.

Carver TLW, Thomas BJ. 1990. Normal germling development by *Erysiphe graminis* on cereal leaves freed of epicuticular wax. *Plant Pathology* **39:** 376–375.

Carver TLW, Thomas BJ, Ingerson–Morris SM, Roderick HW. 1990. The role of abaxial leaf surface waxes of *Lolium spp.* in resistance to *Erysiphe graminis*. *Plant Pathology* **39:** 376–390.

Carver TLW, Ingerson-Morris SM, Thomas JB, Gay AP. 1994. Light-mediated delay of primary haustorium formation by *Erysiphe graminis* f.sp *avenae*. *Physiological and Molecular Plant Pathology* **45:** 59-79.

Carver TLW, Ingerson SM, Thomas BJ. 1996. Influences of host surface features on development of *Erysiphe graminis* and *Erysiphe pisi*. In: *Plant Cuticles*, BIOS Scientific Publishers Ltd., Oxford U.K., pp. 255–266.

Chen W, Provart N, Glazebrook *et al.* 2002. Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. *The Plant Cell* **14:** 559-574.

Chen X, Goodwin M, Boroff M, Liu VL, Jenks MA. 2003. Cloning and characterization of the *WAX2* gene of *Arabidopsis* involved in cuticle membrane and wax production. *The Plant Cell* **15:** 1170- 1185.

Christopher DA, Li X, Kim M, Mullet JE. 1997. Involvement of protein kinase and extraplastidic serine/threonine protein phostphatases in signalling pathways regulating plastid transcription and the psbD blue light-responsive promoter in barley. *Plant Physiology* **113(4):** 1273-1282.

Clarke DD. 1982. The accumulation of cinnamic acid amides in the cell walls of potato tissue as an early response to fungal attacks. In: *Active defense mechanisms in plants*. Edited by Wood RKS, Plenum, New York, pp. 321-332.

Close Tj. 1996. Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. *Physiologia Plantarum* **97:** 795-803.

Close Tj. 1997. Dehydrins: a commonality in the response of plants to dehydration and low temperature**.** *Physiologia Plantarum* **100:** 291-296.

Corey EJ, Suggs JW. 1975. Pyridinium chlorochromate – efficient reagent for oxidation of primary and secondary alcohols to carbonyl-compounds. *Tetrahedron Letters* **31:** 2647-2650.

Cutt JR, Klessig DF. 1992. Pathogenesis-related proteins. In: *Genes involved in plant defense*. (eds. Boller T, Meins F), Springer-Verlag, Berlin/ New York, pp. 209-243.

Danyluk J, Perron A, Houde M *et al.* 1998. Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. *Plant Cell* **10:** 623-638. recherchieren!

Dixon M, Le Thiec D, Garrec JP. 1997. An investigation into the effects of ozone and drought, applied singly and in combination on the quantity and quality of the epicuticular wax of Norway spurce. *Plant Physiology and Biochemistry* **35(6)**: 447-454.

Edwards HH. 1993. Light affects the formation and development of primary haustoria of *Erysiphe gaminis hordei* in leaf epidermal cells of *Hordeum vulgare. Physiological and Molecular Plant Pathology* **42:** 299-308.

Edwards HH. 2002. Development of primary germ tubes by conidia of *Blumeria graminis* f. sp. *hordei* on leaf epidermal cells of *Hordeum vulgare*. *Canadian Journal of Botany* **80**: 1121–1125.

Efremova N, Schreiber L, Bär S *et al.* 2004. Functional conservation and maintenance of expression pattern of *FIDDLEHEAD*-like genes in *Arabidopsis* and *Antirrhinum*. *Plant Molecular Biology* **56:** 821-837.

Egerton-Walburton LM, Balsamo RA, Close TJ. 1997. Temporal accumulation and ultrastructural localization of dehydrins in *Zea mays* L. *Physiologia Plantarum* **101:** 545-555.

Francis SA, Dewey FM, Gurr SJ. 1996. The role of cutinase in germling development and infection by *Erysiphe graminis* f.sp. *hordei*. *Physiological and Molecular Plant Pathology* **49**: 201–211.

Francois LE, Mass EV. 1999. Crop response and management of salt-affected soils. In: *Handbook of plant and crop stress*. 2nd edn., (ed. Pessarakli M), Mercel-Dekker, New York, pp. 169-201.

Fricke W, Peters WS. 2004. The biophysics of leaf growth in salt-stressed barley. A study at the cell level. *Plant Physiology* **129:** 374-388.

Fricke W, Akhiyarova G, Wei W et al. 2006. The short-term growth response to salt of the developing barley leaf. *Journal of Experimental Botany* **57(5):** 1079-1095.

Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M. 2000. *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell* **12:** 393-404.

Giese BN. 1975. Effects of light and temperature on the composition of epicuticular wax of barley leaves. *Phytochemistry* **14**: 921-929.

Giese BN. 1976. Roles of the cer-j and cer-p loci in determining the epicuticular wax composition on barley seedling leaves. *Hereditas* **82:** 137-148.

Gniwotta F, Vogg G, Gartmann V, Carver TLW, Riederer M, Jetter R 2005. What do microbes encounter at the plant surface? Chemical composition of pea leaf cuticular waxes. *Plant Physiology* **139**: 519–531.

Gordon DC, Percy KE, Riding RT. 1998. Effects of u.v.-B radiation on epicuticular wax production and chemical composition of four Picea species. *New Phytologist* **138:** 441-449.

Gou TR, Zhang GP, Zhang YH. 2007. Physiological changes in barley plants under combined toxicity of aluminium, copper and cadmium. *Colloids and Surfaces B: Biointerfaces* **57:** 182-188.

Green JR, Carver TLW, Gurr SJ. 2002. The formation and function of infection and feeding structures. In: *Powdery mildews: a comprehensive treatise.* (eds. Belanger RR, Bushnell WR, Dik AJ, Carver TLW), APS Press, St Paul, MN, USA, pp. 66–82.

Gregersen PL, Thordal-Christensen H, Forster H, Collinge DB. 1997. Differential gene transcript accumulation in barley leaf epidermis and mesophyll in response to attack by *Blumeria graminis* f.sp. *hordei* (syn. *Erysiphe graminis* f.sp. *hordei*). *Physiology and Molecular Plant Pathology* **51:** 85-97.

Grossi M, Gulli M, Stanca AM, Cattivelli L. 1995. Characterization of two barley genes that respond rapidly to dehydration stress. *Plant Science* **105:** 71-80.

Günthard-Georg MS, Keller T. 1987. Some effects of long-term ozone fumigation on Norway spruce. *Trees* **1:** 145-150.

Hannoufa A, McNevin JP, Lemieux B. 1993. Epicuticular wax of *eceriferum* mutants of *Arabidopsis thaliana. Phytochemistry* **33:** 851-855.
Hannoufa A, Negruk V, Eisner G, Lemieux B. 1996. The CER3 gene of *Arabidopsis thaliana* is expressed in leaves, stems, roots, flowers and apical meristems. *The Plant Journal* **10:** 459–467.

Heath MC, Skalamera D. 1997. Cellular interactions between plants and biotrophic fungal parasites. In *Advances in Botanical Research Incorporating Advances in Plant Pathology.* **24:** 195-225. London:Academic.

Hedge Y, Kolattukudy PE. 1997. Cuticular waxes relieve self–inhibition of germination and appressorium formation by the conidia of *Magnaporthe grisea*. *Physiological and Molecular Plant Pathology* **51:** 75–84.

Hendriks T, Meijer EA, Thoma S, Kader JC, De Vries SC. 1994. The carrot extracelluar lipid transfer protein EP2: quantitative role in cutin synthesis. In: *Plant Molecular Biology* (eds. Coruzzi G, Puigdomenech P), Springer-Verlag, Berlin, pp. 85-94.

Hejgaard J, Jacobsen S, Bjorn SE, Kragh KM. 1992. Antifungal activity of chitin-binding-PR-4 type proteins from barley grain and stressed leaf. *FEBS Letters* **307(3):** 389-392.

Hollenbach B, Schreiber L, Hartung W, Dietz K-J. 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. 1969. Chemistry of leaf waxes in relation to wetting. *Journal of the Science of Food and Agriculture* **20:** 124–128.

Holloway PJ. 1970. Surface factors affecting the wetting of leaves. *Pest Management Science* **1:** 156–163.

Iwamoto M, Takeuchi Y, Takada Y, Yamaoka N. 2002. Coleoptile surface cuticle of barley is involved in survival and penetration of *Blumeria graminis*. *Physiological and Molecular Plant Pathology* **60:** 31–38.

Iwamoto M, Takeuchi Y, Takada Y, Kohno S, Matsumoto I, Yamaoka N. 2007. Coleoptile cuticle of barley is necessary for the increase in appressorial turgor pressure of *Blumeria graminis* for penetration. *Journal of General Plant Pathology* **73**: 38-40

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, *et al.* 1998. Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. *Science* **280:** 104-106.

Jasinski M, Stukkens Y, Degand H *et al.* 2001. A plant plasma membrane ATP binding cassette-type transporter is involved in antifungal terpenoid secretion. *The Plant Cell* **13:** 1095-1107.

Jasinski M, Ducos E, Martinoia E, Boutry M. 2003. The ATP-binding cassette transporters: structure, function, and gene family comparison between rice and *Arabidopsis*. *Plant Physiology* **131:** 1169–1177.

Jeffree CE. 1986. The cuticle, epicuticular waxes and trichomes of plants, with reference to their structure, functions and evolution. In: *Insects and the plant surface* (eds Juniper BE, Southwood TRE), Edward Arnold, London, pp. 23–64.

Jenks MA, Tuttle HA, Eigenbrode SD, Feldmann KA.1995. Leaf epicuticular waxes of the *eceriferum* mutants in *Arabidopsis*. *Plant Physiology* **108:** 369-377.

Jenks MA, Andersen L, Teusink RS, Williams MH. 2001. Leaf cuticular waxes of potted rose cultivars as affected by plant development, drought and paclobutrazol treatments. *Physiologia Plantarum* **112:** 62-70**.**

Jetter R, Riederer M. 1994. Epicuticular crystals of nonacosan–10–ol: In–vitro reconstitution and factors influencing crystal habits. *Planta* **195**: 257–270.

Jetter R, Riederer M. 1995. In vitro reconstitution of epicuticular wax crystals: formation of tubular aggregates by long chain secondary alkendiols. *Botanica Acta* **108**: 111–120.

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 & Environment* **23**: 619–628.

Jetter R, Schäffer S. 2001. Chemical composition of the *Prunus laurocerasus* leaf surface. Dynamic changes of the epicuticular wax film during leaf development. *Plant Physiology* **126**: 1725–1737.

Jørgensen JH. 1988. *Erysiphe graminis*, powdery mildew of cereals and grasses. *Advances in Plant Pathology* **6**: 137–157.

Kader J-C. 1996. Lipid-transfer proteins in plants. *Annual Reviews of Plant Physiology and Plant Molecular Biology* **47:** 627-654.

Kang HA, Hershey JWB. 1994. Effect of initiation factor 5A depletion on protein synthesis and proliferation of *Saccharomyces cerevisiae*. *Journals of Biological Chemistry* **269:** 3934-3940.

Kerstiens G. 1996. Cuticular water permeability and its physiological significance. *Journal of Experimental Botany* **47:** 1813–1832.

Kerstiens G. 2006. Water transport in plant cuticles: an update. *Journal of Experimental Botany* **57**: 2493–2499.

Khudsar T, Uzzafar M, Iqbal M. 2001. Cadmium induced changes in leaf epidermis, photosynthetic rate and pigment concentrations in *Cajanus cajan. Biologia Plantarum* **44(1):** 59-64.

Kilian J, Whitehead D, Horak J, *et al.* 2007. The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *The Plant Journal* **50:** 347-363.

Kim M, Thum KE, Morishige DT, Mullet JE. 1999. Detailed architecture of the barley chloroplast psbD-psbC blue light-responsive promoter. *Journal of Biological Chemistry* **274(8):** 4684-4692.

Kinane J, Dalvin S, Bindslev L, Hall A, Gurr S, Oliver R. 2000. Evidence that the cAMP pathway controls emergence of both primary and appressorial germ tubes of barley powdery mildew. *Molecular Plant-Microbe Interactions* **13**: 494–502.

Knight H, Knight MR. 2001. Abiotic stress signalling pathways: specificity and cross-talk. *TRENDS in Plant Science* **6(6):**262-267.

Kobayashi I, Tanaka C, Yamaoka N, Kunoh H. 1991. Morphogenesis of *Erysiphe graminis* conidia on artificial membranes. *Transactions of the Japanese Mycological Society* **32**: 187–198.

Koch K, Neinhuis C, Ensikat HJ, Barthlott W. 2004. Self assembly of epicuticular waxes on living plant surfaces imaged by atomic force microscopy (AFM). *Journal of Experimental Botany* **55:** 711–718.

Koch K, Barthlott W, Koch S *et al.* 2006. Structural analysis of wheat wax (*Triticum aestivum*, c.v. 'Naturastar' L.): from the molecular level to three dimensional crystals. *Planta* **223:** 258-270.

Kolattukudy PE. 1966. Biosynthesis of wax in *Brassica oleracea*. Relation of fatty acid to wax. *Biochemistry* **5:** 2265–2275.

Kollatukudy PE. 1967a. Mechanisms of synthesis of waxy esters in broccoli (Brassica oleracea). *Biochemistry* **6:** 2705-2717.

Kolattukudy PE.1967b. Biosynthesis of paraffins in Brassica oleracea: fatty acid elongation–decarboxylation as a plausible pathway. *Phytochemistry* **6:** 963–975.

Kolattukudy PE. 1971. Enzymatic synthesis of fatty alcohols in *Brassica oleracea*. *Archives of Biochemistry and Biophysics* **142:** 701–709.

Kolattukudy PE, Buckner JS. and Brown L. 1972. Direct evidence for a decarboxylation mechanism in the biosynthesis of alkanes in *B. oleracea*. *Biochemical and Biophysical Research Communications*. **47: 1306**–1313.

Kunoh H, Yamaoka N, Yoshioka H, Nicholson RL. 1988. Preparation of the infection court by Erysiphe graminis. II. Contact-mediated changes in morphology of the conidium surface. *Experimental Mycology* **12:** 325-335.

Kunoh H, Nicholson RL, Yoshioka H, Yamaoka N, Kobayashi I. 1990. Preparation of the infection court by Erysiphe graminis: Degradation of the host cuticle. *Physiology and Molecular Plant Pathology* **36:** 397-407.

Kunst L, Samuels AL. 2003. Biosynthesis and secretion of plant cuticular wax. *Progress in Lipid Research* **42:** 51-80.

Kunst L, Jetter R, Samuels AL. 2005. Biosynthesis and transport of plant cuticular waxes. In: *Biology of the plant cuticle*. (eds Riederer M, Müller C), Blackwell Publishing, Oxford U.K., pp 181-206

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*. *The Plant Journal* **36:** 55-66.

Langridge P, Paltridge N, Fincher G. 2006. Functional genomics of abiotic stress tolerance in cereals. *Briefings in Functional Genomics and Proteomics* **4(4):** 343-354.

Lardizabal KD, Metzs JG, Sakamoto T *et al.* 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 Physiology* **122:** 645-655.

Leide J, Hildebrandt U, Reussing K, Riederer M, Vogg G. 2007. The developmental pattern of tomato fruit wax accumulation and its impact on cuticular transpiration barrier properties: effects of a deficiency in a betaketoacyl-CoA synthase (LeCER6). *Plant Physiology* DOI:10.1104/pp.107.099481

Lemieux B, Koornneef M, Feldman KA. 1994. Epicuticular wax and *eceriferum* mutants. In: *Arabidopsis* (eds Meyerowitz EM, Somerville CR). Cold Spring Harbor Press, New York, pp. 1031-1047.

Li Z-S, Alfenito M, Rea P, Walbot V, Dixon RA. 1997. Vacuolar uptake of the phytoalexin medicarpin by the gluthathione conjugate pump. *Phytochemistry* **45:** 689-693.

Lundqvist U, von Wettstein–Knowles P, von Wettstein D. 1968. Induction of *eceriferum* mutants in barley by ionizing radiations and chemical mutagens. II. *Hereditas* **59**: 473–504.

Lundqvist U, Franckowiak JD. 1997. BGS 523; *Eceriferum*-yj. *Barley Genetics Newsletter* **26**: 450.

Mass EV. 1984. Salt tolerance of plants. In: *The handbook of plant science in agriculture*. (ed Christie BR) CRC, Boca Raton, FL., pp. 55-75.

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.

Molina A, Segura A, García-Olmedo F. 1993. Lipid transfer proteins (nsLTPs) from barley and maize leaves are potent inhibitors of bacterial and fungal plant pathogens. *FEBS Letters* **316(2):** 119-122.

Molina A, García-Olmedo F. 1993. Developmental and pathogen-induced expression of three barley genes encoding lipid transfer proteins. *The Plant Journal* **4(6):** 983-991.

Moose SP, Sisco PH. 2007. Glossy15, an APETALA2-like gene from maize that regulates leaf epidermal cell identity. *Genes & Development* **10:** 3018-3027.

Moreau P, Bessoule JJ, Mongrand S, Testet E, Vincent P, Cassagne P. 1998. Lipid trafficking in plant cells. *Progress in Lipid Research* **37 : 371.**

Muradov A, Petrasovits L, Davidson A, Scott KJ. 1993. A cDNA clone for a pathogenesis-related protein 1 from barley**.** *Plant Molecular Biology* **23:** 439-442.

Mouradov A, Mouradova E, Scott KJ. 1994. Gene family encoding basic pathogenesis-related 1 proteins in barley. *Plant Molecular Biology* **26:** 503-507.

Neinhuis C, Barthlott W. 1998. Seasonal changes of leaf surface contamination in beech, oak and ginkgo in relation to leaf micromorphology and wettability. *New Phytologist* **138:** 91-98.

Nicholson RL, Yoshioka H, Yamaoka N, Kunoh H. 1988. Preparation of the infection court by *Erysiphe graminis*. II. Release of esterase enzyme from conidia in response to a contact stimulus. *Experimental Mycology* **12:** 336-349.

Nicholson RL, Kunoh H, Shiraishi T, Yamada T. 1993. Initiation of the infection process by *Erysiphe graminis*: Conversion of the conidial surface from hydrophobicity to hydrophilicity and influence of the conidial exudates on the hydrophobicity of the barley leaf surface. *Physiology and Molecular Plant Pathology* **43:** 307–318.

Ohlrogge JB, Jaworski JG, Post-Beittenmiller D. 1993. *De novo* fatty acid biosynthesis. In: *Lipid Metabolism in Plants* (ed. Moore TS), CRC Press, Boca Raton, pp. 3-32

Ohlrogge JB, Browse J. 1995. Lipid biosynthesis. *The Plant Cell* **7:** 957-970**.**

Ozturk ZN, Talamé V, Deyholos M, *et al.* 2002. Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. *Plant Molecular Biology* **48:** 551-573.

Park MH *et al.* 1993. Hypusine: its post-translational formation in eukaryotic initiation factor 5A and its potential role in cellular regulation. *Biofactors* **4:** 95-104.

Pighin JA, Zheng H, Balakshin LJ *et al.* 2004. Plant cuticular lipid export requires an ABC transporter. *Science* **306:** 702-704.

Pohl A, Devaux PF, Herrmann A. 2005. Function of prokaryotic and eukaryotic ABC proteins in lipid transport. *Biochemica et Biophysica Acta* **1733:** 29-52.

Post-Beittenmiller D. 1996. Biochemistry and molecular biology of wax production in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*. **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. *Journal of Experimental Botany* **42:** 167-171.

Pruitt RE, Vielle-Calzada J-P, Ploense SE, Grossniklaus U, Lolle SJ. 2000. *FIDDLEHEAD*, a gene required to suppress epidermal cell interactions in *Arabidopsis*, encodes a putative lipid biosynthetic enzyme**.** *Proceedings of the National Academy of Science* **97(3):** 1311-1316.

Pyee J, Yu H, Kollatukudy PE. 1994. Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (*Brassica oleracea*) leaves. *Archives of Biochemistry and Biophysics* **311:** 460-468.

Ramagopal S. 1987. Differential mRNA transcription during salinity stress in barley. *Proceedings of the National Academy of Science USA* **84:** 94-98.

Rashotte AM, Jenks MA, Ross AS, Feldmann KA. 2004. Novel eceriferum mutants in Arabidopsis thaliana. *Planta* **219:** 5-13.

Rea PA, Li Z-S, Lu Y-P, Drosdowicz YM. 1998. From vacuolar GS-X pumps to multispecific ABC transporters. *Annual Review of Plant Physiology and Plant Molecular Biology* **49:** 727-760.

Reisige K, Gorzelanny C, Daniels U, Moerschbacher BM. 2006. The C28 aldehyde octacosanal is a morphogenetically active component involved in host plant recognition and infection structure differentiation in the wheat stem rust fungus. *Physiological and Molecular Plant Pathology* **68**: 33–40.

Rhee Y, Hlousek-Radojcic A, Ponsamuel J, Liu D, Post-Beittenmiller D. 1998. Epicuticular wax accumulation and fatty acid elongation activities are induced during leaf development of leeks. *Plant Physiology* **116:** 901–911.

Richardson A, Franke R, Kerstiens G, Jarvis M, Schreiber L, Fricke W. 2005. Cuticular wax deposition in growing barley (*Hordeum vulgare*) leaves commences in relation to the point of emergence of epidermal cells from the sheaths of older leaves. *Planta* **222**: 472–483.

Riedel M, Eichner A, Jetter R. 2003. Slippery surfaces of carnivorous plants: composition of epicuticular wax crystals in *Nepenthes alata* Blanco pitchers. *Planta* **218(1)**: 87-97.

Riederer M, Schreiber L. 1995. Waxes – The transport barriers of plant cuticles. In: *Waxes: Chemistry, Molecular Biology and Functions* (ed. Hamilton RJ) The Oily Press, West Ferry, pp. 131–156.

Riederer M, Schreiber L. 2001. Protecting against water loss: analysis of the barrier properties of plant cuticles. *Journal of Experimental Botany* **52:** 2023–2032.

Riederer M, Müller C. 2005. *Biology of the plant cuticle*. (eds Riederer M, Müller C), Blackwell Publishing, Oxford U.K. pp 181-215.

Rogers DP, Bankaitis VA. 2000. Phospholipid transfer proteins and physiological functions. *International Review of Cytology* **197:** 35–81.

Rubiales D, Ramirez MC, Carver TLW, Niks RE. 2001. Abnormal germling development by brown rust and powdery mildew on *cer* barley mutants. *Hereditas* **135**: 271–276.

Sase H, Takamatsu T, Yoshida T. 1998. Variation in amount and elemental composition of epicuticular wax in Japanese cedar (Cryptomeria japonica) leaves associated with natural environmental factors. *Canadian Journal of Forest Research* **28:** 87-97.

Schulze-Lefert P, Panstruga R. 2003. Establishment of biotrophy by parasitic fungi and reprogramming of host cells for disease resistance. *Annual Review of Phytopathology* **41:** 641-667.

Shepherd T, Robertson GW, Griffiths DW, Birch ANE, Duncan G. 1995. Effects of environment on the composition of epicuticular wax from kale and swede. *Phytochemistry* **40:** 407–417.

Shepherd T, Griffiths DW. 2006. The effects of stress on plant cuticular waxes. *New Phytologist* **171:** 469-499.

Seki M, Narusaka M, Abe H *et al.* 2001. Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. *The Plant Cell* **13:** 61-72.

Seki M, Ishida J, Narusaka M, *et al.* 2002a. Monitoring the expression pattern of ca. 7000 *Arabidopsis* genes under ABA treatments using a full-length cDNA microarray. *Functional and Integrative Genomics* **2: 282-291.**

Seki M, Narusaka M, Ishida J, *et al.* 2002b. Monitoring the expression profiles of 7000 Arabidopsis genes under drought cold and high-salinity stresses using a full-length cDNA microarray. *The Plant Journal* **31(3):** 279-292.

Shinozaki K, Yamaguchi-Shinozaki K, Seki M. 2003. Regulatory network of gene expression in the drought and cold stress responses. *Current Opinion in Plant Biology* **6:** 410-417.

Singh KB, Foley RC, Oňate-Sánchez L. 2002. Transcription factors in plant defense and stress responses. *Current Opinion in Plant Biology* **5:** 430-436.

Stangl M. 2005. Die Rolle epikutikulärer Wachse bei der Pilz-Pflanzen-Interaktion von Gerstenmehltau (*Erysiphe graminis*) und Gerste (*Hordeum vulgare*). Zulassungsarbeit, Würzburg

Stark RE, Yan B, Ray AK, Chen Z, Fang X, Garbow JR. 2000. NMR studies of structure and dynamics in fruit cuticle polyesters. *Solid State NMR* **16:** 37-45.

Steinmüller D, Tevini M. 1985. Action of ultraviolet radiation (UV-B) upon cuticular waxes in some crop plants. *Planta* **164:** 557-564.

Sterk P, Booij H, Schellekens GA, Van Kammen A, De Vries SC. 1991. Cell-specific expression of the carrot EP2 lipid transfer protein gene. *The Plant Cell* **3:** 907-921.

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 Physiology* **137:** 478-489.

Talamé V, Ozturk NZ, Bohnert HJ, Tuberosa R. 2006. Barley transcript profiles under dehydration shock and drought stress treatments: a comparative analysis. *Journal of Experimental Botany* **58(2):** 229-240.

Tian S. 2005. Molecular structures of natural polymers: cutin, suberin, and melanin. Ph.D. Dissertation, City University of New York.

Thoma S, Kaneko Y, Somerville C. 1993. A non-specific lipid transfer protein from *Arabidopsis* is a cell wall protein. *The Plant Journal* **3:** 427–436.

Thoma S, Hecht U, Kippers A, Botella J, De Vries S, Somerville C. 1994. Tissue specific expression of a gene encoding a cell wall localized lipid transfer protein from *Arabidopsis*. *Plant Physiology* **105:** 35-45.

Thompson JE, Hopkins MT, Taylor C, Wang T-W. 2004. Regulation of senescence by eukaryotic translation initiation factor 5A: implications for plant growth and development. *Trends in Plant Science* **9(4):** 174-179.

Thum KE, Kim M, Morishige DT, Eibl C, Koop HU, Mullet JE. 2001. Analysis of barley chloroplast psbD light-responsive promoter elements in transplastomic tobacco. *Plant Molecular Biology* **47(3):** 353-366.

Tsuba M, Katagiri C, Takeuchi Y, Takada Y, Yamaoka N. 2002. Chemical factors of the leaf surface involved in the morphogenesis of *Blumeria graminis*. *Physiology and Molecular Plant Pathology* **60**: 51–57.

Uchiyama T, Tanaka H, Ogasawara N, Amano K. 1989. Electron microscopic observations of powdery mildew fungi (*Erysiphe graminis* f.sp. *hordei*) on the barley leaf surface and changes in the wax composition of infected leaves. *Nippon Nögeikagaku Kaishi* **63(11):** 1771-1774.

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 ß–ketoacyl–CoA synthase. *Journal of Experimental Botany* **55**: 1401–1410.

von Wettstein–Knowles P. 1971. The molecular phenotypes of the *eceriferum* mutants. In: *Barley Genetics.* 2nd Intl Barley Genetics Symp (1969), (ed. Nilan RA), II Proc., Pullman, USA, Washington State Universal Press, pp. 146–193.

von Wettstein–Knowles P. 1974. Gene mutation in barley inhibiting the production and use of C_{26} chains in epicuticular wax formation. *FEBS Letters* **42**: 187–191.

von Wettstein–Knowles P, Avato P, Mikkelsen JD. 1980. Light promotes synthesis of the very long fatty acyl chains in maize wax. In: *Biogenesis and Function of Plant Lipids*. (eds. Mazliak P, Benveniste C, Douce R, Douce C), Elsevier/ North-Holland Biomedical Press, pp. 271-274.

von Wettstein-Knowles PM. 1982. Elongase and epicuticular wax biosynthesis, *Physiologie Végétale* **20:** 797–809.

Walia H, Wilson C, Wahid A, Condamine P, Cui X, Close TJ. 2006. Expression analysis of barley (*Hordeum vulgare* L.) during salinity stress. *Functional and Integrative Genomics* **6(2):** 143-56.

Walia H, Wilson C, Condamine P, Liu X, Ismail AM, Close TJ. 2007a. Large-scale expression profiling and physiological characterization of jasmonic acid-mediated adaptation of barley to salinity stress. *Plant, Cell and Environment* **30:** 410-421.

Walters DR. 2006. Disguising the leaf surface: the use of leaf coatings for plant disease control. *European Journal of Plant Pathology* **114:** 255-260.

Walther-Larsen H, Brandt J, Collinge DB, Thordal-Christensen H. 1993. A pathogen-induced gene of barley encodes a HSP90 homologue showing striking similarity to vertebrate forms resident in the endoplasmic reticulum. *Plant Molecular Biology* **21-** 1097-1108**.**

Whitecross MI, Armstrong DJ. 1972. Environmental effects on epicuticular waxes of *Brassica napus* L.. *Australian Journal of Botany* **20:** 87–95.

Wu W-Y, Moreau RA, Stumpf PK. 1981. Studies of biosynthesis of waxes by developing jojoba seed. III. Biosynthesis of wax esters from acyl-CoA and long chain alcohols. *Lipids* **6:** 897-902.

Wu F-B, Chen F, Kang W, Zhang G-P. 2004. Effect of cadmium on free amino acid, glutathione and ascorbic acid concentrations in two barley genotypes (*Hordeum vulgare* L.) differing in cadmium tolerance. *Chemosphere* **57:** 447-454.

Xu HI, Gauthier L, Gosselin A. 1995. Stomatal and cuticular transpiration of greenhouse tomato plants in response to high solution electrical conductivity and low soil water content. *Journal of American Society for horticultural Science* **120(3):** 417-122.

Xu X, Dietrich CR, Lessire R, Nikolau BJ, Schnable PS. 2002. The endoplasmic reticulumassociated maize gl8 protein is a component of the acyl-CoA elongase involved in the production of cuticular waxes. *Plant Physiology* **128:** 924–934.

Yamamoto Y, Kobayashi Y, Devi SR, Rikiishi S, Matsumoto H. 2002. Aluminium toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. *Plant Physiology* **128:** 63-72.

Yang SL, Ellingboe AH. 1972. Cuticle layer as a determining factor for the formation of mature appressoria of *Erysiphe graminis* on wheat and barley. *Phytopathology* **62**: 708–714.

Zeyen RJ, Carver TLW, Lyngkjær MF. 2002. The papilla response. In: *The Powdery Mildews: A Comprehensive Treatise* (eds. Bélanger RR, Dik AJ, Bushnell WR, Carver TLW), APS Press, Minnesota, pp. 107-125.

Zheng H, Rowland O, Kunst L. 2005. Disruptions of the *Arabidopsis* enoyl-CoA reductase gene reveal an essential role for very-long-chain fatty acid synthesis in cell expansion during plant morphogenesis. *The Plant Cell* **17:** 1467–1481.

SUMMARY

In order to test the effects of environmental factors on different characteristics of plant leaf waxes, barley plants (*Hordeum vulgare*) were abiotically stress treated (exposure to darkness, heavy metal, high salt concentrations and drought), and biotically stressed by the infection with powdery mildew (*Blumeria graminis* f.sp. *hordei*; *Bgh*). Different wax parameters like amount, chemical composition, and micromorphology of epicuticular wax crystals, were investigated. Etiolated leaves of barley showed distinctly reduced wax amounts and modifications in their relative composition. The alterations of these wax parameters might be a result of a developmental delay, which could have been caused by a decreased availability of energy for cellular processes, due to lack of light. Cadmium exposure led to a 1.5-fold increase of wax amount, while chemical composition was unaffected. In drought- and salt-stressed plants, all investigated leaf wax parameters remained unaltered. In each of the abiotic treatments, the microstructure of epicuticular wax crystals, formed as typical platelets, was not modified. Even after 6d infection with powdery mildew (*Bgh*), neither locally nor systemically enforced modifications of wax features were revealed.

The analyzed leave surfaces, resulting from these four abiotic and the biotic treatment (phenotypic approach), were compared to altered leaf surfaces' characteristics of 18 analyzed eceriferum (*cer*-) wax mutants (genotypic approach). Within the screening, 5 mutants were selected which distinctly differed from the wild-type in wax amount, portions of epi- and intracuticular wax fraction, relative chemical composition, crystal morphology, and surface wettability (hydrophobicity).

Apart from quantitative and qualitative effects on the leaf waxes, environmentally enforced modifications in cuticular waxes might be reflected in molecular processes of wax biogenesis. Therefore, a barley wax-microarray was established. 254 genes were selected, which are putatively involved in processes of *de novo* fatty acid biosynthesis, fatty acid elongation, and modification, and which are supposed to take part in lipid-trafficking between cell compartments, and transport of wax components to the outer cell surface. The regulations within the expression pattern evoked by the respective treatments were correlated with the corresponding analytical wax data, and the observed molecular effects of a 3d powdery mildew infection were compared with succeeding fungal morphogenesis. Etiolation and cadmium exposition pointed to transcriptional modifications in the *de novo* fatty acid synthesis, and in the screened, transport-related mechanisms, which correlate with respective alterations in surface wax characteristics. Moderate changes in the gene expression pattern, evoked by drought- and salinity-stress, might give hints for evolved adaptations in barley to such common habitat stresses. The

invasion of powdery mildew into the epidermal host cells was reflected in the regulation of several genes. Beside other functions, these genes take part in pathogen defense, and intracellular component transport, or they encode transcription factors. The different modifications within the molecular responses evoked by the investigated abiotic treatments, and the effects of powdery mildew infection representing a biotic stressor, were compared between the different treatments.

In order to test the potential impact of different wax parameters on *Bgh*, conidia germination and differentiation was comparably investigated on leaf surfaces of abiotically stressed wild-type and *cer*-mutants, isolated cuticles, and further artificial surfaces. The rates of conidial development were similar on each of the leaf surfaces resulting from the abiotic treatments, while a significant reduction of the germination and differentiation success was revealed for the wax mutant *cer*-yp.949. Compared to the wild-type, developmental rates on isolated cuticles and extracted leaf waxes of the mutant *cer*-yp.949 indicated a modified embedding of cuticular waxes, and a possibly changed three-dimensional structure of the *cer*-yp.949 cuticle, which might explain the reduced conidial developmental rates on leaf surfaces of this particular mutant.

Experiments with *Bgh conidia* on mechanically de-waxed leaf surfaces (selective mechanical removal of the epicuticular leaf waxes with glue-like *gum arabic*, followed by an extraction of the intracuticular wax portion with chloroform) demonstrated the importance of the wax coverage for the germination and differentiation of the fungal conidia. On all dewaxed leaf surfaces, except those of *cer*-yp.949, the differentiation success of the germlings was significantly reduced, by about 20% (“wax-effect”). This result was verified through an artificial system with increased conidia developmental rates on glass slides covered with extracted leaf waxes. Further comparative tests with the major components of barley leaf wax, hexacosanol and hexacosanal, showed that the germination and differentiation of powdery mildew conidia not only depends on the different chemistry, but is also influenced by the respective surface hydrophobicity. Compared to hexacosanol, on hexacosanal coated glass surfaces, higher germination and differentiation rates were achieved, which correlated with increased levels of surface hydrophobicity. Developmental rates of conidia on hydrophobic foils demonstrated that hydrophobicity, as a sole surface factor, may stimulate the conidial germination and differentiation processes. Moreover, the survival of conidia on artificial surfaces is determined by additional surface derived factors, e.g. the availability of water, and a pervadable matrix.

ZUSAMMENFASSUNG

Abiotische und biotische Umweltfaktoren können sowohl die Struktur als auch die chemischen Eigenschaften pflanzlicher Oberflächenwachse beeinflussen. In der vorliegenden Studie wurde vergleichend untersucht, inwiefern sich verschiedene Parameter von Gersten (*Hordeum vulgare*) Blattwachsen, wie deren Menge, chemische Zusammensetzung und die Morphologie epikutikulärer Wachskristalle unter dem Einfluss unterschiedlicher abiotischer Stressoren (Wachstum in Dunkelheit, Schwermetallbelastung, erhöhte Salzkonzentrationen, Trockenheit) und eines biotischen Stressors, dem Befall von Gerstenmehltau (*Blumeria graminis* fsp. *hordei; Bgh*), verändern können. Die Aufzucht ohne natürliches Licht führte zu etiolierten Blättern, die deutlich reduzierte Wachsmengen und quantitative Veränderungen in der Zusammensetzung einzelner Wachskomponenten zeigten. Die Veränderung dieser Wachscharakteristika könnte das Resultat einer pflanzlichen Entwicklungsverzögerung darstellen, die auf den Mangel an Licht, und damit einem Mangel an Energie für die zelluläre Triebkraft zurückzuführen ist. Cadmium-Belastung führte zu einer 1.5-fach erhöhten Wachsauflage der Versuchspflanzen bei unveränderter Chemie. Unter Trocken- und Salzstress zeigten sich keine Veränderungen der untersuchten Wachsparameter. Die plättchenförmige Kristallstruktur der epikutikulären Wachse blieb in jeder der untersuchten abiotischen Behandlungen unverändert.

Um Auswirkungen von biotischem Stress auf die Wachsauflage zu testen, wurde eine Infektion mit Gerstenmehltau (*Bgh*) vorgenommen. Nach sechstägiger Pilzinfektion konnten hierbei weder lokale, noch systemische Veränderungen der unterschiedlichen Wachsparameter der Gerstenblätter detektiert werden. Zusätzlich zu solchen Blattoberflächen, die aus den vier abiotischen und der biotischen Behandlung resultierten (phänotypischer Ansatz), wurden die Oberflächenwachse von 18 *cer*-Wachsmutanten untersucht (genotypischer Ansatz). Hieraus wurden diejenigen fünf Mutanten ausgewählt, die die stärksten Abweichungen an Wachsmengen, Mengenverteilung zwischen epi- und intrakutikulärer Wachsfraktion und/ oder Anteilen der Einzelkomponenten, der Morphologie der epikutikulären Wachskristalle, und somit auch in der davon abhängenden Oberflächenbenetzbarkeit (Hydrophobizität) gegenüber dem Wildtyp aufwiesen.

Um zu überprüfen, inwiefern sich die Modifizierung der Oberflächenwachse durch Umweltfaktoren in den zugrunde liegenden molekularen Prozessen der Wachsbiosynthese widerspiegelt, wurde ein Gerstenwachs-Microarray etabliert. Eine Auswahl von 254 Genen wurde zusammengestellt, denen potentiell Funktionen in der Fettsäurebiosynthese, Kettenverlängerung von Fettsäuren und deren Modifikation zu Wachskomponenten, sowie im Transport von Lipid- und Wachskomponenten zwischen den Zellkompartimenten zukommen. Die Veränderung der Expression einzelner Gene während der abiotischen Stress-Behandlungen wurde mit den Daten der Wachsanalyse dieser Behandlungen korreliert, sowie der Einfluss einer dreitägigen Mehltauinfektion auf molekulare Prozesse der Wirtspflanze mit der Pilzmorphogenese kombiniert. Hinweise auf

transkriptionelle Veränderungen in der *de novo* Fettsäuresynthese und den untersuchten Transportmechanismen, die mit den Veränderungen der Oberflächenwachse korrelieren, waren insbesondere unter Cadmiumstress und durch Etiolierung zu verzeichnen. Die durch Trocken- und Salzstress verursachten, moderaten Veränderungen im Expressionsmuster untersuchter Gene könnten auf eine erworbene Toleranz von Gerste gegenüber solcher Art von Umweltstress hinweisen. Das Eindringen des Pilzes in die Epidermis spiegelte sich in zahlreichen Genregulierungen wider, die besonders der Pathogenabwehr und dem intrazellulären Komponententransport dienen, oder Transkriptionsfaktoren codieren. Der Einfluss der untersuchten abiotischen Faktoren und der Pilzbefall als biotischer Stressor lösten unterschiedliche Modifikationen der molekularen Antworten aus, die miteinander verglichen wurden.

Um zu klären welche Wachsparameter die Auskeimung und Differenzierung der Konidien von *B. graminis* beeinflussen, wurden alle analysierten Blattoberflächen (abiotisch gestresster Wildtyp und *cer*-Mutanten) und weitere artifizielle Oberflächen in Biotests eingesetzt. Auf allen Blattoberflächen des abiotisch behandelten Wildtyps war die Konidienentwicklung gleichermaßen erfolgreich, während sich innerhalb des genotypischen Ansatzes eine signifikante Reduktion des Keimungs- und Differenzierungserfolgs auf Blättern der Mutante *cer*-yp.949 zeigte. Durch vergleichende Untersuchungen mit isolierten Kutikeln und extrahierten Blattwachsen von Wildtyp und der Mutante *cer*-yp.949 konnte gezeigt werden, dass der herabgesetzte Entwicklungserfolg der Konidien auf Blättern der Mutante *cer*-yp.949 auf eine modifizierte Einbettung der Wachse und eine veränderte dreidimensionale Struktur der *cer*-yp.949 Kutikula zurückzuführen sein könnte. Untersuchungen zur Entwicklung von Konidien auf sukzessiv entwachsten Blattoberflächen von Wildtyp und Mutanten (zunächst mechanisches Abheben der epikutikulären Wachskristalle durch *Gummi arabicum*, gefolgt von einer Extraktion der intrakutikulären Wachse mit Chloroform) zeigte die Bedeutung des Vorhandenseins von Wachs für die Auskeimung und Differenzierung der Konidien. Mit Ausnahme der Mutante *cer*-yp.949 wurde der Differenzierungserfolg der Konidien auf allen Blattflächen durch Wachsextraktion signifikant um ca. 20% reduziert („Wachseffekt“).

Durch die Beschichtung von Glasobjektträgern mit extrahierten Blattwachsen konnte dieses Ergebnis auf ein artifizielles System übertragen und bestätigt werden. Zusätzliche Tests mit den Hauptkomponenten der Gerstenwachse Hexacosanol und Hexacosanal zeigten, dass Auskeimung und Differenzierung von Mehltau-Konidien von der unterschiedlichen Chemie, und auch von der Hydrophobizität der Oberfläche beeinflusst werden. Im Vergleich mit Hexacosanol zeigten Hexacosanal beschichtete Glasoberflächen sowohl den größten Keimungs- und Differenzierungserfolg, als auch die höchste Hydrophobizität. Entwicklungsraten auf hydrophoben Folien bestätigten, dass allein die Hydrophobizität der Oberfläche die Auskeimung und die Differenzierung der Konidien stimulieren kann. Das Überleben der Konidien auf artifiziellen Oberflächen wird darüber hinaus durch weitere Faktoren wie Wasserverfügbarkeit und eine durchdringbare Matrix bestimmt.

DANKSAGUNG

Ich danke Prof. Markus Riederer für die Bereitstellung eines außerordentlich gut ausgestatteten Arbeitsplatzes, für seine Unterstützung in der Absicherung meiner Zeit am Institut und seine motivierende Aufgeschlossenheit meiner Arbeit gegenüber. Für die Finanzierung dieser Arbeit danke ich dem SFB 567.

Dr. Gerd Vogg gilt mein besonderer Dank für seine optimistische und offene Art, die für viel Motivation und Arbeitseifer innerhalb der Arbeitsgruppe gesorgt hat.

Meinen Dank auch an Dr. Ulrich Hildebrandt, der nach dem Personalwechsel für die Betreuung des Projektes zuständig war.

Den drei guten Seelen des Instituts Michaela Jäger, Wilma Kreßmann und Monika Noak herzlichen Dank. Wilma vor allem für die „blitz“-schnelle Hilfe in Sachen digitaler Fotografie und lebensnotwendiger Literatur, sowie Moni in sämtlichen organisatorischen Dingen. Vor allem durch ihr ausgeglichenes Wesen, haben sie mich alle durch steinige Täler dieser Arbeit geführt.

Jutta Winkler-Steinbeck danke ich für die aufopfernde und behutsame Handhabung meiner Pflanzen und für die beharrliche Geduld im Umgang mit anhänglichen Pilzsporen.

Danke auch an die Zulassungskandidatin Michaela Stangl, die studentischen Hilfskräfte Werner Pfaff und Mirjam Schelter, sowie die Auszubildende Jessica Dick, die in den jeweiligen Phasen des Projektes für eine konstante Datenflut gesorgt haben.

Großen Dank and Lena Schuster, die allem voran mit besonderem Biss und außerordentlicher Ausdauer für die saubere Etablierung der molekularen Methoden gesorgt hat. Von ihrer stets sorgfältigen und höchst verantwortungsbewussten Arbeitsweise im Labor hat die gesamte Arbeitsgruppe über alle Maßen profitiert.

Aufrichtigen Dank richte ich an meine Zimmergenoss(inn)en: Jana Leide, Dr. Kerstin Reifenrath, Dr. Christian Popp, Nora Travers-Martin und Christian Kinzler, die u.a. im Alltag, sowie auf Kongressen für großen Zusammenhalt und eine belustigende Landschulheim-Atmosphäre gesorgt haben. Vor allem Jana hat mir auf der langen Durststrecke zum Ziel immer loyal zur Seite gestanden und ist mir (nicht nur) deshalb sehr ans Herz gewachsen. Über ihre Statistikkenntnisse und Korrekturarbeiten hinaus, bin ich Kerstin besonders für ihre objektive Sichtweise dankbar, die mich bei nervlichen Instabilitäten stets erfolgreich zurecht gerückt hat. Die Zeit in Würzburg wird besonders durch sie alle unvergesslich bleiben, ihn ihnen habe ich (hoffentlich immerwährende) gute Freunde gefunden.

Meinen Dank auch an Natascha Sieling und viele weitere Mitarbeiter des Institutes für Botanik II. Ich habe fachlich und menschlich viel von ihnen allen gelernt.

Ulrike Gruhn möchte ich nicht nur für ihr Korrekturarbeiten meinen Dank aussprechen. Ihr Vertrauen in meine Fähigkeiten, haben mir über alle Stadien hinweg geholfen, das Ziel und mich selbst nicht aus den Augen zu verlieren.

Aus tiefstem Herzen Dankeschön an meinen Mann Franz Zabka, der nach dieser Zeit immer noch mit mir verheiratet sein will. Er hat stets an mich geglaubt, seine tröstende Schulter zum Halt geboten und mir für die wesentlichen Dinge im Leben die Augen geöffnet.

Ein inniges Dankeschön meiner ganzen Familie, besonders meinen Eltern und Großeltern, die bestimmt bis heute nicht verstehen können, warum ich diesen Weg gewählt habe, aber trotzdem immer hinter mir standen und meine Begeisterung teilten.

ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und dabei keine anderen als die hier angegebenen Quellen und Hilfsmittel verwendet habe.

Ferner erkläre ich, dass ich diese Arbeit weder einer anderen Prüfungsbehörde vorgelegt, noch anderweitig mit oder ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.
Ich erkläre, dass ich bisher keine akademischen Grade erworben oder zu erwerben versucht habe.

Würzburg, den 30.10.07

Vanessa Zabka

CURRICULUM VITAE

Vanessa Zabka geborene Gartmann am 24. April 1977 in Wuppertal

- *seit Februar 2004*

Dissertation im Institut für Botanik II, AG Dr. Hildebrandt an der Julius Maximilians-Universität Würzburg

- *Oktober 2003- Januar 2004*

Folgevertrag als Aushilfskraft in der Abteilung PMQ Biology, Hoffmann La Roche AG, Grenzach/ Wyhlen

- *August 2003- Oktober 2003*

Forschungsreise in Südafrika zur Taxonomie von Schwarzkäferarten (Tenebrionidae) geleitet durch Dr. Sven Geiselhardt

- *April 2003- August 2003*

Aushilfskraft in der Abteilung PMQ Biology, Hoffmann La Roche AG, Grenzach/ Wyhlen

- *Februar 2003*

Abschluss des Diploms an der Albert-Ludwigs-Universität Freiburg

- *Mai 2002 - Januar 2003*

Diplomarbeit: „Kutikulare Kohlenwasserstoffe bei *Aleochara*-Arten (Coleoptera, *Staphylinidae*)“ betreut durch Prof. Dr. Klaus Peschke, Institut für Biologie I (Zoologie) an der Albert-Ludwigs-Universität Freiburg

- *Oktober 1999- April 2002*

Hauptstudium Biologie/ Diplom an der Albert-Ludwigs-Universität Freiburg

- *Juni 2002 – Dezember 2002*

Wissenschaftliche Hilfskraft bei Prof Dr. Ralf Reski, Lehrstuhl für Pflanzenbiotechnologie, Institut für Biologie II (Botanik), Albert-Ludwigs-Universität Freiburg

- *April 2001-April 2003*

Assistenz zur Zoologischen Exkursionen und Bestimmungsübungen bei Dr. Odwin Hoffrichter, Institut für Biologie I (Zoologie), Albert-Ludwigs-Universität Freiburg

- *April 2000-April 2003*

Kursassistenz im biologischen Grundpraktikum 1B (Wirbellose), Institut für Biologie I (Zoologie), Albert-Ludwigs-Universität Freiburg

- *Oktober 2000- Dezember 2000*

Projekt zur Untersuchung von potentiellen Pheromonen bei *Aleochara bilineata* (Coleoptera, Staphylinidae) bei Prof. Dr. Klaus Peschke, Institut für Biologie I (Zoologie), Albert-Ludwigs-Universität Freiburg

- *Oktober 1999- Februar 2000*

Wissenschafltiche Hilfskraft, Institut für Biologie II (Botanik), Albert-Ludwigs-Universität Freiburg

- *April 1999*

Diplom-Vorprüfung, Johannes-Gutenberg-Universität Mainz

- *Oktober 1997- Oktober 1999*

Grundstudium Biologie/ Diplom, Johannes-Gutenberg-Universität Mainz

- *September 1988- Juni 1996* Abitur, Hildegardisgymnasium Bingen am Rhein

- *August 1987- August 1988*, Leibniz-Gymnasium Wiesbaden

PUBLIKATIONEN

Zabka V, Stangl M, Bringmann G, Vogg G, Riederer M, Hildebrandt U. 2007. Host surface properties affect pre–penetration processes in the barley powdery mildew fungus. *New Phytologist* . DOI:10.1111/j1469-8137.2007.02233.x

Gniwotta F, Vogg G, Gartmann V, Carver TLW, Riederer M, Jetter R. 2005. What do microbes encounter at the plant surface? Chemical composition of pea leaf cuticular waxes. *Plant Physiology* **139:** 519–531.

Ruf D, Zabka V, Hildebrandt U, Rostás M. 2008. Plant epicuticular wax affects host choice in herbivores and detection of „chemical footprints“ by parasitoids. In preparation.

TAGUNGS- UND LEHRBEITRÄGE

Gartmann V, Riederer M, Hildebrandt U, Vogg G. Relevance of cuticular wax properties for the interspecific interaction of powdery mildew with its host plant barley *Tagung des Sonderforschungsbereich 567 Mechanismen der interspezifischen Interaktion von Organismen*. Würzburg, Oktober 2005

Gartmann V, Stangl M, Riederer M, Hildebrandt U, Vogg G. Relevance of cuticular wax properties for the interspecific interaction of powdery mildew with its host plant barley. *International Botanical Congress*, Wien, Österreich, Juli 2005

Gartmann V, Gniwotta F, Vogg G, Riederer M. Plant epicuticular waxes and their function in an interspecific interaction with biotrophic fungi. *Botanikertagung,* Braunschweig, September 2004

Stangl M. 2005. Die Rolle epikutikulärer Wachse bei der Pilz-Pflanzen-Interaktion von Gerstenmehltau (*Erysiphe graminis*) und Gerste (*Hordeum vulgare*)