REGULATION AND FUNCTION OF EXTRACELLULAR INVERTASES OF TOMATO

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ABBREVIATIONS

ABA	abscisic acid
ASA	acetyl salicylic acid
BRs	brassinosteroids
CBS	CCA1 binding site
CCA1	Circadian Clock Associated 1
2,4-D	2,4-dichlorophenoxyacetic acid
EE	Evening Element
E-FOL	elicitor preparation of the necrotrophic fungus *Fusarium oxysporum lycopersici*
Fru	fructose
GA	gibberellic acid
Glc	glucose
GUS	β-glucuronidase
IAA	indoleacetic acid
ipt	isopentenyltransferase
JA	jasmonic acid
LHY	Late Elongated Hypocotyl
MAP	mitogen-activated protein
MeJA	methyl jasmonate
MU	4-methylumbelliferone
ORF	open reading frame
PAL	phenylalanine ammonia-lyase
PGA	polygalacturonic acid
PR	pathogenesis related
RNAi	RNA interference
SA	salicylic acid
SAG	senescence activated gene
Suc	sucrose
TE	transposable element
TIR	terminal inverted repeat
TPase	transposase
QTL	quantitative trait locus
UTR	un-translated region
WT	wild type

SUMMARY

Because of growth and development, plant tissues are characterised by a permanent change in source-sink relations. Tissues with a net carbohydrate export (source) or import (sink) have to adopt their actual demand for assimilates according to the developmental status. Furthermore, plants, as sessile life forms, have developed regulatory mechanisms that enable a flexible response of assimilate partitioning to specific requirements of the habitat, like biotic and abiotic stress factors and changing light conditions. The distribution of assimilates involves specific enzyme functions including sugar transporters and sucrose cleaving enzymes and is regulated by a variety of stimuli. Extracellular invertases cover an essential function in apoplastic phloem unloading and play an important role in regulating source-sink relations. This property is reflected by the occurrence of different invertase isoenzymes with specific expression and regulation patterns that enable a co-ordination of the carbohydrate metabolism in diverse tissues, at different developmental stages, and under varying environmental conditions. Improved knowledge of extracellular invertase function might allow altering growth, development or pathogen resistance of crop plants in a specific way.

The present study is aimed at elucidating the regulation patterns and functions of three members of the extracellular invertase gene family of tomato, *Lin5*, *Lin6,* and *Lin7*. Detailed promoter analysis revealed a tissue- and developmental-specific expression of isoenzymes and corresponding regulation patterns. *Lin5* shows a developmental regulated expression in fruits. *Lin6* is expressed in early developmental stages starting in germinating seeds; in grown up plants *Lin6* is solely expressed in pollen and upon wound-stimulation. *Lin7* is exclusively expressed in tapetum and pollen tissue. The hormonal regulation of all three isogenes was analysed in detail, whereby known GA- and JA-mediated flower phenotypes could be correlated with invertase functions. In addition, an important role of Lin7 invertase in pollen germination was demonstrated in a functional approach. This is the most profound analysis of extracellular invertases in the delicate process of floral organ development that includes three tomato isoenzymes. In particular, dissection of the individual roles of Lin5, Lin6, and Lin7 reveals novel insights in carbohydrate supply during flower and fruit development. The analysed tissue-specific promoters are profitable tools in plant biotechnology, which in particular applies to the pollen-specific *Lin7* promoter.

It has been demonstrated that the *Lin6* promoter serves as target for hormonal-, sugar-, and wound-mediated signalling pathways. Moreover, a functional interaction of circadian oscillator elements of *A. thaliana* with the *Lin6* promoter and a diurnal rhythm of

Lin6 expression have been substantiated. This complex regulation pattern is reflected by the identification of many well-defined *cis*-acting elements within the *Lin6* promoter. This feature supports an integration of various stimuli mediated via extracellular invertase expression resulting in a co-ordinated cellular response to changing internal and external conditions. As sugars on their part induce *Lin6* expression, this could result in signal amplification via a positive feedback loop. Furthermore, the extensive appearance and constellation of *cis*-acting elements within the *Lin6* promoter provides the basis to answer questions in signal cross-talk and signal integration in plant gene expression.

In addition, the *Lin6* promoter was successfully used as an inducible expression system. In transgenic tobacco lines an invertase inhibitor was expressed under control of the cytokinin-inducible *Lin6* promoter. Thereby, a causal relationship between cytokinin and extracellular invertase for the delay of senescence was demonstrated. This study emphasises the importance of inducible expression systems to address specific questions on a molecular basis.

The above-mentioned promoter sequences were obtained via sequential genome walks. Hereby two interesting structural features appeared. First, *Lin5* and *Lin7* genes are arranged in a direct tandem repeat on the genome. Second, a CACTA-like transposon insertion in intron I of the *Lin5* gene was revealed. A primer pair deduced from the transposase region of this transposon allowed the amplification of similar sequences of various Solanaceae species.

ZUSAMMENFASSUNG

Wachstum und Entwicklung pflanzlicher Gewebe bedingen eine fortwährende Veränderung von Source-Sink Beziehungen. Gewebe mit einem Nettoexport (Source) oder -import (Sink) von Kohlenhydraten müssen ihren aktuellen Bedarf an Assimilaten entsprechend dem Entwicklungsstadium anpassen. Darüber hinaus haben Pflanzen als ortsgebundene Lebewesen Regulationsmechanismen entwickelt, die eine flexible Antwort der Assimilatverteilung auf spezielle Anforderungen des Habitats, wie biotische oder abiotische Stressfaktoren und wechselnde Lichtbedingungen, ermöglichen. Die Assimilatverteilung ist vielfältig reguliert und erfordert spezifische Enzymfunktionen, wie Zuckertransporter und saccharosespaltende Enzyme. Extrazelluläre Invertasen nehmen eine essentielle Funktion in der apoplastischen Phloementladung und in der Regulation von Source-Sink Übergängen ein. Dies spiegelt sich in dem Auftreten verschiedener Invertase-Isoenzyme mit speziellen Expressions- und Regulationsmustern wider, welche eine Koordination des Kohlenhydratmetabolismus in unterschiedlichen Geweben, zu unterschiedlichen Entwicklungsstufen und unter sich ändernden Umweltbedingungen ermöglichen. Ein detailliertes Wissen über die Funktion extrazellulärer Invertasen könnte eingesetzt werden, um Wachstum, Entwicklung oder Pathogenresisitenz von Nutzpflanzen gezielt zu verändern.

In der vorliegenden Studie wurden die Regulationsmuster und die Funktion dreier extrazellulärer Invertasen aus Tomate, Lin5, Lin6 und Lin7 untersucht. Durch umfangreiche Promotorstudien konnte eine gewebe- und entwicklungsspezifische Expression dieser Isoenzyme und entsprechende Regulationsmuster offengelegt werden. *Lin5* zeigt eine entwicklungsabhängige Expression in Früchten. *Lin6* wird in frühen Entwicklungsstadien, beginnend mit der Samenkeimung, exprimiert; in ausgewachsenen Pflanzen ist eine *Lin6* Expression nur in Pollen oder nach Verwundungsinduktion nachweisbar. *Lin7* wird ausschließlich in Tapetum-Gewebe und Pollen exprimiert. Die hormonelle Regulation der Isogene wurde im Detail untersucht, hierbei konnten bekannte Phänotypen, welche durch Gibberellinsäure und Jasmonate bedingt werden, mit Invertasefunktionen in Korrelation gebracht werden. Darüber hinaus konnte in einem funktionalen Ansatz gezeigt werden, dass *Lin7* eine wichtige Rolle in der Pollenkeimung zukommt. Die vorliegende Arbeit stellt die umfassendste Untersuchung extrazellulärer Invertasen während der Blütenentwicklung dar, an der drei Isoenzyme aus Tomate beteiligt sind. Dadurch, dass den einzelnen Invertasen Lin5, Lin6 und Lin7 individuelle Funktionen zugewiesen werden konnten, eröffnen sich neue Erkenntnisse über die Kohlenhydratversorgung während der Blüten- und Fruchtentwicklung.

Für die untersuchten gewebespezifischen Promotoren eröffnen sich zudem Anwendungsmöglichkeiten in der Biotechnologie, was insbesondere für den pollenspezifischen *Lin7* Promotor zutrifft.

Es konnte gezeigt werden, dass der *Lin6* Promotor das Ziel von hormon-, zucker- und verwundungsvermittelten Signalwegen ist. Darüber hinaus konnte nachgewiesen werden, dass Elemente des circadianen Oszillators von *A. thaliana* mit dem *Lin6* Promotor funktionell interagieren und die *Lin6* Expression einem diurnalen Rhythmus unterliegt. Dieses komplexe Regulationsmuster spiegelt sich in vielen *cis*-aktiven Elementen wider, die im *Lin6* Promotor vorgefunden wurden. Durch dieses Merkmal wird die These gestützt, dass verschiedene Stimuli über die extrazelluläre Invertase integriert werden und so eine koordinierte Zellantwort auf sich ändernde interne und externe Bedingungen ermöglicht wird. Nachdem Zuckermoleküle ihrerseits die Expression von *Lin6* induzieren, wird dadurch eine Amplifikation von Signalen über eine positive Rückkopplungsschleife ermöglicht. Die Vielzahl an *cis*-aktiven Elementen und deren Anordnung im *Lin6* Promotor stellen ein ideales Modellsystem dar, um Fragen in Bezug auf Signalinteraktion und -integration zu untersuchen.

In einer umfangreichen Studie wurde der *Lin6* Promotor erfolgreich als induzierbares Expressionssystem eingesetzt. Hierbei wurde ein Invertaseinhibitor unter der Kontrolle des cytokinininduzierbaren *Lin6* Promotors in transgenen Tabakpflanzen exprimiert. Mit diesem Ansatz ist es gelungen einen kausalen Zusammenhang zwischen dem Hormon Cytokinin und extrazellulären Invertasen in der Seneszenzverzögerung herzustellen. Diese Studie zeigt, dass induzierbare Expressionssysteme essentiell sind, um spezifische Fragestellungen auf molekularer Ebene klären zu können.

Bei der Klonierung obig genannter Promotorsequenzen haben sich zudem zwei interessante strukturelle Besonderheiten ergeben. Zum einen sind die Gene von *Lin5* und *Lin7* in einem Tandem auf dem Genom angeordnet, zum anderen konnte eine Transposoninsertion im Intron I des *Lin5* Gens gezeigt werden. Mit einem Primerpaar, das aus der Transposaseregion dieses Transposons abgeleitet wurde, konnten entsprechende Sequenzen von mehreren Solanaceae Spezies gewonnen werden.

1. GENERAL INTRODUCTION

Growth and development of plant tissues and organs requires a permanent change in source-sink relations. Tissues with a net carbohydrate export (source) or import (sink) have to adopt their actual demand of assimilates depending on the current developmental and carbohydrate status. As sessile life forms, plants have developed regulatory mechanisms that enable a flexible response of assimilate partitioning to specific requirements of the habitat, such as biotic and abiotic stress factors and changing light conditions. Photosynthesis is mainly restricted to leaves, which have been optimised for assimilate production. Therefore, one major task a plant has to solve is the distribution of assimilates from source tissues to a multitude of different sink tissues according to the actual demand. This process involves specific enzyme functions including sugar transporters and sucrose cleaving enzymes and turns out to be highly regulated.

1.1 *Carbohydrate partitioning between source and sink tissues*

Plant tissues can be divided into two major categories, net exporters of sugars (carbon sources) and net importers of sugars (carbon sinks). In higher plants, CO_2 fixation occurs predominantly in mesophyll cells of mature leaves. Photosynthetically active leaves produce more carbohydrates than they consume on their own, therefore, they represent carbon sources. On the other hand there are different types of sink tissues known that rely on a supply of sugars (Ho, 1988). Young leaves, stems or green fruits are photosynthetically active, but they are dependent on an additional carbohydrate import. Other sink tissues, like roots or flowers completely depend on an exogenous assimilate supply. Furthermore, sink tissues can be divided according to the way of carbohydrate utilisation. Storage sinks save most of the imported metabolites in specific organs, such as seeds or tubers. Utilisation sinks, such as roots or meristems consume most of the imported carbohydrates to sustain growth and just little to build up storage compounds.

The sink strength of plant tissues is not static, but it varies during plant development and in response to external and internal stimuli. Starting form a heterotrophic embryo higher plants evolve a mosaic of source and sink tissues. Seeds for example consume high amounts of carbohydrates during seed development and formation of storage compounds. In this stadium seeds show high sink strength. During germination they provide metabolites to the developing seedling and thus, represent source tissues. Source-sink transitions are assumed to be regulated by complex regulation mechanisms, including hormones, carbohydrates, and stress stimuli (Geiger et al., 1996).

In higher plants sucrose represents the main transport form of assimilated carbon. Long-distance transport of sucrose from source to sink tissues is mediated via the phloem sieve elements and is driven by differences in osmotic potentials (Ho, 1988). This transport mechanism includes sucrose export from mesophyll cells, phloem loading in source tissues, and unloading of sucrose in sieve elements of sink tissues (Frommer and Sonnewald, 1995; Lalonde et al., 2003). Unloading of sucrose at the sink tissue might occur either symplastically or apoplastically. Symplastic transport is mediated via plasmodesmata that link neighbouring cells (Lucas et al., 1993; Russin et al., 1996). In storage organs or fully differentiated tissues phloem unloading is assumed to occur mainly symplastically. Other sinks, such as embryo or pollen, are symplastically isolated and sucrose is delivered from the phloem into the apoplast by a postulated sucrose exporter. Unloaded sucrose can be taken up by the sink cell either directly, via sink-specific sucrose transporters, or it is cleaved by extracellular invertase and resulting hexoses are taken up into the sink cell via hexose transporters (Büttner and Sauer, 2000). Sucrose has to be cleaved in hexose monomers prior utilisation, a reaction that is mediated either by invertases or cytoplasmic sucrose synthase. Sucrose synthase is a glycosyl transferase, which cleaves sucrose in a reversible reaction yielding UDP-glucose and fructose. UDP-glucose can be further metabolised via glycolysis or used as precursor for numerous compounds including starch and cellulose (Sturm et al., 1999). Invertases are hydrolases, which irreversibly cleave sucrose in glucose and fructose. Plant invertases include three isoforms, which can be categorised in terms of their biochemical properties and subcellular localisation: (1) acid, vacuolar invertases; (2) neutral, cytoplasmatic invertases; and (3) acid, extracellular invertases (Tymowska-Lalanne and Kreis, 1998a; Sturm, 1999). Sucrose cleaving enzymes, invertase and sucrose synthase, are important determinants of sink strength as they generate a sucrose gradient that supports the unloading of sucrose from phloem due to the steepened concentration gradient. Proposed functions of invertases and sucrose synthase are depicted in Table 1 according to Sturm and Tang (1999), emphasising the dominant role of extracellular invertase in mediating defence responses and differentiation/developmental processes.

1.2 *The role of extracellular invertase in apoplastic phloem unloading*

According to the model of apoplastic phloem unloading (Eschrich, 1980) sucrose is released into the apoplast via sucrose transporters. Apoplastic sucrose is the substrate for cell-wall bound extracellular invertase that hydrolyses sucrose yielding glucose and fructose monomers. Resulting hexoses are imported into the sink cell by monosaccharide transporters (Figure 1). Because of the involvement of three protein-mediated steps (two transporters and invertase) the process of assimilate partitioning can be efficiently regulated.

Table 1. Proposed functions of invertases and sucrose synthase

According to Strum and Tang (1999), with modifications

Extracellular invertase
- Sucrose partitioning between source and sink organs
- Response to wounding and infection
- Control of cell differentiation and plant development

Vacuolar invertase
- Osmoregulation
- Control of sugar composition in fruits and storage organs
- Response to cold

Cytoplasmic invertase
- Largely unknown, probably involved in channeling sucrose into metabolism

Sucrose synthase
- Channeling of sucrose into metabolism
- Sucrose partitioning between source and sink organs
- Response to anaerobis and cold

The enzymatic properties of extracellular invertases support the idea that they function as key regulator of apoplastic phloem unloading. The K_m value of extracellular invertase is in the mM range whereas the K_m value of hexose transporters is in the µM range. Therefore, sucrose cleavage by extracellular invertase represents the limiting step (Roitsch et al., 2003). Moreover, sucrose cleavage by extracellular invertase is the only irreversible step of the apoplastic phloem-unloading pathway. Thus, activities of extracellular invertase and hexose transporters are important for the ability of the sink cell to retrieve carbohydrates from the phloem and for determining the sink strength of the corresponding tissue (Roitsch and Tanner, 1996). This functional coupling of hexose transporters and extracellular invertase is supported by a co-ordinated induction after cytokinin application as shown in *C. rubrum* suspension cultures (Ehneß and Roitsch, 1997) and co-expression of extracellular invertase and hexose transporters in the endospermal transfer cell layer of barley seeds (Weschke et al., 2003). An essential role for extracellular invertase in assimilate partitioning, source-sink regulation, and developmental processes has been shown in several studies including overexpression of a yeast invertase in the apoplast of transgenic tobacco plants (von Schaewen et al., 1990), the analysis of an invertase deficient maize mutant (Miller and Chourey, 1992; Cheng et al., 1996), the determination of developmental regulation during seed development (Weber et al., 1995), antisense suppression of extracellular invertase in transgenic carrot plants (Tang et al., 1999), and antisense suppression of an anther-specific isoenzyme of tobacco (Goetz et al., 2001).

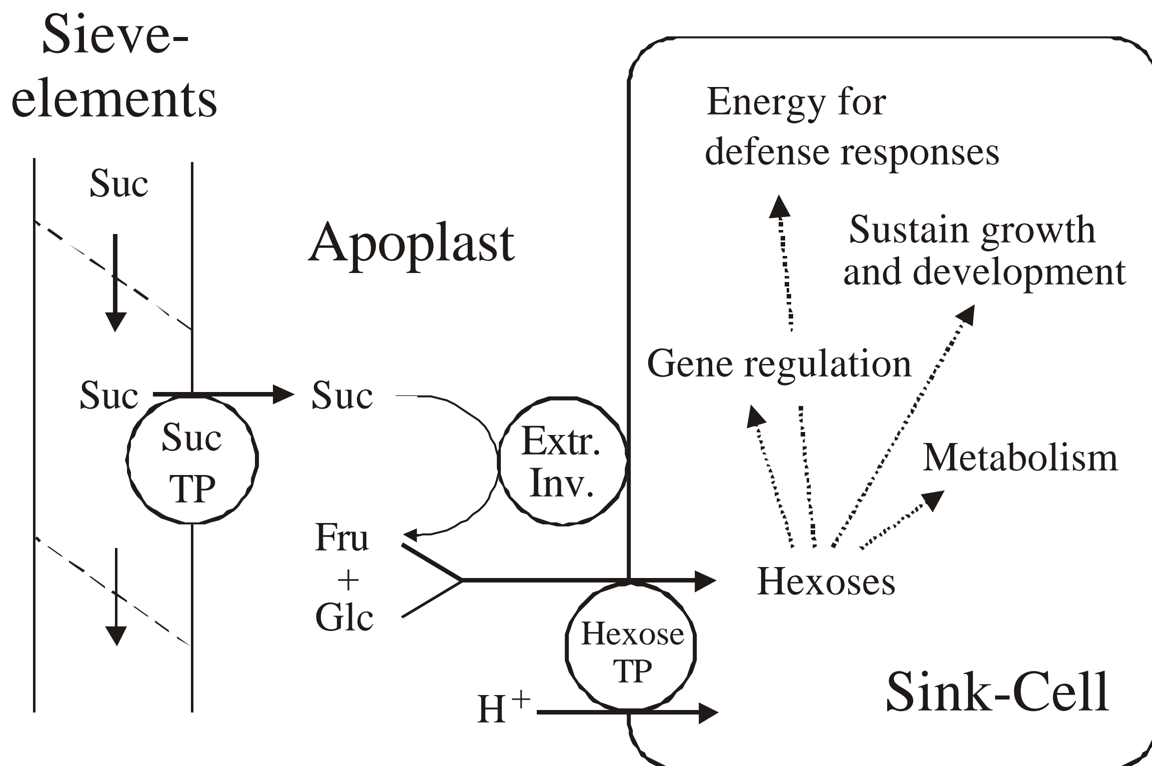

Figure 1. Model for apoplastic phloem unloading in sink tissues. Sucrose (Suc) is unloaded from the phloem sieve elements into the apoplast by a sucrose transporter (SucTP) and hydrolysed by an extracellular invertase (Extr. Inv.) in fructose (Fru) and glucose (Glc). Resulting hexose monomers are taken up from the sink cell by monosaccharide transporters (Hexose TP). According to Roitsch and Tanner (1996), with modifications.

1.3 *Extracellular invertases are organised in gene families comprising several isoenzymes with tissue- and developmental-specific expression patterns*

A common feature of invertases is the organisation in small gene families comprising isogenes with unique spatial and temporal expression patterns. Three genomic clones of extracellular invertases, *InvDc1*, *InvDc2* and *InvDc3*, have been isolated form **carrot**. The steady state levels of transcripts were determined and turned out to show tissue- and developmental-specific expression patterns. High transcript levels of *InvDc1* were found in leaves and roots of young plants and reproductive organs, such as flower buds and flowers. In contrast, no *InvDc2* and *InvDc3* transcripts were found in vegetative organs. *InvDc2* was exclusively expressed in flower buds whereas *InvDc3* was shown to be expressed in very low levels in suspension cultures (Lorenz et al., 1995).

In **maize** four extracellular invertases have been characterised, Incw1, Incw2, Incw3, and Incw4. Expression of *Incw1* was detected in roots, shoots, and kernels; *Incw2* mRNA is present in kernels and shoots but not in roots (Taliercio et al., 1999). *Incw3* is expressed in

young seedling roots, in 4-day-old seedling shoots, anthers, and pollen whereas no expression was observed in young or mature leaves. In contrast, *Incw4* was expressed in all vegetative and reproductive organs tested indicating a constitutive expression (Kim et al., 2000).

The ***A. thaliana*** extracellular invertase gene family comprises 6 isogenes with individual expression patterns. All six genes show distinct levels and spatial patterns of expression (for review see: Sherson et al., 2003).

In **potato** four extracellular invertases have been cloned so far. Hedley et al. (1993; 1994) analysed the expression pattern of *pCD111* and *pCD141*, two extracellular invertases that turned out to be expressed in stems, leaves, and tubers. Maddison et al. (1999) reported on two extracellular invertase genes that are associated with sexual and vegetative growth cycles in potato. *InvGF* expression was detected exclusively in anther and pollen tissue, *invGE* is expressed in nodes in the stem, in a narrow cell layer extending around the stem, and in roots at sites where laterals branch, and floral tissue including anthers and pollen.

Godt and Roitsch (1997) cloned four extracellular invertase genes of **tomato** (*Lin5*, *Lin6*, *Lin7*, and *Lin8*). A detailed characterisation of three isoenzymes of the tomato extracellular invertase gene family is outlined in this study.

The organisation of extracellular invertases in gene families comprising multiple isogenes with individual expression and regulation patterns reflects the diversity of functional roles of invertase enzymes in assimilate partitioning. In particular, invertase isoenzymes with distinct functions, such as modulating defence reactions and supporting growth processes during organ development, confer flexibility to the regulation of carbohydrate metabolism.

1.4 *Specific function of extracellular invertases in providing carbohydrates to floral organs*

An expression of extracellular invertases in floral organs has been reported for a variety of plant species, thus indicating an important role of extracellular invertase in floral development. All six isoenzymes of *A. thaliana* extracellular invertases are expressed, albeit at different levels, in floral organs including one flower-specific isoenzyme (Sherson et al., 2003). *InvGE* and *invGF*, extracellular invertase genes cloned from potato, are expressed in floral organs/vegetative buds and pollen, respectively (Maddison et al., 1999). A flower bud-specific isoenzyme in carrot was analysed by Lorenz et al. (1995) and extracellular invertase expression in developing anthers was shown for *V. faba* (Weber at al., 1995).

The identification of anther- and pollen-specific isoenzymes in invertase gene families (as detailed above) indicates a crucial function of extracellular invertase in providing carbohydrates to the male gametophyte. A functional link between extracellular sucrose cleavage and anther and pollen development has been demonstrated for the tobacco

invertase Nin88 (Goetz et al., 2001). Expression of a *Nin88* antisense construct under the control of the *Nin88* promoter results in a block during pollen development. These results are in line with the characterisation of male gametophyte-specific monosaccharide transporters. Truernit et al. (1999) report on a male gametophyte-specific monosaccharide transporter AtSTP2 that was shown to be expressed in developing pollen after the onset of symplastic isolation of the microspore. Two further pollen-specific monosaccharide transporters in *A. thaliana*, AtSTP6 and AtSTP9, were characterised by Scholz-Starke et al. (2003) and Schneidereit et al. (2003), respectively. Likewise, monosaccharide transporters expressed in anthers have been demonstrated in petunia (Ylstra et al., 1998) and tobacco (Goetz M, Roitsch T, unpublished observations). It has to be assumed that a co-ordinated expression of pollen-specific extracellular invertases and monosaccharide transporter isoenzymes represents an essential mechanism for carbohydrate supply of pollen tissue.

1.5 *Extracellular invertases, key metabolic enzymes and modulators of the stress response display a multiple regulation pattern*

As detailed above, extracellular invertases are assumed to be key regulators of apoplastic phloem unloading. Accordingly, a large number of stimuli that are known to affect carbohydrate requirements, such as phytohormones, sugars, stress related stimuli, and light have been demonstrated to affect expression levels of extracellular invertases (Zouaghi and Rollin, 1976; Sturm and Chrispeels, 1990; Wu et al. 1993a/b/c; Roitsch et al., 1995; Zhang et al., 1996/1997). In particular, the effects of hormones, sugar, and stress stimuli on assimilate partitioning have been addressed as detailed below. Conditions that are characterised in an increased demand of carbohydrates have been shown to go along with enhanced mRNA levels of specific invertase isoenzymes. Further mechanisms involved in regulation of extracellular invertase expression or activity are differential transcript formation (Cheng et al., 1999), exon skipping (Bournay et al., 1996), and inhibition by proteinaceous inhibitors (Krausgrill et al., 1996; Greiner et al., 1998; Rausch and Greiner, 2004). Nevertheless, regulation of extracellular invertase activity appears to take place mainly on the transcriptional level; posttranslational modifications mediating invertase activity are not known (Tymowska-Lalanne and Kreis, 1998a; Roitsch et al., 2000; Roitsch et al., 2003). The high number of regulatory mechanisms supports the central role of extracellular invertase for assimilate partitioning.

1.5.1 *Hormonal regulation of extracellular invertase expression*

For long it has been speculated that plant growth regulators are involved in phloem unloading (Tanner, 1980), regulation of sink strength (Kuiper, 1993), and carbohydrate partitioning (Brenner and Cheikh, 1995). Phytohormones that play an integral role in controlling growth, differentiation, and development of plants turned out to be potent compounds of invertase regulation (Tymowska-Lalanne and Kreis, 1998a; Roitsch et al., 2003) thereby indicating that invertases are involved in mediating the corresponding hormone responses. In particular, increased extracellular invertase levels could satisfy the increased carbohydrate demand of growth-stimulated tissues.

Cytokinins play a major role in regulating active growth processes and promote cell division. As those processes are characterised by an enhanced demand for carbohydrates, a link to the regulation of assimilate partitioning has been suggested (Kuiper, 1993; Roitsch and Ehneß, 2000). Tissues with elevated activities of extracellular invertase are known to contain enhanced cytokinin concentrations supporting a physiological significance of cytokinin-mediated regulation of assimilate partitioning. This idea was corroborated by the observation of Mothes and Engelbrecht (1963) that radioactive labelled nutrients are accumulated in cytokinin treated tissue showing that cytokinins effectively modulate source-sink patterns. In cell culture experiments an up-regulation of extracellular invertases *Cin1* and *Lin6* in response to different cytokinins was shown (Ehneß and Roitsch, 1997; Godt and Roitsch, 1997). Moreover, hexose transporters of *C. rubrum* were co-induced with the extracellular invertase *Cin1* upon cytokinin treatment (Ehneß and Roitsch, 1997). The co-ordinated induction of the two key enzymes in apoplastic phloem unloading by cytokinin supports the idea that apoplastic sucrose cleavage represents an essential mechanism in supplying carbohydrates for cytokinin induced growth.

Gibberellins are hormones that control growth, promote cell elongation, and are important for a variety of developmental processes in particular in seed and floral organs (Richards et al., 2001). GA_3 has been reported to increase invertase activity in several plant organs. A significant role of GA in regulating invertase levels and thus, providing a mechanism for increasing the level of soluble carbohydrates needed for GA promoted growth is supposed (Tymowska-Lalanne and Kreis 1998a). In shoot of dwarf pea plants an induction of invertase expression after GA_3 treatment was observed (Wu et al., 1993c). GA induction of the flower-specific invertase *Lin5* is presented in this study (Chapter 2), thereby discussing a possible link between known GA effects on flower development and invertase function.

Auxin has been reported to stimulate the activity of extracellular invertase (Weil and Rausch, 1990). Morris and Arthur (1984, 1986) could demonstrate that indoleacetic acid promotes growth and invertase activity in segments of young *P. vulgaris* internodes. An

asymmetric induction of invertase mRNA in oat (Wu et al., 1993b) seems to be mediated via gravi-stimulated auxin redistribution.

Brassinosteroids that show high structural similarity to animal steroid hormones (Grove et al., 1979) induce a variety of growth processes in plant tissues (Li and Chory, 1999; Müssig and Altmann, 1999). Goetz et al. (2000) could correlate a localised growth response of the hypocotyl of tomato seedlings with a specific induction of *Lin6* mRNA in corresponding tissues.

Abscisic acid and **jasmonic acid**, hormones that are known to modulate defence/stress responses, were shown to increase the expression level of a pea extracellular invertase (Zhang et al., 1996) and tomato extracellular invertase *Lin6* (this study). The observed jasmonic acid mediated induction is of particular interest as Park et al. (2002) could substantiante a dual role of jasmonates in wound-induced defence and pollen development. In both processes extracellular invertases have been demonstrated to play an essential role (Sturm and Chrispeels, 1990; Goetz et al., 2001).

In contrast to previous stated hormones that all induce expression of extracellular invertase, **ethylene** represses the mRNA level as shown for *Cin1* in autotrophic cell cultures of *C. rubrum* (Linden et al., 1996). Ethylene, a hormone that is synthesised in tissues undergoing senescence or ripening (Davies, 1995), is the only stimulus known to down-regulate invertase expression.

1.5.2 *Sugars modulate invertase activity and provide a mechanism for feed forward regulation*

In plants soluble sugars provide the energy and carbon currencies, but also act as signalling molecules to regulate gene expression, growth, and development (Jang and Sheen, 1997; Smeekens and Rook, 1997). The term „metabolic regulation“ was introduced by Karrar and Rodriguez (1992). Many sugar-regulated genes that cover different physiological pathways, including extracellular invertases, are known from plants (Koch,1996; Roitsch, 1999; Sheen et al., 1999). Glucose and sucrose increase the level of an extracellular invertase of pea (Zhang et al., 1997) and *C. rubrum* (Roitsch et al., 1995). One isogene of tobacco (Krausgrill et al., 1996), *A. thaliana* (Tymowska-Lalanne and Kreis, 1998b), and tomato (Godt and Roitsch, 1997; Sinha et al., 2002) extracellular invertase was shown to be up-regulated by glucose. Sugar induced expression of extracellular invertase is of particular interest, as this mechanism allows the cell to maintain or to amplify the initial stimulation of invertase by diverse signals via feed forward regulation (Figure 2).

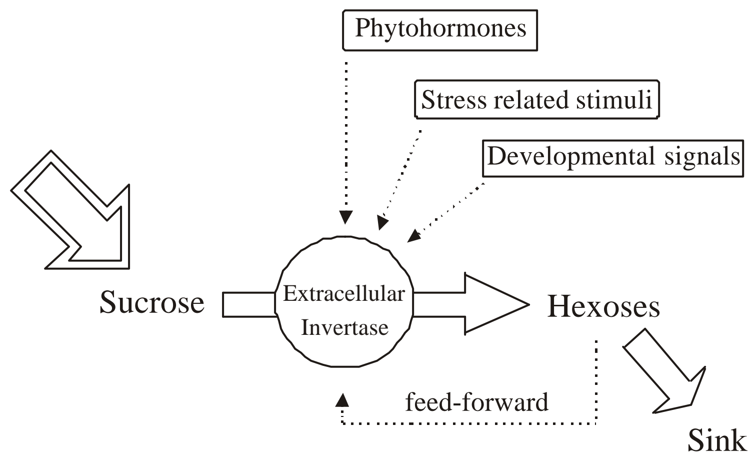

Figure 2. Feed forward regulation of extracellular invertase by sugars. Due to sugar induction of extracellular invertase, initial stimulation by diverse signals will be maintained or amplified via feed forward regulation. This will result in an intensified retention of carbohydrates and triggering of sugar modulated gene regulation. According to Roitsch et al. (2000), with modifications.

1.5.3 *Modulation of stress responses by extracellular invertase*

Much effort has been put in understanding how plants respond to pathogen infection and wounding. Hereby it became clear that induction of defence reactions is preceded by a fast increase of sink metabolism. Wounding, caused by mechanical injury or herbivore attack, is a severe environmental stress factor. Mechanical wounding has been shown to induce *Cin1* (Roitsch et al., 1995) and an extracellular invertase of pea (Zhang et al., 1996). Wounding or infection of carrot roots and leaves with the bacterial pathogen *E. carotovora* results in a fast increase in transcript level of an extracellular invertase (Sturm and Chrispeels, 1990). An important biotic stress for plants is the infection by fungi. This stress factor can be mimicked by the use of fungal elicitors as shown for the necrotrophic fungus *Fusarium oxysporum lycopersici*. Tomato suspension cultures treated with this elicitor preparation showed an up-regulation in *Lin6* extracellular invertase expression (Sinha et al., 2002). Up-regulation of extracellular invertase in response to stress stimuli could be essential to mediate the observed localised increase in sink strength. The identification of monosaccharide transporters that are regulated by environmental stimuli, as pathogen infection or wounding, suggests a close relationship between apoplastic sucrose hydrolysis and hexose uptake during the stress response (Büttner and Sauer, 2000; Fotopoulos et al., 2003). The increased attraction of carbohydrates could serve as metabolic energy for activating an appropriate cascade of defence reactions. Moreover, a number of defence related genes have been found to be induced by soluble sugars (Herbers et al., 1996). Infection of tobacco plants with potato virus Y leads to inhibition of sugar export, induction of defence responses, and a higher resistance towards viral attack (Herbers et al., 2000). Based on this finding, Herbers et al. (2000) proposed a model for sugars to act as amplifiers for defence responses during plant-pathogen interaction. According to this model, viral

infection will cause an apoplastic sucrose accumulation. Sucrose will be hydrolysed to glucose and fructose by extracellular invertase and the hexoses formed will be taken up by mesophyll cells. Because of the feed forward regulation by glucose (see Figure 2) this will increase the expression of extracellular invertase, which results in an intensified retention of carbohydrates and triggers defence responses.

1.6 *Extracellular invertase covers different functions to meet the requirements of assimilate partitioning*

Based on experimental data detailed above, extracellular invertases are supposed to be pivotal regulators of assimilate partitioning. In particular, apoplastic sucrose cleavage has an important impact on cell differentiation, plant development, and mediation of the defence response. The central position of extracellular invertase in these processes is reflected by a spectrum of different functions covered by this enzyme (Roitsch et al., 2000).

1. <u>Carbohydrate supply of sink tissues:</u> Extracellular invertases cover a key function in apoplastic phloem unloading and assimilate supply of sink tissues (Tang et al, 1999; Weber et al., 1995; Goetz et al., 2001). This is reflected by the organisation of invertases in gene families comprising isoenzymes with specific expression- and regulation-patterns. Induction of extracellular invertase mediated by growth stimulating hormones and stress stimuli, representing conditions that require an increased demand of carbohydrates, underlines the above-mentioned finding.

2. <u>Regulation of source-sink-transitions:</u> Altered source-sink relations in transgenic plants overexpressing a yeast invertase (von Schaewen et al., 1990) and induction of extracellular invertase expression in autotrophic cell cultures after metabolic induction (Godt and Roitsch, 1997; Ehneß et al., 1997; Sinha et al., 2002) indicate a crucial role for extracellular invertases in mediating source-sink transitions.

3. <u>Signal amplification in source-sink transitions:</u> Since invertases are transcriptionally induced by sugars (Roitsch et al., 1995; Godt and Roitsch, 1997; Krausgrill et al., 1996; Tymowska-Lalanne and Kreis, 1998b), there will be an amplification of signals that initially up-regulate extracellular invertase mediated by a positive sugar feedback circuit.

4. <u>Integration of signals that regulate source-sink transitions and defence responses:</u> Based on the central position in apoplastic phloem unloading and the huge number of different stimuli that influence extracellular invertase expression, this enzyme can serve as

an integration point of internal and external stimuli resulting in a co-ordinated cellular response. This is in particular true, as sugars serve as signals for further regulatory mechanisms, such as gene expression (Koch, 1996) and defence responses (Herbers et al., 1996; 2000).

1.7 *CACTA-elements, ubiquitous transposable elements in plant genomes*

Transposable elements (TEs) are ubiquitous components of most plant genomes and play an essential role in the structure, size, variability and adaptive evolution of genomes (McClintock, 1984; McDonald, 1995; Kidwell and Lisch, 1997). Depending on their mode of transposition, transposons are divided into two categories. Class 1 elements (retrotransposons) move via an RNA intermediate by a mechanism that is dependent on reverse transcription (Kumar and Bennetzen, 1999). Class 2 elements, transposons, move via a DNA intermediate and terminate in conserved sequence motives (terminal inverted repeats, TIRs) that serve as recognition sites for the transposase (Lewin, 1997). TIRs in combination with subterminal repeated sequences are essential for autonomous transposition, whereas proteins encoded by the internal part of the element, as transposase or DNA binding protein, can also be provided in *trans*. Excision from the genome and reintegration depends on transposase and is mediated via a cut-and-past mechanism (Saedler and Nevers, 1985). As reviewed by Kunze et al. (1997) plant transposons are classified as Activator- (Ac), Enhancer / Suppressor-mutator- (En/Spm) or Mutator (Mu) elements. Members of the En/Spm-family are flanked by short TIRs of 10 to 28 bp that terminate in the CACTA motif and therefore, they are also named CACTA-elements. Those elements are present as autonomous elements and non-autonomous deletion derivatives. The latter depend for their transposition on protein factors encoded by autonomous copies in the same genome (Bennetzen, 2000). Pereira et al. (1986) report on the first characterisation of a CACTA-element, En-1 from maize, at the molecular level. Active CACTA-elements were characterised from a variety of species such as *A. thaliana* (Miura et al., 2001), petunia (Snowden and Napoli, 1998), snapdragon (Nacken et al., 1991), sorghum (Chopra et al., 1999) and carrot (Ozeki et al., 1997). Recently, CACTA-like elements have been detected with the help of computer-aided database searches (Wicker et al., 2003; Wang et al., 2003) and PCR-based approaches (Staginnus et al., 2001).

Transposons are powerful contributors to plant genome/gene structure and evolution (Bennetzen, 2000; Fedoroff, 2000; Gray, 2000). Furthermore, it is known that TEs contribute to genomic plasticity in response to diverse environmental conditions. A number of plant TEs show activation upon stress conditions (Hirochika, 1993; Arnault and Dufournel, 1994; Wesser, 1996). He et al. (2000) isolated a cDNA (*Rim2*) that was strongly induced by the rice

fungal pathogen *M. grisea* and harboured a putative transposase coding open reading frame. Besides their biological function, TEs have become useful tools in molecular biology. Phenotypic variations due to transposon insertion have been used for the identification and isolation of many genes (Kunze et al., 1997). Moreover, mobile elements allow functional studies of identified genes, are valuable tools for mapping, sequencing, and the analysis of evolutionary relationships between plant species.

In this study we report on a CACTA-like transposon insertion in intron I of the *Lin5* gene and the identification of transposase-like sequences in different Solanaceae species. Albeit the impact of the transposon insertion on gene expression or function has not been addressed so far, it could be speculated that the transposon insertion results in an altered isoenzyme function within the tomato extracellular invertase family.

1.8 *Scope of Work*

Plant tissues can be divided into two major categories, net exporters of sugars (carbon sources) and net importers of sugars (carbon sinks). The sink strength of plant tissues is not static, but it varies during plant growth and development and in response to specific requirements of the habitat, such as biotic and abiotic stress factors and changing light conditions. Assimilate partitioning in higher plants is a complex and strictly regulated process, which involves a co-ordinated regulation of several enzyme functions. One critical step is phloem unloading. Extracellular invertase, which irreversibly cleaves sucrose to yield glucose and fructose, is the key step for the uptake of sucrose from the apoplast (Sturm and Tang, 1999; Roitsch et al., 2000). The activity of this enzyme regulates sucrose mass flow to areas of consumption. Because of its central position in the mediation of source-sink transitions, extracellular invertase is regulated by a variety of stimuli and represents a target of various signalling pathways. Different invertase isoenzymes with specific expression and regulation patterns enable a co-ordinated carbohydrate supply of diverse tissues, at different developmental stages, and under varying environmental conditions.

Previously, primary investigations on steady state mRNA levels of the tomato extracellular invertase gene family have been performed (Godt and Roitsch, 1997). Thereby, two flower-specific isoenzymes and a highly regulated invertase turned out to be of particular interest for further detailed analysis. The major task of this study was to elucidate the specific functions of those isoenzymes within the tomato invertase gene family. In a first step the spatial and temporal patterns of gene expression have been addressed using reporter gene fusions with the cloned promoters of Lin5, Lin6 and Lin7 invertases. As regulation of extracellular invertase activity appears to take place mainly at the transcriptional level, a further topic of interest was to test potential regulators of invertase promoter activity, such as

hormones, sugars, and stress stimuli (Chapters 2, 3, and 5). Thereby, the focus was directed towards the *Lin6* promoter as it is characterised by numerous well-established *cis*-acting elements. This basic characterisation revealed several interesting features. First, a diurnal regulation of *Lin6* mRNA levels was observed. Therefore, an attempt was made to shed light on the molecular basis of this unexpected regulation pattern (Chapter 5). Second, *Lin7* expression turned out to be restricted to anther and pollen tissue and the sequence analysis of *Lin7* promoter revealed several GA-response boxes. Thus, further research was focused on a possible function of *Lin7* in growth and development of the male gametophyte and the underlying regulatory mechanisms (Chapter 3). A detailed understanding of this process could provide new opportunities in plant biotechnology with regard to establishing methods for pollination control via modifying the carbohydrate supply of pollen. Third, a transposon insertion in intron I of the *Lin5* gene was revealed. The identification of a transposase-like sequence made it possible to screen for transposon sequences throughout Solanaceae species (Chapter 4).

Since long it has been speculated that cytokinin is involved in regulating carbohydrate partitioning (Mothes and Engelbrecht, 1963) and mediating delay of senescence (Richmond and Lang, 1957). Using a transgenic approach, Gan and Amasino (1995) reproduced this phenomenon via expression of a cytokinin biosynthetic gene under control of a senescence-activated promoter. Considering the potential of extracellular invertase in mediating changes in source-sink relations the idea arose that an up-regulation of extracellular invertase by cytokinin could provide a mechanism for a localised induction of sink metabolism in senescent leaves. The accelerated sucrose cleavage should induce assimilate transport to, as well as inhibit export from these tissues, resulting in delay of senescence (Roitsch and Ehneß, 2000). Following a fundamental characterisation, the cytokinin-inducible *Lin6* promoter was used in a functional approach to elucidate the physiological role of invertases in cytokinin-mediated delay of senescence (Chapter 6). An understanding of the underlying mechanisms is of great scientific interest with regard to cell differentiation and action of plant hormones. Beyond it, the potential regulation of senescence could provide agricultural benefits, such as improved crop yield and minimised post-harvest losses of crops.

The present thesis comprises three publications and three manuscripts, which are arranged into six independent chapters. Generalised remarks, which assemble the chapters into the scope of the work, are presented at the end of the thesis.

1.9 References

Arnault C and Dufournel I (1994) Genome and stresses: reactions against aggressions, behavior of transposable elements. Genetica 93: 149-160

Bennetzen JL (2000) Transposable element contributions to plant genome evolution. Plant Mol Biol 42: 251-269

Bournay AS, Hedley PE, Maddison A, Waugh R and Machray GC (1996) Exon skipping induced by cold stress in a potato invertase gene transcript. Nucleic Acid Res 24: 2347-2351

Brenner ML and Cheikh N (1995) The role of phytohormones in photosynthate partitioning and seed filling. In: Davies PJ, ed. Plant hormones. Kluwer Academic Press. Dordrecht, Boston, London, pp 649-670

Büttner M and Sauer N (2000) Monosaccharide transporters in plants: structure, function and physiology. Biochimica et Biophysica Acta 1465: 263-274

Cheng WH, Taliercio EW and Chourey PS (1996) The Miniature-1 Seed Locus of maize encodes a cell wall invertase required for normal development of endosperm and maternal cells in the pedicel. Plant Cell 8: 971-983

Cheng WH, Taliercio EW and Chourey PS (1999) Sugars modulate an unusual mode of control of the cell-wall invertase gene (Incw1) through its 3´ untranslated region in a cell suspension culture of maize. Proc Natl Acad Sci USA 96: 10512-10517

Chopra S, Brendel V, Zhang J, Axtell JD and Peterson T (1999) Molecular characterisation of a mutable pigmentation phenotype and isolation of the first active transposable element from *Sorghum bicolor*. Proc Natl Acad Sci USA 96: 15330-153335

Davies PJ (1995) Plant Hormones. Physiology, Biochemistry and Molecular Biology. Kluwer Academic Publishers, Dordrecht

Ehneß R and Roitsch T (1997) Co-ordinated induction of mRNAs for extracellular invertase and a glucose transporter in *Chenopodium rubrum* by cytokinins. The Plant Journal 11: 539-548

Eschrich W (1980) Free space invertase, its possible role in Phloem unloading. Ber Dtsch Bot Ges 93: 363-378

Fedoroff N (2000) Transposons and genome evolution in plants. Proc Natl Acad Sci USA 97: 7002-7007

Fotopoulos V, Gilbert MJ, Pittman JK, Marvier AC, Buchanan AJ, Sauer N, Hall JL and Williams LE (2003) The monosaccharide transporter gene, AtSTP4, and the cell-wall invertase, Atβfruct1, are induced in Arabidopsis during infection with the fungal biotroph Erysiphe cichoracearum. Plant Physiol 132: 821-829

Frommer WB and Sonnewald U (1995) Molecular analysis of carbon partitioning in *solanaceous* species. J Exp Bot 46: 587-607

Gan S and Amasino RM (1995) Inhibition of leaf senescence by autoregulated production of cytokinin. Science 270: 1986-1988

Geiger DR, Koch KE and Shieh W-J (1996) Effect of environmental factors on whole plant assimilate partitioning and associated gene expression. J Exp Bot 47: 1229-1238

Godt DE and Roitsch T (1997) Regulation and Tissue-Specific Distribution of mRNA for Three Extracellular Invertase Isoenzymes of Tomato Suggests an Important Function in Establishing and Maintaining Sink Metabolism. Plant Physiol 115: 273-282

Goetz M, Godt DE and Roitsch T (2000) Tissue-specific induction of the mRNA for an extracellular invertase isoenzyme of tomato by brassinosteroids suggests a role of steroid hormones in assimilate partitioning. Plant Journal 22: 515-522

Goetz M, Godt DE, Guivarch A, Kahmann U, Chriqui D and Roitsch T (2001) Induction of male sterility in plants by metabolic engineering of the carbohydrate supply. Proc Natl Acad Sci USA 98: 6522-6527

Gray Y (2000) It takes two transposons to tango. Trends Genet 16: 461-468

Greiner S, Krausgrill S and Rausch T (1998) Cloning of tobacco apoplasmic invertase inhibitor: Proof of function of the recombinant protein and expression analysis during plant development. Plant Physiol 116: 733-742

Grove MD, Spencer GF, Rohwedder WK, Mandava N, Worley JF, Warthen Jr JD, Steffens GL, Flippen-Anderson JL and Cook Jr JC (1979) Brassinolide, a plant growth-promoting steroid isolated from *Brassica napus* pollen. Nature 281: 216-217

He ZH, Dong HT, Dong JX, Li DB and Ronald PC (2000) The rice *Rim*2 transcript accumulates in response to *Magnaporthe grisea* and its predicted protein product shares similarity with *TNP*2-like proteins encoded by CACTA transposons. Mol Gen Genet 264: 2-10

Hedley PE, Machray GC, Davies HV, Burch L and Waugh R (1993) cDNA cloning and expression of a potato (*Solanum tuberosum*) invertase. Plant Mol Biol 22: 917-922

Hedley PE, Machray GC, Davies HV, Burch L and Waugh R (1994) Potato (*Solanum tuberosum*) invertase-encoding cDNAs and their different expression. Gene 145: 211-214

Herbers K, Meuwly P, Frommer WB, Metraux JP and Sonnewald U (1996) Systemic acquired resistance mediated by the ectopic expression of invertase: possible hexose sensing in the secretory pathway. Plant Cell 8: 793-803

Herbers K, Takahata Y, Melzer M, Mock H-P, Hajirezaei M and Sonnewald U (2000) Regulation of carbohydrate partitioning during the interaction of potato virus Y with tobacco. Mol Plant Pathol 1: 51-59

Hirochika H (1993) Activation of tobacco retrotransposons during tissue culture. EMBO J 12: 2521-2528

Ho LC (1988) Metabolism and compartimentation of imported sugars in sink organs in relation to sink strength. Annu Rev Plant Physiol Plant Mol Biol 39: 355-378

Jang J-C and Sheen J (1997) Sugar sensing in higher plants. Trends in Plant Science 2: 208-214

Karrar EE and Rodriguez RL (1992) Metabolic regulation of rice α-amylase and sucrose synthase genes in planta. The Plant Journal 2: 517-523

Kidwell MG and Lisch D (1997) Transposable elements as source of variation in animals and plants. Proc Natl Acad Sci USA 94: 7704-7711

Kim J-Y, Mahé A, Guy S, Brangeon J, Roche O, Chourey PS and Prioul J-L (2000) Characterisation of two members of the maize gene family, *Incw3* and *Incw4*, encoding cell-wall invertases. Gene 245: 89-102

Koch KE (1996) Carbohydrate-modulated gene expression in plants. Annu Rev Plant Physiol Plant Mol Biol 47: 509-540

Krausgrill S, Sander A, Greiner S, Weil M and Rausch T (1996) Regulation of cell-wall invertase by a proteinaceous inhibitor. J Exp Bot 47: 1193-1198

Kuiper D (1993) Sink strength: established and regulated by plant growth regulators. Plant Cell and Environment 16: 1025-1026

Kumar A and Bennetzen JL (1999) Plant retrotransposons. Ann Rev Genet 33: 479-532

Kunze R, Saedler H and Lönnig WE (1997) Plant transposable elements. Adv Bot Res 27: 331-470

Lalonde S, Tegeder M, Throne-Holst M, Frommer WB and Patrick JW (2003) Phloem loading and unloading of sugars and amino acids. Plant, Cell and Environment 26: 37-56

Lewin B (1997) Transposons. In B Lewin, Genes VI. Oxford University Press, Inc., New York, pp 563-595

Li J and Chory J (1999) Brassinosteroide actions in plants. J Exp Bot 50: 275-282

Linden JC, Ehneß R and Roitsch T (1996) Ethylene regulation of apoplastic invertase expression in autotrophic cells of *Chenopodium rubrum*. Plant Growth Regul 19: 219-222

Lorenz K, Lienhard S and Sturm A (1995) Structural organisation and differential expression of carrot β-fructofuranosidase genes: identification of a gene coding for a flower bud-specific isoenzyme. Plant Mol Biol 28: 189-194

Lucas W, Olesinski A, Hull RJ, Haudenshield JS, Deom CM, Beachy RN and Wolf S (1993) Influence of the tobacco mosaic virus 30kD-movement protein on carbon metabolism and photosynthete partitioning in transgenic tobacco plants. Planta 190: 88-96

Maddison AL, Hedeley PE, Meyer RC, Aziz N, Davidson D and Machray GC (1999) Expression of tandem invertase genes associated with sexual and vegetative growth cycles in potato. Plant Mol Biol 41: 741-751

McClintock B (1984) The significance of responses of the genome to challenge. Science 226: 792-801

McDonald JF (1995) Transposable elements: possible catalysts of organismic evolution. Trends Ecol Evol 10: 123-126

Miller EM and Chourey PS (1992) The maize invertase-deficient miniature-1 seed mutant is associated with aberrant pedicel and endosperm development. Plant Cell 4: 297-305

Miura A, Yonebayashi S, Watanabe K, Toyama T, Shimada H and Kakutani T (2001) Mobilization of transposons by a mutation abolishing full DNA methylation in *Arabidopsis*. Nature 411: 212-214

Morris DA and Arthur ED (1984) Invertase and auxin-induced elongation in internodal segments of *Phaseolus vulgaris*. Phytochemsitry 23: 2163-2167

Morris DA and Arthur ED (1986) Stimulation of acid invertase activity by indolyl-3-acetic acid in undergoing cell expansion. Plant Growth Regul 4: 259-271

Mothes K and Engelbrecht L (1963) On the activity of a kinetin-like root factor. Life Sci 11: 852-857

Müssig C and Altmann T (1999) Physiology and molecular mode of action of brassinosteroids. Plant Physiol 37: 363-372

Nacken WKF, Piotrowiak R, Saedler H and Sommer H (1991) The transposable element Tam1 from *Antirrhinum majus* shows structural homology to the maize transposon En/Spm and has no sequence specificity of insertion. Mol Gen Genet 228: 210-208

Ozeki Y, Davies E and Takeda J (1997) Somatic variation during long term subculturing of plant cells caused by insertion of a transposable element in a phenylalanine ammonia-lyase (PAL) gene. Mol Gen Genet 254: 407-416

Park J-H, Halitschke R, Kim HB, Baldwin IT, Feldmann KA and Feyereisen R (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in *Arabidopsis* due to a block in jasmonic acid biosynthesis. Plant J 31: 1-12

Pereira A, Cuypers H, Gierl A, Schwarz-Sommer Z and Saedler H (1986) Molecular analysis of the En/Spm transposable element system in *Zea mays*. EMBO J 5: 835-841

Ramloch-Lorentz K, Knudsen S and Sturm A (1993) Molecular characterisation of the gene for carrot cell wall ß-fruktosidase. Plant J 4: 545-554

Rausch T and Greiner S (2004) Plant protein inhibitors of invertases. Biochimica et Biophysica Acta 1696: 253-261

Richards DE, King KE, Ait-ali T and Harberd NP (2001) How Gibberellin regulates plant growth and development: A molecular genetic analysis of Gibberellin signaling. Annu Rev Plant Physiol Plant Mol Biol 52: 67-88

Richmond AE and Lang A (1957) Effect of kinetin on protein content and survival of detached *Xanthium* leaves. Science 125: 650-651

Roitsch T, Bittner M and Godt DE (1995) Induction of Apoplastic Invertase of *Cenopodium rubrum* by D-Glucose and a Glucose Analogue and Tissue-Specific Expression Suggest a Role in Sink-Source Regulation. Plant Physiol 108: 285-294

Roitsch T and Tanner W (1996) Cell wall invertase: bridging the gap. Bot Acta 109: 90-93

Roitsch T (1999) Source-sink regulation by sugar and stress. Curr Opin Plant Biol 2: 198-206

Roitsch T and Ehneß R (2000) Regulation and source/sink relations by cytokinins. Plant Growth Regul 32: 359-367

Roitsch T, Ehneß R, Götz M, Hause B, Hofmann M, and Sinha A (2000) Regulation and function of extracellular invertase from higher plants in relation to assimilate partitioning, stress responses and sugar signalling. Aust J Plant Physiol 27: 815-825

Roitsch T, Balibrea ME, Hofmann M, Proels R and Sinha AK (2003) Extracellular invertase: key metabolic enzyme and PR protein. J Exp Bot 54: 513-524

Russin WA, Evert RF, Vanderveer PJ, Sharkey TD and Briggs SP (1996) Modification of a specific class of plasmodesmata and loss of sucrose export ability in the sucrose export *defective1* maize mutant. Plant Cell 8: 645-658

Saedler H and Nevers P (1985) Transposition in plants: a molecular model. EMBO J 4: 585-590

Schneidereit A, Scholz-Starke J and Büttner M (2003) Functional characterisation and expression analyzes of the glucose-specific AtSTP9 monosaccharide transporter in pollen of *Arabidopsis*. Plant Physiol 133: 182-190

Scholz-Starke J, Büttner M and Sauer N (2003) AtSTP6, a new pollen-specific H^+-monosaccharide symporter from *Arabidopsis*. Plant Physiol 131: 70-77

Sheen J, Zhou L and Jang JC (1999) Sugars as signaling molecules. Curr Opin Plant Biol 2: 410-418

Sherson SM, Alford HL, Forbes SM, Wallace G and Smith SM (2003) Roles of cell-wall invertases and monosaccharide transporters in the growth and development of *Arabidopsis*. J Exp Bot 54: 525-531

Sinha AK, Römer U, Köckenberger W, Hofmann M, Elling L and Roitsch T (2002) Metabolizable and non-metabolizable sugars activate different signal transduction pathways in tomato. Plant Physiol 128: 1480-1489

Smeekens S and Rook F (1997) Sugar sensing and sugar-mediated signal transduction in plants. Plant Physiol 115: 7-13

Snowden KC and Napoli CA (1998) PsI: a novel Spm-like transposable element from *Petunia hybrida*. Plant J 14: 43-54

Staginnus C, Huettel B, Desel C, Schmidt T and Kahl G (2001) A PCR-based assay to detect En/Spm-like transposon sequences in plants. Chromosome Res 9: 591-605

Sturm A (1999) Invertases. Primary Structures, Functions, and Roles in Plant Development and Sucrose Partitioning. Plant Physiol 12: 1-7

Sturm A, Lienhard S, Schatt S and Hardegger M (1999) Tissue-specific expression of two genes for sucrose synthase in carrot (Daucus carota L.). Plant Mol Biol 39: 349-360

Sturm A and Tang G-Q (1999) The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. Trends in Plant Science 4: 401-407

Sturm A and Chrispeels MJ (1990) cDNA cloning of carrot extracellular β-fructosidase and its expression in response to wounding and bacterial infection. Plant Cell 2: 1107-1119

Taliercio EW, Kim J-Y, Mahe A, Shanker S, Choi J, Cheng W-H, Prioul J-L and Chourey PS (1999) Isolation, characterisation and expression analyses of two cell wall invertase genes in maize. J Plant Physiol 155: 197-204

Tanner W (1980) On the possible role of ABA on phloem unloading. Ber Dtsch Bot Ges 93: 349-351

Tang G-Q, Lüscher M and Sturm A (1999) Antisense repression of vacuolar and cell wall invertase in transgenic carrot alters early plant development and sucrose partitioning. Plant Cell 11: 117-189

Truernit E, Stadler R, Baier K and Sauer N (1999) A male gametophyte-specific monosaccharide transporter in *Arabidopsis*. Plant J 17: 191-201

Tymowska-Lalanne Z and Kreis M (1998 a) The plant invertases: Physiology, biochemistry and molecular biology. Adv Bot Res 28: 71-117

Tymowska-Lalanne Z and Kreis M (1998 b) Expression of the *Arabidopsis thaliana* invertase gene family. Planta 210: 259-265

von Schaewen A, Stitt M, Schmidt R, Sonnewald U and Willmitzer L (1990) Expression of a yeast-derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. EMBO J: 3033-3044

Wang GD, Tian PF, Cheng ZK, Wu G, Jiang JM, Li DB, Li Q and He ZH (2003) Genomic characterisation of *Rim2/Hipa* elements reveals a CACTA-like transposon superfamily with unique features in the rice genome. Mol Gen Genomics 270: 234-242

Weber H, Borisjuk L, Heim U, Buchner P and Wobus U (1995) Seed coat-associated invertases of fababean control both unloading and storage functions. Cloning of cDNAs and cell type-specific expression. Plant Cell 7: 1835-1864

Weil M and Rausch T (1990) Cell wall invertase in tobacco crown gall cells. Plant Physiol 94: 1575-1581

Weschke W, Panitz R, Gubatz S, Wang, Q, Radchuk R, Weber H and Wobus U (2003) The role of invertases and hexose transporters in controlling sugar ratios in maternal and filial tissues of barley caryopses during early development. Plant J 33: 395-411

Wessler SR (1996) Plant retrotransposons: turned on by stress. Curr Biol 6: 959-961

Wicker T, Guyot R, Yahiaoui N and Keller B (2003) CACTA transposons in Triticeae. A diverse family of high-copy repetitive elements. Plant Physiol 132: 52-63

Wu LL, Song I, Karupiach N and Kaufman PB (1993a) Kinetic induction of oat shoot pulvinus invertase mRNA by grvistimulation and partial cDNA cloning by polymerase chain reaction. Plant Mol Biol 21: 1175-1179

Wu LL, Song I, Kim D and Kaufman PB (1993b) Molecular basis of the increase in invertase activity elicited by gravistimulation of oat-shoot pulvini. J Plant Physiol 142: 179-183

Wu L-L, Mitchell JP, Cohn NS and Kaufman PB (1993c) Gibberellin (GA-3) enhances cell wall invertase activity and mRNA levels in elongating dwarf pea (*Pisum sativum*) shoots. Int J of Plant Science 154: 280-289

Ylstra B, Garrido D, Busscher J and Tunen AJ (1998) Hexose transport in growing Petunia pollen tubes and characterisation of a pollen-specific, putative monosaccharide transporter. Plant Physiol 118: 297-304

Zhang L, Cohn NS and Mitchell JP (1996) Induction of a pea cell-wall invertase gene by wounding and its localised expression in phloem. Plant Physiol 112: 1111-1117

Zhang L, Cohn NS and Mitchell JP (1997) A pea cell-wall invertase gene (PsInv-1) with tissue-specific expression. Plant Physiol Bichem 35: 751-760

Zouaghi M and Rollin P (1976) Phytochrome control of β-fructosidase activity in radish. Phytochemistry 15: 897-901

Chapter 2

Novel Mode of Hormone Induction of Tandem Tomato Invertase Genes in Floral Tissues

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Novel Mode of Hormone Induction of Tandem Tomato Invertase Genes in Floral Tissues

Abstract. The genomic organisation of two extracellular invertase genes from tomato (*Lin5* and *Lin7*), which are linked in a direct tandem repeat, and their tissue-specific and hormone-inducible expression are shown. Transient expression analysis of *Lin5* promoter sequences fused to the ß-glucuronidase (GUS) reporter gene (*uidA*) demonstrates a specific expression of *Lin5* during tomato fruit development. A *Lin5* promoter fragment was fused to the truncated *nos* promoter to analyse hormone induction via GUS reporter gene activity in transiently transformed tobacco leaves. A specific up-regulation of GUS activity conferred by this *Lin5* promoter fragment in response to gibberellic acid (GA), auxin and abscisic acid (ABA) treatment was observed, indicating a critical role of the regulation of *Lin5* by phytohormones in tomato flower and fruit development. *In-situ* hybridisation analysis of *Lin7* shows a high tissue-specific expression in tapetum and pollen. These results support an important role for *Lin5* and *Lin7* extracellular invertases in the development of reproductive organs in tomato and contribute to unravel the underlying regulatory mechanisms.

INTRODUCTION

Assimilate partitioning in higher plants between source tissues, such as photosynthetically active leaves, and sink tissues, such as roots, flowers and fruits, is a tightly regulated process. The sink strength of plant tissues is not static, but it varies during plant development and in response to external and internal stimuli. Therefore, a strict regulation of hexose-supplying processes and enzymes is required. Among them, the extracellular invertase cleaves sucrose, the main transport form of carbohydrates in higher plants, irreversibly into glucose and fructose that are taken up by hexose transporters into sink cells. As sucrose cleavage is assumed to be the rate-limiting step in this process (Eschrich, 1980; Evert, 1982), extracellular invertases need to be specifically regulated to cover the actual carbohydrate demands of different sink tissues. Plant extracellular invertase genes represent gene families consisting of several members with specific expression patterns (Roitsch et al., 2000). A huge variety of external and internal stimuli regulating extracellular invertase expression, like plant hormones, sugar levels, elicitors, and wounding have been reported (for review see Tymowska-Lalanne and Kreis, 1998a; Roitsch et al., 2000). The tomato *Lin6* gene, coding for an extracellular invertase, was shown to be induced by glucose, zeatin, brassinosteroide, elicitors and wounding (Godt and Roitsch, 1997; Goetz et al., 2000). Gibberellic acid (GA), auxin and ethylene are also known as regulators of extracellular invertases (Wu et al., 1993; Weil and Rausch, 1990; Linden et al., 1996).

In many plant species several genes coding for extracellular invertases have been cloned and analysed. It was shown that isoforms are expressed in a highly tissue-specific manner. In particular, several flower-specific isoforms are known, thus indicating an important role for extracellular invertases in flower development. Tymowska-Lalanne and Kreis (1998b) reported on a flower-specific expression of an extracellular invertase in *Arabidopsis thaliana*. *InvGE* and *invGF*, extracellular invertases in potato, are expressed in floral tissues/vegetative buds and in pollen, respectively (Maddison et al., 1999). A flower bud-specific isozyme in carrot was analysed by Lorenz et al. (1995). Extracellular invertase expression in developing anthers was shown for *Lilium longiflora* (Clément et al., 1996; Ranwala and Miller, 1998), *Vicia faba* (Weber et al., 1996), maize (Xu et al., 1996; Kim et al., 2000), and tobacco (Goetz et al., 2001).

In a recent study on tobacco, the essential function of an extracellular invertase, Nin88, in the development of the male gametophyte was shown (Goetz et al., 2001). *Nin88* expression follows a distinct spatial and temporal pattern during pollen development and maturation. Tissue-specific antisense repression of *Nin88* via its own tapetum and pollen-specific promoter turned out to be an efficient method to engineer male-sterile plants by

reducing the carbohydrate supply of tapetum tissue and pollen, thereby blocking pollen development.

In this study we describe full genomic sequences and expression patterns of two genes for extracellular invertases in tomato, *Lin5* and *Lin7*, which are organised in a direct tandem repeat. For the first time an induction of the *Lin5* promoter by growth stimulating hormones as GA and auxin, the stress hormone ABA and its regulation during fruit development is shown. The tissue-specific expression patterns of both genes in tomato flower organs and fruit (*Lin5*) and tapetum/pollen grains (*Lin7*) pinpoint the critical role of extracellular invertases in the development of reproductive organs in plants.

MATERIALS AND METHODS

Cloning and sequence analysis

Genomic sequences of *Lin5*/*Lin7* were cloned using the Genome Walker System (Clontech, Heidelberg, Germany) according to the instructions of the manufacturer. Briefly, genomic DNA was isolated from tomato leaves (*Lycopersicon esculentum* cv. Moneymaker) with the NucleoBond System (Macherey und Nagel, Düren, Germany) and digested with different restriction enzymes, to gain corresponding libraries. Beside the recommended restriction enzymes, *Hpa*I, *Sma*I, *Ssp*I and *Eco72*I were used to create additional libraries. After ligation with the adaptors provided, the libraries were used as template for nested PCR. Starting out of a known region of exon I of *Lin5* (accession number AJ 272304) and exon III of *Lin7* (X91391), several sequential walks were carried out. At each stage an overlap of up to 220 bp of identical sequence of the preceding genome walk fragment confirmed the identity of successive clones. Clone 5-3-1, covering sequences of *Lin5* exon I to III, was obtained by PCR on genomic DNA using reverse and forward primers that were designed according to a database sequences of *Lin5* (accession numbers X91389 and AJ272304). To get the complete genomic sequence and 3´ flanking regions of *Lin7* (Clone 7-G-4), one 3´ walk was performed. Clones, corresponding primer sequences and the libraries used are shown in Table 1.

The PCR products obtained were cloned with the Ins T/A clone PCR Product Cloning Kit (MBI-Fermentas, St. Leon-Rot, Germany) and sequenced by SEQLAB (Göttingen, Germany). For sequence analysis the Vector NTI Suite (Infor Max, Bethesta, USA) was used. Putative regulatory elements were identified by sequence alignments to known *cis*-acting elements.

Clone	gene specific primers (GSPs) in 5´to 3´orientation		Library
5-5-3	GSP 1	GCCGTATTTTTCTTACGCGTGACTTCTC	*EcoR*V
	GSP 2	AAAGAGAAGAGCGAGGAGGGGTCATGTG	
5-4-1	GSP 1	CATACCATTAATCCAATGTTTAGGAGGTTG	*Eco*72I
	GSP 2	CACTAATAGCACTTGAAGATTGCAAGTCC	
5-3-1	forward	GGACTTGCAATCTTCAAGTGCTATTAGTG	genomic DNA
	reverse	CAGATAGGTTAGCCGGGATGGCGTAGTTC	
7-3-1	GSP 1	CTCAATTTGGAGAAGATGTGAACTTCGATC	*Ssp*I
	GSP 2	ACCTTCTAGCATCTGAGCACATGAGAATC	
7-1-4	GSP 1	CTTCGTCATACTTTAGGTGCCCTACCG	*Eco*72I
	GSP 2	CTCGTGGTACAATCGATTCGATATCG	
7-1-1	GSP 1	GGTAGGATGGTTGCTGATCCAGACCAGG	*EcoR*V
	GSP 2	CCGAATTGATCAAATGGCTTTGATGGGT	
7-G-4	GSP 1	CGGTGAGGAACACAAGTACGTGCTGAAG	*Hpa*I
	GSP 2	CTCAATTTGGAGAAGATGTGAACTTCGATC	

Table 1. Primer sequences used for PCR based cloning of *Lin5/Lin7* direct tandem repeat. Libraries that yielded the corresponding PCR fragment are indicated. Clones are detailed in Figure 1. GSP1 = gene specific primer 1, GSP2 = gene specific primer 2 (nested primer).

Plasmid construction

For transient GUS analysis the *nos* truncated promoter was cut out of the plasmid pLP62 with the restriction sites *EcoR*I and *Kpn*I and subcloned with these restriction enzymes in pPR97 upstream of the *uidA* gene. This vector (pPR97-62) was used to clone a *Lin5* promoter fragment that was released from clone 5-5-3 by *Hind*III/*Xba*I digest upstream of the truncated *nos* promoter yielding construct pPR97-62-5.

To analyse the *Lin5* full length promoter a GUS reporter gene construct was designed by a three fragment ligation using the *Hind*III-*BseR*I fragment excised from clone 5-5-3 and a *BseR*I-*Xba*I fragment that was obtained by PCR re-amplification on a plasmid-carrying fragment 5-4-1. The reverse primer (5´-TCTAGTCTAGATTCCATTTTTTTTTTTGTGATTTTGGAG-3´) was designed to introduce an *Xba*I restriction site 3´ of *Lin5* translation start site, thereby fusing *Lin5* start codon in frame to the *uidA* translation start. As forward primer the adaptor primer 2, provided by the GenomeWalker System (Clontech) was used. *Hind*III-*BseR*I and *BseR*I-*Xba*I fragments were cloned in pPR97 with *Hind*III/*Xba*I restriction sites yielding construct pPR97-5fl (Figures 1and 2).

Plant materials

For transient expression assays *Nicotiana tabacum* (cv. Samsun) and *Lycopersicon esculentum* (cv. Micro-Tom) plants were grown under greenhouse conditions at 25 °C with 16 h of light and 8 h of darkness. Plants were transferred to the growth chamber (25 °C, 16/8 light/dark cycle) two days before the onset of experiments.

To perform *in-situ* hybridisation, *Lycopersicon esculentum* (cv. Moneymaker) plants were grown under greenhouse conditions as described above.

Preparation of Agrobacterium suspension and agroinfiltration

Agrobacterium tumefaciens strain LBA 4404 containing corresponding constructs were treated as described by Yinong et al. (2000) using kanamycin (100 mg/l) and streptomycin (100 mg/l) as selection markers. Leaf infiltrations of 6-week old tomato plants were carried out as described by Yinong et al. (2000). The construct pPR97-62-5 and the empty vector pPR97-62 were infiltrated in one and the same leaf. Infiltration areas of different constructs were separated by the middle vein. Each set of induction data (water, GA, Abscisic acid (ABA), 2,4-dichlorophenoxyacetic acid (2,4-D) infiltration) was derived from explants of one leaf.

Red and green tomato fruits were equally infiltrated with *Agrobacteria*-containing construct pPR97-5fl using a syringe applied with a thin needle to minimise wounding as described by Spolaore et al. (2001). Infiltrated fruit were kept on the plant, and after two days GUS activity was determined.

Hormone treatment

Two days after agroinfiltration tobacco leaf discs of ca. 6 cm² were excised and infiltrated via a needle less syringe with hormone (GA_3, ABA, 2,4-D, each 10μM; Sigma, St. Louis, MO) and control solutions (water). Leaf disks were kept for 15 h in petri dishes applied with two layers of moistened filter paper and sealed with Parafilm. For incubation, the same growth chamber conditions were used as for growing the plants.

Determination of GUS activity

Leaf discs were reduced to 3 cm² by cutting the outer parts of the disc (to remove the wounded areas), collected in 1.5 ml eppendorf tubes and ground in 300 µl GUS extraction buffer (50 mM sodium phosphate pH 7, 10 mM Na_2 EDTA, 0.1 % Triton X100, 0.1 % sodium

lauroyl sarcosine, 10 mM 2-mercaptoethanol). About 600 mg of tomato fruit explants were ground in 300 µl GUS extraction buffer. After centrifugation for 10 min (15000 x g) at 4°C, 100 µl of supernatant was mixed with 100 µl GUS assay solution (2 mM 4-methylumbelliferyl-D-glucuronide in extraction buffer). 50 µl were immediately removed and transferred to stop solution (final concentration 0.3 M Na_2CO_3) to be used as control. The rest of the mixture was incubated at 37 °C for 1 h and stopped in 0.3 M Na_2CO_3. GUS activity was determined using a luminescence spectrometer (Perkin Elmer LS 30, Langen, Germany) and protein concentration of tissue homogenates was determined with the Bradford reagent (Bradford, 1976) using bovine serum albumin as standard.

In-situ hybridisation

Small flower buds and dissected anthers from open flowers were fixed with 3% (w/v) paraformaldehyde/0.2 % (v/v) glutaraldehyde in PBS (135 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4). After dehydration by a graded series of ethanol, material was embedded in Paraplast (Sigma, Deisenhofen, Germany). Cross-sections (8 µm thick) were mounted on poly-L-lysine coated slides, deparaffinised and rehydrated. After rinsing in 10 mM Tris-HCl (pH 8.0), sections were incubated with 20 µg/ml Proteinase K (in TE) at 37 °C for 20 min and then blocked with 1% bovine serum albumin (BSA) in 10 mM Tris-HCl (pH 8.0) for 1 h. After acetylation, sections were dehydrated by graded series of ethanol and air-dried. For hybridisation a solution of 5x SSC, 0.5 % SDS, 5x Denhardt's reagent, 50% formamide, and 200 U/ml RNase inhibitor containing denaturated DIG-labelled sense or antisense RNA was applied and sections were incubated in a humid box at 45°C overnight. After incubation with 20 µg/ml RNase A at 37°C for 30 min, sections were washed three times with 0.2x SSC at 50°C for 30 min each. Immunological detection of DIG-labelled RNA hybrids was performed with anti-DIG-fab fragment conjugated with alkaline phosphatase (Roche Diagnostics, Mannheim, Germany) according to the supplier's protocol. Staining procedure was performed with NBT and BCIP. Finally, sections were analysed by bright field microscopy with an Axioskop microscope (Zeiss, Jena, Germany). Pictures were taken by a CCD camera (Sony, Japan) and processed through the Photoshop 4.0 program (Adobe, Seattle, MA).

RESULTS

Cloning of Lin5 / Lin7 direct tandem repeat

In order to isolate the 5' sequences responsible for regulating expression of *Lin5* and *Lin7*, 5' genome walks were performed on genomic libraries of tomato starting from exon I of *Lin5* and exon III of *Lin7*. In addition, the full length of the *Lin7* gene was obtained by a 3' walk starting at exon III. *Lin5* full-length sequence as obtained by PCR on genomic DNA with primer corresponding to exon I and exon III. In total, 13 kb of genomic sequence were cloned and analysed representing *Lin5* and *Lin7* genes including 5´- and 3´-flanking sequences. Interestingly, we found that *Lin5* and *Lin7* are organised in a direct tandem repeat with *Lin5* located 5´and *Lin7* located 3´ (Figure 1). These genes are separated by a region of 1.5 kb that contains the *Lin7* promoter.

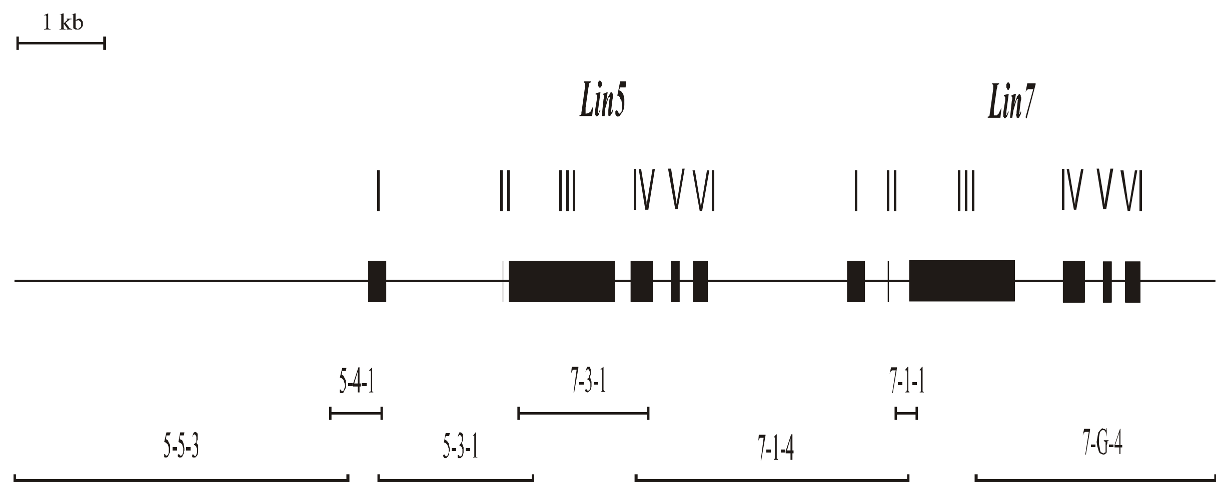

Figure 1. Exon/Intron organisation of tomato *Lin5/Lin7* tandem invertase genes. Exons are depicted by black boxes and exon numbers are shown. Below obtained clones are indicated. All clones, except 5-3-1 (PCR on genomic DNA) and 7-G-4 (3´walk), were achieved by sequential 5´walks, starting out of known sequences (*Lin5* Exon I and *Lin7* Exon III).

Exon/intron organisation and deduced amino acid sequence of Lin5 and Lin7

Sequence comparison with known invertase genes revealed that both genes are composed of six exons (Figure 1). This structure differs from the majority of plant invertase genes which contain seven exons (Tymowska-Lalanne and Kreis, 1998a), two of these exons are fused yielding exon III in *Lin*5 and *Lin7*. A similar gene structure and remarkable identity was found to two potato genes, *invGE* and *invGF*. These genes are also arranged in

tandem with a spacer region of 1.7 kb and are composed of six exons. Corresponding genes show high sequence identities of 90% for *Lin5/invGE* and 91% for *Lin7/invGF,* while the sequence identity of *Lin5* and *Lin7* is 76%. A detailed comparison is shown in Table 2. *Lin5* and *Lin7* show a 9 bp mini-exon II that is characteristic for plant invertase genes; this mini-exon is 100% identical in sequence between the corresponding potato and tomato genes (*Lin5/invGE*; *Lin7/invGF*). The length of four out of five exons of the corresponding genes are identical, the exceptions being exon III for *Lin5/invGE* and exon I for *Lin7/invGF*. The amino acid sequences of *Lin5* and *Lin7* are 70 % identical. *Lin5/invGE* and *Lin7/invGF* show an amino acid identity of 88% and 94% respectively. All four invertases show the same WECPD motive that is characteristic for extracellular invertases (Goetz and Roitsch, 1999). Interestingly, the putative N-terminal signal sequence of *Lin7* shows two additional amino acids compared to *invGF.*

It is noteworthy that even intron sequences are highly conserved between the tomato and potato genes but show partly substantial differences in length (Table 2).

	Size (bp)	Nucleotide identity (%)	Size (bp)	Nucleotide identity (%)	Size (bp)	Nucleotide identity (%)	Size (bp)
	invGE	*invGE/Lin5*	*Lin5*	*Lin5/Lin7*	*Lin7*	*Lin7/invGF*	*invGF*
Exon I	199	94	199	73	199	93	193
Exon II	9	100	9	78	9	100	9
Exon III	1025	88	1022	76	1019	87	1019
Exon IV	245	93	245	79	245	92	245
Exon V	100	97	100	70	97	98	97
Exon VI	180	89	180	73	183	93	183
Intron I	1450	73	1286	12	257	65	219
Intron II	108	52	77	13	333	24	108
Intron III	296	48	179	13	727	8	89
Intron IV	198	78	189	27	98	76	86
Intron V	128	86	131	32	75	63	109

Table 2. Exon and intron similarity of two direct tandem repeats of invertase genes (*invGE/Lin5*, *Lin5/Lin7* and *Lin7/invGF*) in tomato and potato. *InvGE* and *invGF* sequences were derived from the NCBI database (accession number AJ133765).

Defining putative TATA-box and transcription start site in the Lin5 and Lin7 promoters

Putative TATA-box sequences in the *Lin5* and *Lin7* genes are located at the same position (-35, position relative to putative transcription start site) and identical for both promoters (TATAAA). Sequence alignments of 150 bp 5´of the start codon show high sequence identity between *Lin5/invGE* (95%) and *Lin7/invGF* (84%). Sequences bordering the putative transcription start site are identical in corresponding promoters and match with

reported consensus sequences (Joshi, 1987). The length of 5´-UTRs (*Lin5* 41 bp/ *invGE* 40 bp and *Lin7* 109 bp/ *invGF* 101 bp) are similar and show common adenosine-rich tracts (A_4TCACA_{10} for *Lin5* and $A_{13}TTA_8$ for *Lin7*) that have been reported for the pollen-specific promoter of the *lat52* gene in tomato (Bate et al., 1996).

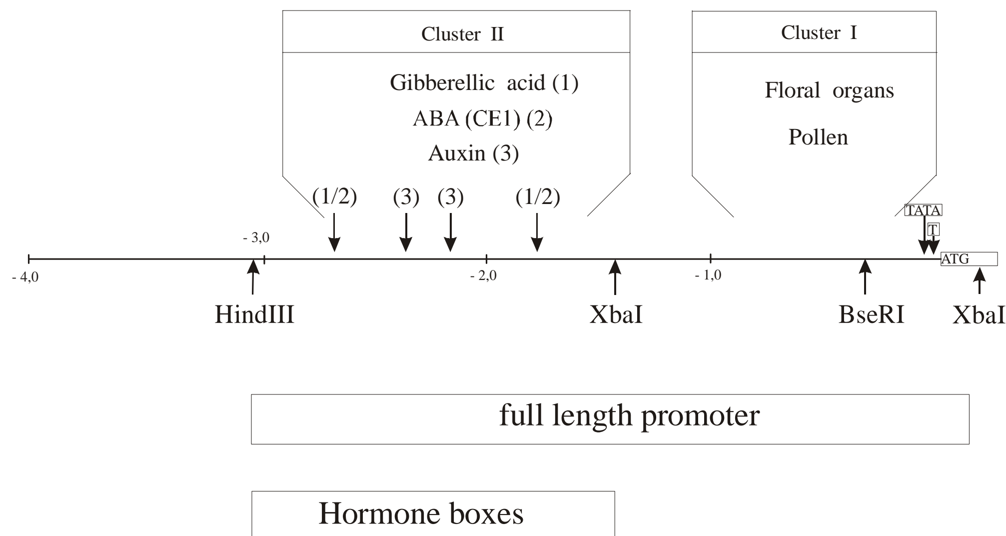

Figure 2. *Lin5* promoter structure. Putative *cis*-acting elements and their clustering are indicated. A putative TATA-box, transcription start site (T), and restriction sites used for different cloning strategies are shown. Promoter fragments used for reporter gene constructs are indicated below.

Lin5 and Lin7 promoter sequences show similarity to known cis-acting elements

Both promoters were screened for the presence of regulatory elements with similarity to known *cis*-acting elements via sequence alignments. The *Lin5* promoter revealed sequences with similarity to floral- and pollen-specific elements. Interestingly, these boxes were clustered in specific promoter regions, with floral and pollen boxes being located mostly within a region up to position -1 kb (cluster I, Figure 2) and hormone response boxes being located within 1.1 kb at positions -2757 to -1658 (cluster II). In cluster I, two identical sequences to the pollen-specific element AAATGA from tobacco (Weterings et al., 1995) are located at positions -298 and -223 relative to the transcription start site. Further pollen-specific elements with high similarity (not more than one base exchange) to the GTCAAA motive in rice (Zou et al., 1994) were detected at positions -1467 and -736. Further, two sequences that show 1 bp exchange/are identical to floral-specific elements are located in 3´-5´orientation at positions -65 (AAAAAACGGTTA, Solano et al., 1995) and -995 (CCTACC, Grotewold et al., 1994). These observations corroborate data of Godt and Roitsch (1997) that show Lin5 expression in different floral tissues, including the tapetum. Cluster II contains several hormone-response boxes. Putative GA-response elements are shown in detail in

Figure 3 in comparison to the GA-response complex of the α-amylase promoter. At -2410 and -2223 two TGTCTC sequences that are described as auxin-response elements (Ulmasov et al. 1999) are located. CE1 box core elements (CACC) that are obligatory for ABA responsiveness (Shen and Ho, 1995) were detected close to GA-response boxes (data not shown) but they are lacking the second *cis*-acting element necessary for high-level ABA induction (G-box elements) in close proximity.

The *Lin7* promoter revealed sequences with similarity to different *cis*-acting elements that have been reported to confer tissue-specific expression in pollen. The pollen-specific element TGTGGT from tomato (Twell et al., 1991) could be found in the *Lin7* promoter with just 1 bp exchange at position -1175. Four elements with no more than 1 bp exchange within the very 5´or 3´ nucleotides compared to the tobacco pollen-specific element AAATGA (Weterings et al., 1995) are located at -1180, -813, -759 and -245. Furthermore, two highly similar/identical sequences to the rice pollen-specific element GTCAAAA are present in the *Lin7* promoter at -372 and -277. The presence of these promoter elements fits in with the reported expression of *Lin7* in anthers (Godt and Roitsch, 1997) and tapetum and pollen reported in the present study (see below).

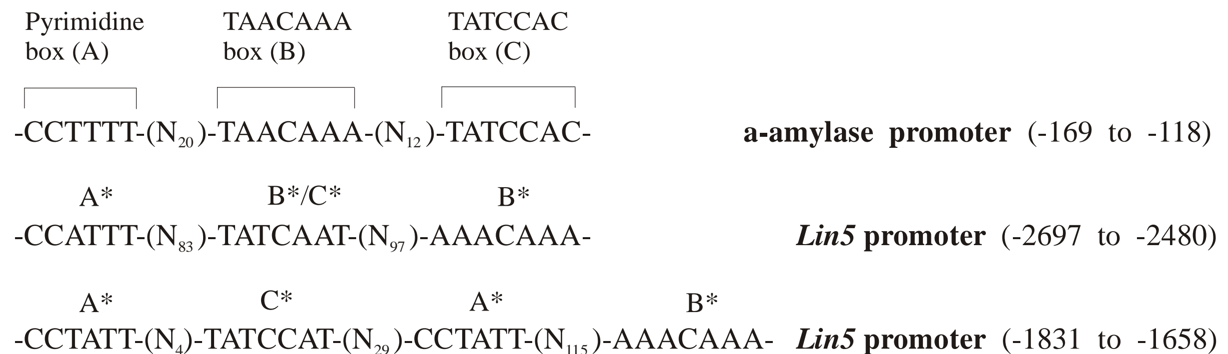

Figure 3. Alignment of GA response complexes of the α-amylase promoter (Gubler et al., 1995) and *Lin5* promoter. Similar boxes in the *Lin5* promoter are indicated with asterisks (*). Numbers in brackets show the position relative to the putative transcription start site.

Lin7 shows a specific spatial and temporal expression pattern

To localise the expression of *Lin7* at different stages of flower development, anthers of tomato plants (*L. esculentum* cv. Moneymaker) were analysed in RNA *in-situ* hybridisation studies. *Lin7* mRNA could be detected in the tapetal cell layer of flower buds, as well as within the cell layers beneath the degraded tapetum cells and pollen grains of open flowers (Figure 4). This spatial and temporal expression pattern indicates an essential function of *Lin7* in the development of the male gametophyte.

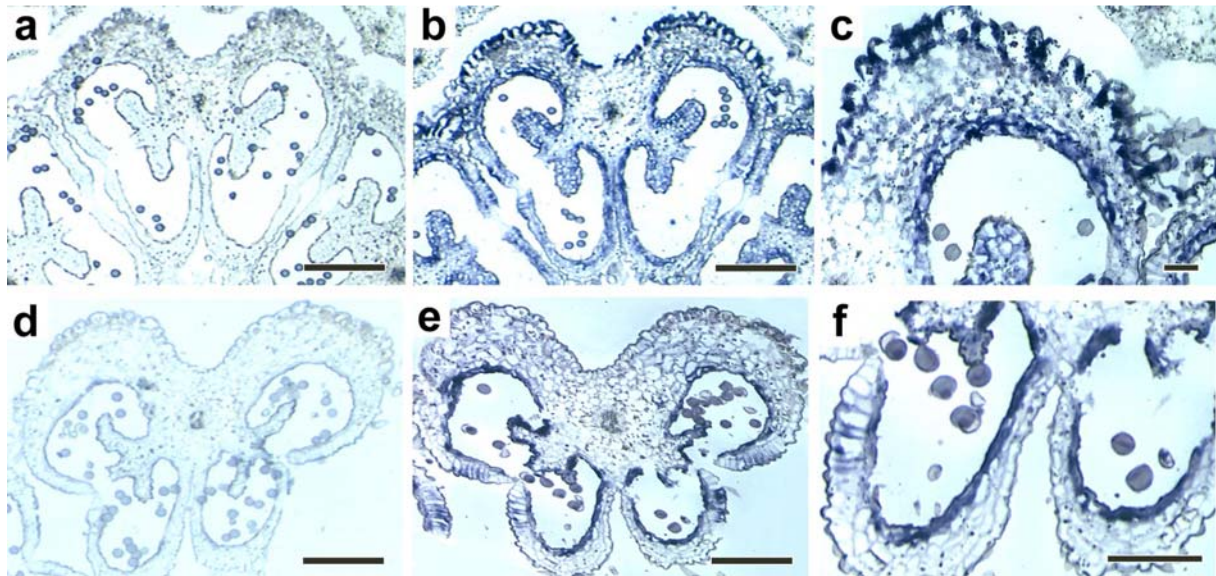

Figure 4. Localisation of transcript accumulation of *Lin7* in tomato anthers. Cross sections of young flower buds (a-c) and of anthers of open flowers (d-e) were processed for *in-situ* hybridisation using DIG-labelled sense (a, d) or antisense (b, c, e, f) RNA. Note the occurrence of positive staining within the tapetum of flower buds (b, c) as well as within the cell layers beneath the degraded tapetum cells and pollen grains of open flowers (e, f). Negative controls performed by using DIG-labelled sense probe do not exhibit label (a, d). Bars represent 200 µm for a, b, d, and e, and 50 µm for c and f.

GUS reporter gene assays show high Lin5 expression in non-mature, green fruits but a reduced expression in mature, red tomato fruits

For analyses of the *Lin5* promoter 3 kb, promoter sequence, including the *Lin5* start codon, were cloned in the vector pPR97 to drive GUS reporter gene expression. The obtained *Lin5* promoter GUS fusion (pPR97-5fl) was transformed into *Agrobacteria* used for transient expression analysis in tomato fruits. In non-mature, green tomatoes (1cm diameter) GUS activity reached up to 2600 pmol MU per mg protein per min. In mature red tomatoes GUS activity was significant lower (up to 40 pmol MU mg $protein^{-1}$ min^{-1}). These results are in agreement with RT-PCR data from Fridman et al. (2002) that show highest *Lin5* expression in non-mature, green and a reduced expression in mature red tomato fruits.

A Lin5 promoter fragment confers GA, auxin and ABA inducibility

In order to analyse the hormone regulation of *Lin5*, a fragment of the *Lin5* promoter that covers the detected GA-, auxin- and ABA-response boxes was cloned upstream of a truncated *nos* promoter-GUS fusion (pPR97-62-5). The vector pPR97 was specifically designed for transient expression analysis, as the *uidA* gene implies an intron sequence to restrict GUS expression to transformed plant cells (Szabados et al., 1995). pPR97-62-5 was used for transient *Agrobacteria*-mediated transformation of tobacco leaves followed by the

analysis of explants for hormone inducibility. As controls, plants were transiently transformed with *uidA* under the control of the truncated *nos* promoter (pPR97-62). As shown in a representative experiment (Figure 5), the *Lin5* promoter fragment conferred a more than 3-fold increase of GUS activity upon treatment with 10 µM GA_3 and a 2-fold increase upon treatment with 10 µM 2,4-D. Treatment with ABA resulted in a small though reproducible reporter gene induction. The weak inducing effect of ABA may be related to the fact that CE1 elements confer a high-level ABA induction only in combination with G-box elements (Shen et al., 1995) that were not present in close proximity to CE1 elements in the *Lin5* promoter. The control showed much lower GUS expression than in pPR97-62-5, and no significant effects of hormone treatment on the expression level of the truncated *nos* promoter were observed. Based on four independent transformations the average hormone inducibility and corresponding standard deviations on pPR97-62-5 and pPR97-62 were determined. Relative to water-infiltrated samples, an increase in GUS activity of 283% ± 29% after GA_3 treatment, 180% ± 12% after 2,4-D treatment and 134% ±21% after ABA treatment was observed for pPR97-62-5. The control (pPR97-62) showed a rather constant expression pattern. Relative to water-infiltrated samples, an increase in GUS activity of 130% ± 27% (GA_3 treatment), 116% ± 25% (2,4-D treatment) and 118% ± 36% (ABA treatment) could be detected with pPR97-62. This data show that the enhancement of GUS expression after application of corresponding hormone solutions is due to the *Lin5* promoter fragment that was cloned 5´of the truncated *nos* promoter.

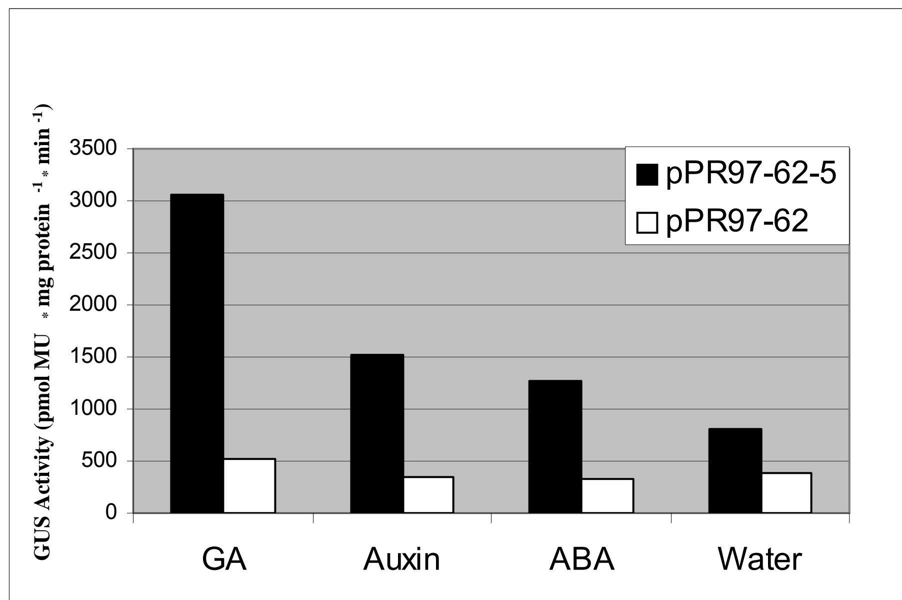

Figure 5. Hormonal regulation of the *Lin5* promoter. Explants of tobacco leaves, transiently transformed with a *HindIII-XbaI* fragment of the *Lin5* promoter fused to a *nos* truncated promoter GUS construct (pPR97-62-5), were treated with hormone solutions (GA_3, ABA, 2,4-D, 10µM each) for 15 h. Water infiltration was used as control. GUS activity of crude extracts was determined fluorometrically using 4-methylumbelliferyl-D-glucuronide as substrate. The vector control pPR97-62 served to rule out an induction conducted by the truncated *nos* promoter. MU = 4-methylumbelliferone.

DISCUSSION

Pollen-specific promoters represent high-potential tools in plant engineering

To investigate the regulation of carbon partitioning, the promoter regions of two genes coding for extracellular invertases in tomato and the tissue specificity and hormonal regulation of expression were analysed.

The most striking feature of the *Lin7* promoter is the occurrence of pollen- and flower-specific elements. Previous northern blot data showed that *Lin7* expression is restricted to the anthers (Godt and Roitsch, 1997). The inspection of the tissue and cell specificity of *Lin7* expression by *in-situ* hybridisation revealed its expression in pollen grains and tapetum. Anther- and pollen-specific extracellular invertases are known throughout various plant species (documented in Introduction), which points towards an important regulatory function in pollen development. In *Lilia* it was shown that carbohydrates play a critical role in anther and pollen development because they function as nutrients to sustain growth, as well as a signal to influence developmental processes (Clément et al., 1996).

In a recent study, the essential function of an extracellular invertase, Nin88, in pollen development of tobacco was shown in detail (Goetz et al., 2001). *Nin88*, like *Lin7*, exhibits a specific expression pattern during tapetum and pollen development. Tissue-specific antisense repression of *Nin88* caused male sterility by reducing the carbohydrate supply of tapetum and pollen. Due to the tissue-specific expression of *Nin88* in tapetum and pollen there was no effect on other plant organs. This offers the possibility to use the *Nin88* promoter to engineer male-sterile plants for hybrid seed production. Now, the corresponding tapetum- and pollen-specific promoter of *Lin7* cloned from tomato offers the possibility to manipulate pollen development in tomato, a plant with high economical relevance in means of vegetable production.

Tissue-specific expression and hormone regulation indicate a crucial role of Lin5 in floral and fruit development

Sequence analysis of the *Lin5* promoter revealed sequences with high identities to flower- and pollen-specific elements, besides hormone-response boxes (GA, auxin, ABA (CE1)). A *Lin5* promoter-GUS fusion was used to analyse the expression mediated by the *Lin5* promoter. Expression in green fruits was higher than expression in mature, red fruits. This is in agreement with northern blot and RT-PCR data which showed that *Lin5* expression, is restricted to reproductive organs and is altered during tomato fruit

development (Godt and Roitsch, 1997; Fridman et al. 2002). High expression was found in early stages of tomato fruit development while mature, red tomatoes show a significant lower *Lin5* expression. Our data using the *Lin5*-GUS fusion indicate that the tested promoter region is sufficient to confer developmental regulation of *Lin5* gene expression.

In addition to this tissue-specific expression, a 1.6 kb *Lin5* promoter fragment was shown to confer hormone inducibility (GA, auxin, ABA) to a truncated *nos* promoter-GUS fusion. A distinct up-regulation of reporter gene activity after applying GA_3 and 2,4-D was observed, suggesting that GA and auxin responsiveness of the *Lin5* promoter are mediated via this promoter region.

The inducibility of *Lin5* gene expression by GA and auxin, in combination with the flower-specific expression of *Lin5*, gives new insights into the mechanism underlying the regulation of flower development. Because of the regulation of developmental and growth processes by auxin and its role in fruit development (reviewed by Berleth and Sachs, 2001), *Lin5* induction by auxin could mediate a higher carbohydrate supply of developing flower and fruit tissue, especially in early developmental stages. Similarly, gibberellins are regulating growth and differentiation processes, especially in respect to floral tissues and seeds (Richards et al., 2001). Thus, the inducibility of the *Lin5* promoter by GA indicates that GA effects might be related to extracellular invertase activity during flower development. This is supported by the temporal expression pattern of genes encoding GA 20-oxidases, enzymes that are presumably sites of regulation of the GA biosynthesis (Rebers et al., 1999), during flower bud and early fruit development. One of this enzymes, Le20ox-3, exhibits increasing transcription levels in the senescing flower, the highest expression level being detected in immature fruits. Abundant transcript levels in immature fruit were also detected for Le20ox-1.

This tissue-specific expression pattern of GA 20-oxidases matches with *Lin5* data, giving a further indication to the close regulation of *Lin5* by GA during flower development.

Further investigations are needed to elucidate the molecular basis of *Lin5* induction by GA in detail and its contribution to flower and early fruit development.

A tandem organisation of invertase genes seems to be a common feature in higher plants

Lin5 and *Lin7* are organised in a direct tandem repeat. A similar tandem organisation of two invertase genes, *invGE/invGF*, showing more than 90% identity in nucleotide sequence to *Lin5/Lin7*, has been found in potato (Madisson et al. 1999). All four genes of these homologous tandem repeats in tomato and potato show the same 6-exon structure that differs from most other plant invertase genes which are composed of 7 exons. Interestingly, corresponding genes of the potato tandem *invGE/invGF* show similar expression patterns to *Lin5/Lin7*. In both species one gene encoding an extracellular invertase shows a relatively broad expression pattern (*Lin5/invGE*) and one gene with an

extremely specified expression pattern does exist (*Lin7/invGF*). *Lin5* is expressed in reproductive organs such as tomato flower and fruit (Godt and Roitsch, 1997). I*nvGE* is expressed in flower tissues, too, but additionally in vegetative organs as lateral nodes in stem, roots and tubers. *Lin7* and *invGF* expression is restricted to tapetum and pollen grains. The high homology in sequence and expression patterns between those genes points towards similar functions of corresponding extracellular invertases in tomato and potato, especially in regard of *Lin7/invGF*. One might assume that the tandem structure originates from a gene duplication of a more generally expressed extracellular invertase gene, assigning a very specific expression to a further gene in order to cover sugar supply of developing pollen.

A tandem organisation of extracellular invertase genes does also exist in the genome of *Arabidopsis thaliana*. A database search revealed that 2414 bp 3´ of *Atßfruct1* (At3g13790) a further putative extracellular invertase gene (At3g13784) is located (Figure 6). *Atßfruct1* was shown to be expressed in root, stem and leaves (Tymowska-Lalanne et al., 1996), thus exhibiting again a rather broad expression pattern of a 5´ located gene. The 3´ located invertase has not been characterised yet. Because pollen-specific elements, similar to those of *Lin7* and *invGF*, are located within its promoter region, it is tempting to speculate that this gene shows a similar expression pattern as *Lin7* and *invGF*. Further investigations are needed to confirm that the expression pattern of these tandem genes is conserved between different plant families. If this expression pattern turns out to be a common theme in plants, the corresponding tapetum- and pollen-specific genes of additional plant species could be easily identified by searching for the 3´ gene within the invertase tandem gene structure.

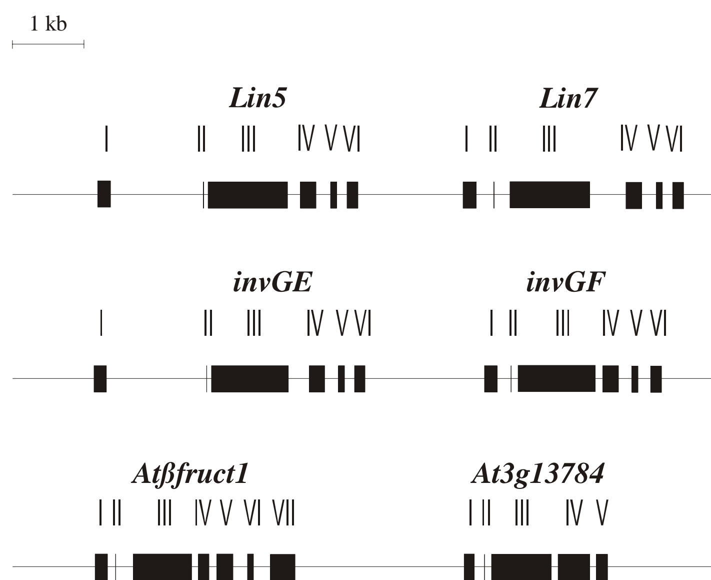

Figure 6. Genomic organisation of three tandem invertase genes that are found in tomato, potato and *Arabidopsis thaliana*. Sequence data for the potato tandem *invGE/invGF* are derived from the NCBI database (accession number AJ133765), the *Arabidopsis* tandem structure was derived from the *Arabidopsis* Genome Database (At3g13790, At3g13784). The structure of the putative mini-exon II of At3g13784 was assembled according to sequence alignments with *Atßfruct1* (At3g13790).

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Note added in proof:

A recent publication by Sherson et al. (Sherson, S.M., Alford, H.L., Forbes, S.M., Wallace, G. and Smith, S.M. 2003. J. Exp. Bot. 54: 525-531) further supports the functional and sequence conservation of tandem invertase genes. The two *Arabidopsis* tandem invertases *Atßfruct1* (AtcwINV1, At3g13790) and At3g13784 (AtcwINV5) show the highest sequence homology among all six cell-wall invertases identified. In addition, the 3´-gene of the *Arabidopsis* invertase tandem (At3g13784) shows the same solely flower specific expression as the corresponding orthologs from the tomato and potato invertase tandems, respectively.

The nucleotide sequence data reported will appear in the GeneBank database under the accession number AY173050.

REFERENCES

Bate, N., Spurr, C., Foster, G.D. and Twell, D. 1996. Maturation-specific translational enhancement mediated by the 5´UTR of a late pollen transcript. Plant J. 10: 613-623.

Berleth, T. and Sachs, T. 2001. Plant morphogenesis: long-distance coordination and local patterning. Curr. Opin. Plant Biol. 4: 57-62.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Clément, C., Burrus, M. and Audran, J.-C. 1996. Floral organ growth and carbohydrate content during pollen development in *Lilium*. Am. J. Bot. 83: 459-469.

Eschrich, W. 1980. Free space invertase, its possible role in Phloem unloading. Ber. Dtsch. Bot. Ges. 93: 363-378.

Evert, R.F.1982. Sieve-tube structure in relation to function. Bioscience 32: 789-795.

Fridman, E., Liu, Y.S., Carmel-Goren, L., Gur, A., Shoresh, M., Pleban, T., Eshed, Y., Zamir, D. 2002. Two tightly linked QTLs modify tomato sugar content via different physiological pathways. Mol. Genet. Genomics 266: 821-826.

Godt, D.E. and Roitsch, T. 1997. Regulation and tissue -specific distribution of mRNA for three extracellular invertase isoenzymes of tomato suggests an important function in establishing and maintaining sink metabolism. Plant Physiol. 115: 273-282.

Goetz, M. and Roitsch, T. (1999) The different pH-optima and substrate specificities of extracellular and vacuolar invertases are determined by a single amino acid substitution. Plant J. 20: 707-711.

Goetz, M., Godt, D.E. and Roitsch T. 2000. Tissue-specific induction of the mRNA for an extracellular invertase isoenzyme of tomato by brassinosteroids suggests a role of steroid hormones in asssimilate partitioning. Plant J. 22: 515-522.

Goetz, M., Godt, D.E., Guivarch, A., Kahmann, U., Chriqui, D. and Roitsch, T. 2001. Induction of male sterility in plants by metabolic engineering of the carbohydrate supply. Proc. Natl. Acad. Sci. USA 98: 6522-6527.

Grotewold, E., Drummond, B.J., Bowen, B. and Peterson, T. 1994. The *myb*-homologous P gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. Cell 76: 543-553.

Gubler, F., Kalla, R., Roberts, J.K., and Jacobsen, J.V. 1995. Gibberellin-regulated expression of a *myb* gene in barley aleuron cells: evidence for myb transactivation of a high-pI α-amylase gene promoter. Plant Cell 7: 1879-1891.

Joshi, C. P. 1987. An inspection of the domain between putative TATA box and Translation site in 79 plant genes. Nuc. Acid. Res. 15: 6643-6653.

Kim, J.-Y, Mahé, A., Guy, S., Brangeon, J., Roche, O., Chourey, P.S. and Prioul, J.-L. 2000. Characterization of two members of the maize gene family, *Incw3* and *Incw4*, encoding cell-wall invertases. Gene 245: 89-102.

Linden, J.C., Ehneß, R. and Roitsch, T. 1996. Ethylene regulation of apoplastic invertase expression in autotrophic cells of *Chenopodium rubrum*. Plant Growth Reg. 19: 219-222.

Lorenz, K., Lienhard, S. and Sturm, A. 1995. Structural organization and differential expression of carrot β-fructofuranosidase genes: identification of a gene coding for a flower bud-specific isoenzyme. Plant Mol. Biol. 28: 189-194.

Maddison, A.L., Hedeley, P.E., Meyer, R.C., Aziz, N., Davidson, D. and Machray, G.C. 1999. Expression of tandem invertase genes associated with sexual and vegetative growth cycles in potato. Plant Mol. Biol. 41: 741-751.

Ranwala, A.P., and Miller, W.B. 1998. Sucrose-cleaving enzymes and carbohydrate pools in *Lilium longiflorum* floral organs. Physiol. Plant 103: 541-550.

Rebers, M., Kaneta, T., Kawaide, H., Yamaguchi, S., Yang, Y.-Y., Imai, R., Sekimoto, H. and Kamiya, Y. 1999. Regulation of gibberellin biosynthesis genes during flower and early fruit development of tomato. Plant J. 17: 341-250.

Richards, D.E., King, K.E., Ait-ali, T. and Harberd, N.P. 2001. How Gibberellin regualtes plant growth and development: A molecular genetic analysis of Gibberellin signaling. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52: 67-88.

Roitsch, T., Ehneß, R., Götz, M., Hause, B., Hofmann, M., and Sinha, A. 2000. Regulation and function of extracellular invertase from higher plants in relation to assimilate partitioning, stress responses and sugar signalling. Aust. J. Plant Physiol. 27: 815-825.

Shen, Q. and Ho, T.-H. D. 1995. Functional dissection of an abscisic acid (ABA)-inducible gene reveals two independent ABA-responsive complexes each containing a G-Box and a novel *cis*-acting element. Plant Cell 7: 295-307.

Solano, R., Nieto, C., Avila, J., Canas, L., Diaz, I. and Paz-Ares, J. 1995. Dual DNA-binding specifity of a petal epidermis-specific MYB transcription factor (MYB.Ph3) from Petunia hybrida. EMBO J. 14: 1773-1784.

Spolaore, S., Trainotti, L. and Casadoro, G. 2001. A simple protocol for transient gene expression in ripe fleshy fruit mediated by *Agrobacterium*. J. Exp. Bot. 52: 845-850.

Szabados, L., Charrier, B., Kondorosi, A., de Bruijn, F.J. and Ratet, P. 1995. New plant promoter and enhancer testing vectors. Mol. Breed. 1: 419-423.

Twell, D., Yamaguchi, J., Wing, R.A., Ushiba, J. and McCormick, S. 1991. Promotor analysis of genes that are coordinately expressed during pollen development reveals pollen-specific enhancer sequences and shared regulatory elements. Genes Dev. 5: 496-507.

Tymowska-Lalanne, Z., Schwebel-Dugué, N., Lecharny, A. and Kreis, M. 1996. Expression and cis-acting elements of the *Atßfruct1* gene from *Arabidopsis* thaliana encoding a cell wall invertase. Plant Physiol. Biochem. 43: 431-442.

Tymowska-Lalanne, Z. and Kreis, M. 1998 a. The plant invertases: Physiology, biochemistry and molecular biology. Adv. Bot. Res. 28: 71-117.

Tymowska-Lalanne, Z. and Kreis, M. 1998 b. Expression of the *Arabidopsis thaliana* invertase gene family. Planta 210: 259-265.

Ulmasov, T., Hagen, G. and Guilfoyle, T.J. 1999. Activation and repression of transcription by auxin-response factors. Proc. Natl. Acad. Sci. USA 96: 5844-5849.

Weber, H., Borisjuk, L., and Wobus, U. 1996. Controlling seed development and seed size in *Vicia faba*: a role for seed coat-associated invertases and carbohydrate state. Plant J. 10: 823-834.

Weil, M. and Rausch, T. 1990. Cell wall invertase in tobacco crown gall cells. Plant Physiol. 94: 1575-1581.

Weterings, K., Schrauwen, J., Willems, G. and Twell, D. 1995. Functional dissection of the promoter of the pollen-specific gene Ntp303 reveals a novel pollen-specific and conserved cis-regulatory element. Plant J. 8: 55-63.

Wu, L.-L., Mitchell, J.P., Cohn, N.S. and Kaufman P.B. 1993. Gibberellin (GA-3) enhances cell wall invertase activity and mRNA levels in elongating dwarf pea (*Pisum sativum*) shoots. Int. J. Plant Sci. 154: 280-289.

Xu, J., Avigne, W.T., McCarty, D.R. and Koch, K.E. 1996. A similar dichotomy of sugar modulation and developmental expression affects both paths of sucrose metabolism: evidence from maize invertase gene family. Plant Cell 8: 1209-1220.

Yinong, Y., Rugana, L., and Min, Q. 2000. *In vivo* analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. Plant J. 22: 543-551.

Zou, J.T., Zhan, X.Y., Wu, H.M., Wang, H. and Cheung, A.Y. 1994. Characterization of a rice pollen-specific gene and its expression. Am. J. Bot. 81: 552-561.

Chapter 3

GA-regulated tomato invertase *Lin7* plays an essential role in pollen germination

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Submitted to Functional Plant Biology

GA-regulated tomato invertase *Lin7* plays an essential role in pollen germination

Abstract. The tomato extracellular invertase family comprises four members with different expression patterns. Three isoenzymes, Lin6, Lin5, and Lin7 invertases, are expressed in floral tissues. Among all isoenzymes, *Lin7* expression is most specific, as it is restricted to tapetum and pollen tissue. In this manuscript we analyse the specific function of Lin7 in pollen tube formation and show evidences for a GA-mediated *Lin7* regulation. The pollen germination efficiency of pollen carrying the RNA silencing construct *Lin7*::*Lin7*-RNAi was significantly reduced relative to nontransgenic pollen, indicating an essential role of Lin7 in pollen germination. The same phenotype was shown by transgenic plants expressing *Arabidopsis* cDNA of *GA2ox2* gene, an enzyme involved in deactivation of biologically active GAs, under the control of the *Lin7* promoter. Histochemical reporter gene analysis in tobacco pollen carrying the *Lin7*::GUS transgene demonstrates *Lin7* expression in pollen and pollen tubes. Application of a GA synthesis inhibitor to *Lin7*::GUS transgenic pollen results in a decrease in GUS activity. This is the first study correlating the GA effect on pollen germination with a specific enzyme function downstream of the GA signalling pathway. An essential function of GA-regulated Lin7 invertase in pollen tube formation is discussed.

INTRODUCTION

Assimilates are produced in photosynthetically active source tissues and transported via the phloem to photosynthetically less active or inactive sink tissues. Sucrose, the major transport sugar in higher plants, is released from the sieve elements of the phloem via sucrose transporters into the apoplast. Extracellular invertase catalyses the irreversible hydrolysis of sucrose into hexose monomers. The resulting hexoses are taken up into the sink cells via hexose transporters and further sucrose is released from the phloem following the steepened concentration gradient (Eschrich 1980). Sucrose cleavage is assumed to be the rate limiting step in this process. This is reflected by complex regulation patterns that allow a strict temporal and spatial expression of extracellular invertase to cover the actual carbohydrate demand of sink tissues (Tymowska-Lalanne and Kreis 1998; Sturm 1999; Roitsch et al. 2003).

Plant extracellular invertase genes represent a gene family consisting of several members with specific expression patterns (Tymowska-Lalanne and Kreis 1998; Roitsch et al. 2003). Tissue-specific expression in combination with inducibility by distinct stimuli confers specific physiological functions to different isoenzymes (Proels et al. 2003). In particular, several flower-specific isoenzymes are known, thus indicating an important role for extracellular invertases in floral development. A flower bud-specific isozyme in carrot was analysed by Lorenz et al. (1995). Extracellular invertase expression in developing anthers was shown for *Vicia faba* (Weber et al. 1995). *InvGE* and *invGF*, extracellular invertase genes in potato are expressed in floral tissues / vegetative buds and pollen, respectively (Maddison et al. 1999). A similar expression pattern is reported for *Lin5* and *Lin7*, extracellular invertases in tomato (Proels et al. 2003). All six isoenzymes of *Arabidopsis* extracellular invertase are expressed in floral organs, albeit at different levels (Sherson et al. 2003). The identification of extracellular invertase isoenzymes that are expressed in anther and pollen tissue supports an essential function of apoplastic sucrose cleavage in providing carbohydrates to the male gametophyte. A functional link between extracellular invertase activity and anther and pollen development has been demonstrated for the tobacco invertase Nin88 (Goetz et al. 2001). Expression of a *Nin88* antisense construct under the control of the *Nin88* promoter results in a block during pollen development. This finding is in line with data on male gametophyte-specific monosaccharide transporters that are functionally linked to extracellular inertases. The presence of three monosaccharide transporters AtSTP4, AtSTP6 and AtSTP9 in *Arabidopsis* pollen (Truernit et al. 1999; Scholz-Starke et al. 2003;

Schneidereit et al. 2003) underlines the high complexity of carbohydrate supply to the male gametophyte.

Gibberellins (GAs) are essential endogenous regulators of plant growth and development. In particular GAs play various roles in growth processes of reproductive organs. In several plant species GA deficiency leads to male sterility because of abnormal anther development (Nester and Zeevaart 1988; Goto and Pharis 1999). GAs are present in developing pollen (Barendse et al. 1970; Mander et al. 1996) and GA application has been shown to affect pollen tube growth (Bhandal and Malik 1979; Viti et al. 1990; Setia et al. 1994; Kimura et al. 1996). Singh et al. (2002) demonstrated that the ectopic expression of a pea *GA2ox2* cDNA under the control of the 35S promoter results in reduced pollen tube growth.

In this study we have addressed the specific function of Lin7 invertase using the hairpin silencing technology. Transgenic tomato plants show reduced pollen germination because of expression of a *Lin7*-RNAi construct under the control of the anther- and pollen-specific *Lin7* promoter. Plants expressing *Arabidopsis GA2ox2* cDNA under the control of the *Lin7* promoter show the same phenotype, pollen tube formation is strongly reduced. Analysis of the *Lin7* promoter demonstrated expression in pollen grains and pollen tubes. A GA synthesis inhibitor could reduce *Lin7* promoter activity in pollen. These data indicate that GA-regulated *Lin7* expression is an essential component for pollen germination. For the first time we substantiate a correlation between the GA effect on pollen germination and a specific enzyme function downstream of the GA signalling pathway.

MATERIALS AND METHODS

Plant material

For pollen analysis and fluorometric GUS assays *Lycopersicon esculentum* (cv Moneymaker) and *Nicotiana tabacum* (vc SR1) plants were grown under greenhouse conditions in Würzburg, Germany at 25°C with additional illumination from 7 a.m. to 7 p.m. of 60 klm m^{-2}. A*rabidopsis thaliana* (ecotype Columbia) plants used for RNA isolation were grown for five weeks under 9h light/15h dark conditions in the climber chamber at 22°C. Then plants were shifted to 15h light/9h dark conditions for flower induction, flowers were harvested after about two weeks.

Plasmid construction

To generate vector pRP712, which carries the GUS reporter gene under the control of 1.3 kb *Lin7* promoter, PCR on genomic DNA of tomato was performed using primers *Lin7*-23 (5´-AAACCCAAGCTTATCAAATGTGTGGTCTTATGTAG-3´) and *Lin7*-24 (5´-ACTAGTCTAGAATCCATAATTTTTATTTTATTTTAATG-3´), thereby introducing restrictions sites *Hind*III and *Xba*I to *Lin7* promoter sequences. Isolation of genomic DNA was performed as previously described (Proels et al. 2003). Oligos were designed according to the genomic sequence of *Lin7* (Acc.Nr. AY173050). The PCR product was digested with *Hind*III/*Xba*I and cloned in the binary vector pBI101+ using the same restriction sites yielding vector pRP712 (*Lin7*::GUS).

GA2ox2 cDNA was cloned using the Revert Aid H Minus cDNA synthesis Kit (MBI Fermentas, St. Leon-Roth, Germany) according to the manufacturers instructions. 3.4 μg of total RNA isolated from *A. thaliana* flowers, essentially according to the method of Chomczynski and Sacchi (1987), served as template in reverse transcription with GA-2 (5´-CGAGCTCATCATGACTACTCTCATACAAGGG-3´) as primer. The resulting product was amplified in a PCR reaction using GA-2 and GA-1 (5´-TCCCGGGCATGGTGGTTTTGCCACAGCCAGTCAC-3´) primers thereby introducing *Cfr*I and *Sac*I restriction sites. Primers were designed according to the mRNA sequence of *A. thaliana GA2ox2* (Acc.Nr. AJ132436). *GA2ox2* cDNA was cloned in pGEM-Teasy vector, sequenced (SEQLAB, Germany) to confirm accuracy of amplification, released by *Cfr*I/*Sac*I digestion and subcloned in pRP712 vector using the same restriction enzymes, thereby replacing the GUS coding sequence by *GA2ox2* cDNA yielding *Lin7*::*GA2ox2* construct.

To obtain the *Lin7*::*Lin7*-RNAi construct, two genomic fragments of *Lin7* were cloned via PCR. First, primers *Lin7*-A1 (5´-CGGGATCCTCAAATGACTCTGCCTTATCCAAAC-3´) and *Lin7*-A2 (5´-GCTCTAGAACCTGGTCTGGATCAGCAACCATC-3´) were used to clone a 1.7 kb fragment covering 0.8 kb of *Lin7* exon III and the complete intron III sequence, thereby introducing restriction sites *BamH*I and *Xba*I, respectively. Second, *Lin7*-A3 (5´-CGGGATCCTCAATCTTCTCCCCATTGTTCATG-3´) and *Lin7*-A4 (5´-TCGAGCTCACCTGGTCTGGATCAGCAACCATC-3´) primers were used to clone the same 0.8 kb fragment of *Lin7* exon III as described above introducing restriction sites *BamH*I and *Sac*I, respectively. Both fragments were cloned in pGEM-T vector, released with corresponding restriction enzymes and cloned in a three fragment ligation in vector pRP712, which was digested with *Sac*I and *Xba*I to replace the GUS coding sequence. The resulting *Lin7*::*Lin7*-RNAi construct expresses complementary 0.8 kb fragments of *Lin7* exon III separated by *Lin7* intron III under the control of the *Lin7* promoter.

Generation of transgenic plants

Construct pRP712 was transformed in tobacco (*Nicotiana tabacum* cv SR1) using standard *Agrobacterium* (LBA4404) transformation procedures (Horsch et al. 1985). Transformation of *Lycopersicon esculentum* with *Lin7*::*Lin7*-RNAi and *Lin7*::*GA2ox2* constructs was done following essentially the protocol of Fillatti et al. (1987). Several independent lines were analysed by PCR for transgenity and additionally by fluorometric GUS assays (pRP712) for functional reporter gene expression and maintained in the greenhouse.

In-vitro pollen germination

Mature pollen was harvested after anthesis and incubated in germination medium (100 mg/L H_3BO_3, 300 mg/L $Ca(NO_3)_2$*$4H_2O$, 200 mg/L $MgSO_4$*$7H_2O$, 100 mg/L KNO_3, 15% w/v PEG4000, 10% w/v sucrose) for 3 h at 26°C. Percentage of germinating pollen showing pollen tubes was determined in three replicates of three independent transgenic lines each.

Flourometric GUS assays

Pollen was harvested, resuspended in water and pollen number was determined. Aliquots were incubated overnight at room temperature in presence of 1.7 μM paclobutrazole or water. Viability of pollen was checked using Tryptan blue solution (Sigma, Deisenhofen, Germany). About 90% pollen from transgene or wild type (WT) plants were viable after overnight incubation. Up to 7000 pollen were used per assay in volumes of 20 to 50 μl. The same volume of GUS assay solution (50 mM sodium phosphate pH 7, 10 mM NaEDTA, 0.1% Trition X100, 0.1% sodium lauroyl sarcosine, 10 mM 2-mercaptoethanol, 2 mM 4-methylumbelliferyl-D-glucuronide) was added, immediately an aliquot was removed and transferred to stop solution (final concentration 0.3 M Na_2CO_3) to be used as control. The rest of the mixture was incubated at 37°C for 30 min and stopped in 0.3 M Na_2CO_3. GUS activity was determined using a luminescence spectrometer (Perkin Elmer LS 30, Langen, Germany).

Histochemical GUS assays

For histochemical GUS analysis the method of Jefferson et al. (1987) was followed. Pollen from mature flowers was harvested, resuspended in pollen germination medium (as outlined above) and incubated for 3 h at 26°C. Germinated pollen were harvested by centrifugation and tissue was fixed with 0.3% formaldehyde for 5 min followed by two

washing steps with 50 mM phosphate buffer pH 7.0. Samples were resuspended in GUS histochemical substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (1 mM) in 50 mM phosphate buffer pH 7.0 and incubated for 8 h at 37°C. Images were created using a microscope (Axioscope 2, Zeiss, Germany).

RESULTS

The Lin7 promoter sequence shows similarity to known GA-response elements

A screen for the presence of regulatory elements with similarity to known *cis*-acting elements revealed pollen-specific elements (Proels et al. 2003) and GA-response elements within the *Lin7* promoter sequence (Figure 1). The *Lin7* promoter contains *cis*-acting elements that are identical or show just one base pair exchange to the GA-response complex described by Gubler et al. (1995). Even the relative position of GA-response elements within the GA-response complex are conserved and the localisation relative to putative transcription start site is similar. Interestingly, in addition to the conserved GA-response complex, *Lin7* promoter shows a cluster of three GA-response elements (B*/B*/CARE*, Figure 1) including a recently identified CAACTC regulatory element (CARE). The latter was characterised by Sutoh and Yamauchi (2003) in the promoter of the GA-inducible *Rep1* gene in rice.

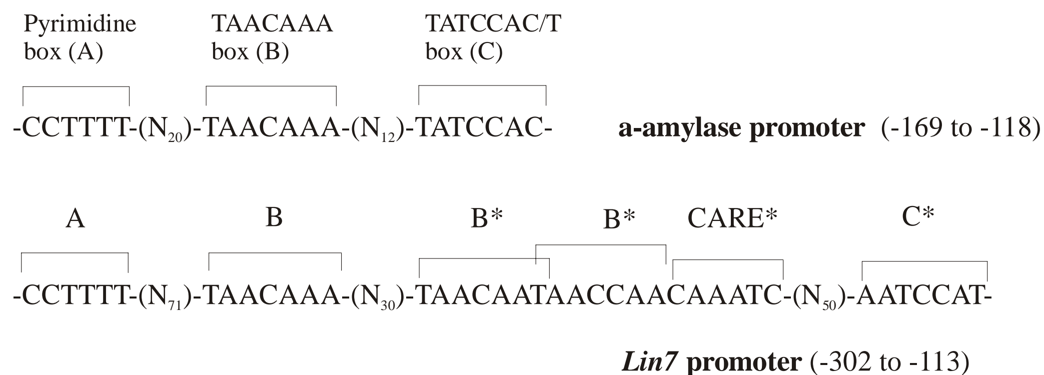

Fig. 1. Alignment of GA-response complexes of the α-amylase promoter (Gubler et al. 1995) and the *Lin7* promoter. A recently identified CARE (Sutoh and Yamauchi 2003) is included in the analysis. Similar boxes (with just one base pair exchange) in the *Lin7* promoter are indicated with asterisks (*). Numbers in parenthesis show the position relative to the putative transcription start site. CARE = CAACTC regulatory element

The Lin7 promoter confers high reporter gene expression in pollen and is responsive to paclobutrazole

Previously, we described the cloning and expression analysis of Lin5 and Lin7, two flower-specific extracellular invertases of tomato. *Lin5* was shown to be expressed predominantly in the gynoecia and fruit; *in-situ* hybridisation data demonstrated expression of *Lin7* in anther and pollen tissue (Godt and Roitsch 1997; Proels et al. 2003). Transient reporter gene studies revealed that a *Lin5* promoter fragment, carrying GA-response boxes, is inducible by exogenous application of GA_3 (Proels et al. 2003). Because of the high tissue-specificity of the *Lin7* promoter, restricting expression to anther and pollen, we did not reach to establish a transient assay for *Lin7* promoter studies. Therefore, transgenic tobacco lines carrying the *Lin7*::GUS transgene were generated. GUS expression in pollen was shown to reach extremely high values of 122 pmol MU/5000pollen/min. Because of this high constitutive expression in pollen, a further induction with exogenously applied GA_3 could not be achieved (data not shown). Treatment of transgenic pollen with a GA synthesis inhibitor (paclobutrazole) results in a reduction of reporter gene activity of 22% ± 3.6% compared to control (Figure 2A). Regarding the high stability of GUS protein, a reduction of more than 20% of GUS activity after paclobutrazole repression reflects a clear effect of GA synthesis inhibition on *Lin7* promoter activity.

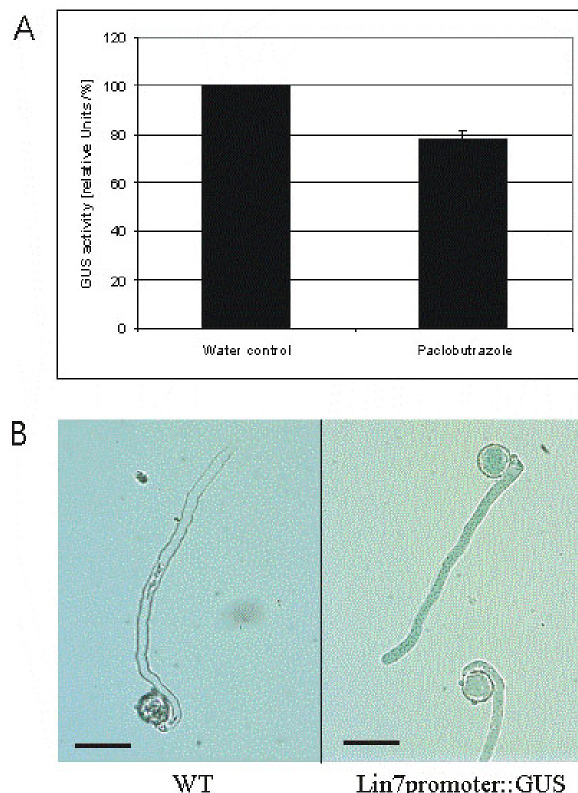

Fig. 2. A) Pollen granules of stable transformed *Lin7*::GUS tobacco plants were collected from mature flowers and emulgated in water. The pollen number per volume was determined and equal amounts of pollen were distributed in 1.5 ml reaction tubes. One set of experiments was used as control the other was treated with 1.7 µM paclobutrazole. After overnight incubation at room temperature GUS activity was measured. Each set of four independent experiments was normalised to the control, standard deviations are indicated. Pollen viability after overnight incubation was determined using tryptan blue staining.
B) GUS histochemical stainings of *Lin7* expression in *Lin7*::GUS (pRP712) transgenic tobacco pollen and WT controls. Pollen were *in-vitro* germinated for 3 h at 26°C, tissue was fixed with 0.3% formaldehyde and histochemical GUS staining was performed for 8 h at 37°C. Bars represent 30 µm.

Histochemical GUS staining shows Lin7 expression in pollen and pollen tubes

To analyse the expression pattern of the *Lin7* promoter in germinating pollen, histochemical GUS stainings were performed on pollen of *Lin7*::GUS transformed tobacco plants. Prior to GUS staining, pollen was *in-vitro* germinated for 3 h. As shown in Figure 2B, staining appears throughout the whole pollen tube and pollen. Experiments were performed with three independent transgenic lines and representative data are shown.

Expression of a Lin7-RNAi construct and GA2ox2 cDNA under the control of the Lin7 promoter reduces pollen tube formation

To investigate the specific function of *Lin7* invertase, we used the hairpin silencing technology (Smith et al. 2000) as an attempt to specifically silence the *Lin7* gene. Therefore, transgenic tomato lines carrying a *Lin7*-RNAi construct under the control of the *Lin7* promoter were generated. As the expression of the *Lin7*-RNAi construct is restricted to anther and pollen tissue, we rule out pleiotrophic effects that might arise once strong constitutive promoters, such as 35S were used. No variations in phenotype were detected in means of anther or pollen physiology compared to WT under the light microscope. Pollen of three independent transformants were analysed in three replicates each in an *in-vitro* pollen germination assay. After 3 h of incubation 4.8% ± 3.7% pollen of the *Lin7*-RNAi expressing line form pollen tubes compared to 42.2% ± 4.7% of WT pollen as shown in Figure 3. This reflects a reduction of about 90% to WT control.

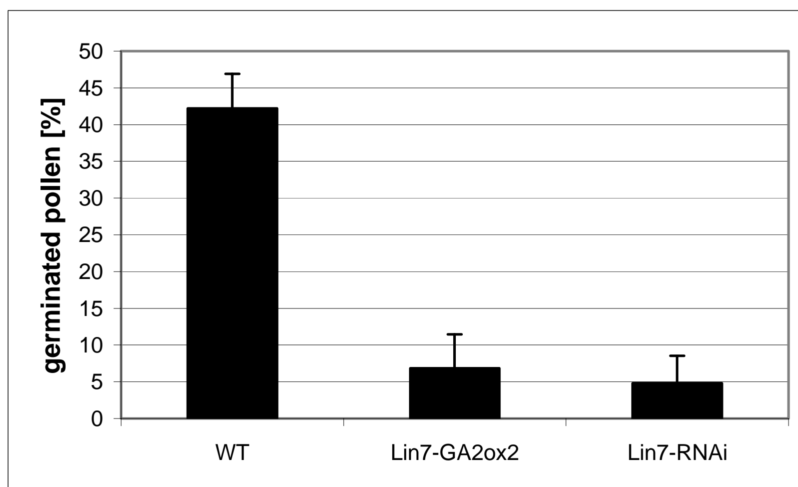

Fig. 3. Transgenic tomato lines expressing *A. thaliana GA2ox2* cDNA and a *Lin7*-RNAi construct under the control of *Lin7* promoter were analysed for pollen germination *in-vitro*. Mature pollen was harvested after anthesis and incubated in germination medium for 3 h at 26°C. Percentage of germinating pollen showing pollen tubes was determined in three replicates of three independent transgenic lines each, standard deviations are indicated.

In a second fuctional approach we created transgenic lines expressing *A. thaliana GA2ox2* cDNA under the control of the *Lin7* promoter. *GA2ox2* represents a flower-specific

enzyme involved in the deactivation of biologically active GAs (Stephen et al. 1999). The scope was to reduce endogenous GA contents at the sites of *Lin7* expression and to compare the resulting phenotypes with *Lin7*::*Lin7*-RNAi lines. Similar to *Lin7*::*Lin7*-RNAi lines we could not detect variations in phenotype in means of anther or pollen physiology compared to WT under the light microscope and just 6.8% ± 4.6% pollen of *Lin7*::*GA2ox2* transgenic lines form pollen tubes after 3 h of incubation compared to 42.2% ± 4.7% of WT pollen.

DISCUSSION

Ectopic expression of GA2ox2 under the control of the Lin7 promoter substantiates an essential role of GA in pollen tube formation

Previously, a physiological role of GA in pollen tube growth was established by expressing a pea *GA2ox2* cDNA in *A. thaliana* under the control of the 35S promoter (Singh et al. 2002). 35S::*GA2ox2* lines showed reduced pollen tube growth and seed abortion. Furthermore, Singh et al. (2002) could show that treatment of pollen with a chemical inhibitor of GA biosynthesis (uniconazole) reduced pollen tube elongation which is consistent with an essential role for GAs in pollen tube growth. Swain et al. (2004) further investigated those transgenic lines and could demonstrate that silencing of *GA2ox2* expression via introducing an RNAi silencing construct could restore pollen tube growth. In our experimental system *GA2ox2* was expressed under the control of the anther- and pollen-specific *Lin7* promoter to restrict transgene expression to corresponding tissues of interest. The only observed phenotype was a reduced pollen germination efficiancy. Thus, using a highly tissue-specific promoter instead of the 35S promoter, we could generate a more subtle and specific phenotype in restricting *GA2ox2* expression mainly to pollen. As *Lin7* promoter activity reaches extremely high levels in pollen, *GA2ox2* expression could be even higher than under control of the 35S promoter. Moreover, several GA response elements are present in the *Lin7* promoter and promoter activity is suppressed by a GA-synthesis inhibitor. Therefore, a close regulation of *GA2ox2* transgene expression mediated by GA could further contribute to the high specificity of the observed phenotype.

Lin7 extracellular invertase plays an essential role in pollen germination

According to northern blot (Godt and Roitsch 1997) and *in-situ* hybridisation data (Proels et al. 2003) showing *Lin7* expression to be restricted to anther and pollen tissue, we supposed a unique function of *Lin7* in growth processes of anther and/or pollen. This idea is further supported by histochemical reporter gene assays demonstrating *Lin7* promoter activity in pollen and pollen tubes. In a transgenic approach we analysed pollen of tomato plants expressing the hairpin silencing construct *Lin7*-RNAi under the control of the *Lin7* promoter in an attempt to specifically silence the *Lin7* gene. Whereas no differences in flower physiology of those transgenic lines could be detected under the light microscope, there is a clear reduction in the formation of pollen tubes *in-vitro*. The fact that transgenic lines are solely affected in pollen germination might be explained with the finding that there are two further extracellular invertases expressed in floral organs. Transgenic lines carrying GUS reporter gene under the control of the *Lin6* promoter reveal considerable GUS activity in pollen, which reached 76 pmol MU/5000pollen/min. This represents 62% compared to *Lin7* mediated GUS expression (Proels RK, Roitsch T, unpublished observation). Godt and Roitsch (1997) could demonstrate that *Lin5*, albeit most strongly expressed in gynoecia and fruit, shows slight expression in stamen as well. *Lin5* and *Lin7* are arranged in a direct tandem repeat on the genome with *Lin5,* showing a broader expression pattern, localised 5´ of the anther- and pollen-specific *Lin7* gene (Proels et al. 2003). A similar tandem organisation was reported in potato (Maddison et al. 1999) with *invGE*, showing a broader expression pattern localised 5´ of *invGF,* which is again restricted to pollen. This finding lead to the speculation that because of a gene duplication a specific isoenzyme function, represented by the anther- and pollen-specific *Lin7* and *invGF* genes, was generated to cover the carbohydrate supply of the male gametophyte. The presence of three extracellular invertases in tomato floral tissues underlines the importance of apoplastic sucrose cleavage to sustain growth and development of reproductive organs.

Pollen germination occurs rapidly in most plants and the rate of tube growth is extremely high, rates up to 160 µm/h are found (Clement et al. 1999). Therefore, pollen tube growth is expected to be an extremely energy consuming process. Expression of extracellular invertases in combination with hexose transporters in growing pollen tubes could cover the accelerated demand for carbohydrates. This idea is corroborated by the identification of pollen-specific monosaccharide transporters, which are functionally linked to extracellular invertases. Ylstra et al. (1998) cloned a monosaccharide transporter from petunia that is specifically expressed in the male gametophyte and shows high levels of mRNA accumulation in mature and germinating pollen. A male gametophyte-specific monosaccharide transporter, AtSTP2, was analysed by Truernit et al. (1999). The *AtSTP2* promoter was shown to be active during pollen maturation as well as in germinating pollen. Scholz-Starke et al. (2003) and Schneidereit et al. (2003) report on a pollen-specific H+-

monosaccharide symporter from *A. thaliana* (AtSTP6) and a glucose specific monosaccharide transporter in pollen of *A. thaliana* (AtSTP9), respectively. Interestingly, AtSTP9 protein was shown to be most prominent in germinating pollen.

To shed further light on the complexity of carbohydrate supply of the male gametophyte, single or multiple gene silencing including Lin5 and Lin6 invertases should be envisaged. Furthermore, the question should be addressed weather individual invertase/monosacharide transporter pairs exist in floral organs and what regulatory mechanisms co-ordinate their expression.

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REFERENCES

Barendse GWM, Rodrigues-Pereira AJ, Berkers PA, Driessen FM, van Eyden-Emons A, Linskens HF (1970) Growth hormones in pollen, styles and ovaries of *Petunia hybrida* and Lilium species. *Acta Botanica Neerlandica* **19**, 175-186.

Bhandal IS, Malik CP (1979) Effect of gibberellic acid, (2-chloroethyl), phosphoric acid, actinomycin-D and cycloheximide on the activity and leaching of some hydrolases in pollen suspension cultures of *Crotalaria juncea. Physiologia Plantarum* **45**, 297-300.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**, 156-159.

Clement C, Pacini E, Audan JC (1999) Anther and Pollen, From Biology to Biotechnology. Springer, Berlin/Heidelberg

Eschrich W (1980) Free space invertase, its possible role in Phloem unloading. *Berichte der Deutschen Botanischen Gesellschaft* **93**, 363-378.

Fillatti JJ, Kiser J, Rose R, Comai L (1987) *Biotechnology* **5**, 726-730.

Godt DE, Roitsch T (1997) Regulation and Tissue-Specific Distribution of mRNA for Three Extracellular Invertase Isoenzymes of Tomato Suggests an Important Function in Establishing and Maintaining Sink Metabolism. *Plant Physiology* **115**, 273-282.

Goetz M, Godt DE, Guivarch A, Kahmann U, Chriqui D, Roitsch T (2001) Induction of male sterility in plants by metabolic engineering of the carbohydrate supply. *Proceedings of the National Academy of Science of the United Sates of America* **98**, 6522-6527.

Goto N, Pharis RP (1999) Role of gibberellins in the development of floral organs of gibberellin-deficient mutant, ga1-1, of *Arabidopsis thaliana. Canadian Journal of Botany* **77**, 944-954.

Gubler F, Kalla R, Roberts JK, Jacobsen, JV (1995) Gibberellin-Regulated Expression of a myb Gene in Barley Aleuron Cells: Evidence for Myb Transactivation of a High-pI α-Amylase Gene Promoter. *Plant Cell* **7**, 1879-1891.

Horsch RB, Fry FB, Hoffmann NL, Eichholtz P, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. *Science* **227**, 1229-1231.

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS-fusions: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *European Molecular Biology Organization Journal* **6**, 3901-3907.

Kimura PH, Okomoto G, Hirano K (1996) Effects of gibberellic acid and streptomycin on pollen germination and ovule and seed development in Muscat Bailey A. *American Journal of Enology and Viticulture* **47**, 152-156.

Lorenz K, Lienhard S, Sturm A (1995) Structural organisation and differential expression of carrot β-fructofuranosidase genes: identification of a gene coding for a flower bud-specific isoenzyme. *Plant Molecular Biology* **28**, 189-194.

Maddison AL, Hedeley PE, Meyer RC, Aziz N, Davidson D, Machray GC (1999) Expression of tandem invertase genes associated with sexual and vegetative growth cycles in potato. *Plant Molecular Biology* **41**, 741-751.

Mander LN, Owen DJ, Croker SJ, Gaskin P, Hedden P, Lewis MJ, Talon M, Gage DA, Zeevaart JA, Brenner ML et al. (1996) Identification of three C20-gibberellins: GA_{97} (2 β-hydroxy-GA_{53}), GA_{98} (2 β-hydroxy-GA_{44}) and GA_{99} (2 β-hydroxy-GA_{19}). *Phytochemistry* **43**, 23-28.

Nester JE, Zeevaart JAD (1988) Flower development in normal tomato and a gebberellin-deficient (ga-2) mutant. *American Journal of Botany* **75**, 45-55.

Proels RK, Hause B, Berger S, Roitsch T (2003) Novel mode of hormone induction of tandem tomato invertase genes in floral organs. *Plant Molecular Biology* **52**, 191-201.

Roitsch T, Balibrea ME, Hofmann M, Proels R, Sinha AK (2003) Extracellular invertase: key metabolic enzyme and PR protein. *Journal of Experimental Botany* **54**, 513-524.

Schneidereit A, Scholz-Starke J, Büttner M (2003) Functional characterization and expression analyzes of the glucose-specific *AtSTP9* monosaccharide transporter in pollen of *Arabidopsis*. *Plant Physiology* **133**, 182-190.

Scholz-Starke J, Büttner M, Sauer N (2003) *AtSTP6*, a new pollen-specific H^+-monosaccharide symporter from *Arabidopsis*. *Plant Physiology* **131**, 70-77.

Setia N, Setia RC, Chhabra N (1994) Interactive effects of growth hormones and calcium antagonists on germination and tube elongation of ground-nut pollen. *Plant Cell Incompatibility Newslett* **26**, 70-80.

Sherson SM, Alford HL, Forbes SM, Wallace G, Smith SM (2003) Roles of cell-wall invertases and monosaccharide transporters in the growth and development of Arabidopsis. *Journal of Experimental Botany* **54**, 525-531.

Singh DP, Jermakow AM, Swain SM (2002) Gibberellins are required for seed development and pollen tube growth in *Arabidopsis. Plant Cell* **14**, 3133-3147.

Smith NA, Singh SP, Wang MB, Stoutjesdijk PA, Green AG, Waterhouse PM (2000) Total silencing by intron-spliced hairpin RNAs. *Nature* **407**, 319-320.

Stephen GT, Phillips AL, Hedden P (1999) Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. *Proceedings of the National Academy of Science of the United Sates of America* **96**, 4698-4703.

Sturm A (1999) Invertases. Primary Structures, Functions, and Roles in Plant Development and Sucrose Partitioning. *Plant Physiology* **12**, 1-7.

Sutoh K, Yamauchi D (2003) Two *cis*-acting elements necessary and sufficient for gibberellin-upregulated proteinase expression in rice seeds. *Plant Journal* **34**, 635-645.

Swain SM, Muller AJ, Singh DP (2004) The *gar*2 and *rga* allels increase the growth of gibberellin-deficient pollen tubes in *Arabidopsis. Plant Physiology* **134**, 694-705.

Truernit E, Stadler R, Baier K, Sauer N (1999) A male gametophyte-specific monosaccharide transporter in *Arabidopsis*. *Plant Journal* **17**, 191-201.

Tymowska-Lalanne Z, Kreis M (1998) The plant invertases: Physiology, biochemistry and molekular biology. *Advances in Botanical Research* **28**, 71-117.

Viti R, Bartolini S, Vitagliano C (1990) Growth regulators on pollen germination in olive. *Acta Horticulturae* **286**, 227-230.

Weber H, Borisjuk L, Heim U, Buchner P, Wobus U (1995) Seed coat-associated invertases of fababean contol both unloading and storage functions. Cloning of cDNAs and cell type-speific expression. *Plant Cell* **7**, 1835-1864.

Ylstra B, Garrido D, Busscher J, Tunen AJ (1998) Hexose transport in growing Petunia pollen tubes and characterization of a pollen-specific, putative monosaccharide transporter. *Plant Physiology* **118**, 297-304.

Chapter 4

Cloning of a CACTA-like transposon insertion in intron I of tomato invertase *Lin5* gene and identification of transposase-like sequences of Solanaceae species

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Cloning of a CACTA-like transposon insertion in intron I of tomato invertase *Lin5* gene and identification of transposase-like sequences of Solanaceae species

Abstract. Very few CACTA-like transposon sequences have been described in Solanaceae species. Sequence information are restricted to partial transposase-like fragments, no target gene of CACTA-like transposon insertion has been described in tomato so far. In this manuscript we report on a CACTA-like transposon insertion in intron I of tomato invertase gene *Lin5* and TPase-like sequences of several Solanaceae species. Primers deduced from the TPase region of tomato CACTA-like transposon allowed the amplification of similar sequences from various Solanaceae species of different subfamilies including Solaneae (*S. tuberosum*), Cestreae (*N. tabacum*) and Datureae (*D. stramonium*). This demonstrates the ubiquitous presence of CACTA-elements in Solanaceae genomes. The obtained partial sequences are highly conserved and allow further detection and detailed analysis of CACTA-like transposons throughout Solanaceae species. CACTA-like transposon sequences will offer the possibility to evaluate their use for genome analysis, functional studies of genes and the evolutional relationships between plant species.

INTRODUCTION

Transposable elements (TEs) are fundamental components of most plant genomes. It is well established that they play an important role in structure, variation, and adaptive evolution of genomes (McClintock, 1984; McDonald, 1995; Kidwell and Lisch, 1997). TEs are grouped into two broad categories due to their mode of transposition. Class 1 elements, retrotransposons, replicate via an mRNA intermediate. The transposition mechanism is dependent on reverse transcription (Kumar and Bennetzen, 1999). The genomic DNA of plants, such as wheat, barley, or maize consists of a large fraction of retrotransposon sequences (SanMiguel and Bennetzen, 1998; Shirasu et al., 2000; Wicker et al., 2001; SanMiguel et al., 2002). Class 2 elements, transposons, move directly as DNA elements and contain terminal inverted repeats (TIRs) (Frey et al., 1990; Kunze et al., 1997). Excision and reintegration depend on proteins, e.g. transposases (TPases), which are encoded by the internal part of the element. Several families of transposons are known, one of them is the CACTA family (also called En/Spm family) because of inverted repeat sequences that terminate in a conserved CACTA motif. CACTA elements comprise autonomous elements and nonautonomous elements that lack proteins that are necessary for transposition. The latter cannot promote their own excision, rather they depend on *trans*-acting factors encoded by independent autonomous copies present in the same genome for their transposition (Gierl et al., 1989; Bennetzen, 2000). Pereira et al. (1986) analysed the first CACTA element, En-1 from maize, at the molecular level. More CACTA elements were characterised from many different species, such as *A. thaliana* (Miura et al., 2001; 2004), carrot (Ozeki et al., 1997), Japanese morning glory (Inagaki et al., 1994), petunia (Snowden and Napoli, 1998), snapdragon (Nacken et al., 1991), and sorghum (Chopra et al., 1999).

CACTA elements are flanked by short TIRs of 10 to 28 bp that terminate in the CACTA motif, which serve as recognition sites for the TPase (Lewin, 1997). As sequence conservation is limited to this motif, it is difficult to identify new elements based on TIR sequence homology. Nevertheless, transposon-like sequences have been detected with the help of computer-aided database searches (Wicker et al., 2003; Wang et al., 2003) and PCR-based approaches (Staginnus et al., 2001) in an increasing number of plant species. Phenotypic variations because of transposon insertion have been used for the identification and isolation of genes. Furthermore, mobile elements allow functional studies of identified genes and the evolutional relationship between plant species. Transposon tagging, which turned out to be practicable in heterologous species, is important for plant genome research

(Chandlee JM, 1990). Therefore, the identification of novel TEs is highly valuable for the plant species of interest.

In this manuscript we describe the identification of a CACTA-like insertion in intron I of tomato extracellular invertase gene *Lin5*. Sequence alignments to a CACTA-like transposable element of *Antirrhinum majus*, Tam1, show high homology, especially to TPase coding sequences. Based on this finding we designed a primer pair within the TPase coding sequence to perform a PCR screen throughout Solanaceae species. Using this primer pair we could amplify and analyse TPase-like sequences of *N. tabacum*, *S. tuberosum* and *D. stramonium,* thus indicating the high sequence conservation throughout Solanaceae species.

MATERIALS AND METHODS

Plant materials

Lycopersicon esculentum (cv Moneymaker) plants were grown under greenhouse conditions in Regensburg (Germany). *Nicotiana tabacum* (cv Samsun) plants were grown under greenhouse conditions in Würzburg (Germany) at 25°C with additional illumination from 7 a.m. to 7 p.m. of 60 klx. *Solanum tuberosum* cv Asparagus and *Datura stramonium* were grown in the botanical garden of Würzburg University (Germany).

Cloning and sequence analysis

Genomic sequences of *Lin5* were cloned using the GenomeWalker System (CLONTECH, Heidelberg, Germany) according to the instructions of the manufacturer. Briefly, genomic DNA was isolated from tomato leaves (*Lycopersicon esculentum* cv Moneymaker) using the NucleoBond System (Macherey und Nagel, Düren, Germany) and digested with different restriction enzymes, to gain corresponding libraries. Besides the recommended restriction enzymes, *Hpa*I, *Sma*I, *Ssp*I and *Eco*72I were used to create additional libraries. After ligation with the provided adaptors the libraries were used as template for PCR and nested PCR. Starting out of a known region of exon III of the *Lin5* gene (Acc.Nr. X91389), two sequential walks were performed to obtain 5273 bp including 439 bp genomic sequence of *Lin5* followed by an abrupt end in sequence homology to *Lin5* sequences, and 4.8 kb that show high homology to CACTA-like transposable elements. An overlap of 252 bp of identical sequence of the preceding genome walk fragment confirmed the identity of successive fragments. Vectors, corresponding primer sequences and the libraries used are shown in Table 1. The obtained PCR products were cloned using the Ins

T/A clone PCR Product Cloning Kit (MBI-Fermentas, St. Leon-Rot, Germany) and sequenced by SEQLAB (Göttingen, Germany). For sequence analysis the Vector NTI Suite (Infor Max, Bethesta USA) was used.

Vector	gene specific primers (GSPs) in 5´ to 3´ orientation		Library
5-2-1 2nd walk	GSP 1	CAACTTTAAGGCCAGCTCAAGTGATCTTG	*Hpa*I
	GSP 2	AGAATCAAAGTAGTCACCATAATCTCTG	
5-1-1 1st walk	GSP 1	GTTATCAGGGACGATCAACGGGTTGTTG	*Dra*I
	GSP 2	CAGATAGGTTAGCCGGGATGGCGTAGTTC	

Table 1. Primers used for PCR based cloning of the 5´ genomic region of *Lin5*. Libraries that yielded the corresponding PCR fragments are indicated. GSP1 = gene specific primer 1, GSP2 = gene specific primer 2 (nested primer).

Sequence analysis of TPase-like sequences of different Solanaceae species

Genomic DNA was isolated from different Solanaceae species. Leaf discs were excised using the lid of a 1.5 ml reaction tube and ground in presence of 400 µl extraction buffer (200mM Tris pH7.5, 250mM NaCl, 25mM EDTA, 0.5% SDS). Debris was removed by centrifugation for 1 min at 13,000 rpm, 300 µl of supernatant was added to 300 µl isopropanol. Samples were incubated for 2 min at room temperature and centrifuged for 5 min at 13,000 rpm to precipitate DNA. The DNA pellet was dried and resuspended in 100µl TE buffer (10mM Tris/HCl pH7.5, 2mM EDTA pH8.0). 3 µl of resuspended genomic DNA were used as template for PCR amplification: annealing temperature 49°C, cycle number 28 to 37, primers used are Lin5-16 (5´-GGTCCATCATCTCCTGGAAATG-3´) and Lin5-17 5´-GAATACATCCGACGCAAATGATTC-3´). Resulting PCR products were precipitated, washed with 70% ethanol, dried, and resuspended in water. 20 to 30 ng of PCR product were sequenced with Lin5-17 as sequencing primer using BigDye Terminator v1.1 Cycle Sequencing Kit and 3100-Avant Genetic Analyser (Applied Biosystems). For sequence analysis the Vector NTI Suite (Infor Max, Bethesta USA) was used. Phylogenetic tree calculation is based on a sequence distance method and utilises the Neighbor-Joining (NJ) algorithm of Saitou and Nei (1987).

RESULTS

Identification of a CACTA-like insertion in intron I of tomato extracellular invertase gene Lin5

In order to clone the promoter sequence of tomato extracellular invertase *Lin5,* a 5´ genome walk was performed on genomic libraries starting from the available exon III sequence of *Lin5*. In total 5273 bp were sequenced, represented by two overlapping clones (Figure 1).

Homology to known invertase sequences terminated 71 bp 5´ of *Lin5* exon II. Sequence similarity searches using the BLAST program revealed that the yet unknown sequence shows high homology to a CACTA-like transposon of *Antirrhinum majus*, Tam1 (Acc.Nr. X 57297). A detailed sequence alignment with *Antirrhinum majus* TPase-like protein Tnp2 revealed a full length TPase-like sequence in intron I of *Lin5* (Figure 1). The orientation of the 2.2 kb TPase-like sequence is 3´ to 5´ in correlation to *Lin5*. The deduced protein sequence shows 43% identity to Tnp2 but includes one frame shift and 12 in-frame stop codons within the potential ORF. This finding lines up with data on CACTA transposons in Triticeae (Wicker et al., 2003). All CACTA elements that were identified by Wicker et al. (2003) appeared to be defective or nonautonomous because of a lack in sufficient coding capacity or coding sequences are interrupted by frame shifts of in-frame stop codons.

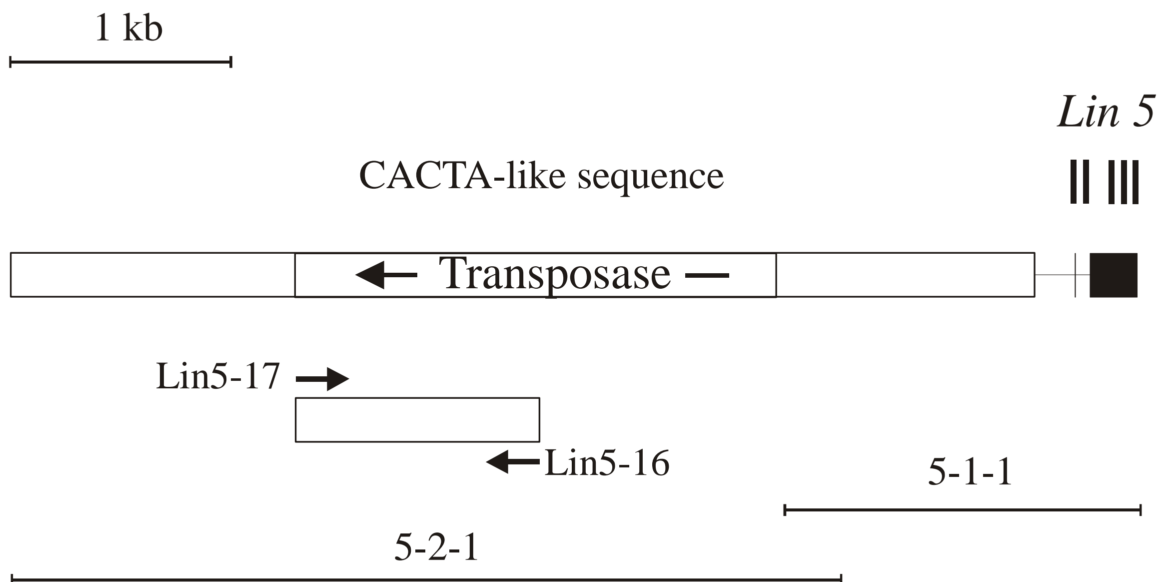

Figure 1. Genomic organisation of a CACTA-like insertion in intron I of tomato invertase gene *Lin5*. Overlapping clones obtained by sequential genome walks, 5-1-1 and 5-2-1, are shown below. Exons II and III of *Lin5*, a sequence homologue to CACTA-like elements, a TPase-like sequence and its orientation are indicated. The primer pair, Lin5-17 and Lin5-16, used for amplification of the conserved TPase 3´ends and the resulting fragment is detailed.

CACTA-like transposons are known to insert preferentially in AT-rich sequences (Nacken et al., 1991; Le et al., 2000; Wang et al., 2003). We analysed intron I sequences of all four members of tomato extracellular invertase genes *Lin5*, *Lin6*, *Lin7*, and *Lin8* (Acc.Nr. AF506004, AY173050) and calculated an AT-content of 75 to 76 %. In detail, intron I of *Lin5* reaches 75% AT-content, supporting the observations mentioned above. Interestingly, just at the integration site we could identify a CACTA sequence, which is known to represent the end of the TIRs of CACTA elements (Nacken et al., 1991), albeit it shows the inverse orientation.

A new and straight forward method for generating sequence information suitable to design primers for PCR screens aimed at TPase-like sequences

Based on the finding that there is high homology within the TPase coding regions of the CACTA-type transposons (Staginnus et al., 2001), we designed the primer pair Lin5-16 and Lin5-17 (Figure 1) for a PCR screen on genomic DNA. Indeed, we got a specific PCR product of the expected 1.1 kb size once this primer pair was used to perform PCR on genomic DNA of *L. esculentum* (Figure 2). The PCR product was used directly for sequence analysis with Lin5-17 as sequencing primer. TPase sequences are known to be present in many variations because of point mutations or deletions (Wicker et al., 2003). Mutations, which occur randomly, will be covered by the dominating non-mutated sequences resulting in a minor signal in the sequencing reaction. Therefore, the resulting sequence is expected to represent several individual TPase sequences that were integrated because of the combined sequencing reaction to yield an abundant sequence. The sequence identity between this abundant sequence and the individually cloned TPase fragment is 96%. As shown in Figure 3B, this abundant sequence of tomato is closer related to TPase-like sequences of other Solanaceae species than the individually cloned tomato sequence, thus putting further evidence for the postulated integration because of the combined sequencing reaction.

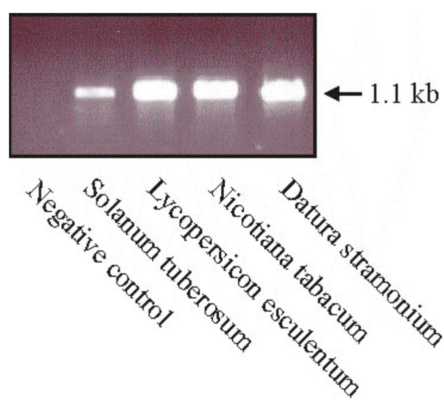

Figure 2. Amplification of TPase-like sequences in Solanaceae species. Genomic DNA from *Solanum tuberosum* (cv Asparagus), *Lycopersicon esculentum* (cv Moneymaker), *Nicotiana tabacum* (cv Samsun), and *Datura stramonium* was used as template for PCR with the primer pair Lin5-16/Lin5-17. PCR products are visualised on an ethidium bromide stained 1% agarose gel. For negative control water was used instead of template DNA.

PCR amplification and analysis of Solanaceae TPase-like sequences

The Lin5-16/Lin5-17 primer pair was used to perform a PCR screen on genomic DNA of several species of different Solanaceae subfamilies. As shown in Figure 2, we could amplify PCR products of 1.1 kp size from *S. tuberosum*, *N. tabacum* and *D. stramonium*. These results show that the sequence homology between Solanaceae subfamilies is high enough to get specific PCR products even without degeneration of primers. The PCR products were used directly for sequence analysis with Lin5-17 as sequencing primer. Sequence alignments of a translated 233 bp fragment of TPase fragments (Figure 3A) demonstrate a high sequence identity among Solanaceae species. In a phylogenetic tree (Figure 3B), which was derived from the sequence alignment, *L. esculentum* and *S. tuberosum* sequences are grouped into one distinct subbranch according to their phylogenetic relation as members of the Solaneae subfamily, whereas *N. tabacum* and *D. stramonium*, representatives of Cestreae and Datureae subfamilies, were excluded.

A

Lycopersicon e. (Genome walk)	SHDSHFISHYLLHVAGRNVLPKNVSLVLIRLGNYFRDKCSKVIRKSDLDMMKAEIVDIECEIEKIFPLSFFDIMTHL
Lycopersicon esculentum	SHDSHFISHYLLHVAVRNVLPKNVSLVLIRLGNYFRAICSKVIRKSDLDKMKAEIVDIECEIEKIFPPSFFDIITHL
Solanum tuberosum	SHDAHFILHYLLQVAVRKVLPKNVSLVLIRLGNYFRAICSKVIKRSDLEKMKAEIIDIECELEKIFPPSFFDIMTHL
Nicotiana tabacum	SHDAHFIMHYMLQVAVIKVLPKNVSLALIRLGNYFRTICSKVIRQSDVDKMQSEIIDIACDLETIFPPTFFDIMTHL
Datura stramonium	SHDAHFIMHYLLQVAVRKALPKNVSLTLIRLGNYFRSICSKVIRKGILIK-WLIS-I-NVALKRFFLQPFLI--HIC
En-1 (*Zea mays*)	SHDYHILIERLVPVMFRGYFSPDVWKIFAELSYFYKQICAKEISKKLMLRFEKEIVVLVCKMEKVFPPGFFNCMQHL
Tnp2 (*Antirrhinum majus*)	SHDHHILMQQLLPIALRKVLPKHVRTPLIKLCTFFRELCSKVLNPQDLVRMGKDIAKTLCDLEKIFPPSFFDIMMHL

B

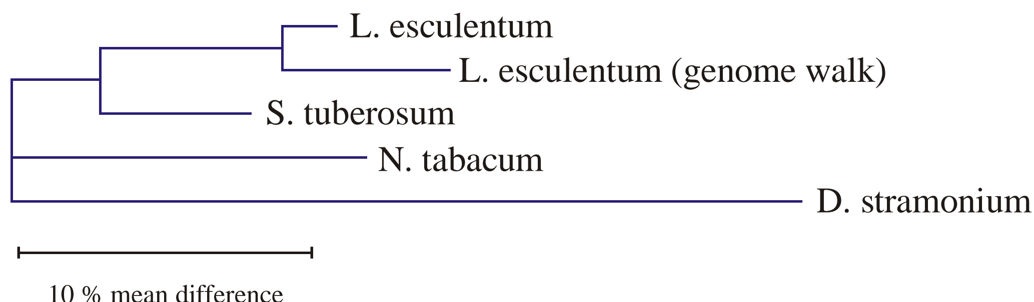

Figure 3. A) Alignment of the predicted amino acid sequence within the TPase region derived from Solanaceae species (*L. esculentum* (genome walk sequence), *L. esculentum*, *S. tuberosum*, *N. tabacum*, *D. stramonium*), En-1 (Acc.Nr. S29329) and Tam-1 (Acc.Nr. S23818). Identical amino acids of all aligned sequences are marked in dark boxes. Identities between Solanaceae sequences are additionally marked with bright boxes.

B) A Neighbor-Joining distance tree is shown, derived from the alignment of TPase-like sequences of Solanaceae species of A). Branch lengths are proportional to percentage sequence difference (scale bar represents 10 % mean difference).

DISCUSSION

Identification of a CACTA-like transposon sequence in intron I of tomato invertase Lin5 gene

Performing a 5´ genome walk approach, we identified a CACTA-like transposon insertion in intron I of tomato extracellular invertase gene *Lin5*. Sequence alignments revealed a full length TPase-like sequence, which is 3´ to 5´orientated in correlation to *Lin5*. However, the TPase-like ORF contains one frame shift and 12 in-frame stop codons. This finding is not surprising, as all CACTA transposons identified in Triticeae by Wicker et al. (2003) appeared to be defective or nonautonomous because of reduced coding capacity, frame shifts, or in-frame stop codons. Obviously, most CACTA-like elements have lost their capacity to move autonomously because of mutations.

At the integration site we identified a CACTA motif, which serves as recognition site for the TPase, albeit in the inverse orientation. This finding shows that the transposon insertion has even lost its capacity to be mobilised in presence of autonomous elements in the genome.

CACTA-like transposons preferentially insert into AT-rich DNA without a consensus motif for insertion (Nacken et al., 1991; Le et al., 2000; Wang et al., 2003). However, there seems to be a preference for insertion into genic regions including exons, introns and the immediate 5´and 3´non-coding termini of genes. 49.5 % of ACATA-like transposon insertions identified by Wang et al. (2003) have inserted into non-coding genic regions including introns. We analysed intron sequences of all four members of tomato extracellular invertases and calculated an AT-content of 75 to 76 %. These findings support the preferential insertion of those elements in AT-rich sequences and make invertase introns potential targets for transposon insertion.

Yau and Simon (2003) describe a 2.5 kb insert in the first and largest intron of the B4367rs (*rs/rs*) carrot acid-soluble invertase isoenzyme II gene. It is speculated that the insertion accounts for the elimination of acid-soluble invertase isoenzyme II transcription in roots and consequently could explain the observed high sucrose accumulation in corresponding tissues.

TEs can alter gene expression in many ways, such as preventing expression, producing splicing products, or providing new regulatory signals. Mutations caused by TEs are mainly gene inactivations (Lönnig and Saedler, 1997). The tomato invertase genefamily comprises four members with different, but overlapping expression patterns (Godt and Roitsch, 1997, Proels et al., 2003). In particular, there are two flower-specific invertases known in tomato, Lin5 and Lin7, which are organised in a direct tandem repeat (Proels et al.,

2003). An altered expression or inactivation of *Lin5* because of TE insertion might be substituted by *Lin7,* thereby changing isoenzyme functions.

It is known that TEs contribute to genomic plasticity in response to diverse environmental conditions. A number of plant TEs show activation upon stress conditions (Hirochita, 1993; Arnault and Dufournel, 1994; Wesser, 1996). *Tnt1* retrotransposon of tobacco was found to be induced by pathogens, microbial elicitors and abiotic factors. Activation of *Tnt1* was discussed as a local and early plant response to microbial stress (Pouteau et al. 1994; Mhiri et al. 1997; Vernhettes et al. 1997). Moreover, transposon insertion and their contribution to the evolution of plant disease resistance genes has been reported by Luck et al. (1998), Henk et al. (1999), and He et al. (2000). As invertases do play a major role in defence responses (Roitsch et al., 2003), CACTA-like insertion in intron I of *Lin5* could add up to stress induced genome modifications as outline above.

The fact that for two plant invertase genes a DNA insertion in the first intron has been demonstrated (Yau and Simon, 2003; this study) make invertase genes interesting candidates to study the functional aspects of transposon insertion.

PCR amplification and analysis of Solanaceae TPase-like sequences

TEs are fundamental components of most plant genomes and play an important role in structure, variation, and adaptive evolution of genomes (McClintock, 1984; McDonald, 1995; Kidwell and Lisch, 1997). As it used to be a difficult and time consuming task to identify transposons, new methods were developed to ease up this process. First, an increasing number of genomes are sequenced allowing computer-aided data mining. A CACTA-like transposon super family in the rice genome with at least 600 members was revealed by Wang et al. (2003) based on data mining and genomic cloning. Wicker at al. (2003) identified CACTA transposons in Triticeae by BLAST search and dot-blot analysis. Second, PCR based methods have been developed to amplify retrotransposon families (Flavell et al., 1992; Suoniemi et al., 1998; Wright et al., 1996) and conserved protein coding regions of transposons via degenerated primer pairs (Staginnus et al., 2001).

In this manuscript we report on the PCR amplification and sequence analysis of TPase fragments of Solanaceae species. Using a primer pair, which has been designed according to a TPase-like sequence of tomato, we could amplify TPase fragments throughout Solanaceae subfamilies, such as Solaneae (*L. esculentum, S. tuberosum*), Cestreae (*N. tabacum*) and Datureae (*D. stramonium*). For the latter two species this is the first report on TPase-like sequences. The presented sequence data, in particular the high sequence conservation (Figure 3A), will allow a detailed analysis of CACTA-like elements throughout Solanaceae species.

Furthermore, we describe a straight forward method that allows obtaining abundant sequences of TPase fragments within plant families or subfamilies. To achieve this goal, we performed PCR on genomic DNA to get variations of TPase fragments and sequenced the PCR product directly instead of cloning individual sequences. The integration effect during the sequencing process, because of covering randomly occurring mutations, results in an abundant sequence of the TPase fragments. This provides the opportunity to design highly specific PCR primer pairs based on such abundant sequences for the cloning and characterisation of TPase-like sequences within plant families or subfamilies.

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The nucleotide sequence data reported will appear in the GenBank database under the accession number AY 639885.

REFERENCES

Arnault C, Dufournel I (1994) Genome and stresses: reactions against aggressions, behavior of transposable elements. Genetica **93**: 149-160

Bennetzen JL (2000) Transposable element contributions to plant genome evolution. Plant Mol Biol **42**: 251-269

Chandlee JM (1990) The utility of transposable elements as tools for the isolation of plant genes. Physiol plantarum **79**: 105-115

Chopra S, Brendel V, Zhang J, Axtell JD, Peterson T (1999) Molecular characterization of a mutable pigmentation phenotype and isolation of the first active transposable element from *Sorghum bicolor*. Proc Natl Acad Sci USA **96**: 15330-15335

Flavell A, Smith D, Kumar A (1992) Extreme heterogenity of *Ty-copia* group retrotransposons in plants. Mol Gen Genet **231**: 233-242

Frey M, Reinecke J, Grant S, Saedler H, Gierl A (1990) Excision of the En/Spm transposable element of *Zea mays* requires two element-encoded proteins. EMBO J **9**: 4037-4044

Gierl A, Saedler H, Peterson PA (1989) Maize transposable elements. Annu Rev Genet **23**: 71-85

Godt DE, Roitsch T (1997) Regulation and Tissue-Specific Distribution of mRNA for Three Extracellular Invertase Isoenzymes of Tomato Suggests an Important Function in Establishing and Maintaining Sink Metabolism. Plant Physiol **115**: 273-282

He ZH, Dong HT, Dong JX, Li DB, Ronald PC (2000) The rice *Rim*2 transcript accumulates in response to *Magnaporthe grisea* and its predicted protein product shares similarity with *TNP*2-like proteins encoded by CACTA transposons. Mol Gen Genet **264**: 2-10

Henk A, Warren R, Innes R (1999) A new *Ac*-like transposon of *Arabidopsis* is associated with a deletion of the RPS5 disease resistance gene. Genetics **151**: 1581-1589

Hirochika H (1993) Activation of tobacco retrotransposons during tissue culture. EMBO J **12**: 2521-2528

Inagaki Y, Hitsatomi Y, Suzuki T, Kasahara K, Iida S (1994) Isolation of a *Suppressor-Mutator/Enhancer*-like transposable element, *Tpn1*, from Japanese morning glory bearing variegated flowers. Plant Cell **6**: 375-383

Kidwell MG, Lisch D (1997) Transposable elements as source of variation in animals and plants. Proc Natl Acad Sci USA **94**: 7704-7711

Kumar A, Bennetzen JL (1999) Plant retrotransposons. Ann Rev Genet **33**: 479-532

Kunze R, Saedler H, Lönnig WE (1997) Plant transposable elements. Adv Bot Res **27**: 331-470

Le QH, Wright S, Yu Z, Bureau T (2000) Transposon diversity in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **97**: 7376-7381

Lewin B (1997) Transposons. In B Lewin, Genes VI. Oxford University Press, Inc., New Yoek, pp 563-595

Lönnig WE, Saedler H (1997) Plant transposons: contributors to evolution? Gene **205**: 245-253

Luck J, Lawrence G, Finnegan E, Jones D, Ellis G (1998) A flax transposon identified in two spontaneous mutant alleles of the *L*6 rust resistance gene. Plant J **16**: 365-369

McClintock B (1984) The significance of responses of the genome to challenge. Science **226**: 792-801

McDonald JF (1995) Transposable elements: possible catalysts of organismic evolution. Trends Ecol Evol **10**: 123-126

Mhiri C, Morel JB, Vernhettes S, Casacuberta JM, Lucas H, Grandbastien MA (1997) The promoter of the tobacco *Tnt1* retrotransposon is induced by wounding and by abiotic stress. Plant Mol Biol **33**: 257-266

Miura A, Yonebayashi S, Watanabe K, Toyama T, Shimada H, Kakutani T (2001) Mobilization of transposons by a mutation abolishing full DNA methylation in *Arabidopsis*. Nature **411**: 212-214

Miura A, Kato M, Watanabe K, Kawabe A, Kotani H, Kakutani T (2004) Genomic localization of endogenous mobile CACTA family transposons in natural variants of *Arabidopsis thaliana*. Mol Gen Genomics **270**: 524-532

Nacken WKF, Piotrowiak R, Saedler H, Sommer H (1991) The transposable element TAM1 from *Antirrhinum majus* shows structural homology to the maize transposon En/Spm and has no sequence specificity of insertion. Mol Gen Genet **228**: 201-208

Ozeki Y, Davies E, Takeda J (1997) Somatic variation during long term subculturing of plant cells caused by insertion of a transposable element in a phenylalanine ammonia-lyase (PAL) gene. Mol Gen Genet **254**: 407-416

Pereira A, Cuypers H, Gierl A, Sommer ZS, Saedler H (1986) Molecular analysis of the En/Spm transposable element system in *Zea mays*. EMBO J **5**: 835-841

Pouteau S, Boccara M, Grandbastien MA (1994) Microbial elicitors of plant defense responses activate transcription of a retrotransposon. Plant J **5**: 535-542

Proels RK, Hause B, Berger S, Roitsch T (2003) Novel mode of hormone induction of tandem tomato invertase genes in floral organs. Plant Mol Biol **52**: 191-201

Roitsch T, Balibrea ME, Hofmann M, Proels R, Sinha AK (2003) Extracellular invertase: key metabolic enzyme and PR protein. J Exp Bot **54**: 513-524

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4**: 406-425

SanMiguel P, Bennetzen JL (1998) Evidence that a recent increase in maize genome size was caused by the massive amplification of intergene retrotransposons. Ann Bot **82**: 37-44

SanMiguel PJ, RamaKrishna W, Bennetzen JL, Busso C, Dubovsky J (2002) Transposable elements, genes and recombination in a 215-kb contig form wheat chromosome 5A(m). Funct Integr Genomics **2**: 70-80

Shirasu K, Schulman AH, Lahaye T, Schulze-Lefert P (2000) A contiguous 66 kb barley DNA sequence provides evidence for reversible genome expansion. Genome Res 10: 908-915

Snowden KC, Napoli CA (1998) PsI: a novel Spm-like transposable element from *Petunia hybrida*. Plant J **14**: 43-54

Staginnus C, Huettel B, Desel C, Schmidt T, Kahl G (2001) A PCR-based assay to detect *En/Spm*-like transposon sequences in plants. Chromosome Res **9**: 591-605

Souniemi A, Taskanen J, Schulman A (1998) *Gypsy*-like retrotransposons are widespread in the plant kingdom. Plant J **13**: 699-705

Vernhettes S, Grandbastien MA, Casacuberta JM (1997) In vivo characterization of transcriptional regulatory sequences involved in the defense-associated expression of the tobacco retrotransposon *Tnt1*. Plant Mol Biol **35**: 673-679

Wang GD, Tian PF, Cheng ZK, Wu G, Jiang JM, Li BD, Li Q, He ZH (2003) Genomic characterization of *Rim2 / Hipa* elements reveals a CACTA-like transposon superfamily with unique features in the rice genome. Mol Gen Genomics **270**: 234-242

Wesser SR (1996) Plant retrotransposons: turned on by stress. Curr Biol 6: 959-961

Wicker T, Stein N, Albar L, Feuillet C, Schlagenhauf E, Keller B (2001) Analysis of a contiguous 211 kb sequence in diploid wheat (*Tricitum monococcum* L.) reveals multiple mechanism of genome evolution. Plant J **26**: 307-316

Wicker T, Guyot R, Yahiaoui N, Keller B (2003) CACTA transposons in Triticeae. A diverse family of high-copy repetitive elements. Plant Physiol **132**: 52-63

Wright D, Ke N, Smalle J, Hauge B, Goodman H, Voytas D (1996) Multiple non-LTR retrotransposons in the genome of *Arabidopsis thaliana*. Genetics **142**: 569-578

Yau YY, Simon PW (2003) A 2.5-kb insert eliminates acid soluble invertase isoenzyme II transcript in carrot (*Daucus carota* L.) roots, causing high sucrose accumulation. Plant Mol Biol **53**: 151-162

Chapter 5

Regulation of source-sink relations by extracellular invertase *Lin6* of tomato: a pivotal enzyme for integration of metabolic, hormonal, and stress signals is regulated by a diurnal rhythm

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Regulation of source-sink relations by extracellular invertase *Lin6* of tomato: a pivotal enzyme for integration of metabolic, hormonal, and stress signals is regulated by a diurnal rhythm

Abstract. Extracellular invertases of tomato (*Lycopersicon esculentum*) are encoded by a gene family comprising four members. To elucidate the function of extracellular invertase Lin6, the corresponding promoter has been cloned and the sink-tissue-specific expression and its regulation by sugars, stress stimuli, growth regulators, and the diurnal rhythm is shown. An analysis in stable transformed tobacco plants carrying a *Lin6* promoter:ß-glucuronidase reporter gene fusion shows *Lin6* expression in pollen grains and a vascular tissue-specific expression in veins and stem. *Lin6* is strongly up-regulated in close proximity to wounded tissue and upon pathogen attack. Moreover, among the global signals known to modulate defence/stress response, methyl jasmonate and abscisic acid up-regulate *Lin6* expression. Salicylic acid on the other hand, as well as acetyl salicylic acid, suppress the wound induction of *Lin6*, supporting that *Lin6* is an inducible compound of the defence/stress response pathway that is antagonistically regulated by jasmonates and salicylates. An induction of the *Lin6* promoter in stable transformed BY2 suspension cultures by sugars and the growth promoting phytohormones cytokinin and auxin, along histochemical expression data, showing *Lin6* expression in germinating seeds and seedlings, indicates a role of Lin6 invertase during growth processes. In addition, *Lin6* is regulated by a diurnal rhythm that drives *Lin6* expression in subjective dawn. Transactivation assays with circadian oscillator elements of *Arabidopsis* Circadian Clock Associated 1 and Late Elongated Hypocotyl demonstrate functional interaction with the *Lin6* promoter.

INTRODUCTION

Carbohydrates are synthesised in photosynthetically active source tissues and exported to photosythetically less active or inactive sink tissues. The sink strength of plant tissues is not static; it varies during plant development and in response to external and internal stimuli. Extracellular invertases catalyse the irreversible hydrolysis of sucrose, the major transport form of carbohydrates in higher plants, to its hexose monomers and contribute to phloem unloading via an apoplastic pathway. The resulting hexoses are taken up into sink cells via hexose transporters, and further sucrose is released from the phloem following the concentration gradient (Eschrich, 1980; Roitsch and Tanner, 1996). As sucrose cleavage is assumed to be the rate-limiting step in this process, extracellular invertases are a potential target of regulation to satisfy the actual carbohydrate demand of different sink tissues. Indeed, the expression of extracellular invertases has been shown to be up-regulated by a number of stimuli that affect source-sink relations, such as plant hormones, sugar levels, abiotic stress, elicitors, and wounding (Tymowska-Lalanne and Kreis, 1998; Sturm, 1999; Sturm and Tang, 1999; Roitsch et al., 2003). The important role of extracellular invertases in assimilate partitioning, regulation of source-sink relations, and developmental processes has been supported by overexpression of yeast derived invertase in the apoplast of transgenic tobacco plants (von Schaewen et a., 1990), the analysis of invertase-deficient maize mutants (Miller and Chourey, 1992), the developmental regulation during seed development (Weber et al., 1995), and antisenes suppression of extracellular invertase in transgenic carrot plants (Tang et al., 1999).

Plant extracellular invertase genes represent small gene families consisting of several members with specific expression patterns (Tymowska-Lalanne and Kreis, 1998; Roitsch et al., 2000; Sherson et al., 2003). Interestingly, flower-specific isoenzymes that are expressed in distinct flower organs, such as anthers, pollen, flower buds, or fruits have been identified (Lorenz et al., 1995; Weber et al., 1995; Godt and Roitsch, 1997; Maddison et al., 1999; Hedley et al., 2000; Goetz et al., 2001). In particular, a number of anther- and pollen-specific invertase isoenzymes have been characterised, thereby emphasising a crucial role of extracellular invertase in providing carbohydrates to the male gametophyte. This idea was corroborated by a functional approach in transgenic tobacco plants. Goetz et al. (2001) demonstrated that antisense suppression of *Nin88* extracellular invertase results in a block during pollen development. These results are in line with the identification of pollen-specific monosaccharide transporters (Truernit et al., 1999; Scholz-Starke et al., 2003; Schneidereit

et al., 2003) and support an essential role of apoplastic sucrose cleavage for providing carbohydrates to pollen tissue.

Wounding and pathogen infection are severe environmental stress factors a plant has to cope with. Analysing how plants respond to stress factors, it became evident that induction of defence responses is accompanied by a fast increase of sink metabolism. Under those conditions a fast and strong up-regulation of extracellular invertase transcript levels is observed (Roitsch et al., 1995; Zhang et al., 1996; Sturm and Chrispeels, 1990; Sinha et al., 2002). The coordinated induction of a monosaccharide transporter and cell-wall invertase during infection with a fungal biotroph (Fotopoulos et al., 2003) confirms an essential role of apoplastic sucrose cleavage in mediating defence responses. Both invertase genes and defence related genes have been found to be coinduced by soluble sugars (Herbers et al., 1996; Zhang et al., 1997; Roitsch et al., 1995, Sinha et al., 2002). Therefore, the increased attraction of carbohydrates could serve as metabolic energy to support appropriate defence responses as well as a self-amplifying signal resulting in an intensified retention of carbohydrates and modulation of gene expression (Roitsch et al., 2000; Herbers et al., 2000). As detailed above, tissue-specific expression in combination with regulation by distinct stimuli confers specific physiological functions to invertase isoenzymes (Lorenz et al., 1995; Weber et al., 1995; Godt and Roitsch, 1997; Kim et al., 2000; Proels et al., 2003; Sherson et al., 2003). Thus, elucidating the expression profiles and regulation patterns of particular isoenzymes within one species is important for a comprehensive understand of extracellular invertase function within the complex regulatory mechanism that coordinates carbohydrate partitioning during development and under different environmental conditions.

Jasmonic acid (JA) and methyl jasmonate (MeJA) have been recognised as plant growth regulators that are involved in various developmentally and environmentally induced changes in gene expression (Creelman and Mullet, 1997; Wasternack and Pathier, 1997). The roles of jasmonates in response to biotic and abiotic stress are well documented (Benedetti et al., 1998; Park et al., 2002). Furthermore, there is evidence for a dual role of JA in both plant development and defence response (Creelman and Mullet, 1997). Recent studies with JA-biosynthetic mutants have shown that jasmonates play an essential role in pollen maturation and wound-induced defence against biotic attacks (Ishiguro et al., 2001; Park et al., 2002). However, there is no downstream gene yet characterised as a potential target of both signalling pathways.

The family of extracellular invertases from tomato (*Lycopersicum esculentum*) is represented by four different enzymes, Lin5, Lin6, Lin7, and Lin8 (Godt and Roitsch, 1997). *Lin5* and *Lin7* were shown to be arranged as a genomic tandem and to be involved in fruit and pollen development, respectively (Fridman et al., 2000; Fridman and Zamir, 2003; Proels et al., 2003). In order to understand the specific function of extracellular invertase Lin6, the

promoter sequence of *Lin6* was cloned and the expression and regulation studied in transiently and stable transformed plant tissues. This allowed in particular addressing both temporal as well as spatial regulation of *Lin6* expression. The *Lin6* promoter confers reporter gene expression in pollen of transgenic tobacco and a strong induction of *Lin6* expression in vegetative tissues upon wounding and pathogen attack was demonstrated. Furthermore, novel modes of *Lin6* regulation in response to growth stimulating hormones and sugars and an oscillation of *Lin6* expression under a diurnal rhythm is shown. The *trans*-acting factors Circadian Clock Associated 1 (CCA1) and Late Elongated Hypocotyl (LHY), which are central circadian oscillator elements of *Arabidopsis* (Alabadi et al., 2002; Carre and Kim, 2002), were shown to functionally interact with *Lin6* promoter elements.

MATERIALS AND METHODS

Cloning and sequence analysis

Genomic sequences of *Lin6* were cloned using the GenomeWalker System (CLONTECH, Heidelberg, Germany) according to the instructions of the manufacturer. Briefly, genomic DNA was isolated from tomato leaves (*Lycopersicon esculentum* cv Moneymaker) with the NucleoBond System (Macherey und Nagel, Düren, Germany) and digested with different restriction enzymes, to gain corresponding libraries. Besides the recommended restriction enzymes, *Hpa*I, *Sma*I, *Ssp*I and *Eco*72I were used to create additional libraries. After ligation with the provided adaptors the libraries were used as template for PCR and nested PCR. Starting out of a known region of exon III of the *Lin6* gene (Acc. Nr. X91390) three sequential walks were performed to obtain 7092 bp including 3.4 kb of the 5´ flanking region of *Lin6*. An overlap of 252 bp or 290 bp of identical sequence of the preceding genome walk fragment confirmed the identity of successive fragments. Vectors, corresponding primer sequences and the libraries used are shown in Table 1. The obtained PCR products were cloned using the Ins T/A clone PCR Product Cloning Kit (MBI-Fermentas, St. Leon-Rot, Germany) and sequenced by SEQLAB (Göttingen, Germany). For sequence analysis the Vector NTI Suite (Infor Max, Bethesta USA) was used. Putative regulatory elements were identified by sequence alignments to known *cis*-acting elements.

Vector	gene specific primers (GSPs) in 5´ to 3´ orientation		Library
6-3-2 3rd walk	GSP 1	TCGGACCCAAGAACTCTGATTAAGGATG	*Eco72*I
	GSP 2	CATGGAACTGTCATTGGATCACGGTAAG	
6-2-2 2nd walk	GSP 1	CCAACCCACGTACCACACCTGGTTTG	*Hpa*I
	GSP 2	GGGTGGGAGGTGGAGTTGACTATAC	
6-1-1 1st walk	GSP 1	GTAGATAGCGGGTTCGAGCGGGATCCAG	*EcoR*V
	GSP 2	GATCAAGTCCTTTGAGACTGAATGAGC	

Table 1. Primers used for PCR based cloning of the 5´genomic region of *Lin6*. Libraries that yielded the corresponding PCR fragments are indicated. GSP1 = gene specific primer 1, GSP2 = gene specific primer 2 (nested primer).

Plasmid construction

For GUS reporter gene analysis two promoter constructs of different size were designed. To get the full length promoter, PCR on genomic DNA was performed using the forward primer Lin6-15 (5'-ATACCCAAGCTTGATGTGGCTCTCACTTTCTCCACGC-3´) to introduce a *Hind*III restriction site and the reverse primer LIN6-2 (5'-TCTAGTCTAGACTCCATCTTTAATTCTTTCTTTTTGTG-3´) to introduce an *Xba*I restriction site. The PCR product was first cloned in pGEM-T, released with *Hind*III and *Xba*I and cloned into the binary vector pBI101+ using *Hind*III and *Xba*I sites, thereby fusing *Lin6* start codon in frame to the *uidA* translation start. The resulting construct pPR631 was partly sequenced to check the integrity of the fusion. To get a shortened promoter fragment of 1 kb size, the forward primer LIN6-1 (5'-ATACCCAAGCTTACCGTGATCCAATGACAGTTCC-3´) was used, the further proceeding was the same as described for pPR631 yielding vector pRP611. For protoplast analysis, the full length promoter GUS fusion cassette of pPR631 was subcloned in pUC19 using the restriction sites *EcoR*I, yielding construct pUC19*Lin6*::GUS.

LHY cDNA cloned in pBluescript was received from G. Coupland (Köln, Germany). LHY cDNA was released from pBluescript and cloned in pRT101 using restriction sites *Xho*I and *BamH*I yielding construct pRT101-LHY. CCA1 cDNA cloned in pBI121 vector (pBCA126) was received from E.M. Tobin (Los Angeles, USA) and used without further manipulations (Wang and Tobin, 1998).

Plant materials

For histochemical staining, fluorometric GUS assays and protoplast isolation *Nicotiana tabacum* (cv Samsun) plants were grown under greenhouse conditions in Würzburg, Germany at 25°C with additional illumination from 7 a.m. to 7 p.m. of 60 klx.

To perform *in-situ* invertase assays, *Lycopersicon esculentum* (cv Moneymaker) plants were grown under greenhouse conditions as described above.

Generation of transgenic tobacco plants

The *Lin6*::GUS constructs pRP611 and pRP631 were transformed in tobacco (*Nicotiana tabacum* cv Samsun) by using standard *Agrobacterium* (LBA 4404) transformation procedures (Horsch et al., 1985). Ten independent lines were analysed by PCR and flourometric GUS assays for transgenity and wound inducibility of reportergene (GUS) and maintained in the greenhouse.

Histochemical GUS assays

For histochemical GUS analysis the method of Jefferson et al. (1987) was followed. Plant material was vacuum infiltrated with the GUS histochemical substrate 5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid (1 mM) and stained for GUS activity (14 h reaction time). Images were created using a binocular (Leica MZFL III, Solms, Germany). Stained samples were stored in 70% ethanol at -20°C until analysis.

Transformation and treatment of BY2 suspension cultures

Tobacco BY2 cells were transformed via *Agrobacterium* (LBA 4404) by 48 h of cocultivation as described by Atanassova et al. (2003). The obtained culture was maintained by weekly dilution of the cells by Linsmaier and Skoog-modified medium (10:80 v/v) supplied with 100 µg/ml kanamycin and 500 µg/ml carbenicillin.

To carry out induction experiments, 5 ml of the culture were inoculated in 80 ml of the medium with a reduced sucrose content of 3 g/l. Cells were responsive to hormonal stimuli 3 to 4 d after subculturing. For each individual experiment 1 ml of culture was distributed in 1.5 ml reaction tube and induced by adding hormones in indicated final concentrations: MeJA 100 µM; ABA, Zeatin, 2,4-D 10 µM each. The cells were incubated for 14 h under permanent shaking in dim light and harvested by centrifugation; GUS activity was determined as described below.

Determination of GUS activity

Leaf discs or BY2 suspension cells were collected in 1.5 ml tubes and ground in 250 µl GUS extraction buffer (50 mM $NaPO_4$ pH 7, 10 mM Na_2 EDTA, 0.1% Triton X100, 0.1 % sodium lauryl sarcosine, 10 mM 2-mercaptoethanol). After centrifugation for 10 min (15,000g) at 4°C, 50 µl of supernatant was mixed with 50 µl GUS assay solution (2 mM 4-methylumbelliferyl-D-glucuronide in extraction buffer). The mixture was incubated at 37°C for 1 to 3 h and stopped in 0.3 M Na_2CO_3. GUS activity was determined using a luminescence spectrometer (Perkin Elmer LS 30, Langen, Germany) and protein concentration of tissue homogenates was determined with the Bradford reagent (Bradford, 1976) with bovine serum albumin as standard.

To determine GUS activity of pollen grains, the corresponding aliquots were harvested by centrifugation, resuspended in 100 µl GUS assay solution (1 mM 4-methylumbelliferyl-D-glucuronide in extraction buffer) and further treated as stated above. Specific GUS activity was determined as cleaved substrate per amount of pollen and min.

Protoplasts were harvested by centrifugation, resuspended in 50 µl of GUS extraction buffer and immediately frozen in liquid nitrogen. After thawing the samples, protoplasts were broken up by vortexing. Samples were centrifuged for 5 min at 4°C at 15,000 rpm and 25µl of the lysate were incubated with 25 µl of 1 mM 4-methylumbeliferyl-D-glucuronide in extraction buffer at 37°C and further treated as stated above.

In-situ invertase analysis

Leaf explants were fixed in a solution of 4% formaldehyde (in phosphate buffer pH 7.0) for 30 min at room temperature. Fixed tissues were incubated at 26°C in a freshly prepared solution consisting of equal volumes of phenazinmethosulfate (0.56 mg/ml), nitrobluetetrazolium (0.96 mg/ml), glucoseoxidase (4.2 mg/ml; 20,000 U/g), and sucrose (80 mg/ml), each buffered at pH 4.0 in 50 mM sodium phosphate buffer, until appearance of a dark staining. In control assays the sucrose was replaced by phosphate buffer, which resulted each time in no detectable staining (data not shown). Stained samples were stored in 70% ethanol at -20 °C.

Treatment of tomato suspension cultures

Photoautotrophic suspension cultures (*L. peruvianum*) were grown for one week under constant light conditions and kept for one further week under a light/dark regime of 12h/12h under constant temperature conditions of 26°C. At indicated time points (LD = 11, 13, 16, 23, 25, 30, 35, 37, 40, 47, 49, 50) 10 ml of cell culture were harvested by centrifugation and immediately frozen in liquid nitrogen and stored at -80 °C until RNA isolation.

RNA isolation and Northern blotting

Total RNA was isolated from ground plant material essentially according to the method of Chomczynski and Sacchi (1987). Northern blot analysis was performed as described (Godt and Roitsch, 1997) by using a radioactive labelled *Lin6*-specific probe. The cloned cDNA fragment of *Lin6* was labelled by using a random primer DNA-labelling kit (MBI Fermentas, St. Leon-Rot, Germany). Signals were detected via a phosphor-imaging system (BAS 2000, FUJIX, Japan).

Expression of CCA1 and LHY in tobacco protoplasts

Preparation of *Nicotiana tabacum* (cv Samsun) mesophyll protoplasts transformation and GUS assays were performed essentially as described by Lyck et al. (1997). 1 to 3 µg of GUS-reporter plasmid and CCA1/LHY expression plasmids in constant molar ratios were used for transformation of 25,000 protoplasts resuspended in 50 µl MES buffered K3M medium. 20 min after mixing with 100 µl 20% Polyethylenglycol (PEG_{6000}, Duchefa, The Netherlands) the suspension was diluted with 900 µl MES buffered K3M and incubated in dim light overnight at 25°C. Protoplasts were harvested by centrifugation and resuspended in GUS extraction buffer. GUS measurements of at least three independent transformation experiments were performed as described above.

RESULTS

Cloning of the promoter of extracellular invertase Lin6 of tomato and defining putative TATA-box and transcription start site

In order to isolate the 5´-sequences responsible for regulating expression of *Lin6*, three 5´-genome walks were performed on genomic libraries of tomato starting from exon III of *Lin6* (Acc.Nr. X91390). In total 7092 bp, represented by three overlapping clones, were obtained, 3414 bp cover sequences 5´ of the translation start site (Fig. 1).

The exon/intron organisation of the cloned sequence was obtained by comparison with the complete cDNA sequence of *Lin6* (Acc.Nr. AB004558). The putative transcription start site is located 49 bp 5´ of the translation start codon and matches with reported consensus sequences (Joshi, 1987). A putative TATA-box (TATAAA) appears 35 bp 5´ of the putative transcription start site. An identical sequence and position for TATA-box motives was found for *Lin5* and *Lin7* extracellular invertases of tomato (Proels et al., 2003).

The *Lin6* promoter was analysed for the presence of regulatory elements with similarity to known *cis*-acting elements via sequence alignments. As shown in Fig. 1, these elements are concentrated in three promoter regions, indicated as cluster I, II and III. The experimental evidence for the functional significance and physiological relevance of these regulatory sequences is outlined below.

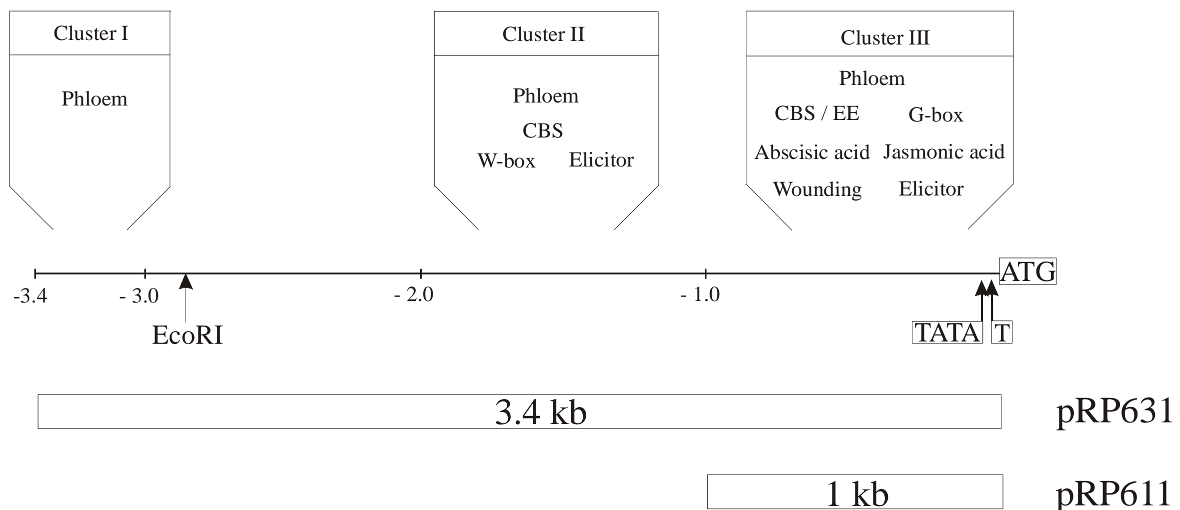

Fig. 1 The *Lin6* promoter structure. Putative *cis*-acting elements and their clustering, a putative TATA box and transcription start site (T) are indicated. Promoter fragments used for reporter gene constructs are shown. An *EcoR*I site, used to subclone the *Lin6*::GUS cassette in pUC19 is indicated. CBS = CCA1 binding site, EE = evening element

The Lin6 promoter is active during early stages of seedling development in vascular tissue

To analyse the tissue-specific expression of the *Lin6* promoter transgenic tobacco plants expressing a reporter gene fusion were generated. 3.4 kb of *Lin6* promoter sequences were subcloned in the binary vector pBI101+ to drive the expression of the ß- glucuronidase (GUS) reporter gene. The obtained *Lin6* promoter*::GUS* fusion on plasmid pRP631 was transformed in *Agrobacterium* that were used to generate transgenic tobacco lines. PCR and fluorometric GUS assays were used to confirm the presence and wound inducibility (GUS) of the transgene in ten independent lines. Tissue explants of those transgenic plants were harvested and used for histochemical detection of GUS activity by an *in-situ* staining procedure.

Seeds derived from *Lin6*::GUS transgenic line were germinated and GUS staining was shown to be present already in the very first stage of development at the beginning of seed germination (Fig. 2a). In young seedlings GUS staining could be detected in veins of cotyledons and the vascular tissue of the stem (Fig. 2b). After approximately further three weeks of development, once first leaves are formed, GUS expression occurs in major and minor veins (Fig. 2c). This expression pattern is in agreement with the appearance of *cis*-acting regulatory elements for phloem expression in *Lin6* promoter. Elements for phloem specific- and clock-regulated expression are most striking features of *Lin6* promoter as we could show via sequence alignments to known *cis*-acting elements. Conserved sequence elements that are implicated in governing phloem-specific expression as described by Yin et al. (1997) are the ASL box ($GCA(N)_{10-17}GCA$), the GATA motif ($A(N)_3GATA$), and the Box II motif (CCCC, flanked by CCA/TGG repeats). The most prevalent element in *Lin6* promoter is the GATA motif that is located in Cluster I (positions: -3298, -3113, -3103), II (positions: -1639, -1365), and III (positions: -502, -434, -338, -274, -238, -159). Cluster I does include an ASL box homologue at position -3247: $GCA\ (N)_{64}GCA(N)_{23}GCA$. A homologue to the box II motif is located at position -885: $TGG(N)_{34}CCCC(N)_{27}CCA(N)_9CCA$.

In adult plants no constitutive expression of the *Lin6* promoter could be detected except for pollen grains, but a strong induction of the *Lin6* promoter upon wounding has been found as detailed below.

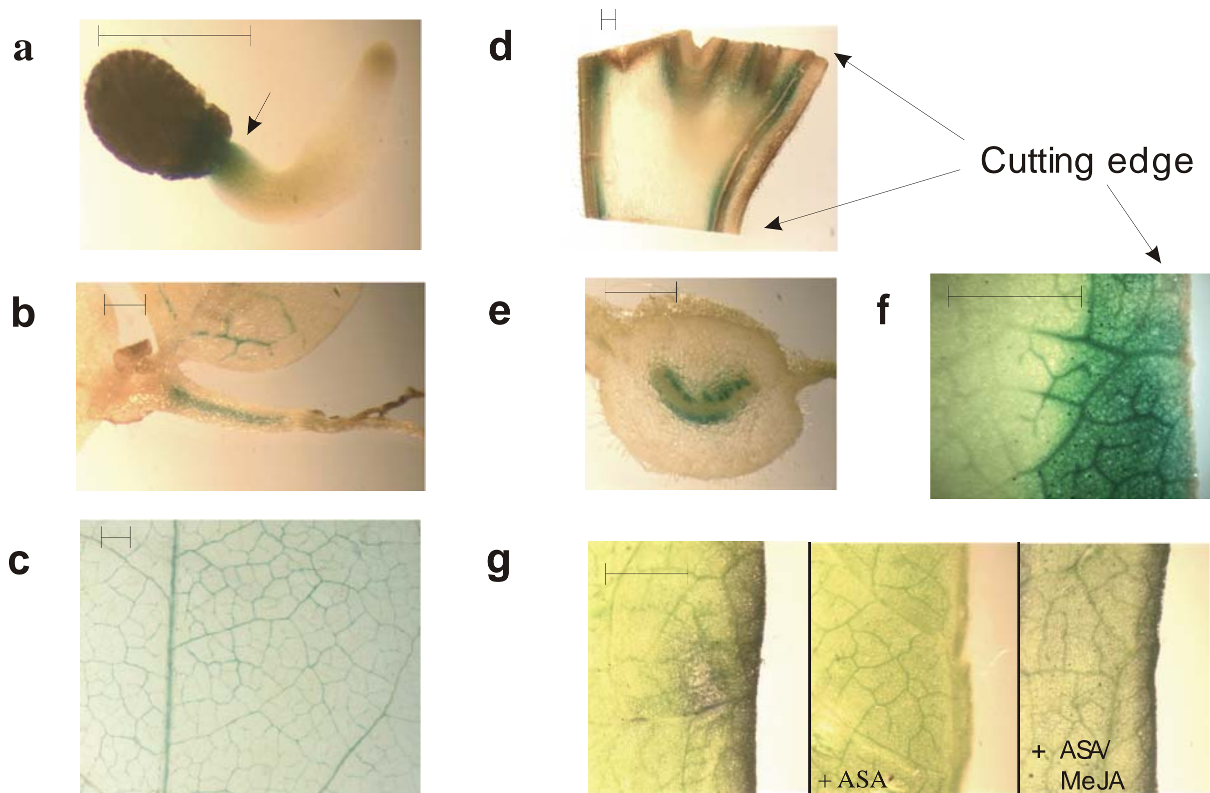

Fig. 2a-g GUS histochemical assays and *in-situ* invertase stainings. Bars represent 1 mm.
a to **f**, GUS histochemical assays of *Lin6* expression in *Lin6*::GUS (pRP631) transgenic tobacco plants. **a**, a germinating seed; **b**, a young seedling with just kotyledons being developed; and **c**, a fraction of a leaf with the major vein and side veins are shown. **d**, stem; **e**, petiol; and **f**, leaf samples were harvested from the plant and thereby injured with a razor blade to apply a wound stimulus. After overnight incubation GUS staining was performed. **g**, *In-situ* invertase assays on wild type tomato plants. Leaf explants derived from the same leaf were incubated overnight in water, 1 mM ASA or 1 mM ASA/ 100µM MeJA followed by *in-situ* invertase staining. The cutting edge is located on the right side in each shown leaf image.

Induction of the Lin6 promoter by sugars and growth stimulating hormones

Actively growing tissues are carbohydrate sinks with elevated demand for carbohydrates and are characterised by a high extracellular invertase activity. To analyse the metabolic regulation by sugars and regulation by growth stimulating phytohormones a *Lin6*::GUS transformed suspension culture line of tobacco was used. Because the *Lin6* promoter is strongly induced by wounding, as detailed below, transgenic suspension cultured cells were generated to circumvent the problems associated with explants. It is possible to take samples and apply stimuli while wounding and stress syndromes are excluded. In addition, the use of tissue cultures for studying the effect of endogenous stimuli has the advantage that growth conditions may be strictly controlled and experiments are not

complicated by differentiation processes. No cuticula or thick cell layers have to be penetrated and any single cell is exposed to the stimulus at virtually the same time.

The tobacco cell suspension line BY2 was transformed by *Agrobacterium* mediated transformation with the same *Lin6*::*GUS* construct, pRP631, which was used to generate stable transformed plants. After selection of transgenic calli on solid medium, transformed cell suspension lines were established with growth characteristics of the parental BY2 wild type cell line (data not shown). Because it is known that sugars induce extracellular invertases (Roitsch et al., 1995), the only drawback of the heterotrophic tobacco cell suspension line is the fact, that they require exogenous sugar for growth. This obstacle could be circumvented by a series of experiments where we analysed the responsiveness of the *Lin6* promoter in a time course experiment (data not shown). The goal was to identify a culture period where the exogenous sugar concentration declined to levels not interfering with *Lin6* expression while the cells were still physiological active and not yet suffering from sugar depletion. The initial sucrose content declines during cultivation because of uptake and metabolisation and ultimately results in a low sugar concentration in the media that allows induction experiments. Usually BY2 cells are grown in the presence of a high initial sucrose concentration of 3 % to avoid sugar depletion even at high cell densities. Therefore, suspension cells were inoculated in fresh BY2 medium with a 90 % reduced sucrose content of 3 g/l compared to the normal cultivation medium. Starting 3 days after subculturing samples were distributed in 1.5 ml reaction tubes, induced with different hormone stimuli and sugars, and analysed after 12 h for GUS activity. It has been found that suspension cultures were sensitive for about 24 h 3 to 5 days after subculturing, depending on the status of the individual pre-culture (data not shown). For the experiments shown in Fig. 3 we determined the status of the culture and carried out the whole set of experiments in the sensitive phase. Fig. 3a demonstrates an induction of the *Lin6* promoter by a factor 2.5 ± 0.25 upon treatment with 10 μM of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) and by a factor of 1.8 ± 0.16 upon treatment with 10 μM of the cytokinin zeatin. In addition a slight induction of 1.4 ± 0.2 after treatment with 30 mM glucose and a 2.2 ± 0.5 fold increase upon treatment with 30 mM sucrose was observed. In control experiments with mannitol no effect on the GUS level was observed (Fig. 3b) ruling out an osmotic effect of the sugar concentrations applied and supporting a specific effect of glucose and sucrose on the *Lin6* promoter.

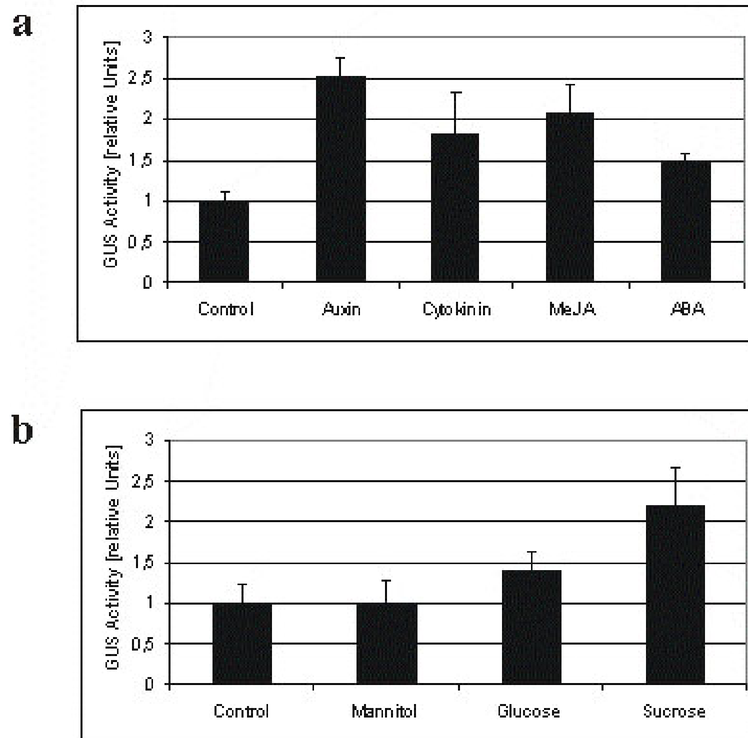

Fig. 3 a, b Relative GUS activity in BY2 suspension cells carrying *Lin6*::GUS (pPR631) in response to different stimuli. The control is the same transformed cell suspension without treatment. Three days after subculturing, samples of 1.5 ml were distributed in reaction tubes and induced with different stimuli for 12 h under permanent shaking. Crude extracts were analysed for GUS activity. The control samples showed an activity of about 240 pmol MU/mg protein/ min. **a**, For hormone treatment cells were induced with 100 µM MeJA, 10 µM zeatin, 10 µM 2,4-D, and 10 µM ABA. **b**, For sugar treatment cells were subcultured in a reduced sugar medium to get a natural sugar depletion due to metabolisation. Cells were then incubated in the presence of 30 mM glucose, sucrose or mannitol.
Data are representative for four to six independent experiments, standard deviations are indicated.

Wounding and pathogen attack strongly up-regulate Lin6 expression

The *Lin6* promoter is characterised by sequences that show high similarity to known elicitor and wound response boxes. In detail, two putative elicitor response elements that show just two base pair exchanges to the ACCTTGCC element were found at the positions -1588 and –624 (Fig. 1). This element was shown by Palm et al. (1990) to confer elicitor responsiveness to the proteinase inhibitor II promoter in potato. Menke et al. (1999) have shown that a 24 bp sequence including a GCC core acts as an important *cis*-element required for JA- and elicitor-responsive gene expression. Two GCC elements are located in close proximity at the positions -639 and -653. A sequence that shows high homology (two base pair exchange) to the wound response element described by Siebertz et al. (1989), TTGTTGAAATATA, is located at position -172. Three W-box elements (GTCA) are located in close proximity at the positions -1748, -1741, and -1732. W-boxes are binding sites for WRKY transcription factors that are involved in plant defence signalling (Eulgem et al., 1999).

To gain insight into the spatial distribution of *Lin6* induction a wound stimulus was applied to source leaves of *Lin6*::GUS plants by cutting the leaf with a razor blade. After 12 h incubation the effect on the *Lin6* promoter was analysed *in-situ* by performing a

histochemical GUS activity staining. As shown in Fig. 2f, the *Lin6* promoter confers GUS expression starting at the cutting edge just adjacent to a thin layer of necrotic cells and reaching approximately 0.5 mm inwards. The expression is not limited to the veins but could be detected in the leaf tissue in proximity to the wounded area, indicating that the wound stimulus is able to broaden the expression of *Lin6* even in non-vascular tissue. However, strongest GUS expression is found within the veins with a higher expression in those veins being located more closely to the cutting edge. Non-wounded leaf areas do not show any GUS expression. Fig. 2d and 2e show in addition cross sections of a stem and petiole, respectively. Both tissues were wounded with a razor blade and incubated for 12 h. GUS staining was performed on the intact stem and petiole explant, later cross sections were analysed. Induction of the *Lin6* promoter could be detected only adjacent to the cutting edge. Again, as seen in the leave section, highest GUS expression is present within the vascular tissue close to the wounded area, supporting a vascular tissue-specific wound effect on *Lin6* promoter activity.

As promoter sequences with high homology to wound response boxes are restricted to the first kb of the *Lin6* promoter (Fig. 1), a second set of transgenic tobacco plants were generated that included only 1 kb of the most 3´ located *Lin6* promoter sequence to drive GUS reporter gene expression. For this purpose tobacco plants were transformed using plasmid pRP611 and transgenic plants were analysed for wound induction as outlined above. The histochemical analyses of GUS reporter gene activity revealed the same temporal and spatial expression pattern described above (data not shown), indicating that the 1 kb promoter fragment is sufficient to drive wound induction and supporting that the identified *cis*-acting regulatory sequence are functional relevant.

To quantify the *Lin6* induction upon wounding, full length *Lin6*::GUS tobacco plants were treated as described above and analysed in a fluorometric GUS assay. As shown in Fig. 4a, wounding does induce *Lin6* promoter more than 50 fold over the control. An even higher induction of about 140 fold over the control was detected in leaf material derived from tobacco plants that were accidentally infected by the tobacco white fly, *Bemisia tabacci,* amounting to 17,000 pmol 4-methylumbelliferone (MU) x mg^{-1} protein x min^{-1}. Those leaves were persistently attacked by the white fly and were visibly damaged. Obviously this permanent stress stimulus did strongly induce sink metabolism in those leaves and consequently, *Lin6*, as a sink-specific gene was dramatically up-regulated. Although the strong increase in GUS activity might be partially because of an accumulation of the rather stable ß-glucuronidase protein, the finding confirms a dramatic and sustained induction of the *Lin6* promoter by long-term herbivore attack.

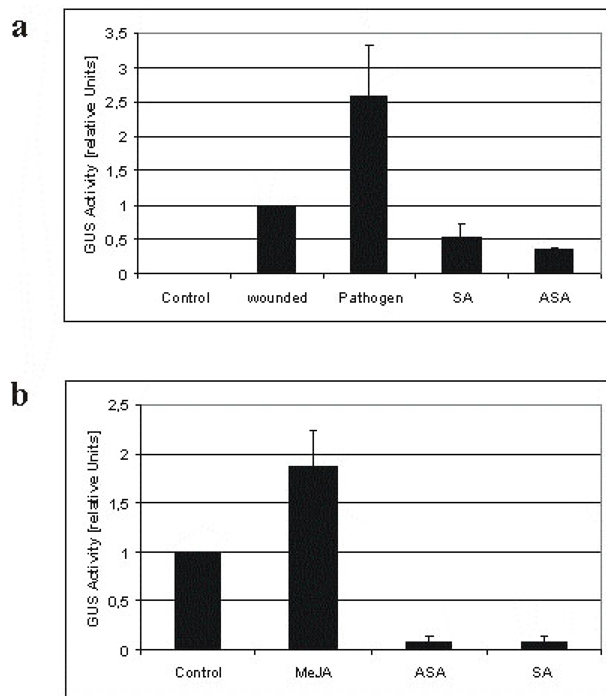

Fig. 4 a, b Relative GUS activity in stable transformed *Lin6*::GUS (pRP631) tobacco plants. **a**, Leaf explants of stable transformed *Lin6*::GUS (pRP631) tobacco plants were immediately analysed for GUS activity (control) or wounded with a razor blade and incubated over night followed by GUS analysis (wounded). Leaf explants of plants that were accidentally contaminated by the tobacco white fly, *Bemisia tabacci*, were included (pathogen). For inhibitor assays, leaf samples were harvested and immediately put in a aqueous solution of 1 mM SA or ASA followed by wounding with a razor blade. After overnight incubation samples were analysed for GUS activity. Three to four independent assays were each normalised to the GUS expression of the wounded sample, standard deviations are indicated. **b**, Pollen granules of stable transformed *Lin6*::GUS (pRP631) tobacco plants were collected from mature flowers, emulgated in water and distributed in 1.5 ml reaction tubes in four equal volumes. One was used as control the others were treated with 100 µM MeJA, 1 mM ASA or 1 mM SA. GUS activity was measured after 15 h, each set of experiments was normalised to the control. Data represent five independent sets of experiments, standard deviations are indicated.

Extracellular invertase Lin6 is a target of defence/stress response pathways

The same transformed BY2 suspension cultures used to show an induction of *Lin6* promoter by growth stimulating hormones and sugars, were used to apply hormones involved in plant stress responses, abscisic acid (ABA) and MeJA. With this experimental system we can show that GUS activity rises by a factor of 2.1 ± 0.3 upon treatment with 100 µM MeJA and by a factor of 1.5 ± 0.1 with 10 µM ABA (Fig. 3a). Regulation of the *Lin6* promoter by ABA is in agreement with the identification of an ABA response cluster as described for several plant promoters (Shen and Ho, 1995), consisting of a CE-1 core element (CACC) at the position -206 and a G-box core motif (ACGT) at the position -127 in the *Lin6* promoter (Fig. 1).

Because jasmonates and other oxidated lipids are involved in regulating plant defence reactions in response to various stress stimuli including mechanical wounding (Benedetti et al. 1998), the signal transduction pathway involved in wound-induced *Lin6* expression has been further characterised. Phenolics, and in particular salicylic acid (SA) and acetyl salicylic acid (ASA), are known to interfere with jasmonate biosynthesis and octadecanoid signalling and frequently an antagonistic interaction is observed in jasmonate mediated signalling of pathogen infection and mechanical wounding (Koiwa et al., 1997).

Because JA was shown to induce *Lin6* expression as outlined above, we have tested the effect of SA and ASA on the wound-induction of *Lin6*. Leaves were harvested, immediately put in an aqueous solution containing 1 mM SA or 1 mM ASA and wounded with a razor blade. Fig. 4a shows that pre-treatment with SA or ASA interfered with *Lin6* induction after wound treatment. GUS expression is reduced by SA and ASA to 54% and 35% of the value of the wounded sample, respectively.

To substantiate whether the transcriptional induction of *Lin6* upon wound treatment is reflected by an increase in invertase activity, we applied the same wound treatment to wild type tomato leaves. The distribution of invertase activity upon wounding was visualised *in-situ* by a histochemical invertase activity stain. Fig. 2g demonstrates that the spatial distribution of the invertase activity in wounded tomato leaves matches the histochemical GUS expression pattern of the transgenic tobacco plants harbouring a *Lin6*::GUS reporter gene construct (Fig. 2f). The presence of ASA efficiently suppresses accumulation of invertase activity and JA can overcome the inhibitory effect of ASA and restores invertase activity (Fig. 2g).

Lin6 expression in pollen grains is regulated by jasmonate

In adult plant the only tissue identified with constitutive *Lin6* expression is pollen. Transgenic plants expressing the *Lin6*::GUS construct accumulate ß-glucuronidase to a high level with an activity of 76 pmol MU x 5000 pollen^{-1} x min^{-1}. As jasmonate is known to be a regulator of pollen development (Park et al., 2002), we tested the effect of 100 µM MeJA on *Lin6* expression. As shown in Fig. 4b the high constitutive expression level is further elevated by MeJA nearly twofold (1.9 $\pm$ 0.4). In contrast, the basal GUS level in pollen is strongly inhibited in the presence of 1 mM SA (0.09 $\pm$ 0.05) and 1 mM ASA (0.09 $\pm$ 0.06) resulting in a 90% reduction. Those results support an important function of the regulation of *Lin6* expression via the octadecanoid pathway and SA signalling for *Lin6* regulation and links *Lin6* invertase expression to developmental processes in reproductive organs.

Lin6 expression is regulated by a diurnal rhythm

Because extracellular invertases regulate source-sink relations the influence of light on the expression of *Lin6* has been determined. Photosynthetically active cell suspension cultures of tomato (*L. peruvianum*), which grow with CO_2 as the sole carbon source, have already been proven as appropriate experimental system to study source-sink regulation (Sinha et al., 2002). The use of this experimental system has the advantage that they are

responsive to light, wound induction of *Lin6* during the harvesting process is circumvented, and it is possible to apply homogenous light conditions to the experimental system. Cell cultures were kept under a 12 h light/12 h dark regime for seven days. The analysis was started 11 h after the onset of a light cycle and *Lin6* expression was followed by northern blot analysis during two full light/dark cycles (48 h). This analysis revealed a periodical variation of *Lin6* expression (Fig. 5a). A clear pronounced minimum of *Lin6* expression could be detected at 11 h to 13 h and 35 h to 37 h, that is at the end of the light-, and the beginning of the dark-phase. Maxima of *Lin6* expression appear 23 h to 25 h and 47 h to 49 h, which is at the end of the dark-, and the beginning of the light-phase. A cycle of *Lin6* expression can be described as follows: *Lin6* starts getting up-regulated in the middle of the dark period, reaches its maximum at the dark-light transition and gets downregulated thereafter.

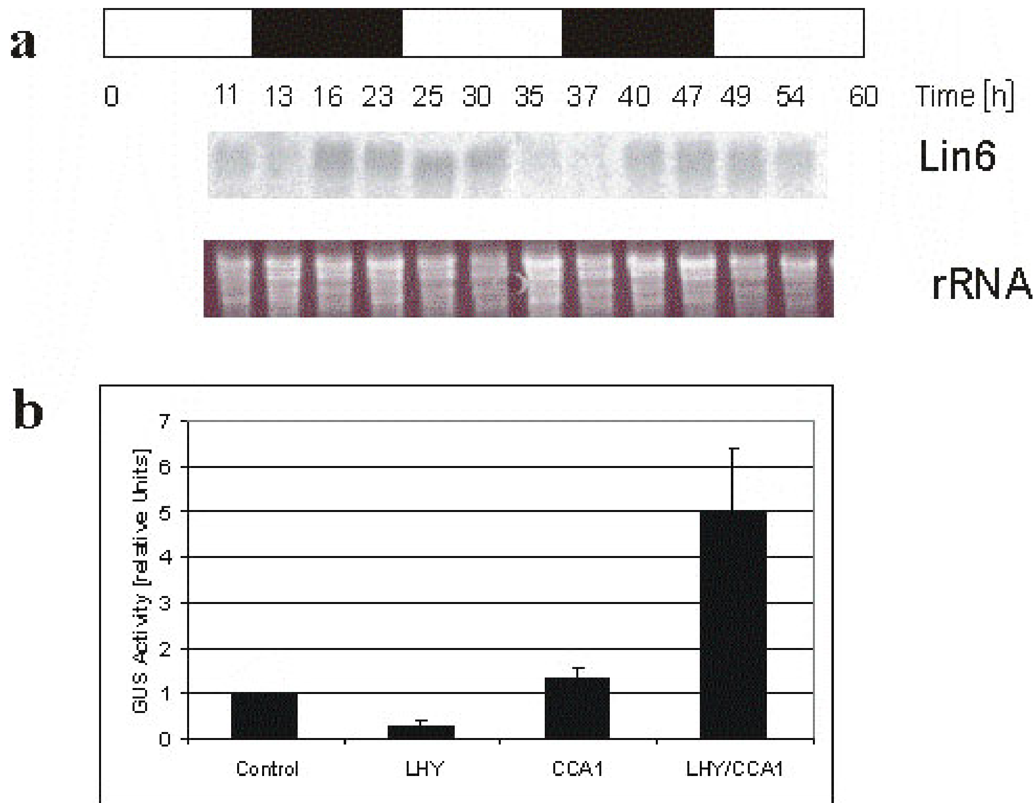

Fig.5 a,b Diurnal and circadian regulation of the *Lin6* promoter. **a**, RNA gel-blot analysis of tomato suspension cultures. Photoautotrophic suspension cultures (*L. peruvianum*) were grown for one week under constant light and kept for one further week under a light/dark regime of 12h/12h under constant temperature conditions of 26°C. At indicated time points samples were harvested and analysed by Northern blot using a radioactive labelled *Lin6*-specific probe. Approximately 25 µg of total RNA for each sample was loaded on the gel and stained with ethidium bromide (rRNA). Open and closed bars represent light and dark photoperiods, respectively. **b**, Transactivation assays with tobacco protoplasts. Tobacco mesophyll protoplasts were transformed with a *Lin6*::GUS effector plasmid (pUC19*Lin6*::GUS) and constructs that carried cDNA of CCA1 or LHY transcription factors under the control of the 35S promoter. After overnight induction in dim light, protoplasts were analysed for GUS activity. Four independent assays were each normalised to *Lin6*::GUS (pRP631) transformed controls, standard deviations are indicated.

CCA1 and LHY, central components of the Arabidopsis circadian clock, do specifically transactivate the Lin6 promoter

The expression pattern of *Lin6* in the photoautotrophic culture resembles northern blot data of CCA1 (Wang and Tobin, 1998), a central component of the *Arabidopsis* circadian clock, suggesting a possible circadian regulation of *Lin6*. In addition, the most striking *cis*-acting elements in *Lin6* promoter, besides phloem-specific boxes, are highly conserved sequences to CCA1 and LHY binding sites, both central elements of the *Arabidopsis* circadian clock. In detail, identical sequences to the CCA1 binding site (CBS), $AA^{A}/_{C}AATCT$, in the Lhcb1*3 promoter of *Arabidopsis* (Wang et al., 1997) are located at the positions -1357 (AAAAATCT) and -92 (AACAATCT). The latter element is flanked by two homologues sequences to the EVENING ELEMENT (EE) (AAAATATCT) that is recognised by CCA1 and LHY in the TOC1 promoter (Alabadi et al., 2001). Those sequences are located at position -324 (AAATTATCT) and -73 (TAATTATCT). Interestingly, the CBS at position -92 shows an overlap with a $CAA(N)_2ATC$ motif (underlined), which is present in most clock controlled *Lhc* promoter regions, and a CAAT box (in bold): CAA**CAAT**CT. An overlap of a CBS with a $CAA(N)_2ATC$ motif (Kellmann et al., 1999) and an overlap of a $CAA(N)_4ATC$ element with a CAAT box (Piechulla et al., 1998) is known for the *Lhca4*1* promoter and the *Lhcb1*2* promoter of tomato, respectively.

To analyse the relation between *Lin6* expression and circadian regulation, the functional interaction between these circadian transcription factors and the *Lin6* promoter were tested by transactivation assays with protoplasts. To get an appropriate high copy target vector the *Lin6* promoter GUS cassette of pRP631 was subcloned in pUC19. The effector plasmids pBCA126 and pRT101-LHY, that carry CCA1 and LHY cDNAs under control of the 35S promoter, were used. Tobacco mesophyll protoplasts were isolated form *N. tabacum* (cv Samsun) plants. The initial experiments revealed that the time point for preparing the protoplasts, performed under the light conditions of a lamina flow bench, had a dramatic influence on the results and thus turned out to be a critical parameter. It became evident that experiments that were done late in the evening, thus extending the period at the early dark phase, did show large variations in promoter activation. Therefore, the time point of harvesting the leaves was chosen at the very end of the light phase of the green house not to disturb the light dark cycle and leaf samples were kept in the dark overnight. Protoplasts were then isolated and transiently transformed during the following afternoon, kept under dim light overnight and harvested for GUS quantification in the early morning, the time point of maximal induction of the *Lin6* promoter based on the northern blot data shown above. The result of the transactivation assays shown in Fig. 5b demonstrate that LHY alone does

repress promoter activity by a factor of 0.3 ± 0.1, that CCA1 alone slightly activates by a factor of 1.4 ± 0.2, and that a combination CCA1 and LHY do results in a strong activating by a factor 5.0 ± 1.4 of the *Lin6* promoter activity. These finding demonstrates a regulation of *Lin6* promoter activity by circadian clock transcription factors CCA1 and LHY via a synergistic interaction of the two regulatory proteins.

DISCUSSION

Lin6 is specifically expressed in vascular tissues and induced by growth stimulating hormones to cover the enhanced carbohydrate demand of growing tissue

Extracellular invertase is the key enzyme of an apoplastic phloem unloading pathway and catalyses the hydrolytic cleavage of the transport sugar sucrose. This mechanism contributes to long-distance assimilate transport and provides the substrate to sustain heterotrophic growth (Eschrich, 1980). According its function, we could show *Lin6* expression being localised in vascular tissues of transgenic tobacco plants via GUS reporter gene assays. In seedlings *Lin6* is constitutively expressed in veins of cotyledons and the stem. Young plants do show in addition an expression of *Lin6* in major and minor veins of leaves, but no constitutive expression could be detected in adult plants except for pollen grains. This finding can be related to the altered sink strength of developing plant tissues. Young seedlings do cover their carbohydrate supply by mobilising storage components provided by the seed. Therefore, the sink-strength of those growing tissues is highest. Once the plant can provide carbohydrates via photosynthesis on its own, high utilisation sink-strength is restricted to actively growing tissues such as expanding leaves, internodes, or inflorescence (Ho, 1988). Adult plants that show no further dramatic growth just have to maintain corresponding heterotrophic tissue and therefore the overall sink-strength, except storage sinks or flowers, is weaker. Apparently, extracellular invertase *Lin6* is expressed just upon a certain threshold of sink-strength in corresponding tissues. This could explain the strong expression in vascular tissue of young seedlings in contrast to a lack of expression in vascular tissue of adult plants.

Plant hormones play an integral role in controlling growth, differentiation, and development of plants. It has been speculated that specific plant growth regulators are particularly involved in regulating sink strength (Kuiper, 1993), carbohydrate partitioning (Brenner and Cheikh, 1995), and phloem unloading (Tanner, 1980). There is accumulating evidence that extracellular invertases are regulated by various phytohormones, which can be

related to the increased carbohydrate demand of growth-stimulated tissues. These findings indicate an important link between primary metabolism and phytohormone action. Elucidation of the relationship between extracellular invertases and phytohormones is therefore important in unravelling the molecular mechanism of physiological phytohormone responses.

In this study we report on a GUS reporter gene analysis in transgenic tobacco plants and suspension cultures. We can show an induction of the *Lin6* promoter upon treatment with the synthetic auxin 2,4-D. A stimulation of extracellular invertase by auxin has been reported by Weil and Rausch (1990). Indole acetic acid promotes both growth and invertase activity in segments of young *P. vulgaris* internodes. The sensitivity to auxin is developmentally regulated and requires mRNA and protein synthesis (Morris and Arthur, 1984, 1986). Moreover, *Lin6* promoter is induced by zeatin, a plant hormone of the cytokinin type. Cytokinins promote cell division and play a major role in the regulation of various processes associated with active growth and thus an enhanced demand for carbohydrates (Kuiper, 1993; Roitsch and Ehness, 2000). The physiological significance of this regulation is supported by the fact that tissues with elevated extracellular invertase activity, such as rapidly growing tissues, are also known to contain elevated cytokinin concentrations (Skoog and Amstrong, 1970). *Agrobacterium* tumors of tobacco and *Arabidopsis* have been reported to be characterised by a high extracellular invertase activity (Weil and Rausch, 1990) and high *Lin6* mRNA levels have been found in tumors on tomato plants (Godt and Roitsch, 1997). Since tumors are characterised by highly elevated levels of both auxin and cytokinin, the identified regulation of the *Lin6* promoter by auxins and cytokinins could be the molecular basis.

The inducing effect of auxins and cytokinins on the *Lin6* promoter along with the histochemical expression analysis of *Lin6* in early stages of plant development, confirm an important role of extracellular invertase *Lin6* in providing carbohydrates to sustain heterotrophic growth.

The dual role of MeJA-regulated Lin6 expression in defence response and pollen development

The transcript level of extracellular invertase has been shown to be up-regulated by different stress-related stimuli (Roitsch et al., 2000). In carrot the induction of extracellular invertase has been demonstrated in wounded or infected leaves (Sturm and Chrispeels, 1990); Fotopoulos et al. (2003) report on a co-induction of a monosaccharide transporter gene and a cell-wall invertase in *Arabidopsis* in response to infection by a fungal biotroph. Furthermore, ectopic expression of an extracellular invertase in tobacco plants resulted in an elevated transcription of defence-related genes and increased resistance towards virus

infection (Herbers et al., 1996). According to these data, extracellular invertases are target genes of stress-related stimuli and contribute to stress signal transduction. In this study we show an up-regulation of *Lin6* promoter due to mechanical wounding and localise the wound induction to a very small area adjacent to the cutting edge. In addition, we could correlate this promoter induction to an induced invertase activity in wounded tomato leaves. Interestingly, wounding does broaden the tissue-specific expression, which is restricted to vascular tissue around the wounded area. This indicates that wounding is able to alter constitutive expression profiles to elicit an appropriate defence response. The potential physiological relevance of the up-regulation of extracellular invertase in response to wounding is a localised increase in the carbohydrate supply, providing additional metabolic energy for the cascade of defence reactions and rebuilding of wounded tissue.

Using BY2 tobacco suspension cultures, stable transformed with *Lin6* promoter GUS construct, we could show an induction of *Lin6* promoter by ABA and MeJA as global signals known to modulate defence/stress responses. The hydroxylated phenolic compound SA and ASA were shown to be potent antagonists of wound induction of the *Lin6* promoter supporting that *Lin6* expression is regulated via the octadecanoid pathway. The same regulation pattern, an inhibition of wound-induced gene expression by SA and ASA has been demonstrated for allene oxide synthase in flax leaves (Harms et al., 1998). As allene oxide synthase is a central component of JA biosynthesis, these data further support the JA mediated wound-induction of *Lin6*.

Moreover, we could show that *Lin6* is expressed constitutively high in pollen of transgenic *Lin6*::GUS (pRP631) plants. This expression could be enhanced by MeJA treatment and strongly inhibited by ASA and SA, which further support an involvement of the octadecanoid pathway in *Lin6* regulation in pollen. One could speculate a link between known effects of jasmonate in pollen development, e.g. the *coi1* JA-insensitive mutant does not produce viable pollen unless supplied with JA (McConn and Browse, 1996), and *Lin6* invertase activity. This is further supported by the fact, that we could previously show the essential role of a tobacco invertase, Nin88, in pollen development (Goetz et al., 2001). This finding is in line with data on a family of male gametophyte-specific monosaccharide transporters that are functionally linked with extracellular invertase. The presence of three monosaccharide transporters AtSTP4, AtSTP6, and AtSTP9 in *Arabidopsis* pollen (Truernit et al., 1999; Scholz-Starke et al., 2003; Schneidereit et al., 2003) supports the importance of extracellular cleavage of the transport sugar sucrose for pollen growth and development.

In a recent study on JA-biosynthetic mutants, Park et al. (2002) could show that jasmonates play an essential role in pollen maturation and wound-induced defence. The MeJA-dependent expression of *Lin6* in pollen and the JA-dependent *Lin6* induction upon wounding gives strong evidence that *Lin6* is a target of both signalling pathways.

Interestingly, Creelman and Mullet (1997) report on several genes being regulated by sugars and JA and speculate about a co-ordinated regulation of target genes via sugars and JA to modulate plant defence under conditions where nutrients are limiting. *Lin6*, as it is sugar regulated, could therefore act at the integration point of developmental signals, sugars, and JA-mediated defence responses.

A diurnal rhythm drives Lin6 expression in subjective dawn

Northern blot analysis of entrained tomato suspension cultures revealed a diurnal rhythm of *Lin6* expression. Expression of *Lin6* is up-regulated in the middle of the dark phase, reaches its maximum at the dark-light transition and then gets downregulated to reach a minimum in the subjective evening. The *Lin6* expression pattern fits in a model of circadian regulated cell elongation, postulated by Harmer et al. (2000). They could show that genes involved in cell expansion are up-regulated at the end of the subjective day and genes involved in cell wall synthesis peak towards the end of the subjective night. *Lin6* is expressed as the latter class of genes and therefore could contribute to cover a higher carbohydrate supply during the reinforcement of the cell wall during cell elongation processes. This is in agreement with the finding that *Lin6* expression correlates with brassinosteroid induced growth (Goetz et al., 2000).

A detailed analysis of *Lin6* promoter revealed the presence of highly conserved binding sites for the circadian regulators CCA1 and LHY, the CBS and EE elements. Multiple copies of these *cis*-acting elements in combination with a G-box element are located within the first 350 bp of *Lin6* promoter. A combination of those elements was postulated by Michael and McClung (2003) to be necessary for clock regulation of genes. Based on this findings we analysed whether CCA1 or LHY transcription factors do functional interact with the *Lin6* promoter. Tobacco mesophyll protoplasts were co-transformed with *Lin6*::GUS reporter construct pUC19*Lin6*::GUS and CCA1 and /or LHY expression constructs. In this system we could show a synergistic effect of CCA1 and LHY for *Lin6* induction. The findings that the *Arabidopsis* circadian regulators were able to induce expression of the *Lin6* promoter indicate that an oscillator output pathway similar to the CCA1/LHY system in *Arabidopsis* might exist in tomato. Likewise, a circadian oscillation of granule-bound starch synthase mRNA levels in *Arabidopsis* leaves and a possible direct involvement of CCA1 and LHY in the underlying control mechanism has been reported (Tenorio et al., 2003).

Lin6, a regulator of source/sink transitions, might work as a pivotal enzyme at the integration point of metabolic, hormonal, and stress signals and the diurnal rhythm

Previously, extracellular invertase has been suggested to function as central modulators of assimilate partitioning in development and defence response (Roitsch et al., 2000). This idea was based on the following findings: 1) Extracellular invertase supply carbohydrates to sink tissues 2) Extracellular invertase regulate source-sink transitions, 3) as extracellular invertase are induced by sugars, there is an amplification of signals that regulate source-sink relations, 4) there is an integration of a huge variety of signals regulating extracellular invertase, such as hormones and stress stimuli.

Data supporting the role of extracellular invertase as a key metabolic enzyme and PR protein were derived from different plant species and isoenzymes (Roitsch et al., 2003). The data on extracellular invertase *Lin6*, which fulfils all the criteria listed above, further support a central role of extracellular invertases. *Lin6* is regulated by sugars, growth stimulating hormones, stress stimuli, and a diurnal rhythm and pinpoints the potential of this enzyme to act as an integration point of all mentioned stimuli. The central function of extracellular invertase in regulating growth processes, sustaining pathogen defence, and adapt the plant to the diurnal rhythm becomes obvious from the *Lin6* expression and regulation pattern.

Further investigations are needed to elucidate the molecular basis that contributes to the multiple regulation pattern of *Lin6*. Based on various regulatory mechanisms that affect *Lin6* expression, it has to be assumed that the *Lin6* promoter is the target of multiple transcription factors. Studying the corresponding molecular mechanism, and in particular the interaction between transcription factors and *cis*-acting elements of the *Lin6* promoter, will not only contribute to elucidate the molecular mechanisms of source/sink regulation but also of transcriptional control of plant genes in general.

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The nucleotide sequence data reported will appear in the GenBank database under the accession number AY 634311.

REFERENCES

Alabadi D, Oyama T, Yanovsky MJ, Harmon FG, Mas P, Kay SA (2001) Reciprocal regulation between TOC1 and LHY/CCA1 within the *Arabidopsis* Circadian Clock. Science **293**: 880-883

Alabadi D, Yanovsky MJ, Mas P, Harmer SL, Kay SA (2002) Critical role for CCA1 and LHY in maintaining circadian rhythmicity in *Arabidopsis*. Curr. Biology **12**: 757-761

Atanasova R, Leterrier M, Gaillard C, Agasse A, Sagot E, Coutos-Thevenot P, Delrot S (2003) Sugar-Regulated Expression of a Putative hexose transporter Gene in grape. Plant Physiol **131**: 326-334

Benedetti CE, Costa CL, Turcinelli SR, Arruda P (1998) Differential expression of a novel gene in response to coronatine, methyl jasmonate, and wounding in the *Coi1* mutant of *Arabidopsis*. Plant Physiol **116**: 1037-1042

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72**: 248-254

Brenner ML, Cheikh N (1995) The role of phytohormones in photosynthate partitioning and seed filling. In: Davies PJ, ed. Plant hormones. Kluwer Academic Press. Dordrecht, Boston, London, pp 649-670

Carre IA, Kim J-Y (2002) MYB transcription factors in the *Arabidopsis* circadian clock. J Exp Bot **53**: 1551-1557

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem **162**: 156-159

Creelman RA, Mullet JE (1997) Biosynthesis and action of jasmonates in plants. Annu Rev Plant Physiol Plant Mol Biol **48**: 355-381

Eschrich W (1980) Free space invertase, its possible role in phloem unloading. Berichte der Deutschen Botanischen Gesellschaft **93**: 363-378

Eulgem T, Rushton PJ, Schmelzer E, Hahlbrock K, Somssich IE (1999) Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors. EMBO J **18**: 4689-4699

Fotopoulos V, Gilbert MJ, Pittman JK, Marvier AC, Buchanan AJ, Sauer N, Hall JL, Williams LE (2003) The monosaccharide transporter gene, AtSTP4, and the cell-wall invertase, Atβfruct1, are induced in Arabidopsis during infection with the fungal biotroph Erysiphe cichoracearum. Plant Physiol **132**: 821-829

Fridman E, Pleban T, Zamir D (2000) A recombination hotspot delimits a wild-species quantitative trait locus for tomato sugar content to 484 bp within an invertase gene. Proc Natl Acad Sc USA **97**: 4718-4723

Fridman E, Zamir D (2003) Functional divergence of a syntenic invertase gene family in tomato, potato, and Arabidopsis. Plant Physiol **131**: 603-609

Godt DE, Roitsch T (1997) Regulation and Tissue -Specific Distribution of mRNA for Three Extracellular Invertase Isoenzymes of Tomato Suggests an Important Function in Establishing and Maintaining Sink Metabolism. Plant Physiol **115**: 273-282

Goetz M, Godt DE, Roitsch T (2000) Tissue-specific induction of the mRNA for an extracellular invertase isoenzyme of tomato by brassinosteroids suggests a role for steroid hormones in assimilate partitioning.

Plant J **22**: 515-522

Goetz M, Godt DE, Guivarch A, Kahmann U, Chriqui D Roitsch T (2001) Induction of male sterility in plants by metabolic engineering of the carbohydrate supply. Proc Natl Acad Sci USA **98**: 6522-6527

Harmer SL, Hogenesch JB, Straume M, Chang H-S, Han B, Zhu T, Wang X, Kreps JA Kay SA (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science **290**: 2110-2113

Harms K, Ramirez II, Pena-Cortes H (1998) Inhibition of wound-induced accumulation of allene oxide synthase transcripts in flax leaves by aspirin and salicylic acid. Plant Physiol **118**: 1057-1065

Hedley PE, Maddison AL, Davidson D, Machray GC (2000) Differential expression of invertase genes in internal and external phloem tissues of potato (*Solanum tuberosum L.*). J Exp Bot **51**: 817-821

Herbers K, Meuwly P, Frommer WB, Metraux J-P, Sonnewald U (1996) Systemic acquired resistance mediated by the ectopic expression of invertase: possible hexose sensing in the secretory pathway. Plant Cell **8**: 793-803

Herbers K, Takahata Y, Melzer M, Mock H-P, Hajirezaei M, Sonnewald U (2000) Regulation of carbohydrate partitioning during the interaction of potato virus Y with tobacco. Mol Plant Pathol **1**: 51-59

Ho LC (1988) Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. Annu Rev Plant Physiol Plant Mol Biol **39**: 355-78

Horsch RB, Fry FB, Hoffmann NL, Eichholtz P, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science **227**: 1229-1231

Ishiguro S, Kawai-Oda A, Ueda J, Nishida I, Okada K (2001) The DEFECTIVE IN ANTHER DEHISCENCE1 gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in *Arabidopsis*. Plant Cell **13**: 2191-2209

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS-fusions: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6**: 3901-3907

Joshi CP (1987) An inspection of the domain between putative TATA box and Translation site in 79 plant genes. Nucleic Acids Res **15**: 6643-6653

Kellmann JW, Hoffrogge R, Piechulla B (1999) Transcriptional regulation of oscillating steady-state *Lhc* mRNA levels: Characterization of two *Lhc* promotor fragments in transgenic tobacco plants. Biol Rhythm Res **30**: 264-271

Kim J-Y, Mahé A, Guy S, Brangeon J, Roche O, Chourey PS, Prioul J-L (2000) Characterisation of two members of the maize gene family, *Incw3* and *Incw4*, encoding cell-wall invertases. Gene **245**: 89-102

Koiwa H, Bressan RA, Hasegawa PM (1997) Regulation of protease inhibitors and plant defence. Trends Plant Sci **2**: 379-384

Kuiper D (1993) Sink strength: established and regulated by plant growth regulators. Plant Cell Environ **16**: 1025-1026

Lorenz K, Lienhard S, Sturm A (1995) Structural organisation and differential expression of carrot β-fructofuranosidase genes: identification of a gene coding for a flower bud-specific isoenzyme. Plant Mol Biol **28**: 189-194

Lyck R, Harmening U, Höhfeld I, Treuter E, Scharf K-D, Nover L (1997) Intracellular distribution and identification of the nuclear localization signals of two plant heat-stress ranscription factors. Planta **202**: 117-125

Maddison AL, Hedeley PE, Meyer RC, Aziz N, Davidson D, Machray GC (1999) Expression of tandem invertase genes associated with sexual and vegetative growth cycles in potato. Plant Mol Biol **41**: 741-751

McConn M, Browse J (1996) The critical requirement for linolenic acid is pollen development, not photosynthesis, in an *Arabidopsis* mutant. Plant Cell **8**: 403-416

Menke FLH, Champion A, Kijne JW, Memelink J (1999) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosnthetic gene Str interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. EMBO J **18**: 4455-4463

Michael TP, McClung CR (2003) Enhancer Trapping Reveals Widespread circadian Clock Transcriptional Control in Arabidopsis. Plant Physiol **132**: 629-639

Miller EM, Chourey PS (1992) The maize invertase-deficient miniature-1 seed mutant is associated with aberrant pedicel and endosperm development. Plant Cell **4**: 297-305

Morris DA, Arthur ED (1984) Invertase and auxin-induced elongation in internodal segments of *Phaseolus vulagaris*. Phytochemsitry **23**: 2163-2167

Morris DA, Arthur ED (1986) Stimulation of acid invertase activity by indolyl-3-acetic acid in undergoing cell expansion. Plant Growth Regul **4**: 259-271

Palm CJ, Cost MA, Gynheung A, Ryan CA (1990) Wound-inducible nuclear protein binds DNA fragments that regulate a proteinase inhibitor II gene from potato. Proc Natl Acad Sci USA **78**: 603-607

Park J-H, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in *Arabidopsis* due to a block in jasmonic acid biosynthesis. Plant J **31**: 1-12

Piechulla B, Merforth N, Rudolph B (1998) Icentification of tomato *Lhc* promoter regions necessary for circadian expression. Plant Mol Biol **38**: 655-662

Proels RK, Hause B, Berger S, Roitsch T (2003) Novel mode of hormone induction of tandem tomato invertase genes in floral organs. Plant Mol Biol **52**: 191-201

Roitsch T, Bittner M, Godt DE (1995) Induction of apoplastic inverstase of Chenopodium rubrum by D-glucose and a glucose analog and tissue-specific expression suggest a role in sink-source regulation. Plant Physiol **108**: 285-294

Roitsch T, Tanner W (1996) Cell wall invertase: bridging the gap. Botanica Acta **109**: 90-93

Roitsch T, Ehness R (2000) Regulation and source/sink relations by cytokinins. Plant Growth Regul **32**: 359-367

Roitsch T, Ehness R, Goetz M, Hause B, Hofmann M, Sinha A (2000) Regulation and function of extracellular invertase from higher plants in relation to assimilate partitioning, stress responses and sugar signalling. Aust J Plant Physiol **27**: 815-825

Roitsch T, Balibrea ME, Hofmann M, Proels R, Sinha AK (2003) Extracellular invertase: key metabolic enzyme and PR protein. J Exp Bot **54**: 513-524

Schneidereit A, Scholz-Starke J, Büttner M (2003) Functional characterization and expression analyzes of the glucose-specific AtSTP9 monosaccharide transporter in pollen of *Arabidopsis*. Plant Physiol **133**: 182-190

Scholz-Starke J, Büttner M, Sauer N (2003) AtSTP6, a new pollen-specific H^+-monosaccharide symporter from *Arabidopsis*. Plant Physiol **131**: 70-77

Shen Q, Ho T-H D (1995) Functional Dissection of an Abscissin Acid (ABA)-Inducible Gene Reveals Two Independent ABA-Responsive Complexes Each Containing a G-Box and a novel *cis*-Acting Element. Plant Cell **7**: 295-307

Sherson SM, Alford HL, Forbes SM, Wallace G, Smith SM (2003) Roles of cell-wall invertases and monosaccharide transporters in the growth and development of *Arabidopsis*. J Exp Bot **54**: 525-531

Siebertz B, Logemann J, Willmitzer L, Schell J (1989) *Cis*-Analysis of the wound-inducible promotor *wun1* in transgenic tobacco plants and histochemical localization of ist expression. Plant Cell **1**: 961-968

Sinha AK, Hofmann MG, Romer U, Kockenberger W, Elling L, Roitsch T (2002) Metabolizable and non-metabolizable sugars activate different signal transduction pathways in tomato. Plant Physiol **128**: 1480-1489

Skoog F, Amstrong DJ (1970) Cytokinins. Annu Rev Plant Physiol **21**: 359-384

Sturm A, Chrispeels MJ (1990) cDNA cloning of carrot extracellular ß-fructosidase and its expression in response to wounding and bacterial infection. Plant Cell **2**: 1107-1119

Sturm A (1999) Invertases. Primary Structures, Functions, and Roles in Plant Development and Sucrose Partitioning. Plant Physiol **12**: 1-7

Sturm A, Tang G-Q (1999) The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. Trends Plant Sci **4**: 401-407

Tang G-Q, Lüscher M, Sturm A (1999) Antisense repression of vacuolar and cell wall invertase in transgenic carrot alters early plant development and sucrose partitioneing. Plant Cell **11**: 117-189

Tanner W (1980) On the possible role of ABA on phloem unloading. Berichte der Deutschen Botanischen Gesellschaft **93**: 349-351

Tenorio G, Orea A, Romero JM, Merida A (2003) Oscillation of mRNA level and activity of granule-bound starch synthase I in *Arabidopsis* leaves during the day/night cycle. Plant Mol Biol **51**: 949-958

Truernit E, Stadler R, Baier K, Sauer N (1999) A male gametophyte-specific monosaccharide transporter in *Arabidopsis*. Plant J **17**: 191-201

Tymowska-Lalanne Z, Kreis M (1998) The plant invertases: Physiology, biochemistry and molecular biology. Adv Botanical Res **28**: 71-117

von Schaewen A, Stitt M, Schmidt R, Sonnewald U, Willmitzer L (1990) Expression of a yeast-derived invertase in the cell wall of toabcco and *Arabidopsis* plants leads to accumulation of cabohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. EMBO J **9**: 3033-3044

Wang ZY, Kenigsbuch D, Sun L, Harel E, Ong MS, Tobin EM (1997) A Myb-related transcription factor is involved in the phytochrome regulation of an *Arabidopsis* Lhcb gene. Plant Cell **9**: 491-507

Wang Z-Y, Tobin EM (1998) Constitutive Expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) Gene Disrupts Circadian Rhythms and Suppresses Its Own Expression. Cell **93**: 1207-1217

Wasternack C, Parthier B (1997) Jasmonate-signaled plant gene expression. Trends Plant Sci **2**: 302-307

Weber H, Borisjuk L, Heim U, Buchner P, Wobus U (1995) Seed coat-associated invertases of fababean contol both unloading and storage functions. Cloning of cDNAs and cell type-speific expression. Plant Cell **7**: 1835-1864

Weil M, Rausch T (1990) Cell wall invertase in tobacco crown gall cells. Plant Physiol 94: 1575-1581

Yin Y, Chen L, Beachy R (1997) Promoter elements required for phloem specific gene expression from the RTBV promoter in rice. Plant J **12**: 1179-1188

Zhang L, Cohn NS, Mitchell JP (1996) Induction of a pea cell-wall invertase gene by wounding and its localised expression in phloem. Plant Physiol **112**: 1111-1117

Zhang L, Cohn NS, Mitchell JP (1997) A pea cell-wall invertase gene (PsInv-1) with tissue-specific expression. Plant Physiol Biochem **35**: 751-760

Chapter 6

Extracellular invertase is an essential component of cytokinin-mediated delay of senescence

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Extracellular invertase is an essential component of cytokinin-mediated delay of senescence

Abstract. Leaf senescence is the final stage of leaf development in which the nutrients invested in the leaf are remobilised to other parts of the plant. Whereas senescence is accompanied by a decline in leaf cytokinin content, exogenous application of cytokinins or an increase of the endogenous concentration delays senescence and causes nutrient mobilisation. The finding that extracellular invertase and hexose transporters, as the functionally linked enzymes of an apolasmic phloem unloading pathways, are coinduced by cytokinins suggested that delay of senescence is mediated via an effect on source-sink relations. This hypothesis was further substantiated in the present study by the finding, that delay of senescence in transgenic tobacco *(Nicotiana tabacum)* plants with autoregulated cytokinin production correlates with an elevated extracellular invertase activity. The finding that the expression of an extracellular invertase under control of the senescence-induced *SAG12* promoter results in a delay of senescence demonstrates that effect of cytokinins may be substituted by this metabolic enzymes. The observation that an increase in extracellular invertase is sufficient to delay leaf senescence was further verified by a complementing functional approach. Localised induction of an extracellular invertase under control of a chemically inducible promoter resulted in ectopic delay of senescence, resembling the naturally occurring green islands in autumn leaves. To establish a causal relationship between cytokinins and extracellular invertase for the delay of senescence, transgenic plants were generated that allowed to inhibit extracellular invertase in the presence of cytokinins. For this purpose, an invertase inhibitor was expressed under control of a cytokinin-inducible promoter. It has been shown that senescence is not any more delayed by cytokinin when the expression of the invertase inhibitor is elevated. This finding demonstrates that extracellular invertase is required for the delay of senescence by cytokinins and that it is a key element of the underlying molecular mechanism.

INTRODUCTION

Cytokinins are a group of plant hormones that promote cell division and play a major role in the regulation of various biological processes associated with active growth, metabolism, and plant development. Because these processes are associated with an enhanced demand for carbohydrates, a link to the regulation of assimilate partitioning (Brenner and Cheikh, 1995), sink strength (Kuiper, 1993), and source-sink relations (Roitsch and Ehness, 2000) has been suggested. This hypothesis is experimentally supported by the observation that radioactively labelled nutrients are preferentially transported and accumulated in cytokinin-treated tissue (Mothes and Engelbrecht, 1963), suggesting that the hormone creates a new source-sink relationship, thus causing nutrient mobilisation. Higher plants consist of a mosaic of photosynthetically active source tissues, such as mature leaves, and photosynthetically less active or inactive sink tissues, such as seeds, flowers, roots, fruits and tubers. The source-sink relations in mature plants are not static, and changes, with respect to the relative sink strength of various organs, number of sinks competing for a common pool of carbohydrates and sink to source transitions, occur during the plant development. The photoassimilates produced in the source organs are transported into the sink organs mostly in the form of sucrose. An apoplasmic phloem unloading of sucrose is mandatory in symplastically isolated tissues, such as embryos or stomata, and seems to be characteristic for actively growing tissues (Eschrich, 1980), and thus, under conditions that may be under the control of cytokinins. In an apoplastic unloading pathways sucrose is released from the sieve elements of the phloem into the apoplast by a sucrose transporter, where it is irreversibly hydrolysed by an extracellular invertase ionically bound to the cell wall. The resulting hexose monomers are then taken up by sink cells through monosaccharide transporters (Roitsch and Tanner, 1996). The extracellular invertase has a crucial function both in source-sink regulation and for supplying carbohydrates to sink tissues, being considered as a central modulator of sink activity (Tang et al.,1999, Goetz et al., 2001, Roitsch et al., 2003). It has been shown that extracellular invertases are up-regulated by a number of stimuli that affect carbohydrate requirements, including growth stimulating phytohormones (Roitsch, 1999; Roitsch et al., 2003). The extracellular invertase activity is usually high in tissues with an elevated cytokinin concentration. A direct link between to cytokinin and the function of invertases was suggested by the stimulation of invertase activity by cytokinins of *in-vitro* cultivated *Chicorium* tissues (Lefebre et al., 1992). This was further substantiated by the finding that extracellular invertases from *Chenopodium rubrum* (Ehness and Roitsch, 1997) and *Lycopersicon esculentum* (Godt and Roitsch, 1997) were induced by

physiological concentrations of different cytokinins. In addition, hexose transporters of *C. rubrum* were coinduced with the extracellular invertase by cytokinins (Ehness and Roitsch, 1997). The co-ordinated induction of the two functionally linked key enzymes of an apoplasmic phloem unloading pathway was shown to result in a higher uptake of hexose sugars. These findings provided the groundwork for elucidating the molecular basis of the mode of cytokinin action, supporting the speculation that the up-regulation of both extracellular invertase and hexose transporters may account for the phenomenon of transport of nutrients to cytokinin-treated tissue as observed by Mothes and coworkers in the 1960s.

The cytokinins are also a key component of plant senescence (Gan and Amasino, 1996, 1997; Buchanan-Wollaston, 1997; Nam, 1997). Leaf senescence is the final stage of leaf development, but is also a recycling process in which the nutrients from those leaves are translocated to other parts of the plant, such as younger leaves, developing seeds, or storage tissues (Gan and Amasino, 1996). The senescing leaves, according to the model proposed for the initiation of leaf senescence, have a photosynthetic rate such that they no longer contribute fixed carbon to the rest of the plant (Hensel et al., 1993). The initiation of leaf senescence is subjected to regulation both by internal and environmental factors. In particular, phytohormones are assumed to be the main internal factors controlling this developmental process. Whereas abscisic acid and ethylene promote senescence, cytokinins typically inhibit senescence (Smart, 1994). Physiological studies have shown that in a variety of monocotyledonous and dicotyledonous plant species, exogenous cytokinin treatment results in delay of leaf senescence (Richmond and Lang, 1957), that the endogenous levels of cytokinins drop along the progression of leaf senescence (Gan and Amasino, 1996), and that differences in tobacco (*Nicotiana tabacum*) leaf senescence are related to differences in endogenous cytokinin content (Singh et al., 1992 a, 1992 b). The so-called green islands in autumn leaves, produced by specific species of caterpillar, fungi, or bacteria, have been shown to be caused by cytokinin secretion (Engelbrecht et al., 1969; Angra and Mandahar, 1993; Chen and Ertl, 1994). The enhanced expression of a bacterial isopentenyltransferase (*ipt*) gene encoding a cytokinin biosynthetic enzyme under the control of the senescence-activated promoter *SAG12* (Noh and Amasino, 1999 a, 1999 b) in tobacco plants produced an efficient retardation in the process of leaf senescence in the mature leaves (Gan and Amasino, 1995), confirming the regulatory role of cytokinins on leaf senescence in tobacco. In addition, the senescence-specific expression of the maize (*Zea mays*) gene *knotted1* encoding a transcription factor in tobacco caused an increase in cytokinins levels and a delayed-senescence phenotype (Ori et al., 1999). However, although the fact that cytokinins cause a delay in senescence were confirmed by elegant transgenic approaches, the underlying molecular basis for this cytokinin effect is still not understood.

In our attempt to elucidate the relation between cytokinin and primary metabolism, the observed increase of extracellular invertase activity in the delayed-senescence leaves of *SAG12:ipt* tobacco plants supported a link between cytokinins, extracellular invertases, and carbohydrate partitioning for the delay of senescence. The suggested link between cytokinins and source-sink relations was further substantiated by complementing functional approaches in transgenic plants. The findings demonstrate that an increase in extracellular invertase activity is not only sufficient to cause a delay of senescence, but that this key enzyme of an apoplasmic phloem unloading pathway is an essential component of the molecular mechanism of delay of senescence by cytokinins.

MATERIALS AND METHODS

Plasmid construction

A construct for senescence-inducible expression of extracellular invertase *Cin1* (*SAG12:Cin1)* was generated in two steps. The *Cin1* cDNA was amplified by PCR from plasmid pMB3 (Roitsch et al., 1995) by primers CIN125Noc (5´-TGCATCGATCAAGTCGATGT-3´) and CIN1SacNco (5´-GCAATTGACTGTAATTCGTACTAATT-3´) that generated *Nco*I sites at both ends and a *Sac*I at the 5´-end of the cDNA. After cutting with *Nco*I, the fragment was cloned into the corresponding site of plasmid pSG499 (Gan and Amasino, 1995) to generate plasmid pRE1171. The expression cassette was subcloned into the binary vector pBI101+ (Goetz et al., 2001) as *Sal*I–*Sac*I fragment to generate plasmid pRE1186-27.

A construct for tetracycline inducible expression of extracellular invertase *Cin1* (*TetR:Cin1)* was generated by initially releasing the complete cDNA of extracellular invertase *Cin1* from plasmid pMB3 as *EcoR*I fragment, filling in the 5´-overhangs by Klenow polymerase, and cloning the fragment into the *Sma*I site of pUC18 to generate pMH06/11. The *Cin1* cDNA was then subcloned as *Kpn*I/*Xba*I fragment into the corresponding sites of the binary vector pBinHygTx (Gatz and Lenk, 1998) to generate pRE697-17.

A fusion between the cytokinin-inducible promoter of extracellular invertase *Lin6* and the apoplasmic invertase inhibitor P17A (*Lin6:P17A*) from tobacco (*Nicotiana tabacum*) was generated by subcloning the invertase inhibitor cDNA as *BamH*I/*Xho*I from plasmid pBK-CMV/P17A (Greiner et al., 1998) plasmid pR6-11 (R. Proels and T. Roitsch, unpublished data) to generate plasmid pTF3-11.

Plant transformation

The constructs were transferred to tobacco (*Nicotiana tabacum* cv Wisconsin 38) by using standard *Agrobacterium tumefaciens* transformation procedures (Horsch et al., 1985). The T1 generation of transgenic plants that was heterozygous for the different transgenes was used for all experiments.

Senescence assay on detached leaves

Detached young leaves from tobacco plants were incubated in a water bath at 49.2°C for 1 min and 30 s, and then transferred to a flask with tap water. For experiments with transgenic lines expressing the tobacco invertase inhibitor P17A under control of the cytokinin-inducible *Lin6* promoter, the water was supplemented with either 30 µg/L of kinetin or an equivalent amount of the solvent (NaOH). The leaves were incubated in a plant growth chamber with 15-h-light/9-h-dark cycle and a constant temperature of 23°C.

For experiments with transgenic lines expressing the extracellular invertase *Cin1* under control of the tetracycline-inducible *TetR* promoter, expression of the transgene was induced by infiltration of a solution containing MS medium (Duchefa, Haarlem, The Netherlands) pH 6.0, 0.02% Silwet, and 10 mg/L of chlorotetracycline. For localised induction, a small area was gently treated with glass paper and then repetitively painted with a brush wetted in the chlortetracycline-containing solution. For mock inoculations the chlortetracycline was omitted.

The leaves were observed for 4 weeks and photographs taken. At least five independent replications with three independent lines were performed.

Chlorophyll determinations

Three frozen leaf disks of each sample analysed, corresponding to ~0.033g of fresh weight material, were extracted and homogenised in 1 ml of 80% acetone and kept thereafter at 4°C. Following homogenisation, the samples were centrifuged at 10,000g, 2 min at 4°C, and 1 mL of the supernatant was used for spectrophotometric determination. The concentration of chlorophylls a/b, as well as the major carotenoides, comprising xantophyll and carotene, was calculated as described by Lichtenthaler (1987). Results were expressed as micrograms of chlorophyll or carotenoids per milliliter. The chlorophyll content of individual samples was determined at least in duplicate, and at least three independent experiments were analysed.

Determination of invertase activity

The activity of extracellular invertase was determined at pH 4.5 as described previously (Roitsch et al., 1995). A Glc test kit (Roche, Indianapolis, IN) was used to determine the amount of Glc liberated. Control reactions were performed using the same volume of extract and water in the reaction mixture instead of sucrose. The concentration of protein in the extracts was determined using the procedure of Bradford (1976). The invertase activity of individual samples was determined at least in triplicate, and at least three independent experiments were analysed.

Soluble sugar determination

For the determination of soluble sugars, 100 mg of ground frozen material were resuspended in 900 µL of distilled water, and after centrifugation at 13,000 rpm for 10 min at 4°C, the supernatant was heated at 105°C for 3 min to denature the enzymes. The resulting supernatant was used for the quantification of soluble sugars using a high-pressure liquid chromatography system coupled with pulsed amperometry detection (Dionex 4500, Dionex Softron, Germering, Germany). The sugar content of individual samples was determined at least in duplicate, and at least three independent experiments were analysed.

Isolation of RNA and RNA gel blot analyses

RNA was obtained in a scaled-down extraction procedure. The material was frozen in liquid nitrogen, and after grinding in a mortar, two replicates of 100 mg of material were used for RNA isolation. Total RNA was isolated according to the methods of Chomczynski and Sacchi (1987). In order to remove the high content of carbohydrates of the RNA-containing samples, the pellets were treated with 500 µL of peqGold Optipure solution (PeqLab, Erlangen, Germany) and maintained at vigorous shaking at 20°C overnight. The samples were then centrifuged at 20,000 g for 30 min at room temperature, and the RNA containing pellet dispersed in 200 µL SDS 0.5% by vigorous shaking at 55°C for 2 h. The samples were extracted with one volume of chloroform, and the upper phase precipitated by the addition of one volume of isopropanol and a final concentration of 0.2 M sodium acetate and incubated at –20°C overnight. The RNA was collected by centrifugation (20,000 g) washed with 85% ethanol and dried by vacuum centrifugation. The pellet was dissolved in 20 µL of diethyl pyrocarbonate-treated water, and the concentration of RNA calculated from the A_{260}.

For RNA gel blot analysis, 15 µg RNA samples containing ethidium bromide, were subjected to electrophoresis on 1.3% agarose formaldehyde gels and transferred to

nitrocellulose filters by capillary transfer, and the cDNA probes labelled by random priming (MBI Fermentas, St. Leon-Rot, Germany). Hybridisation was performed in 50% formamide, 5x SSC (750 mM NaCl and 75 mM sodium citrate, brought to pH 7.0 with HCl), 0,1% SDS and 5x Denhardt's solution (0,1% Ficoll 400, 0,1% BSA, and 0,1% polyvinylpyrrolidone) at 42°C for 18 h. The membranes were washed with decreasing salt concentration at 42°C. A final washing step was performed in 0.2x SSC and 0.1% SDS.

Samples from at least three different experiments were analysed by RNA gel blots analyses.

RESULTS

Delay of senescence by cytokinins correlates with an increase in extracellular invertase activity

The observed induction of extracellular invertases from different plant species by cytokinin (Ehness and Roitsch, 2000) suggested that this regulatory mechanism may be responsible for the delay of senescence in leaves. The induction of sink activity would result in the attraction of metabolites, leading to a decreased remobilisation from the senescing leave into the stem. To further substantiate this hypothesis, we have analysed plants with autoregulated delay of senescence. It has been shown that controlling the expression of *ipt,* a gene encoding isopentenyltransferase, catalysing the rate limiting step in cytokinin biosynthesis, with the senescence-inducible promoter of the *SAG12* gene results in the delay of leaf senescence in tobacco (Gan and Amasino, 1995),lettuce (McCabe et al., 2001) and corolla senescence in petunia (*Petunia hybrida*)(Chang et al., 2003). The *SAG12* gene from *Arabidopsis thaliana* encodes a putative Cys protease expressed in senescing leaves and stems (Noh and Amasino, 1999b; Grbic, 2002), and promoter regions responsible for the senescence-specific expression have been identified (Noh and Amasino, 1999a). The activity of the extracellular invertase isoenzyme has been determined in 17-week-old wild-type plants (*N. tabacum* cv Wisconsin 38, [W38]) and plants with autoregulated increase in cytokinins in senescing leaves (*SAG12:ipt;* Gan and Amasino, 1995). In the wild-type plants, the bottom leaves, and to a lesser extend the leaves in the middle of the plants, showed senescence symptoms, such as an evident degradation of chlorophyll and starting necroses, whereas the top leaves showed no apparent symptoms. By contrast, none of the leaves of the transgenic plants showed signs of senescence, and no apparent influence of the leave position was evident. In control plants, the level of extracellular invertase activity was not significantly affected in middle leaves and reduced by 46% in the bottom leaves with respect to the

extracellular invertase activity in the top leaves of these plants (Figure 1). By contrast, the leaves of the *SAG12:ipt* plants showed an inverse distribution of the extracellular invertase activity. The extracellular invertase activity of the *SAG12:ipt* plants was slightly higher in the middle leaves and 139% higher in the bottom leaves compared to the top leaves of these transgenic plants. Thus, the extracellular invertase activity in the bottom leaves of the *SAG12:ipt* plants was 400% higher compared with the activity in the bottom leaves of senescing wild-type plants. This finding demonstrates that the delay of leave senescence in the *SAG12:ipt* plants correlates with an increase in extracellular invertase activity and supports the hypothesis that cytokinin induction of extracellular invertase contributes to the delay of senescence.

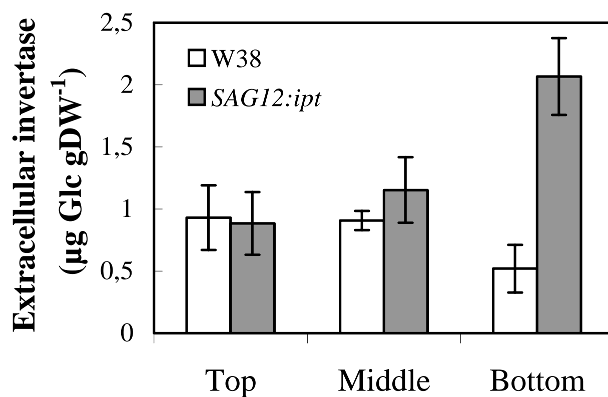

Figure 1. Cytokinin-mediated delay in senescence correlates with an increase in the activity of extracellular invertase.
Extracellular invertase activity has been determined in top, middle, and bottom leaves of tobacco plants expressing the *ipt* gene under control of the senescence-activated *SAG12* promoter (*SAG12:ipt; Gan and Amasino, 1995*) and of wild-type plants (W38). Bars represent the mean value of three independent replications ± SE. DW, dry wight.

Senescence-induced expression of extracellular invertase results in delay of senescence

To substantiate a link between the extracellular invertase activity and delay of senescence by cytokinins, it has been tested whether an increase in extracellular invertase activity may replace the effect of endogenously or exogenously applied cytokinins on the delay of senescence. For this purpose, an extracellular invertase was expressed under control of the *SAG12* promoter in transgenic tobacco plants to test the effect of senescence-induced increase in extracellular invertase activity. Among the numerous extracellular invertases cloned so far, only the cDNA of *Cin1* of *C. rubrum* was proven to encode an biological active enzyme by heterologous expression (Roitsch et al., 1995) and functional analyses (Goetz and Roitsch, 1999). A *SAG1:Cin1* construct was engineered and transformed into tobacco line W38. About 50% of the 70 transgenic lines obtained were characterised by a delayed senescence phenotype both at the whole plant level as well as in senescence assays. For further analyses, the three transgenic lines NT58-5, NT58-15, and NT58-69 were characterised in detail and representative results are shown for line NT58-5. After 17 weeks of development, the *SAG12:Cin1* plants showed a similar phenotype as the

SAG12:ipt plants (Gan and Amasino, 1995) with a clear delay of senescence in the bottom leaves in comparison with the wild-type line (Figure 2A). Analysis of detached young leaves from line NT58-5 incubated in the light for 4 weeks showed that these leaves remained green in comparison to the senescent wild-type leaves (Figure 2B).

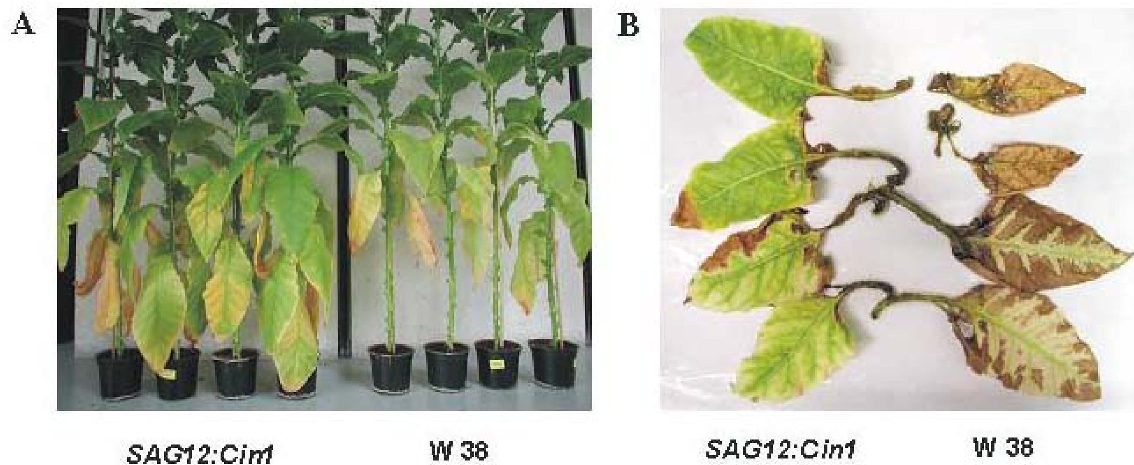

Figure 2. Senescence-induced expression of extracellular invertase *Cin1* results in a delay of senescence
(A) Phenotype of the transgenic *SAG12:Cin1* and of the wild-type (W38) tobacco plants 17 weeks after sawing. The transgenic plants show a delay in senescence of mature leaves in respect to the loss of mature leaves in the wild-type plants.
(B) Delay in senescence of detached young leaves from the transgenic *SAG12:Cin1* and wild-type (W38) tobacco plants incubated in the light for 4 weeks.
The results have been reproduced in five independent experiments with three independent transgenic lines and representative results obtained with line NT58-5 are shown.

The analyses of the invertase activity during development revealed that the extracellular invertase activity specifically increased in the bottom leaves of the *SAG12:Cin1* plants starting in the twelfth week of development and showing the highest levels of 50 μg Glc min^{-1} grams of fresh weight $(FW)^{-1}$ at week 15 (Figure 3A). The onset of the increase in extracellular invertase activity coincided with the induction of the *SAG12* promoter as revealed by the analysis of ß-glucuronidase activity in *SAG12:uid* reporter gene plants (data not shown).

The vacuolar invertase activity was higher in the top leaves compared with the bottom leaves both of W38 plants and of *SAG12:Cin1* plants (Figure 3B). Whereas the vacuolar invertase activity of the control plants decreased during development, this intracellular invertase activity increased at week 15 and 17 in the *SAG12:Cin1* plants.

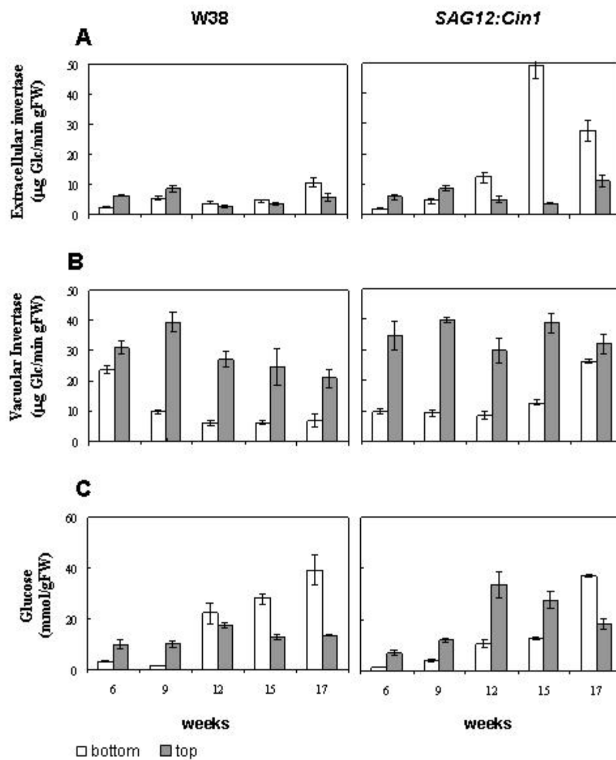

Figure 3. The increase in extracellular invertase activity in transgenic tobacco plants expressing extracellular invertase under control of the senescence-activated promoter *SAG12* is specific and does not result in an increased glucose concentration

(A) Extracellular invertase activity measured in bottom and top leaves of *SAG12:Cin1* and wild-type (W38) plants 17 weeks after sawing. Bars represent the mean value of three independent replications ± SE.

(B) Vacuolar invertase activity measured in bottom and top leaves of *SAG12:Cin1* and wild-type (W38) plants 17 weeks after sawing. Bars represent the mean value of three independent replications ± SE.

(C) Glucose contents of bottom and top leaves of *SAG12:Cin1* and wild-type (W38) plants 17 weeks after sawing. Bars represent the mean of three independent replications leaves ± SE.

The results have been reproduced with three independent transgenic lines and representative results obtained with line NT58-5 are shown.

Analyses of the concentration of soluble sugars revealed that the glucose content in the bottom and top leaves from *SAG12:Cin1* and W38 plants showed an inverse distribution during the different stages of development (Figure 3C). The glucose levels in the senescing bottom leaves of the wild-type plants exceeded the level in the top leaves at the onset of senescence and then further increased, whereas the level remained relative constant in the top leaves. By contrast, in line NT58-5, the onset of senescence resulted in an inverse distribution of the glucose content compared to the wild-type plants. At week 12 and 15, the glucose content in the top leaves of line NT58-5 was 150% and 105% higher, respectively, than in the bottom leaves. Only in samples harvested at week 17, characterised by senescence symptoms also in the bottom leaves of the transgenic line, the glucose content in the bottom leaves increased and exceeded the level in the top leaves. There were no apparent differences between wild-type plants and line NT58-1 with respect to the concentrations of fructose and sucrose (data not shown).

The results obtained with transgenic line NT58-5 have been reproduced in long-term experiments performed with the two independent transgenic lines NT58-15 and NT58-69 also expressing extracellular invertase *Cin1* under control of the *SAG12* promoter. Similar results have been obtained with respect to the delayed senescence at the whole plant level and in detached leaves, activities of extracellular and vacuolar invertase, and concentrations

of soluble sugars (data not shown). In particular, these data support the unexpected finding that invertase activity and hexose levels are not positively correlated and that in *SAG12:Cin1* plants glucose levels are lower in bottom leaves compared to wild-type plants.

Localised chemical induction of extracellular invertase expression results in ectopic delay of senescence

The conclusion derived from the senescence-induced expression of extracellular invertase that extracellular invertase may substitute the cytokinin stimulus was further verified by a complementing experimental approach involving an inducible promoter system. For this purpose, a tetracycline-inducible (derepression) system was chosen (Gatz and Lenk, 1998). Extracellular invertase has been engineered under control of the corresponding promoter and transformed into W38 tobacco plants expressing the required repressor gene. Approximately 40 transgenic plants have been obtained, the three transgenic lines NT17-1, NT27-5, and NT35-7 were analysed in detail and representative data obtained with line NT35-7 are shown.

The effect of the chemical induction of the extracellular invertase expression was studied in leaf detachment assays. The induction of the *Cin1* gene, by applying tetracycline to leaves by petiole feeding, produced a marked delay of senescence visible after 4 weeks of incubation when the control leaves showed already a senesced phenotype (Figure 4A). This retarded-senescence phenotype was related to a higher photosynthetic capacity as determined by chlorophyll fluorescence measurements (Figure 4B) and a higher chlorophyll content (data not shown). In addition, the simultaneous application of tetracycline and kinetin resulted in a higher retardation phenotype and chlorophyll content (data not shown), possibly because of the simultaneous induction of an endogenous tobacco extracellular invertase.

To verify that the delayed senescence was because of the induction of *Cin1,* we performed RNA gel blot analysis. The results show a strong induction of the transcripts for the transgene already after 2 h of incubation with tetracycline (Figure 4C). Induction of the transgene resulted in an average 16-fold higher extracellular invertase activity in tetracycline-treated leaves showing a delay of senescence compared to senescing control leaves. Whereas the mean value of the activity of extracellular invertase of tetracycline-treated leaves was 159.8 milliunits/g FW (SE +/- 24.2), the corresponding value of control leaves was 12.3 milliunits/g FW (SE +/- 3.6) as determined in four different sets of samples. These results further support a positive correlation between extracellular invertase activity and delay of senescence and demonstrate that the induction of the expression of extracellular invertase can effectively substitute the cytokinin action on the delay of senescence.

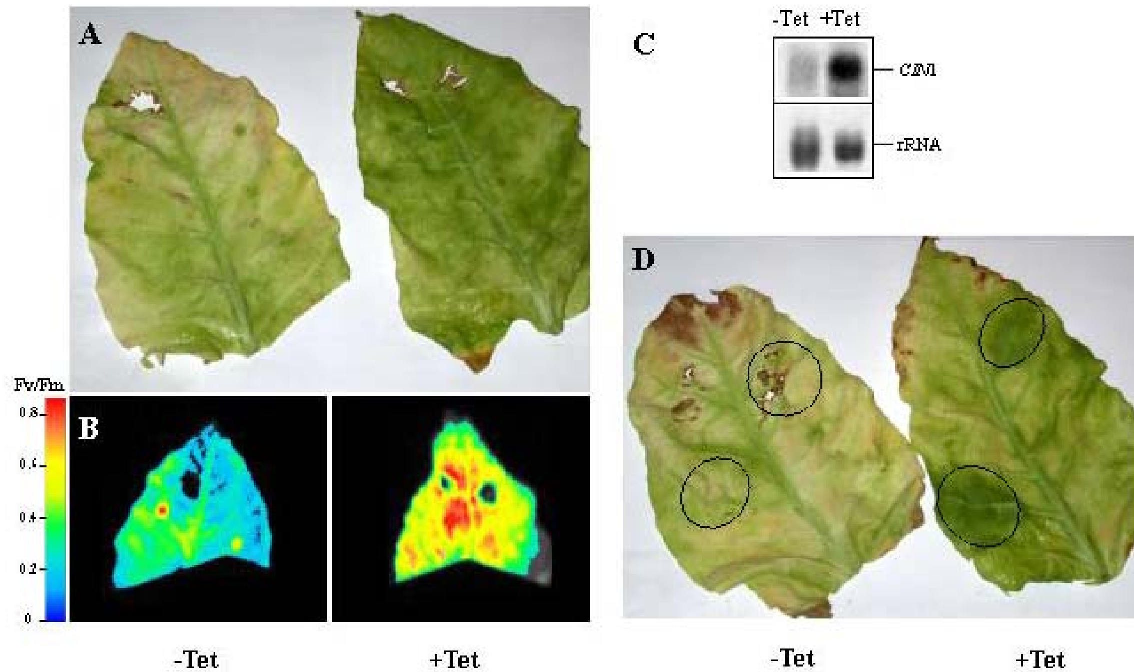

Figure 4. Chemical induction of extracellular invertase Cin1 results in delay of senescence

(A) Effect of the infiltration of tetracycline into detached leaves of a transgenic line expressing the extracellular invertase *Cin1* under control of the tetracycline-inducible *TetR* promoter. The left leaf was infiltrated with a MS control solution, whereas the right one was infiltrated with the same solution containing tetracycline at a final concentration of 10 µg/L.

(B) Chlorophyll fluorescence image of the leaves shown in **(A)**.

(C) RNA gel blot showing the accumulation of the *Cin1* transcripts in leaves after 2 h of treatment with tetracycline in comparison to control leaves.

(D) Effect of the localised induction of *Cin1* by spotting chlortetracycline onto transgenic leaves. A MS solution containing either Silwet 0.02% (left control leaf) or the detergent plus chlortetracycline (10 µg/L) (right leaf) was spotted onto the marked zones.

The results have been reproduced in five independent experiments with three independent transgenic lines and representative results obtained with line NT35-7 are shown.

The effect of tetracycline was further studied by local induction of gene expression in the transgenic leaves. The localised tetracycline application leads to the appearance of green islands similar to those observed in autumn leaves (Figure 4D). The amount of chlorophyll in the green areas was two to six times higher than in the corresponding zones of control leaves. A simultaneous increase of extracellular invertase activity in the treated zones was detected (data not shown), further supporting a causal relationship between the appearance of green island and an increased leaf invertase activity.

Senescence is not delayed by cytokinins when extracellular invertase activity is inhibited

The results obtained with the previously described transgenic approaches prove that an increase in extracellular invertase is sufficient for delay of senescence. Because these data do not rule out the activation of a cytokinin-independent pathway, a further functional approach was performed to answer the question of whether extracellular invertase activity is essential for the delay of senescence by cytokinins. The rational for this approach was to inhibit the extracellular invertase activity in the presence of cytokinins by the expression of an invertase inhibitor under control of a cytokinin-inducible promoter. For this purpose, a tobacco apoplasmic invertase inhibitor (Greiner et al., 1998) was cloned behind the promoter of the cytokinin-inducible invertase gene *Lin6* (Godt and Roitsch, 1997) and transformed into the tobacco cultivar W38. Approximately 40 transgenic plants have been obtained, the three transgenic lines NT71-9, NT71-25, and NT71-29 were analysed in detail, and representative data obtained with line NT71-29 are shown.

Leaf-detachment assays demonstrate that kinetin results in a marked delay of senescence in leaves of wild-type plants. By contrast, this is not the case in leaves of the transgenic line, where the senesced phenotype was observed both in the presence and absence of cytokinin (Figure 5A). The failure of cytokinin to delay senescence was related to the induction of the transgene, as shown by the RNA blot analysis. Invertase inhibitor transcripts were detected both in wild-type plants and line NT71-29 because of the fact that the transgene used in this construction corresponds to the endogenous tobacco gene. However, whereas in the wild-type leaves its expression is down-regulated by kinetin, in the transgenic lines the levels of transcripts are increased, demonstrating an induction of the transgene under control of the cytokinin-inducible promoter (Figure 5B, bottom panel). Because the mRNA level of the endogenous invertase inhibitor gene is repressed by cytokinin, and thus inversely regulated than the transgene under control of the *Lin6* promoter, the treatment results in a highly elevated level of the invertase inhibitor mRNA level in the transgenic plant compared to the corresponding control incubation. To substantiate that cytokinin result in the assumed induction of an endogenous extracellular invertase of tobacco under the experimental conditions used, the mRNA level of tobacco extracellular invertase NtßFruct1 has been determined. Treatment with kinetin induced the expression of *Ntßfruct1* in the wild type and transgenic line already after 1 d (Figure 5B, top panel), supporting the finding that an extracellular invertase is induced by cytokinin in tobacco. To analyse whether the induction of the invertase inhibitor gene under control of the cytokinin-inducible promoter indeed results in an posttranslational inhibition of the activity of the endogenous extracellular invertase coinduced by cytokinin, sugar levels have been determined. Table 1 shows that

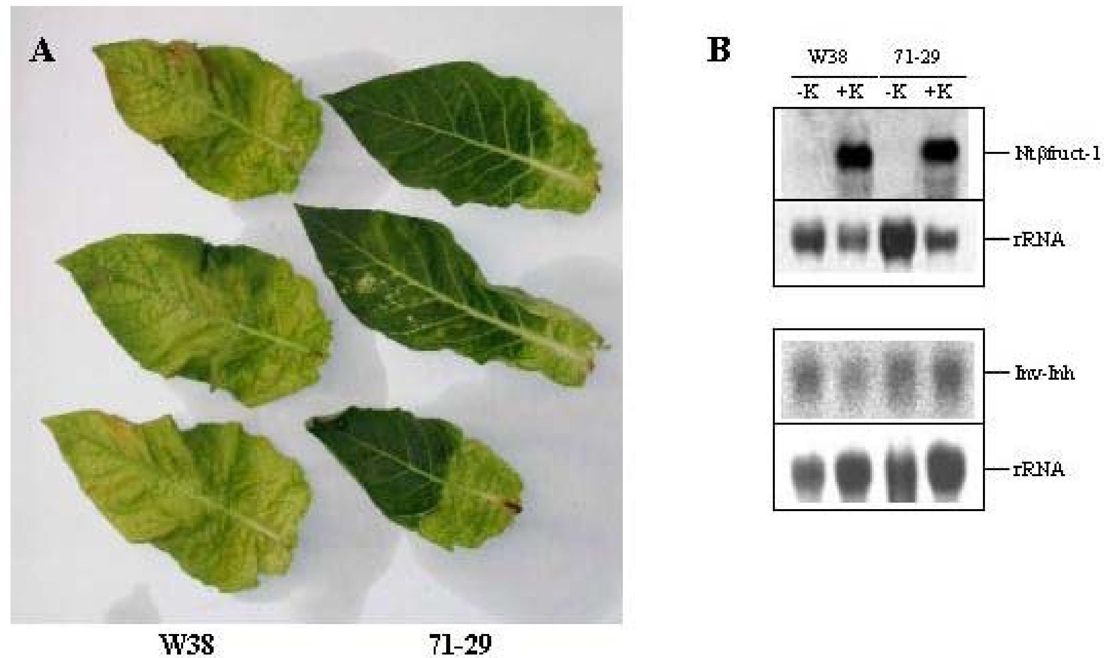

Figure 5. Effect of the inhibition of extracellular invertase activity on the delay of senescence by kinetin.

(A) Detached leaves of a transgenic tobacco line expressing the tobacco invertase inhibitor P17A under control of the cytokinin-inducible promoter *Lin6* (*Lin6:P17A*) and wild-type plants (W38) were infiltrated with water containing kinetin at a final concentration of 30 µg/L.

(B) RNA gel blot analysis showing the accumulation of the transcripts for extracellular invertase Ntßfruct1 and the invertase inhibitor P17A in wild-type (W38) and transgenic (*Lin6:P17A*) plants after 1 and 3 d of treatment with kinetin (K), respectively.

The results have been reproduced in five independent experiments with three independent transgenic lines, and representative results obtained with line NT71-29 are shown.

kinetin induction of the transgene results in a average reduction of the concentration of hexose sugars by 55%, whereas the concentrations in the control incubations were not significantly affected. This finding demonstrates that the products of the enzymatic reaction catalysed by invertase are specifically reduced. Because the endogenous extracellular invertase NtßFruct1 was induced in wild-type leaves and leaves of the transgenic line to similar levels (Fig. 5B), the data demonstrate a posttranslational inhibition of the invertase activity by the invertase inhibitor coinduced by cytokinin. Therefore, the results of this loss-of-function experiment provides further proof for a positive correlation between extracellular invertase activity and delay of senescence. When the extracellular invertase activity is inhibited, the delay of senescence phenotype is no longer observable despite the presence of cytokinin.

Table 1. Kinetin induction of the invertase inhibitor P17A results in inhibition of sucrose cleavage.

Line	Time	Treatment	Glucose (mM/gFW)	Fructose (mM/gFW)	Sucrose (mM/gFW)
W38	1 d	None	19.2 (2.4)	13.5 (1.6)	6.7 (1.2)
		Kinetin	19.7 (2.9)	18.5 (2.8)	9.1 (1.7)
	3 d	None	42.5 (0.4)	41.3 (1.2)	9.0 (4.4)
		Kinetin	47.5 (1.7)	46.7 (1.7)	9.1 (2.4)
Lin6:P17A	1 d	None	14.0 (6.1)	11.6 (5.8)	8.1 (2.1)
		Kinetin	6.0 (1.3)	4.2 (1.6)	6.6 (1.9)
	3 d	None	46.5 (3.5)	33.9 (2.4)	8.8 (0.1)
		Kinetin	19.9 (5.6)	20.1 (5.4)	9.1 (2.6)

Detached leaves of a transgenic tobacco line expressing the tobacco invertase inhibitor P17A under control of the cytokinin-inducible promoter *Lin6* (*Lin6:P17A*) and wild-type plants (W38) were infiltrated with water containing kinetin at a final concentration of 30 μg/L and concentrations of soluble sugars have been determined. The values represent the mean value of three independent replications ± SE obtained with line NT71-29. Similar results have been obtained with line NT71-9.

These data demonstrate that extracellular invertase is an essential component of the mechanism of delay of senescence by cytokinins. Cytokinins fails to delay senescence under conditions when invertase activity is inhibited.

DISCUSSION

Despite the importance of cytokinins for plant growth and development, this class of plant hormone is least understood with respect to the mode of action. Cytokinins are involved in many processes associated with active growth and enhanced metabolic activity. One particular cytokinin effect is the delay of senescence, and this study demonstrates that nutrient mobilisation via an extracellular invertases is an essential components of the underlying regulatory mechanism. This demonstrates that the regulation of leaf senescence is related to changes in source-sink relations and that a direct link between cytokinin action and primary metabolism exists.

The induction by cytokinins of extracellular invertase in suspension cell cultures of *C. rubrum* was shown to be paralleled by a co-ordinated induction of a hexose transporter, which resulted in a twofold increase of sucrose uptake (Ehness and Roitsch, 1997). This greatly supported the hypothesis of an essential link between the molecular mechanism of this phytohormone and primary metabolism (Roitsch et al., 2003), which was further substantiated in this study. It has been shown that the delay of leave senescence in

transgenic plants with autoregulated cytokinin synthesis under control of the *SAG12* promoter (Gan and Amasino, 1995) correlates with an increase of extracellular invertase activity. Thus, extracellular invertases could be a direct mediators of cytokinin in the regulation of this process.

An increase in extracellular invertase activity is sufficient to cause a delay of senescence

Because the cytokinin-mediated delay of senescence is paralleled by a change in the source-sink relationship by affecting extracellular invertase activity, the question arises whether extracellular invertases could replace the effect of endogenous increase or exogenous application of cytokinins. To test this hypothesis, transgenic tobacco plants that expressed the extracellular invertase gene from *C. rubrum* (Ehness and Roitsch, 1997) under control of the senescence-activated *SAG12* promoter have been generated. The *SAG12:Cin1* plants had a clear delayed-senescence phenotype, comparable to that observed in *SAG12:ipt* plants (Gan and Amasino, 1995). The bottom leaves of the 17-week-old transgenic lines were still green, whereas the wild-type plants displayed a clear progression of leaf senescence from bottom to top leaves. It has been previously shown that young healthy leaves detached from wild-type tobacco plants show symptoms of senescence already after 10 d of detachment. The analysis of the cell-wall invertase activity in the leaves of the transgenic line showed an increase of activity specifically in the bottom leaves at the stage in which the activation of the promoter occurs, as shown by the analysis of reporter gene plants. However, no increase in extracellular invertase activity was detected in the leaves of wild-type plants at any of the stages analysed. Thus, it is possible to substitute the cytokinin stimulus by an metabolic enzyme demonstrating a causal relationship. The increased sink strength of bottom leaves in the transgenic line, because of the enhanced expression of extracellular invertase, is sufficient to cause delay in senescence. As a secondary effect, this could account for the observed increase in vacuolar invertase activity. Furthermore, the hydrolysis of sucrose into the hexose monomers not only increases the local sink strength but will also exclude the sugar from phloem loading and transport or remobilisation into the stem.

In general, source and sink metabolism are inversely regulated by various stimuli, including the metabolic regulation by sugars (Roitsch, 1999). An activation of sink metabolism via the induction of sink-specific enzymes, including extracellular invertase, is usually coupled to feedback inhibition of photosynthetic gene expression by carbohydrates (Ehness et al., 1997). The unexpected finding that an increase in extracellular invertase in bottom leaves of *SAG12:Cin1* plants does not results in an increase in glucose steady state concentrations provides an explanation for why photosynthetic activity may be maintained

despite the activation of sink metabolism. Therefore, the delay of senescence induced by extracellular invertases may be related to an activation of the metabolic carbohydrate flux. The resulting higher rate of sugar utilisation causes a decrease of glucose content in the transgenic plants. This ensures that, despite the activation of sink metabolism, the hexose concentration does not reach the threshold level that would result in the feedback-inhibition of photosynthetic gene expression. This provides a mechanism to uncouple the usually observed inverse and co-ordinated regulation of source and sink metabolism. The analyses of transgenic *Arabidopsis* plants with modulated hexokinase activity support the suggestion that the metabolic flux is related to the regulation of senescence (Xiao et al., 2000). Apparently there is a fine-tuned interaction and balance between extracellular hydrolysis by the cell wall-bound invertase and the metabolic flux of the sink cell to avoid the accumulation of carbohydrates. This could explain the contradictory findings that overexpressing a yeast invertase in transgenic *Arabidopsis*, tobacco, and tomato plants results in the accumulation of carbohydrates, an inhibition of photosynthesis, and symptoms that resemble premature senescence (Dickinson et al., 1991; Ding et al., 1993). The strong overexpression of the fungal invertase results in a disturbance of the delicate system that may not be counterbalanced by the plant metabolism because of the fact that the enzymatic and biochemical properties of the heterologous invertase is distinctly different from the plant extracellular invertases.

Sugars are not only substrates for heterotrophic growth but also important signals to regulate various processes in higher plants (Rolland et al., 2002). According to the generally accepted model, leaf senescence is initiated when the photosynthetic rate drops below a certain threshold. Because sugars are primary products of photosynthesis, it has been proposed that sugar levels could be part of the signalling system leading to senescence (Gan and Amasino, 1997; Quirino et al, 2000, Yoshida et al., 2002). However, the analyses of the regulation of leave senescence by sugars so far have been inconclusive. A stimulation of senescence by high sugar levels is suggested by studies with tobacco plants that showed that the levels of glucose and fructose, but not sucrose, increase as the leaves progress through senescence (Wingler et al., 1998). Likewise, a relationship between elevated hexose levels and premature senescence has been concluded from the analyses of transgenic lettuce plants expressing a *SAG12:ipt* construct. By contrast, the activation of the senescence pathways by low sugar levels is supported by the repression of the senescence-activated *SAG12* gene by exogenous application of sugars (Noh and Amasino, 1999a) and its induction because of sugar deprivation (Quirino et al., 2000). This hypothesis is supported by the accelerated leaf senescence of transgenic tomato plants overexpressing the *Arabidopsis* hexokinase with reduced contents of glucose of fructose in comparison with wild-type leaves (Dai et al., 1999). This study and the references cited above indicate that

there is mounting evidence that sugar levels can influence senescence, but more studies are required to determine the causal relationship and variation among species. A solution to the contradictory data with respect to the relation between sugars and senescence are provided by the conclusion that two types of leaves senescence occur in maize leaves: senescence because of assimilate starvation and senescence because of excessive assimilate accumulation (Tollenaar and Daynard, 1982). In addition, the interaction between sugar and hormone signalling (Leon and Sheen, 2003) or the relative ratio between hexose sugars and sucrose rather than the absolute concentration (Wobus and Weber, 1999) may be important as signals to initiate senescence.

It has been observed that the longevity of cut flowers is improved by feeding sugar solutions (Nichols, 1973) and that this effect has been related to an inhibition of the enzymatic activities required for ethylene biosynthesis (Mayak and Borochov, 1984). Ethylene could provide a feedback loop to carbohydrate metabolism because it has been shown that inhibition of ethylene biosyntheses results in the elevation of soluble sugars and improvement of vase life of cut flowers (Ichimura and Hisamatsu, 1999; Zhang and Leung, 2001), that invertase expression is repressed by ethylene (Linden et al., 1996), and that cross-talk between sugar and ethylene signalling exists (Zhou et al., 1998; Yanagisawa et al., 2003).

In a complementing functional approach addressing the role of invertases in the cytokinin-induced delay of senescence, transgenic plants harbouring *C. rubrum* cell wall invertase under the control of a tetracycline-inducible promoter were generated. The delay of senescence induced by the application of tetracycline to detached leaves provides independent experimental evidence that extracellular invertase can effectively substitute the cytokinin action. However, the detection of higher chlorophyll contents and more evident senescence-delayed phenotype in leaves treated simultaneously with kinetin plus tetracycline suggest the existence of additional components in the cytokinin-induced delay of senescence. In addition, the use of this chemically inducible promoter allowed to study the effect of localised induction of extracellular invertase that results in a specific delay of senescence in the treated zones in comparison to the rest of the leaf. Thus, the higher sink strength of these areas because of the expression of extracellular invertase would result in a nutrient mobilisation to these zones. The green islands of autumn leaves have been shown to be caused by cytokinin secretion (Engelbrecht et al.,1969; Angra and Mandahar, 1993; Chen and Ertl, 1994). Because radioactively labelled nutrients are preferably transported to cytokinin-treated areas (Mothes and Engelbrecht, 1963), an altered sink-source relation has been suggested to be responsible for the formation of green islands. The observation that a localised increase of extracellular invertase produces zones with a delay of senescence supports this hypothesis. The detection of increased invertase activity in green islands of

autumn leaves of different species (Z. Novalic and T. Roitsch, unpublished data) confirms this assumption.

Extracellular invertase is essential for cytokinin mediated delay of senescence

Current investigations of the kinetics of expression of senescence activated genes (*SAG*) under natural senescence and under induction of accelerated senescence have suggested that multiple pathways exist that activate distinct set of genes, forming a regulatory network for leaf senescence. Certain genes are likely to be shared by different pathways and may be involved in the execution of senescence, whereas others seem to be unique to specific pathways and may be upstream regulatory genes that affect components of the senescence program. This plasticity of leaf senescence implies that blocking a particular pathway may not necessarily have a significant effect on the progression of senescence (Gan and Amasino, 1997; Chandlee, 2001). To address the question of whether extracellular invertase is an essential component delaying senescence induced by cytokinin, transgenic plants were generated that allowed cytokinin-inducible inhibition of extracellular invertase activity. Transgenic tobacco plants harbouring the invertase inhibitor gene from tobacco under the control of the cytokinin-induced promoter of extracellular invertase *Lin6* from tomato (Godt and Roitsch, 1997) were generated. The presence of putative invertase inhibitor proteins has been shown for a number of sink tissues (Krausgrill et al., 1998), including carnation petals of natural senescing flowers (Halaba and Rudnicki, 1989), where the enzyme has been postulated to control the translocation of sucrose to other organs of the flower (Halaba and Rudnicki, 1988). Recently, the inhibitor from tobacco was cloned, and its functionality and specificity of binding to extracellular invertases demonstrated (Greiner et al., 1998). The effective inhibition of tobacco cell wall invertase reported for the recombinant protein represents a potent tool to study the effect of inhibiting extracellular invertase activity on the cytokinin-induced delay of senescence. The incubation of detached leaves with kinetin should (1) activate the endogenous cytokinin pathway for the delay of senescence, (2) induce the expression of endogenous cytokinin-inducible extracellular invertase, and (3) simultaneously result in the inhibition of extracellular invertase activity by the invertase inhibitor expressed under control of the cytokinin-inducible promoter. The finding that senescence is not any longer delayed in the transgenic plants in the presence of cytokinin demonstrates that an increase in extracellular invertase activity is required for the cytokinin-mediated delay in senescence. The presence of transcripts for the invertase inhibitor in detached young leaves is in agreement with the results obtained by Greiner et al. (1998), where transcripts of this gene are detected in source leaves of 7- and 15-week-old plants. The results reported show a decrease in expression of the invertase inhibitor in leaves from

the bottom to the top of the plant, being high in the old source leaves and reaching the highest levels in senescent leaves (Greiner et al., 1998). This distribution and the findings of this study that the endogenous invertase inhibitor gene is repressed by cytokinin indirectly support a requirement for extracellular invertase activity under conditions of delayed senescence. In addition, the observed repression of the invertase inhibitor demonstrates the regulation of an invertase inhibitor by a plant hormone.

Whereas this study demonstrates the importance of extracellular invertase for only one particular effect of cytokinins, this key enzyme of an apolasmic phloem unloading pathways also could be also involved in other cytokinin-mediated responses. The active growth of tissues depends on the function of the cell division cycle. Because it has been shown that specific D-type cyclins, as important regulators of the cell cycle, are carbohydrate responsive, the extracellular invertase may have a dual function. It provides substrate to satisfy the increased carbohydrate demand of actively growing tissues and generates a metabolic signal to stimulate the cell cycle. Thus, invertases could contribute to the increase in sink size by inducing cell division via a sugar signalling mechanism.

The reported induction of extracellular invertases by sugars (Roitsch et al., 1995; Krausgrill et al., 1996; Godt and Roitsch, 1997; Tymowska-Lalanne and Kreis, 1998, Sinha et al., 2002) could provide a feed forward mechanism to amplify or maintain the cytokinin signal. An initial up-regulation of the extracellular invertase will result in an increased sugar concentration that will then further induce the invertase or keep the invertase induced even if the initial stimulus is not present anymore.

Senescence is a type of programmed cell death that constitutes the final phase of leaf development. Although there has been extensive research focused on whole plant/leaf senescence, the molecular events that induce or contribute to the process have been investigated only recently (Chandlee, 2001). As a result, only a partial picture of the molecular basis for the regulation and progress of this process has emerged, and many questions remained answered. A further understanding of the underlying mechanisms will provide fundamental information about aspects of cell differentiation and the regulation of cellular events through the action of plant hormones and other signals (Gan and Amasino, 1996; Grbic, 2002). The potential regulation of the delay of leaf senescence would provide practical benefits, maximising the crop yield and minimising the postharvest and postproduction losses of fruits, vegetables, flowers, and other crops.

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REFERENCES

Angra S.R., and Mandahar C.L. (1993). Involvement of carbohydrates and cytokinins in pathogenicity of *Helminthosporium carbonum*. Mycopathologia **121**, 91-99.

Bradford M.M. (1976). A rapid and sensitive method for the determination of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248-254.

Brenner M.L., and Cheikh N. (1995). The role of phytohormones in photosynthate partitioning and seed filling. In *Plant Hormones* (Davies, P.J., ed.). Dordrecht: Kluwer Academic Press, pp. 649-670.

Buchanan-Wollaston V. (1997). The molecular biology of leaf senescence. J. Exp. Bot. **48**, 181-199.

Chandlee J.M. (2001). Current molecular understanding of the genetically programmed process of leaf senescence. Physiol. Plant. **113**, 1-8.

Chang H., Jones M., Banowetz G. M., and Clark D. G. (2003) Overproduction of cytokinins in petunia flowers transformed with PSAG12-IPT delays corolla senescence and decreases sensitivity to ethylene. **132**, 2174-2183.

Chen C.-M., and Ertl J.R. (1994). Cytokinin biosynthetic enzymes in plants and slime mold. In: Mok. D.W.S. and Mok M.C. (eds), Cytokinins: Chemistry, activity and function. CRC Press, Boca Raton, pp. 81-85.

Chomczynski P., and Sacchi N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**, 156-159.

Dai N., Schaffer A., Petreikov M., Shahak Y., Giller Y., Ratner K., Levine K., and Granot D. (1999). Overexpression of *Arabidopsis hexokinase* in tomato plants inhibits growth, reduces photosynthesis, and induces rapid senescence. Plant Cell 11, 1253-1266.

Dickinson C.D., Altabella T., and Chrispeels M.J. (1991). Slow-growth phenotype of transgenic tomato expressing apoplastic invertase. Plant Physiol. **95**, 420-425.

Ding B., Haudenshield J.S., Willmitzer L., and Lucas W.J. (1993). Correlation between arrested secondary plasmodesmal development and onset of accelerated leaf senescence in yeast acid invertase transgenic tobacco plants. Plant J. **4**, 179-189.

Ehness R., Ecker M., Godt D., and Roitsch T. (1997) Glucose and stress independently regulate source/sink relations and defense mechanisms via signal transduction pathways involving protein phosphorylation. Plant Cell **9**, 1825-1841.

Ehness R., and Roitsch T. (1997). Co-ordinated induction of mRNAs for extracellular invertase and a glucose transporter in *Chenopodium rubrum* by cytokinins. Plant J. **11**, 539-548.

Engelbrecht L., Orban U., and Heese W. (1969). Leaf –miner caterpillars and cytokinins in the „green islands" of autumn leaves. Nature **223**, 319-321.

Eschrich W. (1980). Free space invertase, ist possible role in phloem unloading. Ber. Dtsch. Bot. Ges. **93**, 363-378.

Gan S., and Amasino R.M. (1995). Inhibition of leaf senescence by autorregulated production of cytokinin. Science **270**, 1986-1988.

Gan S., and Amasino R.M. (1996). Cytokinins in plant senescence: from spray and spray to clone and play. BioEssays **18**, 557-565.

Gan S., and Amasino R.M. (1997). Making sense of senescence. Plant Physiol. **113**, 313-319.

Gatz C., and Lenk I. (1998). Promoters that respond to chemical inducers. Trends Plant Sci. **3**, 352-358.

Godt D.E., and Roitsch T. (1997). Regulation and tissue-specific distribution of mRNAs for three extracellular invertase isozymes of tomato suggests an important function in establishing and mantaining sink metabolism. Plant Physiol. **115**, 273-282.

Goetz M, Godt D.E., Guivarch A, Kahmann U, Chriqui D, and Roitsch T. 2001. Induction of male sterility in plants by metabolic engineering of the carbohydrate supply. Proc. Nat. Acad. Sci. (USA) **98**, 6522-6527.

Goetz, M., and Roitsch, T. (1999). The different pH-optima and substrate specificities of extracellular and vacuolar invertases are determined by a single amino acid substitution. Plant J. **20**, 707-711.

Grbic, V. (2002) Spatial expression pattern of *SAG12:GUS* transgene in tobacco (*Nicotiana tabacum*). Physiol. Plant. **116**, 416-422.

Greiner S., Krausgrill S., and Rausch T. (1998). Cloning of a tobacco apoplasmic invertase inhibitor: Proof of function of the recombinant protein and expression analysis during plant development. Plant Physiol. **116**, 733-742.

Halaba J., and Rudnicki R.M. (1988). Invertase inhibitor-control of sucrose transport from carnation petals to other flower parts. Plant Growth Reg. **7**, 193-200.

Halaba J., and Rudnicki R.M. (1989). Invertase inhibitor in wilting flower petals. Scientia Horticulturae **40**, 83-90.

Hensel L.L., Grbic V., Baumgarten D.A., and Bleecker A.B. (1993). Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in Arabidopsis. Plant Cell **5**, 553-564.

Horsch R.B., Fry J.B., Hoffmann N.L., Wallroth M., Eichholtz D., Rogers S.G., and Fraley R.T. (1985). A simple and general method for transferring genes into plants. Science **227**, 1229-1231.

Ichimura K., and Hisamatsu T. (1999) Effects of continuous treatment with sucrose on the vase life, soluble carbohydrate concentrations, and ethylene production of cut snapdragon flowers. J. Japan. Soc. Horticultural Sci. **68**, 61-66.

Krausgrill S., Greiner S., Köster U., Vogel R., and Rausch T. (1998). In transformed tobacco cells the apoplasmic invertase inhibitor operates as a regulatory switch of cell wall invertase. Plant J. **13**, 275-280.

Krausgrill S., Sander A., Greiner S., Weil M., and Rausch T. (1996). Regulation of cell-wall invertase by a proteinaceous inhibitor. J. Exp. Bot. **47**, 1193-1198.

Kuiper D. (1993). Sink strength: Established and regulated by plant growth regulators. Plant Cell Environment **16**, 1025-1026.

Lefebre R., Vasseur J., Backoula E., and Coullerot J.P. (1992). Participation of carbohydrate metabolism in the organogenic orientation of *Chicorium intybus* tissues cultivated in vitro. Can. J. Bot. **70**, 1897-1902.

Leon P., and Sheen J. (2003) Sugar and hormone connections. Trends in Plant Sci. **8**, 110-116.

Lichtenthaler (1987). Chlorophyll fluorescence signatures of leaves during the autumnal chlorophyll breakdown. J. Plant Physiol. **131**, 101-110.

Linden J.C., Ehneß R., and Roitsch T. (1996) Regulation by ethylene of apoplastic invertase expression in *Chenopodium rubrum* tissue culture cells. Plant Growth Regul. **19**, 219-222.

Mayak, S., and Borochov, A. (1984). Nonosmotic inhibition by sugars of the ethylene-forming activity associated with microsomal membranes from carnation petals. Plant Physiol. **76**, 191-195.

McCabe M.S., Garratt L., Schepers F., Jordi W.J.R.M., Stoopen G.M., Davelaar E., van Rhijn J.H.A., Power J.B., and Davey M. (2001) Effects of PSAG12-IPT gene expression on development and senescence in transgenic lettuce. **127**, 505-516.

Mothes K., and Engelbrecht L. (1963). On the activity of a kinetin-like root factor. Life Sci. **11**, 852-857.

Nam H.G. (1997). The molecular genetic analysis of leaf senescence. Curr. Opin. Biotechnol. **8**, 200-207.

Nichols, R. (1973). Senescence of cut carnation flower: respiration and sugar status. J. Hort. Sci. **48**, 111-121.

Noh Y.-S., and Amasino R.M. (1999a). Identification of a promoter region responsible for the senescence-specific expression of *SAG12*. Plant Mol. Biol. **41**, 181-194.

Noh Y.-S., and Amasino R.M. (1999b). Regulation of developmental senescence is conserved between *Arabidopsis* and *Brassica napus*. Plant Mol. Biol. **41**, 195-206.

Ori N., Juarez M.T., Jackson D., Yamaguchi J., Banowetz G.M., and Hake S. (1999). Leaf senescence is delayed in tobacco plants expressing the maize homeobox gene *k notted1* under the control of a senescence-activated promoter. Plant Cell **11**, 1073-1080.

Richmond A.E., and Lang A. (1957). Effect of kinetin on protein content and survival of detached *Xanthium* leaves. Science **125**, 650-651.

Rolland F., Moore B., Sheen J. (2002). Sugar sensing and signaling in plants. Plant Cell **14** suppl., S185-S205.

Roitsch T. (1999). Source-sink regulation by sugars and stress. Curr. Op. Plant Biol. **2**, 198-206.

Roitsch T., Balibrea M.E., Hofmann M., Proels R., and Sinha A.K. (2003). Extracellular invertase: key metabolic enzyme and PR protein. J. Exp. Bot. **54**, 513-524.

Roitsch T., Bittner M., and Godt D.E. (1995). Induction of apoplastic invertase of Chenopodium rubrum by D-glucose and a glucose analog and tissue-specific expression suggest a role in sink-source regulation. Plant Physiol. **108**, 285-294.

Roitsch T., and Ehness R. (2000). Regulation of source/sink relations by cytokinins. Plant Growth Regul. **32**, 359-367.

Roitsch T., and Tanner W. (1996). Cell wall invertase: Bridging the gap. Botanica Acta **109**, 90-93.

Quirino B.F., Noh Y.-S., Himelblau E., and Amasino R.M. (2000). Molecular aspects of leaf senescence. Trends Plant Sci. **5**, 278-282.

Singh S., Letham D.S., and Palni L.M.S. (1992a). Cytokinin biochemistry in relation to leaf senescence. V. Endogenous cytokinin levels and metabolism of zeatin riboside in leaf discs from green and senescent tobacco (*Nicotiana rustica*) leaves. J. Plant Physiol. **139**, 279-283.

Singh S., Letham D.S., and Palni L.M.S. (1992b). Cytokinin biochemistry in relation to leaf senescence. VII. Endogenous cytokinin levels and exogenous applications of cytokinins in relation to sequential leaf senescence of tobacco. Physiol. Plant. **86**, 398-406.

Sinha A.K., Römer U., Köckenberger W., Hoffmann M., Elling L., and Roitsch T. (2002). Metabolizable and non-metabolizable sugars activate different signal transduction pathways in tomato. Plant Physiol. **128**, 1480-1489.

Smart C.M. (1994). Gene expression during leaf senescence. New Phytol. **126**, 419-448.

Tang G.Q., Lüscher M., and Sturm A. (1999) Antisense repression and vacuolar and cell wall invertase in transgenic carrot alters early plant development and sucrose partitioning. Plant Cell **11**, 1-14.

Tollenaar M. and Daynard T.B. (1982) Effect of source sink ratio on dry matter accumulation and leaf senescence of Zea mays. Can. J. Plant Sci. **62**, 855-860.

Tymowska-Lalanne Z. and Kreis M. (1998). Expression of the *Arabidopsis thaliana* invertase gene family. Planta **207**, 259-265.

Wingler A., Schaewen A., Leegood R.C., Lea P.J. and Quick W.P. (1998). Regulation of leaf senescence by cytokinins, sugars, and light. Plant Physiol. **116**, 329-335.

Wobus U. and Weber H. (1999) Sugars as signal molecules in plant seed development. Biological Chem. **380**, 937-944.

Xiao W., Sheen J., Jang J.C. (2000) The role of hexokinase in plant sugar signal transduction and growth and development. Plant Mol. Biol. **44**, 451-461.

Yanagisawa S., Yoo S.D., Sheen J. (2003) Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. Nature (London) **425**, 521-525.

Yoshida S., Ito M., Nishida I., Watanabe A. (2002) Identification of a novel gene HYS1/CPR5 that has a repressive role in the induction of leaf senescence and pathogen-defence responses in Arabidopsis thaliana. Plant J. **29**, 427-437.

Zhang Z. and Leung D (2001) Elevation of soluble sugar levels by silver thiosulfate is associated with vase life improvement of cut gentian flowers. **75**, 85-90.

Zhou L., Jang J.C., Jones T.L., Sheen J. (1998) Glucose and ethylene signal transduction crosstalk revealed by an Arabidopsis glucose-insensitive mutant. Proc. Nat. Acad. Sci. (USA) **95**, 10294-10299.

Chapter 7

Extracellular invertase:

key metabolic enzyme and PR protein

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Extracellular invertase: key metabolic enzyme and PR protein

Abstract. Extracellular invertase is the key enzyme of an apoplasmic phloem unloading pathway and catalyses the hydrolytic cleavage of the transport sugar sucrose released into the apoplast. This mechanism contributes to long distance assimilate transport, provides the substrate to sustain heterotrophic growth and generates metabolic signals known to effect various processes of primary metabolism and defence responses. The essential function of extracellular invertase for supplying carbohydrates to sink organs was demonstrated by the finding that antisense repression of an anther-specific isoenzyme provides an efficient method for metabolic engineering of male sterility. The regulation of extracellular invertase by all classes of phytohormones indicates an essential link between the molecular mechanism of phytohormone action and primary metabolism. The up-regulation of extracellular invertase appears to be a common response to various biotic and abiotic stress-related stimuli such as pathogen infection and salt stress in addition to specific stress-related reactions. Based on the observed co-ordinated regulation of source/sink relations and defence responses by sugars and stress-related stimuli, the identified activation of distinct subsets of MAP kinases provides a mechanism for signal integration and distribution within such complex networks. Sucrose derivatives not synthesised by higher plants, such as turanose, were shown to elicit responses distinctly different from metabolisable sugars and are rather perceived as stress-related stimuli.

INTRODUCTION

Carbohydrate partitioning between the autotrophic source tissues and a variable number of sink tissues competing for a common pool of carbohydrates is a highly dynamic process that accompanies all stages of growth and development of higher plants. This physiological mosaic is also known to be affected by exogenous factors such as pathogen infection or abiotic stress stimuli that require fast induction of sink metabolism for the ability to activate a cascade of defence responses and to mediate physiological adaptations. The supply of the transport sugar, sucrose, is a limiting step for the growth of sink tissues (Farrar, 1996) and sucrose-metabolising enzymes are important determinants of sink capacity by generating a sucrose gradient to support unloading of sucrose from the phloem. For these reasons, the enzymes responsible for the first metabolic reaction of sucrose are likely critical links between photosynthate production in source leaves and growth capacity of sink organs (Farrar, 1996; Balibrea et al., 2000).

Extracellular invertase is a cell-wall-bound enzyme that catalyses the irreversible cleavage of sucrose released into the apoplast via sucrose transporters. The resulting hexose monomers are then imported into the sink cell by monosaccharide transporters. Supplying carbohydrates via an apoplasmic phloem unloading pathway provides a mechanism for flexible and fast adjustment of the carbohydrate supply according to wide variations in demand. Due to inclusion of three protein-mediated steps, the process of assimilate partitioning can be efficiently regulated according to the current metabolic requirements. The tremendously large surface area of the total apoplasmic space surrounding all sink cells provides the possibility to allow very high uptake rates if required.

Extracellular invertase is in particular suited as a key regulator of apoplasmic phloem unloading due to its enzymological properties. Whereas the K_m value of hexose transporters is in the µM range, the K_m value of extracellular invertases is in the mM range and thus limiting unloading. In addition, extracellular invertase catalyses the only irreversible step of the apoplasmic phloem unloading pathway. Accordingly, a large number of stimuli have been identified that affect the mRNA levels of specific isoenzymes. In addition, various further levels of regulation have been determined for extracellular invertases such as tissue-specific expression, differential transcript formation (Cheng et al., 1999), exon skipping (Bournay et al., 1996), and inhibition by a proteinaceous inhibitor (Krausgrill et al., 1996). The evolution of such a variety of regulatory mechanisms further supports the key role of extracellular invertase for assimilate partitioning.

Since the physiological relevance of extracellular sucrose cleavage has been addressed by molecular approaches (Sturm and Chrispeels, 1990; von Schaewen et al.,

1990; Miller and Chourey, 1992) both extracellular and intracellular invertases have attracted a lot of attention (Tymowska-Lalanne and Kreis, 1998a; Sturm, 1999; Roitsch et al. 2000). Progress is rapid in the field and this review focuses on recent advances and addresses specific aspects of the transcriptional regulation of genes encoding extracellular invertases and the underlying signal transduction pathways. The essential function in sink tissues is evident from transgenic approaches on isogenes that are specifically expressed in distinct flower organs (Goetz et al., 2001). The regulation by all classes of phytohormones indicates that extracellular invertases are involved in mediating the corresponding hormone responses. The induction of extracellular invertase by both abiotic and biotic stress stimuli supports the suggestion that extracellular invertase is not only a key modulator of assimilate partitioning, but is also an important component of various stress responses. Based on the observed co-ordinated regulation of source/sink relations and defence responses (Ehness et al., 1997), recent findings on sugar sensing (Sinha et al., 2002) and MAP kinase function (Link et al., 2002) are discussed in respect of the mechanism of the underlying signal transduction pathways and the function of the cellular regulatory network.

Differential expression of extracellular invertases in floral organs and engineering male sterility by antisense repression of anther-specific isoenzymes

An expression of extracellular invertases in floral organs has been reported for different plant species, namely carrot (Lorenz et al., 1995), *Arabidopsis thaliana* (Tymowska-Lalanne and Kreis, 1998b), *Vicia faba* (Weber et al., 1996), *Lilium longiforum* (Clément et al., 1996), maize (Xu et al., 1996; Kim et al., 2000a), potato (Maddison et al., 1999), tobacco (Goetz et al., 2001), and tomato (Godt and Roitsch, 1997). For the latter six species anther-expressed isoenzymes were reported, a finding, that indicates a crucial function of extracellular invertases in providing carbohydrates to the male gametophyte. The importance of supplying assimilates via an apoplasmic pathway involving cleavage of sucrose by invertase is further supported by the identification of anther-specific hexose transporters. In *Arabidopsis* a monosaccharide transporter was characterised that is expressed in developing pollen after the onset of symplasmic isolation of the microspore (Truernit et al., 1999). Likewise, monosaccharide transporters specifically expressed in anthers have been demonstrated in *Petunia* (Ylstra et al., 1998) and tobacco (M Goetz and T Roitsch, unpublished observations).

A transgenic approach recently demonstrated an essential role of extracellular invertase Nin88 of tobacco in pollen development (Goetz et al., 2001). A highly specific spatial and temporal expression pattern of Nin88 during anther development and pollen maturation was shown. At early developmental stages the Nin88 protein was observed only

in the tapetal cell layer. Once the tapetum starts getting degraded, the Nin88 protein can be detected in the tetrads and when the tapetum is completely degraded, Nin88 is found in the developing microspores. Expression of a *Nin88* antisense construct under the control of its own promoter in transgenic tobacco plants resulted in a block during pollen development. The germination efficiency of immature pollen, derived from *Nin88*-antisense plants, was drastically reduced and correlates with the reduction in invertase enzyme activity. These results demonstrate a critical role of extracellular invertase in pollen development and support the essential function of extracellular sucrose cleavage for supplying carbohydrates to sink tissues via the apoplast. In addition, both sucrose concentrations and sucrose/hexose ratios are affected. Since they are known as metabolic signals, the antisense repression is likely to also affect sugar sensing and signalling which is supported by *in-vitro* rescue experiments (M Goetz and T Roitsch, unpublished data). As the expression of the antisense construct was controlled by the corresponding, highly tissue-specific promoter, the phenotypic effects were restricted to the male gametophyte. *Nin88*-antisense plants are normal in any aspect of plant growth and development except for failure to produce functional pollen. Thus, anther-specific antisense repression of extracellular invertase provides an efficient method to induce male sterility by metabolic engineering of the carbohydrate supply for hybrid seed production or as biological safety method to avoid outcrossing of transgenes.

Antisense repression of extracellular carrot invertase under a constitutive promoter in carrot was shown to result in highly pleiotrophic phenotypes possibly due to numerous secondary effects that confirms a crucial role of this enzyme during various stages of plant growth and development (Tang et al., 1999). The highly tissue-specific repression of invertase activity provides an alternative to generalised antisense repression or knock out mutants to address the biological function of specific extracellular invertases without the complication of unspecific effects.

Up to now, no functional analysis of extracellular invertases in other floral reproductive organs than the male gametophyte has been performed. Different expression patterns for extracellular invertases in ovaries (Kim et al., 2000b) and gynoeceum (Godt and Roitsch, 1997) are reported. Specific antisense approaches could help to clarify the function of extracellular invertases in those floral organs, especially in respect to the early development of the female gametophyte.

The development of reproductive organs has to be a tightly regulated process and was shown to be related to the sugar status. A strictly controlled availability of carbohydrates, mediated by an apoplasmic sucrose cleavage and uptake of resulting sugars via hexose transporters, could be an important determinant in this regulatory mechanism. Therefore,

studies on corresponding flower-specific invertases are expected to be valuable to elucidate the regulatory mechanisms during flower development in general.

Regulation of extracellular invertases by phytohormones

Plant hormones play an integral role in controlling growth, differentiation and development of plants. It has been speculated that specific plant growth regulators are particularly involved in regulating sink strength (Kuiper, 1993), carbohydrate partitioning (Brenner and Cheikh, 1995) and phloem unloading (Tanner, 1980). There is accumulating evidence that extracellular invertases are regulated by various phytohormones which can in most cases be related to the increased carbohydrate demand of growth-stimulated tissues. These findings indicate an important link between primary metabolism and phytohormone action. Elucidation of the relation between extracellular invertases and phytohormones is therefore important in unravelling the molecular mechanisms of physiological phytohormone responses.

Several studies support the theory that gibberellic acid plays a significant role in regulating invertase levels (Tymowska-Lalanne and Kreis, 1998a,b). Gibberellic acid (GA_3) promotes cell elongation, is important for flower induction and has been reported to increase invertase activity in several plant organs such as sugar cane stem (Sacher et al., 1963), Jerusalem artichoke tubers (Edelman and Hall, 1964), beet roots (Palmer, 1966), lentil epycotils (Seitz and Lang, 1968), and internodes of bean (*Phaseolus vulgaris*; Morris and Arthur, 1985) and oat (Kaufman et al., 1968). Invertase mRNA from shoots of dwarf pea plants (*Pisum sativum*) was induced after GA_3 treatment, indicating that the expression of the pea shoot cell-wall invertase gene could be regulated by GA_3 at transcriptional and/or translational levels (Wu et al., 1993a). In suspension-cultured tomato cells (*Lycopersicon esculentum L.*) the addition of GA_3 had no effect on the mRNA for the two invertase genes expressed in flower organs. Although this finding suggests that the function of GAs in flower induction seems to be unrelated to sucrose metabolism (Godt and Roitsch, 1997) a solely tissue-specific GA induction of the corresponding invertase genes can not be ruled out.

It has been reported that the activity of extracellular invertase is stimulated by auxin (Glasziou, 1969; Weil and Rauch, 1990). Experimental manipulation of tissue expansion growth by hormones seems to be mediated by invertases. Auxin appears to play a key role in the regulatory mechanism. IAA promotes both growth and invertase activity in segments of young *P. vulgaris* internodes. The sensitivity to auxin is developmentally regulated and requires mRNA and protein synthesis (Morris and Arthur, 1984, 1986). Gravi-stimulated auxin redistribution seems to be involved in asymmetric induction of invertase mRNAs in oat (Wu et al., 1993b) and maize (Long et. al., 2002).

Cytokinins are a group of phytohormones that promote cell division and play a major role in the regulation of various processes associated with active growth and thus an enhanced demand for carbohydrates, suggesting a link to the regulation of assimilate partitioning (Kuiper, 1993; Roitsch and Ehneß, 2000). The physiological significance of this regulation is supported by the fact that tissues with elevated activities of extracellular invertase, such as rapidly growing tissues, are also known to contain elevated cytokinin concentrations (Godt and Roitsch, 1997). In autotrophic cell cultures, the extracellular invertases from *Chenopodium rubrum Cin1* (Ehneβ and Roitsch, 1997) and from tomato *Lin6* (*Lycopersicon esculentum*) (Godt and Roitsch, 1997) are highly up-regulated in response to physiological concentrations of different cytokinins. These data from suspension cell cultures could be confirmed in different tissues of *Chenopodium* plants. The stimulation of invertase activity in response to cytokinin has also been reported for *in-vitro*-cultivated *Chichorium* tissues (Lefebre et al., 1992). Cytokinins are also considered as key components of plant senescence (Singh et al., 1992; Gan and Amasino, 1996, 1997; Buchanan-Wollaston, 1997; Nam, 1997), based on the ability to delay senescence by modification of sink-source relations (Jordi et al., 2000). The up-regulation of extracellular invertase by cytokinin could provide the molecular basis for such a localised induction of sink metabolism (Roitsch and Ehneß, 2000). A relation between extracellular invertase and cytokinin-mediated growth responses is supported by a transgenic tobacco line (BIK62) expressing the *ipt* cytokinin biosynthetic gene under control of a tagged-promoter in auxiliary buds after the floral transition (Guivarc´h et al., 2002). The modifications of the endogenous cytokinin balance resulted in specific morphological changes due to fast rates of leaf initiation and meristem reactivation, cell cycle activation, and higher amounts of extracellular invertases. These data support the hypothesis of links between morphological cytokinin responses and extracellular invertases by influencing source-sink relations and sugar signals known to regulate the cell cycle (Roitsch and Ehneß, 2000).

Brassinosteroids (BRs) are a group of growth-promoting substances in plants with high structural similarity to animal steroid hormones (Grove et al., 1979). These compounds induce a variety of growth responses when exogenously applied to plant tissues (Li and Chory, 1999; Müssig and Altmann, 1999). The addition of BRs to autotrophic tomato suspension culture cells (*Lycopersicon peruvianum*) specifically elevates the activity of cell-wall-bound invertase, whereas the intracellular invertase activities were not affected (Goetz *et al.*, 2000). This enhanced enzyme activity correlated with the induction of the mRNA of extracellular invertase *Lin6*, whereas the mRNA levels of the other three extracellular invertase isoenzymes were not affected. A correlation between a localised growth response of the hypocotyl of tomato seedlings and a specific induction of *Lin6* mRNA in this tissue support the physiological significance of the regulation of extracellular invertase by BRs as a

prerequisite for BR-induced growth responses. This finding demonstrates a role of BRs in tissue-specific source-sink regulation (Goetz *et al.*, 2000).

Abscisic acid was shown to increase the activity of extracellular invertase in seed tissues of avocado (Richings *et al.*, 2000). The significance of the regulation of extracellular invertase by abscisic acid is further supported preliminary evidence obtained with transgenic tobacco plants expressing a fusion between the promoter of the extracellular invertase *Lin6* of tomato and the ß-glucuronidase reporter gene. The observed induction of the *Lin6* promoter by abscisic acid was in agreement with the identification of abscisic acid responsive *cis* elements in the *Lin6* promoter (R Proels and T Roitsch, unpublished observations).

Ethylene induces fruit ripening, leaf abscission and promotes senescence. By contrast with the previous hormones, ethylene represses the mRNA level for extracellular invertase Cin1 in autotrophic *C. rubrum* in parallel with a reduction in their specific activity (Linden et al., 1996). A general physiological significance can be a down-regulation of enzymes associated with active growth, such as extracellular invertase, in favour of induction of enzymes required for fruit maturation by ethylene (Roitsch et al., 2000). Ethylene is the only stimulus known to down-regulate the expression of extracellular invertase.

Regulation of invertases by abiotic stress

Partitioning of assimilates and its effect on dry matter distribution is influenced by several environmental factors such as temperature, drought, salinity, and nutrient availability (Wardlaw, 1990). In particular, drought and salinity are the major abiotic stresses that limit plant productivity. Although these two abiotic stresses are clearly different in their physical nature, they activate some common reactions in plants (Zhu, 2001). Engineering plants with a greater ability to adapt to these adverse situations is one of the strategies to decrease their negative agronomic impact. In this respect, only limited information about source-sink regulation by stress tolerance is available, despite being directly involved in plant growth and crop productivity. For example, a high root/shoot ratio is considered to be an important adaptive response to drought and salinity (Vartanian, 1996; Balibrea et al., 2000) that permits the plant the recovery of the functional equilibrium by alleviating the stress (Van der Werf, 1996; Geiger et al., 1996). This dry matter redistribution is closely associated with carbohydrate allocation to the roots (Cakmak et al., 1994). Therefore, the processes involved in carbon metabolism and energy production are expected to have priority between the groups of genes or proteins affected by water and saline stresses with potential to improve tolerance.

The photoassimilates produced under salt stress are used to support crucial, mutually processes such a growth, maintenance and osmotic adjustment. The competition of sink organs for the limited carbon supplies under salinity significantly affects overall plant growth,

dry matter distribution and crop yield (Munns and Termaat, 1986; Daie, 1996). As a consequence, the different growth responses to salinity can be interpreted as resulting from changes in the allocation and partitioning of photoassimilates (Poljakoff-Mayber and Lerner, 1994). In general, salinity causes a reduction in sink enzyme activities, leading to an increase in sucrose in source leaves with a decrease in photosynthesis rate by feedback inhibition (Stitt, 1991; Poljakoff-Mayber and Lerner, 1994).

The growth capacity of tomato plants under salinity have been related to the increase in sink activity of young leaves and roots by the induction of vacuolar acid invertase and sucrose synthase activities (Balibrea et al., 2000). However, only extracellular invertase activity was affected by salinity in the same way as the redistribution of dry matter, decreasing in the young leaves and increasing in the root tips (ME Balibrea and F Pérez-Alfocea, unpublished data). Moreover, this activity was much higher in the roots of salt-tolerant wild species *Lycopersicon pennellii* than in those of domestic *L. esculentum* plants. The role of extracellular invertase in the control of assimilate allocation could be specially important in those species including an apoplasmic step in the phloem unloading of sucrose, such as it occurs in tomato. The vacuolar acid invertase and the cytoplasmic sucrose synthase and neutral invertase could have a major role in this process when the phloem unloading pathway is mainly symplasmic or when the extracellular invertase is impaired (Eschrich, 1980; Sturm et al., 1995). Indeed, an inverse relationship between extracellular invertase and cytoplasmic sucrolytic activities has been observed in different organs of tomato plants (Balibrea et al., 1996, 1999) and different genotypes differing in tolerance to salinity and fruit size (ME Balibrea and F Pérez-Alfocea, unpublished data). Wang et al. (2000) reported increases in the cytoplasmic sucrolytic activities (neutral invertase and sucrose synthase) in response to osmotic stress in cultured sweet potato cells. Tymowska-Lalanne and Kreis (1998b) also found an inverse relationship in the expression of the cell-wall invertase and sucrose synthase and vacuolar invertase genes in *Arabidopsis thaliana* in different growth conditions. These authors reported that the higher root development under aeroponic growth conditions was related to the expression of cell-wall invertase, while the sucrose synthase was more expressed in the smaller roots from the soil-cultured plants.

Water deficits in plants lead to physiological modifications, such as photosynthesis reduction, transcriptional and post-transcriptional regulation of various genes, protein turnover and osmolyte biosynthesis (Bohnert et al., 1995). Water stress induces large alterations in source-sink relations with source limitations resulting in a decreased export of assimilates and, therefore, in a decreased crop load (Berman and Dejong, 1996). In maize, the increase of soluble and insoluble invertase activities during pollination and early kernel development was blocked by water stress conditions (Zinselmeier et al., 1995). It has also been shown that induction of male sterility in wheat by meiotic stage water deficit is preceded

by a decline in vacuolar invertase activity (Dorion et al., 1996). By contrast, it was shown that a marked accumulation of hexoses was correlated to an increase of vacuolar invertase activity in mature maize leaves under drought, but it does not affect the cell wall invertase one (Pelleschi *et al.*, 1997, 1999). In vegetative sink and source organs of water-stressed maize plants, the organ-specific induction of acid invertase activity was correlated with an increase in the *Ivr2* gene transcripts and in the vacuolar invertase proteins (Kim *et al.*, 2000a).

Cold-induced stalk elongation in tulip (*Tulipa gesneriana*) is mediated by the induction of invertase expression, but not by sucrose synthase (Balk and de Boer, 1999). By contrast, two sucrose synthase genes from *Arabidopsis thaliana* were found to be differentially up-regulated in leaves exposed to the environmental stresses (cold, drought and O_2-defficiency). The differential stress-responsive regulation of these genes in leaves might represent part of a general cellular response to the allocation of carbohydrates during acclimation processes (Dejardin et al., 1999). Low oxygen stress decreases invertase expression (*Ivr1* and *Ivr2*), but does not affect sucrose synthase, decreasing the invertase/sucrose synthase ratio in maize root tips (Zeng et al., 1999). These responses have an important implication in acclimation to low oxygen stress by the conservation of sucrose and ATP and reducing the hexose-based sugar-signalling system. The shift from hydrolytic sucrose cleavage by invertase to the sucrolytic cleavage by sucrose synthase as adaptation to hypoxic conditions is also supported by Biemelt et al. (1999).

Stress signals and carbohydrate sensing versus metabolic regulation

The transcript level of extracellular invertase has been shown to be up-regulated by different stress-related and sugar stimuli (Roitsch, 1999; Roitsch *et al.,* 2000). Non-metabolisable isoforms of sucrose have been introduced to address the extracellular sensing mechanism of sugars in plants and the data obtained are critically evaluated with respect to stress and carbohydrate dependant regulation of extracellular invertases.

The transcript level of extracellular invertase Cin1 of *C. rubrum* was induced by different stress-related stimuli such as phosphatase inhibitor and benzoic acid (Ehness *et al.* 1997) and mechanical wounding (Roitsch *et al.,* 1995). Lin6, an extracellular invertase of tomato, was reported to be up-regulated in response to the elicitor polygalacturonic acid in suspension cultures and wounded green leaves (Godt and Roitsch, 1997). Recently, *Lin6* was also shown to be up-regulated in the photoautotrophic suspension cell cultures of tomato in response to treatment with an elicitor preparation of the necrotrophic fungus *Fusarium oxysporum lycopersici* (E-FOL; Sinha *et al.* 2002). Also, in carrot, the induction of extracellular invertase has been shown in wounded or infected leaves (Sturm and Chrispeels 1990). The potential physiological relevance of the up-regulation of extracellular invertase in

response to stress-related stimuli is a localised increase of the carbohydrate supply, providing additional metabolic energy for the cascade of defence reactions to get activated (Roitsch, 1999).

Sugars, known to act as signalling molecules regulating a variety of genes in different physiological pathways (Koch, 1996; Roitsch, 1999; Sheen *et al.,* 1999), also induce extracellular invertase. The increase in enzyme activity of extracellular invertase by glucose and sucrose in *C. rubrum* was shown to be parallel with the increased level of mRNA of *Cin1* (Roitsch *et al.,* 1995). One of the isoforms of extracellular invertase was also shown to be up-regulated by glucose in tobacco (Krausgrill *et al.,* 1996), *Arabidopsis* (Tymowska-Lalanne and Kreis, 1998b) and also with sucrose in tomato (Godt and Roitsch, 1997; Sinha *et al.,* 2002). In a recent study, Sinha et al. (2002) have shown up-regulation of *Lin6* from tomato suspension cell cultures also with non-metabolisable sucrose analogues such as palatinose (6-O-α-d-glucopyranosyl-Fru), turanose (3-O-α-d-glucopyranosyl-Fru) and fluorosucrose (1-deoxy-1-fluorofructofuranosylα-d-glucopyranosid). While palatinose and turanose can neither be recognised by sucrose transporters nor can be cleaved by any known plant enzymes (M'Batchi and Delrot, 1988; Li *et al.,* 1994; Sinha *et al.,* 2002), fluorosucrose can be transported inside the cells (Thom and Maretzki, 1992) and is only slowly metabolised (Hitz *et al.,* 1985). Fernie *et al.* (2001) have shown that application of palatinose to discs of potatoes increases the invertase activity and result in a shift in favour of starch synthesis. The authors have proposed the existence of an extracellular sugar-sensing mechanism/factor. This speculation was derived from the fact that palatinose exerts the same response in potato tubers as sucrose and that they are not taken up by the cells. Lalonde et al. (1999) and Fernie et al. (2000) have already speculated that such a factor exists, though to date there is no direct experimental evidence. In another study, Loreti et al. (2000) have shown independent glucose and disaccharide-sensing processes modulating α-amylase in barley embryos. They show the importance of the fructose moiety in the non-metabolisable disaccharide modulating the expression of α-amylase. They assumed the presence of a putative sugar sensor at the level of the plasma membrane and independent of sucrose transporters. A more detailed comparative analysis of the effect of the metabolisable sugars glucose and sucrose, and the sucrose derivatives palatinose, turanose and fluorosucrose, revealed distinct differences (Sinha et al., 2002). The sucrose derivatives had no effect on RbcS expression, resulted in transient induction of extracellular invertase, and elicited fast and transient activation of mitogen-activated protein (MAP) kinases. By contrast, the metabolisable sugars resulted in repression of RbcS, induction of extracellular invertase and failed to activate MAP kinase activity. These results, summarised in Table 1, suggest that the effect of the sucrose derivatives not synthesised by higher plants resembles the effect of the fungal elicitor E-FOL and are distinctly different from metabolisable sugars. The

differential effect of non-metabolisable sugars and glucose were also reported in transcript stability of α-amylase by Loreti et al. (2000). These observation questions the suitability of sucrose isomers as tools to study sugar-sensing mechanisms. It seems that such sugar derivatives are perceived as stress-related stimuli rather than specific sugar molecules. This conclusion is further supported by preliminary experimental evidence obtained with additional sucrose derivatives that are not naturally occurring in plants such as thiosucrose and glucosyl-alpha-1,1-mannitol (AK Sinha, T Roitsch, unpublished observations). The physiological significance of these findings is reflected by the fact that turanose and palatinose are synthesised by plant pathogens.

The fact that both metabolisable sugars and stress-related carbohydrate stimuli studied in different systems regulate extracellular invertase, makes this gene an important candidate to be used as a marker gene for the analyses of converging signalling pathways.

	Transcript level			Enzyme activity
	RbcS	CWI	PAL	MAPK
Glucose	↓	↑	↑	-
Sucrose	↓	↑	↑	-
Turanose	-	[↑]	↑]*	[↑]
Palatinose	-	[↑]	↑]*	[↑]
Fluorosucrose	-	↑]*	↑]*	↑]*
Elicitor	[↓]	[↑]	↑]*	[↑]

Table 1. Differential effect of metabolisable sugars (sucrose, glucose), non-metabolisable sucrose derivatives (turanose, palatinose, fluorofructose), and a fungal elicitor (E-FOL) on the transcript level of the small sub-unit of Rubisco (RbcS), extracellular invertase (CWI) and phenylalanine ammonia-lyase (PAL) and enzyme activity of mitogen activated protein kinase (MAPK) in photoautotrophic suspension cell cultures of tomato.
Arrows pointing up (↑) and down (↓) represent induction and repression, respectively, (-) represent no effect. [ ] indicates a transient nature, and]* indicates that only one time point has been analysed and no data are available on the time course of the response.

Signal integration and distribution by MAP kinases

The observed co-ordinated regulation of source/sink relation and defence reactions in response to sugars and various stress-related stimuli (Ehness et al., 1997) raises the question about the mechanisms whereby diverse signals may be integrated to result in co-ordinated responses while simultaneously maintaining the possibility for unique signal-specific downstream effects.

MAP kinases have been shown to play a major role in the initiation and co-ordination of a plant´s defence response to various biotic and abiotic stimuli. Their role in the response

to elicitors (Zhang et al., 1998), cold and drought (Jonak et al., 1996), wounding (Stratmann and Ryan, 1997), and endogenous signals (Zhang and Klessig, 1997) is well established. Likewise, the defence response against a number of pathogens has been extensively characterised, although those components that determine the specificity of the response remain to be elucidated. Analysis of the number of the known MAP kinases and those encoded in the genome which have no assigned function yet, demonstrates that these MAP kinases are positioned upstream of a far bigger number of cellular responses that range from the induction of sink metabolism (Ehness *et al.*, 1997), the activation of different defence responses to cytoskeletal or cell wall rearrangements (Gross et al., 1993; Jonak et al., 1995; Bögre et al., 1999). However, the induction of suitable cellular reactions against an attacking pathogen also requires an appropriate sensing of the type of the pathogen. The information derived from these receptors then has to be allocated to a limited amount of intracellular transducers. Hence, the question arises of how and where the corresponding signal transduction pathways are integrated to produce the observed co-ordinated and differential effects after pathogen attack.

Using a non-biased biochemical approach we could recently show that a particular stress-related stimulus results in the simultaneous activation of several MAP kinases in photoautotrophic tomato cell cultures (Link et al., 2002). In addition, different stimuli were shown to activate distinctly different subsets of MAP kinases. By chromatographic separation of crude extracts from treated tomato cells, we were able to show that, upon elicitor treatment up to four different MAP kinases are activated. Interestingly, only a crude elicitor preparation from E-FOL resulted in activation of all four MAP kinases studied. When PGA or chitosan, molecules that can be expected to only naturally occur in a complex mixture with other eliciting compounds, were applied to the cells, only certain subsets of these four MAP kinases were activated. Based on this information, it is now possible to assign different putative functions to the activated MAP kinases, without being restricted to an initial identification such as by using antibodies against known MAP kinases. As an example, the results we obtained for three different stimuli are shown in Fig. 1. The *in-gel* kinase assays shown reflect a different degree of `overall` MAP kinase activation which is highest in E-FOL treated samples, moderate after PGA treatment and low after application of salicylic acid. As marker genes for defence response and induction of sink metabolism, the transcriptional activation of phenylalanine ammonia-lyase (PAL) and extracellular invertase (Lin6) was monitored. Both genes are activated by all three stimuli, though to a different degree. Interestingly, the extend of transcriptional activation of the two marker genes reflects the observed degree of MAPK activation. As an additional physiological marker, the production of H_2O_2 which is only induced by E-FOL treatment was determined.

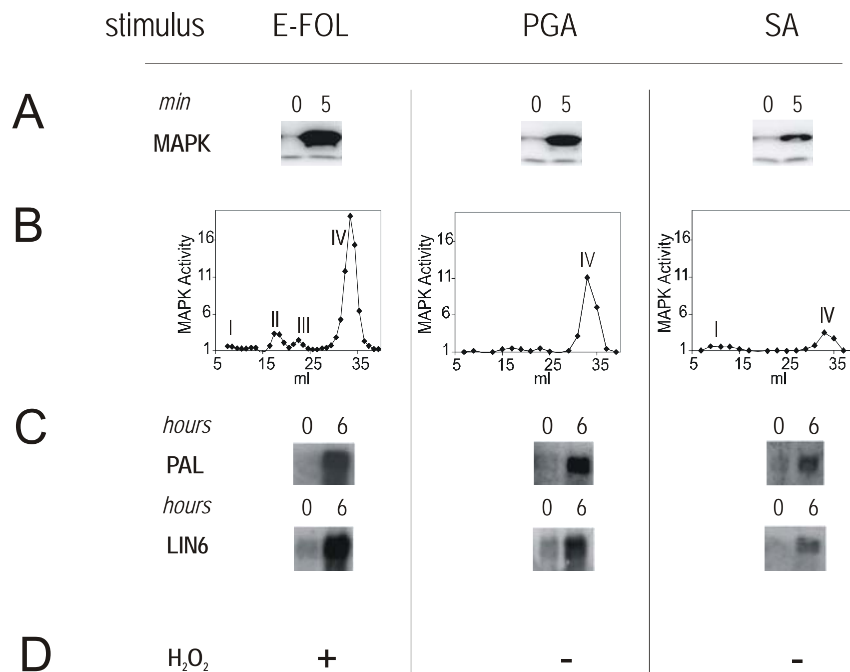

Fig. 1. The differential effect of the fungal elicitor E-FOL, polygalacturonic acid (PGA), and salicylic acid (SA) on the pattern of MAP kinases activated in tomato suspension-cultured cells is compared to different downstream effects. (A) MAPK *in-gel* assays of crude extracts 5 min after treatment that were subjected to further purification. (B) Elution profile of MAP kinase activity from an anion exchange column. (C) Northern blot analysis of mRNA for phenylalanine ammonia-lyase (PAL) and extracellular invertase (Lin6) 6 hours after treatment. (D) Release of H_2O_2 into the culture medium 20 min after the treatment.

The panel of Figure 1B shows the corresponding activity-profiles of the chromatograms from the separation of crude extracts on an anion exchange column. The different MAP kinases are assigned as peaks I to IV, which are only present in E-FOL-treated samples. PGA- and salicylic acid treated samples only show activation of MAP kinases contributing to peak IV (PGA) and peak I and IV (SA). The comparison of the peak patterns now allows different functions tentatively to be assigned to the corresponding MAP kinases. Since only E-FOL treatment results in H_2O_2 production, this response can be assigned to the presence of activated MAP kinases that contribute to peaks II and III. Accordingly, the concomitant activation of a defence response and sink metabolism, monitored by *PAL* and *Lin6* can be assigned to peak IV, since this is the only MAP kinase contributing to the response to PGA. By immunoprecipitation, SIMK and MMK2 homologues in peak II were found and the MAP kinase constituting peak IV was grouped as a SAMK homologue. It will now be interesting, whether differential cellular responses can be mimicked by the concomitant expression of constitutively active mutant isoforms of these MAP kinases.

In an additional study, it has been shown in alfalfa cells treated with a yeast elicitor, that four different MAP kinases (SIMK, SAMK; MMK2 and MMK3; Cardinale et al., 2000) are

activated. Deeper analysis demonstrated, that these MAP kinases are differentially activated by the presence of distinguished components of the yeast elicitor preparation. In this case, chitin resulted in the strong activation of SIMK, MMK2 and MMK3, whereas the activation of SAMK was only weak. Ergosterol on the other hand strongly activated SIMK, MMK3 and SAMK, but showed only little effect on MMK2 activation. β-Glucan, however, activated all four MAP kinases studied. Finally, it has been shown, that different components of an elicitor preparation activate different MAP kinases to a different extend and with different time-courses.

It thus appears that MAP kinases serve as integration and distribution points that enable the cell to react accordingly, not only to a presumably vast amount of different eliciting molecules, but also complex mixtures of those. By the concerted action of different combinations of MAP kinases, that will result in common as well as different downstream effects, the plant cell is able to \`score\` an invading pathogen and thus take appropriate counteractions.

Extracellular Invertase: metabolic enzyme or PR protein?

A number of studies demonstrate that a common response in plant-pathogen interaction is an increase in extracellular invertase activity in addition to the activation of defence-related responses that are directly involved in coping with the pathogen attack such as induction of pathogenesis related (PR) proteins. Corresponding results were obtained by analysing the infection by various plant pathogens such as biotrophic (Hall and Williams, 2000) and necrotrophic fungi (Benhamou *et al.*, 1991), bacteria (Sturm and Chrispeels, 1990), and viruses (Herbers *et al.*, 2000). One problem in such experimental approaches is to distinguish between the invertase activity of the invading pathogen and the invertase of the plant host (Ruffner *et al.*, 1992).

A link between the carbohydrate status, in general, and pathogen responses is also evident from the literature. This includes the phenomenon of high sugar resistance (Horsfall and Dimond, 1957), the finding that various key pathogenesis related genes are sugar inducible (Roitsch, 1999), and that overexpression of a yeast invertase in the plant apoplast confers increased resistance against virus infection and an increased expression of PR proteins (Herbers *et al.*, 2000).

A further indirect link between pathogen responses and invertases may exist via phytohormones. Phytohormones are known to be involved in various plant-pathogen interactions (Jameson, 2000) and the expression of various defence-related genes was shown to be affected by phytohormones (Shinshi *et al.*, 1997; Memelink *et al.*, 1987). Thus, the regulation of extracellular invertase by phytohormones as outlined above could also contribute to plant pathogen responses.

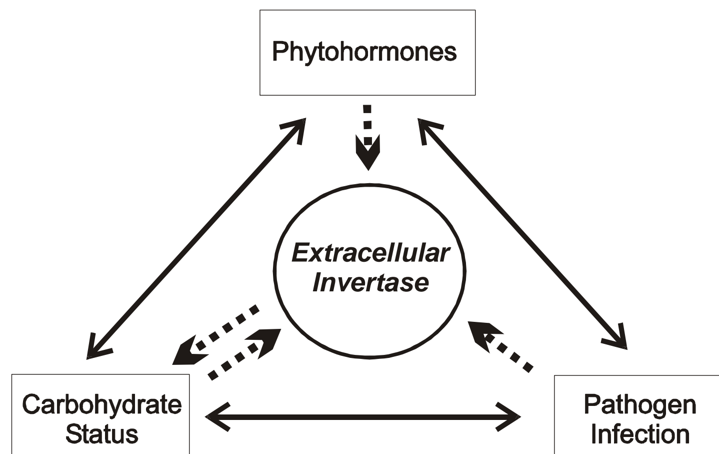

Fig. 2. Function of extracellular invertase in plant-pathogen interactions. Contribution of the transcriptional induction of cell wall invertase by phytohormones, the carbohydrate status and pathogens to the interaction between the different signals.

Extracellular invertase is not only regulated by a similar set of stimuli that induce defence-related genes but the regulation is also co-ordinated. It has been shown that both metabolisable sugars and defence-related stimuli co-ordinately regulate source/sink relations and defence responses (Ehness *et al.*, 1997). Based on the differential effect of the kinase inhibitor staurosporin it has been shown that sugars and stress-related stimuli initially activate independent signal transduction pathways ruling out the activation of extracellular invertase as prerequisite for the regulation of photosynthetic genes and defence-related genes as suggested before (Jang and Sheen, 1994). However, the fact that extracellular invertase is induced by sugars (Roitsch *et al.*, 1995) provides a positive feed-back mechanism: up-regulation of extracellular invertase will elevate the sink strength and thus the sugar concentration that will (further) induce PR genes and repress photosynthetic genes (Roitsch, 1999).

What is the physiological significance of the involvement of extracellular invertase in plant-pathogen interaction? The activation of a cascade of defence reactions requires additional energy. Thus a localised increase in sink strength by an elevated invertase activity can satisfy the increased demand for carbohydrates as energy source of the tissues invaded by a pathogen. In addition, the increase in carbohydrates will generate a metabolic signal that induces the expression of defence-related genes and repression of photosynthesis in addition to signals derived from the pathogen. The finding that treatment of photoautotrophic tomato suspension cultures with E-FOL results in only transient effects on the expression of extracellular invertase and a photosynthetic gene supports such a sugar-sensing mechanism (Sinha et al., 2002). The fungal elicitor induces a source/sink transition and activation of defence-related genes. Since this sink-induced batch culture is not linked to an additional source tissue, there is no increase in the apoplasmic sugar status. The cells are apparently able to sense that the sugar concentration is not increased, despite the increase in extracellular invertase activity, and the original source status is restored.

The available data support that extracellular invertase is not only a key metabolic enzyme to contribute to growth and development of higher plants, but also is an important part of the adaptations to cope with pathogen infections as outlined in Fig. 2 and can thus be considered also as an important pathogenesis-related protein.

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REFERENCES

Balibrea ME, Dell´Amico J, Bolarín MC, Pérez-Alfocea F. 2000. Carbon partitioning and sucrose metabolism in tomato plants growing under salinity. *Physiologia Plantarum* **110,** 503-511.

Balibrea ME, Parra M, Bolarín MC, Pérez-Alfocea F. 1999. Cytoplasmic sucrolytic activity controls tomato fruit growth under salinity. *Australian Journal of Plant Physiology.* **26,** 561-568.

Balibrea ME, Santa-Cruz AM, Bolarín MC, Pérez-Alfocea F. 1996. Sucrolytic activities in relation to sink strength and carbohydrate composition in tomato fruit growing under salinity. *Plant Science.* **118,** 47-55.

Balk PA, de Boer D. 1999. Rapid stalk elongation in tulip (*Tulipa gesneriana* L. cv. Apeldoorn) and the combined action of induced invertase and the water-channel protein γTIP. *Planta* **209,** 346-354.

Benhamou N, Grenier J, Chrispeels MJ. 1991. Accumulation of beta fructosidase in the cell walls of tomato roots following infection by a fungal wilt pathogen. *Plant Physiology* 97(2), 739-750.

Berman ME, Dejong TM. 1996. Water stress and crop load effects on fruit fresh and dry weights in peach (*Prunus persica*). *Tree Physiology* 16, 859-864.

Biemelt S, Hajirezaei MR, Melzer M, Albrecht G, Sonnwald U. 1999. Sucrose synthase activity does not resctrict glycolysis in roots of transgenic potato plants under hypoxic conditions. *Planta* **210,** 41-49.

Bögre L, Calderini O, Binarova P Mattauch M, Till S, Kiegerl S, Jonak C, Pollaschek C, Barker P, Huskisson NS, Hirt H, Heberle-Bors E. 1999. A MAP kinase is activated late in plant mitosis and becomes localized to the plane of cell division. *The Plant Cell* **11**, 101-114.

Bohnert HJ, Nelson DE, Jense RG. 1995. Adaptations to environmental stresses. *The Plant Cell* 7, 1099-1111.

Bournay AS, Hedley PE, Maddison A, Waugh R, Machray GC. 1996. Exon skipping induced by cold stress in a potato invertase gene transcript. *Nucleic Acids Research* **24(12),** 2347-2351.

Brenner ML, Cheikh N. 1995. The role of phytohormones in photosynthate partitioning and seed filling. In: Davies PJ, ed. *Plant Hormones*, Dordrecht, Boston, London, Kluwer Academic Press, 649-670.

Buchanan-Wollaston V. 1997. The molecular biology of leaf senescence. *Journal of Experimental Botany* **48,** 181-199.

Cakmak I, Hengeler Ch, Marschner H. 1994. Partitioning of shoot and root dry matter and carbohydrates in bean plants suffering from phosphorous, potassium and magnesium deficiency. *Journal of Experimental Botany* **45,** 1245-1250.

Cardinale F, Jonak C, Ligterink W, Niehaus K, Boller T, Hirt H. 2000. Differential activation of four specific MAPK pathways by distinct elicitors. *Journal of Biological Chemistry* **275**, 36734-36740.

Cheng WH, Taliercio EW, Chourey PS. 1999. Sugars modulate an unusual mode of control of the cell-wall invertase gene (Incw1) through its 3´untranslated region in a cell suspension culture of maize. *Proceedings of the National Academy of Sciences of USA* **96(18)**, 10512-10517.

Clément C, Burrus M, Audran, J-C. 1996. Floral organ growth and carbohydrate content during pollen development in *Lilium*. *American Journal of Botany* **83**, 459-469.

Daie J. 1996. Metabolic adjustments, assimilate partitioning, and alterations in source-sink relations in drought-stressed plants. In: Zamski E, Schaffer AA, eds. *Photoassimilate Distribution in Plants and Crops. Source-Sink Relationships,* New York, Inc: Marcel Dekker, 407-420.

Dejardin A, Sokolov LN, Kleczkowski LA. 1999. Sugar/osmoticum levels modulate differential abscisic acid-independent expression of two stress-responsive sucrose synthase genes in Arabidopsis. *Biochemical Journal* **2,** 503-509.

Dorion S, Lalonde S, Saini HS. 1996. Induction of male sterility in wheat by meiotic-stage water deficit is preceded by a decline in invertase activity and changes in carbohydrate metabolism in anthers. *Plant Physiology* **111,** 137-145.

Edelman J, Hall MA. 1964. Effect of growth hormones on the development of invertase associated with cell wall. *Nature* **201,** 296-297.

Ehness R, Ecker M, Godt DE, Roitsch T. 1997. Glucose and stress independently regulate source/sink relations and defense mechanisms via signal transduction pathways involving protein phosphorylation. *The Plant Cell* **9,** 1825-1841.

Ehneß R, Roitsch T. 1997. Co-ordinated induction of mRNAs for extracellular invertase and a glucose transporter in *Chenopodium rubrum* by citokinins. *The Plant Journal* **11,** 539-548.

Eschrich W. 1980. Free space invertase, its possible role in phloem unloading. *Berichte der deutschen Botanischen Gesellschaft* **93,** 363-378.

Farrar J. 1996. Regulation of shoot-root ratio is mediated by sucrose. *Plant and Soil* **185,** 13-19.

Fernie AR, Riesmeier JW, Martiny A, Ramalingam S, Willmitzer L, Trethewey RN. 2000. Consequences of the expression of a bacterial glucokinase, both in combination with and independently of a yeast-derived inveratse, in potato tubers. *Australian Journal of Plant Physiology* **27**, 827-833.

Fernie AR, Rossner U, Geigenberger P. 2001. The sucrose analog palatinose leads to a stimulation of sucrose degradation and starch synthesis when supplied to discs of growing potato tubers. *Plant Physiology* **125,** 1967-1977.

Gan S, Amasino RM. 1996. Cytokinins in plant senescence: from spray and pray to clone and play. *Bioessays* **18,** 557-565.

Gan S, Amasino RM. 1997. Making sense of senescence. Molecular genetic regulation of leaf senescence. *Plant Physiology* **113,** 313-319.

Geiger DR, Koch KE, Shieh WJ. 1996. Effect of environmental factors on whole plant assimilate partitioning and associated gene expression. *Journal of Experimental Botany* **47,** 1229-1238.

Glasziou KT. 1969. Control of enzyme formation and inactivation in plants. *Annual Reviews Plant Physiology* **20,** 63-88.

Godt DE, Roitsch T. 1997. Regulation and tissue-specific distribution of mRNAs for three extracellular invertase isoenzymes of tomato suggests an important function in establishing and maintaining sink metabolism. *Plant Physiology* **115,** 273-282.

Goetz M, Godt DE, Guivarch A, Kahmann U, Chriqui D, Roitsch T. 2001. Induction of male sterility in plants by metabolic engineering of the carbohydrate supply. *Proceedings of the National Academy of Sciences of USA* **98,** 6522-6527.

Goetz M, Godt DE, Roitsch T. 2000. Tissue-specific induction of the mRNA for an extracellular invertase isoenzyme of tomato by brassinosteroids suggests a role for steroid hormones in assimilate partitioning. *The Plant Journal* **22**, 515-522.

Gross P, Julius C, Schmelzer E, Hahlbrock K. 1993. Translocation of cytoplasm and nucleus to fungal penetration sites is associated with depolymerization of microtubules and defence gene activation in infected, cultured parsley cells. *EMBO Journal* **12**, 1735-1744.

Grove MD, Spencer GF, Rohwedder WK, Mandava N, Worley JF, Warthen JD, Jr, Steffens GL, Flippen-Anderson JL, Cook JC, Jr. 1979. Brassinolide, a plant growth-promoting steroid isolated from Brassica napus pollen. *Nature* **281,** 216-217.

Guivarc´h A, Rembur J, Goetz M, Roitsch T, Noin, M, Schmülling T, Chriqui D. 2002. Local expression of the ipt gene in transgenic tobacco (*Nicotiana tabacum* L. cv. SR1) axillary buds establishes a role for cytokinins in tuberization and sink formation. *Journal of Experimental Botany* **53,** 621-629.

Hall JL, Williams LE. 2000. Assimilate transport and partitioning in fungal biotrophic interactions. *Australian Journal of Plant Physiology* **27,** 549-560.

Herbers K, Takahata Y, Melzer M, Mock HP, Hajirezaei M, Sonnewald U. 2000. Regulation of carbohydrate partitioning during the interaction of potato virus Y with tobacco. *Molecular Plant Pathology* **1,** 51-59.

Hitz W, Schmitt MR, Card PJ, Giaquinta RT. 1985. Transport and metabolism of 1' -fluorosucrose, a sucrose analog not subject to invertase hydrolysis. *Plant Physiology* **77,** 291-295.

Horsfall JG, Dimond AE. 1957. Interactions of tissue sugar, growth substances, and disease susceptibility. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* **64,** 415-421.

Jameson P. 2000. Cytokinins and auxins in plant-pathogen interactions: An overview. *Plant Growth Regulation* **32,** 369-380.

Jang JC, Sheen J. 1994. Sugar sensing in higher plants. *The Plant Cell* **6,** 1665-1679.

Jonak C, Kiegerl S, Ligterink W, Barker PJ, Huskisson NS, Hirt H. 1996. Stress signaling in plants: a mitogen-activated protein kinase pathway is activated by cold and drought. *Proceedings of the National Academy of Sciences of USA* **93,** 11274-11279.

Jonak C, Kiegerl S, Lloyd C ,Chan J, Hirt H. 1995. MMK2, a novel alfalfa MAP kinase specifically complements the yeast MPK1 function. *Molecular Genetics and Genomics* **248**, 686-694.

Jordi W, Schapendonk A, Davelaar E, Stoopen GM, Pot CS, De Visser R, van Rhijn JA, Gan S, Amasino RM. 2000. Increased cytokinin levels in transgenic P_{SAG12}-IPT tobacco plants have large direct and indirect effects on leaf senescence, photosynthesis and N partitioning. *Plant, Cell and Environment* **23,** 279-289.

Kaufman PB, Ghosheh NS, LaCroix JD, Soni SL, Ikuma H. 1973. Regulation of invertase levels in Avena stem segments by gibberellic acid, sucrose, glucose, and fructose. *Plant Physiology* **52,** 221-228.

Kim JY, Mahé A, Brangeon J, Prioul JL. 2000. A maize vacuolar invertase, IVR2, is induced by water stress. Organ/tissue specifity and diurnal modulation of expression. *Plant Physiology* **124,** 71-84.

Kim J-Y, Mahé A, Guy S, Brangeon J, Roche O, Chourey PS, Prioul J-L. 2000. Characterization of two members of the maize gene family, *Incw3* and *Incw4*, encoding cell-wall invertases. *Gene* **245**, 89-102.

Koch KE. 1996. Carbohydrate-modulated gene expression in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47,** 509-540.

Krausgrill S, Sander A, Greiner S, Weil M, Rausch T. 1996. Regulation of cell-wall invertase by a proteinaceous inhibitor. *Journal of Experimental Botany* **47,** 1193-1198.

Kuiper D. 1993. Sink strength: Established and regulated by plant growth regulators. *Plant Cell and Environment* **16,** 1025-1026.

Lalonde S, Bolos E, Hellmann H, Barker L, Patrick JW, Frommer WB, Ward JM. 1999. The dual function of sugar carriers: transport and sugar sensing. *The Plant Cell* **11,** 707-728.

Lefebre R, Vasseur J, Sackoula E, Coullerot JP. 1992. Participation of carbohydrate metabolism in the organogenic orientation of Chichorium intybus tissues cultivated in vitro. *Canadian Journal of Botany* **70,** 1897-1902.

Li J, Chory J. 1999. Brassinosteroid actions in plants. *Journal of Experimental Botany* **50,** 275-282.

Li ZS, Noubhani AM, Bourbouloux A, Delrot S. 1994. Affinity purification of sucrose binding proteins from the plant plasma membrane. *Biochimica Biophysics Acta* **1219,** 389-397.

Linden JC, Ehneß R, Roitsch T. 1996. Regulation by ethylene of apoplastic invertase expression in *Chenopodium rubrum* tissue culture cells. *Plant Growth Regulation* **19,** 219-222.

Link VL, Hofmann MG, Sinha AK, Ehness R, Strnad M, Roitsch T. 2002. Biochemical Evidence for the activation of distinct subsets of mitogen activated protein kinases by voltage and defense related stimuli. *Plant Physiology* **128**, 271-281.

Long JC, Zhao W, Rashotte AM, Muday GK, Huber SC. 2002. Gravity stimulated changes in auxin and invertase gene expression in maize pulvinal cells. Plant Physiology **128**, 591-602.

Lorenz K, Lienhard S, Sturm A. 1995. Structural organisation and differential expression of carrot β-fructofuranosidase genes: identification of a gene coding for a flower bud-specific isoenzyme. *Plant Molecular Biology* **28**, 189-194.

Loreti E, Amedo A, Perata P. 2000. Glucose and disaccharide mechanisms modulate the expression of a-amylase in barley embryos. *Plant Physiology* **123,** 939-948.

M´Batchi B, Delrot S. 1988. Stimulation of sugar exit from leaf tissues of *vicia faba* L. *Planta* **174,** 340-348.

Maddison AL, Hedeley PE, Meyer RC, Aziz N, Davidson D, Machray GC. 1999. Expression of tandem invertase genes associated with sexual and vegetative growth cycles in potato. *Plant Molecular Biolology* **41**, 741-751.

Memelink J, Hoge JHC, Schilperoort RA. 1987. Cytokinin stress changes the developmental regulation of several defence-related genes in tobacco. *EMBO Journal* **6,** 3579-3583.

Miller EM, Chourey PS. 1992. The maize invertase-deficient *miniature-1* seed mutation is associated with aberrant pedicel and endosperm development. *The Plant Cell* **4,** 297-305.

Morris DA, Arthur ED. 1984. Invertase and auxin-induced elongation in internodal segments of *Phaseolus vulgaris. Phytochemistry* **23,** 2163-2167.

Morris DA, Arthur ED. 1985. Effects of gibberellic acid on patterns of carbohydrate distribution and acid invertase activity in *Phaseolus vulgaris. Physiologia Plantarum* **65,** 257-262.

Morris DA, Arthur ED. 1986. Stimulation of acid invertase activity by indolyl -3-acetic acid in undergoing cell expansion. *Plant Growth Regulation* **4**, 259-271.

Munns R, Termaat A. 1986. Whole-plant responses to salinity. *Australian Journal of Plant Physiology* **13,** 143-160.

Müssig C, Altmann T. 1999. Physiology and molecular mode of action of brassinosteroids. *Plant Physiology* **37,** 363-372.

Nam HG. 1997. The molecular genetic analysis of leaf senescence. *Current Opinion in Biotechnology* **8,** 200-207.

Palmer JM. 1966. The influence of growth regulating substances on the development of enhanced metabolic rates in thin slices of betroot storage tissue. *Plant Physiology* **41,** 1173-1178.

Pelleschi S, Guy S, Kim JY, Pointe C, Mahe A, Barthes L, Leonardi A, Prioul JL. 1999. IVR2, a candidate gene for a QTL of vacuolar invertase activity in maize leaves. Gene-specific expression under water stress. *Plant Molecular Biology* **39,** 373-380.

Pelleschi S, Rocher JP, Prioul JL. 1997. Effect of water restriction on carbohydrate metabolism and photosynthesis in mature maize leaves. *Plant, Cell and Environment* **20,** 493-503.

Poljakoff-Mayber A, Lerner HR. 1994. Plants in saline environments. In: Pessarakli M, ed. *Handbook of plant and crop stress*, New York, Marcel Dekker Inc, 65-96.

Ranwala AP, Miller WB. 1998. Sucrose-cleaving enzymes and carbohydrate pools in *Lilium longiflorum* floral organs. *Physiologica Plantarum* **103**, 541-550.

Richings EW, Cripps RF, Cowan AK. 2000. Factors affecting "Has" avocado fruit size: Carbohydrate, abscisic acid and isoprenoid metabolism in normal and phenotypically small fruit. Physiologia Plantarum **109**, 81-89.

Roitsch T. 1999. Source-sink regulation by sugars and stress. *Current Opinion in Plant Biology* **2,** 198-206.

Roitsch T, Bittner M, Godt DE. 1995. Induction of apoplastic invertase of *Chenopodium rubrum* by D-glucose and a glucose analogue and tissue specific expression suggest a role in sink source regulation. *Plant Physiology* **108,** 285-294.

Roitsch T, Ehneß R. 2000. Regulation of source/sink relations by cytokinins. *Plant Growth Regulation* **32,** 359-367.

Roitsch T, Ehneß R, Goetz M, Hause B, Hofmann M, Sinha AK. 2000. Regulation and function of extracellular invertase from higher plants in relation to assimilate partitioning, stress responses and sugar signalling. *Australian Journal of Plant Physiology* **27,** 815-825.

Ruffner HP, Geissmann M, Rast DM. 1992. Plant and fungal invertases in grape berries infected with botrytis-cinerea. *Physiological and Molecular Plant Pathology* **40,** 181-189.

Sacher JA, Hatch MD, Glasziou KT. 1963. Regulation of invertase synthesis in sugar cane by auxin and sugar-mediated control system. *Physiology Plantarum* **16,** 836-842.

Seitz K, Lang A. 1968. Invertase activity and cell growth in lentil epicotyls. *Plant Physiology* **43,** 1075-1082.

Sheen J, Zhou L, Jang JC. 1999. Sugars as signaling molecules. *Current Opinion in Plant Biology* **2,** 410-418.

Shinshi H, Mohnen D, Meins F. 1987. Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. *Proceedings of the National Academy of Sciences of USA* **84**, 89-93.

Singh S, Letham DS, Palni LMS. 1992. Cytokinin biochemistry in relation to leaf senescence. *Physiologia Plantarum* **86,** 388-406.

Sinha AK, Römer U, Köckenberger W, Hofmann M, Elling L, Roitsch T. 2002. Metabolizable and non-metabolizable sugars activate different signal transduction pathways in tomato. *Plant Physiology* **128,** 1480-1489.

Stitt M. 1991. Rising CO_2 levels and their potential significance for carbon flow in photosynthetic cells. *Plant, Cell and Environment* **14,** 741-762.

Stratmann JW, Ryan CA. 1997. Myelin basic protein kinase activity in tomato leaves is induced systemically by wounding and increases in response to systemin and oligosaccharide elicitors. *Proceedings of the National Academy of Sciences of USA* **94,** 11085-11089.

Sturm A. 1999. Invertases. Primary structures, functions, and roles in plant development and sucrose partitioning. *Plant Physiology* **121,** 1-7.

Sturm A, Chrispeels MJ. 1990. cDNA cloning of carrot extracellular ß-fructosidase and its expression in response to wounding and bacterial infection. *The Plant Cell* **2,** 1107-1119.

Sturm A, Sebkova V, Lorenz K, Hardegger M, Lienhard S, Unger C. 1995. Development and organ-specific expression of the genes for sucrose synthase and three isoenzymes of acid β-fructofuranosidase in carrot. *Planta* **195,** 601-610.

Tanner W. 1980. On the possible role of ABA on phloem unloading. *Berichte der deutschen Botanischen Gesellschaft* **93,** 349-351.

Tang GQ, Lüscher M, Sturm A. (1999) Antisense repression and vacuolar and cell wall invertase in transgenic carrot alters early plant development and sucrose partitioning. The *Plant Cell* **11**, 1-14

Thom M, Maretzki A. 1992. Evidence of direct uptake of sucrose by sugarcane stalk tissue. *Journal of Plant Physiology* **139,** 555-559

Truernit E, Stadler R, Baier K, Sauer N. 1999. A male gametophyt-specific monosaccharide transporter in *Arabidopsis. The Plant Journal* **17**, 191-201.

Tymowska-Lalanne Z, Kreis M. 1998a. The Plant Invertases: Physiology, Biochemistry and Molecular Biology. *Advances in Botanical Research* **28,** 71-117.

Tymowska-Lalanne Z, Kreis M. 1998b. Expression of the *Arabidopsis thaliana* invertase gene family. *Planta* **207,** 259-265.

Van der Werf A. 1996. Growth analysis and photoassimilate partitioning. In: Zamski E, Schaffer AA, eds. *Photoassimilate distribution in plants and crops. Source-Sink Relationships*, New York, Marcel Dekker Inc, 1-20.

Vartanian N. 1996. The drought rhizogenesis. In: Waisel Y, Eshel A, Kafkafi U, eds*. Plant roots, the hidden half*, New York, Marcel Dekker Inc, 471-482.

von Schaewen A, Stitt M, Schmidt R, Sonnewald U, Willmitzer L. 1990. Expression of yeast-derived invertase in the cell wall of tobacco and Arabidopsis plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly growth influences growth and phenotype of transgenic tobacco plants. *EMBO Journal* **9,** 3033-3044.

Wang HL, Lee PD, Chen WL, Huang DJ, Su JC. 2000. Osmotic stress-induced changes of sucrose metabolism in cultured sweet potato cells. *Journal of Experimental Botany* **51,** 1991-1999.

Wardlaw IF. 1990. The control of carbon partitioning in plants. *New Phytologist* **116,** 341-381.

Weber H, Borisjuk L, Wobus U. 1996. Controlling seed development and seed size in *Vicia faba*: a role for seed coat-associated invertases and carbohydrate state. *The Plant Journal* **10**, 823-834.

Weil M, Rausch T. 1990. Cell wall invertase in tobacco crown gall cells. *Plant Physiology* **94,** 1575-1581.

Wu LL, Mitchel JP, Cohn NS, Kaufman PB. 1993a. Gibberellin (GA_3) enhaces cell wall invertase activity and mRNA levels in elongating dwarf pea (*Pisum sativum*) shoots. *International Journal of Plant Sciences* **154,** 280-289.

Wu LL, Song I, Kim D, Kaufmann PB. 1993b. Molecular basis of the increase in invertase activity elicited by gravistimulation of oat-shoot pulvini. *Journal of Plant Physiology* **142**, 179-183.

Xu J, Avigne WT, McCarty DR, Koch KE. 1996. A similar dichotomy of sugar modulation and developmental expression affects both paths of sucrose metabolism: evidence from maize invertase gene family. *The Plant Cell* **8**, 1209-1220.

Ylstra B, Garrido D, Busscher J, Van-Tunen AJ. 1998. Hexose transport in growing Petunia pollen tubes and characterization of a pollen-specific, putative monosaccharide transporter. *Plant Physiology* **118,** 297-304.

Zeng Y, Wu Y, Avigne WT, Koch KE. 1999. Rapid repression of maize invertases by low oxygen. Invertase/sucrose synthase balance, sugar signaling potential, and seedling survival. *Plant Physiology* **121,** 599-608.

Zhang S, Du H, Klessig DF. 1998. Activation of the tobacco SIP kinase by both a cell wall-derived carbohydrate elicitor and purified proteinaceous elicitins from *Phytophthora spp. The Plant Cell* **10,** 435-450.

Zhang S, Klessig DF. 1997. Salicylic acid activates a 48-kD MAP kinase in tobacco. *The Plant Cell* **9,** 809-824.

Zhu JK. 2001. Cell signalling under salt, water and cold stresses. *Current Opinion in Plant Biology* **4,** 401-406.

Zinselmeier C, Schussler JR, Jone RJ, Westgate ME. 1995. Low water potential disrupts carbohydrate metabolism (*Zea may* L.) ovaries. *Plant Physiology* **107,** 385-391.

8. GENERALISED DISCUSSION AND PERSPECTIVES

Assimilate partitioning in higher plants is a complex and strictly regulated process that involves a co-ordinated regulation of specific enzyme functions. One critical step is phloem unloading, a process that is mediated either symplastically or via the apoplast. Apoplastic phloem unloading via sucrose cleavage mediated by extracellular invertase turned out to play a central role in tissues of active growth, embryo tissues, the male gametophyte, and in modulating defence responses (Sturm and Tang, 1999; Roitsch et al., 2003). The central position of extracellular invertase in these processes is reflected by complex regulation mechanisms controlling invertase expression and function. In the last decade numerous invertase isoenzymes of different species have been cloned and characterised. It has been shown that invertases are organised in small gene families comprising isoenzymes with specific expression and regulation patterns (Tymowska-Lalanne and Kreis, 1998). However, the physiological roles of individual members of the invertase gene family within one species are rarely established. The present thesis describes a detailed analysis of Lin5, Lin6, and Lin7 extracellular invertase isoenzymes of tomato and provides data on their physiological functions. Interestingly, all three isogenes are expressed in floral tissues, thus indicating a crucial function of apoplastic sucrose cleavage in carbohydrate supply of floral organs. In addition, the cloned invertase promoters were successfully used in transgenic approaches and harbour high potentials in applied biotechnology.

8.1 *Tomato extracellular invertases are organised in a gene family comprising several isoenzymes with tissue- and developmental-specific expression patterns*

The organisation of invertase genes in small gene families and the complex regulation of their expression confer flexibility to plants in regulating the carbohydrate metabolism. In tomato four partial sequences of extracellular invertases have been cloned (Godt and Roitsch, 1997) and primary investigations on steady state mRNA levels were performed. Thereby, two flower-specific isoenzymes (Lin5, Lin7) and an invertase, which is regulated by a variety of different stimuli (Lin6), appeared to be of particular interest for further detailed analysis. A first step in understanding the functions of different isoenzymes of extracellular invertases is the dissection of the spatial and temporal patterns of gene expression. Therefore, the promoters of *Lin5*, *Lin6*, and *Lin7* invertase genes were cloned and reporter gene fusions designed for plant transformation. As detailed in Chapters 2, 3, and 5 those promoters turned out to be regulated in a developmental- and tissue-specific manner and are responsive to different stimuli, such as phytohormones, sugars, and stress

stimuli. Following the basal characterisation, the promoters of *Lin7* and *Lin6* invertase genes were used in functional approaches (Chapters 3 and 6) to elucidate physiological roles of invertases in pollen germination and cytokinin-mediated delay of senescence.

8.1.1 *Specific function of Lin5, Lin6, and Lin7 extracellular invertases in providing carbohydrates to floral organs*

One striking feature is the presence of several flower-specific isoenzymes within the invertase gene family of different plant species (documented in General Introduction). Moreover, monosaccharide transporters that are specifically expressed in the male gametophyte have been described (Truernit et al., 1999; Scholz-Starke et al., 2003; Schneidereit et al., 2003). Thus, an essential role of apoplastic sucrose cleavage in carbohydrate supply of floral organs, especially the male gametophyte, has been supposed.

Data presented in Chapters 2, 3, and 5 address the role of extracellular invertases in providing carbohydrates to the male gametophyte. *In-situ* hybridisation and GUS reporter gene assays revealed high *Lin7* and *Lin6* promoter activity in pollen. *Lin6* expression in pollen turned out to be regulated by methyl jasmonate, thereby providing a link of *Lin6* expression with known jasmonate-mediated effects on pollen development. A block in JA biosynthesis results in male sterile *Arabidopsis* plants due to defects in anther and pollen development as demonstrated by Park et al. (2002). A functional approach was performed to analyse the role of Lin7 in growth processes of the male gametophyte. Pollen germination efficiency of pollen carrying the *Lin7*-RNAi silencing construct under the control of the *Lin7* promoter was significantly reduced, thus, corroborating an essential role of apoplastic sucrose cleavage in pollen tube formation. This observation is in line with the identification of pollen-specific monosaccharide transporters in *Arabidopsis thaliana* (Truernit et al., 1999; Scholz-Starke et al., 2003; Schneidereit et al., 2003) and underlines the high complexity of carbohydrate supply to the male gametophyte. The strong pollen-specific GUS expression mediated by the *Lin7* promoter declined upon treatment with a GA-synthesis inhibitor, indicating a link between GA-mediated growth processes of reproductive organs with invertase activity. For the first time we could substantiate a correlation between the GA effect on pollen tube formation and a specific enzyme function downstream of the GA signalling pathway.

In contrast to Lin6 and Lin7, the flower-specific Lin5 invertase shows highest steady state mRNA levels in the gynoecia and fruit, a minor expression level was detected in stamen (Godt and Roitsch, 1997). As presented in Chapter 2, the *Lin5* promoter is regulated during fruit development and displays higher expression levels in early developmental stages. Two out of three tomato hexose transporters, which are functionally linked to

invertases, are predominantly expressed in young fruits (Gear et al., 2000). A fragment of the *Lin5* promoter has been demonstrated to confer GA and auxin inducibility to the *nos* minimal promoter. Both phytohormones are known to play important roles in regulating growth processes and differentiation of floral tissues (Berleth and Sachs, 2001; Richards et al., 2001). Therefore, *Lin5* induction by auxin and GA could mediate a higher carbohydrate supply of developing flower tissue, especially in early developmental stages. This idea is supported by the temporal expression pattern of GA biosynthesis genes (Rebers et al., 1999), which matches *Lin5* data, giving a further indication of a close *Lin5* induction by GA during flower and fruit development. An important role of Lin5 in fruit sugar composition was suggested by Fridman et al. (2000; 2002) based on a QTL, which was delimited within the *Lin5* gene and modifies the sugar content of fruits. Several alterations in *Lin5* sequences of the analysed tomato lines *Lycopersicon pennellii* and *Lycopersicon esculentum* were found. This includes a deletion and a different number of repeat sequences both localised in the third intron. That *Lin5* introns are prone to structural modification is further substantiated by the detection of a CACTA-like transposon insertion in intron I of the *Lin5* gene as detailed in Chapter 4. The obtained sequence made it possible to demonstrate the ubiquitous appearance of CACTA-like elements throughout Solanaceae species. The impact of the CACTA-like transposon insertion in *Lin5* intron I on gene expression or function has not been addressed so far. As TEs contribute to genomic plasticity in response to environmental conditions (documented in General Introduction), it could be speculated that the transposon insertions results in an alteration of isoenzyme functions within the tomato extracellular invertase gene family. Interestingly, Yau and Simon (2003) describe a 2.5 kb insert, probably derived from a mobile genetic element, in intron I of a carrot soluble invertase isoenzyme. The observed elimination of invertase transcription and high sucrose accumulation in corresponding tissues might be caused by the DNA insertion thereby contributing to a phenotypic variation (Yau and Simon, 2003).

Performing sequential genome walks, it was found that *Lin5* and *Lin7* are organised in a direct tandem repeat on the genome with *Lin5* being located in 5´ position separated by 1.5 kb from the 3´ located *Lin7* gene (Chapter 2). As *Lin5* and *Lin7* represent the two flower-specific isogenes of tomato extracellular invertases it was assumed that the tandem structure originated from a gene duplication. Gene duplication followed by divergence in the regulatory regions is a potent source of genes with novel functions (Ohno, 1970). Because of the broader expression pattern of *Lin5* compared to the strict tapetum and pollen specificity of *Lin7*, one could speculate that due to this gene duplication the specific function of carbohydrate supply of pollen was shifted to a further isoenzyme, Lin7. The feature of a tandem organisation of two extracellular invertases with a broader expressed isoenzyme in

5´ position and a strictly anther- and pollen-specific isoenzyme in 3´ position is conserved in potato (Maddison et al., 1999; Fridman and Zamir, 2003).

In summary, the flower/fruit-specific expression of *Lin5* and *Lin7*, alongside *Lin6* expression in pollen and corresponding regulatory mechanisms support an essential function of extracellular invertases in growth and development of floral organs. One specific function of Lin7, the GA-mediated formation of pollen tubes, has been addressed in a transgenic approach. Data presented in Chapters 2, 3, and 5 are the most profound analysis of invertase function in the delicate process of floral organ development. The expression and regulation patterns of three invertase isoenzymes of tomato have been detailed. In particular, dissection of the individual roles of flower-specific invertase isoenzymes and corresponding hexose transporters provides new possibilities in understanding regulatory mechanisms in flower development. As Lin5 invertase is assumed to have an impact in crop yield (Fridman et al., 2000; 2002), the understanding of Lin5 function in fruit development could contribute to agricultural benefits. A particular goal would be to manipulate competition between different sinks. The flower- and fruit-specific *Lin5* promoter offers the opportunity to modulate crop yield and sugar composition in fruits. Furthermore, a clear understanding of carbohydrate supply of the male gametophyte is of great interest. Therefore, the question should be addressed whether individual invertase/monosaccharide transporter pairs exist and what regulatory mechanisms co-ordinate their expression. In addition, single or multiple gene silencing, including *Lin5* and *Lin6*, is a promising approach to unravel the complexity of carbohydrate supply of the male gametophyte in tomato. In doing so, a detailed understanding of this process will open up new possibilities in plant biotechnology with regard to establishing methods for pollination control via modifying the carbohydrate supply of the male gametophyte.

8.1.2 *Lin6 extracellular invertase, which is regulated by a variety of stimuli, could serve as integration point of metabolic, hormonal, light, and stress signals*

The activity of invertases can be modulated by organ- and developmental-specific gene expression and by various internal and external stimuli (Roitsch et al., 2003). It has been shown that biotic and abiotic stress factors, plant hormones, sugars, and light regulate invertase expression (for review see: Tymowska-Lalanne and Kreis, 1998; Roitsch et al., 2003). However, analysis of individual invertase isoenzymes was mainly restricted to a small section of possible regulators. To get an insight in the multitude of regulatory mechanisms acting on invertase gene expression, the Lin6 tomato invertase was chosen as model system. As detailed in Chapter 5, *Lin6* turned out to be developmentally regulated. Interestingly, the *Lin6* promoter is not active in fully developed plants, except in pollen tissue.

A strong induction of the *Lin6* promoter in mechanically wounded leaves was shown to be localised in a small region adjacent to the applied wound stimulus. In addition, it was substantiated that *Lin6* is an inducible compound of the defence/stress response pathway. To address the more subtle regulation of *Lin6* by phytohormones and sugars, *Lin6*::GUS transformed tobacco suspension cultures were established. In this system an up-regulation of *Lin6* promoter activity by cytokinin, auxin, jasmonate, abscisic acid, and sugars has been shown. Furthermore, a diurnal regulation of *Lin6* and a functional interaction of circadian oscillator elements of *A. thaliana*, CCA1 and LHY, with the *Lin6* promoter have been demonstrated. The *Lin6* expression pattern fits in a model of circadian regulated cell elongation (Harmer et al., 2000) and supposes a function of Lin6 in covering the higher carbohydrate demand during reinforcement of the cell wall during the cell elongation process.

Lin6 induction by growth stimulating hormones, sugars, and stress stimuli emphasises the central role of extracellular invertase as a key metabolic enzyme and a modulator of defence responses. In particular, increased *Lin6* levels could satisfy the enhanced carbohydrate demand of growth-stimulated tissues and modulate defence responses via a sugar-mediated feed forward circuit. The complex regulation pattern of *Lin6* supports the idea of extracellular invertases as integration points of various stimuli resulting in a co-ordinated cellular response to changing internal and external conditions (Roitsch et al., 2003). The *Lin6* promoter, as it is regulated by a variety of stimuli, is an ideal model system for dissecting signal cross-talk and integration in plant gene expression. The identification of many well characterised *cis*-acting elements that are organised in three clusters on the *Lin6* promoter sequence in different, sometimes overlapping, constellations give reason to assume that a concert of transcription factors acts on the *Lin6* promoter to confer the observed regulation pattern. A challenging task is to screen for transcription factors operating on the *Lin6* promoter and to analyse their potential interaction and regulation profiles. This will provide a rational basis for the final, although far reaching goal to determine the molecular properties of hormone and stress response pathways involved in invertase regulation. Getting a link to stress induced MAP kinases that have been profoundly characterised in the group of Prof. Roitsch (Link et al., 2002a; Link et al., 2002b; Sinha et al., 2002) could help to get a comprehensive insight of stress response output pathways.

8.2 *Tissue-specific and hormone-inducible promoters as tools in molecular biology*

The promoter is the key *cis*-acting regulatory region that controls the transcription of adjacent coding regions into RNA. Promoters show a modular structure and contain a number of *cis*-acting elements, each of which may contribute to one or more determinants of the expression profile. Transcription is performed by RNA polymerase II, which works in

conjunction with other transcription factors that recognise sequence elements embodied in the promoter region. Thereby, a distinct set of transcription factors bind to these sites to give rise to higher-order complexes that have been called enhanceosomes (for review see: Carey, 1998). Specific interactions between proteins that form an enhanceosome can lead to cooperativity in DNA binding and transcriptional synergy (as shown for CCA1 and LHY transcriptions factors in Chapter 5). These multiple protein-protein and protein-DNA interactions will determine transcriptional activity of a given promoter under a specific set of conditions (Singh, 1998).

There are three types of promoters used in biotechnology, constitutive, tissue-specific, and inducible promoters. Whereas constitutive promoters act independent of environmental and developmental factors, the latter two control gene expression in a specific tissue type or at certain stage of development and in response to chemical or physical stimuli (Padidam, 2003; Tang et al., 2004). Non-constitutive promoters have the advantage that transgene expression is restricted to specific tissues or certain inducing conditions leaving the remaining plant tissues unmodified. In addition, there is a new generation of promoters emerging, synthetic promoters, which have been engineered to comprise consensus sequences from common elements of natural promoters. One example are pathogen-inducible synthetic promoters. Those promoters are of big interest for research and harbour great potentials in plant biotechnology due to their well-defined properties (Rushton et al., 2002).

Cloning of the promoter sequences of plant invertases Lin5, Lin6, and Lin7 allowed a detailed analysis of the expression and regulation patterns of three individual members of a tomato invertase gene family. In doing so, two flower-specific promoters (*Lin5*/*Lin7*) and a promoter, which is regulated by a variety of stimuli (*Lin6*) have been characterised in transgenic approaches as detailed below.

8.2.1 *The tissue-specific Lin7 promoter serves as molecular tool to modulate transgene expression in pollen*

The unique property of the *Lin7* promoter to confer high expression rates specifically restricted to pollen tissue was utilised to manipulate gene expression in transgenic plants. Hereby the idea of expressing a *Lin7*-RNAi silencing construct and *GA2ox2* cDNA under the control of the *Lin7* promoter offered the possibility to restrict transgene expression in a narrow developmental- and tissue-specific manner (Chapter 3). This turned out to be of big advantage, as pleiotrophic effects, observed in studies using the TMV 35S promoter (Tang et al., 1999; Singh et al., 2002) were circumvented thereby sharpening the observed phenotypes. The use of specific promoters is of great interest in molecular biology as it offers

the possibility to modify expression profiles in a subtle manner. The *Lin7* promoter, as it confers pollen-specific expression (a pattern that is conserved in *A. thaliana*: Proels R, Roitsch T, unpublished observation), is of particular interest for plant biotechnology. Defining *cis*-acting elements for pollen-specific expression and designing synthetic promoters could help to develop corresponding expression systems. Tissue-specific or synthetic promoters are valuable tools for elucidating gene functions, in addition, they are increasingly relevant for the improvement of crops by genetic engineering. In particular, the *Lin7* promoter could be beneficial in designing pollination control systems, which are essential for hybrid seed production. High-level transgene expression in pollen mediated by *Lin7* promoter sequences could be a feasible method to engineer male sterile plants.

8.2.2 *The multiple regulated Lin6 promoter was successfully used as an inducible expression system*

Besides tissue specificity, there is a further property that makes promoters interesting molecular tools. Promoter sequences have been shown to mediate gene expression in response to a huge variety of stimuli. This feature was used to generate inducible expression systems for chemical inducers, phytohormones, and wound-induction. Moreover, a combination of well-defined *cis*-acting elements serves as pool to design synthetic promoters with unique properties as detailed above.

The potency of the *Lin6* promoter to be used as an inducible expression system in plants was established in a study focused on the role of extracellular invertase in cytokinin-mediated delay of senescence (Chapter 6). To address this question on a functional basis, the cytokinin-responsive *Lin6* promoter served as an inducible system for tobacco apoplasmic invertase inhibitor expression. In transgenic tobacco lines expressing the invertase inhibitor under control of the *Lin6* promoter the delay of senescence phenotype is no longer observable beside the presence of cytokinin. Therefore, this expression system based on the *Lin6* promoter was successfully used to show a causal relationship between cytokinin and extracellular invertase for the delay of senescence. This study emphasises the importance of inducible expression systems to specifically address questions on a molecular level. In particular, *Lin6* promoter sequences could be used to generate smart reporter systems for cytokinin and brassinosteroid, or as sink-marker. Furthermore, the multitude of *cis*-acting elements that is present in the *Lin6* promoter provides the basis for dissecting signal integration mechanisms in plant gene expression.

8.3 References

Berleth T and Sachs T (2001) Plant morphogenesis: long-distance coordination and local patterning. Curr Opin Plant Biol 4: 57-62

Carey M (1998) The enhanceosome and transcriptional synergy. Cell 92: 5-8

Fridman E, Pleban T and Zamir D (2000) A recombination hotspot delimits a wild-species quantitative trait locus for tomato sugar content to 484 bp within an invertase gene. Proc Natl Acad Sci USA 97: 4718-4723

Fridman E, Liu YS, Carmel-Goren L, Gur A, Shoresh M, Pleban T, Eshed Y and Zamir D (2002) Two tightly linked QTLs modify tomato sugar content via different physiological pathways. Mol Genet Genomics 266: 821-826

Fridman E and Zamir D (2003) Functional divergence of a syntenic invertase gene family in tomato, potato, and *Arabidopsis*. Plant Physiol 131: 603-609

Gear ML, McPhillips ML, Patrick JW and McCurdy DW (2000) Hexose transporters of tomato: molecular cloning, expression analysis and functional characterisation. Plant Mol Biol 44: 687-697

Godt DE and Roitsch T (1997) Regulation and Tissue-Specific Distribution of mRNA for Three Extracellular Invertase Isoenzymes of Tomato Suggests an Important Function in Establishing and Maintaining Sink Metabolism. Plant Physiol 115: 273-282

Harmer SL, Hogenesch JB, Straume M, Chang H-S, Han B, Zhu T, Wang X, Kreps JA and Kay SA (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science 290: 2110-2113

Link VL, Hofmann MG, Sinha AK, Ehness R, Strnad M and Roitsch T (2002a) Biochemical evidence for the activation of distinct subsets of mitogen-activated protein kinases by voltage and defence-related stimuli. Plant Physiol 128: 271-281

Link L, Sinha AK, Vashista P, Hofmann MG, Proels RK, Ehness R and Roitsch T (2002b) A heat-activated MAP kinase in tomato: a possible regulator of the heat stress response. FEBS Letters 531: 179-183

Maddison AL, Hedeley PE, Meyer RC, Aziz N, Davidson D and Machray GC (1999) Expression of tandem invertase genes associated with sexual and vegetative growth cycles in potato. Plant Mol Biol 41: 741-751

Ohno S (1970) Evolution by gene duplication. Springer Verlag, Berlin

Padidam M (2003) Chemically regulated gene expression in plants. Curr Opin Plant Biol 6: 169-177

Park J-H, Halitschke R, Kim HB, Baldwin IT, Feldmann KA and Feyereisen R (2002) A knock out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in *Arabidopsis* due to a block in jasmonic acid biosynthesis. Plant J 31: 1-12

Rebers M, Kaneta T, Kawaide H, Yamaguchi S, Yang Y-Y, Imai R, Sekimoto H and Kamiya Y (1999) Regulation of gibberellin biosynthesis genes during flower and early fruit development of tomato. Plant J 17: 341-250

Richards DE, King KE, Ait-ali T and Harberd NP (2001) How Gibberellin regulates plant growth and development: A molecular genetic analysis of Gibberellin signaling. Annu Rev Plant Physiol Plant Mol Biol 52: 67-88

Roitsch T, Balibrea ME, Hofmann M, Proels R and Sinha AK (2003) Extracellular invertase: key metabolic enzyme and PR protein. J Exp Bot 54: 513-524

Rushton PJ, Reinstädler A, Lipka V, Lippok B and Somssich IE (2002) Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen- and wound-induced signaling. Plant Cell 14: 749-762

Schneidereit A, Scholz-Starke J and Büttner M (2003) Functional characterisation and expression analyzes of the glucose-specific AtSTP9 monosaccharide transporter in pollen of *Arabidopsis*. Plant Physiol 133: 182-190

Scholz-Starke J, Büttner M and Sauer N (2003) AtSTP6, a new pollen-specific H^+-monosaccharide symporter from *Arabidopsis*. Plant Physiol 131: 70-77

Singh KB (1998) Transcriptional regulation in plants: The importance of combinatorial control. Plant Physiol 118: 1111-1120

Singh DP, Jermakow AM and Swain SM (2002) Gibberellins are required for seed development and pollen tube growth in Arabidopsis. Plant Cell 14: 3133-3147

Sinha AK, Hofmann MG, Römer U, Köckenberger W, Elling L and Roitsch T (2002) Metabolizable and non-metabolizable sugars activate different signal transduction pathways in tomato. Plant Physiol 128: 1480-1489

Sturm A and Tang G-Q (1999) The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. Trends in Plant Science 4: 401-407

Tang G-Q, Lüscher M and Sturm A (1999) Antisense repression of vacuolar and cell wall invertase in transgenic carrot alters early plant development and sucrose partitioning. Plant Cell 11: 117-189

Tang W, Luo X and Samuels V (2004) Regulated gene expression with promoters responding to inducers. Plant Sci 166: 827-834

Truernit E, Stadler R, Baier K and Sauer N (1999) A male gametophyte-specific monosaccharide transporter in *Arabidopsis*. Plant J 17: 191-201

Tymowska-Lalanne Z and Kreis M (1998) The plant invertases: Physiology, biochemistry and molecular biology. Adv Bot Res 28: 71-117

Yau YY and Simon PW (2003) A 2.5 kb insert eliminates acid soluble invertase isoenzyme II transcript in carrot (*Daucus carota* L.)roots, causing high sucrose accumulation. Plant Mol Biol 53: 151-162

Publications

Link V, Sinha A, Vashista P, Hofmann M, Proels R, Ehness R and Roitsch T (2002) A heat activated MAP kinase in tomato: A possible regulator of the heat stress response. FEBS Lett 531: 179-183.*

Proels RK, Hause B, Berger S and Roitsch T (2003) Novel mode of hormone induction of tandem tomato invertase genes in floral tissues. Plant Mol Biol 52: 191-201.

Roitsch T, Balibrea ME, Hofmann M, Proels R and Sinha AK (2003) Extracellular invertase: key metabolic enzyme and PR protein. J Exp Bot 54: 513-524.

Balibrea Lara ME, Gonzalez Garcia MC, Fatima T, Ehness R, Lee TK, Proels R, Tanner W and Roitsch T (2004) Extracellular invertase is an essential component of cytokinin-mediated delay of senescence. Plant Cell 16: 1276-1287.

Proels RK and Roitsch T. Regulation of source/sink relations by extracellular invertase *Lin6* of tomato: a pivotal enzyme for integration of metabolic, hormonal, and stress signals is regulated by a diurnal rhythm. (submitted to Planta)

Proels RK and Roitsch T. GA-regulated invertase *Lin7* plays an essential role in pollen germination. (submitted to Functional Plant Biology)

Proels RK and Roitsch T. Cloning of a CACTA-like transposon insertion in intron I of tomato invertase *Lin5* gene and identification of transposase-like sequences of Solanaceae species. (submitted to Journal of Plant Physiology)

Hofmann MG, Sinha AK, Proels RK and Roitsch T. Phosphorylation of WRKY transcription factors: Transient activation of two protein kinases during the elicitation of defence responses in tomato. (in preparation) *

Publications marked with an asterisk are not part of this thesis

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Erklärung

gemäß § 4 Abs. 3 Ziff 3, 5 und 8

der Promotionsordnung der Fakultät für Biologie der

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Hiermit erkläre ich ehrenwörtlich, die vorliegende Arbeit in allen Teilen selbständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt zu haben.

Diese Dissertation hat weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen.

Des weiteren erkläre ich, dass ich früher weder akademische Grade erworben habe, noch zu erwerben versucht habe.

Würzburg, den