Inhibition of SNF1-Related Protein Kinase1 Activity and Regulation of Metabolic Pathways by Trehalose-6-Phosphate

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Trehalose-6-phosphate (T6P) is a proposed signaling molecule in plants, yet how it signals was not clear. Here, we provide evidence that T6P functions as an inhibitor of SNF1-related protein kinase1 (SnRK1; AKIN10/AKIN11) of the SNF1-related group of protein kinases. T6P, but not other sugars and sugar phosphates, inhibited SnRK1 in Arabidopsis (Arabidopsis thaliana) seedling extracts strongly (50%) at low concentrations (1–20 μM). Inhibition was noncompetitive with respect to ATP. In immunoprecipitation studies using antibodies to AKIN10 and AKIN11, SnRK1 catalytic activity and T6P inhibition were physically separable, with T6P inhibition of SnRK1 dependent on an intermediary factor. In subsequent analysis, T6P inhibited SnRK1 in extracts of all tissues analyzed except those of mature leaves, which did not contain the intermediary factor. To assess the impact of T6P inhibition of SnRK1 in vivo, gene expression was determined in seedlings expressing Escherichia coli otsA encoding T6P synthase to elevate T6P or otsB encoding T6P phosphatase to decrease T6P. SnRK1 target genes showed opposite regulation, consistent with the regulation of SnRK1 by T6P in vivo. Analysis of microarray data showed up-regulation by T6P of genes involved in biosynthetic reactions, such as genes for amino acid, protein, and nucleotide synthesis, the tricarboxylic acid cycle, and mitochondrial electron transport, which are normally down-regulated by SnRK1. In contrast, genes involved in photosynthesis and degradation processes, which are normally up-regulated by SnRK1, were down-regulated by T6P. These experiments provide strong evidence that T6P inhibits SnRK1 to activate biosynthetic processes in growing tissues.

Suc and trehalose are widespread nonreducing disaccharides that function as translocated carbon sources and stress protection compounds. Plants and cyanobacteria are the only organisms in which the pathways of trehalose and Suc synthesis coexist. In the majority of plants, trehalose occurs in trace amounts only, prohibiting a function as a carbon source. This raises the question of the role of the trehalose pathway in plants, given the large number and ubiquity of putative genes encoding enzymes for trehalose synthesis (Avonce et al., 2006; Lunn, 2007). Many of these genes are subject to a high level of regulation at the transcription (Avonce et al., 2006) and posttranslational (Harthill et al., 2006) levels, suggesting an important function. This is confirmed in transgenic and mutant plants with modified trehalose pathway gene expression, which show a range of phenotypes. For example, a trehalose-6-P synthase (TPS1) has been shown to be essential for embryo development (Eastmond et al., 2002) and for normal vegetative growth and the transition to flowering (Van Dijken et al., 2004). Overexpression of TPS from different species, for example, otsA encoding Escherichia coli TPS, produces effects on sugar utilization in seedlings (Schluepmann et al., 2003) and on vegetative and photosynthetic phenotypes (Pellny et al., 2004; Almeida et al., 2007; Stiller et al., 2008), opposite in nature to seedling and photosynthetic phenotypes of plants expressing E. coli otsB encoding trehalose-6-P phosphatase (TPP; Schluepmann et al., 2003; Pellny et al., 2004). The phenotype of the maize (Zea mays) ramosa3 mutant has been attributed to a knockout of a TPP gene normally expressed in discrete domains subtending axillary meristems (Satoh-Nagasawa et al., 2006). This gives rise to an inflorescence architecture that aids the efficient packing and harvesting of seeds. Trehalose-6-P (T6P) has been found to stimulate starch synthesis via redox activation of ADP-Glc pyrophosphorylase (Kolbe et al., 2005), and T6P responds to light and sugar in relation to carbon
status (Lunn et al., 2006). This supports a role for T6P in signaling the sugar status of the cytosol to the chloroplast and thereby activating starch synthesis. However, most of the phenotypes produced where the pathway has been genetically modified in mutant and transgenic lines, which also extend to effects on abiotic stress resistance (Garg et al., 2002; Avonce et al., 2004), cell division, cell walls (Gómez et al., 2006), and cell shape (Chary et al., 2008), cannot be explained simply in terms of the effect on starch metabolism but rather support a more central function.

In some fungi, T6P inhibits hexokinase, an enzyme implicated in sugar signaling in plants and other organisms (Moore et al., 2003), but there is no evidence that T6P affects plant hexokinases (Eastmond et al., 2002). Another important signaling route in plants is through SNF1-related protein kinase1 (SnRK1) of the family of calcium-independent Ser/Thr protein kinases that includes AMPK of mammals and SNF1 of yeast (Hardie, 2007; Polge and Thomas, 2007). These conserved kinases perform a fundamental role in transcriptional, metabolic, and developmental regulation in response to energy limitation and starvation of the carbon source (Hardie, 2007). SnRK1 in plants is thought to consist of a heterotrimeric complex, as in AMPK and SNF1, composed of an AKIN10 or AKIN11 catalytic $\alpha$-subunit and $\beta$- and $\gamma$-subunits together with a number of additional interacting and regulatory factors (Pierre et al., 2007; Polge and Thomas, 2007; Ananieva et al., 2008). Recent work established that AKIN10 catalytic activity regulates 1,000 or so target genes involved in the response of metabolism and growth to starvation (Baena-González et al., 2007). It was shown that SnRK1 activates genes involved in degradation processes and photosynthesis and inhibits those involved in biosynthetic processes and, by so doing, regulates metabolism and growth in response to available carbon (Baena-González et al., 2007). SnRK1 can phosphorylate class II TPSs (Glinski and Weckwerth, 2005; Harthill et al., 2006) and regulate their transcription (Baena-González et al., 2007), although the importance of this is not clear. SnRK1 transcript increased in response to 100 mM trehalose feeding (Schlüpmann et al., 2004) and was decreased 2-fold in tps1 mutants (Gómez et al., 2006). Given a possible interaction between the two pathways, we went on to determine whether T6P affects the catalytic activity of SnRK1.

Here, we provide evidence for a function of T6P as an inhibitor of SnRK1 activity. First, we show that low micromolar concentrations of T6P inhibit SnRK1 activity in Arabidopsis (Arabidopsis thaliana) seedling extracts and other young plant material, but not in mature leaves. Second, we show that T6P inhibits SnRK1 at a site distinct and separable from the SnRK1 catalytic site via an intermediary factor. This as yet unknown intermediary factor was not found in mature leaves. Third, we establish effects on gene expression in seedlings with elevated T6P consistent with inhibition of SnRK1 in vivo. Overall, the data provide strong evidence for a function of T6P as an inhibitor of SnRK1 to promote biosynthetic reactions in growing tissues.

RESULTS

T6P Inhibits SnRK1 in G-25 Desalted Extracts

SnRK1 activity was measured using desalted tissue extracts with and without T6P in the assay and in comparison with other sugars and sugar phosphates. Inhibition of SnRK1 activity from Arabidopsis seedling extracts by T6P was observed at 1 $\mu$M T6P; at 20 $\mu$M T6P, SnRK1 activity was inhibited by 50% (Fig. 1A). In SnRK1 assays of a range of tissues from Arabidopsis (Fig. 1B) and other plant species (Supplemental Fig. S1), T6P inhibited SnRK1 activity in all except fully grown leaves. No inhibition of SnRK1 by T6P was observed from representatives of the nonplant species yeast (Saccharomyces cervisiae), house fly (Musca domestica), soil nematode (Caenorhabditis elegans), and fresh liver (Supplemental Fig. S1). Arabidopsis seedlings were then focused on as a system in which to study inhibition of SnRK1 by T6P. Minimal effects of 1 mM Glc, Suc, trehalose, Suc-6-P, and Fru-2,6-bisP were observed in comparison with 1 mM T6P (Fig. 1C). Glc-6-P (G6P; 1 mM) inhibited SnRK1 by 15% (Fig. 1C) and 10 mM G6P by 70% (data not shown), as reported previously (Toroser et al., 2000).

In Vivo Evidence of Inhibition of SnRK1 by T6P

We then sought evidence for the inhibition of SnRK1 by T6P in vivo, making use of marker gene expression as a footprint of SnRK1 activity. In a recent study using a mesophyll protoplast expression system to transiently overexpress SnRK1 (AKIN10), Baena-González et al. (2007) established approximately 1,000 genes as markers of SnRK1. We used seedlings of plant lines (Schlüpmann et al., 2003) with contrasting T6P content through constitutive expression of otsA and otsB to elevate T6P and otsB to decrease T6P (Supplemental Fig. S2A). We expected that expression of otsA would result in opposite changes in gene expression compared with expression of otsB. Furthermore, it was expected that changes in otsA would be opposite to the effect of overexpression of SnRK1 (Baena-González et al., 2007). Given that the system that established SnRK1 markers (Baena-González et al., 2007) was a transient expression system in mesophyll protoplasts, parallels in effects on gene expression rather than identical changes were expected. There were no large changes in SnRK1 activity in these seedlings (measured with no T6P in assays; Supplemental Fig. S2B) and small changes in expression of genes encoding the catalytic subunit of SnRK1, AKIN10, and AKIN11 (Supplemental Fig. S2C) in the opposite direction to the expected effect of T6P in vivo. In support of an impact of T6P on SnRK1 in vivo, quantitative reverse transcription (Q-RT)-PCR analysis of a range of SnRK1 markers including genes involved in metabolism and hormone.
signaling showed changes in otsA that were opposite to those in otsB (Fig. 2). TPS5, although not classified as a marker by Baena-González et al. (2007), was included for comparison with TPS8 to TPS11.

We then went on to perform a gene expression profiling experiment using microarrays. otsA and otsB were compared with the wild type, and otsA data were compared with data obtained from overexpression of KIN10 (Baena-González et al., 2007). Microarray data were filtered using a t test with Benjamini and Hochberg multiple test correction (P < 0.05) with a 2-fold change cutoff. Changes in gene expression in otsB were small, with most changes less than 2-fold (Supplemental Table S1) in accordance with Q-RT-PCR data. In otsA, changes in gene expression were far larger. Of the markers repressed by SnRK1 that were established by Baena-González et al. (2007), 296 showed significant induction in otsA and only four showed repression (Fig. 3A). This would be predicted if T6P inhibited SnRK1 in vivo. Of markers normally induced by SnRK1, 316 showed repression compared with the wild type in otsA (Fig. 3B) and only 12 genes showed a greater than 2-fold change in the opposite direction. These data support an effect in otsA that is consistent with inhibition of SnRK1 by T6P in vivo.

To gain a more complete indication of changes in gene expression in otsA, further analysis was conducted using FatiGO (http://fatigo.bioinfo.cipf.es; Al-Shahrour et al., 2004) to determine overrepresentation or underrepresentation of specific biological processes. In comparison with the wild type, it was found that genes of the “biosynthetic process” category (Supplemental Fig. S3; Supplemental Table S2) were overrepresented among the genes up-regulated in otsA transgenics, whereas genes of the “regulation of biological process” category were underrepresented. These data were compared with those obtained by Baena-González et al. (2007) for KIN10 overexpression in the transient protoplast expression system. The same pattern was found for the genes that are down-regulated by KIN10 (Baena-González et al., 2007), indicating opposite effects of otsA and KIN10 overexpression. Further comparison of otsA and KIN10 transgenics was performed using MapMan analysis to display gene expression data on metabolic pathways (Usadel et al., 2005). Clear effects were observed in plants expressing otsA (Fig. 4A), including down-regulation of photosynthetic genes (light reactions, Calvin cycle, and photorespiration), starch breakdown, gluconeogenesis/glyoxylate cycle, and an overall in-

Figure 1. SnRK1 activity in Arabidopsis in the presence of T6P and other sugars and sugar phosphates. Data are expressed as percentage activity compared with no T6P. A, Seedling extracts (n = 6). B, Arabidopsis leaves from mature (35–50 mm) to youngest (5 mm) leaves with stages in between (28–36, 24–28, and 13–17 mm) and seedlings. Values for leaves at 24 to 28 mm, 13 to 17 mm, and smallest leaves are measurements of pooled extracts from four plants. Other values are of three biological replicates. C, Seedling extracts with 1 mM Glc (Glc), Suc, G6P, trehalose (Tre), Suc-6-P (S6P), Fru-2,6-bisP (F26BP), and T6P (n = 4). Error bars represent se of the mean.
duction of genes involved in the tricarboxylic acid (TCA) cycle, mitochondrial electron transport, and nucleotide synthesis. Other effects include the up-regulation of aromatic amino acid and purine synthesis and the down-regulation of branched-chain amino acid degradation. These changes were opposite to the effect of Kin10 overexpression (Fig. 4B). The PageMan program was then employed to display data in terms of functional ontology (Usadel et al., 2006), confirming MapMan analysis showing opposite regulation of genes in photosynthesis, glycolysis, TCA cycle, mitochondrial electron transport, amino acid, protein, and nucleotide metabolism in otsA-expressing and Kin10-overexpressing plants (Fig. 5). Genes not categorized as SnRK1 markers (Baena-González et al., 2007) were also affected (Supplemental Table S1), particularly genes involved in metabolism, which were induced in otsA transgenics (e.g. UDP-glucoronyosyl/UDP-glucosyl transferase, ADP-Glc pyrophosphorylase, Trp synthase), consistent with the induction of biosynthetic reactions in otsA transgenics. Dre2B (At2g38340), a transcription factor gene shown to induce drought tolerance (Wang et al., 2008), was also strongly induced (Supplemental Table S1). Overall, the data strongly support the inhibition of SnRK1 by T6P.

**T6P Inhibition of SnRK1 Is Noncompetitive with Respect to ATP at a Site Distinct and Physically Separable from the Catalytic Site**

In further analysis of SnRK1 activities in seedlings, effects of ATP concentration were determined and data were fitted to kinetic models (Segel, 1993; GenStat, 2007). ATP concentration had little impact on inhibition, indicating noncompetitive inhibition by T6P with respect to ATP (Fig. 6). At higher T6P concentrations up to 4 mM, SnRK1 activity did not tend to zero, indicating that noncompetitive inhibition was partial (Segel, 1993). When fitted to enzyme kinetic models using nonlinear regression, the data best fitted partial noncompetitive mixed-type inhibition ($r^2 = 0.991$; Supplemental Fig. S4; Segel, 1993; GenStat, 2007). In this

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**Figure 2.** Transcript abundance (log2) of SnRK1 marker genes measured by Q-RT-PCR in otsA (black bars) and otsB (white bars) seedlings relative to the wild type. A and B, SnRK1-repressed markers in otsA and otsB: TPS5 (TPS5, trehalose-P synthase), ADK (ADK1, nucleotide metabolism; adenosine kinase 1), DXP (DXP, isoprenoid metabolism; 1-o-deoxysucrose-P synthase), HDT (HDT1, RNA transcription regulation; histone deacetylase HD2 family), MYB (MYB75, anthocyanin synthesis; transcription factor), EXP (EXP10, cell wall expansin), SPP (SPP2, Suc-P phosphatase), RNS (RNS1, ribonuclease), MDH (MDH1, TCA cycle; malate dehydrogenase), and CYT (CYT, cytochrome P450). C and D, SnRK1-induced markers in otsA and otsB: TPS8 to TPS11 (TPS8 to -11, trehalose-P synthase), AXP (AXP, auxin signaling), SEN (SENS, auxin signaling), LIP (LIP, triacylglycerol lipase-like protein), UDG (UDG, UDP-Glc epimerase), PGP (PGPD14, RNA regulation; pollen germination-related protein), and UBQ (UBIQUITIN, protein degradation). Error bars denote se (n = 3). All values for otsA and otsB transgenics are statistically significantly different from each other (t test, P < 0.05). See Supplemental Table S3 for gene Arabidopsis Genome Initiative codes.
model, the substrate, ATP, and the inhibitor, T6P, combine independently and reversibly with SnRK1 at different sites. Both SnRK1 and SnRK1 inhibitor complex bind substrate with different affinities, and the subsequent complexes form product at different rates. $K_p$, the dissociation constant of the enzyme-inhibitor complex, was calculated (Segel, 1993) as 7.53 μM; $K_i$, the dissociation constant of the enzyme-substrate-inhibitor complex, was calculated as 10.5 μM; $K_m$ ATP was calculated as 139 μM; $K_m$ ATP (where inhibitor is bound) was calculated as 195 μM; $V_{max}$ was calculated as 7.21 nmol mg$^{-1}$ protein min$^{-1}$; and $V_{max}$ with 1 mM T6P was calculated as 1.44 nmol mg$^{-1}$ protein min$^{-1}$.

SnRK1 was then assayed in supernatant of seedling extracts after immunoprecipitation with antibodies to AKIN10 and AKIN11 of the catalytic component of SnRK1. AKIN10 antibody removed 57.1% of SnRK1 activity, and AKIN11 antibody removed 38.3% of SnRK1 activity (95.4% of total, confirmed when both antibodies were added together; Fig. 7A). The activities remaining in the supernatant contributed by AKIN10 and AKIN11 were still inhibited by T6P, indicating that T6P inhibits both AKIN10 and AKIN11.

The pellet precipitated by AKIN10 and AKIN11 antibodies from seedling extracts was then resuspended and assayed for SnRK1 activity. SnRK1 activity was recovered (>90%) but was no longer inhibited by T6P (Fig. 7B). To determine whether this was due to a requirement for a factor not precipitated by AKIN10 and AKIN11 antibodies, supernatant from immunoprecipitated extract was added back to resuspended pellet. This restored inhibition by T6P to a large extent (Fig. 7B). Inhibition of SnRK1 by T6P was also lost during anion-exchange chromatographic purification of SnRK1 (Fig. 7C) but could be restored by adding supernatant from immunoprecipitated seedling extracts. Boiled supernatant from seedlings did not restore inhibition by T6P (Fig. 7C). These experiments show that an intermediary factor separable from SnRK1 activity is necessary for inhibition of SnRK1 by T6P.

SnRK1 Inhibition by T6P and Intermediary Factor Are Not Found in Mature Leaves

To establish if the difference in T6P inhibition between seedlings and mature leaves could be attributed to the intermediary factor, experiments were performed using immunoprecipitated SnRK1 and supernatant from seedlings and mature leaves and recombining SnRK1 activity in immunoprecipitate from one with supernatant from the other (Fig. 7D). It was found that mature leaf supernatant could not restore T6P inhibition when added to resuspended immunoprecipitated SnRK1 from seedlings, but seedling supernatant added to resuspended immunoprecipitated SnRK1 from mature leaves did result in inhibition by T6P (Fig. 7D). This shows that an additional factor necessary for T6P inhibition of SnRK1 is present in seedling extracts but not in mature leaf extracts, which can explain the lack of T6P inhibition in mature leaves.

DISCUSSION

In plants, the pathways for the synthesis of the two widespread nonreducing disaccharides in nature, tre-
Figure 4. Comparison of the effects of over-expression of KIN10 (Baena-González et al., 2007) and otsA on metabolism using MapMan analysis (Usadel et al., 2005). A, otsA transgenic seedlings. B, KIN10-overexpressing cells (Baena-González et al., 2007). CHO, Carbohydrate; OPP, oxidative pentose-P pathway.
Figure 5. Comparison of overexpression of KIN10 (Baena-González et al., 2007) with otsA using PageMan analysis (Usadel et al., 2006). Ontological groups with a change in gene expression different from the change in genes from other ontological groups are shown in color. Blue indicates an increase in gene expression, and red indicates a decrease, with darker colors representing more significant P values (Wilcoxon test). Gray indicates no data available, and white indicates no significant effect.
Inhibition of SnRK1 Activity by T6P

Figure 6. Impact of ATP concentration (60–500 μM) on the inhibition of SnRK1 by T6P (20 μM–4 mM; n = 6). Error bars represent ± of the mean.

Halose and Suc, coexist (Paul et al., 2008). While the function of Suc as a carbon source in plants is known, the function of the trehalose pathway has been more enigmatic. Here, we provide evidence that T6P, the precursor of trehalose, functions as an inhibitor of SnRK1 (AKIN10/AKIN11) of the SNF1-related group of protein kinases in plants.

In seedlings and young plant material, SnRK1 activity was inhibited by low micromolar concentrations of T6P. In seedlings, which provided a convenient system to study T6P inhibition of SnRK1 in more detail, activity was inhibited by up to 50% between 1 and 20 μM T6P (Fig. 1A). Inhibition of SnRK1 by 1 and 10 mM G6P by 15% (Fig. 1C) and 70% (data not shown), respectively, was also found. This confirms the findings of Toroser et al. (2000), who established the first evidence that G6P could provide a mechanism for metabolic regulation of SnRK1. Our work shows that T6P is a more effective regulator than G6P. Effects in the low millimolar (1–10 mM for G6P) and micromolar (1–20 μM for T6P) ranges reflect likely tissue concentrations of these metabolites (Toroser et al., 2000; Lunn et al., 2006; Supplemental Fig. S2A). The distribution of T6P between cell types is not yet known, and current estimates of T6P concentrations represent an average of all cells. Local concentrations of T6P higher than those predicted from measurements of whole seedling extracts are likely, given that expression patterns of trehalose pathway genes are strongly cell specific, for example, the TPS gene responsible for RAMOSA3 (Satoh-Nagasawa et al., 2006) and TPS and TTP genes in root cells (Birnbaum et al., 2003; Brady et al., 2007; Schluepmann and Paul, 2009).

SnRK1 was recently shown to affect the transcript abundance of approximately 1,000 genes in Arabidopsis in a central role in the response to starvation (Baena-González et al., 2007). If T6P inhibits SnRK1 in vivo, we would expect an effect on SnRK1 target gene expression in Arabidopsis seedlings expressing E. coli trehalose pathway genes, otsA encoding T6P synthase to elevate T6P and otsB encoding T6P phosphatase to decrease T6P. Furthermore, inhibition of SnRK1 by T6P in otsA would produce effects opposite to those when KIN10 was overexpressed. Despite the different experimental systems used, we found a surprisingly strong agreement of gene expression patterns. In microarray experiments, of the 1,000 or so SnRK1 markers, transcript abundances of 612 were affected statistically significantly more than 2-fold in otsA. Of these, 596 (97%) were changes in the direction to be predicted from inhibition of SnRK1 by T6P in planta (Fig. 3). The majority of changes in otsB transgenics were less than 2-fold (Supplemental Table S1), but changes were nevertheless in the opposite direction to changes in otsA determined by Q-RT-PCR (Fig. 2). Small changes in otsB may be because constitutive expression of otsB results in longer term pleiotropic effects and secondary regulation of gene expression that dampens primary effects mediated through SnRK1. We have previously shown higher G6P content in otsA than in otsB (Schluepmann et al., 2003), and given that G6P inhibits SnRK1, this could have compensated for low T6P in otsB. The impact of lowering T6P in otsB may have been less than that of increasing T6P in otsA. Analysis of the cellular distribution of T6P concentrations in vivo in relation to SnRK1 activity would answer this. Nevertheless, these data provide evidence that T6P regulates target genes of SnRK1.

Comparison of microarray data of otsA with cells overexpressing KIN10 (Baena-González et al., 2007) using FatiGO analysis (Al-Shahrour et al., 2004) showed a general up-regulation of biosynthetic processes in plants expressing otsA opposite to the effect in KIN10-overexpressing plants (Supplemental Fig. S3; Supplemental Table S2), consistent with inhibition of SnRK1 in vivo. The MapMan and PageMan programs showed that biosynthetic pathways, degradation pathways, and photosynthesis were regulated in the opposite way in otsA transgenics compared with plants overexpressing KIN10 (Figs. 4 and 5). This provides evidence that T6P counters the activation by SnRK1 of degradation pathways and photosynthesis and the inhibition by SnRK1 of biosynthetic reactions. Particularly strongly affected were genes involved in photosynthesis, mitochondrial electron transport, nucleotide and amino acid metabolism, and the TCA cycle. Other biosynthetic genes not previously categorized as SnRK1 markers were also up-regulated in otsA transgenics (Supplemental Table S1), for example, genes involved in starch and amino acid synthesis and the utilization of UDP-Glc. The APL3 gene (At4g39210) encoding ADP-Glc pyrophosphorylase, the key enzyme of starch synthesis, was strongly up-regulated. T6P also redox activates ADP-Glc pyrophosphorylase (Kolbe et al., 2005), indicating that it is regulated by T6P at two levels to promote starch synthesis. Reasons for the effects on genes not formally categorized as markers are likely to be the different
experimental systems used and the fact that longer term changes in gene expression result from the expression of otsA. Furthermore, the filtering used in the analysis of KIN10 markers (Baena-González et al., 2007) was stringent ($P < 0.0004$), and it is possible that the number of SnRK1 markers extends farther than those formally categorized as such (Baena-González et al., 2007). Additionally, our data do not exclude other effects of T6P in addition to a direct impact on SnRK1, but there was no indication that whole pathways were affected by T6P that were not also regulated by SnRK1. Overall, these data support the inhibition of SnRK1 activity by T6P in vivo as a promoter of biosynthetic reactions and inhibition of photosynthesis and degradation pathways.

Further analysis of seedling extract SnRK1 activity data obtained at varying T6P and ATP concentrations showed that inhibition of SnRK1 by T6P was noncompetitive with respect to ATP (Fig. 6). Kinetic modeling of these data predicted partial noncompetitive mix-type inhibition where T6P and ATP interact independently at different sites (Supplemental Fig. S4). This analysis was performed on kinetic data obtained with G-25 desalted extracts to remove small $M_r$ compounds such as T6P. Ideally, kinetic analysis would be performed with a purified SnRK1 complex. However, a SnRK1 complex complete with interacting factors has never been purified or expressed from plants. In further work, it was possible to substantiate and extend the prediction of the kinetic model by showing that the component responsible for T6P inhibition is distinct and physically separable from SnRK1 catalytic activity. This was done by precipitating SnRK1 activity with AKIN10 and AKIN11 antibodies and through partial purification of SnRK1 activity by anion-exchange chromatography. Activities purified in these ways were no longer inhibited by T6P (Fig. 7, B and C). An intermediary factor necessary for T6P inhibition was separated in the supernatant of seedling extracts from SnRK1 activity in the immunoprecipitate. When it was added back to immunoprecipitated SnRK1 and anion-exchange purified SnRK1, T6P inhibition of SnRK1 activity was restored (Fig. 7, B and C). The identity of this intermediary factor has not been established. Being heat labile (Fig. 7C) and not retained on G-25 Sephadex, it is likely to be a protein. It is becoming increasingly clear that the regulation of SnRK1 is particularly complex, involving a growing number of interacting proteins (Polge and Thomas, 2007; Ananieva et al., 2008). In mammals, the formation of a heterotrimer consisting of $\alpha$, $\beta$, and $\gamma$-subunits is necessary for activity. Less is known in plants, but given that the intermediary factor is readily separated from SnRK1 activity, the intermediary factor

![Figure 7. Seedling SnRK1 activities at 0 (white bars) and 1 mM (black bars) T6P after immunoprecipitation with antibodies to AKIN10 and AKIN11. A, SnRK1 activity in supernatant. B, SnRK1 activity in immunoprecipitate after resuspension of combined AKIN10 and AKIN11 pellet and after recombination with supernatant at varying pellet-supernatant ratios. C, Activity of anion-exchange-purified SnRK1 and after addition of supernatant from AKIN10 and AKIN11 immunoprecipitated seedling extracts. D, Immunoprecipitated SnRK1 activity from seedlings recombined with supernatant from mature leaves and immunoprecipitated mature leaf SnRK1 recombined with supernatant from seedlings. Data are means of four biological replicates. Error bars represent $\pm$ of the mean.](https://www.plantphysiol.org/doi/10.1104/pp.149.1868)
is unlikely to be a core part of the complex. It was not found in mature leaves, as mature leaf supernatant separated from SnRK1 activity in the same way could not restore T6P inhibition (Fig. 7D). This can explain the lack of inhibition of SnRK1 by T6P in mature leaves (Fig. 1B). Mature leaf SnRK1 could be made sensitive to T6P by adding back the factor from seedling supernatant to immunopurified mature leaf SnRK1 (Fig. 7D), again suggesting that the intermediary factor is not a core part of the complex. There is evidence that SnRK1 is regulated differently in different tissues, for example, of β-type subunits, where expression varies according to organ, developmental stage, and environmental conditions (Polge et al., 2008). The intermediary factor may mediate T6P inhibition of SnRK1 in tissues that are actively growing. In mature leaves, this function may be absent because, being fully expanded, mature leaves have no large requirement for biosynthetic reactions.

T6P inhibition was not found for SnRK1 equivalents of the heterotrophs surveyed (Supplemental Fig. S1). This study was not exhaustive, so it does not rule out the possibility that regulation of a similar nature exists in organisms other than plants. In plants, we provide the possibility that regulation of a similar nature exists. This study was not exhaustive, so it does not rule out the lack of inhibition of SnRK1 by T6P in mature leaves, where T6P regulates starch synthesis via redox activation of AGPase (Kolbe et al., 2005; Lunn et al., 2006). As T6P does not inhibit SnRK1 in mature leaves, T6P regulation of starch synthesis may be a major function of T6P in this tissue.

Overall, our data provide evidence that T6P is another part of the complex network that regulates SnRK1 activity in plants, where it counters SnRK1 activity in young tissues to promote biosynthetic reactions. In plants, unlike other organisms, trehalose is not a major end product except in a few resurrection species. Hence, T6P is removed from major pathway flux and can fluctuate without compromising other functions, unlike central intermediates (e.g. G6P), which have a limited dynamic range. Suc has been shown to lead to a large (30-fold) and rapid (minutes to hours) increase in T6P levels (Lunn et al., 2006). Therefore, T6P could signal the availability of Suc and thus inhibit SnRK1 activity and the starvation response that SnRK1 mediates to promote biosynthetic reactions and growth in response to Suc availability. Specific communication mediated by Suc has been found previously, for example, Suc-mediated transcriptional regulation of phloem-specific Suc symporter (Vaughn et al., 2002) and Suc-induced repression of translation (Wiese et al., 2004). It is not known whether T6P responds to other sugars such as Glc. Glc mediates signaling through hexokinase-1, which regulates photosynthetic gene expression in particular (Moore et al., 2003). Interestingly, unlike T6P, which is synthesized from G6P and UDP-Glc, hexokinase-1 signaling occurs independently of hexose-Ps (Moore et al., 2003). Hence, T6P-dependent signaling through SnRK1 represents a route distinct to that of hexokinase-1-dependent Glc signaling. In agreement, there is no evidence of significant cross talk between the two signaling pathways (Baena-González and Sheen, 2008).

Through defining a signaling route for T6P, we provide a mechanistic basis for the function and potency of T6P in plants as an inhibitor of the central regulator SnRK1. Further work is necessary to identify and characterize the intermediary factor necessary for T6P inhibition of SnRK1 activity and to determine the cell specificity of the mechanism.

**MATERIALS AND METHODS**

**Biological Material**

Seeds of Arabidopsis wild type (*Arabidopsis thaliana* ecotype Col-0) and expressing otsA encoding an *Escherichia coli* TTP (A19.3) or otsB encoding an *E. coli* TTP (B12.1; Schleupmann et al., 2003) driven by the cauliflower mosaic virus 35S promoter were surface sterilized and grown for 7 d in 0.5X Murashige and Skoog medium (Sigma M0404) and 0.5% Suc with gentle shaking at 23°C/16-h day, 150 μmol quanta m⁻² s⁻¹. Spinach (*Spinacia oleracea*) and wheat (*Triticum aestivum*) were grown as described previously (Sugden et al., 1999). Broccoli (*Brassica oleracea*) and cauliflower (*Brassica oleracea*) were bought fresh. Yeast (*Saccharomyces cerevisiae*) strain W303-1A was grown at 30°C to an optical density at 530 nm of 1.0 in 200 mL of yeast nitrogen base medium supplemented with 2% Suc, 39 mM ammonium sulfate, adenine, and uracil (10 mL 1⁻¹, Leu, His, and Trp (20 mg mL⁻¹). *Caenorhabditis elegans* wild type was obtained from the Caenorhabditis Genetics Centre and maintained in the laboratory under standard conditions on OP50 *E. coli* as described previously (Sulston and Hodgkin, 1988).

**SnRK1 Assays**

Plant total soluble protein was extracted in 2 mL (per gram fresh weight tissue) of ice-cold homogenization buffer of 100 mM Tricine-NaOH, pH 8, 25 mM NaF, 5 mM dithiothreitol, 2 mM tetradsodium pyrophosphate, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM protease inhibitor cocktail (Sigma P9599), phosphatase inhibitors (PhosStop; Roche), and insoluble polyvinylpyrrolidone to 2% (w/v). Homogenate was centrifuged at 13,000g at 4°C. Supernatant (200 μL) was spin desalted (2.5-ml Sepadex G-25 medium columns; GE Healthcare) and preequilibrated with hexokinase assay buffer. Eluant was supplemented with protease inhibitor cocktail and okadaic acid to 2.5 μM before freezing in liquid N₂, SnRK1 was assayed using the established procedure (Davies et al., 1989; Wekes et al., 1993; Dale et al., 1995) in 25 mL in duplicate in microtiter plate wells at 30°C. Assay medium was 40 mM HEPES-NaOH, pH 7.5, 5 mM MgCl₂, 200 μM ATP containing 12.5 kBq [γ⁻³²P]ATP (GE Healthcare), 200 μM AMARA peptide (Ala-Met-Ala-Arg-Ala-Leu-Ala-Leu-Ala-Leu-Ala-Leu-Ala-Phe-Ile), 5 mM dithiothreitol, 1 μM okadaic acid, 1 μM pepstatin A, 10 μM E64, and 7 μM chymostatin. Assays were started with extract (10 μg of protein). After 4 min, 15 μL was transferred to 4-cm² squares of Whatman P81 phosphocellulose paper, immediately immersed in 1% phosphoric acid, then washed with four 800-mL volumes of 1% phosphoric acid, immersed in acetone, dried, and transferred to liquid scintillation vials. Assays were linear over time (5 min) and extract volume and were highly reproducible. Kinetic modeling data are representative of more than six separate experiments.

For assays of SNF1, yeast cells were pelleted, washed three times with sterile distilled water, and frozen in liquid N₂. For assays of AMPK, adult *C. elegans* were snap frozen and a pellet of *C. elegans* of mixed stages was used for protein extraction. Liver, *S. cerevisiae*, *M. domestica*, and *C. elegans* were extracted as described previously (Davies et al., 1989; Wekes et al., 1993; Sugden et al., 1999). Assays were conducted using the established procedure (Davies et al., 1989; Wekes et al., 1993; Dale et al., 1995) in 25 mL in duplicate in microtiter plate wells at 30°C. In the assay of AMPK, 200 μM AMP was included. Two experiments were performed, including three biological replicates on each occasion.
Immunoprecipitation of SnRK1

Antiserum to AKIN10 and AKIN11 peptides raised in rabbits were affinity purified by Eurogentec. AKIN10 peptide was Arg-Ala-Ser-Ser-Gly-Tyr-Leu-Gly-Ala-Glu-Phe-Gln-Glu-Thr-Thr-Met, and AKIN11 peptide was Thr-Thr-Asp-Ser-Ser-Arg-Met-Arg-Thr-Pro-Glu-Ala-Gly (Zhang et al., 2008). Antibody-protein complex was formed by mixing 100 μg of antibody with 50 μL of protein A Sepharose beads (48, fast-flow; Sigma P4924) at 4 °C with gentle shaking for 2 h. After pelleting and then washing four times with homogenization buffer, protein A Sepharose beads bound with antibody were mixed with 100 μL of protein extract (200 μg of protein) for 2 h at 4 °C with gentle agitation. Sepharose beads bound with the immunocomplex were pelleted and washed thoroughly with homogenization buffer. Immunoprecipitate was resuspended in 50 μL (final volume) of homogenization buffer without protease, phosphatase inhibitors, or insoluble polyvinylpyrrolidone. Immunoprecipitate and supernatant were assayed for SnRK1 activity separately and together at different ratios after reconstituting for 2 h at 4 °C with gentle agitation.

Anion-Exchange Chromatography

SnRK1 activity was extracted and purified 192-fold relative to crude extract exactly as described by Su et al. (1999) by successive rounds of anion-exchange chromatography, including two final HPLC fractions employing a Mono-Q HR column (10 mm diameter, 10 cm long; GE Healthcare).

Microarrays

Arabidopsis seedlings were grown as above without Suc under continuous light. Procedures were followed according to the Galbraith protocol using the 29,000-element version 3.0 Arabidopsis Oligonucleotide Microarrays. RNA was extracted using a small-scale phenol-free total RNA isolation kit (Ambion), mRNA amplified using the amino allyl message Amp II aRNA amplification kit (Ambion), labeling using Alexa Fluor 647 (green) and 555 (red) dye (Invitrogen), and microarray immobilization, hybridization, and washing were performed exactly according to the Galbraith protocol (http://ag.arizona.edu/microarray/) using the 29,000-element version 3.0 Arabidopsis Oligonucleotide Microarrays (Agp3.6.2). Slides were scanned using the Gene Pix 4000B scanner. Data were analyzed using GeneSpring GX 7.3.1, normalized per spot and per chip using intensity-dependent (Lowess) normalization, and transformed to ignore low-intensity probes less than 0.01. Dye swaps were performed on each replicate of three biological replicates. otsA and otsB transgenic were compared with the same wild-type sample of three biological replicates. Data were filtered using a t test in combination with FatiGO (Supplemental Fig. S3).

Q-RT-PCR

Total RNA was isolated from seedlings using the Trizol method (Invitrogen). RNA was quantified on the Nanodrop Spectrophotometer (ND-1000), and its integrity was determined by agarose gel electrophoresis. Genomic DNA was removed with RNase-free DNase using a TURBO DNA-free kit (Ambion). cDNA was synthesized by reverse transcribing 2.5 μg of RNA using SuperScript III reverse transcriptase (Invitrogen catalog no. 18080-044) and random synthetic hexamers (Promega C1181). Gene expression was quantified using SYBR Green chemistry on a 7500 real-time PCR system (Applied Biosystems) in 10 μL of each reaction, containing 5 μL of Power SYBR Green Master Mix (Applied Biosystems), 1 μL of cDNA, and 0.25 μM primers. PCR used an initial denaturing stage of 95°C for 5 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR was performed with three technical replicates repeated on three biological replicates. Data were normalized using a protein phosphatase 2A subunit (Czechowski et al., 2005). Primers (Supplemental Table S3) were designed using the Primer Express software version 2.0 (Applied Biosystems).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. SnRK1 activity in extracts from different species.

Supplemental Figure S2. T6P amount and SnRK1 activity and transcript abundance in Arabidopsis seedlings expressing otsA and otsB.

Supplemental Figure S3. FatiGO analysis for overrepresentation or underrepresentation of biological processes.

Supplemental Figure S4. Data from Figure 3 fitted to curves predicted by the partial noncompetitive mixed-type inhibition model (Segel, 1993; GenStat, 2007; r^2 = 0.991).

Supplemental Table S1. Genes more than 2-fold affected compared with the wild type.

Supplemental Table S2. Biological processes that are overrepresented or underrepresented among the significantly changing genes as analyzed by FatiGO (Supplemental Fig. S3).

Supplemental Table S3. Primer sequences for Q-RT-PCR.

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