Extracellular invertase: key metabolic enzyme and PR protein

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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 synthesized by higher plants, such as turanose, were shown to elicit responses distinctly different from metabolizable sugars and are rather perceived as stress-related stimuli.

Key words: Extracellular invertase, MAP kinase, plant–pathogen interaction, phytohormones, salt stress, source/sink regulation, sugar sensing.

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-metabolizing 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 probably critical links between photosynthetic 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 apoplasm 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 apoplastic space surrounding all sink cells provides the possibility to allow very high uptake rates if required.

Extracellular invertase is particularly suited as a key regulator of apoplastic phloem unloading due to its enzymological properties. Whereas the $K_m$ value of hexose transporters are in the $\mu$M range, the $K_m$ value of extracellular invertases are in the mM range and thus are limiting unloading. In addition, extracellular invertase catalyses the only irreversible step of the apoplastic 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 with respect to 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 (Lorentz et al., 1995), Arabidopsis thaliana (Tymowska-Lalanne and Kreis, 1998b), Vicia faba (Weber et al., 1996), Lilium longiflorum (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 (Godb 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 apoplastic pathway involving cleavage of sucrose by invertase is further supported by the identification of anther-specific hexose transporters. In Arabidopsis a monosaccharide transporter was characterized that is expressed in developing pollen after the onset of symplasmic isolation of the microspore (Truernt et al., 1990). Likewise, monosaccharide transporters specifically expressed in anthers have been demonstrated in Petunia (Ylstra et al., 1998) and tobacco (M Goetz, 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 also likely to affect sugar sensing and signalling, which is supported by in vitro rescue experiments (M Goetz, 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 a biological safety method to avoid the 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 generalized 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 floral reproductive organs other than the male gametophyte has been performed. Different expression patterns for extracellular invertases in ovaries (Kim et al., 2000b) and gynoecium (Godt and Roitsch, 1997) are reported. Specific antisense approaches could help to clarify the function of extracellular invertases in those floral organs, especially with 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 apoplastic 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 in elucidating 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 relationship 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 (GA3) 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 epicotyls (Seitz and Lang, 1968), and internodes of bean (Phaseolus vulgaris; Morris and Arthur, 1985) and oat (Kaufman et al., 1973). Invertase mRNA from shoots of dwarf pea plants (Pisum sativum) was induced after GA3 treatment, indicating that the expression of the pea shoot cell-wall invertase gene could be regulated by GA3 at transcriptional and/or translational levels (Wu et al., 1993a). In suspension-cultured tomato cells (Lycopersicon esculentum L.) the addition of GA3 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 the 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 Ehneb, 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 (Ehneb 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). Cytokinin expressions are also considered as key components of plant senescence (Singh et al., 1992; Gan and Asamasino, 1996, 1997; Buchanan-Wollaston, 1997; Nam, 1997), based on the ability to delay senescence by the 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 localized induction of sink metabolism (Roitsch and
A relationship between extracellular invertase and cytokinin-mediated growth responses is supported by a transgenic tobacco line (BIK62) expressing the *ipt* cytokinin biosynthetic gene under the control of a tagged-promoter in axillary 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-cultured 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 localized 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 by preliminary evidence obtained with transgenic tobacco plants expressing a fusion between the promoter of the extracellular invertase Lin6 of tomato and the β-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, 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 CN1 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 to recover 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 processes such as 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, F Pérez-Alfocea, unpublished data). Moreover, this activity was much higher in the roots of the salt-tolerant wild species *Lycopersicon pennellii* than in those of the domestic *L. esculentum* plants. The role
of extracellular invertase in the control of assimilate allocation could be specially important in those species including an apoplastic 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, 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 (1998) also found an inverse relationship in the expression of the cell-wall invertase and sucrose synthase and vacuolar invertase genes in Arabidopsis thaliana under 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 (Bohner 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 (Zinselmieier 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 with an increase of vacuolar invertase activity in mature maize leaves under drought, but it does not affect the cell wall invertase activity (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 environmental stresses (cold, drought and O₂-deficiency). 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 an 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 stimuli and sugars (Roitsch, 1999; Roitsch et al., 2000). Non-metabolizable 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 the carbohydrate-dependent 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 (Godd 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-POL; 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 localized increase in 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 parallel the increased level of mRNA of Cin1 (Roitsch et al., 1995). One of the isoforms of extracellular invertase was shown to be up-regulated by glucose in tobacco (Krausgrill et al., 1996), Arabidopsis
(Tymowska-Lalanne and Kreis, 1998b) and 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 with non-metabolizable sucrose analogues such as palatinose (6-O-α-D-glucopyranosyl-Fru), turanose (3-O-α-D-glucopyranosyl-Fru) and fluorosucrose (1-deoxy-1-fluorofructofuranosyl-α-D-glucopyranoside). While palatinose and turanose can neither be recognized 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 metabolized (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-metabolizable 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 metabolizable 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 metabolizable sugars resulted in repression of RbcS, induction of extracellular invertase and failed to activate MAP kinase activity. These results, summarized in Table 1, suggest that the effect of the sucrose derivatives not synthesized by higher plants resembles the effect of the fungal elicitor E-FOL and are distinctly different from metabolizable sugars. The differential effect of non-metabolizable sugars and glucose were also reported in transcript stability of α-amylase by Loreti et al. (2000). These observations question 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,

Table 1. Differential effect of metabolizable sugars (sucrose, glucose), non-metabolizable sucrose derivatives (turanose, palatinose, fluorosucrose), and a fungal elicitor (E-FOL) on the transcript level of the small subunit 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

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<tr>
<th>Transcript level</th>
<th>Enzyme activity</th>
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<tr>
<td>RbcS</td>
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<td>Glucose</td>
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<td>Sucrose</td>
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<td>Turanose</td>
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<td>Fluorosucrose</td>
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<td>Elicitor</td>
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T Roitsch, unpublished observations). The physiological significance of these findings is reflected by the fact that turanose and palatinose are synthesized by plant pathogens.

The fact that both metabolizable 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 analysis of converging signalling pathways.

**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 characterized, 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), to 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 pathogen. The information derived from these receptors has then 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 it has recently been shown 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, it was possible 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 the activation of all four MAP kinases studied. When PGA or chitosan, molecules that can be expected only to occur naturally 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 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 the 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 extent of transcriptional activation of the two marker genes reflects the observed degree of MAPK activation. As an additional physiological marker, the production of \( \text{H}_2\text{O}_2 \) which is only induced by E-FOL treatment was determined.

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 peaks I

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**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 h after treatment. (D) Release of \( \text{H}_2\text{O}_2 \) into the culture medium 20 min after the treatment.
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 H₂O₂ 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 to discover 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 extent 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 to 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 the 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 distinguishing 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 results in 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., 1987; Memelink et al., 1987). Thus, the regulation of extracellular invertase by phytohormones as outlined above could also contribute to plant pathogen responses.

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 metabolizable 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 a 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 feedback 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 localized increase in sink strength by an elevated invertase activity can satisfy the increased demand for carbohydrates as an energy source for 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 acti-
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viation 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 the facts that extracellular invertase is not only a key metabolic enzyme that contributes to the growth and development of higher plants, but also is an important part of the adaptations that cope with pathogen infections as outlined in Fig. 2 and can thus be considered as an important pathogenesis-related protein.

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