Trehalose Mediated Growth Inhibition of Arabidopsis Seedlings Is Due to Trehalose-6-Phosphate Accumulation1[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 the expression of trehalose phosphate synthase homologs for growth. Expression analysis of genes from the Arabidopsis trehalose metabolism further supports this: Suc rapidly induces expression of trehalose phosphate synthase homologs 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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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, add2 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 G1c-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-phosphatase-phosphatase (TPP) and -hydrolase (TPH) on trehalose. Growth of TPP expressors is not improved over wild

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**Table 1. Putative Arabidopsis T6P phosphatases containing the TPP domain but not the TPS domain**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Arabidopsis Genome Initiative Number</th>
<th>Expression Tag</th>
<th>Reference</th>
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<tr>
<td>AtTPPA</td>
<td>At5g51460</td>
<td>Yes</td>
<td>Vogel et al. (1998)</td>
</tr>
<tr>
<td>AtTPPB</td>
<td>At1g78090</td>
<td>Yes</td>
<td>Vogel et al. (1998)</td>
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<td>AtTPPC</td>
<td>At1g22110</td>
<td>Yes</td>
<td></td>
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<td>AtTPPD</td>
<td>At1g35910</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>AtTPPE</td>
<td>At2g22190</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>AtTPPF</td>
<td>At4g12430</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>AtTPPG</td>
<td>At4g25990</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>AtTPPH</td>
<td>At4g39770</td>
<td>Yes (putative pseudogene)</td>
<td></td>
</tr>
<tr>
<td>AtTPPI</td>
<td>At5g10100</td>
<td>Yes</td>
<td></td>
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<tr>
<td>AtTPPJ</td>
<td>At5g65140</td>
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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 \( \Delta H^o \) of \(-5 \) kJ mol\(^{-1}\) 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, \( \Delta H^o \) 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).

### 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).
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 transgenic seedlings with altered T6P levels. Seedlings with modified T6P levels by way of transgene expression were TPS expression line 19.3 with 3-fold increased T6P, TPP expression line B12.1 with 4-fold reduced T6P, TPH expression line 16.2 with 0.7-fold reduced T6P, and trehalase expression line 46.2 with unchanged levels of T6P (Schluepmann et al., 2003). Transgenic 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 AtTTPA and B, and the TPP homologs TPPE, F, G, and H (Table 1), 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 TPS 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
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 compared to wild type. Expression is shown for all genes with probes on the Affymetrix 8.3K gene 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.

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 Affymetrix 8.3K gene 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.
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, PR4, Pro dehydrogenase PRO1, S-locus protein kinase At4g27300, Cytochrome P450 91A, Ser/Thr kinase At4g23180, AtKIN11, and GAD2. Genes in this list, such as PR4 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 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.
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 AtTP5 within 30 min (Fig. 5). Accumulation of T6P in the absence of metabolizable carbon increases expression of five from the six AtTP 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 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 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.
Table II. Cluster of genes with expression levels correlating with T6P levels

Clustering of microarray data with MA55 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.

<table>
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<tr>
<th>Affymetrix</th>
<th>TPS/wt</th>
<th>TPP/wt</th>
<th>tre/sorb</th>
<th>suc/sorb</th>
<th>AGI</th>
<th>TAIR May-03</th>
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<tbody>
<tr>
<td>15616_s_at</td>
<td>3</td>
<td>0.4</td>
<td>100.9</td>
<td>76.6</td>
<td>At1g21250</td>
<td>Wall-associated kinase 1; expressed in leaves and stems &amp; induced by pathogen infection (PMID:10380805)</td>
</tr>
<tr>
<td>17917_s_at</td>
<td>2.5</td>
<td>0.5</td>
<td>15.6</td>
<td>45.1</td>
<td>At2g41090</td>
<td>Calmodulin-like calcium binding protein (CaBP-22); identical to calmodulin-like-calcium-binding protein (CABP-22) (Arabidopsis)</td>
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<td>14763_at</td>
<td>2.5</td>
<td>0.5</td>
<td>5.7</td>
<td>5.4</td>
<td>At3g09010</td>
<td>Protein kinase family; contains protein kinase domain, Pfam:PF00069</td>
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<tr>
<td>12880_at</td>
<td>1.6</td>
<td>0.7</td>
<td>5.6</td>
<td>1.5</td>
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<td>AIG2-related protein; similar to AIG2 protein GB:P54121 from (Arabidopsis)</td>
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<tr>
<td>20570_at</td>
<td>1.8</td>
<td>0.6</td>
<td>5.2</td>
<td>0.6</td>
<td>At4g12430</td>
<td>Trehalose-6-phosphate phosphatase, putative; similar to trehalose-6-phosphate phosphatase (AtTPPB) (Arabidopsis) GI:2944180; contains Pfam profile PF02358: Trehalose-phosphatase</td>
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<tr>
<td>17045_at</td>
<td>2.2</td>
<td>0.5</td>
<td>5.1</td>
<td>2</td>
<td>At1g78090</td>
<td>Trehalose-6-phosphate phosphatase (TPPB); identical to trehalose-6-phosphate phosphatase (AtTPPB) GI:2944180 (Arabidopsis)</td>
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<tr>
<td>17901_at</td>
<td>2.3</td>
<td>0.5</td>
<td>4</td>
<td>2</td>
<td>At2g44670</td>
<td>Senescence-associated protein -related; similar to senescence-associated protein SACG102 (GI:22331931) (Arabidopsis)</td>
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<tr>
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<td>1.8</td>
<td>0.9</td>
<td>3.1</td>
<td>6.2</td>
<td>At1g72930</td>
<td>Disease resistance protein (TIR class), putative; domain signature TIR exists, suggestive of a disease resistance protein.</td>
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<tr>
<td>16140_s_at</td>
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<td>0.6</td>
<td>3.1</td>
<td>2.4</td>
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<td>Wall-associated kinase 2; induced by salicylic acid or INA (PMID:10380805)</td>
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<tr>
<td>16413_s_at</td>
<td>2.3</td>
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<td>At4g39210</td>
<td>Glc-1-phosphate adenylyltransferase, large subunit 3 (ADP-Glc pyrophosphorylase) (APL3); identical to SPjP55231</td>
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<td>2.5</td>
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<td>At4g21050</td>
<td>Def zinc finger protein; PBF protein, Triticum aestivum, EMBL:A012284</td>
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<td>Expressed protein; similar to yeast hypothetical protein SPjP53965/YND2_YEAST</td>
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<td>1.6</td>
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<td>Peroxidase, putative; identical to peroxidase (Arabidopsis) gi</td>
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<td>12790_s_at</td>
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<td>0.6</td>
<td>2</td>
<td>1.4</td>
<td>At4g22690</td>
<td>Cytochrome P450 family; flavonoid 3’,5’-hydroxylase Hf1, Petunia x hybrida, PIR2:S38985</td>
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<tr>
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<td>2</td>
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<td>At3g04720</td>
<td>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</td>
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<td>3.4</td>
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<td>Ribosomal-protein S6 kinase (ATPK19)-related; identical to putative ribosomal-protein S6 kinase (ATPK19) GB:DA2061 (Arabidopsis) (FEBS Lett. 358 (2), 199–204 (1995))</td>
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<td>15124_s_at</td>
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<td>Expressed protein</td>
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<td>1.6</td>
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<td>13880_s_at</td>
<td>1.7</td>
<td>1</td>
<td>1.6</td>
<td>1.7</td>
<td>At4g25900</td>
<td>Aldose 1-epimerase family; similar to asparagine-associated protein C; APOC (Chlamydomonas reinhardii) Gl:697004 Pfam profile PF01263; Aldose 1-epimerase family</td>
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<td>19789_at</td>
<td>2.3</td>
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<td>At4g17770</td>
<td>Trehalase phosphatase family; contains Pfam profile: PF02358 trehalase-phosphatase</td>
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<td>0.8</td>
<td>1.5</td>
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<td>3</td>
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<td>1.4</td>
<td>9.9</td>
<td>At2g42620</td>
<td>MADS-box protein; strongly similar to GB:O22456, MADS-box protein, Location of EST gb</td>
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<tr>
<td>13550_at</td>
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<td>1.3</td>
<td>0.4</td>
<td>At4g23180</td>
<td>Ser/Thr kinase -related protein; Ser/Thr kinase, Brassica oleracea</td>
</tr>
</tbody>
</table>

(Table continues on following page.)
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 TTPB. TPPB is a functional T6P phosphatase as shown by

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

<table>
<thead>
<tr>
<th>Affymetrix</th>
<th>TPS/wt</th>
<th>TPP/wt</th>
<th>tre/sorb</th>
<th>suc/sorb</th>
<th>AGI TAIR May-03</th>
<th>TAIR May 2003</th>
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<td>2.4</td>
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<td>homeodomain protein, BELL1 (BEL1)</td>
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<tr>
<td>12286_s_at</td>
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<td>0.9</td>
<td>1.3</td>
<td>0.6</td>
<td>At3g29160</td>
<td>Snf1-related protein kinase KIN11 (AKIN11); identical to protein kinase Akin11 GI:1729444 from (Arabidopsis)</td>
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<td>14730_s_at</td>
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<td>0.9</td>
<td>1.2</td>
<td>0.6</td>
<td>At1g65960</td>
<td>Glu decarboxylase 2 (GAD 2); similar to Glu decarboxylase (gad) GI:2294111 from (Petunia hybrida)</td>
</tr>
<tr>
<td>17865_at</td>
<td>1.4</td>
<td>1</td>
<td>1.2</td>
<td>1.4</td>
<td>At1g75280</td>
<td>Isolavone reductase, putative; identical to SP</td>
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<td>1.4</td>
<td>0.9</td>
<td>1.2</td>
<td>0.3</td>
<td>At2g05520</td>
<td>Gly-rich protein (GRP); identical to Gly-rich protein; atGRP (GI:259447) (Arabidopsis)</td>
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<tr>
<td>18222_at</td>
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<td>0.9</td>
<td>1.2</td>
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<td>hZIP family transcription factor; contains a hZIP transcription factor basic domain signature (PDOC000036)</td>
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<td>12052_at</td>
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<td>Trehalose-6-Phosphate in Arabidopsis</td>
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<td>17423_s_at</td>
<td>1.7</td>
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<td>1.1</td>
<td>2.5</td>
<td>At2g27190</td>
<td>Iron(III)-zinc(II) purple acid phosphatase (precursor); identical to iron(III)-zinc(II) purple acid phosphatase (precursor) SP:Q38924 from (Arabidopsis)</td>
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</table>

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**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 I 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...
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 Schluemmann 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 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 (Wayne Rashand, 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 pouring 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, the neutralized with 5 M KOH in 1 M triethanolamine solution to convert T6P to trehalose. The pH of the sample was then adjusted to 25 mM sugar without or with further addition of 50 mM trehalose. Plates of medium were kept vertical, which yields straight roots. Root growth was measured by using enzyme-linked assays as in Schluepmann et al. (2003). Trehalose was from Sigma-Aldrich (St. Louis) or from Hayashibara (Okayama, Japan). RNA extractions from seedlings were using the Plant Mini kit (Qiagen, Hilden, Germany). 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-ttcgtctcagatccctccggcttcc-TAMRA), the forward primer gcctgaccaaccagtaga, and reverse primer tcttcgttctccacgttgga. To detect AtTPS1, we used (FAM-ttcttcgttctcagatccctccggcttcc-TAMRA), the forward primer ttggtcgtactcgcaaatc, and reverse primer tcccttgcgccttcatttg. To detect AtTPS5, we used (FAM-ctctccgtctcagatccctccggcttcc-TAMRA), the forward primer tttggtcgtactcgcaaatc, and reverse primer tttggtcgtactcgggttcatttg. To detect trehalase AtTRE1, we used (FAM-ttcgtctcagatccctccggcttcc-TAMRA), the forward primer tttggtcgtactcgcaaatc, and reverse primer tttggtcgtactcgggttcatttg. To detect AtTRE1, we used (FAM-ttcgtctcagatccctccggcttcc-TAMRA), the forward primer ttggtcgtactcgcaaatc, and reverse primer tttggtcgtactcgggttcatttg. Q-PCR probes detected mRNA linearly over the range tested and had an efficiency nearing 2.

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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 (Schluemmann 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 spectroscopic 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).

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