Trehalose Mediated Growth Inhibition of Arabidopsis Seedlings Is Due to Trehalose-6-Phosphate Accumulation[1][w]

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Trehalose-6-phosphate (T6P) is required for carbon utilization during Arabidopsis development, and its absence is embryo lethal. Here we show that T6P accumulation inhibits seedling growth. Wild-type seedlings grown on 100 mM trehalose rapidly accumulate T6P and stop growing, but seedlings expressing *Escherichia coli* trehalose phosphate hydrolase develop normally on such medium. T6P accumulation likely results from much-reduced T6P dephosphorylation when trehalose levels are high. Metabolizable sugars added to trehalose medium rescue T6P inhibition of growth. In addition, Suc feeding leads to a progressive increase in T6P concentrations, suggesting that T6P control over carbon utilization is related to available carbon for growth. Expression analysis of genes from the Arabidopsis trehalose metabolism further supports this: Suc rapidly induces expression of trehalose phosphate synthase homolog *AtTPS5* to high levels. In contrast, T6P accumulation after feeding trehalose in the absence of available carbon induces repression of genes encoding T6P synthases and expression of T6P phosphatases. To identify processes controlled by T6P, we clustered expression profile data from seedlings with altered T6P content. T6P levels correlate with expression of a specific set of genes, including the S6 ribosomal kinase *ATPK19*, independently of carbon status. Interestingly, Suc addition represses 15 of these genes, one of which is *AtKIN11*, encoding a Sucrose Non Fermenting 1 (SNF1)-related kinase known to play a role in Suc utilization.

In microbes, trehalose accumulation is a crucial defense mechanism that stabilizes proteins and biological membranes under a variety of stress conditions, including increased temperature, hydrostatic pressure, desiccation, nutrient starvation, osmotic or oxidative stress, and exposure to toxic chemicals (Attfield, 1987; De Virgilio et al., 1990; Wiemken, 1990; Singer and Lindquist, 1998; Iwahashi et al., 2000; Benaroudj et al., 2001). Trehalose synthesis in microbes, as in plants, occurs mostly via the phosphorylated precursor, T6P, with a trehalose phosphate synthase (TPS) and a trehalose phosphate phosphatase (TPP). Signal transduction from stress perception to trehalose synthesis, however, regulates the expression of enzymes metabolizing T6P in both *Escherichia coli* and yeast. For example, two crucial steps for triggering the heat shock response in yeast appear to be the initiation of sphingolipid signaling and the relocation of MSN2 and MSN4 transcription factors from cytosol to nucleus leading to transcriptional activation of *TPS2*, a TPP enzyme, among other enzymes (Boy-Marcotte et al., 1999).

In most plants, trehalose is hardly detectable at 0.15 mg g^{-1} dry weight or lower, depending on tissue and species (Garcia et al., 1997; Roessner et al., 2000; Vogel et al., 2001). Therefore, it is not a stress protectant except in certain specialized resurrection species that accumulate the compound quantitatively. However, plants have more genes encoding homologs of T6P metabolizing enzymes than microbes such as yeast. Particularly surprising is the remarkable radiation of genes with TPP domains in Arabidopsis. This domain is found in seven TPS homologs, *AtTPS5* to *AtTPS11*, as well as in ten TPP homologs (Leyman et al., 2001; Eastmond et al., 2003); a nomenclature for TPP genes is proposed in Table I. The Arabidopsis genome does not appear to contain sequences with homology to trehalose phosphate hydrolase (TPH).

We have previously shown that the metabolic precursor of trehalose, T6P, is essential for carbon utilization in Arabidopsis and that its control may affect similar steps in glycolysis and beyond as in yeast (Schluepmann et al., 2003). Eastmond et al. (2002) previously observed that the *tps1* mutant is embryo lethal; this is most likely due to reduced T6P levels and, as a consequence, an apparent inability to utilize sugars.

Moreover, T6P levels have been shown to influence photosynthetic capacity per leaf area (Pellny et al., 2004). Therefore, the role of the many TPP enzymes

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Table I. *Putative Arabidopsis T6P phosphatases containing the TPP domain but not the TPS domain*

Gene	Arabidopsis Genome Initiative Number	Expressed Sequence Tag	Reference
AtTPPA	At5g51460	Yes	Vogel et al. (1998)
AtTPPB	At1g78090	Yes	Vogel et al. (1998)
AtTPPC	At1g22210	Yes	
AtTPPD	At1g35910	Yes	
AtTPPE	At2g22190	No	
AtTPPF	At4g12430	Yes	
AtTPPG	At4g22590	Yes	
AtTPPH	At4g39770	Yes (putative pseudogene)	
AtTPPI	At5g10100	Yes	
AtTPPJ	At5g65140	Yes	

might be restricted to regulating levels of T6P in the plant. Alternatively, these enzymes might mediate control over trehalose levels in the plants, and altered levels of this molecule could function as a signal. The role of trehalose has been studied very little in plants.

Seedlings on 100 mM trehalose germinate but do not develop primary leaves; their cotyledons become dark green with a red rim, and root growth ceases (Wingler et al., 2000). Interestingly, after 7 d on trehalose cotyledons accumulate starch and *ApL3* expression appears particularly high (Wingler et al., 2000). In addition, *adg2* mutant seedlings that are unable to produce starch are less growth-inhibited by trehalose than wild type (Fritzius et al., 2001). Metabolizable sugar rescues growth inhibition on trehalose.

Trehalose, therefore, might interfere with carbon allocation by way of forcing available carbon into starch in source organs, there being insufficient metabolizable carbon for root growth (Wingler et al., 2000).

Trehalose feeding leads to symptoms that resemble those obtained by over expression of *E. coli* TPS; such plants have increased T6P content, but trehalose is below detection levels (Schluepmann et al., 2003). We show here that growth arrest by trehalose leads to T6P accumulation. This arrest can be overcome by expression of *E. coli* TPH in Arabidopsis seedlings. TPH hydrolyzes T6P to Glc and Glc-6-phosphate, bypassing trehalose. We conclude that T6P accumulation is growth inhibitory. Growth inhibition cannot be overcome by expression of *E. coli* TPP, suggesting that endogenously synthesized T6P is inefficiently dephosphorylated when trehalose concentrations are high in the cell.

T6P-mediated growth inhibition can be overcome if metabolizable sugar is supplied in addition to trehalose, suggesting that seedlings need to relate T6P levels with available carbon. Seedlings respond to T6P accumulation in the absence of carbon by inducing TPP and reducing TPS gene expression. Conversely, Suc addition leads to a rapid induction of *AtTPS5*. The data uncovers a signal transduction mechanism relating T6P with available carbon by means of transcriptional regulation of T6P metabolizing enzymes.

To identify possible target processes of T6P interference with carbon utilization or supply, we used cluster analysis of expression profiling data. A total of 35 genes were identified with an expression profile correlating with T6P levels. Half of these genes are related to stress responses suggestive of a link between T6P metabolism and stress responses in Arabidopsis. The other genes encode T6P metabolizing enzymes, or are part of signal transduction processes. One of the latter is *AKIN11*, coding a Sucrose Non Fermenting 1 (SNF1)-related kinase known to be involved in signal transduction affecting sugar utilization (Bhalerao et al., 1999).

RESULTS

Trehalose-6-Phosphate Mediates Trehalose-Induced Growth Inhibition

Trehalose supplied in the medium inhibits growth of Arabidopsis seedlings (Wingler et al., 2000). At 100 mM trehalose in the medium, growth of wild-type Col.0 seedlings is completely arrested (Fig. 1). Expressing *E. coli* cytosolic trehalase, *Tref*, allows seedlings to develop long roots on trehalose. These roots are as long as on control osmotic (Fig. 1). TREF does not have a secretory signal and is hence cytosolic in these transgenic plants. Exogenously supplied trehalose is therefore efficiently imported by plant cells.

Trehalose accumulating in the cytosol may be toxic. Alternatively, its accumulation may affect levels of T6P. Because of the similar appearance of wild-type seedlings on trehalose and of TPS-expressing seedlings, we tested the latter hypothesis by growing seedlings expressing *E. coli* trehalose 6-phosphate-phosphatase (TPP) and -hydrolase (TPH) on trehalose. Growth of TPP expressors is not improved over wild

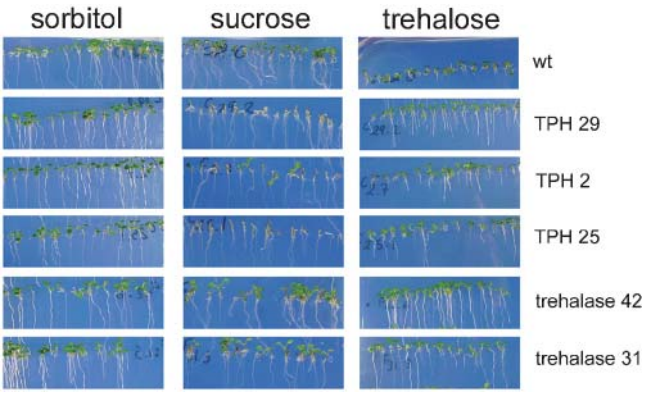

Figure 1. Trehalase and TPH-expressing seedlings of Arabidopsis grown on 100 mM trehalose. Seedlings expressing *E. coli treF* or TPH (*treC*), behind the cauliflower mosaic virus *(CaMV) 35S* promoter (Schluepmann et al., 2003) were grown on medium with either sorbitol (200 mM), Suc (200 mM), or trehalose (100 mM). wt, Col-0 wild type; TPH 29, 2, and 25 are three independent TPH expressor lines; trehalase 42 and 31 are two independent trehalase expressor lines.

type on 100 mM trehalose (data not shown). Interestingly, TPH expressors grow on 100 mM trehalose (Fig. 1), suggesting that accumulating T6P causes growth arrest. TPP and TPH enzymes are active in the lines shown because seedlings of these lines are sensitive to supplied Suc (Fig. 1 and Schluepmann et al., 2003). TPP is not effective for rescue on trehalose because of the catalysis dependence on product accumulation. Under standard conditions phosphate cleavage has an approximate ΔH° of -5 kJmol^{-1} at 311 K and neutral pH (Tewari et al., 1988; Goldberg and Tewari, 1994; Alberty, 1996), but when trehalose accumulates 10^3 fold, that is from micro to millimolar concentrations, $\Delta H'$ becomes positive and T6P hydrolysis by TPP enzymes is no longer thermodynamically favored. TPH catalysis is not limited by trehalose accumulation since the products of catalysis are Glc and Glc-6-phosphate. TPH expressors grow slower than trehalase expressors on 100 mM trehalose (Fig. 1). Trehalase activity releases Glc that is used for growth. In the TPH plants, however, trehalose is not phosphorylated to T6P since under the low phosphate conditions the TPP reaction is irreversible. This is consistent with lack of rescue by trehalose of embryos deficient in AtTPS1 but that express TPP genes (Eastmond et al., 2002). TPH activity, therefore, cleaves only T6P accumulating due to inefficient dephosphorylation of endogenously synthesized T6P, and these seedlings do not benefit from the carbon in trehalose. To confirm the genetically implied accumulation of T6P we measured T6P levels in seedlings fed with various sugars.

Trehalose Feeding Results in T6P Accumulation

T6P levels in wild-type seedlings grown on 100 mM sorbitol are typically 2 nmol g^{-1} FW. T6P levels accumulate to 8 nmol g^{-1} FW half an hour after supply of 100 mM trehalose, and then decrease to about 2-fold the levels on sorbitol after 24 h (Fig. 2A). Metabolite measurements further show that trehalose feeding results in a drop of Glc-phosphate pools compared to sorbitol feeding (Fig. 2B–D). This is consistent with results obtained from transgenic lines with increased T6P levels by way of TPS expression (Schluepmann et al., 2003).

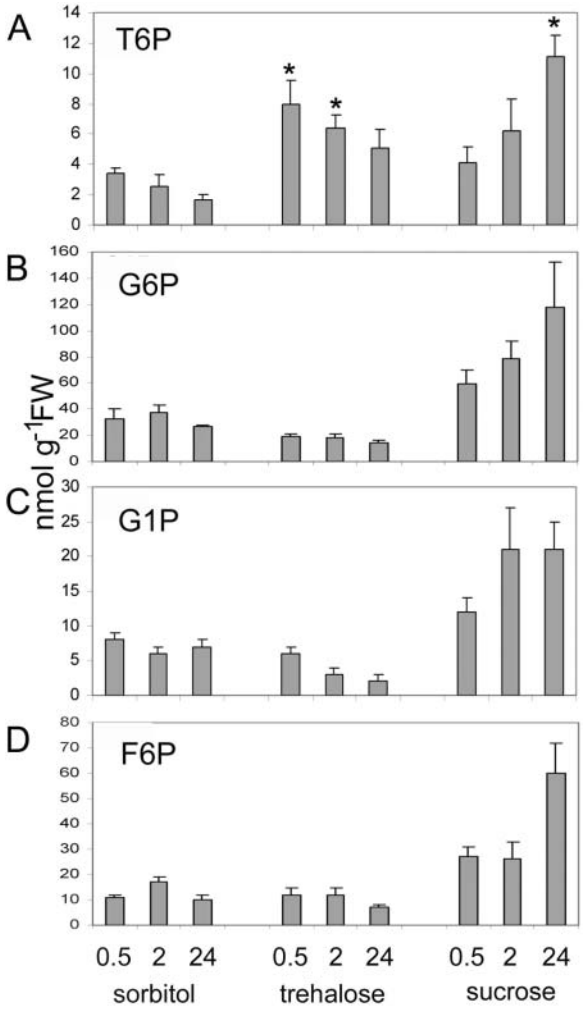

Figure 2. T6P and hexose phosphate levels in seedlings fed with sorbitol, Suc, or trehalose. Wild-type Col-0 seedlings were grown in rotating liquid culture under constant light in half-MS medium, then supplied with sugar prior to harvest on day 7. 100 mM of either sorbitol, Suc, or trehalose was supplied for 0.5, 2, and 24 h prior to harvest. Seedlings were collected on a sieve, washed, and snap frozen, then T6P and Glc- and Fru-phosphates were determined as described in Methods. A, T6P; B, Glc-6-phosphate; C, Glc-1-phosphate; and D, Fru-6-phosphate levels in seedlings. * Represent significantly different values ($P < 0.05$) compared to the sorbitol control.

T6P Inhibits Growth in the Absence of Metabolizable Carbon

Growth on high trehalose can be restored if sugar is supplied simultaneously with trehalose (Fig. 3). Supply of sorbitol osmoticum does not restore growth; neither does supply of 3-O-methyl Glc, a Glc analog not entering glycolysis. Addition of Glc together with Fru, Suc, and maltose is equally effective at restoring growth. Seedlings on trehalose remain sensitive to Man and 2-deoxyglucose suggesting that accumulating T6P does not affect hexokinase activities phosphorylating these sugars. Metabolizable carbon thus rescues T6P-mediated growth arrest. This result implies that T6P control over carbon utilization be related with available carbon for growth. We therefore measured T6P levels after Suc feeding as well as after trehalose feeding (Fig. 2A).

Suc feeding induces T6P levels in seedlings to 11 nmol g^{-1} FW after 24 h, five times the level in seedlings supplied with osmotic control. Suc-induced T6P accumulation is progressive and reaches 6 nmol g^{-1} FW already after 2 h. This is consistent with previous results demonstrating that T6P is required for sugar utilization (Schluepmann et al., 2003). Suc feeding leads to an increase in hexose- and Fru-phosphate pools (Fig. 2B).

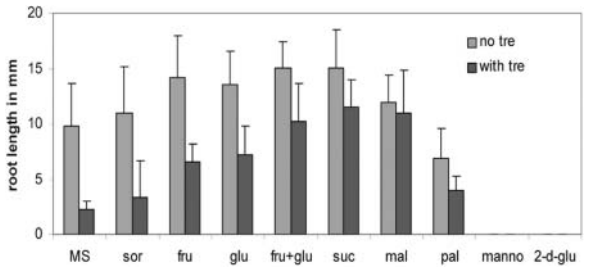

Figure 3. Root growth on trehalose media. Wild-type Col-0 seed was germinated and seedlings grown under long day conditions on agar-solidified half-MS medium with 50 mM sugar without (no tre) or with 50 mM trehalose (with tre). Root length was measured after 7 d. Sugars tested are sorbitol (sor), Glc (glu), Fru (fru), 50 mM Fru plus 50 mM Glc (Fru + Glu), Suc (suc), maltose (mal), palatinose (pal), Man (manno), and 2-deoxy Glc (2-d-glu).

Regulated Expression of Genes From the Arabidopsis T6P Metabolism

To address whether gene expression is important in signal transduction leading to altered T6P levels, we generated expression profiles of wild-type seedlings after feeding different sugars. We additionally generated expression profiles of transgene seedlings with altered T6P levels. Seedlings with modified T6P levels by way of transgene expression were TPS expressor line 19.3 with 3-fold increased T6P, TPP expressor line B12.1 with 4-fold reduced T6P, TPH expressor line 16.2 with 0.7-fold reduced T6P, and trehalase expressor line 46.2 with unchanged levels of T6P (Schluepmann et al., 2003). Transgene seedlings and wild-type parent control were grown in half-Murashige and Skoog (MS) medium supplemented with 10 mM Suc. Sugar feeding of wild-type seedlings was after 6 d in half-MS medium and during 24 h. Expression profiles were obtained by hybridizing seedling cRNA probes to the Affymetrix 8200 gene chip (Affymetrix, Santa Clara, CA) as described in ''Materials and Methods''. This 8200 gene chip contains oligonucleotide probes for *AtTPS1, 4, 5, 8, 10,* and *11*. It also contains probes for *AtTPPA* and *B*, and the TPP homologs *TPPE, F, G,* and *H* (Table I), as well as for the trehalase *AtTRE1*. Figure 4 compares expression data obtained from array hybridization to oligonucleotides of these genes using cRNA from transgenics with modified T6P levels and from wild type fed with 100 mM sorbitol, Suc, or trehalose. Note that expression values are compared only with the control for each of the two experiments, that is with wild-type expression values for the transgenics and with expression values of wild type grown on sorbitol for the sugar feeding experiment.

Expression of five out of six TPP genes is induced in seedlings fed with trehalose compared to sorbitol (Fig. 4A) and in seedlings expressing *E. coli* TPS compared to wild type (Fig. 4B). Expression levels of TPP genes are higher when trehalose feeding than when expressing *E. coli* TPS, consistent with the initial higher T6P accumulation after trehalose feeding. In the case of *TPPB*, a gene with demonstrated TPP activity (Vogel et al., 1998), trehalose feeding induces expression 3-fold more than *E. coli* TPS expression. Expression of these genes is reduced in TPP expressors. Suc feeding does not similarly induce these TPP genes (Fig. 4A).

Expression of Arabidopsis TPS homologs, on the other hand, is generally induced in seedlings expressing *E. coli* TPP compared to wild type. Similarly, trehalose feeding reduces expression of three out of six TPS homologs (Fig. 4C). These findings point to a regulatory feedback mechanism that adjusts expression of T6P metabolizing genes to T6P levels. Seedlings supplied with Suc do not show such feedback, suggesting that this mechanism is suppressed when metabolizable carbon is supplied.

Suc Rapidly Induces High Levels of *AtTPS5* Expression

Interestingly, expression of *AtTPS5* is induced 21-fold after Suc feeding (Fig. 4C). The Suc effect on *AtTPS5* is not mimicked by trehalose feeding (Fig. 4C) and is thus a Suc or available carbon-specific transcriptional response relating T6P metabolism with available carbon. To verify results obtained from microarray analysis and to study the kinetics of mRNA accumulation after sugar feeding, we repeated the feeding experiments under identical conditions but including 0 min, 30 min, 1 h, 4 h, and 24 h time points and followed expression of *AtTPS5*, *AtTPS1,* and *AtTRE1* by quantitative (Q-) PCR (Fig. 5). Sorbitol osmotic (100 mM) does not affect expression of *AtTPS5* compared to the 0 h time point control. Suc feeding induces *AtTPS5* expression 3-fold within 30 min and induction continues to 90-fold after 24 h (Fig. 5A). Suc does not affect *AtTPS1* expression as early, yet it induces this TPS after 24 h marginally compared to *AtTPS5* (Fig. 5C). Trehalose does not induce *AtTPS5* expression although it does marginally induce expression of the trehalase *AtTRE1* after 24 h (Fig. 5B). Q-PCR measurements hence confirm the results obtained using microarray hybridization.

Very rapid induction of *AtTPS5* expression suggests that signaling processes leading to altered *AtTPS5* expression are an early response to Suc feeding. The massive induction of *AtTPS5* over the first 24 h also coincides with T6P accumulation during the first 24 h after Suc feeding.

Transcriptional Profiling Reveals a Cluster of Genes With Expression Correlating With T6P Levels in the Seedlings

T6P is required to utilize available carbon, but it also is growth inhibitory when accumulating in the absence of available carbon. This is consistent with a role of T6P as a regulator of metabolic flux. We resorted to cluster analysis of transcription profiles to identify processes controlled by T6P and further characterize growth inhibition due to T6P accumulation in the

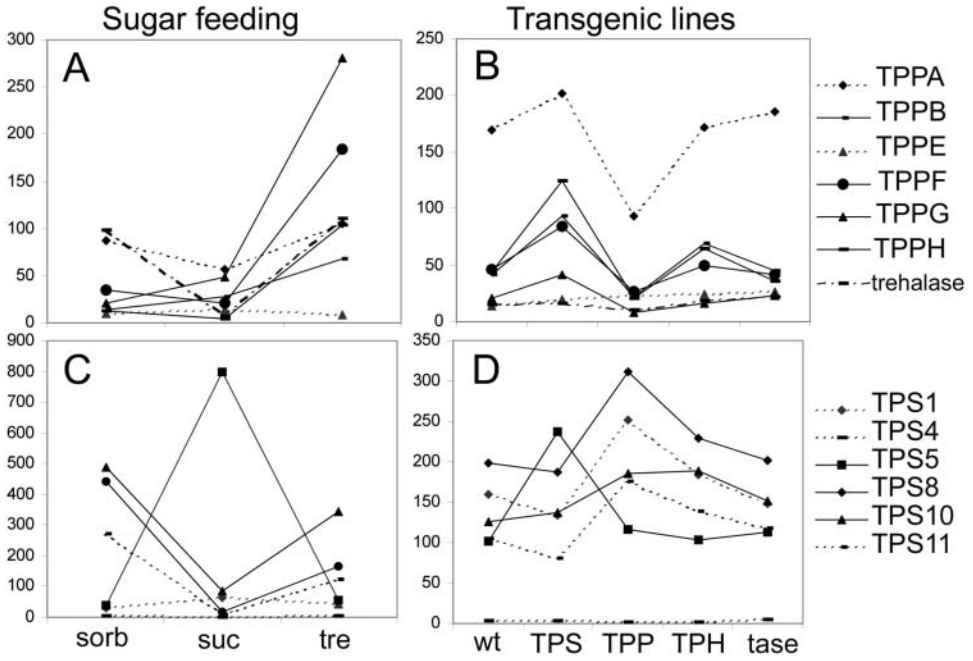

Figure 4. Expression of Arabidopsis trehalose metabolism genes in transgenics with altered T6P levels and after 24-h sugar feeding. Wild-type Col-0 seedlings were grown in rotating liquid cultures of 50 mL half-MS medium for 6 d and then fed with 100 mM of either sorbitol (sorb), Suc (suc), or trehalose (tre) for 24 h. Transgenic seedlings were grown for 7 d on agar solidified half MS with 10 mM Suc. RNA extraction, probe labeling, and microarray hybridization are described in Methods. wt, Col-0 wild type; TPS, line 19.3 expressing *E. coli* TPS with 3-fold increased T6P levels over wild type; TPP, line 12.1 expressing *E. coli* TPP with 4-fold reduced T6P levels over wild type; TPH, line 16.2 expressing *E. coli* TPH with 0.7-fold the amount of T6P found in wild type; tase, line 42.6 expressing *E. coli* cytosolic trehalase with unchanged T6P levels compared to wild type. Expression is shown for all genes with probes on the Affymretrix 8.3Kgene chip; we used the TAIR annotation dated from May 2003 for annotation of the Affymetrix probes. A, Expression of TPP genes and trehalase after sugar feeding; B, Expression of TPP genes in lines expressing *E. coli* genes; C, Expression of TPS genes after sugar feeding; D, Expression of TPS genes in lines expressing *E. coli* genes.

absence of metabolizable carbon. The experimental design to identify genes regulated by changes in T6P levels is summarized in Figure 6A. The underlying assumption of this approach is that T6P regulation of a process depends directly on T6P concentrations and that this is reflected quantitatively in gene expression.

A general description of the microarray results can be found in Supplemental Data 1. When clustering data from all array results generated, clusters obtained are dominated by the large transcriptional changes after sugar feeding, particularly after Suc (Supplemental Data 2). To better understand the effect of changed T6P levels, cluster analysis was therefore first restricted to data obtained from transgenic lines with altered T6P levels. Cluster analysis by correlation coefficient using MAS 5.0 software yields only two clusters (Fig. 6B): a cluster of 43 genes with expression correlating with T6P levels in the plants and a cluster of 81 genes essentially induced in TPP plants. Similar but not identical results were obtained using clustering by Self Organizing Map algorithms of MAS 5.0 or Genespring 6 software (Silicon Genetics, Redwood City, CA; data not shown).

Expression of 35 genes in the 43-gene cluster correlating with T6P levels is also induced when wild-type seedlings are fed with trehalose compared with sorbitol (Table II). Of these 35 genes, 15 are induced more than 2-fold in TPS versus TPP expressors and when wild-type seedlings are fed trehalose compared to sorbitol. Presence of two genes encoding TPP enzymes in this group (TPPB and F) lends support to the proposed regulatory feedback loop. Presence of several receptor kinases such as *WAK*1; *WAK*2; *At3g09010*, a LRR VIII receptor kinase; and *At1g72930*, a TIR receptor, would suggest activation of phosphorylation cascades. Identity of these genes and presence of peroxidase, an *AIG2* related gene, and cytochrome P450 flavonoid hydroxylase indicate some kind of defense response activation correlating with T6P levels. Presence of *CaBP-22* suggests activation of calcium signaling. The group further comprises Pro dehydrogenase, a senescence associated *SAG2* like protein, *At2g4760* and the barely detectable *At4g21050* zinc finger proteins of unknown functions. Enzymes related to central carbon metabolism in Table II are restricted to *APL3* and an aldose 1-epimerase (*At4g25900*). *APL3*, however, responds much more strongly to Suc, as seen from the Suc feeding control, than to T6P accumulation (Table II, and Q-PCR data not shown). Clustering of gene expression data thus indicates that evolutionary old connections linking trehalose synthesis with stress responses have

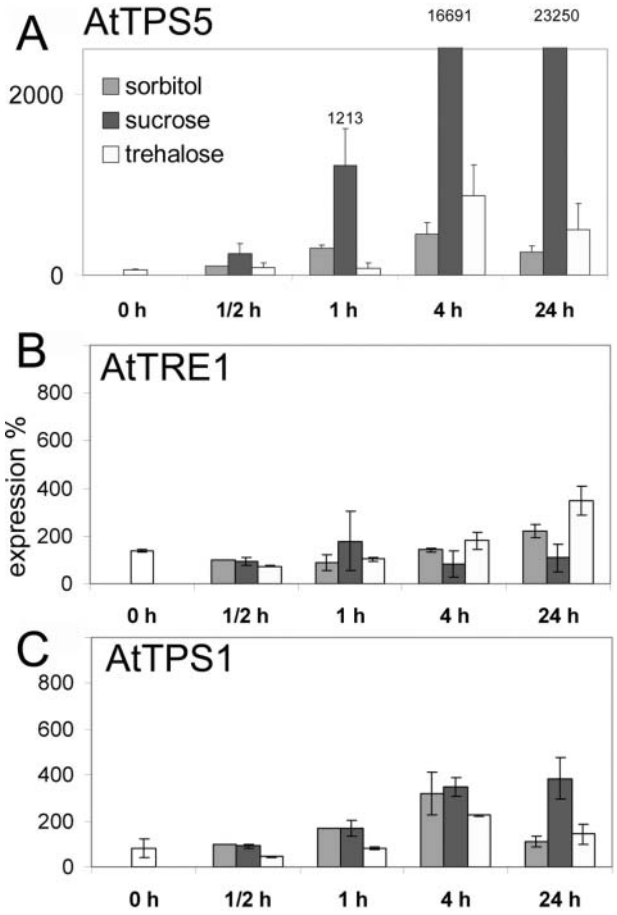

Figure 5. Induction kinetics of *AtTPS5*, *AtTPS1*, and *AtTRE1* after sugar feeding. Seedlings were grown in rotating liquid culture for a total of 7d. Before harvest they were fed with 100 mM of either sorbitol, Suc, or trehalose for 1/2, 1, 4, and 24 h. RNA extraction, DNA removal, cDNA synthesis, and Q-PCR analysis are described in Methods. Expression values are given in % relative to the value obtained after 1/2 h sorbitol feeding. To show early changes in *AtTPS5* expression, the scale in (A) was restricted to 20-fold induction, numbers above the Suc columns at 1, 4, and 24 h correspond to expression values for these columns.

been retained in Arabidopsis. The link in plants covers genes known from biotic and abiotic stress responses.

Suc feeding, which after 24 h leads to a greater accumulation of T6P than trehalose feeding, leads to further induction compared to trehalose feeding of eight genes from the 35 genes in Table II. Correlation with T6P levels is convincing for only six genes: *CaBP22*, TIR class resistance protein At1g72930, *ATPK19*, putative tyr phosphatase *At4g03960*, GPI anchored protein At3g52370, and plastocyanin-like domain containing protein *At4g12880* (Fig. 6C). Four of these genes belong to signal transduction pathways, the remaining two lack functional annotation. T6P levels hence correlate with transcriptional activation of signal transduction processes that include calcium signaling as well as signal perception and transduction by phosphorylation. The function of these signaling processes is only experimentally documented for *ATPK19* as this S6 ribosomal kinase is induced by cold and salinity (Mizoguchi et al., 1995, 1996). Correlation with T6P levels of the expression of the six genes in Figure 6C occurs independently of the seedling's carbon status, since they are induced by trehalose and Suc. Presence of *ATPK19* in this group suggests that the T6P connection with stress responses in Arabidopsis may not result from nutrient starvation.

T6P Levels Correlate with Expression of Central Metabolism Regulator *AtKIN11*

To determine T6P-induced processes responsible for the growth inhibition observed when T6P accumulates in the absence of added Suc, we identified genes with expression correlating with T6P levels in all samples but suppressed when seedlings were fed Suc. These are most interesting with regard to the role of T6P in growth arrest and carbon utilization. We hence selected genes among the 35 genes, correlating with T6P levels in Table II that are induced by trehalose but repressed by Suc. This yields a list of 11 genes, which include TPP homolog *AtTPPF*, hypothetical protein *At2g47560*, Dof-zinc finger protein *At4g21050*, *PR-4*, Pro dehydrogenase *PRO1*, S-locus protein kinase *At4g27300*, Cytochrome P450 91A, Ser/Thr kinase *At4g23180*, *AtKIN11*, and *GAD2*. Genes in this list, such as *PR-4* and *PRO1*, characterize a stress response. Interestingly, these genes are known from biotic and abiotic stress responses. The list also includes Glu dehydrogenase *GAD2* and *AtKIN11* associated with processes in central metabolism. Quantitative PCR analysis confirms a 5-fold induction of *AtKIN11* after trehalose feeding of wild-type seedlings (Fig. 7A). Quantitative PCR also confirms induction of *AtKIN11* in TPS versus TPP-expressing seedlings grown in constant light and further shows that changes in T6P levels change *AtKIN11* response to light or carbon fixed (Fig. 7B). *AtKIN11* transcript levels are generally higher in seedlings with high T6P levels than in wild type, and they remain at the high levels reached during light even after 24 h darkness. In seedlings with low T6P, *AtKIN11* transcript levels are generally lower than in wild type, and, after a period of darkness, fail to raise to light levels as quickly as in wild type.

Trehalose induction of *AtKIN11* under light is comparatively slow as it becomes significant after 24 h only. *AtKIN11* is not induced by Suc feeding over the 24 h of our experiment, yet *AtKIN11* has been implicated in regulation of sugar uptake (Bhalerao et al., 1999).

Remarkably, many genes encoding enzymes from central metabolism are induced after Suc feeding, but these are not found clustering with T6P levels (Supplemental Data 2, List of Suc-Induced Genes). T6P, therefore, may control primary metabolism either directly as an allosteric regulator of enzymes or as a signal on transduction cascades that regulate activity of these enzymes to bring about carbon utilization.

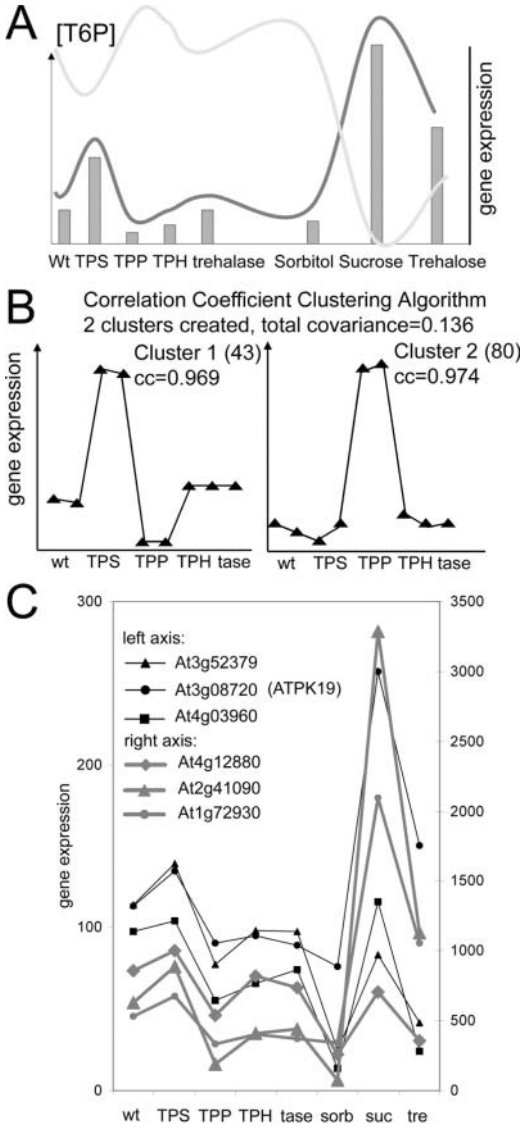

Figure 6. Clustering data from microarray analysis. A, Experimental design to determine genes with an expression profile correlating with T6P levels. Bars represent T6P levels in wild type, transgenic lines expressing *E. coli* TPS, TPP, TPH, or trehalase and in wild type fed with sorbitol, Suc, or trehalose for 24 h. The dark line represents the expression profile of a gene that correlates positively with T6P levels in the seedlings. The light line represents the expression profile of a gene that correlates negatively with T6P levels in the seedlings. B, The only two clusters obtained after correlation coefficient clustering of data from transgenics with altered T6P. Correlation clustering in MAS 5.0 was carried out on data obtained from transgenics expressing *E. coli* enzymes to identify genes responsive to varying T6P. Data from the sugar-feeding experiments were excluded since the large changes in gene expression obtained would dominate clustering. Correlation coefficient clustering identified only the two clusters with the profiles shown. Cluster 1 (43), a cluster containing 43 genes with an expression profile positively correlating with T6P levels. Cluster 2 (80), a cluster of 80 genes with increased expression in plants expressing *E. coli* TPP. CC is the correlation coefficient calculated. Graphs represent gene expression of biological duplicates of wild-type and *E. coli* TPS, TPP, and TPH, as well as a single array of *E. coli* trehalase-expressing seedlings. C, Genes correlating with T6P levels in the plants over all data collected. Expression of genes in Table II was averaged from the duplicate data sets of wild type, TPS, TPP, and TPH samples. Graphs were then made from each gene in Table II, and genes with an expression profile correlating with T6P levels after 24 h sugar feeding were chosen. Left and right axes have different scales.

DISCUSSION

We have previously shown that low T6P levels limit carbon utilization in Arabidopsis (Schluepmann et al., 2003). We observe here that carbon available induces T6P accumulation, and this is consistent with our previous findings. We also find that T6P accumulation without external supply of metabolizable carbon inhibits growth of Arabidopsis seedlings. Expression analysis further uncovered that seedlings relate T6P levels to available carbon by way of a rapid signaling process controlling transcription of T6P-metabolizing genes. Suc induces *AtTPS5* within 30 min (Fig. 5). Accumulation of T6P in the absence of metabolizable carbon increases expression of five from the six *AtTPP* genes tested. There are 21 T6P metabolism genes in Arabidopsis compared to only three in yeast, a remarkable radiation given that Arabidopsis has only one gene-encoding trehalase (Leyman et al., 2001). Results from cluster analysis of transcription profiles from seedlings with modified T6P levels reveal a link between T6P levels and expression of genes from nutrient, biotic, and abiotic stress responses. Thus radiation of T6P-metabolizing enzymes in plants could be explained if stress stimuli were integrated at the level of transcription of T6P metabolizing genes to adjust carbon utilization to the physiological requirements of the different stress responses. Expression analysis further reveals that T6P levels override the light- or carbon-controlled expression of *AKIN11* (Fig. 7).

Trehalose Feeding Leads to Accumulation of its Biosynthetic Precursor, T6P, and This Inhibits Growth

The effects of exogenous trehalose are multiple, yet growth arrest after 100 mM trehalose feeding is due to T6P accumulation because it is overcome in seedlings expressing *E. coli* TPH. T6P accumulation is not due to phosphorylation of trehalose as the reaction catalyzed by TPP is irreversible, given the low phosphate level in the cells. *tps1* mutant embryo rescue by *E. coli* TPS expression, but not by supply of trehalose, supports this conclusion (Eastmond et al., 2002; Schluepmann et al., 2003). So does the reduced growth rate of TPH expressors compared to trehalase expressors. TPH cleaves endogenously synthesized T6P that accumulates in the presence of high trehalose concentrations due to much reduced T6P dephosphorylation. Trehalase expressors thrive on Glc released from cleavage of the supplied trehalose, but TPH expressors do not benefit from carbon available in trehalose supplied. Feedback inhibition of AtTPS enzymes as well as the observed repression of *AtTPS* gene expression could therefore explain the measured drop to 2-fold control

Table II. *Cluster of genes with expression levels correlating with T6P levels*

Clustering of microarray data with MAS5 gave a cluster of 43 genes with expression correlating with T6P levels in plants with altered T6P by way of *E. coli* enzyme expression. This table consists of genes from this cluster that are also induced by trehalose compared to sorbitol feeding. Annotation of the Affymetrix probe sets was using the TAIR ftp file from May 2003. TPS/wt, ratio of expression means from TPS-expressing seedlings and wild type; TPP/wt, ratio of expression means from TPP-expressing seedlings and wild type; tre/sorb, ratio of expression means from wild-type seedlings on trehalose versus sorbitol; suc/sorb, ratio of expression means from wild-type seedlings on Suc versus sorbitol.

Affymetrix	TPS/wt	TPP/wt	tre/sorb	suc/sorb	AGI TAIR May-03	TAIR May 2003
15616_s_at	3	0.4	100.9	76.6	At1g21250	Wall-associated kinase 1; expressed in leaves and stems & induced by pathogen infection (PMID:10380805)
17917_s_at	2.5	0.5	15.6	45.1	At2g41090	Calmodulin-like calcium binding protein (CaBP-22); identical to calmodulin-like calcium-binding protein (CABP-22) (Arabidopsis)
14763_at	2.5	0.5	5.7	5.4	At3g09010	Protein kinase family; contains protein kinase domain, Pfam:PF00069
12880_at	1.6	0.7	5.6	1.5	At3g28930	AIG2-related protein; similar to AIG2 protein GB:P54121 from (Arabidopsis)
20570_at	1.8	0.6	5.2	0.6	At4g12430	Trehalose-6-phosphate phosphatase, putative; similar to trehalose-6-phosphate phosphatase (AtTPPB) (Arabidopsis) GI:2944180; contains Pfam profile PF02358: Trehalose-phosphatase
17045_at	2.2	0.5	5.1	2	At1g78090	Trehalose-6-phosphate phosphatase (TPPB); identical to trehalose-6-phosphate phosphatase (AtTPPB) GI:2944180 (Arabidopsis)
17901_at	2.3	0.5	4	2	At2g44670	Senescence-associated protein -related; similar to senescence-associated protein SAG102 (GI:22331931) (Arabidopsis);
18003_at	1.8	0.9	3.1	6.2	At1g72930	Disease resistance protein (TIR class), putative; domain signature TIR exists, suggestive of a disease resistance protein.
16140_s_at	1.6	0.6	3.1	2.4	At1g21270	Wall-associated kinase 2; induced by salicylic acid or INA (PMID:10380805)
16413_s_at	2.3	0.9	3	254.5	At4g39210	Glc-1-phosphate adenylyltransferase, large subunit 3 (ADP-Glc pyrophosphorylase) (APL3); identical to SP|P55231
13807_at	2.4	0.8	3	0.6	At2g47560	Hypothetical protein
19297_at	4.2	0.3	2.5	0.8	At4g21050	Dof zinc finger protein; PBF protein, Triticum aestivum, EMBL:AJ012284
13978_at	1.4	0.6	2.3	1.2	At2g32960	Expressed protein; similar to yeast hypothetical protein SP|P53965|YND2_YEAST
12752_s_at	1.6	0.8	2.2	1.3	At4g21960	Peroxidase, putative; identical to peroxidase (Arabidopsis) gi|1402904|emb|CAA66957
12790_s_at	1.4	0.6	2	1.4	At4g22690	Cytochrome P450 family; flavonoid 3′,5′-hydroxylase Hf1, Petunia x hybrida, PIR2:S38985
15162_at	1.8	0.8	2	0.8	At3g04720	Hevein-related protein precursor (PR-4); identical to hevein-like protein precursor GB:P43082 (Arabidopsis), similar to wound-induced protein (WIN2) precursor GB:P09762 (Solanum tuberosum); Pfam HMM hit: chitin_binding proteins
15680_s_at	1.5	1	2	3.4	At3g08720	Ribosomal-protein S6 kinase (ATPK19)-related; identical to putative ribosomal-protein S6 kinase (ATPK19) GB:D42061 (Arabidopsis) (FEBS Lett. 358 (2), 199–204 (1995))
15124_s_at	4.4	0.2	1.9	0.3	At3g30775	Osmotic stress-induced Pro dehydrogenase (pro1); annotation temporarily based on supporting cDNA gi|1778014|gb|U59508.1|ATU59508
17636_at	1.5	0.8	1.8	8.6	At4g03960	Expressed protein
13598_at	1.6	0.9	1.7	3.3	At3g52370	Predicted GPI-anchored protein; annotation temporarily based on supporting cDNA gi|26450295|dbj|AK117608.1|
12270_at	1.4	0.8	1.6	0.3	At4g27300	S-locus protein kinase, putative; similar to receptor protein kinase (Ipomoea trifida) gi|836954|gb|AAC23542; contains S-locus glycoprotein family domain, Pfam:PF00954
13880_s_at	1.7	1	1.6	1.7	At4g25900	Aldose 1-epimerase family; similar to apospory-associated protein C; APOC (Chlamydomonas reinhardtii) GI:6970044 Pfam profile PF01263: Aldose 1-epimerase
19789_at	2.3	1.1	1.5	20.9	At4g17770	Trehalose phosphatase family; contains Pfam profile: PF02358 trehalose-phosphatase
17522_at	1.5	0.8	1.5	0.8	At5g36220	Cytochrome P450 91A1; Identical to Cytochrome P450 (SP:Q9FG65) (Arabidopsis);
16949_s_at	1.6	0.9	1.4	2.7	At4g12880	Plastocyanin-like domain containing protein
16614_s_at	3	0.7	1.4	9.9	At1g24260	MADS-box protein; strongly similar to GB:O22456, MADS-box protein, Location of EST gb|H37053
13550_at	1.4	0.8	1.3	0.4	At4g23180	Ser/Thr kinase -related protein; Ser/Thr kinase, Brassica oleracea

(*Table continues on following page.*)

Table II. (*Continued from previous page.*)

Affymetrix	TPS/wt	TPP/wt	tre/sorb	suc/sorb	AGI TAIR May-03	TAIR May 2003
16162_s_at	1.6	0.9	1.3	2.4	At5g41410	homeodomain protein, BELL1 (BEL1)
12286_s_at	1.3	0.9	1.3	0.6	At3g29160	Snf1-related protein kinase KIN11 (AKIN11); identical to protein kinase AKin11 GI:1729444 from (Arabidopsis)
14730_s_at	1.3	0.9	1.2	0.6	At1g65960	Glu decarboxylase 2 (GAD 2); similar to Glu decarboxylase (gad) GI:294111 from (Petunia hybrida)
17865_at	1.4	1	1.2	1.4	At1g75280	Isoflavone reductase, putative; identical to SP|P52577 Isoflavone reductase homolog P3 (EC 1.3.1.-) {Arabidopsis}; contains Pfam profile PF02716: isoflavone reductase
12763_s_at	1.4	0.9	1.2	0.3	At2g05520	Gly-rich protein (GRP); identical to Gly-rich protein; atGRP (GI:259447) (Arabidopsis)
18222_at	1.4	0.9	1.2	0.3	At2g18160	bZIP family transcription factor; contains a bZIP transcription factor basic domain signature (PDOC00036)
12052_at	2.1	0.5	1.1	0.1	At3g54880	Expressed protein; hypothetical protein, Picea mariana, AF051204
17423_s_at	1.7	1	1.1	2.5	At2g27190	Iron(III)-zinc(II) purple acid phosphatase (precursor); identical to iron(III)-zinc(II) purple acid phosphatase (precursor) SP:Q38924 from (Arabidopsis)

levels in T6P accumulation of seedlings after 24 h on 100 mM trehalose.

Growth arrest due to T6P accumulation occurs only when no additional metabolizable sugar is supplied. T6P was shown to be limiting for carbon utilization in Arabidopsis seedling growth (Schluepmann et al., 2003). Here we show that in the absence of carbon T6P accumulation is growth inhibiting. From the yeast model, we would expect that T6P accumulation represses influx of metabolizable Glc into glycolysis and leads to a depletion of hexose phosphates (Bonini et al., 2003). Metabolite measurements presented in Figure 2 only show a trend toward reduced hexose phosphates in seedlings accumulating T6P on trehalose, whereas reduction of hexose phosphates is significant in seedlings accumulating T6P by way of *E. coli* TPS expression (Schluepmann et al., 2003). Rescue of the T6P-mediated growth arrest by supply of Glc or Fru, however, shows that entry of these monosaccharides into metabolism is not inhibited by accumulating T6P. These results are consistent with previous results suggesting that T6P may regulate a step beyond hexokinase both in yeast and in plants (Noubhani et al., 2000; Bonini et al., 2003; Schluepmann et al., 2003). Mutants capable of growth on 100 mM trehalose have been obtained that are being used to further our understanding of how T6P accumulation arrests seedling growth.

Seedlings Relate T6P Levels With Available Carbon by Regulating Transcription of T6P-Metabolizing Enzymes

Supply of Suc leads to progressive accumulation of T6P in seedlings. This agrees with previous findings showing that seedlings require T6P for carbon utilization and that plants with increased T6P levels by way of *E. coli* TPS enzyme expression grow faster than wild type on medium supplemented with metabolizable carbon (Schluepmann et al., 2003). Supply of Suc also rapidly induces a massive accumulation of *AtTPS5* transcript (Fig. 5), suggesting involvement of this TPS homolog in rising T6P levels following Suc addition. Supply of trehalose, on the other hand, leads to an immediate peak of T6P that decreases over the 24 h period of our analysis.

Trehalose also induces expression of *TPPB*. TPPB is a functional T6P phosphatase as shown by

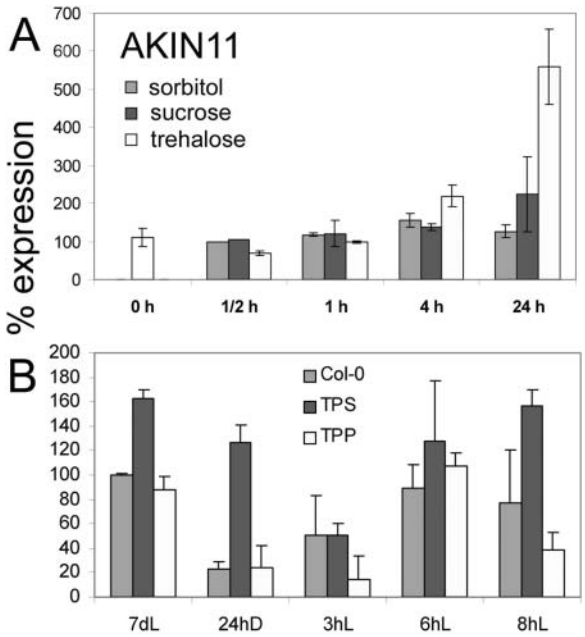

Figure 7. Expression analysis of *AtKIN11*. A, Induction kinetics of *AtKIN11* expression after sugar feeding. Q-PCR was carried out on RNA from 7 d seedlings fed with 100 mM of either sorbitol, Suc, or trehalose for 0.5, 1, 4, and 24 h. B, Induction of *AtKIN11* expression after a prolonged dark period in plants with altered T6P levels. Seedlings of wild-type Col-0 (Col-0), TPS expressor line 19.3 (TPS), and TPP expressor line 12.1 (TPP) were grown on agar-solidified medium for 7 d under continuous light, then transferred to the dark for 24 h before transfer to continuous light again. RNA for quantitative PCR was extracted at the end of the 7 d continuous light period (7 dL), at the end of the 24 h dark period (24 hD), and after 3, 6, and 8 h after the subsequent light period (3 hL, 6 hL, and 8 hL, respectively).

complementation of TPP-defective yeast (Vogel et al., 1998). These observations imply a mechanism that relates T6P levels with available carbon. This mechanism regulates expression of T6P-metabolizing enzymes and has previously been proposed to operate also in response to nitrogen levels (Wang et al., 2003). Nitrate feeding in the presence of 15 mM Suc induces expression of *TPPB* and *TPPJ* 14 and 4-fold, respectively, after 20 min only. *TPPB* is also induced in seedlings accumulating T6P by way of trehalose feeding or *E. coli* TPS expression, suggesting that this enzyme is important for T6P catabolism. The data from Wang et al. (2003) and ours therefore may suggest that nitrogen and carbon nutrient cues are integrated, or balanced, at the level of T6P accumulation. The surprising speed of this transcriptional regulation suggests a direct signaling path between sensing nutrient and transcriptional regulation of T6P metabolizing genes, be it for nitrogen or carbon-related nutrient cues.

Transcriptional Profiling Associates T6P With Stress Responses

We have used expression profiling as a means to identify processes controlled by the T6P metabolite. The underlying assumption of this approach is that gene expression, from genes of processes in which T6P plays a role, correlates with T6P levels over the eight conditions tested. Our approach is validated here because correlation coefficient clustering confirms the earlier observation that T6P levels affect expression of T6P metabolizing genes (Table II). Correlating metabolite profiles with gene expression profiles is an emerging area of systems biology (Sweetlove et al., 2003). This approach has already been applied to identify correlations, for example between Glu decarboxylase and 4-aminobutiric acid, WRKY6 and Lys, as well as a CONSTANS-like protein versus ascorbate during potato tuber development (Urbanczyk-Wochniak et al., 2003). These correlations are no proof of causal relationships but allow establishing hypotheses either verified in the literature or open to experimental testing. The results discussed should hence be viewed with this perspective.

Expression profiling of an average 6500 Arabidopsis genes in seedlings over eight conditions did not uncover a correlation between T6P levels and expression of enzymes of primary metabolism with exception of an aldose 1-epimerase-like encoding gene (*At4g25900*). Cluster analysis of the expression data obtained associates T6P with stress signal transduction. The cluster of six genes with an expression positively correlating with T6P levels, irrespective of available carbon, contains *ATPK19*, a kinase known to be induced by salt and cold, and proteins of calcium and phosphorylation signaling cascades (Mizoguchi et al., 1995; Mizoguchi et al., 1996). Both these signaling cascades are known to be active in response to abiotic stresses (Cheong et al., 2003). Tobacco- and rice- expressing TPS or TPS/TPP hybrid enzymes have indeed enhanced resistance to abiotic stress (Holmstrom et al., 1996; Romero et al., 1997; Garg et al., 2002; Jang et al., 2003). Enhanced resistance was generally assigned to trehalose, but levels of trehalose in the transgenic crops could not be related to resistance phenotypes. Resistance to stress might therefore be due to increased T6P levels in the plants. Cluster analysis further reveal that genes known to belong to biotic stress responses correlate positively with T6P levels. These include *PR-4*, wall associated kinases 1 and 2, an AIG2-related protein, as well as a TIR-class disease resistance protein (Table II). We have indeed been able to confirm resistance of Arabidopsis plants with T6P accumulation to *Peronospora parasitica* (H. Schluepmann, unpublished data), and trehalose spray application to wheat has previously been shown to protect wheat from powdery mildew infection (Reignault et al., 2001).

Remarkably, amounts of T6P correlate with expression of *AKIN11,* which links T6P to a kinase-controlling signal transduction response to Suc (Bhalerao et al., 1999; Ferrando et al., 2001). Plant SNF-related kinase AKIN11 is homologous to, and can functionally complement, yeast SNF1 kinase. AKIN11 activity is enhanced after feeding Suc in a Pleiotropic Regulatory Locus 1 independent way, which is probably independent of hormone and hexokinase effects. The situation in plants differs from yeast in that Suc promotes activity of this complex in plants, whereas Glc represses its activity in yeast. Transcription of *AKIN11* depends on light; therefore, AKIN11 complexes may relate carbon supplied by the chloroplast and activity of SnRK target enzymes. In TPS expressors *AKIN11,* expression remains induced in the absence of light- or carbon-fixed, whereas in TPP expressors it is further repressed during prolonged dark periods.

To conclude, results from cluster analysis suggest that plants have retained ancestral connections that relate trehalose metabolism with stress in fungi and bacteria. In response to stress or nutrient deprivation, primary metabolism of yeasts is rerouted via T6P toward quantitative synthesis of trehalose. We propose

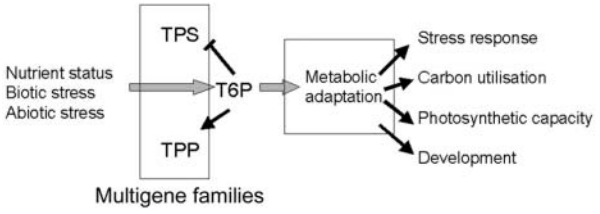

Figure 8. Model of the role of T6P metabolism in plants. Different stresses, be it nutrient, biotic, or abiotic stress, are sensed and transduction paths activated that change expression and activity of the many TPS and TPP homologs which changes levels of T6P in the cells. The different levels of T6P alter central metabolism, which brings about altered photosynthethic capacity (Pellny et al., 2004), carbon utilization and growth (Schluepmann et al., 2003), disease resistance (Schluepmann et al., unpublished), and mediates development (Eastmond et al., 2002).

that plants have retained connections between nutrient, abiotic, as well as biotic stress perception and T6P metabolism (Fig. 8). Radiation of the TPS and TPP families in plants could reflect the different roles of these genes in response to the different stresses. There are 11 TPS homologs and eight TPP homologs in Arabidopsis, and genes of these families are differentially expressed as our data and that from the Garnet Affymetrix database show. In plants, quantitative synthesis of trehalose as stress protectant has been replaced by compounds such as Pro and Suc.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Plant material was Arabidopsis Col.0. Transgenic lines expressing enzymes of *Escherichia coli* trehalose metabolism are described in Schluepmann et al. (2003). Trehalose was from Sigma-Aldrich (St. Louis) or from Hayashibara (Okayama, Japan). Seedlings were generally grown in half-strength MS medium (Murashige and Skoog, 1962). Media were prepared as 2-fold concentrate then diluted with water and filter-sterilized sugar stock solutions to obtain the indicated sugar concentrations. Seed was stratified during 48 h at 4°C before transfer to 22°C and long-day growth conditions (16 h light per day) or, where indicated, constant light.

Root growth measurements

Wild-type seedlings were grown on solid half-MS medium containing 50 mM sugar without or with further addition of 50 mM trehalose. Plates of medium were kept vertical, which yields straight roots. Root growth was monitored after 3 and 7 d by determining the root length on digital images using Image J (Wayne Rasband, National Institutes of Health, Bethesda, Maryland).

Metabolite analysis

Seedlings were grown under continuous light at a density of 1000 seed per 50 mL liquid half-MS medium, shaking. Seedlings were all harvested within 1 h on day 7.

Harvest was by poring the liquid medium over a sieve, washing the seedlings with 250 mL water, and absorbing the remaining water on paper. Seedlings were then weighed, snap frozen, and ground in liquid N2. Powder (50–100 mg of tissue) was extracted in 3 vol 5% perchloric acid on ice for 30 min, then neutralized with 5 M KOH in 1 M triethanolamine and metabolites measured by using enzyme-linked assays as in Schluepmann et al. (2003). T6P was assayed in a procedure adapted from van Vaeck et al. (2001). Extract (400 μL) was passed through 3-mL NH2-SPE columns (International Sorbent Technologies, Mid-Glamorgan, UK) to remove neutral compounds including sugars. Columns had previously been washed with 3 mL methanol, twice with 3 mL 0.5 M acetic acid and finally with 3 mL water. After applying extract to the column, it was washed with 3 mL water. After this, T6P was eluted with 2 mL 2.5 M ammonia solution. Samples were then freeze dried and resuspended in 400 μL 100 mM imidazole buffer pH 8 containing 1 mM $MgCl_2$.

Alkaline phosphatase was added and samples incubated for 2 h at room temperature to convert T6P to trehalose. The pH of the sample was then reduced to 6, samples boiled for 30 min to destroy phosphatase. After cooling and spinning, samples were incubated for 1 h with Glc oxidase to remove Glc produced from Glc-6-phosphate in the phosphatase reaction. Samples were boiled for 30 min to destroy Glc oxidase. Then, after cooling and spinning, trehalase was added. Glc produced was determined using the assay of Jones et al. (1977) using a dual-wavelength spectrophotometer (340 nm/ 410 nm) comparing to aliquots of samples taken before trehalase treatment as blanks. This method could resolve T6P levels down to 1 nmol g^{-1} FW.

Oligonucleotide Microarray Expression and Cluster Analysis

Wild type, a transgenic line containing more T6P by way of *E. coli* TPS expression, and two lines containing less T6P expressing, respectively *E. coli* TPP and TPH, were used. A line expressing *E. coli* cytosolic trehalase was used as control for changed cytosolic trehalose. These transgenic lines have previously been described (Schluepmann et al., 2003). Alternatively T6P levels in the seedlings were changed by feeding wild-type seedlings with 100 mM trehalose, Suc, or sorbitol osmotic control.

Seedlings grown for 24-h sugar treatment were treated as described in metabolite measurements. Frozen tissue was ground using two 3-mm diameter glass beads in Eppendorf tubes using a Dismembranator (Braun, Melsungen, Germany). RNA extractions from seedlings were using the Plant Mini kit (Qiagen, Hilden, Germany).

To anneal probes to Affymetrix microarrays, RNA concentrations were adjusted to 25 μg RNA per labeling reactions using both the photo spectrometric and capillary electrophoresis methods (RNA Lab-on-a-chip from Caliper Technologies, Mountain View, CA). cDNA synthesis, biotin labeling of RNA probe and annealing of the probe to the chip with subsequent washing and staining carried out as described in the Affymetrix Gene Chip Expression Analysis Technical Manual (Affymetrix). Scanning was with a Hewlett-Packard Gene array scanner (Hewlett-Packard, Palo Alto, CA). Results were normalized, quantified, and analyzed using Micro Array Suite 5.0 software (Affymetrix). Data was then exported into Genespring 6 and analysis carried out again with this software for comparison.

Q-PCR

RNA extractions from Arabidopsis seedlings were using the Plant Mini Kit (Qiagen).

RNA obtained was then treated with DNAseI (DNA-FreeTM, Ambion, Austin, TX) and efficiency of the treatment tested by PCR. cDNA was synthesized using M-MLV Reverse Transcriptase (Promega, Madison, WI) and primer odT16V (Invitrogen, Carlsbad, CA) as per manufacturer's protocol. Q-PCR was then carried out with the ABI-prism 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) as per manufacturer's protocol (PE-Applied Biosystems).

To detect expression of the *AtACT2* reference gene, we used (FAM-aagtcttgttccagccctcgtttgtgc-TAMRA), forward primer gctgagagattcagatgccca, and reverse primer atggaagctgctggaatccac. To detect *AtTPS5*, we used (FAM-tcccaagaatatcgtgtacctcgtcagtgg-TAMRA), the forward primer ccgcgaaacaatcgaaatct, and reverse primer ttcccagtctgttccatcatttg. To detect *AtTPS1*, we used (FAM-atctccttggctcacctgacgacgtc-TAMRA), the forward primer tgggtcgtactcgcaccaa, and reverse primer tttgcttccttgagaagctcg. To detect trehalase *AtTRE1*, we used (FAM-ttcgtctcagatccctccggcttcc-TAMRA), the forward primer gctgcaccacgaaccagtaga, and reverse primer ttcttcgttctccacgttgga. Q-PCR probes detected mRNA linearly over the range tested and had an efficiency nearing 2.

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LITERATURE CITED

Alberty RA (1996) IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (JCBN). Recommendations for nomenclature and tables in biochemical thermodynamics. Recommendations 1994. Eur J Biochem **240:** 1–14

Attfield PV (1987) Trehalose accumulates in Saccharomyces cerevisiae during exposure to agents that induce heat shock response. FEBS Lett **225:** 259–263

Benaroudj N, Lee DH, Goldberg AL (2001) Trehalose accumulation during cellular stress protects cells and cellular proteins from damage by oxygen radicals. J Biol Chem **276:** 24261–24267

Bhalerao RP, Salchert K, Bako L, Okresz L, Szabados L, Muranaka T, Machida Y, Schell J, Koncz C (1999) Regulatory interaction of PRL1 WD protein with Arabidopsis SNF1-like protein kinases. Proc Natl Acad Sci USA **96:** 5322–5327

Bonini BM, Van Dijck P, Thevelein JM (2003) Uncoupling of the glucose growth defect and the deregulation of glycolysis in Saccharomyces cerevisiae Tps1 mutants expressing trehalose-6-phosphate-insensitive

hexokinase from Schizosaccharomyces pombe. Biochim Biophys Acta **1606:** 83–93

Boy-Marcotte E, Lagniel G, Perrot M, Bussereau F, Boudsocq A, Jacquet M, Labarre J (1999) The heat shock response in yeast: differential regulations and contributions of the Msn2p/Msn4p and Hsf1p regulons. Mol Microbiol **33:** 274–283

Cheong YH, Kim KN, Pandey GK, Gupta R, Grant JJ, Luan S (2003) CBL1, a calcium sensor that differentially regulates salt, drought, and cold responses in arabidopsis. Plant Cell **15:** 1833–1845

De Virgilio C, Simmen U, Hottiger T, Boller T, Wiemken A (1990) Heat shock induces enzymes of trehalose metabolism, trehalose accumulation, and thermotolerance in Schizosaccharomyces pombe, even in the presence of cycloheximide. FEBS Lett **273:** 107–110

Eastmond PJ, Li Y, Graham IA (2003) Is trehalose-6-phosphate a regulator of sugar metabolism in plants? J Exp Bot **54:** 533–537

Eastmond PJ, van Dijken AJ, Spielman M, Kerr A, Tissier AF, Dickinson HG, Jones JD, Smeekens SC, Graham IA (2002) Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for Arabidopsis embryo maturation. Plant J **29:** 225–235

Ferrando A, Koncz-Kalman Z, Farras R, Tiburcio A, Schell J, Koncz C (2001) Detection of in vivo protein interactions between Snf1-related kinase subunits with intron-tagged epitope-labelling in plants cells. Nucleic Acids Res **29:** 3685–3693

Fritzius T, Aeschbacher R, Wiemken A, Wingler A (2001) Induction of ApL3 expression by trehalose complements the starch-deficient Arabidopsis mutant adg2-1 lacking ApL1, the large subunit of ADP-glucose pyrophosphorylase. Plant Physiol **126:** 883–889

Garcia AB, Engler J, Iyer S, Gerats T, Van Montagu M, Caplan AB (1997) Effects of Osmoprotectants upon NaCl Stress in Rice. Plant Physiol **115:** 159–169

Garg AK, Kim JK, Owens TG, Ranwala AP, Choi YD, Kochian LV, Wu RJ (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci USA **99:** 15898–15903

Goldberg RN, Tewari YB (1994) Thermodynamics of Enzyme-Catalyzed Reactions. 2. Transferases. J Phys Chem Ref Data **23:** 547–617

Holmstrom KO, Mantyla E, Welin B, Mandal A, Palva ET (1996) Drought tolerance in tobacco. Nature **379:** 683–684

Iwahashi H, Nwaka S, Obuchi K (2000) Evidence for contribution of neutral trehalase in barotolerance of Saccharomyces cerevisiae. Appl Environ Microbiol **66:** 5182–5185

Jang IC, Oh SJ, Seo JS, Choi WB, Song SI, Kim CH, Kim YS, Seo HS, Choi YD, Nahm BH, Kim JK (2003) Expression of a bifunctional fusion of the Escherichia coli genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. Plant Physiol **131:** 516–524

Jones MGK, Outlaw WH, Lowry OH (1977) Enzymic assay of 10-7 to 10-14 moles of sucrose in plant tissues. Plant Physiol **60:** 379–383

Leyman B, Van Dijck P, Thevelein JM (2001) An unexpected plethora of trehalose biosynthesis genes in Arabidopsis thaliana. Trends Plant Sci **6:** 510–513

Mizoguchi T, Hayashida N, Yamaguchi-Shinozaki K, Kamada H, Shinozaki K (1995) Two genes that encode ribosomal-protein S6 kinase homologs are induced by cold or salinity stress in Arabidopsis thaliana. FEBS Lett **358:** 199–204

Mizoguchi T, Irie K, Hirayama T, Hayashida N, Yamaguchi-Shinozaki K, Matsumoto K, Shinozaki K (1996) A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in Arabidopsis thaliana. Proc Natl Acad Sci USA **93:** 765–769

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant **15:** 473–497

Noubhani A, Bunoust O, Rigoulet M, Thevelein JM (2000) Reconstitution of ethanolic fermentation in permeabilized spheroplasts of wild-type and trehalose-6-phosphate synthase mutants of the yeast Saccharomyces cerevisiae. Eur J Biochem **267:** 4566–4576

Pellny TK, Ghannoum O, Conroy JP, Schluepmann H, Smeekens S, Andralojc J, Krause KP, Goddijn O, Paul MJ (2004) Genetic modification of photosynthesis with E. coli genes for trehalose synthesis. Plant Biotechnol J **2:** 71–82

Reignault P, Cogan A, Muchembled J, Sahraoui ALH, Durand R, Sancholle M (2001) Trehalose induces resistance to powdery mildew in wheat. New Phytol **149:** 519–529

Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L (2000) Technical advance: simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. Plant J **23:** 131–142

Romero C, Belles JM, Vaya JL, Serrano R, Culianez-Macia FA (1997) Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: Pleiotropic phenotypes include drought tolerance. Planta **201:** 293–297

Schluepmann H, Pellny T, van Dijken A, Smeekens S, Paul M (2003) Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in Arabidopsis thaliana. Proc Natl Acad Sci USA **100:** 6849–6854

Singer MA, Lindquist S (1998) Thermotolerance in Saccharomyces cerevisiae: the Yin and Yang of trehalose. Trends Biotechnol **16:** 460–468

Sweetlove LJ, Last RL, Fernie AR (2003) Predictive metabolic engineering: a goal for systems biology. Plant Physiol **132:** 420–425

Tewari YB, Steckler DK, Goldberg RN, Gitomer WL (1988) Thermodynamics of hydrolysis of sugar phosphates. J Biol Chem **263:** 3670–3675

Urbanczyk-Wochniak E, Luedemann A, Kopka J, Selbig J, Roessner-Tunali U, Willmitzer L, Fernie AR (2003) Parallel analysis of transcript and metabolic profiles: a new approach in systems biology. EMBO Rep **4:** 989–993

van Vaeck C, Wera S, Van Dijck P, Thevelein JM (2001) Analysis and modification of trehalose 6-phosphate levels in the yeast Saccharomyces cerevisiae with the use of Bacillus subtilis phosphotrehalase. Biochem J **353:** 157–162

Vogel G, Aeschbacher RA, Muller J, Boller T, Wiemken A (1998) Trehalose-6-phosphate phosphatases from Arabidopsis thaliana: identification by functional complementation of the yeast tps2 mutant. Plant J **13:** 673–683

Vogel G, Fiehn O, Jean-Richard-dit-Bressel L, Boller T, Wiemken A, Aeschbacher RA, Wingler A (2001) Trehalose metabolism in Arabidopsis: occurrence of trehalose and molecular cloning and characterization of trehalose-6-phosphate synthase homologues. J Exp Bot **52:** 1817–1826

Wang R, Okamoto M, Xing X, Crawford NM (2003) Microarray analysis of the nitrate response in Arabidopsis roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. Plant Physiol **132:** 556–567

Wiemken A (1990) Trehalose in yeast, stress protectant rather than reserve carbohydrate. Antonie Van Leeuwenhoek **58:** 209–217

Wingler A, Fritzius T, Wiemken A, Boller T, Aeschbacher RA (2000) Trehalose induces the ADP-glucose pyrophosphorylase gene, ApL3, and starch synthesis in Arabidopsis. Plant Physiol **124:** 105–114