Effects of trehalose on gene expression in *Arabidopsis thaliana* seedlings: a genome-wide analysis

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Summary

Trehalose (α-D-glucopyranosyl-[1,1]-α-D-glucopyranoside), a non reducing disaccharide consisting of two glucose units is present in a large variety of organisms such as bacteria, fungi and invertebrate animals where it may serve as a storage compound or stress protectant. Trehalose also accumulates in a few desiccation-tolerant 'resurrection plants' and may have similar functions there. However, in almost all higher plants, including the model plant *Arabidopsis thaliana*, trehalose is only present in hardly detectable amounts. Nevertheless, plants contain several genes for each of the two enzymes required for trehalose synthesis, and an *Arabidopsis* mutant lacking one of these genes is embryo-lethal. It is therefore interesting to investigate the possible physiological roles of trehalose in plants. For this purpose *A. thaliana* seedlings were incubated with trehalose, and the effects on gene transcript levels were studies at the whole-genome scale. Some of the transcripts most strongly induced by trehalose were selected for a detailed analysis, and their expression was studied by quantitative PCR.

It has recently been shown by others, working also with *A. thaliana*, that several effects of exogenous application of trehalose can be mimicked by genetic manipulations leading to enhanced endogenous trehalose-6-phosphate (T6P) levels, and that trehalose application indeed rises the endogenous T6P level. (This effect might result from a feedback inhibition of trehalose-6-phophate phosphatase when intracellular trehalose levels are high.) Thus, the changes in gene expression triggered by trehalose application may be due to an increase in intracellular T6P concentrations rather than to an increase in trehalose levels *per se*.

A problem of trehalose application, as used by others and initially also in this thesis, is the ubiquitous occurrence of trehalase activity in plants, an enzyme most likely present in the cell wall. This enzyme reduces exogenous trehalose levels and generates glucose, making it difficult to interpret data on exogenous trehalose application. To prevent this, in the main part of the thesis, trehalose was applied in combination with the potent trehalase inhibitor validamycin A (val). In the presence of validamycin A, application of 2-4 mM trehalose was sufficient to alter gene expression in a similar way as application of 25 mM alone.

In this work it is shown that 25 mM trehalose added in combination with val (tre/val) caused a two fold or more up or down regulation of 2277 genes of which more than a third was also regulated by abscisic acid (ABA), linking trehalose metabolism to ABA metabolism or ABA signalling. In the ABA deficient Arabidopsis mutant *aba1*, selected genes inducible by tre/val and ABA still could be induced by tre/val, indicating ABA and ABA metabolism not to be necessary to induce ABA regulated genes upon tre/val treatment. The qualitative difference of the kinetic induction curve of gene expression over time after tre/val application for the genes inducible by tre/val and ABA compared to the induction curve after ABA treatment further supports the hypothesis the tre/val induction is independent of ABA. Interestingly, the induction of some of these by tre/val and ABA inducible genes was suppressed in *abi2-1*, a mutant impaired in the ABA response. Therefore, it can be suggested that ABI2 is necessary for the regulation of at least some genes induced by tre/val. The results obtained by the two ABA mutants suggest the signalling cascade triggered by tre/val affects the ABA signalling pathway downstream of the ABA binding site but upstream of ABI2 activity.

Classification of the genes up regulated by tre/val but not by ABA revealed a set of genes involved in disease resistance and secondary metabolism indicating trehalose in combination with validamycin A may act as an elicitor in plants.

Sugars such as sucrose (suc), glucose and fructose serve in plants not only as important intermediates in the primary metabolism, carbon sources or substrates for storage compounds like starch and cellulose, but can also act as signalling molecules in a similar way as trehalose/trehalose-6-phosphate. Plant cells can sense these sugars via either a hexokinase-dependent, or -independent system. The signal transduction pathways of both these systems include protein phosphatases (PPs) and protein kinases (PKs).

One gene that is readily induced by suc is the one encoding sucrose:fructan 6-fructosyltransferase (6-SFT) in barley, a key enzyme in fructan synthesis. The promoter of this gene was studied in excised barley leaves and transformed Arabidopsis bearing a GUS reporter gene driven by a region of the barley 6-SFT promoter. The broad-spectrum kinase inhibitor K252a as well as genistein (GEN), an inhibitor thought to be specific for protein tyrosine kinases in animal systems, were able to reduce the Suc induced activation of the 6-SFT promoter in both systems, indicating that PKs are involved in Suc mediated regulation. Interestingly, staurosporine (STAU), an inhibitor of PKs similar to K252a did not affect Suc induction, indicating a degree of specificity of these inhibitors. A strong reduction of Suc triggered induction of 6-SFT expression was caused by 1 μM of the potent PP inhibitor okadaic acid (OK). This suggests that PP2A activity is also involved in the Suc mediated regulation of the 6-SFT promoter.

Scope of this thesis

The objective of this dissertation is to gain new insights in the role of trehalose metabolism in *Arabidopsis thaliana* seedlings by analysing gene expression responses upon trehalose treatment at the whole genome level. The connection between trehalose metabolism and abscisic acid (ABA) was investigated using ABA mutants. Additionally, in the context of sugar sensing, components of the sucrose-mediated regulation of the 6-SFT promoter of barley were analysed.

This thesis contains 3 chapters and two appendixes, starting with a general introduction. An attempt has been made to introduce the reader to the basic information on trehalose metabolism in plants and the current progress made in the field has been reviewed.

Chapter 2 and Appendix II describe the experimental work undertaken. These sections are presented in the form of independent research articles. Chapter 3 as appendix II contains an introduction to provide a background to the work that follows.

Chapter 2 has the title: "Trehalose induces ABA regulated genes in wild type Arabidopsis and in the ABA deficient mutant *aba1*" and involves contributions from David Brodmann, Andres Wiemken and Thomas Boller. This study extends the previous work of gene expression analysis upon trehalose supply to Arabidopsis seedlings (Wingler et al., 2000) by using a whole genome expression analysis approach. Gene expression results are presented indicating trehalose metabolism to be involved in carbohydrate metabolism, abiotic stress and pathogen defence. An additional approach with ABA mutants reveals the ability of trehalose metabolism to influence ABA signalling.

Excised barley leaves and transgenic Arabidopsis plants carrying the 6-SFT promoter driving the expression of a GUS reporter gene were used to study the signalling events controlling the activity of the 6-SFT promoter (appendix II). Transformation of Arabidopsis involved collaboration with Dr. Sjef Smeekens, University of Utrecht, The Netherlands. Studies involving the regulation of promoter activity using inhibitors of protein phophatases and protein kinases were done with valuable input from Tita Ritsema and Vinay Janthakahalli Nagaraj

Chapter 3 is a general discussion of all the new findings reported in this dissertation. The open questions related to the work done and future scenarios for trehalose metabolism research are also dealt with in this chapter. Appendix I displays two tables belonging to the work presented in chapter 2. The complete list of references cited in the various parts of the thesis can be found after Chapter 3.

Supplementary data is enclosed to the thesis on a CD-ROM. This CD contains the raw data of the two transcription profiles that were performed in collaboration of the Torrey Mesea Research Institue (TMRI), Syngenta, USA. The raw data are displayed on two Excel spreadsheets.

Chapter 1:

General introduction

Chemical structure of trehalose

Trehalose is a disaccharide consisting of two subunits of glucose bound by an α:1→1 linkage (α -D-glucopyranosyl-[1,1]- α -D-glucopyranoside) (Fig. 1.)

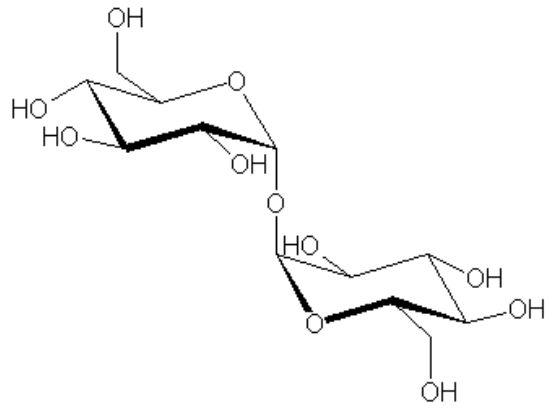


Figure 1. Chemical structure of Trehalose

Occurrence of trehalose

Trehalose is a common sugar in both prokaryotic and eukaryotic organism, such as bacteria, fungi and invertebrates (Elbein, 1974) (Crowe et al., 1984). In these organisms, trehalose often accumulates in large quantities. In plants, an accumulation of substantial amounts of trehalose is very rare. Among the lower plants, a few desiccation tolerant 'resurrection' plants including the ferns *Selaginella lepidophylla* ("rose of Jericho") (Adams et al., 1990), and *S. sartorii* (Iturriaga et al., 2000) accumulate relatively large

amounts of trehalose. Until recently, it was thought that flowering plants were unable to produce trehalose (Muller et al., 1995). The only well-established exception was the desiccation tolerant angiosperm *Myrothamnus flabellifolius*, which accumulates considerable amounts of trehalose (Bianchi et al., 1993). However, with GC-MS techniques, trehalose could be identified unequivocally in axenically grown wild type *Arabidopsis thaliana* (Vogel et al., 2001) growing on the potent trehalase inhibitor validamycin A (val) (Asano et al., 1990). Metabolic profiling using GC-MS analysis has also led to the identification of trehalose in potato (Roessner et al., 2000). These results together with the finding of a large, almost entirely expressed, trehalose biosynthesis gene family in Arabidopsis (Leyman et al., 2001) and the expression of homologues of all these genes in a diverse set of other plant species (Eastmond et al., 2003) suggest that virtually all higher plants have the capacity to synthesize of trehalose, but only in very small amounts.

Chemical properties of trehalose

Trehalose remains stable at elevated temperatures and at low pH and does not undergo the Maillard reaction with proteins (Colaco et al., 1995). Trehalose has been shown to stabilise proteins and membranes under stress conditions, especially during desiccation and heat stress (Crowe et al., 1984; Wiemken, 1990). Trehalose prevents the denaturation of proteins by replacing the water and binding via hydrogen bridges to polar residues of the protein. By the same mechanism the disaccharide prevents the fusion of membranes. In addition, trehalose forms glasses (vitrification) in the dry state, a process that may be required for the stabilisation of dry macromolecules (Crowe et al., 1998).

Trehalose metabolism in micro-organisms and invertebrates

In bacteria, fungi and insects, trehalose is usually synthesized in two steps. First, trehalose-6-phosphate synthase (TPS) catalyses the synthesis of trehalose-6-phosphate (T6P) from glucose-6-phosphate (Glc6P) and UDP-glucose (UDPGlc). Subsequently trehalose-6-phosphate phosphatase (TPP) removes the phosphate yielding trehalose (Elbein, 1974). The catabolism of trehalose occurs by the action of the hydrolytic enzyme trehalase, yielding two glucose units (Muller et al., 1999). Interestingly, *S. cerevisiae* and related yeasts contain two different types of trehalase: the neutral trehalases (*NTH1* and *NTH2*) localized in the cytosol, and the acidic trehalase (*ATH1*) that is found in the vacuole (Argüelles, 2000). Alternatively, degradation and synthesis of trehalose may be achieved by trehalose phosphorylase, a reversible enzyme present in *Euglena gracilis* and *Pichia fermentans*, that converts trehalose with inorganic phosphate to glucose (Glc) and Glc6P (Belocopitow and Maréchal, 1970; Schick et al., 1995). In *Escherichia coli* the catabolism of trehalose occurs either by the periplasmic trehalase *treA*, the cytoplasmic trehalase *treF* or by phosphorylation of trehalose to T6P and subsequent hydrolysis by the trehalose-6-phosphate hydrolase (TPH) activity *treC* to Glc and Glc6P (Rimmele and Boos, 1994; Horlacher et al., 1996).

Role of trehalose in bacteria, fungi and animals

In *E. coli*, trehalose accumulates in response to high osmotic strength and is thought to act in this species as an active osmolyte. In *Bacillus subtilis* trehalose is not involved in osmoprotection and serves exclusively as a carbon source (Argüelles, 2000). In some bacteria trehalose occurs as structural compound located as cell wall glycolipid called

"cord factor", consisting of a unit of trehalose esterified to two mycolic acid residues, which gives rise to α,α-trehalose 6,6'-dimycolates. Cord factor elicits immunogenic responses in the host and acts as a toxic element causing pathogenic lesions by mycobacteria (Spargo et al., 1991). A protective role for trehalose has been postulated in dormant spores under dehydration conditions in *Streptomyces*, where it acts as a substitute for water (Martin et al., 1986).

Several studies proposed trehalose to be a major reserve compound in yeasts and other fungi since vegetative resting cells as well as dormant and reproductive structures are able to store large amounts of trehalose. This interpretation is controversial since it has been shown that trehalose is predominantly synthesized at the onset of reduced growth periods and not when there is an excess of exogenous energy source. It was suggested that in yeast, trehalose is a stress protectant rather than a strict reserve carbohydrate. This hypothesis is based on the demonstration that trehalose is a crucial factor in the adaptive response to a variety of stresses, namely, those induced by nutrient starvation, heat shock, dehydration or oxidative agents (Wiemken, 1990). However, the bulk of trehalose stored during preconditioning treatments is not sufficient to account for the adaptive response. Therefore it is likely that trehalose acts together with other components, like e.g. heat shock proteins, to structurally protect the cell contents. Compared to the situation in bacteria, the role of trehalose as osmoprotectant in yeast is probably less important (Argüelles, 2000).

Several species of insects contain trehalose in the hemolymph, which is quickly mobilized during flight. Also in higher animals, enzymes involved in trehalose metabolism are present, although their role is not well understood. In humans, trehalase

has been located both in the border membranes of epithelial cells of the small intestine. It may be involved in sugar transport across the membrane and/or hydrolysis of ingested trehalose. However, the physiological role of trehalase found in human kidney and serum remains a mystery, because trehalose is not found in blood (Muller et al., 1995; Argüelles, 2000).

Roles of trehalose biosynthesis in plants

a) Trehalose metabolism in plants

The comparison of the *E. coli* and yeast derived *tps* and *tpp* genes with sequences in plant databases revealed the presence of homologous EST clones derived from Arabidopsis and rice. Functional genes encoding enzymes of the trehalose anabolism i.e. TPS and TPP have been identified in Arabidopsis (Blazquez et al., 1998; Vogel et al., 1998). Vogel *et al.* (1998) used an elegant assay to complement a heat-sensitive yeast *tps2* mutant lacking TPP activity by expressing an Arabidopsis cDNA library in this mutant. The two Arabidopsis genes *AtTPPA* and *AtTPPB*, both complementing the yeast mutant by encoding TPP, were identified. Blazquez *et al.* (1998) identified the Arabidopsis tps gene (*AtTPS1*) by complementing the *tps1* mutant from *S. cerevisiae*, which is unable to grow on glucose due to an uncontrolled influx of glucose in the glycolysis in the absence of T6P. *AtTPS1*, *AtTPPA* and *AtTPPB* are expressed at low levels in Arabidopsis.

The effort of systematic sequencing of the Arabidopsis genome (Initiative, 2000) revealed the existence of a family of eleven *TPS* genes that is divided in two classes, class I and class II, depending on their homology to S. cerevisiae *TPS1* and *TPS2* (which encode for TPS and TPP activity, respectively) (Leyman et al., 2001) and a family of ten

TPP genes (class III) (Eastmond et al., 2003). Almost all of the genes of the classes I-III seem to be expressed at mRNA level in Arabidopsis (Eastmond et al., 2003) and are therefore potentially encode for active enzymes. However, two genes of class II, *AtTPS7* and *AtTPS8* did not complement yeast mutants deficient in TPS or TPP (Vogel et al., 2001). One possible explanation for the presence of homologues of TPS and TPP that lack catalytic activity is that they play a role in the formation of a complex. In *S. cerevisiae* for example, trehalose synthesis is carried out by a holoenzyme complex consisting out of four homologous proteins (i.e. TPS1, TPS2, TPS3 and TSL1). It has been shown that TPS3 and TSL1 cannot complement for TPS1 or TPS2 but are required for optimal enzymatic activity of the latter (Reinders et al., 1997; Bell et al., 1998).

Since none of the Arabidopsis trehalose synthesis genes appears to contain clear sub-cellular targeting signals, trehalose metabolism probably occurs in the cytosol (Eastmond and Graham, 2003).

Activity of trehalase, the enzyme that hydrolyses trehalose, is ubiquitous in higher plants (Muller et al., 2001). Trehalase genes have been identified and functionally characterized from soybean (*GMTRE1*) and Arabidopsis (*AtTRE1* (At4g24040)) (Aeschbacher et al., 1999; Muller et al., 2001). *AtTRE1* seems to be the only trehalase of its kind in Arabidopsis, since no homologue is present in the genome. It is likely that trehalase is the sole route of trehalose breakdown in Arabidopsis as trehalose accumulates in the presence of the specific trehalase inhibitor validamycin A (Muller et al., 2001). Analyses of cell cultures have shown that the major part of trehalase activity is extracellular (Muller et al., 1995). Taking all these information together and considering the absence

of TPH and trehalose phosphorylase homologues, trehalose metabolism is thought to occur in plants as shown in Figure 2.

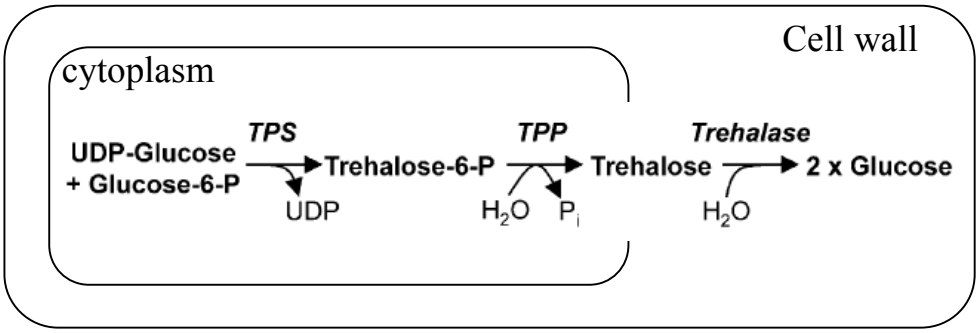


Figure 2. Proposed trehalose metabolism in plants. TPS: trehalose-6-phosphate synthase; TPP: trehalose-6-phosphate phosphatase (Figure and legend from Wingler, 2000)

b) Trehalose/T6P responses

Trehalose levels found in plants are generally very low with the exception of resurrection plants. Thus it is unlikely that trehalose is directly involved in stress protection. The trehalose levels are also much too low to act as a reserve compound. This raises the question of the function of trehalose in plants. If it had no function, it would be unlikely that trehalose biosynthesis would have survived in plants since its production would just be an unnecessary, wasteful energy consuming process for the plant. One possibility is that trehalose (or its precursor, trehalose-6-phosphate) serves as signal molecule.

Recently, it has been reported that exogenous trehalose application to *Arabidopsis thaliana* results in an enhanced T6P concentration in the cell. This effect is thought to result from a reduced T6P dephosphorylation by the TPP enzyme, which is feedback inhibited in activity when intracellular trehalose levels are high (Fig. 3) (Schluepmann et

al., 2004). That would mean, when trehalose is added to the plant, the resulting response might be partially due to regulation of T6P and not exclusively to an enhanced trehalose pool. Thus, in this case one could speak about a 'trehalose/T6P' ('Tre/T6P') dependent regulation. Numerous studies used the system of exogenous application of trehalose in order to investigate the role of trehalose in plants. These studies indicate a role of Tre/T6P in regulation of carbohydrate metabolism/allocation and sugar sensing. In barley for example, externally supplied trehalose induces the mRNA level and the activity of sucrose:fructan-6-fructosyl-transferase, an enzyme of fructan biosynthesis, whereas the fructan content is not raised (Muller et al., 2000). In soybean, trehalose enhances sucrose synthase activity (Muller et al., 1998) whereas in Arabidopsis seedlings it strongly induces the expression of *ApL3*, a gene encoding a large subunit of ADP-glucose pyrophosphorylase, which is a crucial enzyme in starch biosynthesis. This induction of *ApL3* expression leads to increased ADP-glucose pyrophosphorylase activity, an over-accumulation of starch in the shoots and decreased root growth, suggesting Tre/T6P to interfere with carbon allocation to the sink tissues by inducing starch synthesis in the source tissues. Already 25 mM of exogenously applied trehalose results in an inhibition of growth and root elongation (Wingler et al., 2000). In addition, *adg2* mutants that are unable to produce starch can be complemented in starch biosynthesis by trehalose feeding (Fritzius et al., 2001).

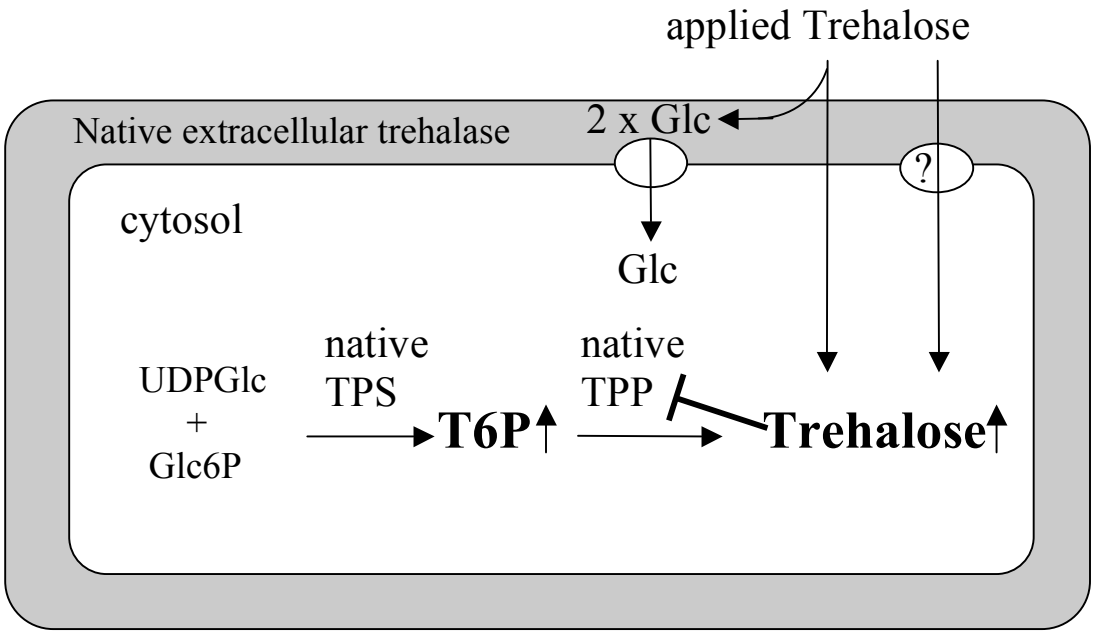


Figure 3: Scheme of trehalose and trehalose-6-phosphate (T6P) accumulation upon trehalose feeding in wild type plants, as proposed by Schluepmann *et al*. 2004. Trehalose is supplied exogenously. It might be transported into the cytosol by an unknown transporter or unknown transporters, or it enters the cytosol without the help of any transporter. The cytoplasmic trehalose levels increase. In addition, much of the trehalose is degraded by the extracellular trehalase activity to two units of glucose (Glc), which enter the cell via hexose transporters. The feedback inhibition of TPP activity by trehalose leads to an enhanced trehalose-6-phosphate (T6P) level. Font of T6P and trehalose and vertical arrows beneath indicate intracellular levels compared to non-treated plants. TPS and TPP stand for the trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase activity of the plant, respectively; UDPGlc is uridindiphophateglucose and Glc6P represents glucose-6-phosphate.

In this thesis, in order to get a clear Tre/T6P response, I tried to block the hydrolysis of exogenously applied trehalose by the addition of the potent plant trehalase inhibitor validamycin A (val) in combination with trehalose. Under these conditions, the formation of Glc is reduced to a minimum, the main path of trehalose is to enter the cell and trigger its effects as molecule *per se*. Indeed, an approximately 10-fold lower trehalose concentration was needed to affect gene expression upon trehalose treatment in combination with val (tre/val) compared to trehalose alone. It also appeared that the induction of gene expression by 25 mM trehalose was much higher when the seedlings were treated with tre/val than with trehalose alone. A possible explanation for this is that in the presence of val a much higher cytoplasmic trehalose concentration can be reached than in plants treated with trehalose only, resulting in a more efficient feedback inhibition of TPP activity. A strong inhibition of TPP subsequently would lead to a high intracellular T6P level resulting in a pronounced Tre/T6P response (Fig. 4).

Besides val, useful tools in research on trehalose metabolism are the transgenic Arabidopsis plants carrying different genes of the *E. coli* trehalose metabolism under the control of the CaMV35S promoter (Schluepmann et al., 2003). Overexpression of the *E. coli* TPS gene *otsA* in Arabidopsis is expected to rise the endogenous T6P and, to a lesser extend, trehalose content. Indeed, the T6P level was increased about 2-3 fold in *otsA* expressors compared to wild type, but the trehalose levels, however, stayed below 2 $nmolg^{-1}$ FW (Fig 5). The plants expressing *otsA* (TPS) have dark green cotyledons with anthocyanin accumulation along the rim; smaller, dark green rosette leaves compared with wild type and show a bushy growing. Seedlings expressing the *E. coli*

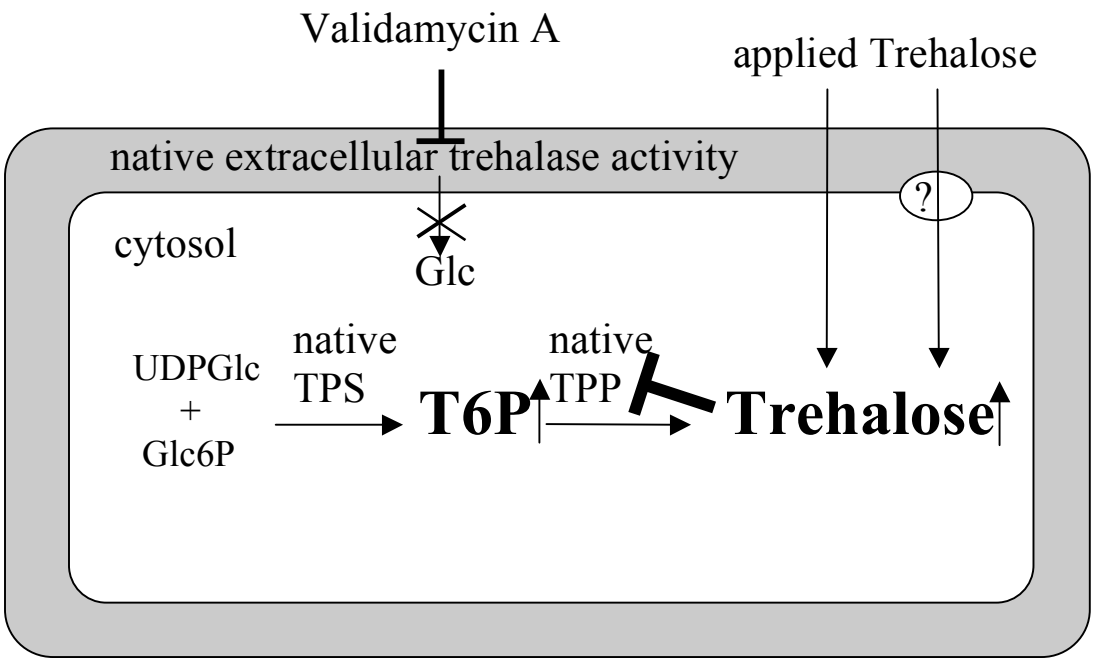


Figure 4: Scheme of trehalose and trehalose-6-phsophate (T6P) accumulation in plants treated with trehalose in combination with the potent trehalase inhibitor validamycin A. Trehalose is supplied exogenously. It might be transported into the cytosol by an unknown transporter or unknown transporters, or it enters the cytosol without the help of any transporter. Due to the presence of the inhibitor, during the process of entering the cell, trehalose is degraded to a much lesser extend to glucose (Glc) compared to plants treated with trehalose only, resulting in a higher extracellular and consequently intracellular trehalose concentration. As proposed by Schluepmann *et al.* 2004, the feedback inhibition of TPP activity by the trehalose leads to an enhanced trehalose-6-phosphate (T6P) level. The T6P levels will therefore likely rise to a higher level than in trehalose only treated plants because of an efficient inhibition of TPP. The font of T6P and trehalose and vertical arrows beneath indicate intracellular levels compared to non-treated plants. TPS and TPP stand for the trehalose 6-phosphate synthase and trehalose 6-

phosphate phosphatase activity of the plant, respectively, UDPGlc is uridindiphosphateglucose and Glc6P represents glucose-6-phosphate.

TPP gene *otsB* are expected to have a decreased T6P and an increased trehalose level. Whereas the T6P content is more than two fold lower in plants expressing *otsB* compared to wild type, the trehalose level also remained below the detection limit of 2 nmol g^{-1} FW (Fig 6). *otsB* (TPP) expressors have cotyledons displaying bleached areas and mature leaves are lighter green and larger than wild type. The mature plants of this line also bolt up to 3 weeks later than wild type and have a pronounced apical dominance. Plants expressing the *E. coli* trehalose-6-phosphate hydrolase (TPH) gene *treC* are expected to have lower T6P and also a lower trehalose level compared to the wild type. It was reported that T6P levels in these plants are more than two fold lower than in wild type, whereas trehalose contents were not distinguishable from wild type (Fig 7). The phenotype of plants expressing *treC* (TPH) is very similar to the phenotype of the plants expressing *otsB* (TPP). The seedlings of *treC* expressors expand and green later compared to the wild type. Seedlings and mature plants show bleached areas and the leaves of mature plants are larger compared to the wild type. The expression of the cytoplasmic *E. coli* trehalase *treF* is expected to reduce trehalose and, to a lesser extend, also T6P levels. However, *treF* expression did not change T6P and trehalose levels compared to wild type (Fig. 8) and yielded plants with a phenotype not distinguishable from wild type when grown on soil. The trehalose content in all transgenic lines was

below the HPLC detection limit of 2 nmol g^{-1} FW, meaning an eventual decrease in trehalose levels could not be reported due to analytical limits (Fig. 5, 6, 7, 8).

There were parallels in symptoms of Arabidopsis seedlings fed with trehalose and those expressing *E. coli* TPS (having primarily enhanced T6P but less induced trehalose level compared to the wild type) like dark green cotyledons. This indicate rather T6P than trehalose may be the active compound causing effects upon trehalose feeding (Schluepmann et al., 2003). In addition, it has been shown recently that enhanced T6P levels in Arabidopsis increases starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase (AGPase), whereas TPP expression prevented the increase in AGPase activation even in response to sucrose or trehalose feeding (Kolbe et al., 2005). The effect of enhanced AGPase activity accompanied by starch accumulation was also observed in wild type Arabidopsis seedlings fed with trehalose (Wingler et al., 2000). These results suggest that many effects observed by adding trehalose are likely due to elevated levels of T6P.

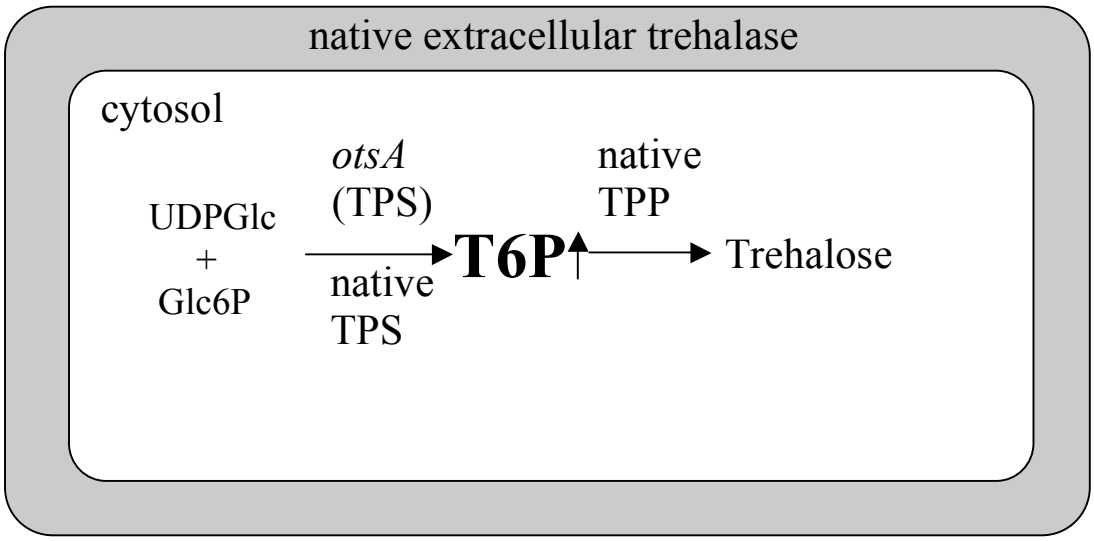


Figure 5: Scheme of trehalose-6-phosphate (T6P) and trehalose levels in transgenic plants expressing the *E. coli* trehalose metabolism gene *otsA*, encoding for a trehalose-6-phosphate synthase (TPS) under the control of the CaMV35S promoter as described in Schluepmann *et al.* 2003 and Schluepmann *et al.* 2004. These transgenic plants have a T6P content enhanced 2-3 fold more compared to wild type plants. Trehalose levels are below 2 nmolg-1 FW as in the wild type. The font of T6P and trehalose and vertical arrows beneath indicate intracellular levels compared to non-treated plants. Native TPP and native TPS stand for the natural trehalose-6-phosphate phosphatase and natural trehlose-6-phosphate synthase activity of the plant, respectively; UDPGlc is uridindisphophateglucose and Glc6P represents glucose-6-phosphate.

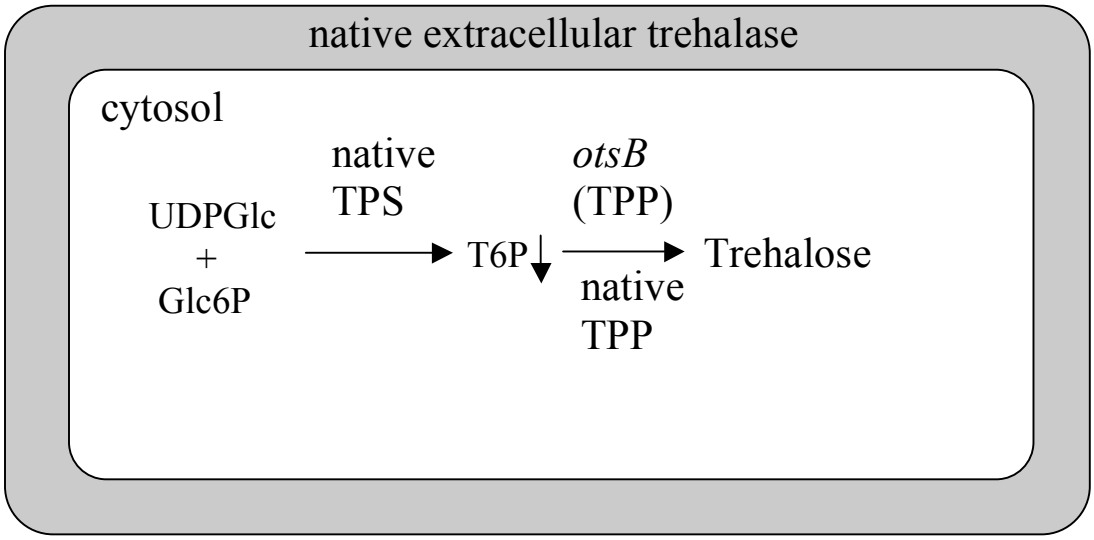


Figure 6: Scheme of trehalose-6-phosphate (T6P) and trehalose levels in transgenic plants expressing the *E. coli* trehalose metabolism gene *otsB*, encoding for a trehalose-6-phosphate phosphatase (TPP) under the control of the CaMV35S promoter as described in Schluepmann *et al.* 2003 and Schluepmann *et al.* 2004. These transgenic plants have a T6P content more than two fold lower compared to wild type plants. Trehalose levels are below 2 $nmolg^{-1}$ FW as in the wild type. The font of T6P and trehalose and vertical arrows beneath indicate intracellular levels compared to non-treated plants. Native TPP and native TPS stand for the natural trehalose-6-phosphate phosphatase and natural trehalose-6-phosphate synthase activity of the plant, respectively; UDPGlc is uridindisphophateglucose and Glc6P represents glucose-6-phosphate.

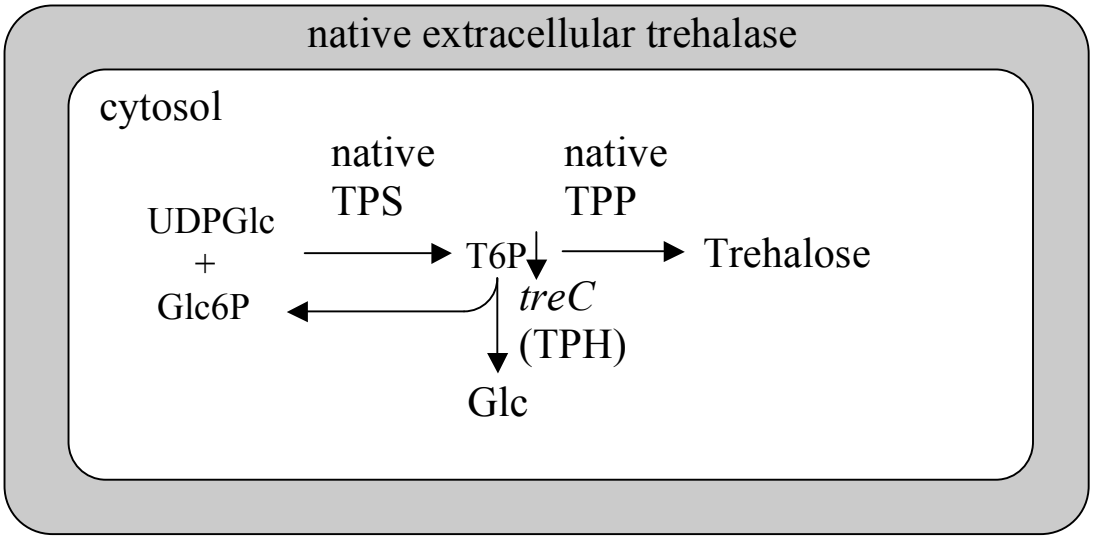


Figure 7: Scheme of trehalose-6-phosphate (T6P) and trehalose levels in transgenic plants expressing the *E. coli* trehalose metabolism gene *treC*, encoding for a trehalose-6-phosphate hydrolase (TPH) under the control of the CaMV35S promoter as described in Schluepmann *et al.* 2003 and Schluepmann *et al.* 2004. TPH catalyses the hydrolization of T6P to glucose (Glc) and glucose-6-phsophate (Glc6P). These transgenic plants have a T6P content more than two fold lower compared to wild type plants. Trehalose levels are below 2 nmolg-1 FW as in the wild type. The font of T6P and trehalose and vertical arrows beneath indicate intracellular levels compared to non-treated plants. Native TPS and native TPP stands for the natural trehalose-6-phosphate synthase and natural trehalose-6-phosphate phosphatase activity of the plant, respectivley; UDPGlc represents uridindisphophateglucose.

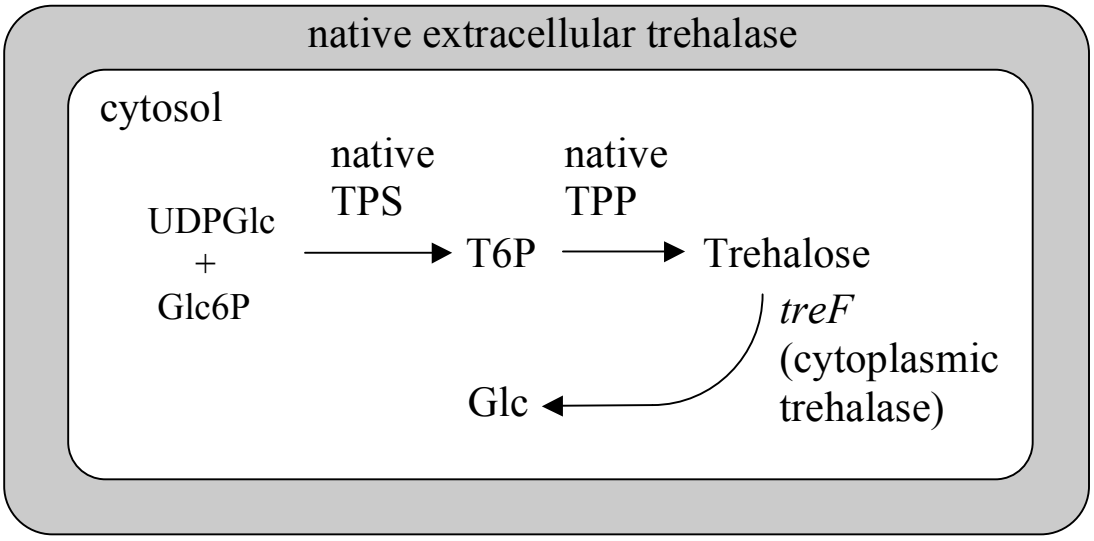


Figure 8: Scheme of trehalose-6-phosphate (T6P) and trehalose levels in transgenic plants expressing the *E. coli* trehalose metabolism gene *treF*, encoding for a cytoplasmic trehalase under the control of the CaMV35S promoter as described in Schluepmann *et al.* 2003 and Schluepmann *et al.* 2004. Trehalase catalyses the hydrolization of trehalose to two glucose (Glc) units. These transgenic plants have the same T6P content as the wild type. Trehalose levels are below 2 nmolg-1 FW as in the wild type. The font of T6P and trehalose and vertical arrows beneath indicate intracellular levels compared to non-treated plants. Native TPS and native TPP stands for the natural trehalose-6-phosphate synthase and natural trehalose-6-phosphate phosphatase activity of the plant, respectivley; UDPGlc is uridindisphophateglucose and Glc6P represents glucose-6-phsophate.

The growth arrest on trehalose of wild type plants can be overcome by expression of *E. coli* trehalose-6-phophate hydrolase (TPH) *treC* in Arabidopsis seedlings, catalysing the hydrolization of T6P to Glc6P and Glc (Fig. 6). This indicates that T6P and not trehalose is the causal agent of growth inhibition. However, the treA gene of *Bacillus subtilis*, which encodes a highly homologous TPH catalysing the hydrolyzation of T6P to Glc and Glc6P (TPH activity) shows also a weak trehalase activity when the purified enzyme is incubated with trehalose (Gotsche and Dahl, 1995). The purified enzyme of *E. coli* encoding for this activity, treC, showed no trehalase activity when incubated with trehalose (Rimmele and Boos, 1994), but considering the results of the enzyme from *B. subtilis*, there might be still a weak trehalase activity not detected by the assay performed by that group. Therefore, it cannot be excluded that the restored growth on trehalose of plants expressing *treC* under the strong CaMV35S promoter is due to the enhanced trehalase activity (Thus, an efficient trehalose hydrolysation) rather than to the TPH activity decreasing T6P levels (Fig. 9B). It might therefore be, that the inhibiting agent of Arabidopsis growth is trehalose not T6P.

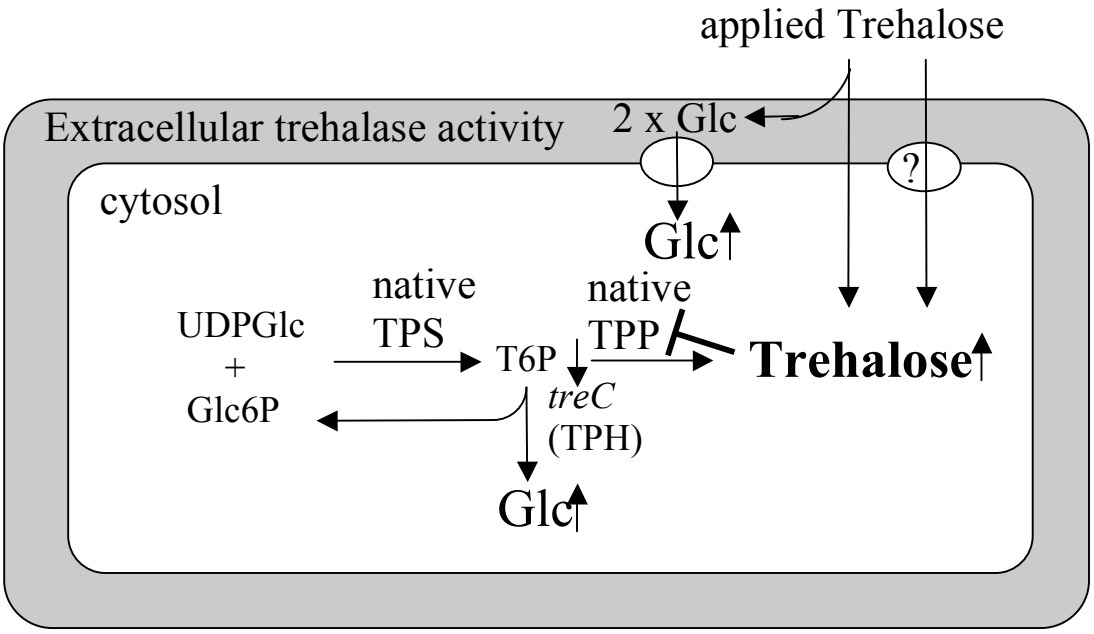


Figure 9A: Scheme of the effect of trehalose addition in trehalose-6-phosphate hydrolase (TPH) overexpressing plants as proposed by Schluepmann *et al.* 2004. The *E. coli* TPH gene *treC* hydrolyses trehalose-6-phosphate (T6P) to glucose (Glc) and glucose-6-phosphate (Glc6P). Trehalose is supplied exogenously. It might be transported into the cytosol by an unknown transporter or unknown transporters, or it enters the cytosol without the help of any transporter. The cytoplasmic trehalose levels increase. In addition, much of the trehalose is degraded by the extracellular trehalase activity to two units of glucose (Glc), which enter the cell via hexose transporters. The trehalose-6-phosphate phosphatase (TPP) activity is feedback inhibited by trehalose. TPH activity keeps cytoplasmic T6P levels low even if TPP activity is reduced by high concentrations of trehalose. Font of T6P, trehalose and Glc, and vertical arrows beneath indicate intracellular levels compared to non-treated plants. Native TPS and native TPP stand for

the natural trehalose-6-phosphate synthase and natural trehalose-6-phosphate phosphatase activity of the plant, respectively. UDPGlc is uridindiphosphateglucose.

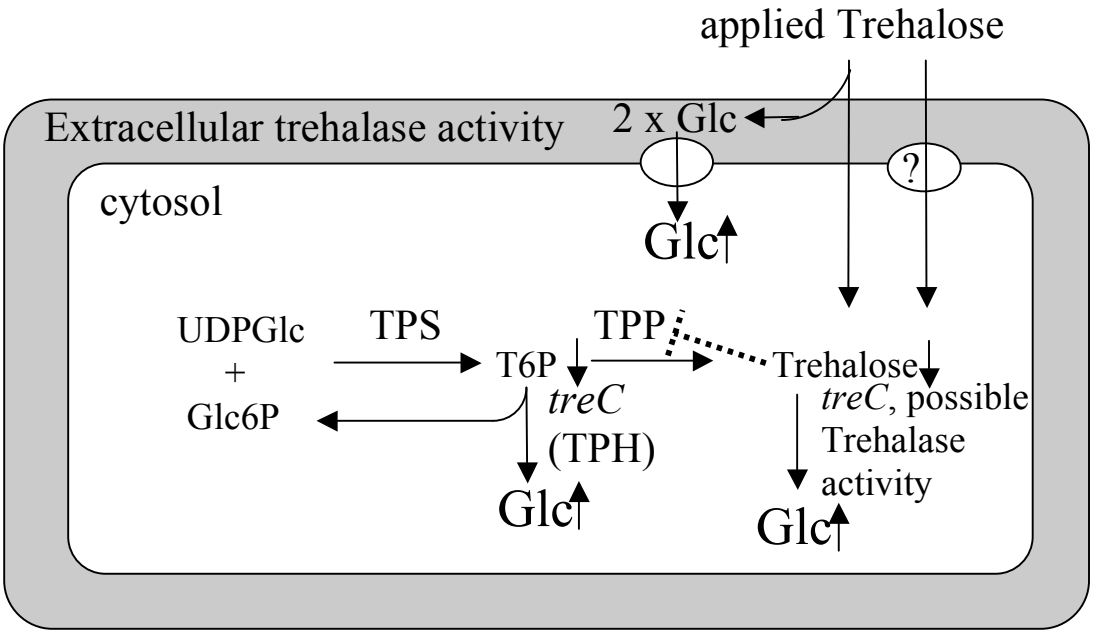


Figure 9B: Alternative interpretation of the effect of trehalose addition in *treC* overexpressing plants. The *E. coli* trehalose-6-phosphate hydrolase (TPH) gene *treC* hydrolyses trehalose-6-phosphate (T6P) to glucose (Glc) and glucose-6-phosphate (Glc6P), but may also have a trehalase side activity. Trehalose is supplied exogenously. It might be transported into the cytosol by an unknown transporter or unknown transporters, or it enters the cytosol without the help of any transporter. Much of the trehalose is degraded by the extracellular trehalase activity to two units of glucose (Glc), which enter the cell via hexose transporters. TPH activity keeps cytoplasmic T6P levels low as proposed by Schluepmann *et al.* 2004, but additionally, intracellular trehalose may be degraded to Glc by the side trehalase activity of the treC enzyme cancelling the inhibition

of TPP activity to a certain degree. Font of T6P, trehalose and Glc, and vertical arrows beneath indicate intracellular levels compared to those in Figure 9A. Native TPS and native TPP stand for the natural trehalose-6-phosphate synthase and natural trehalose-6-phosphate phosphatase activity of the plant, respectively. UDPGlc is uridindiphosphateglucose.

Trehalose metabolism has apparently a crucial role in plant development: The Arabidopsis *tps1* mutant, deficient for the AtTPS1 protein is embryo lethal (Eastmond et al., 2002). Since this mutant can be rescued by expression of the *E. coli* TPS but not by addition of trehalose, this indicates again that T6P rather than trehalose or the AtTPS1 protein is indispensable for development in Arabidopsis (Schluepmann et al., 2003). Interestingly, the embryo lethal *tps1* mutant can be partially recovered by reducing external Suc concentrations (Eastmond et al., 2002). Consistent with this finding, T6P levels seem to regulate the accumulation of respiratory intermediates like glucose-6-phosphate (Glc6P), fructose-6-phosphate (Fru6P) and citrate upon sugar feeding (Schluepmann et al., 2003). Thus, T6P seems to regulate the influx of sugars into respiration. This led to the assumption of a regulatory role of T6P in Arabidopsis similar to yeast where this molecule inhibits hexokinase II *in vitro* at physiological concentrations (Blazquez et al., 1993) and is therefore considered as an important component of glycolytic regulation. In yeast and animal systems hexose phosphorylation by hexokinase (HXK) is suggested to be an important sugar sensing mechanism. Somehow the active hexokinase initiates a signalling cascade that leads to altered gene expression. It was proposed that the yeast Hxk2 protein is itself located in the nucleus as

part of a DNA-protein complex that binds to Glc-repressed genes. In plants a similar HXK-dependent sugar sensing mechanism seems to control many processes and metabolic pathways. HXK-dependent sensing of Suc occurs through binding of its product upon hydrolysis (Glc) by invertase. Sugar-induced feedback inhibition of photosynthesis has been described for many plant species and this overrides regulation by light, tissue type, and developmental stage (Smeekens, 2000). However, the two well-known hexokinases of *Arabidopsis*, AtHXK1 and AtHXK2, were not inhibited by T6P *in vitro* (Eastmond et al., 2002). Nevertheless, the possibility that T6P acts on HXK cannot be fully dismissed because, although AtHXK1 appears to be the predominant HXK isoform in developing seeds, the effects of T6P on every active enzyme of the whole HXK gene family has not been tested yet in Arabidopsis (Eastmond et al., 2003). The control of the respiratory pathway through HXK or components further downstream may also explain how T6P levels regulate the usability of available sugars as shown in the study of Schluepmann *et al.* 2003. There, TPS overexpression improves growth on sugar over wild type. Another feat of plants overexpressing TPS activity is the improvement of photosynthetic capacity, an effect that seems to be closely related to the activity and amount of Rubisco (Garg et al., 2002; Pellny et al., 2004). Remarkably in this context, amounts of T6P correlate with expression of *AKIN11* (a plant sucrose non fermenting (SNF)-related kinase able to complement yeast SNF1 kinase), which links T6P to a kinase controlling signal transduction response to Suc (Schluepmann et al., 2004). In addition, the T6P induced AGPase activity is also dependent on a SNF-related kinase, namely *SnRK1* (Kolbe et al., 2005). All these results suggest that in plants T6P acts as a

key regulator in sugar sensing and, thus, the expression of genes associated with carbon metabolism.

c) Improving abiotic stress tolerance in plants by genetic engineering of trehalose metabolism

There is a growing interest in the application of trehalose metabolism to engineer drought tolerant crops (Penna, 2003). Strikingly, although all attempts to overproduce trehalose in amounts comparable with those present in 'resurrection plants' failed, most of the plants transformed with enzymes of trehalose metabolism from *E. coli* and yeast, showed enhanced resistance to drought, desiccation and other abiotic stresses. For example, in tobacco, introduction of the yeast *TPS1* gene, under transcriptional control of the Rubisco small subunit promoter, results in the accumulation of low levels of trehalose (0.08-0.32% of the dry weight) (Holmström et al., 1996). The transgenic lines showed retarded growth but also a reduced water loss in detached leaves compared to the control lines. A similar experiment was performed with tobacco plants bearing the yeast *TPS1* gene driven by the CaMV 35S promoter. The transgenic Tobacco plants accumulated low amounts of trehalose (up to 0.017% of fresh weight), but showed various pleiotrophic effects like small, dark and lancet-shaped leaves (Romero et al., 1997). This observation is consistent with studies in transgenic tobacco expressing the *otsA* (TPS) gene derived from *E. coli* (Goddijn et al., 1997). In rice, transformation of a fusion gene containing the *E. coli* trehalose biosynthetic genes *otsA* (TPS) and *otsB* (TPP) gene under the control of either tissue-specific or stress-dependent promoters resulted in trehalose contents up to 10 times higher in transgenic lines than in control plants (Garg et al., 2002). However, in all

the described transgenic plants the endogenous trehalose concentration was too low, that trehalose *per se* could act as a protectant of cellular structures. It is more likely that secondary effects of T6P or trehalose overproduction (Tre/T6P responses) make the plants more resistant to abiotic stresses. The effects are Tre/T6P regulated, since the overproduction of trehalose in these plants is achieved via T6P. Important players might be ABA metabolism and ABA regulated genes. T6P indeed has also been reported to be involved in ABA signalling: plants over expressing AtTPS1 show Glc- and ABA-insensitive phenotypes. These effects seem to be at least in part due to an altered regulation of genes involved in Glc and ABA signalling during seedling vegetative growth (Avonce et al., 2004). Additionally, transcription analysis of seedlings with modified T6P concentrations link T6P levels to the expression of genes involved in abiotic stress (Schluepmann et al., 2003).

d) Role of trehalose metabolism in plant symbiosis and plant disease

It is possible that trehalase activity in plants keeps cellular trehalose concentrations low in order to prevent an interference of trehalose with T6P levels by inhibitory feedback of TPP activity (See section "Trehalose/T6P responses" of this chapter). Such a role of trehalase may be of importance in interactions of plants with trehalose-producing microorganisms. In support of this hypothesis, trehalase activity was found to be induced in Arabidopsis plants infected with the trehalose-producing pathogen *Plasmodiophora brassicae* (Brodmann et al., 2002) and in symbiotic relationships of plants with trehalose producing microorganisms, such as arbuscular mycorrhizal fungi (Schubert and Wyss, 1996) and rhizobia (Muller et al., 1994). Trehalase expression is probably not induced by

its substrate trehalose because trehalase activity increases before the accumulation of trehalose in Arabidopsis infected with *P. brassicae* (Brodmann et al., 2002). It is more likely that it is regulated by auxin. As shown previously, treatment of the roots of soybean plants with trehalose had no effect on trehalase activity, whereas trehalase activity was strongly increased in auxin-treated roots (Muller et al., 1995).

Trehalose produced by the pathogen can only influence the regulation of plant metabolism when it is released into the plant and when the amounts of trehalose accumulating in the plant cells are sufficiently high. In *Saccharomyces cerevisiae*, export of trehalose occurs via a high-affinity proton symporter (Stambuk et al., 1996). This export of trehalose from the cytosol to the external environment is essential for survival during dehydration and during germination (Cuber et al., 1997). Export of trehalose may happen in a similar way in fungi invading plants. Alternatively, trehalose could be released passively during cell death of the microorganisms (Ludwig-Muller, 1999).

In addition to trehalose, hexoses and starch accumulated in the roots and hypocotyls of with *P. brassicae* infected plants (Brodmann et al., 2002). However, there is no evidence of a causal relationship between these changes in carbohydrate pools and Tre/T6P response, but it might be that the Tre/T6P response is a tool for the fungi to manipulate the carbon household of the plant to his own favour (supply of carbohydrates of the plant to the pathogen's location).

Transcription profiles of seedlings with modified T6P levels reveal a link between T6P levels and expression of genes involved in abiotic stress responses (Schluepmann et al., 2003; Schluepmann et al., 2004). Indeed, unpublished data confirm resistance of Arabidopsis plants with T6P accumulation to *Peronospora parasitica* (Schluepmann et

al., 2004). In addition, trehalose spray application to wheat has previously been shown to protect wheat from powdery mildew infection (Reignault et al., 2001).

Chapter 2:

Trehalose Induces ABA Regulated Genes in Wild Type Arabidopsis and in the ABA Deficient Mutant *aba1*

David Brodmann, Andres Wiemken, Thomas Boller

Abstract

To gain insight into the role of trehalose metabolism in plants the effects of 25 mM trehalose application in combination with 10 μM of the trehalase inhibitor validamycin A (tre/val) on mRNA expression were tested. Arabidopsis seedlings were used to study the effects on gene expression at whole genome level. The investigation revealed a regulation (more than two fold up or down) of 2277 genes by tre/val of which 746 were also regulated by 20 μM abscisic acid (ABA). The biggest groups formed by classification of known genes induced by tre/val and ABA treatment were transcription, carbohydrate metabolism, response to abiotic stress and cell wall metabolism. The predominant groups of known genes up regulated by tre/val but not by ABA were signal transduction, disease resistance, secondary metabolism, carbohydrate metabolism and transcription. The up-regulation of genes involved in disease resistance and secondary metabolism indicates that trehalose acts as an elicitor in plants. A trehalose concentration of 2-4 mM in combination with validamycin A is necessary to affect gene expression. We used the Arabidopsis mutants *aba1* and *abi2-1* that are deficient in ABA synthesis and have an impaired response to ABA, respectively, to examine the effects of these mutations on gene expression changes by tre/val treatment. In *aba1* mutants selected genes up

regulated by tre/val and ABA could still be induced by tre/val application whereas in *abi2-1* mutants the enhanced gene expression caused by tre/val was partially repressed suggesting the signal cascade triggered by tre/val affects the ABA signaling pathway downstream of the ABA binding site but upstream of ABI2 activity.

Introduction

Trehalose (α-D-glucopyranosyl-[1,1]-α-D-glucopyranoside) is a non reducing disaccharide widely spread in nature (Elbein, 1974) that has remarkable properties as a stress protectant saving membrane and protein structures from damage during various kinds of stresses (Wiemken, 1990; Goddijn and van Dun, 1999). In plants, large trehalose accumulation is restricted to some desiccation tolerant plants such as some ferns and the angiosperm *Myrothamnus flabellifolia* (Muller et al., 1995). However, trehalose has been detected in Arabidopsis in minor amounts after application of the potent trehalase inhibitor validamycin A (Vogel et al., 2001). Homologues of the trehalose biosynthesis genes trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) have been found in the genome of Arabidopsis (Leyman et al., 2001) of which almost all are expressed (Eastmond et al., 2003) and some proven to be functional active (Blazquez et al., 1998; Vogel et al., 1998). Trehalose-6-phosphate (T6P), whose formation is catalyzed by TPS, has been shown to be strongly involved in carbohydrate utilization (Schluepmann et al., 2003), able to alter photosynthesis (Pellny et al., 2004), essential for embryo maturation (Eastmond et al., 2002) and probably regulating sugar metabolism in plants (Eastmond et al., 2003). Over expressing TPS and TPP in tobacco and rice enhances drought tolerance (Holmström et al., 1996; Garg et al., 2002; Jang et

al., 2003). Trehalose application to plants has been shown to change carbohydrate pools and expression of enzymes involved in carbohydrate metabolism. In barley it induces the activity of sucrose:fructose-6-fructosyl transferase, an enzyme of fructan biosynthesis (Muller et al., 2000). In Arabidopsis it induces the expression of *ApL3*, a gene encoding a large subunit of ADP-glucose pyrophosphorylase, which is an important enzyme in starch biosynthesis. This induction of *ApL3* leads to an accumulation of starch in the shoots of seedlings (Wingler et al., 2000; Fritzius et al., 2001). Here we tried to elucidate the possible physiological role of trehalose in plants by extending the view on the effects of 25 mM trehalose in combination with 10 μM validamycin A (tre/val) to mRNA expression to a global level using whole genome microarray techniques. We also tried to elucidate the connection of exogenous trehalose application and ABA using the ABA deficient mutants *aba1* and the *abi2-1* impaired in ABA synthesis and ABA signaling respectively. Furthermore a trehalose concentration and time series were performed to analyze the gene expression response to tre/val quantitatively and kinetically.

Results

Global mRNA expression analysis revealed a set of genes up regulated by tre/val and ABA as well as a set of genes up regulated by tre/val only

To get more insight in the changes of gene expression in plants in response to trehalose, a genome wide gene expression analysis has been carried out with the Affymetrix GeneChip Arabidopsis ATH1 Genome Array carrying over 24.000 unique gene sequences. We wanted to compare trehalose (tre) treatment or trehalose treatment in combination with the potent trehalase inhibitor validamycin A (tre/val) with other treatments. Therefore 12 days old wild type Col-0 Arabidopsis seedlings were treated for two days with either 25 mM trehalose (tre) or 25 mM trehalose + 10 µM validamycin A (tre/val). In addition 25 mM sucrose (suc) or 25 mM glucose (glc) or 20 µM abscisic acid (ABA) were tested. The phytohormone ABA was chosen because earlier expression array assays of plants treated with tre/val unexpectedly revealed a regulation of a set of genes known to be regulated also by ABA (data not shown). By comparing the expression profile of tre/val and ABA treated plants we were hoping to find synergies helping us to understand the kind of connection between tre and ABA treatment. As controls 25 mM mannitol (man) (osmotic control) and 10 µM validamycin A (val) were used. Tre/val treatment resulted in a dramatic change in expression profile (2277 genes were more than two fold up or down regulated) (Fig. 1.). Tre/val affected more genes than tre alone showing that inhibition of trehalase activity allows trehalose to display a much larger array of effects in the plant before it is broken down to glucose (Fig. 1.). A set of 115 genes was regulated by tre but not by tre/val or glc (data not shown). This was

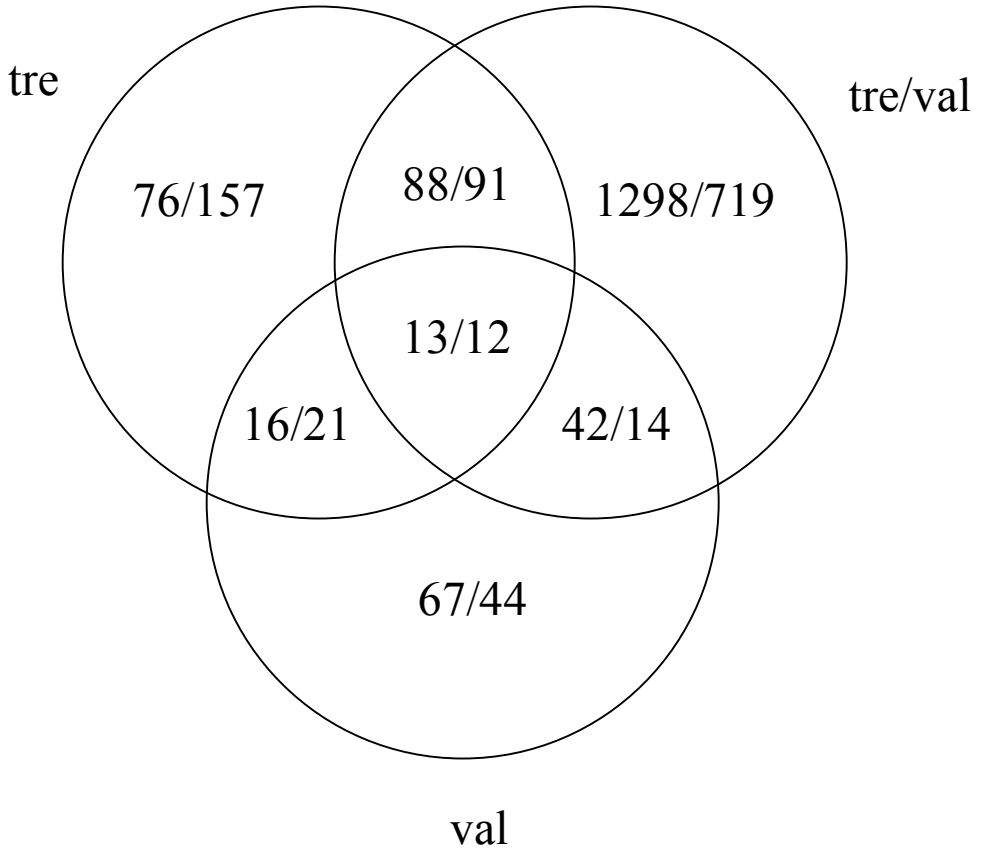


Figure 1. Effects of tre, tre/val and val on gene expression in seedlings analysed by Affymetrix microarray. The Venn diagram shows the number of genes affected (induced/repressed) more than two fold by treatment of 12 days old Arabidopsis seedlings.

unexpected since the genes regulated by tre in theory are supposed to be a subset of the tre/val or glc regulated genes. However, if the threshold of regulation is set to four fold up or down, all the genes up regulated solely by tre become a subset of either glc or tre/val, and only three genes are still down regulated by tre but not by tre/val of glc (data not shown). The specific effects of tre seem to diminish if the threshold is set higher respectively lower showing that these effects are more due to small differences in expression than to a expression pattern divergent to those of glc and tre/val.

Val treatment alone had only little effects on gene expression profiling indicating the differences in the expression profile between tre and tre/val treatments are not caused by val itself (Table I & 2, Appendix I). Suc treatment shared 25.3% differentially regulated genes with genes regulated by tre/val. Glc treatment shared 24 % of its regulated genes with the tre/val regulated genes (Fig. 2). This could be explained by an insufficient inhibition of trehalase activity by val causing a breakdown of a certain amount of trehalose entering the plant. Suc is sharing 25.3 % of regulated genes with tre/val treatment. This could also be due to the exposure of the plant to glc since exogenously applied suc can be hydrolysed to glc and fructose by invertase activity. This is supported by the fact that most (67.9 %) of the genes regulated by suc and tre/val are also regulated by glc (Fig. 2). Man did share only 2.2-15.7 % of all genes regulated with all other treatments (data not shown) indicating most of the observed changes in expression are not due to osmotic reasons.

Out of 2040 genes regulated by ABA 746 (i.e. 36.6 %) genes were found to be also regulated by tre/val treatment (Fig. 2). An explanation of this finding could be the induction of the expression of genes required for ABA synthesis upon tre/val treatment.

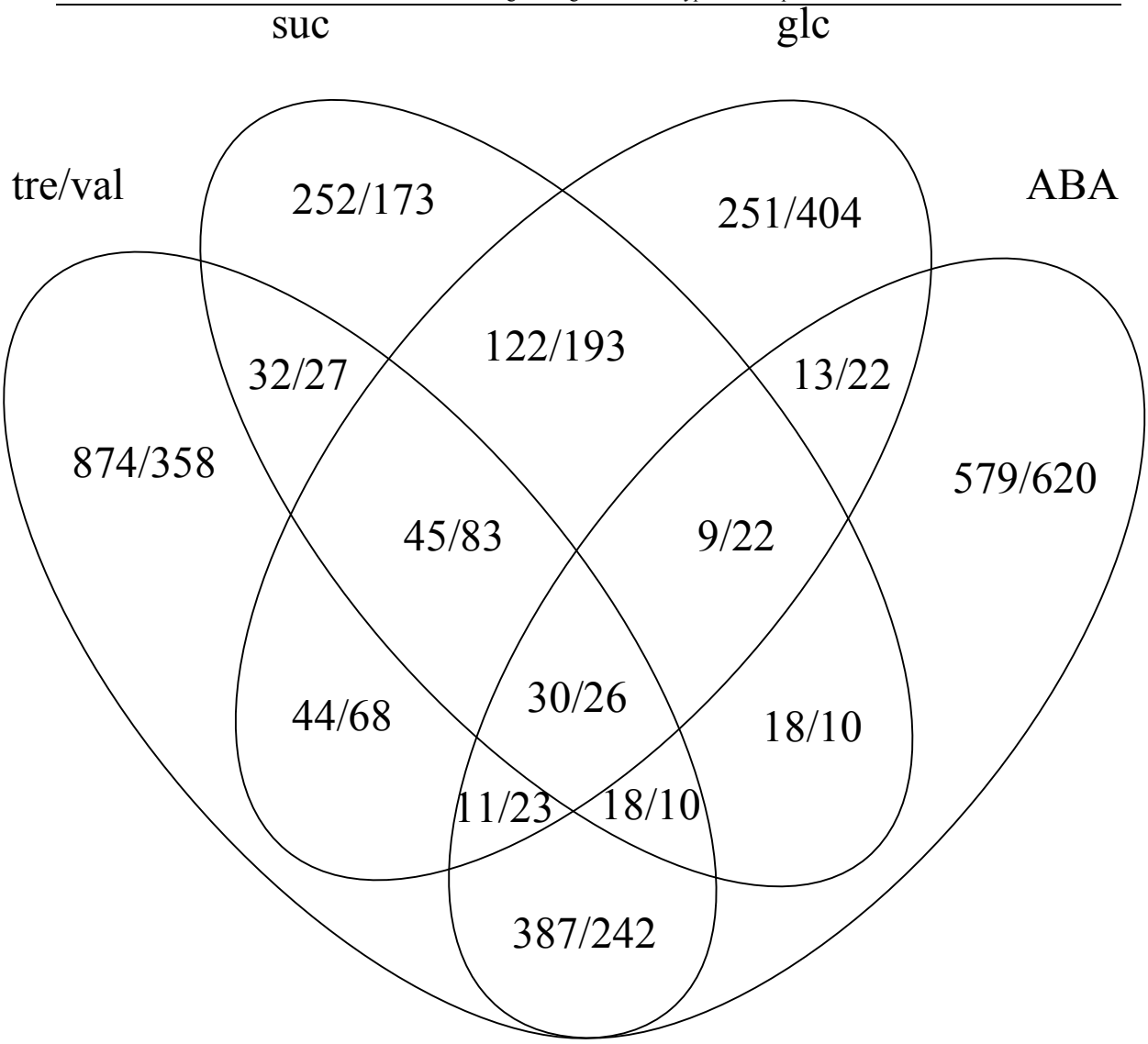


Figure 2. Effects of tre/val, suc, glc and ABA on gene expression in seedlings analysed by Affymetrix microarray. The four-way Venn diagram shows the number of genes affected (induced/repressed) more than two fold by treatment of 12 days old Arabidopsis seedlings.

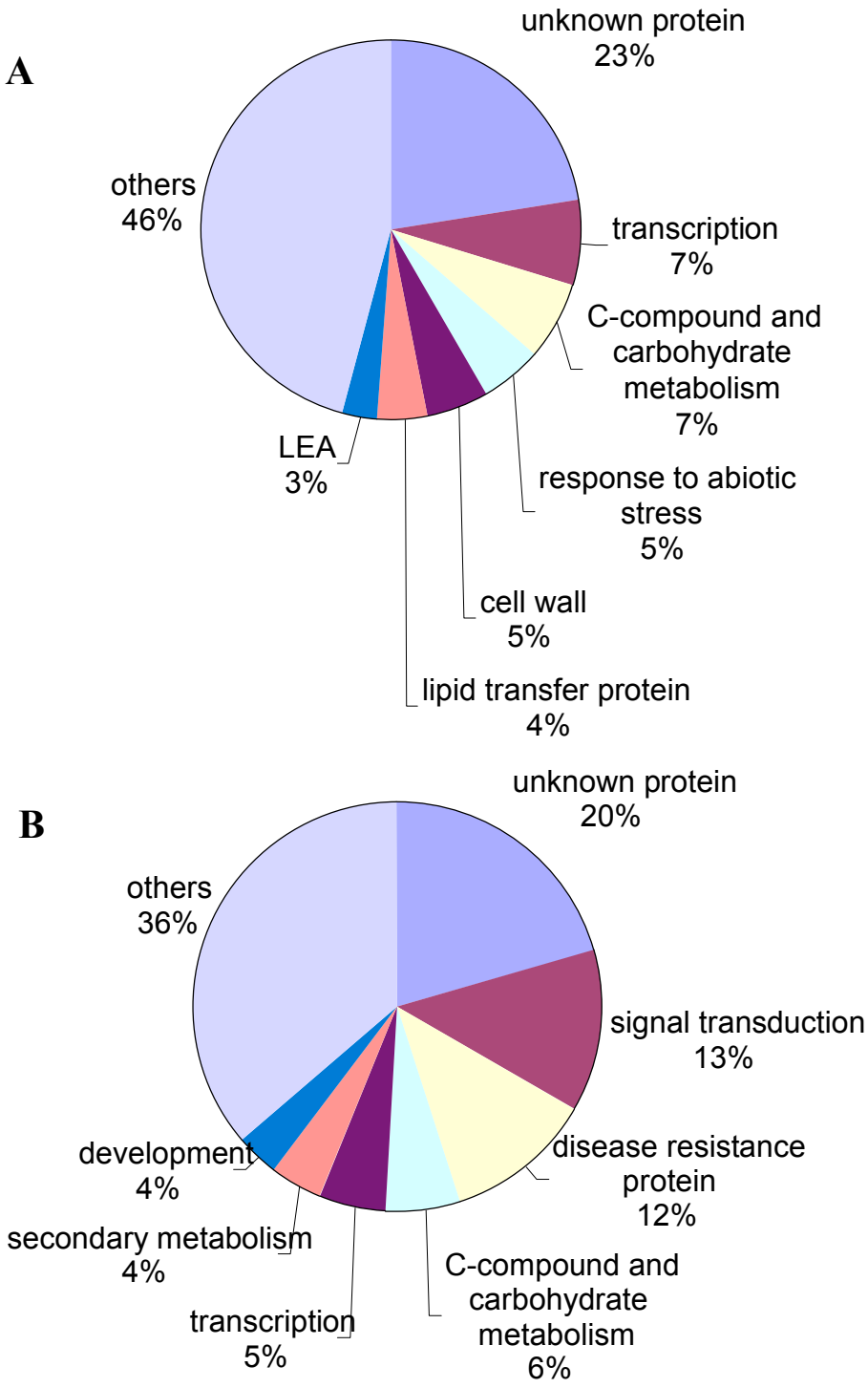

A
unknown protein
23%
transcription
7%
C-compound and
carbohydrate
metabolism
7%
response to abiotic
stress
5%
cell wall
5%
lipid transfer protein
4%
LEA
3%
others
46%
B
unknown protein
20%
signal transduction
13%
disease resistance
protein
12%
C-compound and
carbohydrate
metabolism
6%
transcription
5%
secondary metabolism
4%
development
4%
others
36%

Figure 3. classification of 151 genes up regulated by 25 mM trehalose + 10 mM validamycin A more than two fold and more than four fold by 20 mM abscisic acid (**A**) and 177 genes up regulated by 25 mM trehalose + 10 mM validamycin A more than four fold but not more than two fold by 20 mM abscisic acid (**B**). Genes unable to form a class > 2% are displayed as "others". mRNA expression of the genes was analyzed with microarray techniques using the ATH1 Affymetrix GeneChip. The classification was performed using the TAIR and the MIPS databases.

AtNCED3, a caroteinoid dioxygenase gene involved in ABA biosynthesis (Finkelstein and Christopher, 2002) was up regulated 13 fold. *ABA1* and another caroteinoid dioxygenase *AtNCED1* are not affected. The genes known to be responsible for the further downstream ABA metabolism were marginally up regulated (between 1.2 and 1.7 fold). Regarding ABA biosynthesis genes ABA treated seedlings showed a similar expression pattern as the plants treated with tre/val. Some changes could also be observed after man treatments and are therefore probably caused by osmotic effects (data not shown). These results indicate that tre/val application could lead to *de novo* synthesis of ABA and subsequently triggering a regulation of ABA regulated genes. In summary, two sets of genes were emerging: a first set that was induced by both tre/val and by ABA and a second set that was up regulated by tre/val but not by ABA (Tables I & II). From the first set the genes up regulated two fold or more by tre/val and four fold or more by ABA and from the second set the genes up regulated four fold or more by tre/val but not up regulated more than two fold by ABA were classified using the TAIR (http://www.arabidopsis.org) and the MIPS (http://mips.gsf.de/proj/thal/db/index.html)

databases. The classification of the first set of genes revealed the following predominant functional categories: “unknown protein” (22.5% of the genes classified in total), “transcription” (7.3%), “carbohydrate metabolism” (6.6%), “response to abiotic stress” (5.3%), “cell wall” (5.3%), lipid transfer protein” (4.0%), “LEAs (late embryonic abundant proteins)” (3.3%) and “others” (45.7%). The classification of the genes of the second set revealed the following functional categories: “unknown protein” (20.5%), “signal transduction” (12.9%), “disease resistance protein” (11.7%), “carbohydrate metabolism” (5.8%), “transcription” (5.3%), “secondary metabolism” (4.1%), development” (3.5%) and “others” (36.3%) (Fig. 3). The strong presence of “disease resistance proteins” in set two could indicate that trehalose can act as an elicitor in plants. For verification of the data genes up regulated more than two fold by tre/val were compared with the expression results of an 8K Affymetrix chip that was performed independently. The treatment not measured with the 8K chip is ABA. Generally the expression data of the same gene from both chips were consistent (Tables I & II, Appendix I).

Selected genes that were highly up regulated by tre/val and ABA or by tre/val but not by ABA were checked for expression by quantitative PCR. Qualitatively the values of the whole genome chip were reproducible, whereas the fold induction of genes measured by real time PCR was with the exception of one case higher than measured with the array (Table III).

		Fold induction			
		ABA	ABA	tre/val	tre/val
Gene	Description	chip	real-time	chip	real-time
AT3G02480	unknown protein, similar to pollen coat protein from *B. oleracea*	141.0	1600.2	109.5	132.5
AT5G59320	nonspecific lipid-transfer protein precursor-like	182.0	2534.4	83.7	254.9
AT5G59310	nonspecific lipid-transfer protein precursor-like	264.2	31741.4	63.4	879.7
AT1G52690	late embryogenesis-abundant protein, putative	96.1	2040.5	40.2	104.4
AT5G15500	putative protein	47.7	330.9	26.7	24.1
AT2G42540	cold-regulated protein cor15a precursor	49.3	140.5	26.5	35.5
AT1G35910	trehalose-phosphatase, putative	1.6	7.8	16.2	49.3
AT4G39210	glucose-1-phosphate adenylyltransferase (*ApL3*)	2.1	21.5	6.2	18.8
AT2G21590	putative ADP-glucose pyrophosphorylase large subunit	1.1	2.4	1.9	4.6

Comparison of fold inductions of selected genes from the set of genes up regulated by tre/val and by ABA upon ABA and tre/val treatment

		Fold induction			
		ABA	ABA	tre/val	tre/val
Gene	Description	chip	real-time	chip	real-time
AT1G43910	unknown protein, belongs to AAA-type ATPase family	-1.5	2.5	94.8	651.5
AT1G21520	hypothetical protein	-1.1	2.3	68.5	396.2
AT5G13320	auxin-responsive - like protein	1.9	1.4	61.7	223.3
AT3G60420	putative protein	1.4	2.6	57.4	90.9
AT3G56400	*AtWRKY70*	-1.3	0.3	36.2	38.6

Comparison of fold inductions of selected genes from the set of genes up regulated by tre/val but not by ABA upon ABA and tre/val treatment

		Fold induction			
		ABA	ABA	tre/val	tre/val
Gene	Description	**Chip**	**Real-time**	**Chip**	**Real-time**
AT4G40040	*Histone H3*	1.4	1.8	1.2	1.1

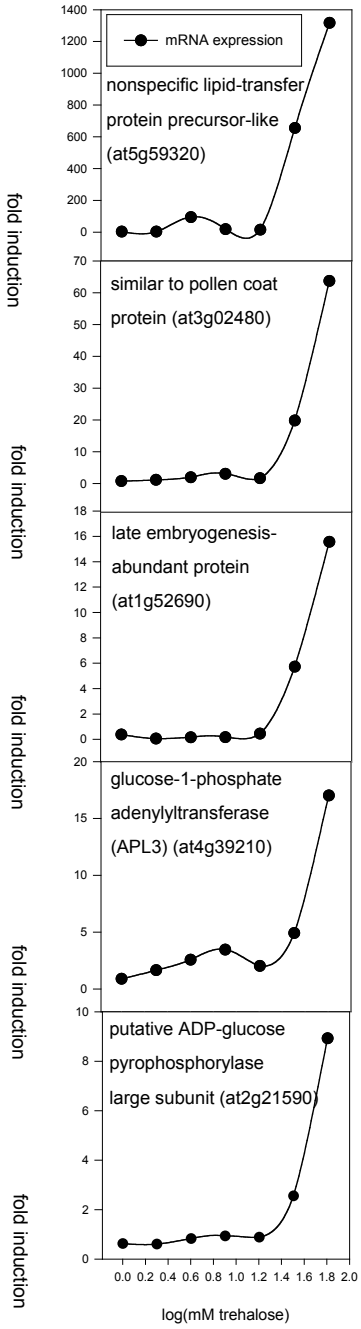
Comparison of mRNA expression levels of the *Histone H3* that was used to standardize all expression values

Table III. Comparison of mRNA levels of selected genes in Col-0 wild type plants treated with 20 mM abscisic acid (ABA) and 25 mM trehalose + 10 mM validamycin A (tre/val) obtained by microarray (chip) and by real-time PCR (real time) analysis

respectively. The expression of Histone H3 (at4g40040) was used to standardize all expression values. For calculation of fold inductions the standardized expressions were compared to thouse of water controls.

Effects of different trehalose concentrations on gene expression

Since tre/val treatment of Arabidopsis seedlings causes strong alterations in gene expression, we were interested in the trehalose concentrations necessary, in combination with validamycin A, to trigger these changes. To address this question a trehalose concentration series was performed. 12 days old Arabidopsis seedlings were incubated for 48 h on different trehalose concentration (1, 2, 4, 8, 16, 32 and 64 mM) whereas 10 μM validamycin A was supplied to all treatments. From the tre/val and ABA induced set of genes (set one) and the tre/val exclusively induced set (set two), nine respectively five genes were selected for quantification of mRNA (Fig. 4 and 5). In set one 1 mM trehalose changed the mRNA level of the genes analyzed between 0.4 and 2.7 fold in expression. The genes at5g59310 and at5g59320 had a peak of mRNA expression at a trehalose concentration of 4 mM, whereas the genes at3g02480, at5g15500, at4g39210 and at2g21590 peaked at 8 mM trehalose. The genes at3g42540 and at1g52690 had no peak at 4 or at 8 mM trehalose. At trehalose concentrations higher than 16 mM the expression level of all genes tested rose constantly in a way that it could be supposed that the expression would continue to rise if more than 64 mM trehalose would be added (Fig. 4). In set two 1 mM trehalose induces the mRNA level of the analyzed genes between 1.5 and 5.7 fold. At3g60420 and at3g56400 revealed a first peak of expression at a trehalose concentration of 4 mM and at1g21520 at a trehalose concentration of 8 mM. The other genes did not show an expression peak but rather a lag phase or did not react at all at these concentrations. At trehalose concentrations higher than 16 mM the expression level of all genes raised constantly like in set one. (Fig. 5).


mRNA expression
nonspecific lipid-transfer protein precursor-like (at5g59320)
similar to pollen coat protein (at3g02480)
late embryogenesis-abundant protein (at1g52690)
glucose-1-phosphate adenylyltransferase (APL3) (at4g39210)
putative ADP-glucose pyrophosphorylase large subunit (at2g21590)
fold induction
log(mM trehalose)

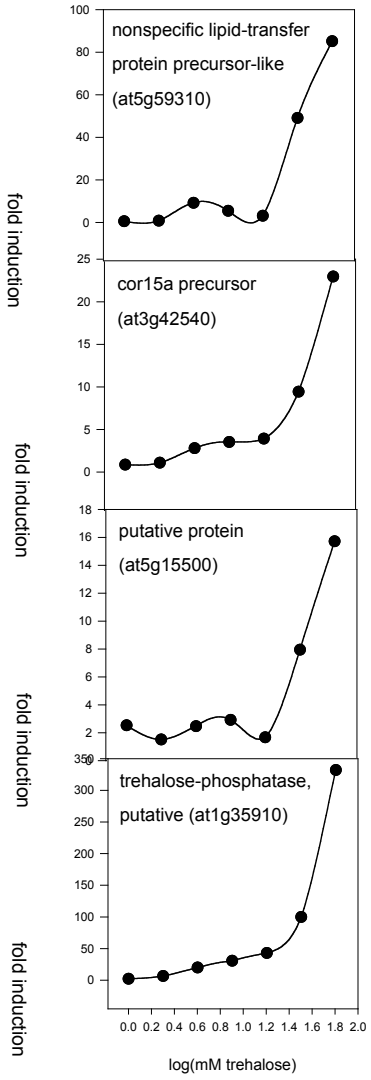
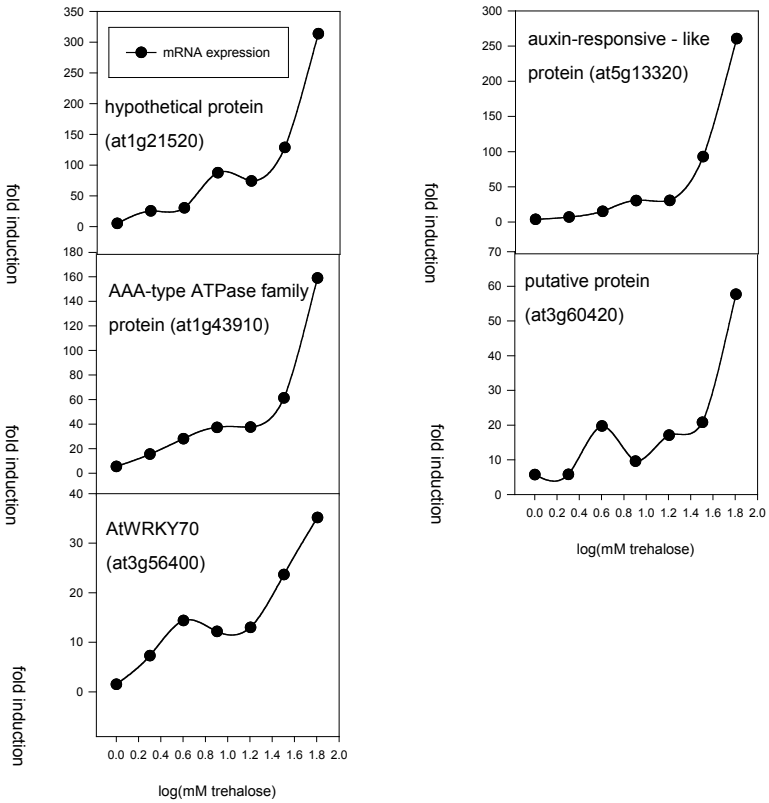

nonspecific lipid-transfer protein precursor-like (at5g59310)
cor15a precursor (at3g42540)
putative protein (at5g15500)
trehalose-phosphatase, putative (at1g35910)
fold induction
log(mM trehalose)

Figure 4. Fold induction of mRNA of selected genes induced by TV and by ABA in Arabidopsis seedlings treated with different concentrations of trehalose (0, 1, 2, 4, 8, 16, 32 and 64 mM) plus 10 mM validamycin A. mRNA expression of genes were standardized with the expression of the histone H3 gene (at4g40040). For the calculation of the fold induction the standardized expressions were compared to a water treated control. The mRNA analysis was performed by quantitative PCR.

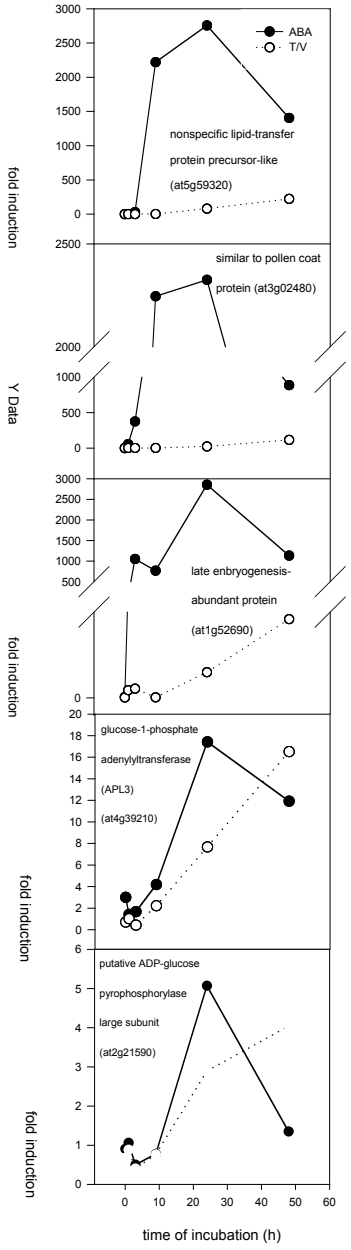
Figure 5. Fold induction of mRNA of selected genes induced by TV but not by ABA in Arabidopsis seedlings treated with different concentrations of trehalose (0, 1, 2, 4, 8, 16, 32 and 64 mM) plus 10 mM validamycin A. mRNA expression of genes were standardized with the expression of the histone H3 gene (at4g40040). For the calculation of the fold induction the standardized expressions were compared to a water treated control. The mRNA analysis was performed by quantitative PCR.


mRNA expression
hypothetical protein
(at1g21520)
AAA-type ATPase family
protein (at1g43910)
AtWRKY70
(at3g56400)
auxin-responsive - like
protein (at5g13320)
putative protein
(at3g60420)
fold induction
log(mM trehalose)

In general the qualitatively expression pattern of the different genes of both sets after trehalose application were similar. In both sets of genes two phases of gene induction could be observed. At trehalose concentrations between 1 and 16 mM some genes showed a peak of expression at a trehalose concentration of 4 or 8 mM, whereas the expression of other genes raised constantly with higher tre concentrations or was not affected. At trehalose concentrations higher than 16 mM, the expression of genes raised uniformly on both sets. These results indicate that trehalose levels exceeding the suggested physiological content by far are necessary to change gene expression and the regulation of expression of all genes tested is similar.

Changes in gene expression in Arabidopsis seedlings treated with tre/val or ABA during 48 hours

To learn about the kinetics of gene expression response of Arabidopsis seedlings followed by tre/val and ABA treatment a time course was performed. 12 days old seedlings were collected after 0, 1, 3, 9, 24 and 48 hours of treatment. tre/val and ABA treatments were compared to a water control. In set one (tre/val and ABA up regulated) generally the point of up regulation of the genes is achieved earlier by ABA than by tre/val treatment. The fold induction of expression by ABA peaked between 9 and 24 hours. After 24h of incubation the steady state level of the mRNA of the genes was decreasing. At 48 hours of ABA treatment the expression was still higher in ABA treated than in tre/val treated plants with the exception of the weak induced genes at4g39210 and at2g21590. In tre/val treated plants the induction of expression begins at approximately 9 hours after the start of incubation and does not stop during the whole incubation time


ABA
T/V
nonspecific lipid-transfer
protein precursor-like
(at5g59320)
similar to pollen coat
protein (at3g02480)
late enbryogenesis-
abundant protein
(at1g52690)
glucose-1-phosphate
adenylyltransferase
(APL3)
(at4g39210)
putative ADP-glucose
pyrophosphorylase
large subunit
(at2g21590)
fold induction
Y Data
time of incubation (h)

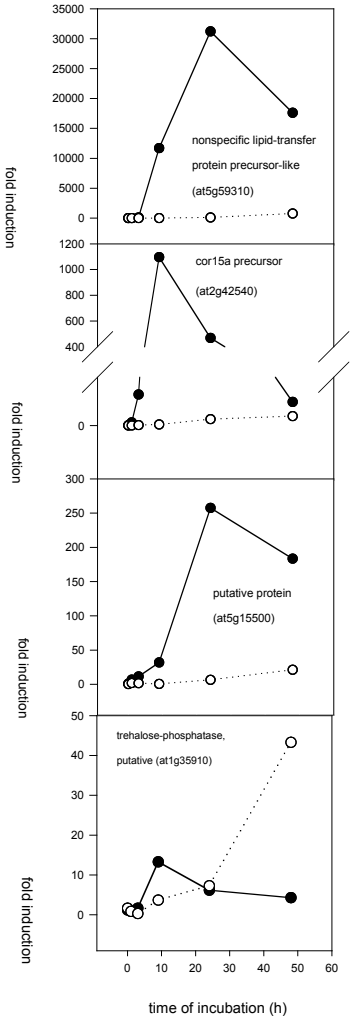
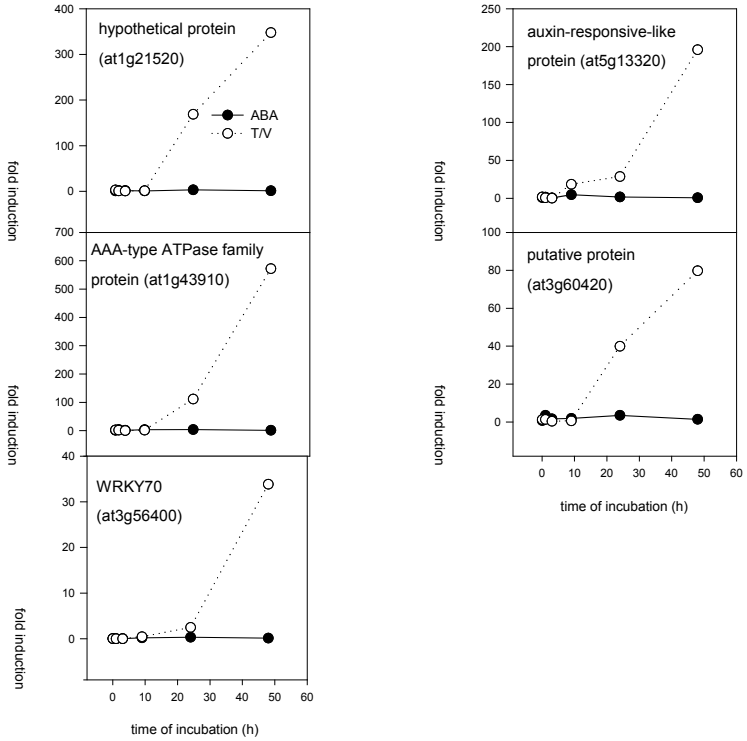

nonspecific lipid-transfer
protein precursor-like
(at5g59310)
cor15a precursor
(at2g42540)
putative protein
(at5g15500)
trehalose-phosphatase,
putative (at1g35910)
fold induction
time of incubation (h)

Figure 6. mRNA expression of selected genes of the set induced by TV and ABA at different time points (0, 1, 3, 9, 24 and 48h). Arabidopsis seedlings were treated with TV, ABA and water (as a control). mRNA expression of genes were standadized with the expression of the Histone H3 gene (at4g40040). For the calculation of the fold induction the standardized expressions were compared to the water control.

Figure 7. mRNA expression of selected genes of the set induced by TV but not ABA at different time points (0, 1, 3, 9, 24 and 48h). Arabidopsis seedlings were treated with TV, ABA and water (as a control). mRNA expression of genes were standadized with the expression of the Histone H3 gene (at4g40040). For the calculation of the fold induction the standardized expressions were compared to the water control.


hypothetical protein
(at1g21520)
ABA
T/V
AAA-type ATPase family
protein (at1g43910)
WRKY70
(at3g56400)
auxin-responsive-like
protein (at5g13320)
putative protein
(at3g60420)
fold induction
time of incubation (h)

tested. It is likely that the peak of expression is reached after 48 hours of incubation (Fig. 6). In set two (tre/val up only) the ABA treatment did not change much the expression during the whole time of incubation, with the putative trehalose-phosphatase (at1g35910) as an exception. The expressions of the genes in the tre/val treated plants started to rise above the one in ABA treated plants at nine hours of treatment, in the case of at1g35910 after 24 hours of incubation. tre/val treatment resulted in a constant increase after 9h of induction in mRNA level that seemed not to peak at 48 hours of incubation (Fig. 7). Qualitatively the expression curves of the genes of both sets looked similar in the plants treated with tre/val.

Effects of trehalose on gene expression in the ABA mutants *aba1* and *abi2-1*

To test whether ABA signalling or ABA itself are part of the signal pathway(s) by which trehalose modulates gene expression, mRNA transcripts of genes in tre/val treated ABA mutants *aba1* and *abi2-1* were investigated. *aba1* mutants (having Ler background) are not able to produce more than 5% of the endogenous ABA content of a wild type plant due to a mutation in *ABA1* (at5g67030), a zeaxanthin epoxydase necessary for ABA anabolism (Koornneef et al., 1982). *abi2-1* mutants (having Col-0 background) on the other hand are deficient in function of the phosphatase ABI2 in the ABA responsive pathway (Koornneef et al., 1984). *aba1* mutants were not able to suppress efficiently the induction of nine genes up regulated by tre/val and ABA (set one) or five genes up regulated by tre/val but not by ABA (set two) after tre/val application.

For set one the *aba1* mutant revealed a mixed expression pattern. By tre/val treatment three genes were more up regulated in *aba1* mutants compared to the wild type whereas

		Fold induction			Fold induction		
Gene	Description	ABA	tre/val	line	ABA	tre/val	line
at3g02480	unknown protein, similar to pollen coat protein from *B. oleracea*	8.0	53.7	wt	46.7	5.3	wt
		12.6	24.2	*aba1*	5.3	1.9	*abi2-1*
at5g59320	nonspecific lipid-transfer protein precursor-like	1240.5	60.8	wt	1473.7	66.3	wt
		2246.0	69.8	*aba1*	521.0	94.7	*abi2-1*
at5g59310	nonspecific lipid-transfer protein precursor-like	7416.0	331.3	wt	38652.5	1124.3	wt
		28071.6	82.8	*aba1*	635.6	463.1	*abi2-1*
at1g52690	late embryogenesis-abundant protein, putative	428.5	16.1	wt	219.5	2.3	wt
		1878.2	6.3	*aba1*	48.7	1.2	*abi2-1*
at5g15500	putative protein	35.7	6.7	wt	180.2	8.5	wt
		148.7	6.3	*aba1*	1.2	0.6	*abi2-1*
at2g42540	cold-regulated protein cor15a precursor	176.6	16.8	wt	199.7	64.1	wt
		35.1	12.3	*aba1*	30.2	25.9	*abi2-1*
at1g35910	trehalose-phosphatase, putative	8.0	53.7	wt	10.5	29.9	wt
		12.6	24.2	*aba1*	3.9	120.7	*abi2-1*
at4g39210	glucose-1-phosphate adenylyltransferase (*ApL3*)	44.1	28.2	wt	7.7	618.7	wt
		18.5	61.4	*aba1*	2.7	134.8	*abi2-1*
at2g21590	putative ADP-glucose pyrophosphorylase large subunit	14.6	4.9	wt	0.7	1.5	wt
		10.4	6.1	*aba1*	1.3	5.5	*abi2-1*

Fold inductions in *aba1* and *abi2-1 mutants* and wt lines of genes up regulated by tre/val and ABA upon ABA and tre/val treatment. Annotation of the genes are according to TAIR and MIPS databases.

		Fold induction			Fold induction		
Gene	Description	ABA	tre/val	line	ABA	tre/val	line
at1g43910	unknown protein, belongs to AAA-type ATPase family	0.9	58.6	wt	0.7	166.9	wt
		1.0	42.2	*aba1*	0.6	78.3	*abi2-1*
at1g21520	hypothetical protein	0.7	37.6	wt	2.5	207.9	wt
		1.3	25.1	*aba1*	0.9	6.7	*abi2-1*
at5g13320	auxin-responsive - like protein	0.8	84.3	wt	1.0	257.6	wt
		0.7	32.3	*aba1*	0.7	272.8	*abi2-1*
at3g60420	putative protein	0.8	30.0	wt	0.9	113.7	wt
		0.4	11.1	*aba1*	1.0	40.3	*abi2-1*
at3g56400	*AtWRKY70*	0.7	78.0	wt	0.2	30.7	wt
		0.9	56.4	*aba1*	0.2	35.0	*abi2-1*

Fold inductions in *aba1* and *abi2-1* mutants and wt lines of genes up regulated by tre/val but not by ABA treatment upon ABA and tre/val treatment. Annotation of the genes are according to TAIR and MIPS databases.

Table IV. mRNA expression of selected genes in *aba1* (*aba1*) and *abi2-1* (*abi2-1*) mutants and the corresponding wild types (wt) Ler and Col-0 treated for 48 hours with 20 mM abscisic acid (ABA) and 25 mM trehalose + 10 mM validamycin A (tre/val) respectively. mRNA expression of genes were standardized with the expression of Histon H3 (at4g40040). For the calculation of fold inductions the standardized expressions were compared to those of water controls. All expressions were measured by quantitative PCR.

six genes were expressed to a lower extend (Table IV A). In set two the *aba1* mutant treated with tre/val showed a lower expression level of all the genes compared to wild type plants (Table IV B), suggesting the lack of ABA makes the *aba1* mutant to be less susceptible to tre/val regarding the modulation of gene expression of genes up regulated by tre/val but not by ABA (set two). After tre/val treatment six genes from set one were expressed at lower levels and three genes were expressed at higher levels in the *abi2-1* mutants than in the wild type (Table IV A). In set two the treatment of tre/val resulted in a lower expression in *abi2-1* mutants of the tested genes compared with the wild type with the exception of at5g13320 and at3g56400 (Table IV). The results obtained by the *aba1* mutants showed that the *aba1* mutation is not able to suppress a positive regulation of genes upon tre/val treatment indicating that ABA is not necessary to mediate the signal for altering gene expression upon tre/val treatment. The *abi2-1* mutation on the other hand was able to suppress partially two thirds of the genes tested for mRNA expression suggesting at least for some genes up regulated by tre/val and ABA, ABI2-1 is involved in triggering induction of gene expression after tre/val treatment.

Discussion

Information about a possible physiological function of trehalose in plants is scarce. In previous studies it has been shown that exogenously trehalose application to Arabidopsis plants changes gene expression and modulates expression in synergy with sucrose (Wingler et al., 2000; Fritzius et al., 2001). Attempts to overproduce trehalose in tobacco by heterologous expression of the trehalose synthesizing enzymes result in altered development of the roots and leaves and stunted growth (Romero et al., 1997). In rice heterologous expression of a fusion protein consisting of the *E. coli* trehalose biosynthetic genes (*otsA* and *otsB*) reveals normal grow and shows enhanced tolerance towards drought. Because the proteins are fused to each other and therefore trehalose-6-phosphate (T6P) is not likely to accumulate, rather trehalose than trehalose-6-phosphate causes the enhancement of tolerance towards abiotic stresses (Garg et al., 2002; Jang et al., 2003). T6P in plants is about to reveal as a crucial factor in carbohydrate metabolism, growth and development (Eastmond et al., 2002; Eastmond and Graham, 2003; Schluepmann et al., 2003; Schluepmann et al., 2004).

Here we performed transcription profiling assays analyzing plants treated with trehalose and validamycin A, a potent trehalase inhibitor in plants (Muller et al., 2001), to get an insight of what the possible function of trehalose could be and through which mechanism trehalose is changing gene expression. The expression profiling experiment revealed that almost the third part of genes enhanced by tre/val treatment is also up regulated by ABA. Therefore, a genetic approach using mutants altered in ABA synthesis and response was used for analyzing this signaling mechanism.

Application of tre/val leads to an induction of plant defense genes and genes that are also up regulated by ABA

In wild type plants, tre/val application causes a regulation of 2277 gene transcripts (Fig.1). In order to look for similarities and differences in mRNA expression between tre/val treated plants and plants fed with other sugars, the genes regulated by tre/val treatment were compared to the ones regulated by tre or suc or glc. Also included was a comparison to ABA treatment because of the involvement of both trehalose metabolism and ABA in abiotic stress management and sugar sensing in plants. Tre/val affected the expression of the plant by far more than tre did in terms of amount of genes altered and quantitative expression changes suggesting val successfully reduced trehalase activity enabling trehalose *per se* to enter the plant. Suc treatment shared more than a quarter of its regulated genes with tre/val treatment. This could be explained by the fact that suc as well as tre/val treatment exposes the plant to glc because of either invertase activity or insufficient trehalase inhibition by val respectively. On the other hand it could be that both molecules can trigger common signals cascades leading to similar alteration of expression of the same genes. Suc effects shared with tre/val effects are almost all shared also by glc treatment. However, since suc can also be built up by glc it cannot be ruled out that tre/val and suc share genes that are specifically regulated by suc and not by glc.

ABA and tre/val treatment shared 746 regulated genes (Fig.2). The 13-fold induction of *AtNCED3*, a crucial gene involved in ABA synthesis would support the theory that tre/val application leads to *de novo* synthesis of ABA in plants. This could explain the finding that plants over expressing trehalose biosynthesis genes are more resistant to drought compared to wild type plants (Holmström et al., 1996; Garg et al., 2002). This hypothesis

is further confirmed by the fact that several genes known to contribute to ABA synthesis (*AtNCED3* (at3g14440), *ABA2* (at1g52340), *ABA3/LOS5* (at1g16540), *CNX2* (at2g31950)) are also up regulated between 1.2 and 1.7 fold by tre/val. There is also a batch of 1199 genes regulated by ABA but not affected in expression by tre/val. This does not support the hypothesis of enhanced ABA production upon tre/val treatment, since in that case the entire set of genes regulated by ABA should also be regulated by tre/val.

Three sets of genes are emerging. A first set of genes regulated by tre/val and ABA, a second set of genes regulated by tre/val but not by ABA and a third set of genes regulated by ABA but not by tre/val.

To address the question what role trehalose might have in plants we decided to look at the genes positively regulated by tre having the focus on genes that are up regulated by both tre/val and ABA (set one) and genes that are up regulated uniquely by tre/val (set two). Both sets revealed genes classified "carbohydrate metabolism" (6.6 % and 5.8% respectively) and "transcription" (7.3% and 5.3% respectively) (Fig.3). The regulation of genes involved in transcription is no surprise considering alteration of expression of 2277 (tre/val) and 2040 (ABA) genes respectively. The induction of two T6P phosphatases (TPP) (at1g35910 and at4g22590) in set two is consistent with the theory that trehalose application leads to an elevated T6P level to which the plant reacts with induction of TPP expression in order to reduce the T6P content (Schluepmann et al., 2004).

Exclusively in set one the genes of the category "response to abiotic stress", "cell wall", "LEAs" (late embryo abundant proteins), and "lipid transfer proteins" are up regulated (table I, fig. 3A). Only in the second set of genes (unique tre/val) were genes classified to

"signal transduction", "disease resistance proteins", "secondary metabolism" and "development" (Table II, fig. 3B). Among the genes involved in signal transduction there are 14 kinases and putative kinases pointing towards an activation of phosphorylation cascades by tre/val. Among the signal transduction genes are the wall-associated kinase 1 (*WAK1*) (at1g21250) and the calmodulin-like calcium binding protein (*CaBP-22*) (at2g41090) which were found before after trehalose feeding (Schluepmann et al., 2004). Among the disease resistance proteins are six WRKY domain containing genes. One such transcription factor that is induced by tre/val treatment, *AtWRKY70,* has been shown to play a crucial role in regulation of the jasmonate- and salicylate-mediated gene expression (Li et al., 2004). The induction of the disease related genes, cell wall metabolizing genes and those involved in secondary metabolism could be put in the context of plant microbe interactions. Various microbes invading plants are known to contain high amounts of trehalose and some plants react by enhancing their trehalase activity (Muller et al., 2001; Brodmann et al., 2002). Trehalose could therefore act as an elicitor in plants.

A trehalose concentration series in combination with 10 μM validamycin A revealed that a concentration of 2-4 mM trehalose applied exogenously is necessary to change gene expression (Fig. 4 and 5). The expression patterns of the genes tested from the two different sets described above after the increasing tre concentration treatment were qualitatively similar suggesting all the genes tested have at least one factor in common that is necessary for triggering gene response after trehalose application. The concentration of trehalose applied to the plant necessary for altering gene expression exceeds the physiological content measured in Arabidopsis Col-0 inflorescences after val

application approximately by a factor of 100. It is possible that such a high concentration of tre has to be added to get a response because of the ubiquitous trehalase activity of the plant.

ABA induces ABA inducible genes faster than tre/val treatment

To get more information about kinetics of the response of gene expression after tre/val or ABA treatment a time course series was performed. The genes of set one (tre/val and ABA up) are much faster induced and reached a higher induction by ABA than by tre/val treatment. After 48 hours the expression of the genes of set one after ABA treatment is already over its peak and starts to decline whereas the expression of the genes after tre/val treatment is at that time at the highest point measured (Fig. 6). After nine hours the genes of set two (tre/val up only) are expressed to a higher fold level by tre/val treatment than by ABA treatment and subsequently rise constantly (Fig. 7). As expected, the genes not induced by ABA as seen on the array are also not changed in expression during 48 hours of treatment (Fig. 7). It is possible that the response is slower and less strong by tre/val than by ABA treatment because tre/val treatment is triggering first ABA synthesis and ABA is then subsequently modulates gene expression. However, it cannot be excluded that other factors independent from ABA are inducing the genes. Since all the genes tested of set one and two show a similar induction curve after tre/val treatment it is likely that trehalose treatment triggers all the genes using the same transduction system. This would support the hypothesis that the change of expression by tre/val of all genes tested is ABA independent. The slow induction of gene expression upon tre/val treatment indicates that it takes a long time for tre to enter the plant or altering of gene expression

needs a prerequisite that has to be produced. One possible candidate could be T6P since it has been shown that application of exogenous 100 mM tre leads to an accumulation of T6P in the plant (Schluepmann et al., 2004).

Induction of ABA inducible genes by tre/val is not dependent on ABA but dependent on ABI2-1

Tre/val application enhanced the expression of genes inducible by tre/val and ABA treatment. To test whether ABA is necessary for this effect to happen, we decided to use the ABA deficient mutant *aba1* (Koornneef et al., 1982). This mutant is impaired in epoxy-carotenoid biosynthesis, and produces only a fractional amount of the physiological ABA content of wild type plants (Rock and Zeevaart, 1991). Selected genes that are inducible by tre/val and ABA (set one) and genes that are only up regulated by tre/val but not by ABA (set two) were analyzed for mRNA expression. The results showed that, in *aba1* plants, thus in absence of ABA, tre/val is able to induce genes inducible by tre/val and ABA (set one) (Table IV A). It seemed that for the genes tested of set one the removal of ABA is not sufficient to repress induction by tre/val. This would supports the hypothesis that tre/val application does not elicit its response via ABA synthesis. One factor of the ABA biosynthetic pathway, 9-cis-epoxy-carotenoid dioxygenase (*ATNCED3*), is induced by tre/val. But this enzyme acts downstream of the *aba* locus in the ABA synthetic pathway (Finkelstein and Christopher, 2002; Seo and Koshiba, 2002) and is unlikely to be able to compensate for the diminished zeaxanthin epoxidase (ZEP) activity in *aba1* plants. The scenario emerges that the genes are induced by affection of the ABA signaling pathways downstream of the ABA perception site. The

response to tre/val treatment seems to be similar in wild type plants as in mutants. The genes of set two are all up regulated as well in *aba1* plants, indicating that this set is also ABA independent in the expression response. The tre/val treatment enhances the induction of all genes tested of set two in Ler wild type plants over the expression in *aba1* plants (Table IV B). These modulation of expression in the *aba1* mutant could indicate that ABA is interplaying with the pathway triggered by tre/val or could be due to pleiotropic effects.

The *abi2-1* mutant is mutated in a protein phosphatase 2C (Leung et al., 1997) required for ABA responsiveness (Koornneef et al., 1984) in processes such as seed dormancy, stomatal closure and growth inhibition (Finkelstein and Christopher, 2002). This plant was used to test whether the expression response by tre/val treatment includes the ABA signal transduction pathway mediated by *ABI2*. The results show that an impaired function of *ABI2* can partially suppress induction of gene expression after tre/val treatment. Six genes of set one are suppressed by tre/val whereas three are induced (table IV A). In set two tre/val treatment raises the expression of the genes tested in Col-0 wild type plants over the mutants with the exception of gene "auxin-responsive-like protein" (at5g13320) and *AtWRKY70* (at3g56400) (Table IV B). ABA treatment revealed that all the genes of set one up regulated more than two fold in wild type are suppressed in *abi2-1* mutants. Since it has been shown that ABI2 forms a complex with ABI1, PROTEIN KINASE SALT-SENSITIVE3 (PKS3) and the Ca^{2+} binding protein SCaBP5 which is negatively regulating the ABA signal relays downstream of cytosolic Ca^{2+} (Guo et al., 2002), the possible influence of the pathway triggered by tre/val treatment is taking place at the level of or upstream of cytosolic Ca^{2+}.

These results suggest that the triggering of induction by tre/val of all genes tested of both sets is not dependent on ABA. We could also show by expression analysis of set one that ABI2 is part of the signal cascades triggered by tre/val. Recapitulating the results indicates that the cascades triggered by tre/val influence the ABA signaling pathway at a unknown point between the ABA binding site and the action site of ABI2.

It will take more expression analysis of mutants impaired for genes which products act downstream of ABA in the ABA responsive pathway to pinpoint the site of affection of this signaling cascade by tre. The same method could be used to unveil the mechanism by which tre regulates gene expression independent from the ABA responsive pathway.

Materials and methods

Plant material

Seeds of wild type Arabidopsis plants (ecotype Col-0 and Ler) and of abscisic acid mutants (*aba1* (Koornneef et al., 1982) and *abi2-1* (Koornneef et al., 1984)) were obtained from the Nottingham Arabidopsis Stock Center (Nottingham, UK). The plants were grown on one-half-strength Murashige and Skoog medium without sucrose (Sigma-Aldrich, Buchs, Switzerland) solidified with 1% (w/v) agar. The agar plates were oriented vertically and were incubated in a daily cycle of 18 h of light (80 μmol $m^{-2}s^{-1}$) at 22 °C and 6 h of darkness at 18 °C. After 12 d of growth, the seedlings were transferred for two days to the medium containing the specific treatments.

Reverse transcriptase reaction

Total RNA was extracted from shoots of the Arabidopsis seedlings and treated with DNAse using the NucleoSpin RNA Plant kit (Macherey-Nagel, Oensingen, Switzerland). One microgram of RNA was reverse transcribed using AMV reverse transcriptase, AMV RT buffer (Promega, Madison, USA) and oligo(dT). The mixture was let at room temperature for ten minutes, heated up to 42°C for 15 minutes and to 56°C for two minutes. Boiling for five minutes stopped the reaction. For quantitative real time PCR the solution was diluted with water 1:4.

Quantitative PCR

1 µl of the diluted cDNA was subjected to the quantitative PCR reaction. The PCR reaction was performed using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). The concentration of the two primers was 2.5 µM for each. All calculated copy numbers of the tested genes were divided through the copy numbers of *Histon H3* (at4g40040) measured in the same simple. Expression of *Histon H3* was used as a constitutive control. The ratio was subsequently used to calculate a fold induction by comparison with the ratio of a control. The genes, primers, and fragment sizes were the following: hypothetical protein (at1g21520) 5'-GTGTGATCCCAAAGGAGAGAGAG-3' and 5'-TCATGGTCACCGTCACCGTCA-3' (127 bp); unknown protein, belongs to AAA-type ATPase family (at1g43910) 5'-CAGCTCATGGCGAGTAAGAACG-3' and 5'-CCTCTTCCTCCACATAGAATC-3' (106 bp); *ApL3* (at4g39210) 5'-ATCTCAAGACCGCCGTGGTGAT-3' and 5'-GGTGATCGGCTGATCACTCTTA-3' (162 bp); putative ADP-glucose pyrophosphorylase large subunit (at2g21590) 5'-GGCCAGAGGAAGGATTCTACAT-3' and 5'-TCAGATTGTTGCTCGGTGGACT-3' (123 bp); *Histon H3* (at4g40040) 5'-CAGGAGGCTGCTGAAGCATACC-3' and 5'-TAAGCACGTTCTCCTCTGATCC-3' (131 bp); putative protein (at3g60420) 5'-GACGCAACTAAGTACGTGGTAG-3' and 5'-GGCGGCTTACAACTACATGATC-3' (160 bp); auxin-responsive-like protein (at5g13320) 5'-GAGGAGAAGGAGACGGCACAGT-3' and 5'-ATCAAGCGACTCCTCCATTACC-3' (81 bp); unknown protein, similar to pollen coat protein from *B. oleracea* (at3g02480) 5'-CCGGCATGAACAAAAGCCACTA-3' and 5'-GAAAAGAAGTCCAAGGTGCTTGC-3' (46 bp); non specific lipid-transfer protein precursor-like (at5g59320) 5'-

AAGTCTAGCCTCTGGCCTTCCT-3' and 5'-AACGCCAAAACGACGACGTAAGC-3' (118 bp); non specific lipid-transfer protein precursor-like (at5g59310) 5'-CCATCTCCACGAGCACCAACTG-3' and 5'-GGCAAATGATGTCGTTATTCCCCAC-3' (64 bp); late embryogenesis-abundant protein, putative (at1g52690) 5'-CTACTGATGCGGTGAAGCACA-3' and 5'-CGAGTAGTGGTCGTAGTAGTAG-3' (99 bp); cold-regulated protein cor15a precursor (at2g42540) 5'-GGTAAAGCAGGAGAGGCTAAGG-3' and 5'-GTGACGGTGACTGTGGATACCAT-3' (202 bp); putative protein (at5g15500) 5'-GGGAATGGGTGCAGTCTAAGAG-3' and 5'-CACATACCGATTAGGCGCAACA-3' (112 bp); *AtWRKY70* (at3g56400) 5'-CATGGTTCGTCCACGGAGAAT-3' and 5'-GACGTAACTGGCCTGATGATG-3' (87 bp); trehalose-phsophatase, putative (at1g35910) 5'-AACTCGTGCAGAAGCTAGAGG-3' and TTCATCCTCCTCATCGGACTC-3' (137 bp). 40 cycles were run for each quantitative PCR assay.

RNA preparation and processing for GeneChip analysis

Isolated total RNA samples were processed as recommended by Affymetrix (Affymetrix GeneChip Expression Analysis Technical Manual, Affymetrix, Inc.). In brief, total RNA was initially isolated using an RNeasy Plant Mini Kit (Qiagen). Total RNAs were quantified with a portion of the recovered total RNA adjusted to a final concentration of 1 μg μL^{-1}. All RNA samples were quality assessed before beginning target preparation/processing steps by running a small amount of each sample on an agarose gel. The following steps from RNA processing until the quantification and analysis of the

results were performed as described (Wang et al., 2003). RNA expression of different samples was quantified using the GeneChip® Arabidopsis ATH1 Genome Array containing probes designed to measure in over 24,000 gene sequences (Affymetrix Inc., U.S.) The array is based on information from the international Arabidopsis sequencing project (Initiative, 2000). Transcription changes more than two fold up or two fold down compared to the control are designated as regulated or differentially regulated; a convention that is used throughout the whole text.

Chapter 3:

General discussion

Sugars such as sucrose, glucose, and fructose not only are essential in plant metabolism, but also have signalling functions. It is, for example, well established that increased levels of glucose or sucrose, the end products of photosynthesis, repress photosynthetic gene expression in source leaves (Rolland et al., 2002). This feedback mechanism, achieved by the sugar signalling system, ensures a tight control of the plant's energy budget. This response of the cell to sugar levels is called sugar sensing. Sugar sensing can be defined as the interaction between a sugar molecule and a sensor protein in such a way that a signal is generated. This signal subsequently initiates signal transduction cascades that result in cellular responses such as altered gene expression and enzymatic activities (Smeekens, 2000). Since hexokinase appeared to be a very important component in hexose sensing in plants (Moore et al., 2003), hexose sensing is distinguished in a hexokinase-independent and a hexokinase-dependent system. Sucrose is sensed via a separate system. In order to integrate the sugar-induced cellular responses in a higher level (tissue, organ, organism), sugar sensing pathways interact with other sensing and signalling pathways (e. g. nitrogen metabolism and phytohormones) (Leon and Sheen, 2003).

Activated sugar sensors initiate a signal transduction cascade resulting in altered gene expression. The downstream components in the signalling pathways of sugar sensing are mostly unknown, it is generally believed that there is an involvement of protein Ser/Thr-kinases and protein Ser/Thr-phosphatases, calmodulin and Ca^{2+} (Smeekens, 2000).

An example of hexokinase independent sugar sensing is the Suc regulated expression of the 6-SFT promoter in barley (Nagaraj, 2004): Suc supply induces the formation of the storage carbohydrate fructan by induction of sucrose:fructan 6-fructosyltransferase (6-SFT), an important enzyme for fructan synthesis, of the level of gene expression (Nagaraj et al., 2001). The work presented here, using different protein kinase and protein phosphatase inhibitors, shows that this regulatory pathway includes protein Ser/Thr-kinases, the protein Ser/Thr-phosphatase 2A and probably some kinase activity inhabitable by genistein (GEN). GEN inhibits Tyr-kinases in certain animal systems. Interestingly, the promoter of 6-SFT is activated by Suc not only in barley but also in *Arabidopsis thaliana* and the inhibitors of protein kinases and protein phosphatases affect induction in similar ways. This finding indicates that the analysed pathway is conserved in both species.

Trehalose metabolism has the first time been connected to sugar sensing in the work of Wagner *et al.* 1986, which showed that trehalose induces enzymes of fructan synthesis in barley. Various other studies confirmed this connection. For example, it has been reported that exogenously applied trehalose induces sucrose synthase in soybean roots (Muller et al., 1998). Trehalose also enhances activity of the ADP-glucose pyrophosphorylase in Arabidopsis seedlings, leading to starch accumulation (Wingler et al., 2000). However, trehalose might not be the regulatory molecule as such. Based on the work of Schluepmann et al. 2004, it appeared the external supply of trehalose to the plant results not only in an elevated level of cytoplasmic trehalose, but also in an elevated level of cytoplasmic trehalose-6-phosphate (T6P) (Fig. 3, General introduction). This is thought to be caused by an inhibitory feedback of trehalose-6-phosphate phosphatase

(TPP) when intracellular trehalose levels are high. This finding has made it unclear, whether the effects observed upon trehalose feeding are a trehalose or T6P response. Recent experiments carried out with transgenic Arabidopsis plants expressing the trehalose metabolism genes *otsA* (trehalose-6-phosphate synthase (TPS)) of *E. coli*, suggest T6P to act as a signal molecule. These plants mimic responses similar to those in plants fed with exogenous trehalose, like enhanced activity of ADP-glucose pyrophosphorylase (Kolbe et al., 2005) and inhibited growth (Schluepmann et al., 2003). In these transgenic plants, trehalose levels remains below the detection limit of HPLC measurements, as in wild type, suggesting rather T6P than trehalose to be the active signal molecule. The work of Schluepmann *et al.* 2003/2004 with transgenic Arabidopsis strongly indicates that T6P is an important regulatory molecule. However, it remains an open question whether trehalose itself has a signalling function in plants. Therefore, in the present work, we speak about a 'trehalose/T6P' ('Tre/T6P') response.

The work presented here shows, that some genes up-regulated in their expression by Suc are also up-regulated by trehalose treatment in combination with the potent trehalase inhibitor validamycin A (tre/val), indicating that some of the gene expression response by Suc involves trehalose or T6P as a signal. In addition, another group of genes is regulated in its expression in a parallel manner by Glc and also by tre/val. Thus, regulation of Glc sensing might also work, in part, via Tre/T6P. However, further experiments have to been carried out to prove this. The regulation of Glc regulated genes by tre/val might in fact be a Glc effect due to incomplete trehalase inhibition by val (Fig. 4 in the introduction assumes complete inhibition). An additional connection between Tre/T6P response and

sugar sensing is the regulation of genes involved in carbohydrate metabolism upon tre/val treatment.

One of the most important findings of my work is the discovery of two groups of genes regulated by tre/val. The first group is completely unaffected by ABA, the second group is also induced by ABA (Fig. 1). Recently, it has been shown that ABA metabolism and ABA signalling is linked to sugar sensing (Leon and Sheen, 2003). Indeed, more than a third of the genes regulated by tre/val are also regulated by ABA. Interestingly, the regulation of these genes by trehalose appears not to depend on ABA as shown by the un-altered regulation in ABA biosynthesis mutants (Fig. 2a). For some of these genes, the *abi2-1* mutant, affected in a component of the ABA signalling pathway, inhibits trehalose induction indicating that trehalose uses, in part, the same signalling components as ABA (Fig. 2b). Further experiments with ABA mutants will be necessary to clarify the pathway inducing ABA regulated genes by trehalose.

Enhanced resistance to drought of plants over expressing trehalose metabolism genes of *E. coli* and yeast might be a pleiotropic effect of an artificially enhanced T6P level in the plant, rather than a feat of the physiological function of T6P in the wild type. However, as shown in this study, Tre/T6P response results in an induction of a vast amount of ABA regulated genes, independent of ABA levels in the plant. Therefore, the constant induction of ABA regulated genes might be partially responsible for the enhanced resistance towards drought in these plants with enhanced trehalose production.

It is also interesting to consider the genes regulated by trehalose but not by ABA. However, some of these may have to do with sugar sensing as indicated above. This includes autoregulation (Schluepmann et al., 2004). In addition, tre/val treatment, as

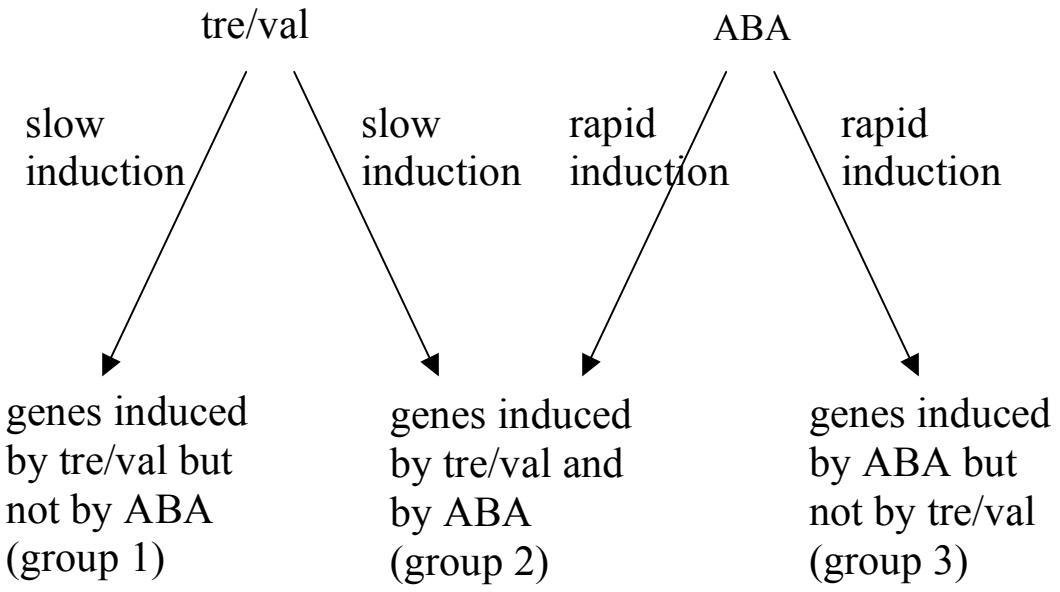


Figure 1: Grouping of different set of genes (group 1, 2 and 3) induced in expression (mRNA level) upon 25 mM trehalose plus 10 μM validamycin A (tre/val) and abscisic acid (ABA) treatment. Whereas tre/val treatment results in a slow induction, ABA treatment causes rapid induction of gene expression.

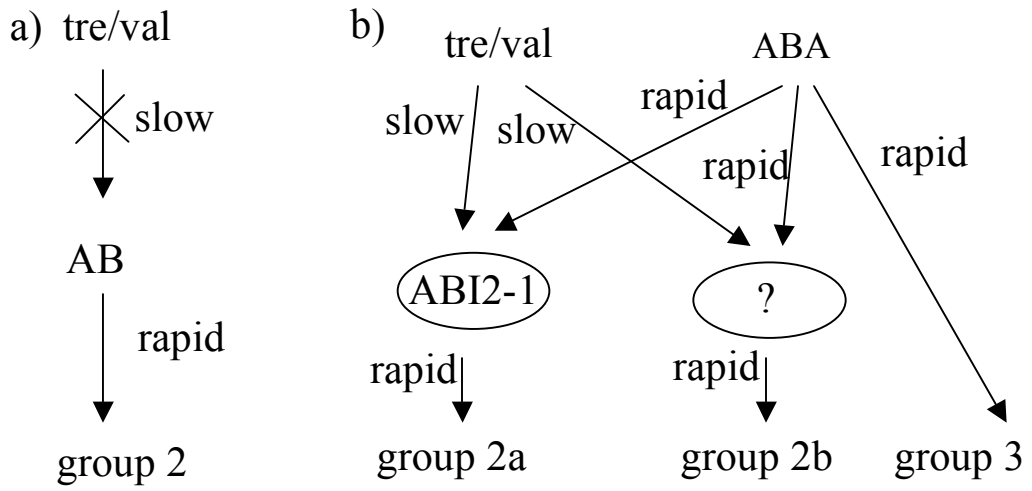


Figure 2: a) Scheme of induction of the set of genes that is induced by 25 mM trehalose plus 10 μM validamycin A (tre/val) and also by abscisic acid (ABA) (group 2). The induction of expression of group 2 upon tre/val treatment turned out not to occur via ABA, since genes of group 2 are also induced in ABA mutants deficient of ABA. b) Proposed scheme of pathways inducting genes upon tre/val and ABA treatment. Tre/val treatment causes a slow activation of ABI2-1, a protein phosphatase necessary for ABA signalling. This protein enables subsequently a rapid induction of expression of genes that are also regulated by ABA (group 2a). Additionally, tre/val treatment is also able to slowly induce genes independent of ABI2-1 regulation (group 2b) via unknown factors symbolized by a question mark. ABA induces an additional set of genes that is independent of tre/val-mediated induction (group 3).

reported in this work, induces some pathogen and defence related genes, which is consistent with the analysis of gene expression in *E. coli otsA* (TPS) gene expressors (Schluepmann et al., 2004). It might be, that the induction of pathogen related genes is elicited by Tre/T6P, helping the plant to defend itself against trehalose producing pathogens.

One of the reasons why plants use trehalose or T6P as signals in sugar sensing may be the fact that these molecules consist of components that are being sensed (Glc) but do not belong to the primary carbohydrate metabolism and are distinct from Fru, Glc and Suc (and other sugars of primary metabolism) because of their unique structure (α,α-1,1 linkage). In addition, the separate metabolic pathway allows a tight control of the levels of T6P and trehalose within the cell, independent from other metabolic activities.

The Arabidopsis mutant deficient for AtTPS1 activity is an embryo lethal. The embryo can be rescued by expression of the *E. coli otsA* (TPS) gene, suggesting T6P or trehalose to be essential for embryo maturation. Interestingly, the mutant can also be partially rescued by reduction of endogenous Suc concentration (Eastmond et al., 2002), suggesting trehalose or T6P to regulate the Suc utilization also in germination in plants.

All these results indicate a prominent role of trehalose metabolism in sugar sensing. However, one of the next steps in research of the trehalose metabolism would be to pinpoint the target to which trehalose or T6P binds. Further experiments will be necessary to understand the mode of action and possible targets of T6P controlling the respiratory chain, as it has been shown that T6P seems to regulate the influx of sugars into the respiration pathway (Schluepmann et al., 2003). It also remains to be tested whether T6P binds to any member of the hexokinase family in Arabidopsis (Eastmond et

al., 2002). Additionally, there is also pressing need to establish, whether the Tre/T6P responses presented in this work are due to the signalling function of trehalose or of T6P.

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Appendix I

Table I. Clustering of microarray data of the genes up regulated four fold or more by tre/val and two fold or more by ABA revealed a set of 128 genes. This table displays also a the expression of these genes modulated by 25 mM glucose (glc), 25 mM trehalose (tre), 25 mM sucrose (suc), 25 mM mannitol (man), 10 mM validamycin A (val), 25 mM trehalose + 10 mM validamycin A (tre/val) and 20 mM abscisic acid (ABA). Annotation of the genes was done by following the databases TAIR and MIPS. The numbers displayed represent fold change relative to a water treated control. ATH1 means full genome chip, 8K means the chip bearing app. 8000 different genes. tre/sorb is the expression ratio of 100 mM trehalose treatment to 100 mM sorbitol treatment from Schluepmann *et al.* 2004.

Table II. Clustering of microarray data of the genes up regulated four fold or more by tre/val but not more than two fold by ABA revealed a set of 179 genes. This table displays also a the expression of these genes modulated by 25 mM glucose (glc), 25 mM trehalose (tre), 25 mM sucrose (suc), 25 mM mannitol (man), 10 mM validamycin A (val), 25 mM trehalose + 10 mM validamycin A (tre/val) and 20 mM abscisic acid (ABA) treatment. Annotation of the genes was done by following the databases TAIR and MIPS. The numbers displayed represent fold change relative to a water treated control. ATH1 means full genome chip, 8K means the chip bearing app. 8000 different genes. tre/sorb is the expression ratio of 100 mM trehalose treatment to 100 mM sorbitol treatment from Schluepmann *et al.* 2004.

ster of genes induced by tre/val and ABA

of microarray data of the genes up regulated four fold or more by tre/val and two fold or more by ABA revealed a set of 128 genes. This table displays also a the expression of these ulated by 25 mM glucose (glc), 25 mM trehalose (tre), 25 mM sucrose (suc), 25 mM mannitol (man), 10 µM validamycin A (val), 25 mM trehalose + 10 µM validamycin A (tre/val) and 20 acid (ABA). Annotation of the genes was done by following the databases TAIR and MIPS. The numbers displayed represent fold change relative to a water treated control. ATH1 genome chip, 8K means the chip bearing app. 8000 different genes. tre/sorb is the expression ratio of 100 mM trehalose treatment to 100 mM sorbitol treatment from Schluepmann et a.

	ATH1 glc	8K glc	ATH1 tre	8K tre	Schl 8K tre/sorb	ATH1 suc	8K suc	ATH1 man	8K man	ATH1 val	8K val	ATH1 tre/val	8K tre/val	ATH1 ABA	classification by TAIR and MIPS MATDB annotation
)_A	1.9		2.2			1.3		-1.1		-1.1		109.5		141.0	ABA-responsive protein-related
)_A	1.0		8.6			3.2		2.2		1.1		83.7		182.0	lipid transfer protein 3 (LTP3)
)_A	1.1		4.0			1.4		1.3		-1.0		63.4		264.2	lipid transfer protein 4 (LTP4)
)_A	1.1	-3.3	2.6	1.0		1.7	-1.8	2.5	-1.1	1.5	-3.3	45.9	6.8	3.3	chitinase, putative
)_A	1.0	2.0	2.0	3.5		-1.2	-1.8	1.2	1.0	1.3	-1.2	40.2	56.7	96.1	late embryogenesis abundant protein
)_A	1.9		2.1			1.8		-1.3		1.1		27.0		2.4	ornithine cyclodeaminase/mu-crystallin family protein
)_A	-1.1		2.6			-1.0		2.0		1.2		26.7		47.7	ankyrin repeat family protein
)_A	1.0		1.5			-1.0		1.9		1.2		26.5		49.3	cold-regulated protein (cor15a)
)_F	1.2		3.0			2.1		-1.2		1.2		22.6		2.8	glutathione S-transferase-like protein (AtGSTF12)
)_A	2.2		2.6			1.6		1.1		1.0		20.1		5.8	galactinol synthase, putative
)_A	1.4	-1.2	1.6	1.8		1.2	-1.4	1.2	-1.4	1.0	-1.6	18.1	1.4	5.9	heavy-metal-associated domain-containing protein
)_A	1.0		2.8			1.2		1.2		1.0		17.6		16.2	protein phosphatase 2C, putative
)_A	-1.4	-2.2	2.1	2.6		-1.3	-2.2	1.8	1.9	-1.7	-2.0	17.4	21.9	28.3	amino acid transporter family protein
)_A	1.2		2.7			1.3		1.5		1.2		14.8		27.5	lipid transfer protein (LTP) family protein
)_A	-1.2	-1.3	1.2	1.8		-1.1	1.1	1.5	-1.3	-1.7	-1.1	14.6	1.8	15.7	Class II AtPUB19 armadillo/beta-catenin repeat family protein
)_A	-1.3		1.0			-1.2		-1.1		1.2		14.0		2.1	AAA-type ATPase family protein, contains Pfam profile: ATPase family
)_F	2.6	2.2	1.4	6.1		2.3	4.7	1.3	3.2	-1.0	2.4	13.8	5.9	3.5	myb family transcription factor (MYB75),
)_A	2.1	1.2	2.7	8.1		2.6	2.9	1.1	2.6	1.1	1.1	13.1	13.5	25.5	nodulin MtN3 family protein
)_A	1.2		2.1			1.3		2.2		-1.0		13.0		5.7	9-cis-epoxycarotenoid dioxygenase (AtNCED3)
)_F	1.3	-2.2	-1.1	-1.7		1.2	-2.4	1.1	-1.4	-1.2	-2.4	12.4	6.7	2.6	putative glutathione S-transferase; ; supported by cDNA: gi 14423533 gb AF387004.1 AF387004
)_A	-1.7	-1.3	1.1	3.1		-1.4	-1.9	1.6	2.3	-1.5	-1.6	11.8	5.4	8.1	expressed protein
)_A	5.3	1.4	2.4	3.3		4.5	4.1	-1.2	1.4	1.2	-3.9	11.4	13.6	6.3	Expressed protein
)_A	1.1	-1.0	2.3	2.8		1.6	1.2	1.6	2.0	-1.4	-1.1	11.0	4.3	19.4	Arabidopsis thaliana homeobox 7 (ATHB-7)
)_F	-1.2	-1.2	1.4	1.8		-1.2	-3.4	-1.4	-1.3	1.1	-1.1	10.8	10.6	32.8	low-temperature-responsive 65 kD protein (LTI65) / desiccation-responsive protein 29B (RD29B)
)_F	1.2	-1.0	1.9	3.0	7.7	-1.0	1.3	1.2	1.9	1.3	-2.5	10.8	13.1	7.6	tyrosine aminotransferase / tyrosine transaminase
)_A	2.2		3.1			1.0		-1.2		1.1		10.7		3.6	unknown protein;;supported by full-length cDNA: Ceres:35675.
)_A	1.2		1.8			1.1		1.8		-1.0		10.7		12.4	CBL-interacting protein kinase 5 (CIPK5)
)_A	1.4	2.2	2.3	4.2		1.6	2.6	-1.4	1.8	-1.2	2.0	10.5	13.0	15.6	late embryogenesis abundant
)_S	1.3	1.6	2.2	1.8		1.8	1.4	1.0	2.2	1.1	-1.4	9.5	12.9	21.2	cold and ABA inducible protein kin1
)_A	-1.2	2.4	1.1	-3.0		1.1	-2.2	2.1	1.3	-1.0	-3.0	9.3	7.4	4.8	putative ABC transporter
)_A	-1.0		2.4			1.7		1.4		1.6		9.2		13.6	unknown protein;;supported by full-length cDNA: Ceres:96702.
)_A	-1.1		1.7			1.1		2.2		1.3		9.0		16.3	expressed protein
)_A	-1.3	-4.0	1.1	3.1		-1.1	-1.4	1.6	1.5	-1.3	1.0	8.4	1.2	7.6	expressed protein
)_A	1.9		1.5			1.2		1.4		1.1		8.3		18.7	homeobox domain-leucine zipper protein ATHB-12
)_A	1.6	1.8	1.6	1.4		1.9	2.8	1.7	1.6	1.3	1.0	8.3	1.4	6.4	Protein phosphatase 2C (PP2C)
)_A	1.3		2.0			1.8		1.5		-1.1		8.2		16.9	protein kinase family protein (MAPKKK18)
)_A	1.2		1.8			1.1		-1.0		-1.1		8.2		3.6	Lipid Transfer Protein type 4
?_A	-1.6		-1.1			-1.2		-1.2		-1.5		8.0		14.5	seven in absentia (SINA) family protein
)_A	1.5		1.9			2.3		2.5		1.9		8.0		3.0	receptor-like protein kinase 5 precursor (RLK5); ; supported by cDNA: gi 166849 gb M84660.1 ATHRLPKC
)_A	2.7	1.6	2.3	2.0		2.0	1.8	1.4	1.6	1.2	-1.3	8.0	6.7	13.8	identical to dehydrin ERD10 (Low-temperature-induced protein LTI45)
)_F	1.1		-1.5			-1.4		1.2		1.4		7.9		40.9	expressed protein
)_A	-1.0		1.8			1.9		2.0		2.2		7.6		2.4	kinase-related, similar to receptor-like protein kinase 4

O_A	-1.7	-1.5	1.4	2.1		1.1	-1.7	1.2	1.6	3.2	-1.3	7.6	4.4	2.9	vegetative storage protein 2 (VSP2);
															putative serine threonine protein kinase;similar to serine threonine-specific
															kinase GB:S68589 (Arabidopsis thaliana); Pfam HMM hits: putative serine
O_A	-2.1		1.3			-1.2		-1.2		-1.0		7.5		2.6	threonine protein kinase, Eukaryotic protein kinase domain
O_A	-1.2		1.2			1.2		-1.0		1.1		7.0		2.1	putative protein;
O_A	1.2	2.0	1.2	1.4		1.3	1.6	-1.0	1.4	1.3	1.6	7.0	4.9	25.6	late embryogenesis abundant domain-containing protein
O_A	1.2	-1.1	1.3	-1.4		-1.0	-1.5	-1.2	-1.1	-1.2	-1.8	7.0	2.1	7.1	lipase class 3 family protein
O_F	-2.2		-1.4			-1.5		1.1		-1.2		7.0		10.6	no apical meristem (NAM) family protein
O_F	3.2		2.0			2.7		-1.2		1.1		7.0		4.7	short-chain dehydrogenase/reductase (SDR) family protein
O_A	1.0		1.1			1.6		-1.0		1.1		6.9		46.9	expansin-related protein 1 precursor (At-EXPR1)
O_A	1.1		1.8			-1.0		-1.2		1.4		6.8		9.5	expressed protein
															unknown protein;ESTs gb H36966, gb R65511, gb T42324 and gb T20569
O_F	1.1	1.5	1.4	1.7		1.4	1.3	2.0	3.0	1.2	2.8	6.7	4.9	3.4	come from this gene
O_A	2.1		2.6			2.1		1.2		1.2		6.5		3.4	glutaredoxin family protein
O_A	-2.0		-1.4			-1.8		-1.1		-1.7		6.5		8.1	sulfotransferase family protein
O_F	-1.5	-1.8	1.4	-1.3		-1.3	-2.1	4.8	1.1	2.3	-1.1	6.4	1.2	13.2	MADS-box protein (AGL20)
O_A	-1.1		1.4			-1.0		1.5		1.2		6.4		7.6	expressed protein
O_A	-1.1		-1.2			-1.6		1.7		-1.5		6.4		2.7	putative protein;hypothetical protein - Arabidopsis thaliana, PID:g2062170
O_F	-2.7	-1.9	2.3	3.2		1.1	1.2	2.7	1.8	1.1	-1.2	6.3	3.5	9.5	delta 1-pyrroline-5-carboxylate synthetase A (P5CS A) also (P5CS1)
															hypothetical protein;contains similarity to PP2 lectin polypeptide GI:410436 from
O_A	-1.0		1.4			1.1		1.5		-1.2		6.3		3.2	(Cucurbita maxima); supported by cDNA: gi 15292968 gb AY050918.1
															glucose-1-phosphate adenylyltransferase (APL3); ; supported by cDNA: gi
O_I	4.4	-1.2	3.1	2.2		2.5	5.7	-2.5	-1.2	1.5	-1.2	6.2	7.1	2.1	16648984 gb AY059862.1
O_A	-1.2	2.6	1.2	1.4	2.8	1.7	1.8	1.0	2.0	1.1	1.0	6.1	7.7	6.6	expressed protein
O_A	1.6	-2.4	1.9	1.7		1.8	-1.6	1.6	1.0	1.0	-1.6	6.1	1.2	3.9	putative protein;
O_A	-1.3	1.0	-1.2	1.4		-1.1	-1.1	1.3	1.2	1.1	1.1	6.0	5.3	11.7	benzodiazepine receptor-related
															hypothetical protein;similar to putative beta-1,3-glucanase GB:AAD26909
O_A	1.2		2.3			1.4		1.3		1.4		6.0		2.2	GI:4662638 from (Arabidopsis thaliana)
O_A	-1.6	-2.6	1.1	2.1		-1.8	-3.4	1.2	1.8	1.1	1.7	5.9	3.9	10.8	calcium-binding protein (RD20)
															LS1-like protein;AT-LS1 product - Arabidopsis thaliana,
O_A	-2.3		-1.4			-1.6		1.2		-1.2		5.9		2.2	EMBL:X58827;supported by full-length cDNA: Ceres:107294.
O_A	-1.2		-1.0			-1.6		1.7		-1.1		5.9		5.0	zinc finger (C3HC4-type RING finger) family protein
															hypothetical protein;similar to reticuline oxidase-like protein GB:CAB45850
O_A	4.9		1.1			2.4		9.4		3.0		5.7		2.6	GI:5262224 from (Arabidopsis thaliana)
O_F	2.9		1.6			2.2		1.6		2.1		5.7		2.4	DSBA oxidoreductase family protein
O_F	1.1	1.1	1.7	-1.6		1.5	-1.6	2.2	-1.2	1.3	-1.3	5.7	1.9	2.4	putative NAM-like protein;
O_I	1.5	-1.9	-1.2	-4.2		-1.2	-2.7	1.5	-1.2	1.1	-1.4	5.6	3.6	7.9	cytochrome P450, putative (CYP76C2)
O_A	2.7	1.1	1.7	1.0		2.5	1.1	-1.2	-1.1	1.0	-1.6	5.6	1.4	7.1	cinnamyl-alcohol dehydrogenase
O_A	2.0	-3.0	1.6	1.7		2.3	2.7	1.3	1.3	1.1	-2.4	5.5	10.1	11.2	basic endochitinase
O_A	1.8	-1.4	1.7	-2.0		1.3	-2.2	-1.2	-1.8	1.2	-2.6	5.4	1.2	2.0	SCARECROW-like protein;
O_A	1.2		1.7			1.0		-1.2		-1.0		5.4		6.1	beta-ketoacyl-CoA synthase family protein
O_A	3.2		2.5			2.3		1.3		1.2		5.4		4.5	glycine-rich protein
O_A	1.6	1.7	2.6	3.5		1.2	1.4	1.3	-1.3	1.4	-4.0	5.4	3.3	7.7	beta-ketoacyl-CoA synthase, putative
O_A	1.1		1.1			-1.2		1.2		1.0		5.3		8.6	expressed protein
O_A	-2.5		1.5			-2.3		1.8		-1.1		5.3		13.3	zinc finger (C3HC4-type RING finger) family protein
O_A	1.2	1.1	1.9	1.4		-1.2	-1.4	-1.3	1.0	1.2	1.2	5.3	1.3	2.2	CCAAT-box binding transcription factor
O_A	1.7		2.1			2.0		1.2		9.0		5.3		3.7	unknown protein;;supported by full-length cDNA: Ceres:38751.
O_A	2.8		1.0			2.5		-1.2		1.2		5.3		18.8	plastocyanin-like domain-containing protein
O_A	-1.2		-1.2			1.6		-1.4		-1.3		5.3		3.4	esterase/lipase/thioesterase family protein
O_F	1.1	-1.6	2.0	2.3		1.4	1.0	1.8	2.1	1.7	1.3	5.3	4.1	6.3	G-box binding factor 3 (GBF3)

O_A	1.2		1.6			1.1		1.5		1.5			5.3						2.1 F-box family protein
O_A	-1.0		-1.1			-1.9		-1.4		-1.4			5.2						5.1 expressed protein
O_A	-1.1	-4.0	1.2	-1.5		-1.5	-2.7	-1.1	1.1	1.3	-1.0	3.0	5.1	3.0					5.4 expressed protein
O_A	1.6	1.0	2.3	2.4		2.4	1.7	1.6	1.1	1.6	1.6	1.5	5.0	1.5					5.9 expressed protein
O_A	2.3	1.8	2.4	3.3		2.6	4.6	-1.9	1.8	1.0	-1.6	3.4	5.0	3.4					4.0 rubber elongation factor (REF) family protein
O_A	-1.1	-1.5	1.6	-1.4		-1.1	-1.8	2.3	-1.2	1.2	-1.1	2.6	5.0	2.6					5.7 late embryogenesis abundant 3 family protein
O_F	-1.0	1.6	1.4	2.6		1.9	2.0	1.6	1.4	1.3	3.4	4.0	5.0	4.0					2.2 MutT-like protein;contains Pfam profile: PF00293 Bacterial mutT protein
O_A	1.0	-1.3	2.5	2.9		1.4	1.6	1.8	3.1	1.2	-1.8	4.5	4.9	4.5					5.7 responsive to dehydration 22 (RD22)
O_A	1.6		1.3			-1.5		1.3		-1.1			4.9						4.1 BTB/POZ domain-containing protein
O_A	1.5		1.6			1.1		-1.2		1.2			4.9						2.4 thioesterase family protein
O_A	-1.0		2.2			1.3		1.3		1.0			4.9						4.0 oticosapeptide/Phox/Bem1p (PB1) domain-containing protein
O_A	2.2		1.3			2.1		-1.5		2.6			4.8						6.3 Lipid Transfer Protein type 5
O_A	1.2		1.8			-1.1		-1.0		1.4			4.8						5.8 AP2 domain-containing transcription factor, putative
O_A	1.8	1.5	1.7	1.1		2.5	1.4	1.2	1.0	1.3	-1.7		4.8	2.2					3.6 hexose transporter, putative
O_A	-1.1		2.3			1.6		1.8		1.7			4.7						3.4 unknown protein;
																			glucose-6-phosphate phosphate-translocator precursor, putative;similar to
																			glucose-6-phosphate phosphate-translocator precursor GI:2997591 from
O_A	11.2	3.9	1.9	1.2		8.7	24.8	1.7	1.2	2.2	1.5	4.6	1.9						3.8 (Pisum sativum); supported by cDNA: gi 14596172 gb AY042874.1
O_A	1.0		1.3			-1.4		1.5		1.1		4.5							2.9 expressed protein
O_F	2.0		2.4			1.4		1.0		1.2		4.5							4.2 cinnamoyl-CoA reductase-related
O_A	2.5	-2.8	1.4	3.5		2.3	2.5	-1.4	1.2	1.1	2.2	4.5	3.5						3.8 unknown protein;
																			low-temperature-responsive protein 78 (LTI78) / desiccation-responsive protein
O_F	1.4	1.8	1.5	3.4		1.1	2.4	-1.6	3.5	1.0	-1.8	4.5	26.9						18.8 29A (RD29A)
O_A	1.6		2.3			2.1		1.2		1.3		4.5							3.7 zinc finger (C3HC4-type RING finger) family protein
O_A	-1.6		1.2			-1.4		1.2		1.0		4.4							5.2 no apical meristem (NAM) family protein
O_A	-1.5		1.3			-1.7		2.2		-1.1		4.4							4.2 expressed protein
O_A	-1.5		1.8			-1.4		-1.0		-1.8		4.4							12.6 invertase/pectin methylesterase inhibitor family protein
O_F	-1.0		1.1			1.1		1.1		1.6		4.4							2.1 S-adenosyl-L-methionine:carboxyl methyltransferase family protein
																			hypothetical protein;similar to hin1 GB:Y07563 GI:1619320 Nicotiana
O_A	1.8		1.0			1.4		1.6		-1.2		4.4							3.5 tabacum;supported by full-length cDNA: Ceres:21639.
																			putative pectinesterase; ; supported by cDNA: gi 13605695 gb AF361829.1
O_A	-1.3	-7.5	-1.6	-4.4		-1.1	-6.7	1.8	-3.6	2.6	-10.9	4.4	1.3						3.9 AF361829
O_A	-2.2		-1.3			-1.9		1.8		-1.1		4.3							6.9 expressed protein
O_A	1.9	-1.7	-1.1	-2.1		1.5	-2.1	1.8	1.1	1.1	-2.5	4.3	-1.3						3.3 putative embryo-abundant protein;
O_A	-1.1	-2.3	-1.1	-3.1		1.1	-4.0	1.5	-1.3	-1.1	-2.2	4.3	1.8						2.0 serine threonine-specific kinase lecRK1 precursor,lectin receptor-like;
O_A	1.1	1.3	1.9	2.2		1.2	1.5	1.7	1.8	1.4	1.4	4.2	3.5						8.6 protein phosphatase 2C (ABI2)
O_A	1.1		2.2			1.6		-1.3		1.1		4.2							5.5 family II extracellular lipase 3 (EXL3)
O_F	1.5	-1.4	2.0	1.2		-1.0	-1.3	1.7	1.5	1.9	1.2	4.2	1.9						5.6 amino acid permease I (AAP1)
O_A	-1.8		-1.2			-2.0		-1.3		-1.7		4.2							3.7 expressed protein
O_A	2.0		2.7			2.1		1.2		1.3		4.1							6.7 phenylalanine ammonia-lyase (PAL)
																			At14a-1 protein;identical to At14a protein GB:AAD26355 GI:4589123
O_S	-2.0		-1.2			-1.1		1.2		-1.2		4.1							3.8 (Arabidopsis thaliana) (Gene 230 (1), 33-40 (1999))
O_A	1.7		1.3			1.4		1.1		1.5		4.1							3.3 prephenate dehydratase family protein
O_A	1.0		1.6			1.3		1.2		1.0		4.1							16.4 heat shock transcription factor family protein
O_A	2.3		3.3			4.1		-1.3		1.2		4.0							5.6 major latex protein-related
O_A	1.6		1.2			1.6		1.6		-1.3		4.0							3.1 hypothetical protein;predicted by genemark.hmm
																			zinc finger protein, putative;contains Pfam profile: PF01760 CONSTANS family
O_A	1.3		1.3			1.1		1.3		-1.3		4.0							2.5 zinc finger;supported by full-length cDNA: Ceres:258241.
																			heat shock transcription factor HSF1, putative;similar to GB:S52641 from
																			(Arabidopsis thaliana) (Plant Mol. Biol. 26, 353-362 (1994)); supported by
O_A	1.4		1.7			1.2		-1.2		-1.1		4.0							2.5 cDNA: gi 15810193 gb AY056111.1
O_A	-2.0		-1.2			-1.2		1.4		-1.1		4.0							6.7 expressed protein
																			putative protein;predicted proteins, Arabidopsis thaliana;supported by full-length
O_A	2.4		1.6			1.4		-1.1		1.2		4.0							2.6 cDNA: Ceres:106702.

uster of genes induced by tre/val but not by ABA

of microarray data of the genes up regulated four fold or more by tre/val but not more than two fold by ABA revealed a set of 179 genes. This table displays also a the expression of these genes by 25 mM glucose (glc), 25 mM trehalose (tre), 25 mM sucrose (suc), 25 mM mannitol (man), 10 μM validamycin A (val), 25 mM trehalose + 10 μM validamycin A (tre/val) and 20 μM abscisic acid ment. Annotation of the genes was done by following the databases TAIR and MIPS. The numbers displayed represent fold change relative to a water treated control. ATH1 means full genome eans the chip bearing app. 8000 different genes. tre/sorb is the expression ratio of 100 mM trehalose treatment to 100 mM sorbitol treatment from Schluepmann et a. 2004.

	ATH1 glc	8K glc	ATH1 tre	8K tre	Schl 8K tre/sorb	ATH1 suc	8K suc	ATH1 man	8K man	ATH1 val	8K val	ATH1 tre/val	8K tre/val	ATH1 ABA	classification by TAIR and MIPS
D_A	2.8		2.9			2.5		1.2		1.2		94.8		-1.5	AAA-type ATPase family protein
D_A	1.5		3.0			1.4		1.2		1.3		68.5		-1.1	expressed protein
D_A	-1.3		1.7			-1.3		2.0		1.3		61.7		1.9	auxin-responsive - like protein. auxin-responsive GH3 family protein
D_A	1.1		1.8			1.1		1.2		1.4		57.4		1.4	expressed protein
D_A	3.3		3.4			1.9		1.1		-1.2		57.3		-1.5	protease inhibitor/seed storage/lipid transfer protein (LTP) family
D_A	-1.0		1.5			-1.3		1.0		1.1		49.9		-1.2	Plant U-box protein (PUB) Class V AtPUB54
D_F	-1.3		1.2			-1.3		1.0		1.0		42.4		-1.2	unknown protein
D_F	1.5	-1.5	3.6	5.3		1.8	1.3	1.2	1.4	1.4	-1.5	42.2	11.9	1.1	accelerated cell death 6 (ACD6)
D_A	-1.1		1.6			1.2		1.1		-1.1		41.4		-1.1	AAA-type ATPase family protein
D_A	1.3		1.3			1.4		1.6		-1.0		38.0		1.2	expansin family protein (EXPR3)
D_F	1.8	1.2	1.6	6.2	100.9	2.6	4.8	-1.4	1.4	1.1	-2.1	37.7	26.2	-1.3	wall-associated kinase 1
D_A	-1.2		1.0			-1.2		-1.0		-1.2		36.3		1.5	expressed protein
D_A	-1.3	-1.9	2.9	2.4		1.1	-1.1	1.1	-1.5	1.8	-2.4	36.2	9.8	-1.3	AtWRKY70
D_A	-1.6	-1.6	1.5	1.6	10.4	-1.0	-2.0	-1.1	1.8	-1.5	-1.8	35.1	41.2	-1.4	protein kinase - like protein, KI domain interacting kinase 1
D_A	1.4		6.1			1.6		-1.2		1.9		33.5		-2.6	protein kinase - like. legume lectin family protein
D_A	-1.1	-2.1	-1.4	-1.3		-1.3	-1.0	-1.7	-1.9	-1.1	-1.5	32.6	6.5	-1.2	peroxidase like protein. Class III peroxidase (PER49)
D_A	-1.1	1.8	1.8	10.3		1.1	3.0	-1.2	1.6	1.4	-1.4	30.8	37.9	1.1	unknown protein
D_A	-1.4		1.1			1.0		-1.4		-1.2		30.7		-1.4	calcium-transporting ATPase (calcium pump), putative
D_A	1.4		2.0			1.2		1.0		1.5		30.6		1.1	ankyrin-repeat-containing protein-related
D_A	2.8	1.2	2.9	4.7		3.9	5.7	-1.7	-1.4	-1.6	1.2	27.5	6.7	-1.7	putative leucoanthocyanidin dioxygenase (LDOX).
D_F	-1.1		1.5			1.6		1.7		1.3		26.1		1.1	unknown protein
D_A	1.1		-1.0			1.0		1.1		1.1		25.7		-1.1	AtWRKY46
D_A	1.6	-1.8	3.1	8.3		2.6	6.0	-2.0	-2.0	-1.5	-2.4	24.7	11.1	-1.8	dihydroflavonol 4-reductase (dihydrokaempferol 4-reductase) (DFR)
D_A	1.2		1.8			1.2		1.4		1.0		24.6		1.7	BCS1 - like protein.AAA-type ATPase family protein
D_A	1.1	-1.5	1.4	5.3		-1.3	1.3	-1.3	1.4	1.1	-1.5	23.4	11.9	1.3	AtWRKY59
D_F	-1.2		1.4			1.5		-1.1		1.3		22.6		-1.2	resistance protein-like (defence). disease resistance family protein
D_A	1.5		1.3			1.8		-1.1		1.1		22.3		-2.5	putative calcium-binding protein
D_A	-1.1	-1.8	-1.1	1.4		-1.1	1.2	2.0	-1.3	-1.2	-1.7	22.3	9.2	-1.7	AIG1 avirulence-responsive protein / avirulence induced gene (AIG1),
D_A	-1.1		1.9			2.5		-1.1		2.0		21.4		1.2	glycosyltransferase family protein
D_A	-1.7		1.2			-1.0		1.1		-1.2		20.3		1.1	Similarity to At4g25835 BCS1 like mitochondrial protein (e Value: 1.1E-96).
D_F	1.2		1.6			1.1		-1.1		1.4		20.1		1.4	putative disease resistance protein. disease resistance family protein / LRR family protein
D_A	3.2		3.1			2.9		-1.4		-1.1		19.4		-1.0	Expressed protein
D_S	1.0		-1.2			-1.4		-1.2		1.1		18.9		-1.4	UDP-glucoronosyl/UDP-glucosyl transferase family protein
D_A	12.8		1.6			9.1		7.3		2.5		17.9		-1.2	glutathione S-transferase, putative AtGSTF6
D_A	1.2	-2.2	2.1	-1.4		1.2	-3.0	1.3	1.4	-1.2	1.4	17.5	1.8	-1.6	aspartyl protease family protein
D_F	-1.1		1.3			1.3		1.3		-1.2		16.4		-1.5	putative mutT domain protein;
D_A	1.0		1.5			1.1		1.1		1.2		16.2		1.6	trehalose-6-phosphate phosphatase - like protein
D_A	-1.1		1.6			-1.3		1.3		1.1		15.4		-1.2	leucine-rich repeat transmembrane protein kinase, putative (LRR XI)
D_A	-1.2		1.3			-1.1		1.4		1.2		15.0		-1.2	AtWRKY50
D_A	1.0	-1.4	1.1	1.2		1.1	-1.2	1.2	1.8	1.2	3.0	14.2	7.3	1.1	expressed protein
D_S	1.7		2.5			2.9		-1.5		-1.0		13.7		-1.3	leucoanthocyanidin dioxygenase (anthocyanidin synthase) (LDOX/ANS), putative
D_F	-1.4		-1.0			-1.2		2.3		-1.1		13.5		1.2	heavy-metal-associated domain-containing protein.
D_F	1.0		1.3			1.3		1.6		-1.0		12.9		1.0	wall-associated kinase 3 (WAKL)
D_A	1.8		1.9			-1.0		1.1		1.5		12.7		-1.1	No apical meristem (NAM) protein family
D_A	-1.1		1.2			1.2		1.1		1.2		12.6		-1.4	isochorismate synthase 1 (isochorismate mutase) (ICS1)
D_A	1.2		1.3			2.7		1.6		1.6		12.4		1.5	expressed protein
D_A	1.8	1.1	2.5	11.7		1.6	2.8	1.2	1.3	1.2	-3.4	12.3	7.8	1.1	unknown protein

_F	-1.8		1.1			-1.5		-1.6		-1.5		12.1		-1.5	leaf senescence-associated protein (SAG101)
_A	1.1		1.7			1.7		-1.1		1.6		11.9		-1.2	heavy-metal-associated domain-containing protein, similar to farnesylated protein ATFP3
_A	2.4		2.3			1.3		1.2		1.8		11.7		1.6	disease resistance protein family. disease resistance family protein, contains leucine rich-repeat (LRR)
_A	1.1		1.2			1.0		1.4		1.6		11.2		1.6	hypothetical protein
_A	2.2		1.2			1.2		1.9		1.5		11.1		1.0	expressed protein
_A	-1.1	-2.2	-3.0	-2.4		-1.8	-3.2	1.0	-1.5	-1.0	-1.8	10.9	-1.8	-1.1	broad-spectrum mildew resistance RPW8 family protein
_A	1.3	1.4	1.6	2.4		1.3	-1.2	1.5	3.8	1.1	-1.8	10.9	8.7	1.1	SigA binding protein
_A	2.4	-4.8	-1.7	-4.9		1.0	-4.5	2.9	-1.3	1.7	-2.9	10.8	1.1	1.6	unknown protein
_A	-1.2	-1.2	1.2	1.3	4.1	-2.5	-1.2	1.1	1.4	1.0	1.0	10.6	7.5	-1.2	phytoalexin-deficient 4 protein (pad4)
_F	-1.4		-1.2			-1.4		-1.2		-1.5		10.3		-1.5	expressed protein
_A	-1.4		-1.3			-1.0		2.3		1.4		9.8		-1.4	ankyrin repeat protein family
_A	1.6	1.6	1.5	1.0		1.4	3.0	1.4	2.0	1.2	-3.0	9.8	16.4	-1.2	enhanced disease susceptibility 5 (Eds5) (salicylic acid induction deficient 1) (Sid1)
_A	1.3	-1.8	1.0	-1.2		1.1	1.0	1.3	2.0	1.2	-1.2	9.8	5.9	1.4	putative anthocyanidin synthase
_I	1.0		1.2			1.2		2.0		1.6		9.8		1.6	serine/threonine kinase - like protein. protein kinase family protein
_A	2.5	1.8	1.7	7.3		1.6	6.8	-3.1	1.3	1.1	-1.4	9.7	7.2	-4.9	calmodulin-like calcium-binding protein, 22 kDa (CaBP-22)
_A	1.3	3.6	1.4	3.4		1.2	3.8	1.2	3.8	-1.0	3.8	9.4	12.6	1.2	jacalin lectin family protein
_F	1.1	-1.5	1.1	-1.1	4.8	-1.2	-1.2	1.7	-1.0	1.1	-1.2	9.2	3.8	1.2	MutT/nudix family protein
_A	1.0		1.1			-1.3		-1.3		-1.5		9.2		-1.5	glutathione transferase AtGSTU22
_A	1.2		1.0			1.2		1.0		1.3		9.0		1.1	disease resistance protein family. Receptor kinase-like protein family
_A	1.4		1.2			2.1		1.8		1.9		9.0		1.2	endochitinase-like protein
_F	1.8		1.2			2.1		1.2		1.2		8.9		1.1	At1g02230 no apical meristem (NAM) family protein, transcrption factor activity. E-value:e-165
_S	7.7	-3.4	1.1	-2.3		6.7	-1.1	4.7	-1.3	1.9	-6.1	8.9	1.1	-1.5	glutathione S-transferase, putative AtGSTF7
_A	-1.8	-1.7	-1.5	-1.2		-1.2	-1.5	2.3	1.6	-1.4	-1.1	8.8	2.0	-1.7	expressed protein
_F	4.4	-2.0	1.8	-1.8		4.1	1.0	2.8	1.1	1.3	-1.8	8.6	2.0	-1.4	putative glutathione S-transferase (AtGSTF3)
_A	1.3		1.0			1.3		-1.0		-1.2		8.6		1.6	unknown
_F	-1.4		1.4			1.0		-1.1		-1.2		8.2		-1.3	plastid heme oxygenase -related
_F	4.5	-1.8	1.6	-1.3		3.3	-1.1	3.0	-1.0	1.5	-1.7	8.1	1.6	-1.2	glutathione S transferase (AtGSTF2)
_F	1.0	-2.8	1.3	2.5	13.7	-1.4	-3.6	1.2	-3.2	2.3	-1.7	8.1	21.9	-1.4	trehalose-6-phosphate phosphatase - like protein
_A	2.0		2.5			1.6		3.2		2.3		8.0		1.4	expressed protein
_F	1.0		1.0			1.0		1.0		1.0		7.8		1.0	heavy-metal-associated domain-containing protein.
_A	1.8		1.4			-1.1		-1.6		1.4		7.7		1.0	Kip-related protein 6; kip-related protein 6 (KRP6) / cyclin-dependent kinase inhibitor 6 (ICK6)
_S	-1.1		-1.6			-2.0		2.3		1.2		7.7		1.0	expressed protein
_A	-1.3		1.2			-1.5		1.3		-1.1		7.5		1.3	expressed protein
_A	2.4	1.2	1.1	-1.2		1.8	-1.1	-1.2	-1.2	1.8	-5.9	7.3	1.4	-1.4	putative chitinase
_F	-1.9	-1.6	1.6	1.5	3.1	-1.7	1.1	-1.7	1.0	1.0	-1.1	7.2	1.8	-1.7	disease resistance protein (TIR class), putative.
_A	-1.7		1.2			-1.2		1.2		1.1		7.2		-2.2	lipase (class 3) family. Lipid Acylhydrolase-like
_A	1.0		1.0			1.0		1.0		1.0		7.2		1.1	expressed protein
_F	-2.0		-1.5			1.7		1.6		-1.6		7.2		-1.7	disease resistance protein family. disease resistance family protein, contains leucine rich-repeat (LRR)
_A	-1.6	-5.9	-1.3	-5.5		-1.2	-4.0	1.9	-1.5	1.3	-2.2	7.1	4.3	-1.3	calmodulin-binding protein
_A	10.7	-2.1	1.5	-1.4		6.5	1.5	2.3	-1.5	1.3	-3.8	7.0	-1.7	-2.7	lectin -related. legume lectin family protein
_A	-1.2	-1.5	1.8	2.0	5.7	1.0	1.0	1.4	1.0	-1.0	-1.6	7.0	2.0	1.1	ORF1, putative
_A	-1.7		-1.4			1.4		1.2		1.0		6.9		1.2	zinc finger (C3HC4-type RING finger) protein family
A	1.1		1.0			1.0		1.5		1.0		6.4		1.0	no AGI TAIR annotation on Array
_F	-1.7	1.2	1.0	1.0		-1.3	1.4	-1.1	2.6	-1.4	1.2	6.3	3.2	1.4	phosphofructokinase family protein
_A	4.1		2.2			1.6		-1.2		-1.1		6.3		1.5	hydroxycinnamoyl/benzoyltransferase-related
_F	-1.2	1.4	1.2	1.6		-1.0	1.4	-1.0	1.6	1.0	1.4	6.3	1.6	1.2	calmodulin-8 (CAM8)
_A	1.1		1.2			1.0		1.6		1.4		6.2		-1.0	disease resistance protein (TIR class)
_I	1.2	-6.6	-1.2	-4.1		-1.9	-3.1	1.5	-2.1	1.6	-4.1	6.2	1.6	1.8	germin-like protein (GLP9)
_F	-1.0		1.1			1.2		1.0		-1.2		6.2		1.1	acireductone dioxygenase (ARD/ARD') family protein

_A	-1.8		1.1			-1.7		-1.4		-1.3		6.2		-1.3	No apical meristem (NAM) protein family, transcription factor activity
_A	1.0	1.6	-1.0	-1.4		1.1	-1.4	1.4	-1.4	1.7	-1.2	6.2	3.6	1.2	calmodulin-binding protein
_A	1.1	3.0	-1.0	2.6		1.9	2.8	1.5	1.6	1.2	1.8	6.1	5.1	1.3	Hypothetical Protein
_A	-1.0		-1.2			-1.6		1.9		-1.0		6.0		1.4	cytochrome P450 CYP71B23
_A	1.3	-1.9	1.5	1.2		1.3	1.2	1.3	1.3	1.2	-1.7	5.9	1.5	1.5	receptor lectin kinase, putative Shiu identifies as (LRK1)
_A	1.1		1.1			1.1		1.3		1.4		5.9		1.3	arabinogalactan-protein (AGP5)
_A	-1.4		1.4			1.5		1.2		-1.0		5.9		-1.4	leucine rich repeat protein kinase family (LRR VII)
_A	1.5	-2.8	1.2	-1.8		1.1	-2.2	-1.1	-3.0	1.2	-3.0	5.9	6.8	-1.1	luminal binding protein 3 (BiP-3) (AtBP3)
_I	-1.3		-1.1			-1.1		1.5		-1.0		5.8		-1.4	glucuronosyl transferase - like protein
_F	2.0	-1.3	1.8	1.2		1.9	-1.2	-1.2	-1.4	1.0	-3.2	5.7	1.6	1.1	AtWRKY60
_F	1.4	2.8	1.3	1.8		-1.1	2.6	-1.3	1.4	-1.1	-1.4	5.7	1.4	1.3	myb family transcription factor (AtMYB90)
_A	1.7		1.3			1.8		2.4		1.5		5.7		1.8	expressed protein
_I	-1.4		-1.7			-2.1		1.4		1.5		5.7		-1.1	expressed protein weak similarity to resistance complex protein I2C-2 (Lycopersicon esculentum)
_A	-1.1		1.5			1.2		1.2		-1.2		5.7		1.9	heavy-metal-associated domain-containing protein, contains Pfam profile PF00403: Heavy-metal-associated domain
_A	-1.5	-1.7	-1.1	-1.1		-1.4	-1.7	1.3	1.2	-1.1	-1.1	5.4	3.1	1.1	leucine-rich repeat transmembrane protein kinase, putative (RLK LRR VII)
_A	-2.7		-2.1			-2.2		1.7		-1.3		5.4		-1.3	zinc finger -like protein. zinc finger (C2H2 type) family protein
_A	-1.5	-2.4	1.0	-1.4		-1.0	-2.2	1.5	-2.2	1.3	-1.2	5.4	1.1	-1.2	putative disease resistance protein
_A	2.2	-1.8	2.2	1.4		2.0	-1.8	1.6	-2.0	1.8	1.3	5.3	-1.8	1.3	unusual floral organ (UFO) (FBX1)
_A	1.0		1.1			1.0		3.1		1.0		5.3		1.0	expressed protein
_F	4.0	-1.3	1.8	-3.0		2.5	-2.2	-1.0	-1.6	1.7	-4.3	5.2	1.3	-2.0	dnaK-type molecular chaperone hsc70.1 - like. heat shock cognate 70 kDa protein 2 (HSC70-2)
_A	-1.0	-1.2	-1.0	1.0		1.5	1.4	1.3	-1.2	1.1	1.0	5.2	10.9	1.1	F-box protein (SKP1 interacting partner 3-related)
_A	1.3		1.0			1.0		1.0		1.1		5.2		1.0	hypothetical protein
_A	-1.0		-1.1			1.1		1.3		-1.1		5.1		-1.3	amino acid permease - like protein
_A	1.3		1.5			1.1		2.0		1.2		5.1		-1.1	probable wall-associated kinase
_A	1.6		1.0			1.3		1.2		1.3		5.1		1.2	unknown gene
_F	-1.1	-1.6	1.2	1.2		1.5	1.1	1.6	1.0	1.3	-3.1	5.0	3.2	-1.3	cyclic nucleotide-regulated ion channel (AtCNGC3)
_I	1.3		-1.0			1.4		1.3		1.4		5.0		1.5	cytochrome P450 monooxygenase (CYP76C6)
_F	1.3		1.5			-1.1		1.2		-1.2		5.0		1.9	disease resistance Cf-2 like protein. disease resistance family protein / LRR family protein
_A	-1.3	2.2	-1.5	2.0		-1.1	1.8	1.1	1.6	1.1	-1.2	5.0	1.0	1.3	cytochrome P450, putative (CYP96A5)
_A	1.0		1.0			1.0		1.0		1.0		5.0		1.0	AAA-type ATPase family. AAA-type ATPase family protein, contains Pfam profile: ATPase family PF00004
_A	-1.5	1.4	-1.2	1.2		-1.0	-1.6	1.0	1.4	-1.2	1.6	4.9	4.0	1.9	disease resistance protein RPS2. disease resistance protein RPS2 (CC-NBS-LRR class)
_A	-1.1		1.1			1.1		1.4		1.4		4.9		-1.0	expressed protein
_A	1.9		2.6			1.9		-1.1		1.4		4.8		-1.6	receptor-related protein kinase (SD-1)
_A	1.4		2.3			1.2		1.1		-1.1		4.8		1.0	Fe-S metabolism associated domain-containing protein
_F	1.3		-1.3			1.2		-1.2		1.1		4.8		-1.6	NAM (no apical meristem)-related protein, transcription factor activity
_F	5.6	2.0	2.6	1.4		3.4	1.8	-1.2	1.0	1.8	1.8	4.7	5.9	-1.5	expressed protein
_A	1.3	-1.8	1.2	-1.4		1.0	-1.8	2.2	1.8	1.0	2.2	4.7	8.7	1.7	curculin-like (mannose-binding) lectin family protein
_I	-1.1		-1.3			-1.2		1.6		1.4		4.7		-1.1	disease resistance protein-like. disease resistance protein (TIR-NBS-LRR class), putative
_A	1.2	-1.6	1.2	1.0		1.2	2.0	1.3	-2.0	1.1	-2.4	4.7	1.4	1.4	senescence-associated protein-related, similar to senescence-associated protein SAG102
_A	-1.8		-1.6			-1.3		-1.5		-1.7		4.7		-1.5	lipase (class 3) family
_F	1.4	1.6	1.1	1.7	2.0	1.3	1.7	1.2	1.2	1.1	1.1	4.7	4.8	1.2	glycosyl hydrolase family 1
_A	1.3	-1.5	1.2	1.2		1.2	1.2	1.3	-1.1	1.6	-1.1	4.7	1.3	1.1	hypothetical protein
_F	1.9	-1.1	1.4	1.1	2.4	1.4	-1.2	1.3	1.5	1.5	-1.3	4.6	1.5	1.1	protein kinase, putative (RLCKVII)
_F	1.2		1.5			2.8		1.2		1.4		4.6		1.3	glycosyl hydrolase family 85 protein
_A	-1.4		1.0			-1.2		1.2		-1.4		4.6		1.9	alternative oxidase, putative.
_A	1.2		1.1			1.0		1.1		1.1		4.6		-1.3	pRIB5 protein
_A	-1.2		-1.0			-1.5		2.4		1.2		4.5		1.4	calmodulin-binding family protein
_F	1.1	-5.5	1.3	-1.6		-1.6	-5.2	1.4	-1.1	1.0	-1.9	4.5	3.5	-1.0	unknown protein
_A	4.2		1.9			3.6		1.3		1.9		4.4		1.5	disease resistance protein family (LRR). disease resistance family protein / LRR family protein

0_A	-2.0	-1.8	1.5	-1.2	-1.0	-1.4	1.4	1.3	2.2	1.7	4.4	2.3	-1.8	transcription factor WRKY 40 (AtWRKY40)
0_A	-1.1		1.4		1.3		1.1		1.2		4.4		-1.0	unknown protein
0_A	1.8	-2.2	-1.1	-1.6	1.0	-2.2	1.1	-1.8	-1.2	-2.0	4.3	-1.2	-1.2	disease resistance protein (TIR class)
0_F	2.8		1.7		2.6		3.6		2.8		4.3		1.7	cytochrome P450, putative CYP71A18
0_F	-1.1		-1.1		2.1		-2.7		1.1		4.3		1.3	glycosyl hydrolase family 1, beta-glucosidase (BG1)
0_F	1.2		1.0		1.0		1.5		1.7		4.3		-1.1	leucine rich repeat protein kinase, putative (LRR I)
0_A	3.2	1.5	1.8	1.6	2.2	1.6	-1.1	1.3	1.6	-1.8	4.3	1.9	1.2	leucine rich repeat protein family
0_A	1.5		1.1		1.2		1.0		-1.4		4.3		1.5	fruktokinase 1. pfkB-type carbohydrate kinase family protein
0_A	1.6		-1.0		1.3		1.8		1.5		4.3		1.3	nucellin protein, putative
0_A	1.1		1.1		1.1		-1.0		-1.0		4.3		1.4	transducin family protein / WD-40 repeat family protein
0_A	1.0		1.4		1.1		1.0		1.2		4.2		-1.0	syntaxin of plants SYP121
0_A	-1.3	-1.3	-1.2	-1.1	1.3	-1.3	1.7	1.0	-1.0	-1.2	4.2	1.7	1.3	putative disease resistance protein. disease resistance protein (CC-NBS-LRR class)
0_A	1.4	1.4	1.6	1.0	2.6	1.0	1.9	2.2	1.7	-1.2	4.2	2.8	1.7	protein phosphatase 2C (PP2C), putative. group B (AthPP2C5)
0_A	1.0		1.3		1.4		-1.0		-1.1		4.2		-1.0	expressed protein
0_A	7.4		1.4		5.0		-1.2		-1.4		4.2		1.4	putative glutathione transferase AtGSTU24
0_A	-1.0		1.4		-1.2		-1.2		-1.1		4.2		1.5	armadillo repeat containing protein [Arabidopsis thaliana
0_A	1.4	1.2	1.6	1.0	-1.0	1.0	1.2	1.2	1.4	-1.4	4.2	2.8	1.6	putative Fe(II)/ascorbate oxidase. Oxidoreductase
0_S	1.0	-1.3	1.1	-1.5	1.1	-1.5	1.5	-1.4	1.3	-1.9	4.2	1.3	1.2	leucine-rich repeat protein kinase family (LRR II)
0_A	1.1	1.0	1.6	2.0	1.7	2.0	1.2	-1.2	1.7	3.0	4.2	8.1	1.4	beta-1,3-glucanase 2 (BG2) (PR-2)
0_A	-1.1		-1.3		-1.4		-1.1		-1.1		4.1		1.3	exocyst subunit EXO70 family protein
0_A	-2.8		-2.0		-2.4		1.8		-1.2		4.1		1.6	NAM-related protein (no apical meristem), transcription factor activity
0_A	-1.3	-1.8	1.3	-1.2	1.3	-1.6	1.7	1.0	1.4	-1.8	4.1	1.8	1.3	aminotransferase class I and II family protein
0_F	1.2		-1.2		-1.3		-1.1		1.2		4.1		-3.8	Putative GPI-anchored protein
0_A	1.0		1.0		1.0		1.0		1.0		4.1		1.0	NPR1/NIM1-interacting protein 1 (NIMIN-1)
0_A	1.3	1.1	1.5	-1.2	1.5	-1.4	1.1	1.1	-1.2	-1.4	4.1	1.1	1.1	chromosome structural maintenance protein-related
0_A	1.0		1.0		1.0		1.3		1.1		4.1		1.1	calmodulin-related protein, putative
0_F	3.7		1.7		2.7		-1.7		2.3		4.1		1.0	male sterility MS5 family protein
0_A	1.5	-2.1	-1.2	-1.6	1.4	-2.1	1.6	-1.3	1.6	-2.4	4.1	1.3	-1.1	xyloglucan fucosyltransferase, putative (AtFUT4)
0_A	1.1		1.1		1.6		-1.2		1.1		4.1		-1.4	FAD-linked oxidoreductase family
0_I	7.6		-1.6		8.5		2.6		2.3		4.1		-1.8	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
0_A	1.4		-1.0		-1.0		1.1		1.1		4.0		-1.8	WAK-like kinase (WLK)(WAKL)
0_A	1.7		-1.1		1.2		1.2		1.5		4.0		1.7	hypothetical protein
0_A	1.5		1.5		1.3		1.4		1.5		4.0		-1.0	Similar to: At2g45430 putative AT-hook DNA-binding protein (e Value: 3.2E-91)
0_A	1.0		-1.1		1.7		-1.2		-1.4		4.0		1.3	hypothetical protein

Appendix II:

Regulation of the barley 6-SFT promoter in barley and transgenic Arabidopsis; Collaborative project with Dr. Tita Ritsema and Dr. Vinay J. Nagaraj

Abstract

Fructan synthesis in excised barley leaves can be induced by continuous illumination or by sucrose (Suc) treatment in the dark. A key enzyme of this process is the sucrose:fructan 6-fructosyltransferase (6-SFT). It has been shown that the promoter of this gene contains the necessary *cis*-acting elements for Suc-mediated and light induction. To characterize the components regulating this promoter, specific protein phosphatase and protein kinase inhibitors were tested for their ability to suppress Suc induced induction of 6-SFT expression in two separate systems. Excised barley leaves were used on one hand and on the other hand *Arabidopsis thaliana* seedlings bearing a GUS reporter gene construct driven by the 6-SFT promoter were investigated. Our results indicate in both systems the involvement of an unknown spectrum of protein kinases as well as the protein phosphatase PP2A in the Suc mediated induction of 6-SFT expression. In addition our results suggest that the barley 6-SFT promoter in Arabidopsis is regulated in a similar way as in barley. The transgenic plants used here might be a valuable tool to elucidate components involved in regulating 6-SFT expression in future projects.

Introduction

Sugars have important signalling functions throughout all stages of the plant's life cycle. For glucose and fructose a hexokinase-dependent and –independent hexose sensing system can be distinguished. Additionally in plants there is a separate sensing system for sucrose (Smeekens, 2000). In contrast to the situation in microorganisms, most downstream components in plant sugar signalling cascades are not well characterized. However, reversible protein phosphorylation is a key mechanism for intracellular signal transduction. It is even the most common mechanism for cellular regulation in eukaryotic cells. And indeed, the use of specific inhibitors has indicated the involvement of a variety of protein kinases (PKs) and protein phosphatases (PPs) in plant sugar signalling (Rolland et al., 2002).

Here the sugar-induced regulation mechanism of the barley promoter of the fructan-synthesizing gene sucrose:fructan 6-fructosyltransferase (6-SFT) is analysed. A hexokinase independent signal transduction pathway (Muller et al., 2000) involving protein kinase and phophatases (Noel et al., 2001) is likely to be involved. Fructans, polymers of fructose, are major non-structural storage carbohydrates present in the vegetative tissues of ca. 15 % of the flowering plant species (Hendry, 1993). Fructans play an important role in assimilate partitioning, plant development and environmental stress tolerance (Ritsema and Smeekens, 2003). Barley (*Hordeum vulgare* L.) accumulates branched fructans with β(2-1) and β(2-6) linkages called graminans (Nagaraj, 2004).

The proposed pathway for graminan synthesis in barley is the 1-SST/6-SFT-model (Wiemken et al., 1995). These fructans are thought to be synthesized from sucrose

through the consecutive action of two enzymes: sucrose:sucrose 1-fructosyltransferase (1-SST), producing the trisaccharide 1-kestose, and sucrose:fructan-6-fructosyltransferase (6-SFT), which adds fructosyl units to 1-kestose to produce first the branched tetrasaccharide bifurcose and then longer-chain graminans (Sprenger et al., 1995; Wiemken et al., 1995; Vijn and Smeekens, 1999). In barley the regulation of transcription suggests 1-SST to be the pace-making enzyme of fructans synthesis in barley (Nagaraj et al., 2004). The barley 6-SFT promoter can be activated by light and sucrose in barley leaves after transient transformation by particle bombardment as demonstrated with a promoter-GUS reporter construct. Additionally several *cis*-acting regulatory elements such as boxes involved in light, cold temperature, abscisic acid (ABA) and drought regulation have been found in the sequence of that promoter (Nagaraj et al., 2001).

In wheat, okadaic acid (OK), an inhibitor of protein Ser/Thr phosphatases 1 (PP1) and 2A (PP2A) and staurosporin (STAU) a general inhibitor of protein Ser/Thr kinases are able to reduce both fructan synthesis and 6-SFT transcription upon sucrose feeding indicating the Suc-induced fructan synthesis to be mediated by protein kinases and protein phosphatases (Noel et al., 2001). Here, the signal transduction events determining the activity of the 6-SFT promoter in response to Suc were investigated further using on one hand excised leaves of wild type barley and on the other hand stably transformed Arabidopsis plants bearing a region of the barley 6-SFT promoter fused to the GUS reporter gene (Nagaraj, 2004). Arabidopsis has been used extensively as a model to investigate sugar sensing in plants, and several mutants with altered sugar sensing and signaling are available (Smeekens, 2000). In addition this plant has also proved to be useful to investigate the regulation of activities of sugar responsive promoters from other

plants (Martin et al., 1997). Here we show that, although Arabidopsis is a non-fructan producing plant, the sugar-regulated activation of the barley 6-SFT promoter is maintained in Arabidopsis. To biochemically dissect the signal transduction events, histochemical assays were performed utilizing specific inhibitors of protein kinase and protein phosphatase activity. Both protein phosphatases, namely PP2A, and several protein kinases appear to be involved in the Suc-induced activity of the 6-SFT promoter.

Results

Modulation of Suc-induced 6-SFT expression in barley by protein kinase and protein phosphatase inhibitors

To analyse the effects of specific inhibitors of protein Ser/Thr kinases, tyrosine kinases and protein Ser/Thr phosphatases, leaflets of one week old barley plants that were kept for 24 hours in the dark were treated in the dark for 24 hours with 100 mM Suc, 100 mM Sor, water and 100 mM Suc + 10 μM K252a or 2 μM STAU or 2 μM GEN or 1 μM OK. The level of 6-SFT transcript in the excised leaflets was then analysed by quantitative PCR, using specific primers for the 6-SFT coding sequence. The expression level of a H3 histone gene of barley was used to normalize the copy number of the 6-SFT transcript. Here, all the obtained ratios are compared with the difference of the ratios of the Suc and Sor treatment that was set to 100 %. The effectiveness of the inhibitors is indicated as a percentage of inhibition of the Suc induced 6-SFT expression. In barley leaves treated with water, the expression of 6-SFT is in the same range of those treated

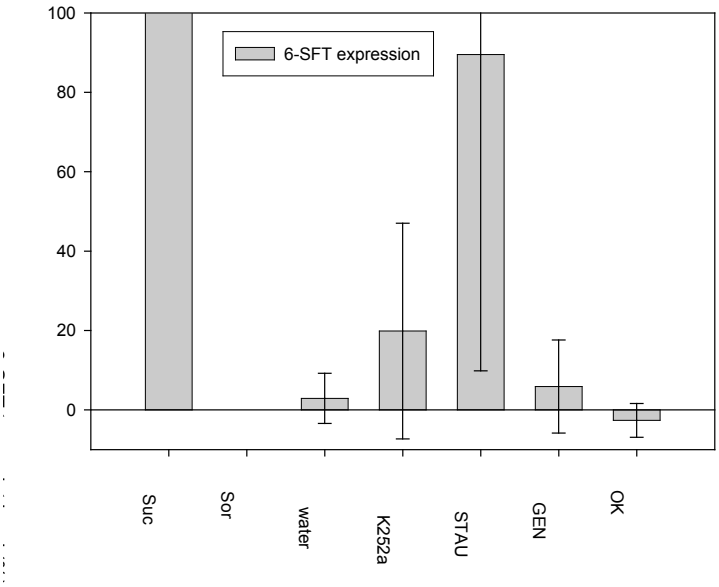

Fig. 1. Induction of mRNA levels of the 6-SFT gene in barley leaflets after 24 hours of different treatments. Transcript quantification has been performed by real time PCR. All values obtained for 6-SFT expression were normalized by the expression of a H3 histone gene. Results of the different treatments are compared to sucrose and sorbitol induction (set as 100% and 0% respectively). The treatments were 100 mM sucrose (Suc), 100 mM sorbitol (Sor), water and 100 mM sucrose + 10 μM K252a (K252a) or 2 μM staurosporine (STAU) or 2 μM genistein (GEN) or 1 μM okadaic acid (OK). The leaflets used were parts of the primary leaf of one-week-old barley, kept for 24 hours in the dark before the harvest. The treatment has been also carried out in the dark. Data are means of between three and five independent RNA extractions +/- standard deviation.

with the osmotic control Sor, suggesting the induction of 6-SFT expression by Suc not being due to osmotic effects.

The broad-spectrum protein kinase inhibitor K252a caused a reduction of the Suc-induced 6-SFT expression in barley leaflets by 80 %, indicating the involvement of protein kinases in the Suc activated signal transduction cascade regulating the 6-SFT promoter (Fig 1.). The inhibitor GEN reduced Suc induced 6-SFT expression by 94 %, suggesting an involvement of GEN-inhibitable protein kinases in sucrose induction of fructan synthesis (Fig 1.). 2 μM STAU inhibited the 6-SFT expression by only 10%. A high variation of values got from this treatment resulted in rather big values of the standard deviation (Fig 1.). OK, an inhibitor of protein phosphatase was reducing the Suc-induced 6SFT expression down to water and Sor levels (Fig 1.). This points to the involvement of protein phosphatases in the sucrose-mediated induction of fructan synthesis in barley. The strong inhibition of fructosyltransferase (FTF) activity and 6-SFT expression at the transcriptional level by OK can also be observed in wheat (Noel et al., 2001).

Screening of transgenic Arabidopsis plants

To confirm the results obtained in barley on signal transduction regulation of the 6-SFT promoter, stably transformed Arabidopsis plants bearing 1.6 kb of the barley 6-SFT promoter fused to the GUS reporter gene were analysed.

In order to get homozygous transgenic Arabidopsis lines of three individual transformation events, the progeny of three T-0 generations, i.e. line III, VII and X, were

sprayed with Basta. A small percentage of seedlings survived. Some of the healthy looking seedlings were transplanted into separate pots. From these plants the seeds were harvested and a small aliquot (approximately 50 seeds) was grown and checked for Basta resistance. From each line of this generation a batch of seeds showing 100 % resistance to Basta and intense blue colouring after GUS staining (data not shown) was selected for further analysis assuming these batches to be homozygous for the transformed 6-SFT promoter construct.

Modulation of 6-SFT promoter regulation in transgenic Arabidopsis plants by kinase and phosphatase inhibitors

In mature transgenic Arabidopsis leaves of line III STAU and OK were able to reduce Suc-induced activation of the 6-SFT promoter (Nagaraj, 2004). Here we report the interference of K252a, STAU, GEN and OK with the Suc triggered regulation machinery of the 6-SFT promoter in seedlings of the transgenic Arabidopsis lines III, VII and X. To quantify the GUS activity in the seedlings, a MUG assay with subsequent fluorescence emission quantification was performed. For the histochemical assay two-weeks old Arabidopsis seedlings were kept for 16-24 hours in the dark and subsequently treated in

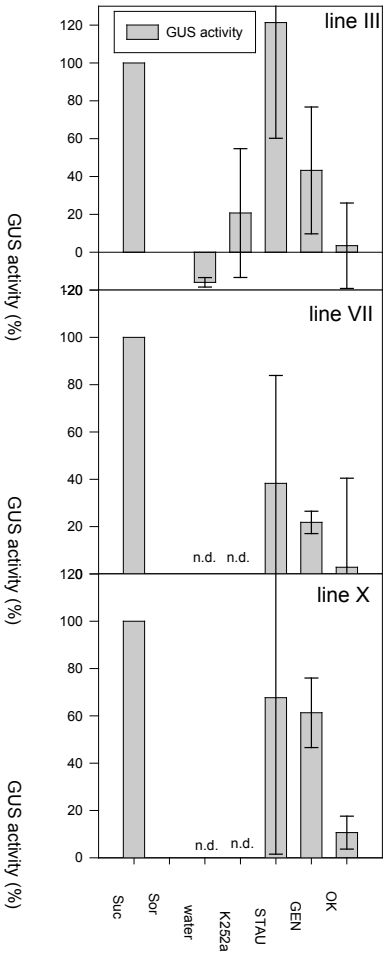

Fig. 2. Induction of GUS activity in three different transgenic Arabidopsis lines bearing a *uidA* gene driven by the barley 6-SFT promoter after 24 hours of different treatments. Quantification of GUS activity has been performed by the MUG assay. For each assay ten two week old seedlings were used. Results of different treatments are compared to sucrose and sorbitol induction (set as 100% and 0% respectively). The treatments were 100 mM sucrose (Suc), 100 mM sorbitol (Sor), water and 100 mM sucrose + 10 μM

K252a (K252a) or 2 μM staurosporine (STAU) or 2 μM genistein (GEN) or 1 μM okadaic acid (OK). The treatments have been carried out in the dark. Water and K252a treatment has not been done for line VII and X (n.d.). Data are means of at least six independent enzyme extractions +/- standard deviation.

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the dark for 16-24 hours with 100 mM Suc, 100 mM Sor, water and 100 mM Suc + 1 μM K252a or 2 μM STAU or 2 μM GEN or 0.5 μM OK. Each average value of the different treatments of line III, VII and X represents at least six independent enzyme extractions.

The similarity of the GUS activity in Sor treated plants to the one in the water treated seedlings in line III suggests the GUS expression in Sor treated plants to be due to a constitutive background expression. It also shows that the promoter is not induced by osmotic stress.

The difference between Suc-induced GUS activity and the GUS activity in Sor treated plants was set as 100% for each line. All applied inhibitors inhibited the Suc induced GUS activity by a certain percentage. In line III the protein Ser/Thr kinase inhibitor K252a reduced the Suc-induced GUS activity by 66% indicating an involvement of protein kinases in the Suc triggered signalling cascade regulating the 6-SFT promoter.

The general kinase inhibitor STAU could only partially inhibit the Suc-induced GUS activity in transgenic Arabidopsis, as was seen in barley (Averages were 21% induction, 62% inhibition and 32% inhibition in line III, VII and X respectively). In addition all lines treated with STAU showed a high variation of inhibition.

GEN was able to reduce the Suc-induced 6-SFT promoter activity in all lines tested between 39% and 78% (Fig. 2.).

In all lines the Suc mediated induction of GUS activity was strongly reduced (between 89% and 97%) by OK, an inhibitor of protein phosphatases class 1 and 2A (Fig. 2.).

Discussion

In plants sugars can be sensed by sugar sensors, which feed information into signal transduction cascades that lead to different plant responses. The involvement of protein kinases, protein phosphatases, Ca^{2+} and calmodulin in these transduction mechanisms has been proposed (Smeekens, 2000). There are numerous studies trying to dissect sugar induced signalling required for regulation of gene expression using specific inhibitors of protein Ser/Thr phosphatases and protein Ser/Thr kinases. It has been shown that the Suc inducible expression of sporamin and β-amylase in sweet potato can be blocked by the PP1 and PP2A inhibitors OK, microcystin-LR and calyculin A (Takeda et al., 1994). It also has been reported that the Suc induced expression of a β-glucuronidase-fusion gene, with the promoter of the gene for the sweet potato β-amylase is Ca^{2+} dependent and inhibited by STAU or K252a in tobacco leaves (Ohto et al., 1995) and that sucrose, glucose or fructose induce a calcium dependent autophosphorylation of several protein kinases that is inhibited by protein kinase inhibitors such as STAU or K252a (Ohto and Nakamura, 1995). All these data indicate that in sugar induced signal transduction protein phosphorylation as well as dephosphorylation are common and required mechanisms.

6-SFT, a key enzyme of fructan synthesis in grasses, is strongly induced upon Suc feeding (Muller et al., 2000; Nagaraj et al., 2004). Affection of 6-SFT expression in grasses by exogenous sugar levels (Wagner et al., 1986; Muller et al., 2000; Pollock et al., 2003), cold stress (Wei et al., 2001) and supply of nitrogen (Morcuende et al., 2004)

have been reported. However, only limited attempt has been made to elucidate the signal transduction events regulating these effects. Studies in wheat and transgenic Arabidopsis plants using inhibitors point to the involvement of protein phosphatases and kinases in the Suc-induced signal transduction pathway regulating 6-SFT expression (Noel et al., 2001; Nagaraj, 2004).

Here, we want to further identify protein kinases and phosphatases activities intervening with the signal transduction pathway triggered upon Suc mediated regulation of the barley 6-SFT promoter. For this purpose two different plant systems were chosen. On one hand dissected barley leaves were used. In these leaflets the activity of the 6-SFT promoter was determined by measuring the mRNA level of the 6-SFT gene by quantitative PCR. On the other hand transgenic Arabidopsis plants bearing a *uidA* reporter gene driven by a 1.6 kb part of the barley 6-SFT promoter were analysed. In these plants the regulation of the 6-SFT promoter was measured by quantifying GUS activity.

To get evidence whether protein kinases are involved in the investigated signalling transduction cascade, three kinase inhibitors differing in target specificity were used.

The broad-spectrum kinase inhibitor K252a was able to strongly reduce the Suc induced activation of the 6-SFT promoter in barley leaflets as well as in transgenic Arabidopsis leaves, indicating that in the signalling cascade of both organisms protein kinases are involved in the regulation of the investigated promoter.

Another inhibitor used here to investigate the Suc mediated induction of the 6SFT-promoter was GEN. GEN is considered a specific inhibitor of Tyr-protein kinases in animals. GEN was able to reduce the Suc mediated induction of activity of the 6-SFT

promoter in transgenic Arabidopsis as well as in excised barley leaves. In contrast to Ser/Thr-kinases, specific Tyr-kinases are not positively known in plants, although their occurrence has often been proposed (Luan, 2002). MAPKKs are clearly mixed Ser/Thr-protein kinases which phosphorylate a Tyr and a Ser residue in their MAPK substrates (Nuhse et al., 2000); they are not inhibited by genistein. Alternatively, GEN might inhibit certain Ser/Thr-protein kinases. These finding may indicate the involvement of putative protein Tyr-kinases in the Suc triggered signalling cascade investigated, or the involvement of Ser/Thr-protein kinases as suggested above. In wheat leaves 100 μM GEN treatment had no effect on Suc induced FTF activity (Noel et al., 2001).

The second general kinase inhibitor used besides K252a was STAU. However, STAU was not able to inhibit the Suc induced 6-SFT expression. It is a surprise that there is a difference between the K252a and the STAU effect since in many plant systems, these two kinase inhibitors have very similar effects (Felix et al., 1991).

At high concentrations (i.e. 10 μM), STAU is a general kinase inhibitor, but at low concentrations (50-100 nM) its first targets are the activity of protein kinase C (PKC) and Ca^{2+}/calmodulin dependent protein kinases (CDPK) in certain animal systems (Wolf and Baggiolini, 1988; Yanagihara et al., 1991), but its targets in plants are poorly defined. Additionally, there are no homologues of protein kinase C in plants.

However, in wheat the same STAU concentration as used here (2 μM) inhibits the Suc-induced induction of FTF activity by 50% and reduces the 6-SFT transcript in corresponding manner (Noel et al., 2001). These data point to an involvement of kinase activity, which is only partially inhibited at the STAU concentration used here.

OK, an inhibitor of protein phosphatases class 1 and 2A reduced the Suc-induced 6SFT expression in barley and the Suc-induced GUS activity in Arabidopsis down to Sor levels (Fig 1. & 2.). This result is further supported by the strong inhibition of the Suc induced FTF activity and the 6-SFT mRNA level by 1μM OK in wheat leaflets (Noel et al., 2001). PP2A has been implicated in sugar signaling in other studies. It has been shown that in intact MCF7 cells treated for three hours with 1 μM OK, the activity of PP2A was abolished almost completely, whereas the PP1 activity was not affected under the same conditions. It also has been reported that only 50 % of PP1 activity was inhibited by 60-600 nM OK in cell extracts, and 50 % of the PP2A activity was inhibited already at an OK concentration of 0.1-2 nM (Cohen, 1991). These results indicate that the OK treatments used here (1 μM for the barley leaflets, 0.5 μM for the Arabidopsis seedlings) inhibit PP2A but not PP1 activity in the cell (Favre et al., 1997). Both hexokinase dependent and independent pathways of sugar sensing are involved in cell cycle regulation, and PP2A seems to be involved in both (Riou-Khamlich et al., 2000).

In conclusion, all the data presented here indicate the following components to be involved in the Suc-induced regulation of the barley 6-SFT promoter: the protein phosphatase PP2A (inhibited by OK), as well as an unknown spectrum of protein kinases (inhibited by K252a) including proposed tyrosine kinases (inhibited by GEN). Whether the involvement of Ca^{2+}/calmodulin-dependent protein kinases can be excluded (no inhibition by STAU) is not clear, since in another monocot (wheat), the same STAU concentration as used in this study was able to inhibit the Suc induced FTF activity and the 6-SFT mRNA level by approximately 50 % (Noel et al., 2001).

However, restrictively, it has to be mentioned, that the interpretation of experiments performed with inhibitors is difficult. It is not possible to distinguish by such experiments whether the inhibitor works direct and specific way, or, even if one is getting a positive inhibition, the inhibitor acts in an indirect, rather unspecific, unknown way. Additionally, if the inhibitor does not give any positive result, the cells might not take it up or the plant decontaminated it before it reached its target.

Material and methods

Barley and Arabidopsis growth conditions

Barley (*Hordeum vulgare* L. cv. Lyric) seeds were soaked for 24 hours in running tap water, planted in a commercial soil mixture (UFA Haus und Garten, Bern, Switzerland) and grown for 7 days in a growth chamber. The conditions were: 16 h light (130 µmole photons $m^{-2}s^{-1}$) at 26°C, 8 h dark at 20°C.

Seeds of Arabidopsis plants were grown on half-strength Murashige and Skoog medium without sucrose (Sigma-Aldrich, Buchs, Switzerland) solidified with 1% (w/v) agar. The agar plates were oriented vertically and were incubated in a daily cycle of 18 h of light (130 µmol photons $m^{-2}s^{-1}$) at 22°C and 6 h of darkness at 18°C. After 12 days of growth the seedlings were subjected to the different treatments.

Plasmid construct, Arabidopsis transformation and selection of transgenic plants

The plasmid pP6SFT3301 was constructed by replacing the 35S promoter region of the binary vector pCAMBIA3301 with the barley 6-SFT promoter (AJ306962) so that the 6-SFT promoter controls the expression of the GUS reporter gene. The pCAMBIA3301

was first digested by *EcoR*I and *Nco*I to delete the 35S promoter region controlling the *uidA* (GUS) reporter gene. A 1.6 kb *EcoR*I and *Nco*I fragment of the 6-SFT promoter was then ligated to the digested pCAMBIA3301 vector so that 6-SFT promoter controls the expression of the GUS reporter gene. The pP6SFT3301 construct was sequenced to check for errors during cloning. The T-DNA region also has the *bar* gene which confers resistance to glufosinate (Basta) and enables selection of plants. The plasmid has a kanamycin resistance gene for selection of bacterial transformants.

The pP6SFT3301 plasmid was introduced into *Agrobacterium tumefaciens* and Arabidopsis plants of the Col-0 ecotype were transformed by dipping the developing floral tissues into a solution containing the *Agrobacterium* culture (Clough and Bent, 1998). The seeds obtained were germinated as described above. Transgenic plants were selected on the basis of resistance to the herbicide Basta (Omya AG, Safenwil, Switzerland). Five days old seedlings were sprayed with 300µM Basta using a mist sprayer. Spraying was repeated every 2 days until healthy green plants were clearly distinguishable from moribund seedlings.

GUS assay

For the GUS assay two weeks old auxenically grown seedlings were washed with water three times and subsequently immersed in the GUS substrate solution in the dark at 37°C for 24 hours. The GUS substrate solution contained 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 100 mM potassium phosphate buffer (pH 7.0). The solution was stored at – 20 °C. X-glc was added to a final concentration of 1 mM freshly before

use. The plantlets were then rinsed in phosphate buffer, kept for 4 hours in the fixing solution containing 2.5% glutaraldehyde and 200 mM sodium cacodylate, pH 7.2. The whole plants were analysed for blue staining under the binocular.

MUG assay

For the quantitative GUS assay 10 transgenic Arabidopsis seedlings were subjected to a 4-methylumbelliferyl-β-D-glucuronide (MUG) assay. 12 days old seedlings were treated with the specific sugar or sucrose plus inhibitor solutions. Afterwards the seedlings were homogenized in 150 μL extraction buffer. The filter sterilized extraction buffer contained 50 mM Na_2PO_4 pH 7.0, 10 mM DTT, 1 mM EDTA, 0.1 % SDS and 0.1 % triton X-100. The homogenized tissue was centrifuged for 5 minutes at maximum speed and 27 μL of the supernatant were put in a 96 well plate for measuring fluorescence. Each sample was run in triplica. 3 μL of 10X MUG solution was added. The MUG stock contained 35 mg MUG per 10 mL extraction buffer. The 96 well plate was sealed, shaken and incubated 3 h at 37°C in the dark. To stop the reaction 250 μL of a 0.2 M Na_2CO_3 solution (stop buffer) was added. The fluorescence was measured using the Fluostar Optima (BMG Labtechnologies) with an excitation of 355 nm and an emission of 460 nm. For the gain adjustment one sample was chosen manually.

Inhibitors

Okadaic acid (OK), an inhibitor of protein phosphatases 1 (PP1) and 2A (PP2A), genistein (GEN), an inhibitor of tyrosine protein kinases, staurosporine (STAU) and

K252a, both general inhibitors of protein kinases were dissolved in dimethylsulfoxide (DMSO) at 100 μM, 10 mM, 200 μM and 2 mM respectively and stored at -20°C.

Induction of 6-SFT expression in barley

In the middle of the light period one-week old barley plants were put in the dark for 24 hours and subsequently harvested. 5 cm of the middle part of the primary leaf was cut in two similar sized pieces. Three of these leaflets were dipped at their cut edges for 24 hours in, 100 mM Suc or 100 mM Sor solutions. In the case of treatment with inhibitors the leaves were pretreated with inhibitors in DMSO for 2 hours before the treatment with Suc or Sor. The following inhibitors solutions were used at the indicated final sample concentration: 1 μM Okadaic acid (OK) (Sigma-Aldrich, Schnelldorf, Germany), 10 μM K252a (Alexis Biochemicals, Switzerland), 2 μM genistein (GEN) and 2 μM staurosporine (STAU). GEN and STAU were a kind gift of Dr. Tita Ritsema. To make sure the inhibitor solutions got in contact with the plants, the leaves were submerged in 900 μL of the inhibitor solutions and subjected to vacuum (98 kPa) for 30s. Then the leaves were half pulled out of the solution to enable a transpiration stream and kept for two hours in the dark. Afterwards 100 μL 1 M Suc or 1M Sor were added and the samples were placed for another 24 hours in the dark at 20°C. After treatments the leaflets were frozen in liquid nitrogen and stored at –80°C.

Induction of the 6-SFT promoter in transgenic Arabidopsis seedlings

In the middle of the light period two-week old transgenic Arabidopsis seedlings were put in the dark for 24 hours. Subsequently ten intact seedlings per sample were submerged for

24 hours in 100 mM Suc or 100 mM Sor solutions. In the case of treatment with inhibitors the seedlings were pretreated with inhibitors in DMSO for 2 hours before the treatment with Suc or Sor. The following inhibitors solutions were used at the indicated final sample concentration: 0.5 μM OK, 2 μM GEN and 2 μM STAU. To make sure the inhibitor solutions got in contact with the seedlings, the leaves were submerged in 900 μL of the inhibitor solutions and subjected to vacuum (98 kPa) for 30s. Then the leaves were for two hours in the dark. Afterwards 100 μL 1 M Suc or 1M Sor were added and the samples were placed for another 24 hours in the dark at 20°C. After treatments the seedlings were frozen in liquid nitrogen and stored at –80°C.

RNA extraction, cDNA synthesis, Real time PCR analysis

The RNA extraction, cDNA synthesis and real time PCR analysis was performed as described in chapter X. Copy numbers were calculated from amplification plots of known standards for the putative histone and the 6-SFT gene. Transcript levels of a putative histone gene were used to normalize the amount of copies of 6-SFT. The primer used to quantify the 6-SFT transcripts and the 6-SFT plasmid standard were 6-SFT (forward): 5'-TCC AAT GAG GAC GAT GGC ATG T-3' and 6-SFT (reverse): 5'-AAT GCA TGC AAG CGA GGT-3'. The sequences of the primers of the putative histone were: (forward) 5'-CGC AAG TAC CAG AAG AGC AC-3' and (reverse) 5'-ATG ATG GTC ACA CGC TTG GC-3'.

The primers for the putative histone were designed after the barley EST HC11F01w found on the barley EST library "CR-EST: The IPK Crop EST Database" (Gatersleben);

website: http://pgrc.ipk-gatersleben.de/cr-est/index.php. The sequence of this barley EST was chosen because it showed the highest similarity (85 %) to the coding sequence of the constitutively expressed Arabidopsis histone gene H3G (at4g40040). Real time PCR analysis showed that the barley EST HC11F01w transcript levels did not change after sugar treatments of the plants (Virginie Galati, personal communication).

Publications

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