

Altering Trehalose-6-Phosphate Content in Transgenic Potato Tubers Affects Tuber Growth and Alters Responsiveness to Hormones during Sprouting^{1[C][W]}

Stefan Debast, Adriano Nunes-Nesi², Mohammad R. Hajirezaei, Jörg Hofmann, Uwe Sonnewald, Alisdair R. Fernie, and Frederik Börnke*

Friedrich-Alexander-Universität, Department of Biology, Division of Biochemistry, 91058 Erlangen, Germany (S.D., J.H., U.S., F.B.); Max-Planck-Institute for Molecular Plant Physiology, 14476 Potsdam-Golm, Germany (A.N.-N., A.R.F.); Leibniz Institut für Pflanzengenetik und Kulturpflanzenforschung, 06466 Gatersleben, Germany (M.R.H.); and ECROPS Erlangen, Center of Plant Sciences, 91058 Erlangen, Germany (U.S., F.B.)

Trehalose-6-phosphate (T6P) is a signaling metabolite that regulates carbon metabolism, developmental processes, and growth in plants. In *Arabidopsis* (*Arabidopsis thaliana*), T6P signaling is, at least in part, mediated through inhibition of the SNF1-related protein kinase SnRK1. To investigate the role of T6P signaling in a heterotrophic, starch-accumulating storage organ, transgenic potato (*Solanum tuberosum*) plants with altered T6P levels specifically in their tubers were generated. Transgenic lines with elevated T6P levels (B33-TPS, expressing *Escherichia coli* osmoregulatory trehalose synthesis A [*OtsA*], which encodes a T6P synthase) displayed reduced starch content, decreased ATP contents, and increased respiration rate diagnostic for high metabolic activity. On the other hand, lines with significantly reduced T6P (B33-TPP, expressing *E. coli* *OtsB*, which encodes a T6P phosphatase) showed accumulation of soluble carbohydrates, hexose phosphates, and ATP, no change in starch when calculated on a fresh weight basis, and a strongly reduced tuber yield. [¹⁴C]Glucose feeding to transgenic tubers indicated that carbon partitioning between starch and soluble carbohydrates was not altered. Transcriptional profiling of B33-TPP tubers revealed that target genes of SnRK1 were strongly up-regulated and that T6P inhibited potato tuber SnRK1 activity in vitro. Among the SnRK1 target genes in B33-TPP tubers, those involved in the promotion of cell proliferation and growth were down-regulated, while an inhibitor of cell cycle progression was up-regulated. T6P-accumulating tubers were strongly delayed in sprouting, while those with reduced T6P sprouted earlier than the wild type. Early sprouting of B33-TPP tubers correlated with a reduced abscisic acid content. Collectively, our data indicate that T6P plays an important role for potato tuber growth.

Cellular homeostasis critically depends on signaling systems that transmit information on the prevailing internal and external conditions. In plants, sugars can act as signaling molecules that relay information about the cell's metabolic status in order to adjust growth and development accordingly (Rolland et al., 2002, 2006). Several independent signaling pathways that control sugar responses in plants have been identified, distinguishing between mainly hexoses and the storage and transport disaccharide, Suc. Recently, trehalose and the

metabolism associated with its synthesis have additionally been proposed to be a component of the plant's sugar signaling system (for review, see Paul, 2007; Paul et al., 2008). Trehalose (α -D-glucopyranosyl-[1,1]- α -D-glucopyranoside) is, like Suc, a nonreducing disaccharide widely distributed in nature (Elbein et al., 2003). By analogy to the formation of Suc, the biosynthesis of trehalose involves the generation of trehalose-6-phosphate (T6P) from Glc-6-P and UDP-Glc by a trehalose-6-phosphate synthase (TPS) and the subsequent dephosphorylation of T6P to trehalose and inorganic phosphate by trehalose-6-phosphate phosphatase (TPP; Cabib and Leloir, 1958). Historically, the presence of trehalose in plants was thought to be confined to some resurrection plants, such as *Selaginella lepidophylla*, where it might serve as a stress protectant (Adams et al., 1990). However, research conducted during the last decade suggests that trehalose metabolism is ubiquitous in plants, although the majority of plants accumulate only trace amounts of this sugar. Sequenced plant genomes contain large families of genes encoding TPS- and TPP-related proteins (Lunn, 2007). From the 11 TPS-like genes encoded by the *Arabidopsis* (*Arabidopsis thaliana*) genome, it appears that only one (AtTPS1) has TPS activity, while the other family members are catalytically inactive TPS-like proteins that might fulfill

¹ This work was supported by the Bundesministerium für Bildung und Forschung in the frame of the GABI-FUTURE program (grant no. FKZ 0315059).

² Present address: Max-Planck Partner Group at the Departamento de Biologia Vegetal, Universidade Federal de Viçosa, 36570-000 Viçosa-Minas Gerais, Brazil.

* Corresponding author; e-mail fboernke@biologie.uni-erlangen.de.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Frederik Börnke (fboernke@biologie.uni-erlangen.de).

[C] Some figures in this article are displayed in color online but in black and white in the print edition.

[W] The online version of this article contains Web-only data.

www.plantphysiol.org/cgi/doi/10.1104/pp.111.179903

specific regulatory functions (Vandesteene et al., 2010). An essential role of trehalose biosynthesis in plants has been established using mutants or transgenic plants with altered expression of trehalose biosynthetic enzymes. For example, disruption of the *AtTPS1* gene in the Arabidopsis *tps1* mutant leads to embryo lethality, results in impaired vegetative growth, and prevents the transition to flowering (Eastmond et al., 2002; van Dijken et al., 2004; Gomez et al., 2010). Evidence that the product of the TPS reaction, T6P, rather than the protein itself plays a regulatory role comes from the facts that the *Escherichia coli* osmoregulatory trehalose synthesis A (*OtsA*) gene can complement the Arabidopsis *tps1* mutant phenotype and that wild-type Arabidopsis plants overexpressing *OtsA* are able to grow faster than wild-type plants on medium supplemented with sugars (Schluepmann et al., 2003). In contrast, plants overexpressing *OtsB*, encoding a TPP, further showed that T6P is indispensable for efficient carbohydrate utilization, as T6P depletion leads to the accumulation of sugar phosphates in Arabidopsis seedlings (Schluepmann et al., 2003).

A direct role of T6P in the redox activation of the chloroplast-localized ADP-Glc pyrophosphorylase (AGPase), the key enzyme of starch synthesis in plastids, has been postulated (Kolbe et al., 2005). Both direct feeding of T6P to isolated pea (*Pisum sativum*) chloroplast and overexpression of TPS in Arabidopsis have been shown to cause redox activation of AGPase, suggesting that T6P is transported into the chloroplast or binds to an envelope protein and thus can transmit a metabolic signal from the cytosol to the plastid. T6P levels in Arabidopsis seedlings have been shown to correlate with Suc content, leading to the proposal that T6P acts as a signal of Suc status in plants (Lunn et al., 2006). Indeed, there appears to be a correlation between increased levels of T6P, redox activation of AGPase, and increased rates of starch synthesis (Lunn et al., 2006).

The diversity of phenotypes created by altering trehalose metabolism in plants indicates that T6P signaling extends well beyond starch synthesis. Recent evidence suggests that T6P inhibits the SnRK1 (AKIN10/AKIN11) family of calcium-independent Ser/Thr protein kinases in Arabidopsis seedlings (Zhang et al., 2009). SnRK1 plays a central role in regulating the cellular response to energy limitation in plants, animals, and yeast (Hardie, 2007; Halford and Hey, 2009). Upon sensing the energy deficit associated with stress, nutrient deprivation, and darkness, SnRK1 triggers extensive transcriptional changes that contribute to restoring homeostasis and longer term responses for adaptation, growth, and development. Transient activation of Arabidopsis AKIN10 has been shown to trigger extensive reprogramming of transcription, affecting approximately 1,000 genes in mesophyll protoplasts (Baena-González et al., 2007). Expression profiling of *OtsA*- and *OtsB*-expressing Arabidopsis seedlings showed that genes normally induced by SnRK1 are repressed by T6P and those repressed by SnRK1 are induced by T6P (Zhang et al.,

2009). Collectively, these data suggest that T6P inhibits SnRK1 activity to activate energy-consuming processes in response to Suc availability.

Genetic approaches have demonstrated the importance of abscisic acid (ABA) in sugar response pathways, with the pathways sharing common signaling components (Rolland et al., 2006). Several studies reported a connection between T6P and ABA signaling. Avonce et al. (2004) showed that Arabidopsis seedlings overexpressing *AtTPS1* exhibit ABA insensitivity, allowing germination in the presence of 2.5 μM ABA. In turn, Arabidopsis mutants carrying weak alleles of *tps1* were hypersensitive to ABA, and the degree of hypersensitivity correlated with the reduction in T6P content (Gomez et al., 2010). Another study suggests that the effects of exogenously supplied trehalose on starch metabolism and growth of Arabidopsis seedlings are mediated by the transcription factor ABSCISIC ACID-INSENSITIVE4 (Ramon et al., 2007).

Although an important role of SnRK1 signaling for potato (*Solanum tuberosum*) tuber development and carbohydrate metabolism has been established (Purcell et al., 1998; Lovas et al., 2003; Tiessen et al., 2003), a possible link to T6P signaling has not yet been investigated. To shed further light on T6P-regulated processes in plants, we used transgenic potato tubers as a model system to analyze T6P signaling in a heterotrophic storage organ of a crop plant. To this end, transgenic potato lines with modulated T6P content in their tubers were generated. A detailed physiological and molecular analysis of the resulting transgenic lines indicates that in the potato tuber T6P acts as an integrator of the metabolic and developmental programs involving SnRK1 signaling.

RESULTS

Generation of Transgenic Potato Plants with Tuber-Specific Expression of Trehalose Biosynthetic Enzymes

With the aim to modulate the T6P content specifically in tubers of transgenic potato plants, two constructs were generated that either express the *E. coli* *OtsA* gene, encoding a TPS, or the *OtsB* gene, encoding a TPP, under the control of the tuber-specific B33 promoter. To this end, the entire coding region of *OtsA* was amplified from *E. coli* K12 genomic DNA and inserted behind the B33 promoter in the vector pBin-B33 (Rocha-Sosa et al., 1989), giving rise to plasmid B33-TPS. Similarly, the *E. coli* *OtsB* gene was amplified and inserted into the same vector to yield plasmid B33-TPP. Both constructs were used to generate transgenic potato plants utilizing *Agrobacterium tumefaciens*-mediated gene transfer. Forty-six regenerated plants from the B33-TPS transformation and 39 plants from B33-TPP transformation were transferred to the greenhouse. After harvest of tuber material, expression of the transgene was investigated using reverse tran-

scription (RT)-PCR. Seventeen B33-TPS plants and eight B33-TPP plants were identified to express the respective transgene (data not shown). For each construct, three lines showing the highest transgene expression (Fig. 1) were propagated by stem cuttings and used for further analysis.

In order to assess whether transgene expression affects T6P content in tuber material, the T6P level was measured in samples from transgenic tubers and the wild-type control using a sensitive HPLC-tandem mass spectrometry (MS/MS) assay. As shown in Table I, B33-TPS tubers displayed a 5-fold (line 16) to a 30-fold (lines 17 and 19) increase in T6P content as compared with the control, while the level of that metabolite was decreased by approximately 50% in B33-TPP tubers in relation to the wild type. An even stronger decrease in T6P levels in B33-TPP tubers could not be excluded, as the levels were close to the detection limit of the method (data not shown). Analysis of leaf material harvested from transgenic plants 4 h into the light period revealed that no significant changes in T6P level occurred in leaves of both transgenic genotypes (Table I), indicating that the modulation of the T6P content was indeed tuber specific. Moreover, the phenotype of aboveground organs of both transgenic lines was indistinguishable from that of the wild-type control (data not shown).

Modulation of the T6P Content Affects Yield, Tuber Morphology, and Carbohydrate Content

Growing potato tubers of B33-TPP lines had a longitudinal shape, while B33-TPS tubers were phenotypically comparable to the wild-type control (Fig. 2A), besides having an increased number of lenticels (Fig. 2, B and C). The number of tubers per plant was significantly increased in lines B33-TPP11 and B33-TPP34, being almost three times higher in the latter

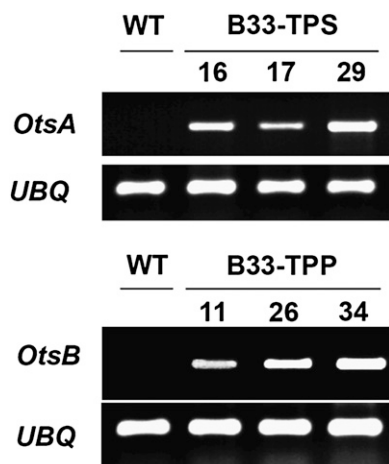


Figure 1. RT-PCR analysis of transgene expression in tubers of selected transgenic potato lines. Ubiquitin served as an internal standard to ensure that equal amounts of cDNA were used for the reactions. WT, Wild type.

Table I. T6P content in growing potato tubers

Samples were taken from growing tubers and immediately frozen in liquid nitrogen before T6P levels were determined using HPLC-MS/MS. Values represent means $\pm$ SD of four to seven individual tubers and leaves from five individual plants per plant line. * Significant difference ($P < 0.05$); ** $P < 0.001$ according to t test (I) compared with the wild-type control.

Plant Line	Tuber Tissue	Leaf Tissue
	<i>nmol g⁻¹ fresh wt</i>	
Wild type Solara	1.21 $\pm$ 0.46	1.06 $\pm$ 0.18
B33-TPS 16	7.03 $\pm$ 1.90**	1.47 $\pm$ 0.29
B33-TPS 17	41.24 $\pm$ 6.53**	1.22 $\pm$ 0.18
B33-TPS 29	45.77 $\pm$ 7.03**	1.17 $\pm$ 0.30
B33-TPP 11	0.63 $\pm$ 0.20*	1.04 $\pm$ 0.28
B33-TPP 26	0.72 $\pm$ 0.15*	1.16 $\pm$ 0.25
B33-TPP 34	0.64 $\pm$ 0.11*	1.00 $\pm$ 0.10

line compared with the control (Table II). Total tuber biomass was significantly decreased in all transgenic lines when compared with a control specimen, irrespective of whether the T6P content was increased or decreased (Table II). The fresh weight-dry weight ratio was significantly increased in two of the B33-TPP lines (lines 26 and 34), while the ratio was decreased in line B33-TPS17 (Table II).

It has previously been established that T6P is a regulator of carbon utilization in Arabidopsis (Schluepmann et al., 2003). In order to investigate if altered T6P levels would affect carbohydrate status in potato tubers, the levels of soluble sugars and starch were determined in both B33-TPS and B33-TPP lines. Expression of TPS led to a significant decrease in tuber starch content in all B33-TPS lines to a level approximately 50% below that of the wild type (Table III). In B33-TPP, there was no clear effect on starch levels when compared with the wild-type control (Table III).

A reduction in T6P content in B33-TPP tubers led to a significant increase in hexose levels as well as in Suc content in all three lines (Table III). In sum, soluble sugars were increased 3.6-fold in B33-TPP line 11, 4.8-fold in line 26, and 5.2-fold in line 34. Given that starch levels were not altered in B33-TPP lines, this led to an increase of total carbohydrates of approximately 20% in these tubers when calculated on a fresh weight basis. In B33-TPS lines, Glc levels were significantly decreased, while a decrease in Suc content was only observed in the two strongest B33-TPS lines (TPS17 and TPS19). Glc-6-P was reduced in B33-TPS lines 17 and 29, while other hexose phosphates and UDP-Glc, the immediate precursor of Suc, were unchanged (Table IV). By contrast, in all B33-TPP lines investigated, hexose phosphates as well as UDP-Glc were consistently about 2-fold up-regulated when compared with the wild-type control (Table IV).

Utilization of External Glc by Tuber Discs of B33-TPS and B33-TPP Transgenic Lines

To relate the information obtained from the analysis of steady-state levels of carbohydrates to actual met-

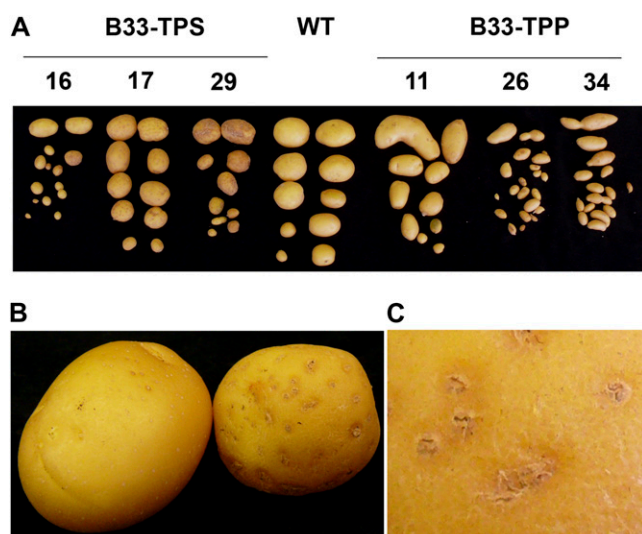


Figure 2. Phenotypic changes of B33-TPS and B33-TPP tubers compared with the wild-type (WT) control. A, Tubers from a single representative plant per line were photographed 2 weeks after harvest. B, Closeup of a tuber taken from line B33-TPS 16. C, Detailed view of lenticels on the tuber surface of the B33-TPS 16 tuber. [See online article for color version of this figure.]

abolic change, we next assessed the major fluxes of carbon metabolism. For this purpose, we incubated tuber discs in 10 mM MES-KOH (pH 6.5) supplemented with 20 mM unlabeled Glc and 2 μ Ci (3 mCi mmol⁻¹) of [U-¹⁴C]Glc. All discs were prepared from growing potato tubers that were removed from the mother plant immediately before the experiment, and incubation was carried out in sealed flasks that allowed CO₂ trapping in alkaline solution. Four hours after incubation, tuber discs were analyzed to determine the distribution of label. As shown in Table V, uptake of label in tubers with altered T6P content did not differ from their respective controls. Also, the partitioning of label between soluble and insoluble material was unaltered in the transgenic tubers as compared with the wild type. B33-TPS tubers showed an increased label incorporation into organic acids, while partitioning into other cellular components was

unaltered. Concomitantly, all B33-TPS-derived tuber discs displayed a significant increase in CO₂ evolution, indicative for a higher respiratory activity in these lines. Accordingly, respiration rates measured on intact B33-TPS tubers were significantly increased at the time of harvest as well as 1 week after storage (Supplemental Fig. S1).

A decrease in T6P content in B33-TPP tubers led to a significant increase in partitioning of label into sugars, which was mainly attributable to an elevated incorporation into Suc (Table V); however, partitioning to other components, including organic acids, was unaltered in these lines.

Modulation of T6P Content Leads to a Decrease of the ATP/ADP Ratio

The ATP/ADP ratio was decreased by 50% to 90% in all transgenic lines as compared with the control irrespective of whether the T6P content was increased or decreased (Table VI). However, this was apparently due to different reasons in B33-TPS and B33-TPP lines. While the total ADP content was increased in all transgenic lines, B33-TPS plants displayed a significant reduction in ATP levels while B33-TPP lines 26 and 34 accumulated significantly more ATP than the wild type. Given the proportionally more drastic increase of ADP in B33-TPP lines as compared with B33-TPS lines, a 2-fold increase in ATP in B33-TPP line 34 still resulted in a decrease of the ATP/ADP ratio by 50% in this line (Table VI).

Transcriptional Changes

To further characterize processes that might be regulated by T6P in potato tubers, we conducted a transcript analysis of B33-TPS and B33-TPP tubers compared with the wild-type control using microarrays. To this end, samples were prepared from storage tissue taken from freshly harvested tubers and transcript profiles were prepared using the Agilent 4 × 44 k array (Kloosterman et al., 2008). Data analysis was performed as described in “Materials and Methods” using the GeneSpring GX7.3.1 software using a 2-fold change cutoff. Changes in gene expres-

Table II. Modulation of the T6P content affects yield-related parameters of the transgenic potato lines

Values represent means $\pm$ SD of five to six individual plants. * Significant difference ($P < 0.05$); ** $P < 0.001$ according to *t* test (I) compared with the wild-type control.

Plant Line	Tuber Biomass per Plant	Tubers per Plant	Dry Weight/Fresh Weight Ratio
Wild-type Solara	289.8 $\pm$ 16.3 ^g	8.0 $\pm$ 2.1	0.21 $\pm$ 0.03
B33-TPS 16	109.9 $\pm$ 20.4**	7.8 $\pm$ 2.0	0.21 $\pm$ 0.00
B33-TPS 17	191.1 $\pm$ 18.2**	7.2 $\pm$ 1.6	0.18 $\pm$ 0.00*
B33-TPS 29	184.9 $\pm$ 23.4**	9.4 $\pm$ 2.7	0.20 $\pm$ 0.02
B33-TPP 11	257.0 $\pm$ 20.6**	12.8 $\pm$ 0.7*	0.23 $\pm$ 0.02
B33-TPP 26	53.4 $\pm$ 13.4**	13.8 $\pm$ 4.2*	0.25 $\pm$ 0.02*
B33-TPP 34	72.1 $\pm$ 17.3**	20.6 $\pm$ 6.6*	0.26 $\pm$ 0.02*

Table III. Changes in carbohydrate content in growing B33-TPS and B33-TPP tubers compared with the wild-type control

Values represent means $\pm$ SD of five to nine individual plants. * Significant difference ($P < 0.05$); ** $P < 0.001$ according to t test (I) compared with control specimens.

Plant Line	Glc	Fru	Suc	Starch
	$\mu\text{mol g}^{-1}$ fresh wt			
Solara wild type	9.0 $\pm$ 1.4	2.1 $\pm$ 0.9	17.3 $\pm$ 0.8	624.7 $\pm$ 66.6
B33-TPS 16	5.4 $\pm$ 0.8**	0.8 $\pm$ 0.7*	17.9 $\pm$ 1.9	300.0 $\pm$ 49.7**
B33-TPS 17	2.3 $\pm$ 0.7**	1.4 $\pm$ 0.4	7.7 $\pm$ 1.0**	452.7 $\pm$ 14.0**
B33-TPS 29	2.5 $\pm$ 0.5**	1.4 $\pm$ 1.2	11.5 $\pm$ 1.2**	421.0 $\pm$ 98.6**
B33-TPP 11	44.0 $\pm$ 5.4**	5.3 $\pm$ 0.5**	45.9 $\pm$ 4.3**	684.2 $\pm$ 90.1
B33-TPP 26	39.2 $\pm$ 10.6**	6.0 $\pm$ 0.9**	83.2 $\pm$ 9.8**	551.3 $\pm$ 114.9
B33-TPP 34	56.9 $\pm$ 10.0**	7.7 $\pm$ 2.3**	73.4 $\pm$ 7.6**	617.9 $\pm$ 40.6

sion in B33-TPS were small, with most changes less than 2-fold in magnitude, and no consistent changes were observed between the transgenic lines (data not shown). In B33-TPP, changes in gene expression were far larger, with 3,770 features found to be consistently differentially expressed in all three transgenic lines in comparison with the wild type (Supplemental Table S1). Therefore, we concentrated our analysis of the transcriptional profiles obtained from B33-TPP tubers. Among the features differentially regulated in B33-TPP tubers, 2,910 were up-regulated while 860 were down-regulated. To gain a complete indication of the changes in gene expression in B33-TPP tubers, the differentially regulated genes were grouped into 21 functional categories (Fig. 3; Hartmann et al., 2011), with two groups, transcripts of unknown function and transcripts that could not be assigned to any other category, accounting for about 53% of the transcripts. A large fraction of the genes found to be down-regulated in B33-TPP lines were associated with storage protein gene expression, mainly encoding patatins and protease inhibitors (Fig. 3; Supplemental Table S1). Another set of down-regulated genes associated with translation mainly encode ribosomal proteins, translation initiation, and elongation factors (Fig. 3; Supplemental Table S1). While 52 transcripts in this category were down-regulated, only 16 showed elevated expression levels in B33-TPP tubers as compared with the wild-type control. Among the 16

features of this category up-regulated, five are predicted to encode plastid-localized proteins; there was only one feature associated with plastid translation among the 52 down-regulated features. This might indicate that translation in plastids is differentially affected by T6P than the same process in the cytosol. The most down-regulated feature of the cell cycle category was assigned to a CDK-activating kinase, while a transcript corresponding to a CDK inhibitor was among the up-regulated features (Supplemental Table S1). This might indicate that cell cycle activity is reduced in B33-TPP tubers. Along that line, a transcript encoding for the translationally controlled tumor protein (TCTP) was significantly down-regulated in all B33-TPP lines (Supplemental Table S1). In Arabidopsis TCPT has been shown to act as a positive regulator of mitotic growth by specifically controlling the duration of the cell cycle (Brioudes et al., 2010).

Functional categories of genes with a higher percentage of up-regulated than down-regulated transcripts in B33-TPP tubers as compared with the wild type were much more diverse. A number of genes controlling the biosynthesis of cellulose (cellulose synthase) and genes involved in cell wall remodeling, such as pectin esterases, polygalacturonases, and expansins (Fig. 3; Supplemental Table S1), were found to be strongly up-regulated in B33-TPP tubers.

Inspection of the energy metabolism category revealed that a number of transcripts likely associated

Table IV. Levels of hexose phosphates and UDP-Glc in growing B33-TPS and B33-TPP tubers compared with the wild-type control

Values represent means $\pm$ SD of six individual plants. * Significant difference ($P < 0.05$) according to t test (I).

Plant Line	Glc-6-P	Fru-6-P	Glc-1-P	UDP-Glc
	nmol g^{-1} fresh wt			
Solara wild type	19.4 $\pm$ 2.0	8.2 $\pm$ 3.1	4.2 $\pm$ 0.6	4.8 $\pm$ 0.9
B33-TPS 16	17.2 $\pm$ 2.9	5.5 $\pm$ 3.2	3.6 $\pm$ 0.6	7.6 $\pm$ 3.5
B33-TPS 17	15.8 $\pm$ 2.4*	4.5 $\pm$ 2.1	4.4 $\pm$ 0.7	6.8 $\pm$ 2.7
B33-TPS 29	13.0 $\pm$ 3.5*	5.5 $\pm$ 1.8	4.0 $\pm$ 0.6	5.5 $\pm$ 1.9
B33-TPP 11	39.2 $\pm$ 7.0*	13.6 $\pm$ 3.9*	7.7 $\pm$ 1.5*	7.4 $\pm$ 1.7*
B33-TPP 26	41.8 $\pm$ 10.5*	14.5 $\pm$ 4.1*	8.6 $\pm$ 1.3*	7.6 $\pm$ 2.3*
B33-TPP 34	51.8 $\pm$ 6.3*	18.5 $\pm$ 7.6*	8.8 $\pm$ 2.0*	8.5 $\pm$ 3.0*

Table V. Redistribution of radiolabel after incubation with [$U\text{-}^{14}\text{C}$]Glc in tuber discs of B33-TPS and B33-TPP lines

Tuber discs were cut from growing tubers of six separate plants per genotype. After 4 h of incubation, the tuber discs were extracted and fractionated. Values represent means $\pm$ SD of measurements of six individual plants per genotype. Values in boldface denote fractions further fractionated into subfractions. Values in italics show significant subfractions of the soluble fraction. * Significant difference ($P < 0.05$) according to Student's t test (II).

Parameter	^{14}C Incorporation			
	Wild Type	B33-TPS 16	B33-TPS 17	B33-TPS 29
Total metabolized (Bq g $^{-1}$ fresh wt)	1,468.4 $\pm$ 111.7	1,649.6 $\pm$ 330.0	1,460.3 $\pm$ 329.0	1,431.6 $\pm$ 176.8
Insoluble (%)	30.8 $\pm$ 6.1	38.1 $\pm$ 7.1	22.7 $\pm$ 5.8	36.9 $\pm$ 10.1
Protein (%)	1.8 $\pm$ 0.5	2.7 $\pm$ 0.7	3.2 $\pm$ 1.3	2.9 $\pm$ 0.7
Starch (%)	17.9 $\pm$ 6.8	22.3 $\pm$ 6.9	9.5 $\pm$ 5.5	20.1 $\pm$ 8.6
Cell wall (%)	11.1 $\pm$ 1.4	13.1 $\pm$ 2.9	10.0 $\pm$ 3.1	14.0 $\pm$ 4.3
Soluble (%)	69.2 $\pm$ 6.1	61.9 $\pm$ 7.1	77.3 $\pm$ 5.8	63.1 $\pm$ 10.1
Amino acid (%)	3.8 $\pm$ 0.7	4.3 $\pm$ 0.9	4.2 $\pm$ 1.2	3.8 $\pm$ 0.3
Sugars (%)	57.5 $\pm$ 7.4	46.2 $\pm$ 8.1	59.8 $\pm$ 5.7	45.1 $\pm$ 9.8
Acidic (%)	7.9 $\pm$ 1.2	11.3 $\pm$ 1.6*	13.2 $\pm$ 5.4	14.1 $\pm$ 1.3*
<i>Organic acids (%)</i>	<i>5.4 $\pm$ 1.0</i>	<i>8.9 $\pm$ 1.1*</i>	<i>10.1 $\pm$ 4.1*</i>	<i>10.7 $\pm$ 1.2*</i>
<i>Released CO₂ (%)</i>	<i>2.7 $\pm$ 0.2</i>	<i>3.6 $\pm$ 0.7*</i>	<i>4.0 $\pm$ 0.8*</i>	<i>4.0 $\pm$ 0.5*</i>

Parameter	^{14}C Incorporation			
	Wild Type	B33-TPP 11	B33-TPP 26	B33-TPS 34
Total metabolized (Bq g $^{-1}$ fresh wt)	631.8 $\pm$ 179.1	733 $\pm$ 111.1	666.2 $\pm$ 188.9	445.9 $\pm$ 55.5
Insoluble (%)	46.3 $\pm$ 21.5	48.1 $\pm$ 19.7	39.1 $\pm$ 13.6	36.9 $\pm$ 8.5
Protein (%)	2.2 $\pm$ 0.8	1.9 $\pm$ 0.9	1.9 $\pm$ 0.5	1.9 $\pm$ 1.2
Starch (%)	32.7 $\pm$ 16.6	33.9 $\pm$ 14.7	27.2 $\pm$ 12.1	25.5 $\pm$ 6.5
Cell wall (%)	11.4 $\pm$ 4.8	12.5 $\pm$ 4.9	10.0 $\pm$ 2.1	9.5 $\pm$ 2.9
Soluble (%)	37.2 $\pm$ 10.0	37.8 $\pm$ 13.7	34.6 $\pm$ 9.4	44.1 $\pm$ 24.5
Amino acid (%)	12.9 $\pm$ 6.8	13.0 $\pm$ 3.2	15.3 $\pm$ 2.2	11.9 $\pm$ 3.6
Acidic (%)	12.2 $\pm$ 6.0	9.5 $\pm$ 5.5	6.7 $\pm$ 5.9	11.2 $\pm$ 6.2
<i>Organic acids (%)</i>	<i>11.7 $\pm$ 1.0</i>	<i>12.1 $\pm$ 1.2</i>	<i>10.9 $\pm$ 0.4</i>	<i>11.1 $\pm$ 0.7</i>
<i>Released CO₂ (%)</i>	<i>3.2 $\pm$ 0.9</i>	<i>2.4 $\pm$ 0.3</i>	<i>3.0 $\pm$ 1.1</i>	<i>3.4 $\pm$ 1.1</i>
<i>Suc (%)</i>	<i>6.7 $\pm$ 1.3</i>	<i>23.6 $\pm$ 6.3*</i>	<i>17.6 $\pm$ 4.4*</i>	<i>25.6 $\pm$ 1.8*</i>
<i>Other sugars (%)</i>	<i>6.3 $\pm$ 1.5</i>	<i>9.0 $\pm$ 3.7*</i>	<i>9.8 $\pm$ 1.0*</i>	<i>5.2 $\pm$ 1.9</i>

with carbon dissimilation and mitochondrial electron transport were up-regulated in B33-TPP tubers. These included features corresponding to Fru-1,6-bisphosphatase, glycerol-3-phosphate dehydrogenase, phosphoglycerate mutase, pyruvate dehydrogenase, and succinate dehydrogenase (Supplemental Table S1).

Taken together, these data indicate that transcripts associated with biosynthetic processes are rather down-regulated in B33-TPP tubers, while catabolism-associated ones are induced. Among the most down-regulated features was a transcript encoding a TPP (16-fold down-regulated), which might indicate a compensatory response to adjust the expression of trehalose-metabolizing genes to T6P levels.

In order to investigate whether SnRK1 signaling might be involved in mediating the transcriptional changes observed in B33-TPP tubers, we compared the set of genes found to be differentially regulated in the transgenic tubers with that of potential SnRK1-regulated genes in Arabidopsis (Baena-González et al., 2007) using the BLASTX annotation of POCI features against Arabidopsis proteins (Kloosterman et al., 2008). It can be expected that a decrease in T6P would have a

similar effect than overexpression of SnRK1. Given that SnRK1 markers were established using transient SnRK1 overexpression in Arabidopsis protoplasts, it can be assumed that effects on gene expression would not be identical in B33-TPP tubers but would rather resemble the situation previously found in Arabidopsis. In addition, both microarray platforms most likely represent an overlapping set of transcripts rather than an identical one. For 646 out of the 1,021 Arabidopsis SnRK1 marker genes proposed by Baena-González et al. (2007), a potato ortholog could clearly be identified as being present on the POCI array. The comparison further identified 133 transcripts (21%) out of the 646 that were regulated in the same direction (either up or down) in both gene sets (Supplemental Table S2). Only 35 genes (5%) out of the 646 SnRK1 marker genes showed opposite regulation as compared with the Arabidopsis gene set (Supplemental Table S2). Among the SnRK1 markers up-regulated in B33-TPP tubers were transcripts assigned to a range of functional categories, such as branched-chain amino acid degradation, trehalose metabolism, lipid metabolism, and cell wall modification. Considerable overlap in SnRK1 markers that were

Table VI. Steady-state levels of adenine nucleotides in B33-TPS and B33-TPP transgenic tubers compared with the wild-type control

Values represent means $\pm$ SD of 10 individual plants. * Significant difference ($P < 0.001$) according to Student's t test (II) compared with the wild-type control.

Plant Line	ATP	ADP	ATP/ADP Ratio
	<i>nmol g⁻¹ fresh wt</i>		
Wild type	21.2 $\pm$ 2.4	3.0 $\pm$ 0.4	7.2 $\pm$ 0.9
B33-TPS 16	6.7 $\pm$ 0.9*	4.7 $\pm$ 0.6*	1.4 $\pm$ 0.1*
B33-TPS 17	13.9 $\pm$ 0.9*	6.1 $\pm$ 0.3*	2.3 $\pm$ 0.2*
B33-TPS 29	4.9 $\pm$ 0.3*	8.3 $\pm$ 1.2*	0.6 $\pm$ 0.1*
B33-TPP 11	20.2 $\pm$ 1.0	7.5 $\pm$ 0.4*	2.7 $\pm$ 0.2*
B33-TPP 26	37.7 $\pm$ 1.7*	24.1 $\pm$ 0.9*	1.6 $\pm$ 0.1*
B33-TPP 34	46.9 $\pm$ 2.2*	14.3 $\pm$ 0.8*	3.3 $\pm$ 0.3*

down-regulated was found among transcripts annotated as ribosomal proteins (Supplemental Table S2).

We next used quantitative real-time (q)RT-PCR to confirm the transcriptional changes observed in the transcriptome analysis and applied this approach to a selected set of bona-fide SnRK1 marker genes from a range of functional categories. Samples for RNA preparation were taken from a set of plants independent from that used for the array experiment. Sucrose synthase (SuSy), although not classified as an SnRK1 marker by Baena-González et al. (2007), was included in the experiment as it has previously been identified as a SnRK1 target in potato (Purcell et al., 1998) and was also up-regulated on the B33-TPP microarray (Supplemental Table S2). We also included *KIN10*, the SnRK1 catalytic subunit, and *KINγ*, an activating subunit of the SnRK1 kinase complex, as there was a clear, although not significant, up-regulation of the respective transcripts on the B33-TPP microarray (Supplemental Table S1). As shown in Figure 4, changes in gene expression as quantified by qPCR reflected those previously observed in the microarray experiment. In addition, there was a significant increase in transcript levels of *SnRK1* and *KINγ* (Fig. 4). In order to investigate whether changes in gene expression in B33-TPS lines would be opposite to those observed in B33-TPP lines, transcript levels of the same set of genes were quantified in material taken from transgenic tubers with increased T6P content. The analyses showed that

a number of transcripts (*FBPase*, *TPS8*, *TPS11*, *UDPG*, and *KINγ*) were significantly down-regulated as compared with the wild-type control, while others (*SuSy2*, *G1P-X*, and *KIN10*) were not significantly changed as compared with the control (Fig. 4).

Taken together, these data are consistent with an activation of SnRK1 signaling in B33-TPP tubers through a reduction of the T6P content in vivo.

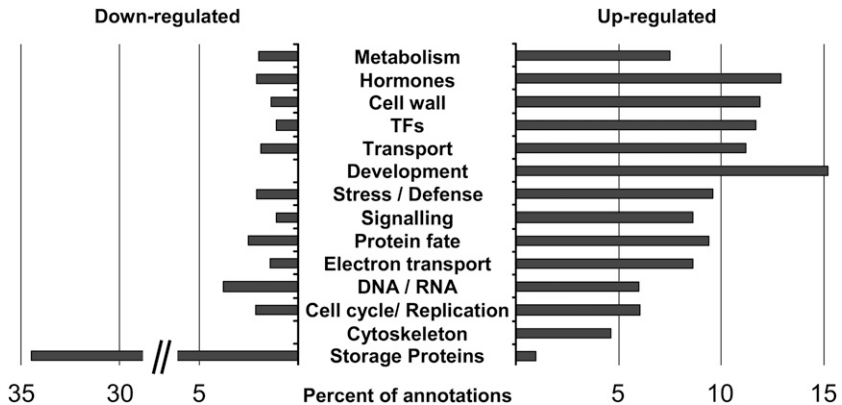
Inhibition of Potato Tuber SnRK1 Activity by T6P

Having established that the changes in transcription in B33-TPP tubers reflect activated SnRK1 signaling, we next sought to investigate whether potato tuber SnRK1 activity is inhibited by T6P in vitro. When 1 mM T6P was added to desalted protein extracts prepared from growing potato tubers, SnRK1 activity was decreased by approximately 30% as compared with the activity measured in the same extract but without the addition of T6P (Fig. 5A). The inhibitory effect of T6P was specific, as addition of 1 mM Suc-6-P had no effect on in vitro SnRK1 activity (Fig. 5A). In order to assess concentration-dependent differences in T6P sensitivity of SnRK1 activity, inhibition was investigated in extracts prepared from mature tubers in the presence of different concentrations of T6P. The measurements revealed that SnRK1 activity was already inhibited when 10 μM T6P was added to the assay and that maximal inhibition was achieved at approximately 100 μM T6P (Fig. 5B). The maximal extractable activity in desalted extracts from B33-TPP and B33-TPS transgenic tubers was largely unchanged (Supplemental Fig. S2A). These data show that SnRK1 activity in potato tubers is prone to inhibition by T6P.

Exogenously Applied Suc Modulates T6P Content and Represses SnRK1 Marker Genes in Potato Tuber Discs

T6P levels have been shown to reflect Suc content in Arabidopsis (Lunn et al., 2006). In order to investigate if Suc supply would have the potential to modulate T6P levels in potato tubers, we fed discs of wild-type potato tubers with different sugars for 4 h and subsequently measured T6P content. Feeding of the hexoses

Figure 3. Functional assignment of transcripts differentially expressed in B33-TPP and wild-type potato tubers. Differentially expressed transcripts were classified into functional groups. Bars illustrate the percentage of transcripts within various categories based on total numbers. The categories “unknown” and “unclassified,” containing about 52% of differentially expressed genes, are not shown for clarity (for more detailed information, see Supplemental Table S1). TFs, Transcription factors.



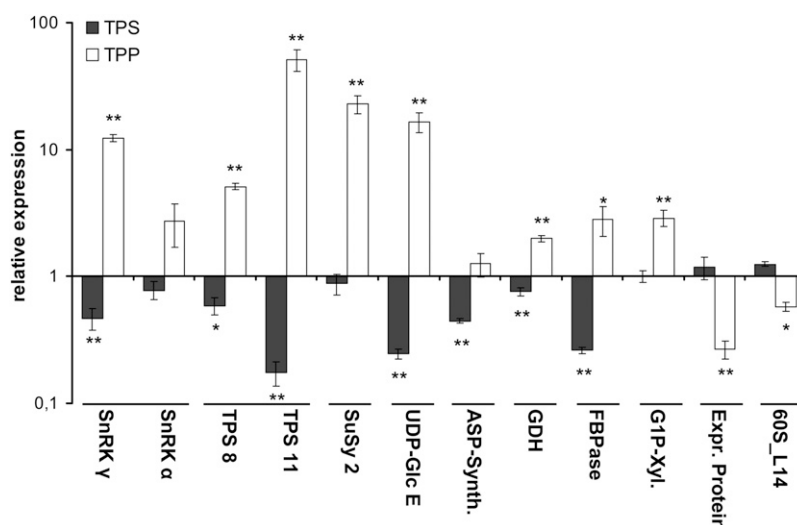


Figure 4. Transcript abundance of SnRK1 subunits and SnRK1 marker genes measured by qRT-PCR in tubers of B33-TPS line 29 (black bars) and B33-TPP line 34 (white bars) transgenic plants relative to the wild type. Transcripts are as follows: SnRK γ (*KIN γ* ; regulatory SnRK subunit; POCL_ID MICRO.804.C1_1507), SnRK α (*KIN α* ; catalytic SnRK subunit; POCL_ID MICRO.13181.C1_878), TPS8 (POCL_ID POABM58TP_861), TPS11 (POCL_ID cSTC3D14TH_235), SuSy2 (POCL_ID STMHE19TV_557), UDP-Glc E (UDP-Glc epimerase; POCL_ID MICRO.7015.C2_1344), ASP-synth. (Asn synthase; POCL_ID MICRO.3150.C2_616), GDH (Gln dehydrogenase; POCL_ID bf_stolxxxx_0055h06.t3m.scf_448), FBPase (cytosolic Fru-1,6-bisphosphatase; POCL_ID MICRO.5872.C2_1120), G1P-Xyl. (Glc-1-P metabolism; Xyl catabolism-related expressed protein; POCL_ID bf_arrayxxx_0091a12.t7m.scf_711), Expr. Protein (expressed protein; POCL_ID MICRO.5525.C1_852), and 60S L14 (ribosomal protein L14; POCL_ID MICRO.1272.C2_547). Data are expressed as $\log_{10}$ of relative expression of the wild-type controls and shown as means $\pm$ SD of three independent samples. Statistical differences as determined by Student's *t* test (II) are indicated (* $P < 0.05$, ** $P < 0.001$).

Glc and Fru as well as of the nonmetabolizable Suc isomer palatinose had no significant effect on the T6P content in tuber discs (Fig. 6). In contrast, exogenous supply of trehalose as well as Suc led to a significant increase in T6P levels as compared with the control (Fig. 6). Trehalose has previously been shown to lead to elevated T6P levels through feedback inhibition of TPP when intracellular trehalose levels are high (Schluepmann et al., 2004) and thus serves as a positive control. The observation that Suc but not the hexoses Glc or Fru results in significant T6P accumulation argues for a Suc-specific sensing mechanism that is involved in T6P signaling. Transcript levels of the SnRK1 marker genes *TPS8* and *GDH* as well as *KIN γ* were significantly down-regulated in Suc fed tuber discs, indicating that SnRK1 signaling also responds to short-term changes in T6P levels in potato tubers (Fig. 7).

Modulation of T6P Content Affects Sprouting of Stored Potato Tubers

We made the initial observation that tubers harvested from B33-TPP transgenic potato plants when stored at room temperature broke dormancy and produced sprouts much earlier than control tubers. This prompted us to investigate the sprouting behavior of transgenic tubers with altered T6P content in more detail. To this end, tubers from B33-TPP and

B33-TPS lines that were harvested at the same time were stored at room temperature in the dark, and sprouting was quantitatively assessed in transgenic tubers as compared with the control over a period of 40 weeks. As shown in Figure 8, wild-type tubers started sprouting at around 10 weeks after harvest, and 100% sprouting was achieved after about 19 weeks. In contrast, B33-TPP tubers started sprouting already about 6 weeks after harvest, and full sprouting was observed in B33-TPP line 11 approximately 11 to 12 weeks after harvest; while B33-TPP lines 34 and 26 required an additional 2 to 3 weeks to achieve complete sprouting, they still sprouted significantly faster than the control tubers (Fig. 8). Strikingly, sprouting in B33-TPS tubers was significantly delayed in comparison with the wild type. B33-TPS line 16 achieved full sprouting at about 25 weeks after harvest, while lines 17 and 29 did not achieve 100% sprouting over the entire observation period of 40 weeks (Fig. 8). The delay in sprouting of B33-TPS lines correlated with the T6P content in these tubers, with line 16 showing the lowest level while line 29 had the highest T6P content among B33-TPS lines (Table I). These data indicate that T6P levels either directly or indirectly affect tuber dormancy.

One of the factors known to regulate potato tuber dormancy and bud break is endogenous plant hormones (Suttle, 2004). GAs and cytokinins (CKs) are thought to be involved in the release of dormancy,

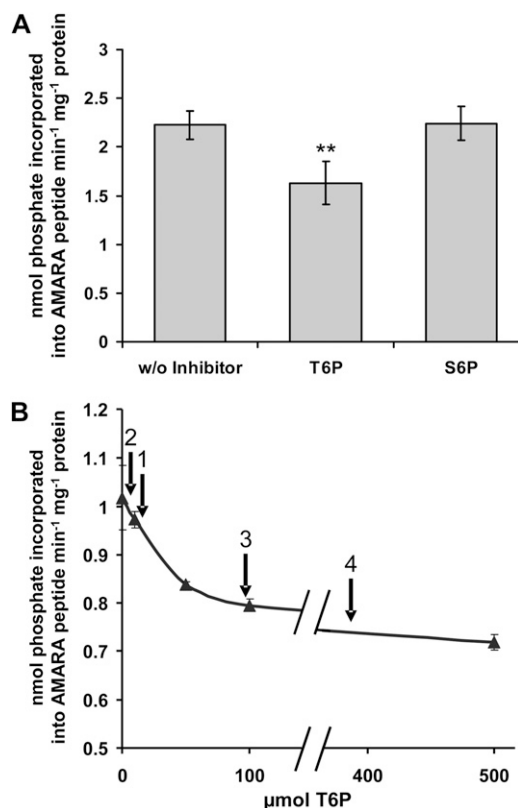


Figure 5. SnRK1 activity is inhibited by T6P in growing potato tubers. **A**, SnRK1 activity in extracts prepared from growing potato tubers was assayed with or without the addition of 1 mM T6P. Addition of 1 mM Suc-6-P (S6P) served as a control. Values represent means $\pm$ SD of three biological replicates. ** Significant difference ($P < 0.001$) compared with the control without T6P or S6P. **B**, SnRK1 activity in extracts from mature potato tubers ($n = 4$; $\pm$ SD) in the presence of increasing concentrations of T6P. Numbered arrows indicate estimated in vivo T6P concentrations: 1, the wild type; 2, B33-TPP tubers; 3, B33-TPS line 16; 4, B33-TPS line 29. All estimations assume T6P residing exclusively in the cytosol and further assume the volume of the cytosol to be 12% of the total cell volume.

whereas ABA has been associated with the maintenance of tuber dormancy (Suttle and Hultstrand, 1994). In order to investigate whether T6P transgenic tubers are altered in the hormonal response, we studied the dormancy-releasing capacity of GA and CK using a recently established in vitro sprout-release assay (Hartmann et al., 2011). This assay is based on isolated tuber buds, which are placed on wet filter paper in closed petri dishes. Upon addition of either 50 μ M GA or 100 μ M 6-benzylaminopurine (a CK), bud growth can be followed over time. Tuber discs were excised from B33-TPP and B33-TPS tubers as well as from control plants 2 weeks after harvest and subjected to a sprout-release assay in the presence of either GA or CK. As observed before (Hartmann et al., 2011), clear signs of sprout induction on wild-type tuber discs became visible 3 d after hormone application, with approximately 30% of the discs having visible sprouts (Fig. 9; Supplemental Fig. S3). After 5

d, more than 80% of the GA-treated and more than 60% of the CK-treated wild-type discs displayed clear sprouting. In contrast, less than 10% of the GA-treated B33-TPS discs had visible signs of sprouting after 5 d, and none of the CK-treated B33-TPS discs showed any bud break at this time point (Fig. 9). However, sprouting occurred much faster in both treatments in the case of B33-TPP tuber discs. Here, more than 60% of the discs showed clearly visible sprouting already at 3 d after treatment and reached 100% sprouting in both cases at the end of the experimental period (Fig. 9). Taken together, these data show that decreased T6P content renders B33-TPP tuber discs hypersensitive toward both hormones, while elevated T6P in B33-TPS tubers leads to CK as well as GA insensitivity. This indicates that T6P might modify a signal that possibly acts upstream of the two hormones tested. Since ABA has previously been assumed to regulate the maintenance of potato tuber dormancy (Suttle and Hultstrand, 1994), it might constitute such a signal. Measurements of the ABA content in tubers directly after harvest revealed that the level of this hormone was reduced by approximately 70% in B33-TPP tubers as compared with the control (Fig. 10A). ABA levels in B33-TPS tubers were not significantly altered as compared with the wild type at this time point. It has previously been hypothesized that a decrease in ABA during tuber storage is primarily regulated through ABA catabolism (Destefano-Beltran et al., 2006). Among the genes differentially expressed in B33-TPP tubers as compared with the wild type was a putative ABA-8'-hydroxylase, the key enzyme of ABA catabolism (8-fold induction; Supplemental Table S1). To further investigate ABA-8'-hydroxylase expression in more detail, qRT-PCR analysis was performed on RNA taken from B33-TPP and B33-TPS tubers directly after harvest. As shown in Figure 10B, a 30-fold induction of ABA-8'-hydroxylase

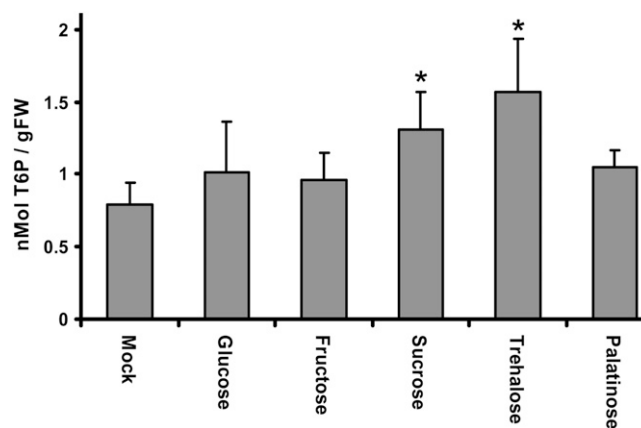


Figure 6. Exogenous application of Suc to excised tuber discs increases T6P levels. Freshly cut discs from tubers stored for 1 week at room temperatures were fed with 100 mM of the indicated sugars. After 4 h, the T6P content was determined using HPLC-MS/MS. Values represent means $\pm$ SD of six independent tubers. * Significant difference ($P < 0.05$) analyzed by *t* test (I). FW, Fresh weight.

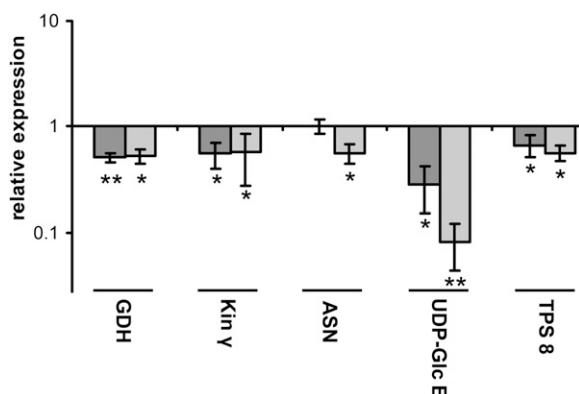


Figure 7. Exogenous application of Suc or trehalose represses SnRK1 marker gene expression in potato tubers. Freshly cut discs from tubers stored for 1 week at room temperature were fed with 100 mM Suc (dark gray bars) or trehalose (light gray bars). After 4 h, total RNA was extracted and converted into cDNA. Relative transcript levels of SnRK1 marker genes were determined by qRT-PCR. Data are expressed as $\log_{10}$ of relative expression of buffer-treated controls and shown as means $\pm$ SD of four independent samples. Statistical differences as determined by Student's *t* test (II) are indicated (* $P < 0.05$, ** $P < 0.001$). ASN, Asn synthase; GDH, Gln dehydrogenase.

transcript levels was observed in B33-TPP tubers, while the expression of this gene was significantly decreased (approximately 5-fold) in B33-TPS tubers as compared with the control (Fig. 10B). Taken together, these data might indicate that decreased T6P content in B33-TPP tubers causes precocious sprouting by lowering ABA content through the induction of ABA catabolism.

DISCUSSION

T6P, the intermediate of trehalose biosynthesis, has emerged as an essential signaling molecule in plants (for review, see Paul, 2007; Paul et al., 2008). Transgenic or mutant plants with altered T6P content

display various phenotypes suggestive for the involvement of T6P signaling in a range of processes related to metabolic and developmental regulation (Goddijn and Smeekens, 1998; Eastmond et al., 2002; Schluepmann et al., 2003, 2004; Avonce et al., 2004; Pellny et al., 2004; van Dijken et al., 2004; Gomez et al., 2010). However, our understanding of the underlying signaling mechanisms is still in its infancy.

Here, we present a detailed molecular and physiological analysis of transgenic potato tubers expressing *E. coli* enzymes to either reduce (B33-TPP) or elevate (B33-TPS) the T6P content in a tuber-specific manner. Our results provide evidence that T6P acts as an integrator of nutritional status and growth in potato tubers.

Manipulation of the T6P Content Affects Carbon Utilization and Energy Metabolism

Diminished T6P levels in B33-TPP tubers as well as elevated levels in B33-TPS eventually led to a decrease in tuber yield in all transgenic lines, although the underlying cause is likely to be different in both cases. Higher soluble sugar levels in B33-TPP tubers are consistent with a model in which T6P is required to efficiently utilize carbohydrates for growth, as was previously shown for *Arabidopsis* plants overexpressing OtsB (Schluepmann et al., 2003). Similar to what was observed in B33-TPP tubers, these transgenic *Arabidopsis* plants accumulated hexose phosphates. The increase in soluble sugars in B33-TPP tubers was not accompanied by obvious changes in carbon partitioning between central metabolic pathways, further supporting the notion that increased sugar levels reflect reduced carbon consumption for tuber growth rather than gross metabolic changes through T6P directly affecting the regulation of metabolic pathways. A range of studies imply that Suc breakdown and starch synthesis are limited by levels of adenine nucleotides, as elevated ATP levels lead to increases in starch synthesis

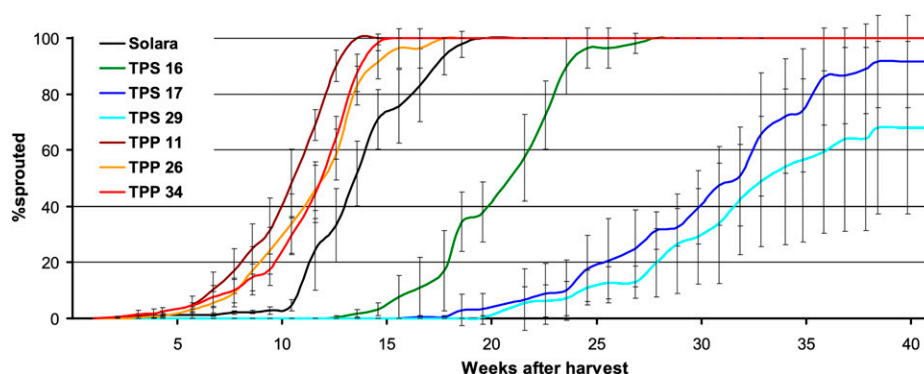
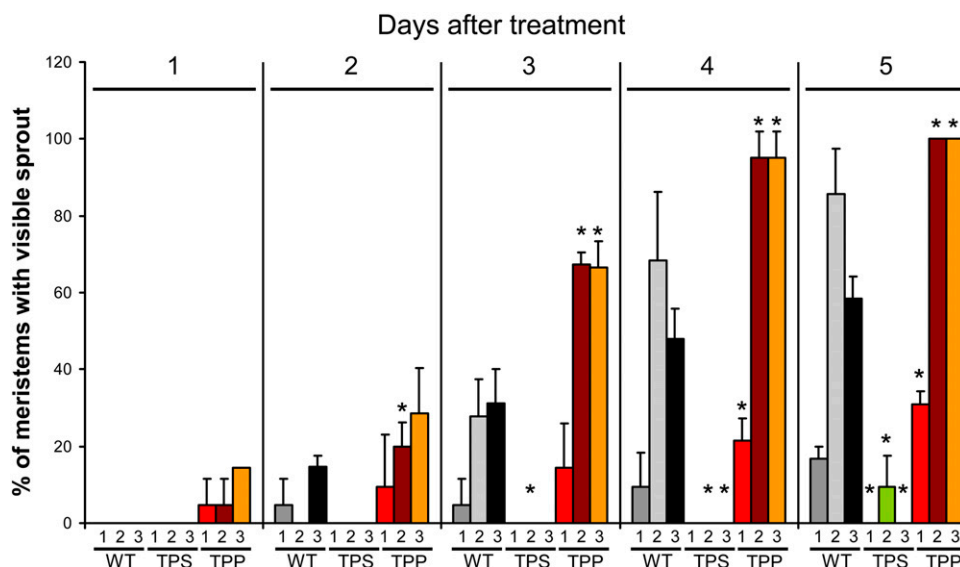


Figure 8. Sprouting of transgenic potato tubers was accelerated in B33-TPP tubers and delayed in B33-TPS tubers relative to the wild type. After harvest, tubers were stored at room temperature. Tubers were periodically scored for visible sprout growth over a period of 40 weeks. The values show mean sprouting rates of four to 12 tubers of five individual plants each $\pm$ SD. Individual comparison of observed time points shows significant differences (II) compared with wild-type tubers in sprouting ($n = 5$; $P < 0.001$) for B33-TPS and B33-TPP tubers.

Figure 9. Modulation of T6P content in transgenic tubers affects hormone responsiveness in an in vitro sprout-release assay. Tuber discs from the different genotypes containing a single bud complex were treated with water (bars 1), 50 μM 6-benzylaminopurine (bars 2), or 50 μM GA₃ (bars 3) and scored daily for visible sprouting. The bars show means of three experiments containing seven to 12 excised buds from at least five individual tubers each $\pm$ SD. * Significant difference ($P < 0.05$) according to Student's *t* test (II). WT, Wild type.



(Loef et al., 2001; Regierer et al., 2002; Oliver et al., 2008). ATP levels are drastically increased in OtsB-overexpressing lines, indicating that ATP availability is not the primary cause for the decrease in carbon utilization observed in these tubers. It has recently been shown that import into the amyloplast of Glc-6-P and ATP via the Glc-6-P/phosphate translocator and the adenylate translocator, respectively, limits starch synthesis (Zhang et al., 2008). Thus, down-regulation of these two transport activities could also be the cause for the accumulation of Glc-6-P and ATP in B33-TPP tubers. However, the available transcript data provide no indication for a transcriptional regulation of these two important transporters in TPP-overexpressing tubers. Reduced T6P content seems to uncouple ATP availability from sugar-utilizing processes.

Uptake and utilization of exogenously applied ^{14}C -labeled Glc was not different in both types of transgenics as compared with the control. This was also true for the distribution of label between the soluble and insoluble fractions. In potato tubers, the latter is mainly represented by starch. It has previously been shown that trehalose redox activates AGPase, the enzyme that catalyzes the committed step of starch synthesis, when fed to potato tuber discs (Kolbe et al., 2005). Although redox regulation of AGPase was not assessed in B33-TPS or B33-TPP tubers, our data indicate that a constitutive alteration of T6P levels in vivo does not change carbon flux into starch in transgenic tubers. Starch levels were even reduced in tubers with elevated T6P content, most likely owing to increased respiration rates that were observed in B33-TPS tubers. The latter is an indicator of high metabolic activity and was likely a compensatory response to the decreased ATP content and the concomitantly decreased energy charge in these tubers. Arabidopsis seedlings expressing OtsA can utilize exogenously supplied sugars more efficiently for growth than untransformed control plants (Schluepmann et al., 2003). However, T6P accumulation

without external supply of metabolizable carbon inhibits the growth of Arabidopsis seedlings (Schluepmann et al., 2004). This resembles the situation found in B33-TPS tubers, where an increase in T6P leads to reduced tuber yield and lower Glc, Suc, and starch contents. A possible explanation for these changes is that elevated T6P levels anticipate the increased demand for ATP to fuel energy-consuming processes and growth, which then leads to a shift of carbon allocation into respiration. This results in an imbalance between sugar supply from source tissues on the one hand and carbon use for energy production in tubers on the other hand, eventually resulting in carbon limitation despite T6P signaling high Suc availability. Although carbon flux in B33-TPS tubers is redirected toward respiration, ATP levels remain low. The reason for that is unclear: either T6P induces an ATP-consuming process that continuously uses newly synthesized ATP, or increased respiration rates do not result in increased ATP synthesis rates because electrons are transferred to oxygen via alternative oxidase, thus bypassing energy conservation (Rasmusson et al., 2009).

Transcriptional Changes in B33-TPP Tubers Are Indicative for T6P-Mediated SnRK1 Signaling

In a recent seminal study, Paul and coworkers (2008) provided strong evidence that T6P, at least partially, exerts its signaling function through the inhibition of SnRK1, a member of the family of calcium-independent Ser/Thr protein kinases that includes AMPK of mammals and SNF1 of yeast (Zhang et al., 2009). These highly conserved protein kinases fulfill fundamental roles in maintaining energy homeostasis, as they safeguard cellular energy level by regulating ATP production and consumption and thereby growth (Baena-González et al., 2007; Hardie, 2007; Halford and Hey, 2009).

SnRK1 in Arabidopsis seedlings is inhibited by micromolar concentrations of T6P in vitro. Inhibition was

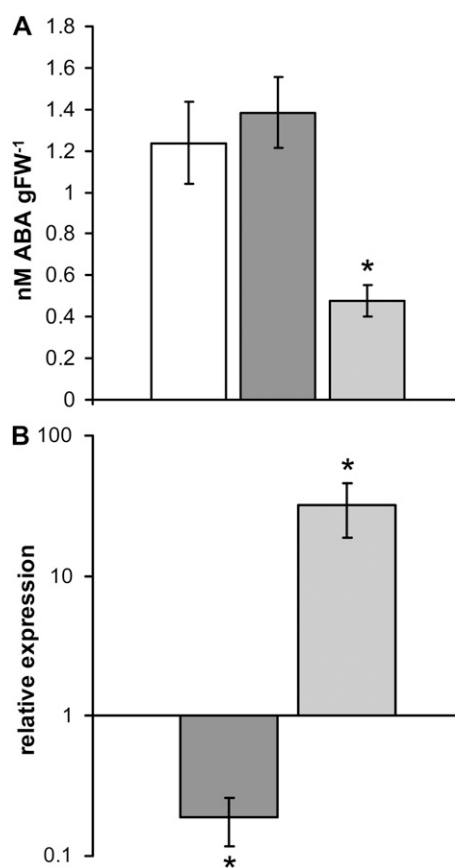


Figure 10. Analysis of ABA levels and ABA-8'-hydroxylase expression in B33-TPP (light gray bars) and B33-TPS (dark gray bars) tubers. A, Endogenous levels of free ABA in freshly harvested tubers from B33-TPP line 34 and B33-TPS line 29 compared with the wild type (white bar). Values represent means of five individual tubers $\pm$ SD. * Significant difference ($P < 0.05$). FW, Fresh weight. B, Relative transcript abundance of ABA-8'-hydroxylase (*StCYP707A2*) in tubers from B33-TPP line 34 (light gray bar) and B33-TPS line 29 (dark gray bar) as analyzed by qRT-PCR. Values represent means of four individual tubers $\pm$ SD. * Significant difference ($P < 0.05$) according to Student's *t* test (II).

dependent on an as yet unidentified auxiliary factor that is not present in mature leaf tissue (Zhang et al., 2009). This indicates that tissue-specific differences exist in the regulation of SnRK1 by T6P. In our experiments, maximal extractable SnRK1 activity (without the addition of T6P to the assay) was in the range that was previously reported for potato tuber (Man et al., 1997). Inhibition of SnRK1 activity by T6P in extracts from potato tubers was slightly higher in growing tubers than in mature tubers. To judge the *in vivo* relevance of this inhibition, the T6P content was estimated in potato tubers assuming that T6P is restricted to the cytosol. The cytosol has been shown to account for approximately 12% of the total cell volume of potato parenchyma tissue (Farré et al., 2001). Thus, T6P concentration was estimated to be 5 μ M or less in B33-TPP tubers, 12 to 15 μ M in wild-type tubers, and between 100 and 380 μ M in B33-TPS tubers. Based on these calculations and the data on *in vitro* T6P inhibition of SnRK1 activity, the modulation of

T6P content in the transgenic tubers most likely has strong repercussions on the *in vivo* SnRK1 activity (Supplemental Fig. S2B).

SnRK1 has been shown to be required for SuSy gene expression in potato tubers (Purcell et al., 1998), and overexpression of SnRK1 in tubers increases the expression of SuSy (McKibbin et al., 2006). Decreased T6P content in B33-TPP tubers should lead to an increase in SnRK1 activity that subsequently induces SuSy expression. Several SuSy isoforms exist in plants that differ in their spatial and temporal expression patterns and likely serve isoform-specific functions (Bieniawska et al., 2007). Our expression analysis indicates that not all SuSy isoforms are equally responsive to SnRK1 signaling, as only SuSy2 was significantly up-regulated in our probe set. A specific function for this SuSy isoform in potato tubers has as yet not been defined, and it appears that induction of its expression does not translate into maximal extractable SuSy activity, which was unchanged in B33-TPP tubers (data not shown). Alteration of SnRK1 activity in Arabidopsis protoplasts triggers extensive transcriptional reprogramming affecting over 1,000 genes (Baena-González et al., 2007), and Zhang et al. (2009) showed that genes induced by SnRK1 are repressed in plants with elevated T6P content and those usually repressed by SnRK1 are induced in plants with decreased T6P content. SnRK1 activates genes involved in nutrient remobilization and represses those involved in biosynthetic processes and storage, consistent with a global metabolic switch induced by SnRK1 to provide alternative sources of energy through amino acid catabolism, Suc, polysaccharide, and cell wall hydrolysis (Baena-González et al., 2007; Baena-Gonzalez and Sheen, 2008; Zhang et al., 2009). The large overlap in transcriptional changes detected in B33-TPP tubers with those found in SnRK1-overexpressing Arabidopsis protoplasts (Baena-González et al., 2007) is consistent with an activation of SnRK1 signaling in T6P-depleted transgenic potato tubers. Intriguingly, in contrast with the results of Zhang et al. (2009), in which a more considerable change at the transcriptomic level was observed in OtsA-expressing Arabidopsis seedlings, in our studies heterotrophic potato tubers displayed an opposite effect (i.e. far greater transcriptomic changes following expression of OtsB than OtsA). A possible explanation for this discrepancy might be differences in the prevailing carbohydrate status between these tissues. Arabidopsis seedlings have relatively low carbon and tend toward starvation mode; hence, the impact of OtsA is greater. However, in potato tubers, there is more carbohydrate and they are more in the feast mode, where OtsB would have greater impact. This possibility notwithstanding, as in Arabidopsis (Zhang et al., 2009), changes in SnRK1 marker gene expression were in the opposite direction in TPS- and TPP-expressing potato transgenics. This suggests that increased expression of SnRK1 and one of its regulatory subunits, KIN γ , in B33-TPP tubers is most likely not the cause of

the activation of SnRK1 signaling; rather, it suggests that T6P regulates SnRK1 target genes in TPP- and TPS-expressing potato lines.

Arabidopsis plants with reduced T6P levels, either through expression of *E. coli* TPP (Schluepmann et al., 2003) or through a mutation in *TPS1* (Gomez et al., 2010), display severely reduced vegetative growth, although carbohydrate levels are high. At least two scenarios could explain the relationship between T6P, carbohydrate levels, and growth. First, T6P could have direct effects on carbon metabolism, restricting the ability to utilize sugars and thus limit energy supply for growth. Alternatively, as has been suggested by Gomez et al. (2010), T6P could decrease growth, which subsequently leads to elevated carbohydrates due to a decreased demand. Data obtained from the analysis of B33-TPP are in support of the latter hypothesis. Carbon flux through central metabolic pathways was not grossly altered in transgenic tubers, which might suggest that the effects of T6P on carbon metabolism are primarily indirect. In mammals, AMPK is known to negatively regulate cell growth and proliferation in response to energy limitation (Hardie, 2007). Activation of AMPK inhibits the growth of cancer cells by inducing the expression of the cyclin-dependent kinase inhibitors p21 and p27 (Rattan et al., 2005). A homolog of p27 was found to be up-regulated in B33-TPP tubers, indicating that a similar mechanism arresting the cell cycle in response to energy deprivation could operate in plants via T6P/SnRK1 signaling. Another mechanism by which AMPK causes cell cycle arrest in mammalian cells is through the negative regulation of TOR (for target of rapamycin), a central protein kinase that promotes cell growth and proliferation. A similar role for TOR in regulating growth-related processes seems to be conserved in plants (Menand et al., 2002); however, little is known about the nutrient regulation of plant TOR and its interplay with other metabolic signaling pathways. An important component of TOR is the TCTP, which acts as the guanine nucleotide exchange factor of the Ras GTPase Rheb that controls TOR activity (Wulfschleger et al., 2006). Recent work in Arabidopsis indicates that TCTP silencing via RNA interference induces slower vegetative development and inhibits leaf expansion due to reduced cell size, establishing TCTP as an important regulator of growth in plants and implying a function of plant TCTP as a mediator of TOR activity similar to that known in nonplant systems (Berkowitz et al., 2008). The down-regulation of TCTP in B33-TPP tubers suggests an antagonistic relationship between TOR and T6P/SnRK1 signaling in these organs. We cannot exclude that T6P in the transgenic tubers affects other processes than SnRK1 signaling or that individual transcriptional changes reflect secondary effects not directly linked to T6P/SnRK1 signaling. However, our data suggest that the primary effect of T6P-mediated SnRK1 signaling in growing potato tubers is decreasing growth, which subsequently affects carbohydrate levels through reduced sink

strength for these metabolites in tubers with decreased T6P content. Because cell growth and proliferation are highly energy-intensive processes, it is not surprising that SnRK1 activation should inhibit them while sufficient carbon supply should promote growth. In Arabidopsis, T6P levels have been shown to reflect Suc content (Lunn et al., 2006) and increased T6P promotes seedling growth on Suc (Schluepmann et al., 2003). The data presented here show that T6P levels in excised tuber discs also respond to exogenous Suc, while feeding hexoses does not affect T6P content. Subsequent qRT-PCR analysis of SnRK1 marker gene expression in Suc-fed tuber discs was diagnostic for an inhibition of SnRK1 signaling. These data fit into a model in which T6P content reflects the Suc supply and suggest that inhibition of SnRK1 by T6P enables the plant to activate growth-promoting processes.

T6P Alters Hormone Responsiveness during Tuber Sprouting

Mutants or transgenic plants with altered trehalose metabolism display effects on a range of developmental transitions, such as embryo maturation or induction of flowering in Arabidopsis (Eastmond et al., 2002; van Dijken et al., 2004; Gomez et al., 2006) and inflorescence branching in maize (*Zea mays*; Satoh-Nagasawa et al., 2006). After formation, potato tubers undergo a period of dormancy that is characterized by the absence of visible bud growth. The length of tuber dormancy and the initiation of sprouting depend on the genetic background and are affected by preharvest and postharvest conditions (Sonnewald, 2001; Suttle, 2004). With the onset of sprouting, the tuber turns into a source organ supporting sprout growth through the provision of carbohydrates. This is associated with metabolic alterations as well as with major changes in gene expression (Campbell et al., 2008; Hartmann et al., 2011). In addition, endogenous plant hormones are supposed to play a critical role in the regulation of tuber dormancy and bud break (Suttle, 2004).

The results presented in this study demonstrate that T6P content affects the length of dormancy in transgenic tubers with altered trehalose metabolism. Reduction of T6P content accelerates dormancy release and sprouting, while an increase in T6P prolongs dormancy and inhibits sprouting in a dose-dependent manner. Directly opposite to what was observed in growing B33-TPP and B33-TPS tubers, it appears that during sprouting lower T6P levels induce growth-related processes while increased T6P inhibits bud break. The question arises whether this is due to a direct effect of T6P signaling or whether it arises as a pleiotropic effect owing to physiological differences of the transgenic tubers as compared with the wild type. The dose dependency of the inhibitory effect of T6P on dormancy release might argue for a more direct effect. A considerable proportion of the transcriptional changes observed in growing B33-TPP tubers appears to reflect an activation of SnRK1 signaling, suggesting

that this central regulatory protein kinase could also be involved in mediating T6P effects on tuber dormancy. In support of this proposal, transgenic potato tubers with antisense SnRK1 expression did not sprout over a period of 2 years when stored at 5°C, strongly suggesting a role of SnRK1 in the control of dormancy (Halford et al., 2003). This strongly reflects the situation found in B33-TPS transgenic tubers, where elevated T6P levels are proposed to inhibit SnRK1 signaling similar to decreasing SnRK1 function by antisense repression. It has been hypothesized that antisense repression of SnRK1 activity impairs the mobilization of starch to support sprout growth (Halford et al., 2003). Although we have no direct data on starch mobilization rates in B33-TPS tubers, this explanation appears unlikely because tubers with higher T6P content had higher respiratory activities (a gross indicator of metabolic activity) and reduced starch levels, suggesting that starch mobilization is not impaired but rather accelerated in these tubers.

T6P signaling has previously been shown to be linked to ABA signaling (Avonce et al., 2004; Ramon et al., 2007; Gomez et al., 2010). The sustained synthesis and action of endogenous ABA is required for both the initiation and maintenance of tuber dormancy, suggestive for an involvement of ABA signaling in these processes (Suttle and Hultstrand, 1994). Inhibition of ABA synthesis in an in vitro microtuber system using the phytoene desaturase inhibitor fluridone resulted in premature sprouting after only 3 to 6 weeks of in vitro culture, which could be suppressed by the inclusion of ABA in the growth medium (Suttle and Hultstrand, 1994). Generally, tuber ABA content declines during storage, although a comparison of six cultivars with varying lengths of dormancy revealed no correlation between final ABA content and sprouting behavior (Biemelt et al., 2000). Thus, it appears that ABA is of particular importance for the induction and maintenance of dormancy during early phases, while later other growth regulators become more important (Suttle, 2007). Recent evidence suggests that ABA tissue levels during dormancy are regulated by a dynamic equilibrium of ABA biosynthesis and degradation, which progressively favors degradation as dormancy proceeds (Destefano-Beltran et al., 2006). Expression analyses of genes encoding proteins catalyzing the final postzeaxanthin steps of ABA biosynthesis and the catabolic enzyme ABA-8'-hydroxylase have demonstrated a highly dynamic pattern of gene expression during dormancy and have identified these enzymatic steps as key regulators of ABA turnover (Destefano-Beltran et al., 2006). In particular, ABA catabolism is temporally correlated with the expression of two of the four putative ABA-8'-hydroxylases present in potato. The expression of one of these, *StCYP707A2*, rose steadily throughout storage, reaching a final level 10-fold greater than the initial value (Destefano-Beltran et al., 2006). The same gene was found to be strongly up-regulated in B33-TPS tubers, coinciding with a marked reduction of ABA content in

bulk tissue. Although ABA-8'-hydroxylases were not identified as potential SnRK1 target genes in *Arabidopsis* (Baena-González et al., 2007), it appears possible that the expression of *StCYP707A2* can be induced by SnRK1 activity in potato tubers, which then leads to lower ABA content and a release of dormancy. Expression of *StCYP707A2* was significantly down-regulated in B33-TPS tubers, which would be expected for a bona-fide SnRK1 target gene. Interestingly, seeds of *Arabidopsis* T-DNA insertion mutants in *AtCYP707A2* have higher ABA content and prolonged dormancy (Kushiro et al., 2004). An interaction between SnRK1 and ABA signaling has previously been suggested to occur in *Arabidopsis*, pea, and tomato (*Solanum lycopersicum*; Bradford et al., 2003; Radchuk et al., 2006, 2010; Jossier et al., 2009).

Bulk tissue ABA levels in B33-TPS tubers were not significantly increased at the time of harvest; however, previous studies have shown that tuber ABA content was highest in meristems and lower in surrounding tissues (Destefano-Beltran et al., 2006). Thus, it remains possible that tissue-specific differences in ABA content and dynamics might exist in B33-TPS lines, which contribute to the prolongation of dormancy in tubers with elevated T6P content.

In an in vitro sprout-release assay, buds excised from B33-TPS tubers were shown to be hypersensitive toward the sprout growth-promoting hormones CK and GA, while treatment of buds excised from B33-TPS tubers did not result in the promotion of sprout growth. CK has been implicated in the termination of tuber dormancy, while GA is not sufficient to break dormancy in the absence of CK but rather has a role in subsequent sprout growth (Turnbull and Hanke, 1985; Hartmann et al., 2011). Immediately after harvest and during the initial period of storage, exogenous CKs have no effect on dormancy. As storage was extended, tubers developed a time-dependent increase in CK sensitivity (Turnbull and Hanke, 1985; Hartmann et al., 2011). Obviously, increased T6P levels and a concomitant inhibition of SnRK1 activity counteract the development of CK sensitivity in B33-TPS tubers. Whether this is related to sustained ABA levels in these tubers will be the subject of future investigations.

In summary, we provide evidence that T6P mediates growth effects through SnRK1 signaling as a central and pivotal mechanism linking growth, development, and metabolism in potato tubers. Future work will have to address the question of to what extent and under which circumstances endogenous T6P levels are modulated in potato tubers in order to reveal the exact nature of the interrelationship between Suc, T6P, SnRK1, and the regulation of growth and metabolism. A recent study on developing wheat (*Triticum aestivum*) grain revealed enormous differences in T6P content during grain development (Martínez-Barajas et al., 2011). T6P levels changed 178-fold 1 to 45 d after anthesis, correlating with Suc content, with a strong decline after onset of the grain-filling phase toward the desiccation stage. In addition, the expression of SnRK1 marker genes corre-

lated with T6P content over the developmental time course. There were also strong differences in T6P content when filial and maternal tissue was compared overall, suggesting a marked effect of tissue type and developmental stage on T6P content in wheat grain. It will be interesting to determine whether potato tubers display similar temporal and spatial resolution of T6P effects on cellular processes.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Potato plants (*Solanum tuberosum* 'Solara') were propagated in tissue culture under a 16-h-light/8-h-dark period on Murashige and Skoog medium (Sigma) containing 2% (w/v) Suc. The selected lines used for the analyses were clonally propagated from stem cuttings and subsequently cultivated in the greenhouse in individual 4-L pots at 50% humidity with 16 h of supplemental light ($150 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) and 8 h of darkness. The temperature regime followed the light/dark cycle with 21°C and 18°C.

Plasmid Construction and Generation of Transgenic Plants

The transgene DNA from the OtsBA operon from the *Escherichia coli* K12 strain DH5 α (accession no. AY587539) was PCR amplified from bacterial genomic DNA with the primers otsA5' containing a 5' *KpnI* restriction site and otsA3' containing a 3' *SalI* restriction site for OtsA and primers SD12 and SD13 for both containing *SalI* restriction sites for OtsB, respectively (primer sequences are given in Supplemental Table S3). The resulting fragments (1,424 bp for OtsA and 818 bp for OtsB) were sequence verified and cloned into a pBin-derived vector containing the tuber-specific B33 promoter (Rocha-Sosa et al., 1989); in the case of OtsB, the direction of insertion was analyzed by *EcoRV* digest. The binary constructs were transformed into *Agrobacterium tumefaciens* strain C58C1 carrying the virulence plasmid pGV2260 (Deblaere et al., 1985) and selected on kanamycin, ampicillin, and rifampicin. Transformation of potato plants was performed as described (Rocha-Sosa et al., 1989). The regenerated plants were screened for tuber-specific expression by RT-PCR analysis using the same pairs of oligonucleotides. Three lines each were selected for further experiments (TPS 16, 17, and 29 and TPP 11, 26, and 34).

Sampling of Tuber Material for Metabolite Measurements

Tubers of about 20 g fresh weight, if available, were used for all analyses. Samples were taken from the apical part of the tuber at defined regions of the storage parenchyma inside the vascular ring (perimedulla) but avoiding the inner part of the central pith.

Sugar Incubation Experiments

Tuber discs of 8 mm diameter were cut from potato tubers 1 week after harvest and incubated for 4 h in 5 mL of 10 mM MES-KOH buffer, pH 6.5, containing 100 mM Glc, Fru, Suc, trehalose, and palatinose with moderate shaking at 100 rpm at 21°C in Erlenmeyer flasks. Tuber discs were subsequently washed two times in incubation buffer and used for further analysis of T6P content via methanol/chloroform extraction and HPLC-MS/MS measurement (six replicates each) and for RNA preparation and subsequent qPCR analysis (four replicates each).

Measurement of SnRK1 Activity

Tuber total soluble protein was extracted in prechilled Tricine-NaOH buffer, pH 8 (100 mM), containing 500 μM EDTA and EGTA, 25 mM NaF, 5 mM dithiothreitol, 2 mM tetrasodium pyrophosphate, and 1 mM benzamidine using mortar and pestle. Protease inhibitor "complete ETDA-free" (Roche) and phosphatase inhibitor "PhosSTOP" (Roche) were freshly added prior to extraction. Phenylmethylsulfonyl fluoride was also added to a final concen-

tration of 1 mM. The extract was centrifuged at 17,000g, and the supernatant was desalted using Sephadex G25 spin columns. The eluent was supplemented with okadaic acid to 2.5 μM and divided into aliquots of 20 μg of total protein each in a volume of 10 μL . SnRK1 was assayed using the established procedure (Davies et al., 1989; Dale et al., 1995; Man et al., 1997) with slight modifications in a total volume of 25 μL at 32°C. The assay medium was composed of 40 mM HEPES-NaOH, pH 7.5, 5 mM MgCl_2 , 4 mM ATP containing 15 kBq of [γ - ^{33}P]ATP (GE Healthcare), 200 μM AMARA peptide (Ala-Met-Ala-Arg-Ala-Ala-Ser-Ala-Ala-Ala-Leu-Ala-Arg-Arg-Arg; kindly provided by Jutta Eichler, University of Erlangen-Nurnberg), 5 mM dithiothreitol, 1 μM okadaic acid, 1 μM pepstatin A, 10 μM E64, and 5 μM chymostatin. The assay was started by adding 15 μL of assay medium to 10 μL of protein extract. After 20 min, 15 μL was transferred to 4-cm 2 squares of Whatman P81 phosphocellulose paper, dried, and subsequently washed for 10 min each with four 500-mL volumes of 1% phosphoric acid with moderate shaking at 100 rpm and once with 100 mL of acetone. After washing, the P81 squares were dried and transferred to liquid scintillation vials. Total AMARA kinase activity was calculated using the specific activity of [γ - ^{33}P]ATP. For inhibition studies, the appropriate inhibitor was added to the extracts 5 min before the reaction was started. During this preincubation phase, the samples were kept on ice.

Measurement of T6P

T6P was extracted from 100 mg of plant tissue with 500 μL of chloroform and 800 μL of methanol:water (5:3). The phases were separated by centrifugation at 12,000g. The chloroform phase was reextracted twice with prechilled MilliQ water. The combined aqueous fraction was diluted to 5% methanol with prechilled MilliQ (10 mL) water and freeze dried. The resulting pellet was dissolved in 300 μL of MilliQ water and filtered through a 10-kD filter (AcroPrep 96; PALL Life Science). A 10- μL aliquot of the filtrate was subsequently analyzed on an ICS3000 HPLC system (Dionex) connected to a QTrap 3200 triple-quadrupole mass spectrometer (AB Sciex) as described previously (Horst et al., 2010). T6P was quantified at molecular weights of 421/241 and 421/97 at a retention time of 18.60 ± 1.10 min.

Determination of the Respiration Rate of B33-TPS Potato Tubers

The respiration rates of freshly harvested and 1-week-stored potato tubers were determined with a combined gas exchange/chlorophyll imaging system (GFS 3000; Walz Messtechnik). Tubers selected for this experiment all had comparable size and a fresh weight of about 20 ± 3 g. Five individual tubers per transgenic line from individual plants each were selected. Assimilation rate (negative values) was measured at $400 \mu\text{L L}^{-1} \text{CO}_2$ without illumination and calculated on a fresh weight basis as described by Evans and von Caemmerer (1996).

Measurement of Starch and Soluble Sugars

Soluble sugars and starch levels were determined in tuber and leaf discs extracted with 80% (v/v) ethanol and 20 mM HEPES, pH 7.5, as described (Stitt et al., 1989) but adapted for determination in a plate reader by direct downscaling the assay to a volume of 200 μL .

Determination of Metabolites Using HPLC-MS/MS

Organic acids and phosphorylated intermediates were extracted from 100 mg of potato tuber tissue powder with 1 mL of 1 M ice-cold perchloric acid as described (Horst et al., 2010). A total of 300 μL of the extract was filtered through a 10-kD filter (AcroPrep 96; PALL Life Science). Ten microliters of the filtrates was analyzed on an ICS3000 HPLC system (Dionex) connected to a QTrap 3200 triple-quadrupole mass spectrometer as described (Horst et al., 2010). Spike experiments were performed by adding a standard solution of all 29 analyzed metabolites to potato tuber tissue prior to extraction to analyze matrix effects in tuber tissue.

[U- ^{14}C]Glc Feeding Experiments

Developing potato tubers were rapidly harvested from 10-week-old potato plants; a 10-mm-diameter latitudinal core was taken, and 2-mm discs were cut from this and washed three times in fresh incubation medium (10 mM MES-KOH, pH 6.5) and then incubated (eight discs in 5 mL of incubation medium

containing [^{14}C]Glc [$1.4 \text{ MBq mmol}^{-1}$] to a final concentration of 10 mM. Samples were incubated for 2 h before washing again three times in unlabeled incubation medium and freezing in liquid N_2 until further analysis, as described by Fernie et al. (2001a).

Determination of Adenosine Nucleotides

Nucleotides were detected employing a highly sensitive fluorescence method according to Haink and Deussen (2003) with some modification. Prior to HPLC separation, an aliquot of the samples used for sugar alcohols was derivatized with 10% (v/v) chloroacetaldehyde in 62 mM sodium citrate and 76 mM potassium dihydroxide phosphate, pH 5.2. The mixture was incubated for 40 min at 80°C, cooled immediately on ice, centrifuged at 14,000 rpm for 1 min, and used for HPLC analysis. Separation was carried out with a reverse-phase HPLC system (Alliance 2795; Waters) consisting of a gradient pump, a degassing module, an integrated microtiter plate autosampler, and a fluorescence detector. The gradient was accomplished with a buffer containing 5.7 mM tetrabutylammonium bisulfate, 30.5 mM potassium dihydroxide phosphate, pH 5.8, and an eluent containing pure acetonitrile (Roti C Solv HPLC; Roth). A single run was set to 4.5 min following a reconditioning of 3 min with the eluent. The excitation wavelength was set at 280 nm, and the emission wavelength was 410 nm. In all cases, chromatograms were integrated using the software package Empower.

Fractionation of ^{14}C -Labeled Material

Tissue was fractionated exactly as described by Fernie et al. (2001b), with the exception that sugars were fractionated enzymatically rather than utilizing thin-layer chromatography, as defined by Carrari et al. (2006). Labeled Suc levels were determined after a 4-h incubation of 200 μL of total neutral fraction with 4 units mL^{-1} hexokinase in 50 mM Tris-HCl, pH 8.0, containing 13.3 mM MgCl_2 and 3.0 mM ATP at 25°C. For labeled Glc and Fru levels, 200 μL of neutral fraction were incubated with 1 unit mL^{-1} Glc oxidase and 32 units mL^{-1} peroxidase in 0.1 M potassium phosphate buffer, pH 6, for a period of 6 h at 25°C. After the incubation time, all reactions were stopped by heating at 95°C for 5 min. The label was separated by ion-exchange chromatography as described by Fernie et al. (2001b). The reliability of these fractionation techniques has been thoroughly documented previously (Runquist and Kruger, 1999; Fernie et al., 2001a; Carrari et al., 2006).

Microarray Analysis

For transcript profiling, RNA was prepared from freshly harvested potato tuber tissue, immediately frozen in liquid nitrogen at harvest. For each of the three TPP lines, tuber material of four individual tubers was pooled (two tubers each pool), and RNA was isolated as described (Logemann et al., 1987) and purified using RNeasy Mini Spin Columns (Qiagen) following the manufacturer's protocol. RNA quantity and quality were assigned as described (Hartmann et al., 2011). Labeling of samples, microarray analysis, and data set extraction were performed according to the manufacturer's protocols (Agilent) and feature extraction software as described (Hartmann et al., 2011). Statistical analysis of features being differentially expressed more than 2-fold was done by volcano plot ($P < 0.05$) and false-positive exclusion by Benjamini-Hochberg multiple test correction. Annotation and functional assignment were performed as described (Hartmann et al., 2011).

qRT-PCR

For qRT-PCR analysis, RNA was isolated from freshly harvested potato tuber tissue, immediately frozen in liquid nitrogen, and stored at -80°C as described (Logemann et al., 1987). RNA was isolated from three or four individual tubers of the strongest overexpressing lines of TPS and TPP for analysis of transgenic potato tubers. For expression analysis in the feeding experiment, RNA was isolated from fed discs from four individual tubers from two independent feeding experiments. The corresponding primers for the amplification of target gene fragments between 75 and 150 bp were designed using Primer3plus software (Untergasser et al., 2007). cDNA was synthesized and qRT-PCR was performed as described previously (Arsova et al., 2010) on the Mx3000P Q-PCR system (Stratagene) in combination with the Brilliant II SYBR Green Q-PCR Master Mix Kit (Stratagene). Oligonucleotides used for the determination of relative mRNA abundance of candidate genes are summarized in Supplemental Table S3.

Sprout-Release Assay

The sprout-release assay was performed on excised potato tuber discs of 5 mm height and 8 mm diameter according to Hartmann et al. (2011). The concentrations of 6-benzylaminopurine and GA_3 used to induce sprouting were 50 μM each.

Measurement of ABA

ABA was extracted with modifications according to Pan et al. (2008). A total of 200 mg of frozen potato tuber tissue was extracted by homogenization with 1 mL of acidic 1-propanol:water:concentrated HCl (2:1:0.002, v/v/v) and agitated for 30 min at 4°C in 15-mL polypropylene tubes. With 2 mL of chloroform instead of dichloromethane and further agitation for 30 min, samples were centrifuged for 10 min at 3,000g. The lower phase was dried in a Speedvac concentrator and reconditioned with 80 μL of methanol. For chromatography, 10 μL was injected to a Luna C18 RP column ($250 \times 4.6 \text{ mm}$; Phenomenex) with precolumn installed in an ICS3000 HPLC system (Dionex). The flow rate was 0.3 mL min^{-1} at 30°C with a binary gradient consisting of buffer A (water/0.75% acidic acid, pH 2.55) and buffer B (acetonitrile/0.75% acidic acid) as follows: 0 to 5 min, 20% B; 5 to 26 min, 46% B; 27 min, 90% B; 27 to 32 min, 90% B; 34 min, 20% B; 34 to 45 min, 20% B. The ABA content was measured with a QTrap 3200 mass spectrometer (ABI; Sciex) with electrospray ionization-MS/MS and negative ionization in multiple reaction monitoring mode at $-4,500 \text{ V}$. Ion source temperature was 600°C. The recorded Q1/Q3 mass transition for ABA was 263/153 D. Dwell time was 75 ms, while potentials for DP and EP were -25 V , for CEP and CE were -4 V , and for CXP was -22 V . Peak areas were quantified by comparison with a standard curve between 0.1 and 500 nM derived from pure ABA purchased from Sigma. Recovery of 3 pmol of spiked pure ABA into 100 mg of tissue was $98\% \pm 15.5\%$.

Statistical Analysis

Statistical analysis for all experiments generating metabolome data was performed using the VANTED Java application (Klukas et al., 2006) using the incremented Welch-Satterthwaite t test assuming a normal distribution of independent samples and unknown SD, referred to in figure legends and tables as (I). Levels of significance were $P < 0.05$ and $P < 0.001$. Statistical analysis for transcript data, enzymatic activity, and sprouting surveys was performed using the algorithm embedded into Microsoft Excel, referred to in figure legends and tables as (II), using the above assumptions and definition of significance.

The transcriptome data discussed here have been deposited at ArrayExpress (E-MEXP-3215).

Supplemental Data

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

Supplemental Figure S1. Respiration rates of intact B33-TPS transgenic tubers compared with the wild type.

Supplemental Figure S2. SnRK1 activity in B33-TPS and B33-TPP transgenic potato tubers.

Supplemental Figure S3. Visible sprout growth in an in vitro sprout-release assay.

Supplemental Table S1. Genes more than 2-fold affected in B33-TPP transgenic lines compared with the wild type.

Supplemental Table S2. Overlap between SnRK1 marker genes (Baena-González et al., 2007) in Arabidopsis and genes more than 2-fold affected in B33-TPP transgenic lines.

Supplemental Table S3. Oligonucleotides used in this study.

ACKNOWLEDGMENTS

We thank Alfred Schmiel for support during metabolite analysis. We further thank Ingrid Schießl and Madlen Nietzsche for their support during plant and tuber harvesting.

Received May 12, 2011; accepted June 10, 2011; published June 13, 2011.

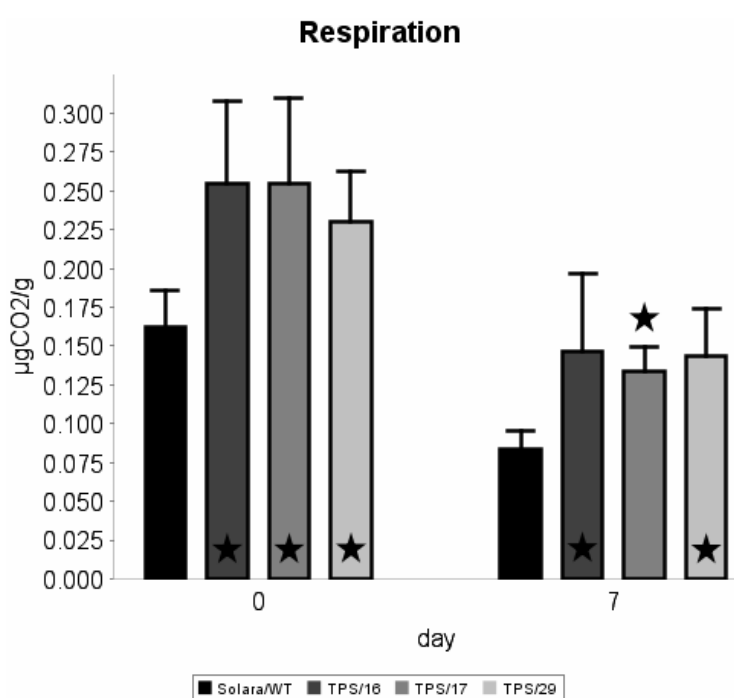
LITERATURE CITED

- Adams RP, Kendall E, Kartha KK (1990) Comparison of free sugars in growing desiccated plants of *Selaginella lepidophylla*. *Biochem Syst Ecol* 18: 107–110
- Arsova B, Hoja U, Wimmelbacher M, Greiner E, Üstün S, Melzer M, Petersen K, Lein W, Börnke F (2010) Plastidial thioredoxin z interacts with two fructokinase-like proteins in a thiol-dependent manner: evidence for an essential role in chloroplast development in *Arabidopsis* and *Nicotiana benthamiana*. *Plant Cell* 22: 1498–1515
- Avonce N, Leyman B, Mascorro-Gallardo JO, Van Dijk P, Thevelein JM, Iturriaga G (2004) The *Arabidopsis* trehalose-6-P synthase AtTPS1 gene is a regulator of glucose, abscisic acid, and stress signaling. *Plant Physiol* 136: 3649–3659
- Baena-González E, Rolland F, Thevelein JM, Sheen J (2007) A central integrator of transcription networks in plant stress and energy signaling. *Nature* 448: 938–942
- Baena-Gonzalez E, Sheen J (2008) Convergent energy and stress signaling. *Trends Plant Sci* 13: 474–482
- Berkowitz O, Jost R, Pollmann S, Masle J (2008) Characterization of TCTP, the Translationally Controlled Tumor Protein, from *Arabidopsis thaliana*. *Plant Cell* 20: 3430–3447
- Biemelt S, Hajirezaei M, Hentschel E, Sonnewald U (2000) Comparative analysis of abscisic acid content and starch degradation during storage of tubers harvested from different potato varieties. *Potato Res* 43: 371–382
- Bieniawska Z, Barratt DHP, Garlick AP, Thole V, Kruger NJ, Martin C, Zrenner R, Smith AM (2007) Analysis of the sucrose synthase gene family in *Arabidopsis*. *Plant J* 49: 810–828
- Bradford KJ, Downie AB, Gee OH, Alvarado V, Yang H, Dahal P (2003) Abscisic acid and gibberellin differentially regulate expression of genes of the SNF1-related kinase complex in tomato seeds. *Plant Physiol* 132: 1560–1576
- Brioudes F, Thierry A-M, Chambrier P, Mollereau B, Bendahmane M (2010) Translationally controlled tumor protein is a conserved mitotic growth integrator in animals and plants. *Proc Natl Acad Sci USA* 107: 16384–16389
- Cabib E, Leclerc LF (1958) The biosynthesis of trehalose phosphate. *J Biol Chem* 231: 259–275
- Campbell M, Segear E, Beers L, Knauber D, Suttle J (2008) Dormancy in potato tuber meristems: chemically induced cessation in dormancy matches the natural process based on transcript profiles. *Funct Integr Genomics* 8: 317–328
- Carrari F, Baxter C, Usadel B, Urbanczyk-Wochniak E, Zanor MI, Nunes-Nesi A, Nikiforova V, Centeno D, Ratzka A, Pauly M, et al (2006) Integrated analysis of metabolite and transcript levels reveals the metabolic shifts that underlie tomato fruit development and highlight regulatory aspects of metabolic network behavior. *Plant Physiol* 142: 1380–1396
- Dale S, Wilson WA, Edelman AM, Hardie DG (1995) Similar substrate recognition motifs for mammalian AMP-activated protein-kinase, higher-plant HMG-CoA reductase kinase-a, yeast Snf1, and mammalian calmodulin-dependent protein-kinase-I. *FEBS Lett* 361: 191–195
- Davies SP, Carling D, Hardie DG (1989) Tissue distribution of the AMP-activated protein-kinase, and lack of activation by cyclic-AMP-dependent protein-kinase, studied using a specific and sensitive peptide assay. *Eur J Biochem* 186: 123–128
- Deblaere R, Bytebier B, De Greve H, Deboeck F, Schell J, Van Montagu M, Leemans J (1985) Efficient octopine Ti plasmid-derived vectors for Agrobacterium-mediated gene transfer to plants. *Nucleic Acids Res* 13: 4777–4788
- Destefano-Beltran L, Knauber D, Huckle L, Suttle J (2006) Chemically forced dormancy termination mimics natural dormancy progression in potato tuber meristems by reducing ABA content and modifying expression of genes involved in regulating ABA synthesis and metabolism. *J Exp Bot* 57: 2879–2886
- Eastmond PJ, van Dijken AH, Spielman M, Kerr A, Tissier AE, Dickinson HG, Jones JDG, 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
- Elbein AD, Pan YT, Pastuszak I, Carroll D (2003) New insights on trehalose: a multifunctional molecule. *Glycobiology* 13: 17R–27R
- Evans JR, von Caemmerer S (1996) Carbon dioxide diffusion inside leaves. *Plant Physiol* 110: 339–346
- Farré EM, Tiessen A, Roessner U, Geigenberger P, Trethewey RN, Willmitzer L (2001) Analysis of the compartmentation of glycolytic intermediates, nucleotides, sugars, organic acids, amino acids, and sugar alcohols in potato tubers using a nonaqueous fractionation method. *Plant Physiol* 127: 685–700
- Fernie AR, Roessner U, Trethewey RN, Willmitzer L (2001a) The contribution of plastidial phosphoglucosyltransferase to the control of starch synthesis within the potato tuber. *Planta* 213: 418–426
- Fernie AR, Roscher A, Ratcliffe RG, Kruger NJ (2001b) Fructose 2,6-bisphosphate activates pyrophosphate:fructose-6-phosphate 1-phosphotransferase and increases triose phosphate to hexose phosphate cycling in heterotrophic cells. *Planta* 212: 250–263
- Goddijn O, Smeekens S (1998) Sensing trehalose biosynthesis in plants. *Plant J* 14: 143–146
- Gomez LD, Baud S, Gilday A, Li Y, Graham IA (2006) Delayed embryo development in the *ARABIDOPSIS* TREHALOSE-6-PHOSPHATE SYNTHASE 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. *Plant J* 46: 69–84
- Gomez LD, Gilday A, Feil R, Lunn JE, Graham IA (2010) AtTPS1-mediated trehalose 6-phosphate synthesis is essential for embryogenic and vegetative growth and responsiveness to ABA in germinating seeds and stomatal guard cells. *Plant J* 64: 1–13
- Haink G, Deussen A (2003) Liquid chromatography method for the analysis of adenosine compounds. *J Chromatogr B Analyt Technol Biomed Life Sci* 784: 189–193
- Halford NG, Hey SJ (2009) Snf1-related protein kinases (SnRKs) act within an intricate network that links metabolic and stress signalling in plants. *Biochem J* 419: 247–259
- Halford NG, Hey SJ, Jhureea D, Laurie S, McKibbin RS, Zhang Y, Paul MJ (2003) Dissection and manipulation of metabolic signalling pathways. *Ann Appl Biol* 142: 25–31
- Hardie DG (2007) AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol* 8: 774–785
- Hartmann A, Senning M, Hedden P, Sonnewald U, Sonnewald S (2011) Reactivation of meristem activity and sprout growth in potato tubers require both cytokinin and gibberellin. *Plant Physiol* 155: 776–796
- Horst RJ, Doeblemann G, Wahl R, Hofmann J, Schmiedl A, Kahmann R, Kämper J, Sonnewald U, Voll LM (2010) *Ustilago maydis* infection strongly alters organic nitrogen allocation in maize and stimulates productivity of systemic source leaves. *Plant Physiol* 152: 293–308
- Jossier M, Bouly JP, Meimoun P, Arjmand A, Lessard P, Hawley S, Hardie DG, Thomas M (2009) SnRK1 (SNF1-related kinase 1) has a central role in sugar and ABA signalling in *Arabidopsis thaliana*. *Plant J* 59: 316–328
- Kloosterman B, De Koeijer D, Griffiths R, Flinn B, Steuernagel B, Scholz U, Sonnewald S, Sonnewald U, Bryan GJ, Prat S, et al (2008) Genes driving potato tuber initiation and growth: identification based on transcriptional changes using the POCI array.
- Klukas C, Junker BH, Schreiber F (2006) The VANTED software system for transcriptomics, proteomics and metabolomics analysis. *J Pest Sci* 31: 289–292
- Kolbe A, Tiessen A, Schlupmann H, Paul M, Ulrich S, Geigenberger P (2005) Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase. *Proc Natl Acad Sci USA* 102: 11118–11123
- Kushiro T, Okamoto M, Nakabayashi K, Yamagishi K, Kitamura S, Asami T, Hirai N, Koshiba T, Kamiya Y, Nambara E (2004) The *Arabidopsis* cytochrome P450 CYP707A encodes ABA 8'-hydroxylases: key enzymes in ABA catabolism. *EMBO J* 23: 1647–1656
- Loef I, Stitt M, Geigenberger P (2001) Increased levels of adenine nucleotides modify the interaction between starch synthesis and respiration when adenine is supplied to discs from growing potato tubers. *Planta* 212: 782–791
- Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. *Anal Biochem* 163: 16–20
- Lovas A, Bimbó A, Szabó L, Bánfalvi Z (2003) Antisense repression of StbGAL83 affects root and tuber development in potato. *Plant J* 33: 139–147
- Lunn JE (2007) Gene families and evolution of trehalose metabolism in plants. *Funct Plant Biol* 34: 550–563
- Lunn JE, Feil R, Hendriks JH, Gibon Y, Morcuende R, Osuna D, Scheible WR, Carillo P, Hajirezaei MR, Stitt M (2006) Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADP-glucose pyrophosphorylase and higher rates of starch synthesis in *Arabidopsis thaliana*. *Biochem J* 397: 139–148

- Man AL, Purcell PC, Hannappel U, Halford NG (1997) Potato SNF1-related protein kinase: molecular cloning, expression analysis and peptide kinase activity measurements. *Plant Mol Biol* **34**: 31–43
- Martínez-Barajas E, Delatte T, Schluepmann H, de Jong GJ, Somsen GW, Nunes C, Primavesi LF, Coello P, Mitchell RA, Paul MJ (2011) Wheat grain development is characterized by remarkable trehalose 6-phosphate accumulation pregrain filling: tissue distribution and relationship to SNF1-related protein kinase1 activity. *Plant Physiol* **156**: 373–381
- McKibbin RS, Muttucumaru N, Paul MJ, Powers SJ, Burrell MM, Coates S, Purcell PC, Tiessen A, Geigenberger P, Halford NG (2006) Production of high-starch, low-glucose potatoes through over-expression of the metabolic regulator SnRK1. *Plant Biotechnol J* **4**: 409–418
- Menand B, Desnos T, Nussaume L, Berger F, Bouchez D, Meyer C, Robaglia C (2002) Expression and disruption of the *Arabidopsis* TOR (target of rapamycin) gene. *Proc Natl Acad Sci USA* **99**: 6422–6427
- Oliver SN, Tiessen A, Fernie AR, Geigenberger P (2008) Decreased expression of plastidial adenylate kinase in potato tubers results in an enhanced rate of respiration and a stimulation of starch synthesis that is attributable to post-translational redox-activation of ADP-glucose pyrophosphorylase. *J Exp Bot* **59**: 315–325
- Pan X, Welti R, Wang X (2008) Simultaneous quantification of major phytohormones and related compounds in crude plant extracts by liquid chromatography-electrospray tandem mass spectrometry. *Phytochemistry* **69**: 1773–1781
- Paul M (2007) Trehalose 6-phosphate. *Curr Opin Plant Biol* **10**: 303–309
- Paul MJ, Primavesi LF, Jhurreea D, Zhang Y (2008) Trehalose metabolism and signaling. *Annu Rev Plant Biol* **59**: 417–441
- 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
- Purcell PC, Smith AM, Halford NG (1998) Antisense expression of a sucrose non-fermenting-1-related protein kinase sequence in potato results in decreased expression of sucrose synthase in tubers and loss of sucrose-inducibility of sucrose synthase transcripts in leaves. *Plant J* **14**: 195–202
- Radchuk R, Emery RJ, Weier D, Vigeolas H, Geigenberger P, Lunn JE, Feil R, Weschke W, Weber H (2010) Sucrose non-fermenting kinase 1 (SnRK1) coordinates metabolic and hormonal signals during pea cotyledon growth and differentiation. *Plant J* **61**: 324–338
- Radchuk R, Radchuk V, Weschke W, Borisjuk L, Weber H (2006) Repressing the expression of the SUCROSE NONFERMENTING-1-RELATED PROTEIN KINASE gene in pea embryo causes pleiotropic defects of maturation similar to an abscisic acid-insensitive phenotype. *Plant Physiol* **140**: 263–278
- Ramon M, Rolland F, Thevelein J, Van Dijck P, Leyman B (2007) ABI4 mediates the effects of exogenous trehalose on *Arabidopsis* growth and starch breakdown. *Plant Mol Biol* **63**: 195–206
- Rasmusson AG, Fernie AR, Van Dongen JT (2009) Alternative oxidase: a defence against metabolic fluctuations? *Physiol Plant* **137**: 371–382
- Rattan R, Giri S, Singh AK, Singh I (2005) 5-Aminoimidazole-4-carboxamide-1- β -D-ribofuranoside inhibits cancer cell proliferation *in vitro* and *in vivo* via AMP-activated protein kinase. *J Biol Chem* **280**: 39582–39593
- Regierer B, Fernie AR, Springer F, Perez-Melis A, Leisse A, Koehl K, Willmitzer L, Geigenberger P, Kossmann J (2002) Starch content and yield increase as a result of altering adenylate pools in transgenic plants. *Nat Biotechnol* **20**: 1256–1260
- Rocha-Sosa M, Sonnewald U, Frommer W, Stratmann M, Schell J, Willmitzer L (1989) Both developmental and metabolic signals activate the promoter of a class-I patatin gene. *EMBO J* **8**: 23–29
- Rolland F, Baena-Gonzalez E, Sheen J (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annu Rev Plant Biol* **57**: 675–709
- Rolland F, Moore B, Sheen J (2002) Sugar sensing and signaling in plants. *Plant Cell* **14**: S185–S205
- Runquist M, Kruger NJ (1999) Control of gluconeogenesis by isocitrate lyase in endosperm of germinating castor bean seedlings. *Plant J* **19**: 423–431
- Satoh-Nagasawa N, Nagasawa N, Malcomber S, Sakai H, Jackson D (2006) A trehalose metabolic enzyme controls inflorescence architecture in maize. *Nature* **441**: 227–230
- 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
- Schluepmann H, van Dijken A, Aghdasi M, Wobbes B, Paul M, Smeekens S (2004) Trehalose mediated growth inhibition of *Arabidopsis* seedlings is due to trehalose-6-phosphate accumulation. *Plant Physiol* **135**: 879–890
- Sonnewald U (2001) Control of potato tuber sprouting. *Trends Plant Sci* **6**: 333–335
- Stitt M, Lilley RMC, Gerhardt R, Heldt HW (1989) Determination of metabolite levels in specific cells and subcellular compartments of plant leaves. *Methods Enzymol* **174**: 518–552
- Suttle JC (2004) Physiological regulation of potato tuber dormancy. *Am J Potato Res* **81**: 253–262
- Suttle JC (2007) Dormancy and sprouting. In D Vreugdenhil, ed, *Potato Biology and Biotechnology: Advances and Perspectives*. Elsevier, Amsterdam, pp 288–309
- Suttle JC, Hultstrand JF (1994) Role of endogenous abscisic acid in potato microtuber dormancy. *Plant Physiol* **105**: 891–896
- Tiessen A, Prescha K, Branscheid A, Palacios N, McKibbin R, Halford NG, Geigenberger P (2003) Evidence that SNF1-related kinase and hexokinase are involved in separate sugar-signalling pathways modulating post-translational redox activation of ADP-glucose pyrophosphorylase in potato tubers. *Plant J* **35**: 490–500
- Turnbull CGN, Hanke DE (1985) The control of bud dormancy in potato tubers: measurement of the seasonal pattern of changing concentrations of zeatin-cytokinins. *Planta* **165**: 366–376
- Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JAM (2007) Primer3Plus, an enhanced Web interface to Primer3. *Nucleic Acids Res* **35**: W71–W74
- Vandesteene L, Ramon M, Le Roy K, Van Dijck P, Rolland F (2010) A single active trehalose-6-P synthase (TPS) and a family of putative regulatory TPS-like proteins in *Arabidopsis*. *Mol Plant* **3**: 406–419
- van Dijken AJH, Schluepmann H, Smeekens SCM (2004) *Arabidopsis* trehalose-6-phosphate synthase 1 is essential for normal vegetative growth and transition to flowering. *Plant Physiol* **135**: 969–977
- Wulschleger S, Loewith R, Hall MN (2006) TOR signaling in growth and metabolism. *Cell* **124**: 471–484
- Zhang L, Häusler RE, Greiten C, Hajirezaei MR, Haferkamp I, Neuhaus HE, Flügge UI, Ludewig F (2008) Overriding the co-limiting import of carbon and energy into tuber amyloplasts increases the starch content and yield of transgenic potato plants. *Plant Biotechnol J* **6**: 453–464
- Zhang Y, Primavesi LF, Jhurreea D, Andralojc PJ, Mitchell RA, Powers SJ, Schluepmann H, Delatte T, Wingler A, Paul MJ (2009) Inhibition of SNF1-related protein kinase1 activity and regulation of metabolic pathways by trehalose-6-phosphate. *Plant Physiol* **149**: 1860–1871

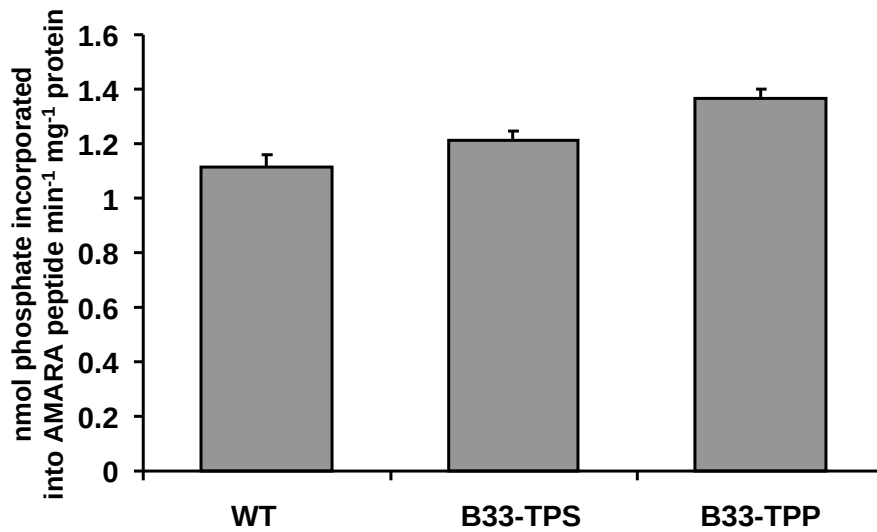
Supplementary Material Debast et al.

Supplementary Figure S1: B33-TPS tubers display increased respiration rates. Tubers selected for this experiment all had a freshweight of about 20g (± 3 g). Tubers were inserted into the cuvette of a PAM-Fluorometer (GFS-3000 and MINI-Imaging-PAM Chlorophyll Fluorometer; Walz Messtechnik GmbH, Effeltrich, Germany) and changes in CO_2 concentration were used to calculate respiration rate per hour and g-freshweight. Data are shown as the mean $\pm$ SD of six plants per line. The same tubers were used for measurement directly after harvest and after one week of storage.*denotes significant difference ($p < 0.05$) to the wild type control.

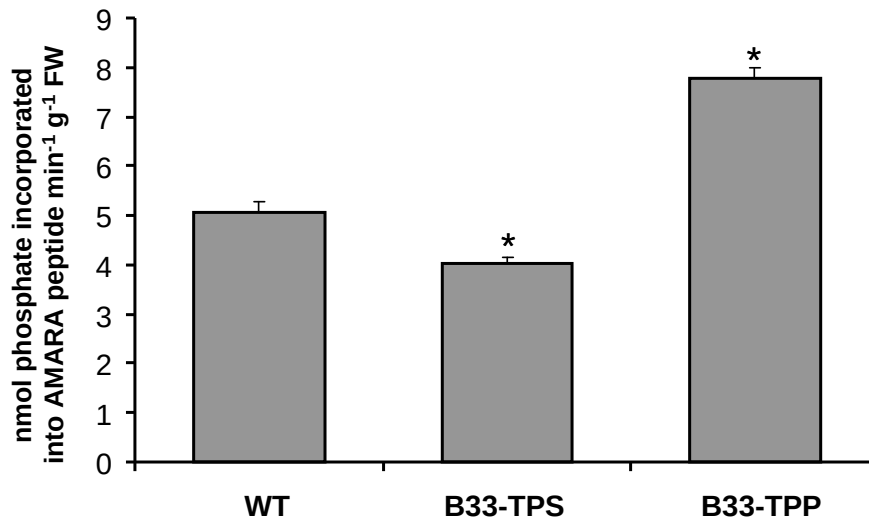


Debast et al. Supplementary Figure S2

A



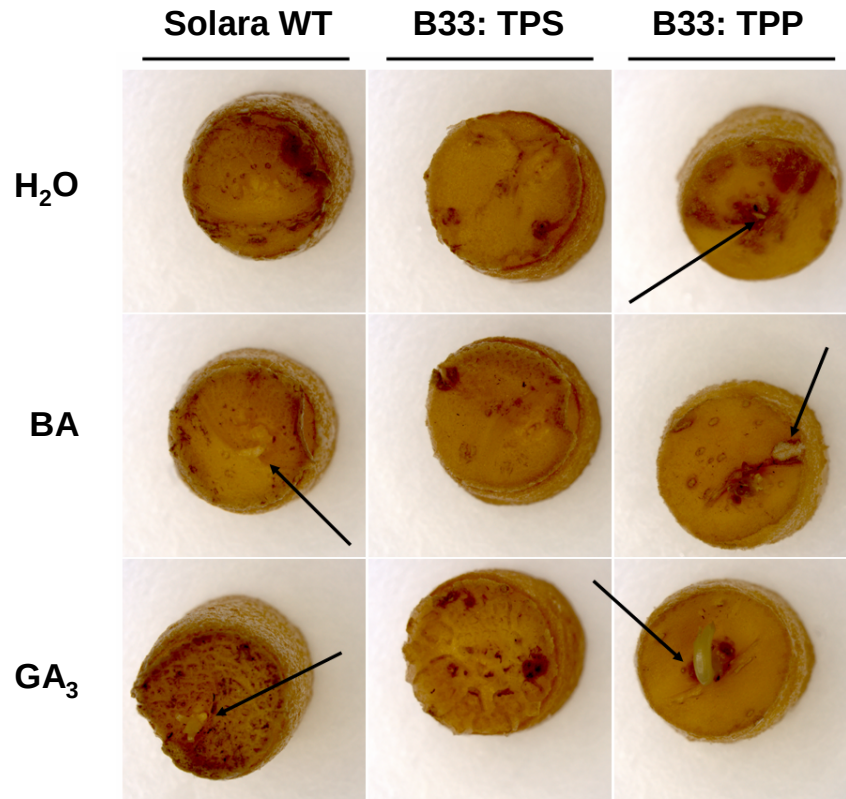
B



Supplementary Figure S2: SnRK1 activity in B33-TPS and B33-TPP transgenic potato lines.

- (A) Maximal extractable SnRK1 activity from B33-TPS line 29 and B33-TPS line 34 mature transgenic tubers compared to WT ($n = 4$, $\pm$ SD).
- (B) Estimated in vivo SnRK1 activity in B33-TPS and B33-TPP mature transgenic tubers compared to WT on a fresh weight basis ($n = 4$, $\pm$ SD). * denotes a significant statistical difference ($P = 0.05$) according to student's *t*-test.

Debast et al., Supplementary Figure S3.



Supplementary Figure S3: In vitro sprout release assay of B33-TPS and B33-TPP transgenic tubers.

Tuber discs from the different genotypes containing a single bud complex were treated with H_2O , 50 μM BA, 50 μM GA_3 and pictured 4 days after beginning of the treatment. Arrows indicate visible sprout growth.

Supplementary Table S3: Oligonucleotides used in this study

Oligo name	Sequence	Gene	Genbank	corresponding "Poci" identifier	resulting fragment length
SF201 (5'-ubi3)	5'ttccgacaccatcgacaatgt3'	Reference gene Ubiquitin3			105bp
SF202 (3'-ubi3)	5'cgaccatcctcaagctgctt3'	Reference gene Ubiquitin3			105bp
SD_Q05	5'GCTTTTGAAAGAGCGTCTGG3'	uridine diphosphate glucose epimerase	AY221085.1	MICRO.7015.C2_1344	194bp
SD_Q06	5'TTTGATTGGTAACCCCAAGG3'	uridine diphosphate glucose epimerase	AY221085.1	MICRO.7015.C2_1344	194bp
SD_Q07	5'AACGGGAAACTGAGGGTTCT3'	fructose 1,6-bisphosphatase	X76946.1	MICRO.5872.C2_1120	196bp
SD_Q08	5'TTTGCTCTTCAGCAGCGTAG3'	fructose 1,6-bisphosphatase	X76946.1	MICRO.5872.C2_1120	196bp
SD_Q11	5'AGATGTGGAGCCCAGTGAAC3'	TPS8 Glycosyltransferase		POABM58TP_861	191bp
SD_Q12	5'GCTGACGGTCAAAACGATCT3'	TPS8 Glycosyltransferase		POABM58TP_861	191bp
SD_Q13	5'GAAGCAGACCCTGACTTTGG3'	TPS11 Glycosyltransferase	AB368491.1	cSTC3D14TH_235	202bp
SD_Q14	5'ACAACACGAAATCGGGAGAC3'	TPS11 Glycosyltransferase	AB368491.1	cSTC3D14TH_235	202bp
SD_Q15	5'GCCAGAGGGTGGTCATTTTA3'	putative adenylate kinase	DQ222521.1	MICRO.4435.C1_1176	195bp
SD_Q16	5'CTGTGCTGAAGATGCAAAGC3'	putative adenylate kinase	DQ222521.1	MICRO.4435.C1_1176	195bp
SD_Q17	5'AAGGACTATGGGAGGCTCGT3'	60S ribosomal protein L14	DQ191666.1	MICRO.1272.C2_547	205bp
SD_Q18	5'TGGACTCCCACTTGGTCTTC3'	60S ribosomal protein L14	DQ191666.1	MICRO.1272.C2_547	205bp

SD_Q25	5'ACCGATACTCCCGACATCAG3'	expressed protein		MICRO.5525.C1_852	203bp
SD_Q26	5'TTTCCTCCTTTCCCAGACAA3'	expressed protein		MICRO.5525.C1_852	203bp
SD_Q27	5'GGGTTGCACTTGCTATTTCGT3'	sucrose synthase 2	AY205084.1	STMHE19TV_557	197bp
SD_Q28	5'GGTTGGTTTGGGAAATGATG3'	sucrose synthase 2	AY205084.1	STMHE19TV_557	197bp
SD_Q35	5'GCCACATGGTTACTTGCAGA3'	KIN gamma	AK320114.1	MICRO.804.C1_1507	197bp
SD_Q36	5'TGGCAAAACACCATCAAAGA3'	KIN gamma	AK320114.1	MICRO.804.C1_1507	197bp
SD_Q45	5'CCACATTGCTGGTATGGACA3'	glucose 1-phosphate metabolism,xylose catabolism	AK321744.1	bf_arrayxxx_0091a12.t7m.scf_711	117bp
SD_Q46	5'GCTCGGGAGTCAAACTCTG3'	glucose 1-phosphate metabolism,xylose catabolism	AK321744.1	bf_arrayxxx_0091a12.t7m.scf_711	117bp
SD_Q47	5'GAGGCTGCTAACCATCCAAC3'	GDH 1(Glutamate Dehydrogenase)	AK325007.1	bf_stolxxxx_0055h06.t3m.scf_448	99bp
SD_Q48	5'GACACCACCCGAGTTAGCAT3'	GDH 1(Glutamate Dehydrogenase)	AK325007.1	bf_stolxxxx_0055h06.t3m.scf_448	99bp
SD_Q49	5'GCTTCTTGGTCGAACAACCT3'	glutamine-dependent asparagine synthetase	FN421442.1	MICRO.3150.C2_616	109bp
SD_Q50	5'GGGTGTCCAAATTCCCATT3'	glutamine-dependent asparagine synthetase	FN421442.1	MICRO.3150.C2_616	109bp
SD_Q57	5'CCCATTTCGCAAAGAGAAAA3'	SNF1-related protein kinase [Solanum tuberosum]	X95996.1	MICRO.13181.C1_878	93bp
SD_Q58	5'TCCAGCAGAGTCCCAAGAAC3'	SNF1-related protein kinase [Solanum tuberosum]	X95996.1	MICRO.13181.C1_878	93bp
SD_Q61	5'GCATTGAATTCTTGGGTTGG3'	ABA Hydroxylase	DQ206631.1	MICRO.18185.C1	117bp
SD_Q62	5'TTCCTTCACATGACCATCCA3'	ABA Hydroxylase	DQ206631.1	MICRO.18185.C1	117bp

OtsA 5'	5'GGTACCAACATGAGTCGTTTAGTCGTA3'	OtsA K12 complete ORF (KpnI)	AY587539		1424bp
OtsA 3'	5'GTCGACCTACGCAAGCTTTGGAAAG3'	OtsA K12 complete ORF (Sall)	AY587539		1424bp
SD12	5'GTCGACAACAATGACAGAACCGTTAACCGAAAC3'	OtsB K12 complete ORF (Sall)	AY587539		818bp
SD13	5'GTCGACTTAGATACTACGACTAAACG3'	OtsB K12 complete ORF (Sall)	AY587539		818bp
Ubifor	5'ATGCAGATYTTTGTGAACAC3'	degenerate Polyubiquitin 5'			220bp *x
Ubirev	5'ACCACCACGRAGACGGAG3'	degenerate Polyubiquitin 3'			220bp *x