Running Head: Metabolite Control Over Growth: A Case For Trehalose-6-Phosphate

Author to whom correspondence should be sent:

Henriette Schluepmann, Padualaan 8, 3584 CH Utrecht, The Netherlands.

Tel +31 30 253 3289 E-mail: h.schlupmann@uu.nl

Journal Research Area:

Signal transduction and Hormone action- Associate Editor Bonnie Bartel
Title:

Growth arrest by trehalose-6-phosphate: an astonishing case of primary metabolite control over growth by way of the SnRK1 signaling pathway

Authors:


Institution addresses:

Molecular Plant Physiology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands (T.D., P.S, H.S); Department of Biomedical Analysis, Utrecht University, PO Box 80082, 3584 CA Utrecht, The Netherlands (T.D., A. deJ., G.S.); Institute for Chemistry and Dynamics of the Geosphere III Phytosphere, Research Centre Juelich, 52425 Juelich, Germany (A.W-K.). Plant Science, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, United Kingdom (L.F.P, M.J.P.). RIKEN Plant Science Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan (Y.K., M.M)
Footnotes

Financial source:

CW-ECHO (T.L. Delatte), NUFFIC-PhD (P. Sedijani), Biotechnological and Biological Sciences Research Council of the United Kingdom (L.F. Primavesi, M.J. Paul), NWO Meervoud and Utrecht University Cooperation Start-up Fund Asia (H. Schluepmann).

Present address of Thierry L. Delatte: Wageningen University Plant Sciences, Droevendaalsesteeg 1, 6708PB, Wageningen.

Correspondence author: Henriette Schluepmann, h.schlupmann@uu.nl
Abstract (250 words)

The strong regulation of plant carbon allocation and growth by the trehalose metabolism is important for our understanding of mechanisms that determine growth and yield with obvious applications in crop improvement. To gain further insight on the growth arrest by trehalose feeding, we first established that starch deficient seedlings of the pgm1 mutant were similarly affected as WT on trehalose. Starch accumulation in the source cotyledons therefore did not cause starvation and consequent growth arrest in the growing zones. We then screened the FOX collection of Arabidopsis expressing full-length cDNAs for seedling resistance to 100 mM trehalose. Three independent transgenic lines were identified with dominant segregation of the trehalose resistance trait that over-express the bZIP11 transcription factor. The resistance of these lines to trehalose could not be explained simply through enhanced trehalase activity or through inhibition of bZIP11 translation. Instead, trehalose-6-phosphate (T6P) accumulation was much increased in bZIP11 over-expressing lines suggesting that these lines may be insensitive to the effects of T6P. T6P is known to inhibit the central stress-integrating kinase SnRK1 (KIN10) activity. We confirmed that this holds true in extracts from seedlings grown on trehalose, then showed that two independent transgenic lines over-expressing KIN10 were insensitive to trehalose. Moreover, expression of marker genes known to be jointly controlled by SnRK1 activity and bZIP11 was consistent with low SnRK1 or bZIP11 activity in seedlings on trehalose. Results reveal an astonishing case of primary metabolite control over growth by way of the SnRK1 signaling pathway involving T6P, SnRK1 and bZIP11.
Trehalose (α, α-1, 1 linked D-glucopyranosyl D-glucopyranoside) inhibits growth and is toxic when fed to seedlings of the dodder vine *Cuscuta reflexa* (Veluthambi et al., 1982a; Veluthambi et al., 1982b) or *Arabidopsis thaliana* (Fritzius et al., 2001; Ramon et al., 2007; Schluepmann et al., 2004; Schluepmann and Paul, 2009; Wingler et al., 2000). This effect is somewhat surprising as trehalose is a common sugar and synthesized in many organisms at high concentrations functioning as a carbon source and stress protection compound. In plants the trehalose pathway is ubiquitous and indispensable, but typically trehalose does not accumulate to high levels. A major function of the pathway in sugar signaling and regulation of growth and development has been elucidated in plants in recent years (Eastmond et al., 2002; Paul et al., 2010; Satoh-Nagasawa et al., 2006; Schluepmann et al., 2003; Zhang et al., 2009). The impact of trehalose feeding on growth is most likely related to this signaling function (Schluepmann et al., 2004). However, the precise mode of action of growth inhibition by trehalose is not known.

A striking feature of Arabidopsis seedlings grown on trehalose is starch accumulation in the source tissues whilst no starch accumulates in the tip of the roots in the columella, representing a reversal of carbon allocation. Starch accumulation in the cotyledons can be explained through upregulation of ADPglucose pyrophosphorylase (AGPase) transcriptionally (Wingler et al., 2000) and through AGPase redox activation (Kolbe et al., 2005) and also by inhibition of starch degradation (Ramon et al., 2007). However, interestingly, this effect is found only in source tissues such as cotyledons and not in sink tissues such as the columella of root tips, which no longer accumulate starch and appear to be starving due to lack of sufficient carbohydrate (Wingler et al., 2000). Feeding metabolisable sugar in combination with trehalose rescues growth and it would appear that the primary effect of trehalose on growth is related to utilization of sugar. This is also a feature of trehalose toxicity in cut dodder shoots where trehalose and sucrose uptake and accumulation were studied (Veluthambi et al., 1982a; Veluthambi et al., 1982b). Radio-labeled trehalose accumulated evenly throughout the shoot. Trehalose accumulation is associated with a decrease in the radio-labeled sucrose accumulation and starch content of shoot tips as well as growth arrest in the growing zone of the shoot tips (Veluthambi et al., 1982b).

Growth arrest of Arabidopsis seedlings on medium with 100 mM trehalose was previously attributed to trehalose 6-phosphate (T6P) accumulation under these particular conditions (Schluepmann et al., 2004). T6P has emerged as a powerful signal molecule in plants, a target of which has been identified as SnRK1 of the SNF1/AMPK group of protein kinases (Martinez-Barajas et al., 2011; Paul et al., 2010; Zhang et al., 2009). SNF1-related protein kinases perform a fundamental role in the
physiological response of cells to energy limitation and starvation of carbon source through regulation of pathways and processes involved in metabolism, growth and development (Halford and Hey, 2009; Hardie, 2007; Polge and Thomas, 2007). SnRK1 integrates stress, sugar and specific developmental signals (Baena-Gonzalez et al., 2007; Baena-Gonzalez and Sheen, 2008; Baena-Gonzalez, 2010). T6P inhibits SnRK1 from all plant tissues so far tested except for mature leaves. This can be explained through the requirement of an intermediary factor present in growing tissues but not in mature leaves and is consistent with the view that T6P promotes anabolic processes associated with growing tissues (Zhang et al., 2009). Whilst T6P promotes growth on sucrose (Paul et al., 2010; Zhang et al., 2009), T6P inhibits growth on trehalose (Schluepmann et al., 2004). SnRK1 activity was proposed to be inactivated by sugars (Baena-Gonzalez et al., 2007), consistent with the observation that sucrose feeding causes a rise in T6P concentrations (Lunn et al., 2006; Schluepmann et al., 2003) which subsequently inhibit SnRK1 activity, promoting growth. The link between SnRK1 and T6P is significant as it establishes T6P as a signaling metabolite integrating carbon metabolism with the activity of enzymes and gene expression reprogramming controlled by this central protein kinase (Paul et al., 2010; Zhang et al., 2009). It is quite possible that growth arrest on trehalose is mediated through SnRK1.

Expression of Asparagine Synthase 1 (ASN1) has previously been used as a reporter of SnRK1 activity; the expression of ASN1 increases when the catalytic subunit of SnRK1, KIN10, is over expressed (Baena-Gonzalez et al., 2007). The promoter of ASN1 was shown to contain a G-box sequence known to be bound by bZIP (basic region/leucine zipper motif) called GBF (G-box Binding Factor) transcription factors. Co-expression of several bZIP transcription factors with KIN10 was shown to potentiate the expression of ASN1 (Baena-Gonzalez et al., 2007). bZIP transcription factors regulate a range of processes in growth and development in relation to the environment including stress responses (Jakoby et al., 2002). A model was proposed by which several stress signals including hypoxia and darkness converge through SnRK1 signaling and are coordinated in part by the bZIP transcription factors 54, 18, 63, 1, 38 and 11 (Baena-Gonzalez et al., 2007). It is not known whether these bZIP proteins are phosphorylation targets of SnRK1 and/or if bZIP gene expression is controlled by SnRK1 directly. bZIP54, 18, 63, 1 and 38 gene-expression is induced whilst bZIP11 is repressed when KIN10 is increased (Supplemental Fig. S1A; Baena-Gonzalez et al., 2007) or when seedlings are starving; the opposite is true when seedlings have increased carbon access through sugar or CO₂ feeding (Supplemental Fig. S1B). Potentiation of KIN10 induced ASN1 expression when KIN10 and bZIP11 are co-expressed suggests that the SnRK1 and bZIP11 controls of ASN1 expression interact.
The changes in gene expression after KIN10 over-expression are comparable to those obtained during carbon starvation regimes and opposite those under high supply of glucose, sucrose or CO$_2$ (Supplemental Fig. S1B). The S1-class of bZIP transcription factors consists of bZIP 1, 2, 11, 44 and 53 and appears to be expressed specifically in sinks such as young leaves, anthers and seeds (Rook et al., 1998; Weltmeier et al., 2009); their expression is also affected by abiotic stresses in a tissue-specific manner (Kilian et al., 2007). S1-class bZIP are generally regulated at the post transcriptional level by Sucrose-Induced Repression of Translation (SIRT) at a conserved uORF located in the 5 prime of the mRNA (Hanson et al., 2008; Weltmeier et al., 2009; Wiese et al., 2004). bZIP11 has been proposed to alter nitrogen metabolism by controlling the expression of ASN1 and Proline Dehydrogenase (PDH; Hanson et al., 2008) and changes in bZIP1 alter the transcriptional response to the carbon/nitrogen ratio (Kang et al., 2010; Obertello et al., 2010). Whilst T6P and SnRK1, and bZIP11 and SnRK1 have been linked, a connection between all three components in the regulation of growth has not previously been established.

The strong regulation of carbon allocation and growth by the trehalose pathway is important for our understanding of mechanisms that determine plant growth and yield with obvious application in crop improvement. To gain further insight on the growth arrest by trehalose feeding, we first established that starch deficient seedlings of the pgm1 mutant are similarly affected as WT on trehalose. Starch accumulation in the cotyledons, the source tissue therefore, does not cause starvation and consequent growth arrest in the growing zones. We then screened the FOX collection of Arabidopsis expressing full-length cDNAs behind the CaMV35S promoter (Ichikawa et al., 2006) for seedling resistance to 100 mM trehalose. Three independent transgenic lines from differing pools of the collection were identified with dominant segregation of the trehalose resistance trait that over-expressed the bZIP11 transcription factor. The resistance of these lines to trehalose could not be explained through enhanced trehalase activity or through inhibition of bZIP11 translation. Instead, T6P accumulation was much increased in bZIP11 over-expressing lines suggesting that these lines may be insensitive to the effects of T6P. T6P is known to inhibit SnRK1 activity and we confirmed that this holds true in extracts from seedlings grown on trehalose, then showed that two independent transgenic lines over-expressing KIN10 were insensitive to trehalose. Moreover, expression of a set of marker genes known to be jointly controlled by SnRK1 activity and bZIP11 was found to be consistent with low SnRK1 or bZIP11 activity in seedlings on trehalose. Results were consistent with the existence of a growth regulating pathway involving T6P, SnRK1 activity and bZIP11, regulating growth in the growing zones of Arabidopsis seedlings.
RESULTS

Primary and secondary screening of the FOX-collection on trehalose

Trehalose at 100 mM in half-strength MS inhibited the growth of Arabidopsis seedlings of all accessions thus far tested. Seedlings germinated on this medium developed short roots less than 3 mm long after 14 d and the leaf primordia did not extend into leaves (Fig. 1A WT tre; Fig. 1B WT) compared to normal development on 100 mM sorbitol osmoticum control (Fig. 1A WT sorb; Fig. 1B WT). Starch accumulated in large amounts in one or both cotyledons in seedlings grown on trehalose (Fig. 1A WT tre); such massive accumulation of carbon as starch in the source tissues of seedlings could possibly cause starvation in the growing zones of seedlings. The starch-less pgm1 mutant (Caspar et al., 1985), however, was inhibited on trehalose in a similar manner to WT (Fig. 1A pgm1 tre). Starch accumulation in the source tissues of seedlings, the cotyledons, thus does not cause growth arrest on trehalose.

To further understand the mechanism of growth arrest on trehalose using a non hypothesis-driven approach, we used a genetic screen: seedlings from the Arabidopsis FOX collection (Ichikawa et al., 2006) were screened for growth on 100 mM trehalose. The entire collection was partitioned in 141 pools each containing 20-30 T1 seed from 100 independent transgenic lines; primary screening was carried out twice with 1000 seed per plate for each 141 pools. The germination frequency varied between 100% and as low as 30% entailing that not all transgenic lines were assayed for their resistance to trehalose and that thus the screen did not test all the cDNA of the FOX collection. T1 seedlings during the primary screening were chosen that displayed significantly longer roots than wild type and leaf primordia (Fig. 1A 93-1 tre). Secondary screening of T2 retained only lines where seedling roots after 14 d of growth on trehalose were at least 3 fold longer than WT and of the same length as WT on osmoticum control (Fig. 1B). In the case of lines 93-1 and 93-32 from pool 93, seedling root lengths were nearly as long on trehalose as they were on sorbitol (Fig. 1B). Seedlings from line 70 were assayed at a later stage because trehalose resistant seedlings obtained from pool 70 were difficult to grow and after re-screening only one plant grew to set seed. The T2 seedlings from lines 93-3, 33-1 and 70 were 100% resistant to trehalose, whilst those from 93-1 were not and thus still segregating.

An obvious cause of resistance to trehalose would be increased trehalase activity. Expression of the E.coli cytosolic trehalase in Arabidopsis seedlings resulted in seedlings with high trehalase activity that thrive on trehalose (Fig. 1B treF and Fig. 1C treF). Therefore activity of the Arabidopsis trehalase
(TRE1) was assayed in lines identified during the secondary screening. We tested several membrane and cell wall preparations from WT seedlings but trehalase activities in these fractions were below detection levels. Instead activity was readily detected in the soluble fraction and was not significantly different in FOX lines compared to WT, except for a small increase from pool 93 (ANOVA P=0.052). These activities were 10-20 fold lower compared to seedlings expressing E.coli trehalase treF (Fig. 1C).

Over-expression of the full-length cDNA of bZIP11 is linked with resistance to trehalose

Seedlings from the FOX lines were back crossed into WT and resistance to trehalose of F1 generation seedlings evaluated for each line. F1 seedlings of lines 33-1, 93-1 and 70 were resistant to trehalose, indicating that the trehalose resistance in these lines segregates as a dominant trait (Fig. 2A 33-1, 93-1). The cDNA present in the T-DNAs in the heterozygous F1 therefore caused trehalose resistance. The cDNA was then amplified using primers on the flanking sequences of the FOX vector for two different plants from each line (Fig. 2B). Fragments obtained from plants of the lines 33-1, 33-3, 70, 93-1 and 93-3 were of the same length. Subsequent cloning and sequencing revealed that these fragments all contained the full-length cDNA of bZIP11. The lines were from 3 differing pools, pools 33, 70 and 93. The lines therefore represented 3 independent transgenic events selected through screening which linked presence of the bZIP11 cDNA with trehalose resistance.

Expression of bZIP11 cDNA was tremendously increased in the FOX lines containing the bZIP11 cDNA compared to WT (Fig. 2C). In seedlings on trehalose, AtTRE1 expression was less than two-fold increased compared to WT (Fig. 2D, ANOVA P=0.014), very much less increased than bZIP11 expression. Over- or antisense-expression of bZIP1 in lines previously characterized (Kang et al., 2010) did not yield resistance to trehalose suggesting that the function of bZIP11 could not be replaced by the other S1-class bZIP bZIP1 (Fig. 2E). bZIP1 expression was high in treF (E.coli trehalase) -expressors on trehalose compared to WT or bZIP11 expressors (Fig.2F). Even though trehalase activity determinations suggested that the mechanism of trehalose resistance in bZIP11 over-expressors differed from that in treF expressors, we still wondered if the in vitro assays of trehalase (Fig. 1C) were conclusive.

AtTRE1 is not required for bZIP11 trehalose resistance

In further confirmation that TRE1 did not underlie resistance to trehalose in the FOX expressing bZIP11, a tre1 knock out was characterized from the SALK collection (Salk 147073c, Alonso et al., 2003). PCR with primers on the left border of the T-DNA and in the start of the gene confirmed that
the T-DNA was located 5 prime to the ATG of TRE1. Sequencing of the insertion site revealed an additional 25-bp insertion in the 5 prime and confirmed the T-DNA insertion site. Gene expression quantification with Q-PCR further verified that the insertion causes a dramatic decrease in TRE1 mRNA (Fig. 3A). Trehalase activity in seedlings and flowers of the tre1-1 line was below detection (Fig. 3B). tre1-1 was more susceptible to 25 and 50 mM trehalose than WT from the Col0 accession (Fig. 3C) showing that TRE1 does contribute to the relative tolerance of WT seedlings to low levels of trehalose. The tre1-1 seedlings were similarly sensitive to trehalose as the previously characterized tre1-2 in the Ler accession (Fig. 3C; Vandesteene, 2009). The F1 seedlings from the cross tre1-1 with the selected FOX lines were resistant to 100 mM trehalose (shown for the cross with FOX 93 in Supplemental Fig. S2). When analyzing the genotype of F2 seedlings with roots at least three-fold longer than WT, F2 seedlings could be found that were homozygous for the tre1-1 T-DNA insertion (Fig. 3D). The trehalose resistance phenotype of seedlings homozygous for tre1-1 and over-expressing bZIP11 is shown in Supplemental Fig. S2. Together the data showed that AtTRE1 may contribute to but is not required for resistance to 100 mM trehalose and cannot explain rescue of seedlings on trehalose by bZIP11.

Translational repression at uORF2 does not occur on trehalose unlike on sucrose

bZIP11 cDNA is known to contain several upstream Open Reading Frames (uORFs) in the 5 prime region (Weltmeier et al., 2009; Wiese et al., 2004). uORF2 is conserved in the plant kingdom and found in all the S1-class bZIP mRNA. uORF2 mediates sucrose-induced repression of translation (SIRT) of the bZIP11 protein. The mechanism may also inhibit bZIP11 protein synthesis in seedlings grown on trehalose. To test this possibility seedlings of the UBQ10:5'UTR-GUS/GFP-I line (Wiese et al., 2004) that express the 5 prime UTR containing uORF2 in front of GUS/GFP were grown for 7 d, then transferred to medium supplemented with either sucrose or trehalose for 48 h before GUS staining. GUS staining confirmed SIRT on sucrose medium supplemented with 100 mM sucrose throughout all tissues of the seedling (Fig. 3E 100 SUC). On trehalose medium, however, repression of translation was not observed (Fig. 3E 100 TRE). We therefore concluded that seedlings from the FOX lines translate bZIP11 unrestricted by SIRT when grown on trehalose.

bZIP11 over-expressing seedlings accumulate T6P

WT seedlings growing on 100 mM trehalose accumulate T6P (Schluepmann et al., 2004) which has been linked to growth arrest under these particular conditions. We wondered whether the FOX line seedlings were insensitive to T6P or had just a low accumulation of T6P. T6P in seedlings grown for 14 d on osmoticum control was 0.6-1 nmol g⁻¹ FW in bZIP11 over-expressors, six- to tenfold higher
than in WT (Fig. 4A). On trehalose medium, T6P accumulated in WT to 1.8 nmol g\(^{-1}\) FW as expected. T6P accumulation in bZIP11 over-expressors was, however, much higher reaching over 60 nmol g\(^{-1}\) FW (Fig. 4B). Data therefore showed that bZIP11 over-expression causes T6P accumulation.

In contrast, T6P levels in seedlings expressing *E. coli* trehalase treF were similar to WT under control growth conditions. On trehalose, T6P levels in seedlings expressing treF accumulated to 1.5 nmol g\(^{-1}\) FW, a somewhat lesser extent than in WT, suggesting that when trehalase is expressed unlikely all of the trehalose is cleaved. The differing accumulation of T6P in bZIP11 compared to treF expressing seedlings further supported the results which showed that trehalose resistance by bZIP11 is independent of trehalase. Instead the results point to the possibility that bZIP11 over-expression renders seedlings less susceptible to T6P accumulation.

T6P is known to inhibit seedling SnRK1 activity when seedlings are grown under normal conditions (Zhang et al., 2009). To test whether SnRK1 is inhibited by T6P in seedlings grown on trehalose, extracts from WT Arabidopsis seedlings grown in 100 mM trehalose were prepared from which small molecular weight compounds were removed using a desalting procedure. SnRK1 activity was then assayed in the absence or presence of 1 mM T6P. Results confirmed that SnRK1 from trehalose grown seedlings was significantly inhibited by T6P (Fig. 4C). In addition, SnRK1 activity assayed from extracts of bZIP11 over-expressing seedlings (93, 33) was similar to that of the WT (Fig. 4C). Over-expression of bZIP11 thus did not cause large changes in SnRK1 activity. We therefore hypothesized that the T6P accumulation and inhibition of SnRK1 in seedlings expressing constitutively high levels of bZIP11 may be overcome because bZIP11 likely controls a subset of genes responsive to SnRK1 activity. To test this possibility we first needed to establish whether SnRK1 activity limits the growth of seedlings on trehalose.

**KIN10 over-expressing seedlings grew on 100 mM trehalose**

To test whether reduced SnRK1 activity underlies the growth inhibition on trehalose, two lines O1 and O2 overexpressing KIN10 with high SnRK1 activity, previously characterized in detail were used (Baena-Gonzalez et al., 2007). Seedlings of both lines were resistant to 100 mM trehalose (Fig. 5A, only O2 shown). SnRK1 activity therefore likely is limiting when WT seedlings are grown on trehalose. The result further indicated that T6P inhibition of SnRK1 likely underlies growth arrest on trehalose and confirmed genetically previous results obtained by combining biochemical assay with gene-expression profiling (Zhang et al., 2009).

As with the bZIP11 over-expressors, KIN10 over-expression did not cause a significant induction of AtTRE1 activity (Fig. 5B) or of trehalase expression in seedling extracts (Fig. 5C). In addition, KIN10
over-expression did not affect the expression of bZIP11 (Fig. 5D); neither did bZIP11 over-expression affect SnRK1 activity (Fig. 4C) or KIN10 expression (Supplemental Fig. S3). Data therefore suggested that the SnRK1 and bZIP11 interaction described previously (Baena-Gonzalez et al., 2007) in seedlings on trehalose is likely post transcriptional with SnRK1 activity changes altering bZIP11 subcellular localization or activity.

**Regulation of targets common to bZIP11 and SnRK1**

ASN1 is one of the known targets of SnRK1 and its expression was much reduced in WT grown on trehalose compared with sorbitol thus confirming that SnRK1 activity is likely inhibited by T6P on trehalose (Fig. 6A ASN1).

It is known that bZIP11 potentiates the induction of DIN6 (ASN1) gene-expression when SnRK1 activity is increased by KIN10 over-expression in protoplasts (Baena-Gonzalez et al., 2007). We compared genes known to be controlled by 6 h transient expression of KIN10 in protoplasts (511 up, 521 down; Baena-Gonzalez et al., 2007) with those known to be controlled in whole seedlings by the nuclear transfer of bZIP11 after 2 h (167 up, 96 down; Hanson et al., 2008). Table I lists the genes jointly controlled by KIN10 and bZIP11. In spite of the differing conditions used to identify regulation of gene expression, a large proportion of the bZIP11 induced genes, 32 out of 167, were also induced by KIN10. These include 4 genes of the 7 confirmed to be regulated by bZIP11 under its endogenous promoter: ASN1 (At3g47340), PRODH (At3g30775), BT2 (At3g43830) and PGPD14 (At5g22920). Nine genes from the 96 genes repressed by bZIP11 were also repressed by KIN10; only 3 genes from the total of 261 genes compared appeared differentially regulated by bZIP11 and KIN10. Results obtained from this comparison were thus consistent with the hypothesis that bZIP11 may be a target of SnRK1 regulation and may mediate a part of the output of SnRK1 signaling; they further showed that bZIP11 does in addition regulate transcription of a set of genes not included in regulation by SnRK1.

To test whether KIN10 and bZIP11-mediated rescue on trehalose may involve genes that are commonly controlled by both, we assayed the expression of a randomly chosen array of jointly induced (8) or repressed (5) genes in WT seedlings grown on trehalose compared to sorbitol (Fig. 6A). All but one of the 8 genes jointly induced by bZIP11 and KIN10 were significantly repressed on trehalose. Furthermore 3 of the 5 jointly repressed genes were significantly induced on trehalose compared to sorbitol. We conclude that a large proportion of genes jointly regulated by KIN10 and bZIP11 respond on trehalose as if KIN10 or bZIP11 were less active.
To compare the downstream events in KIN10 and bZIP11 over-expression, the levels of soluble sugars (Glc, Fru and Suc) were compared with those found in WT and in treF expressors (Fig. 6B). Similar results were found for hexoses and sucrose and data are presented as total soluble sugar. Soluble sugars accumulated in WT Col.0 or Ler seedlings grown on trehalose and in treF seedlings which were included for comparison. The data were consistent with carbon accumulation in source tissues whilst sugars are not used for sink growth. Strikingly, bZIP11 over-expression lines 93 or 33 had 10-20 times higher soluble sugar than WT (Fig. 6B 93, 33) irrespective of the medium. Sugars were also elevated (threefold compared to WT) in KIN10 over-expressors compared to WT and this increase was even more on trehalose (Fig. 6B O1, O2).

Arrest of hypocotyl elongation when wild type seedlings were grown in continuous darkness was another effect of feeding trehalose (Fig. 6C, D WT tre). We thus concluded that trehalose feeding not only stopped use of carbon fixed by photosynthesis but also of carbon from seedling reserves needed for the for hypocotyl growth in the etiolation response. Over-expression of bZIP11 (Fig. 6C) or KIN10 (Fig. 6D) was able to suppress hypocotyl growth inhibition by trehalose in the dark. The observation suggested that KIN10 and bZIP11 rescue T6P inhibition of SnRK1 regardless of the source of carbon fueling the growth of sinks. Therefore convergence of the pathways involving KIN10 and bZIP11 in the control of carbon utilization for growth is likely.
DISCUSSION

Trehalose, a widespread disaccharide synthesized in all non vertebrate organisms functions widely as a carbon source and stress protection compound. When fed exogenously to plants, however, it can have a surprisingly strong inhibitory effect on growth and carbon allocation (Veluthambi et al., 1982a; Veluthambi et al., 1982b; Wingler et al., 2000). The trehalose pathway in plants has evolved a role that is distinct from other organisms in the regulation of metabolism in relation to growth and development (Paul et al., 2010; Schluepmann and Paul, 2009; Zhang et al., 2009). The signal transduction mechanisms involved in trehalose signaling therefore are of great interest as potential targets for crop improvement. Here we provide evidence that growth arrest by trehalose-6-phosphate is an astonishing case of primary metabolite control over growth by way of the SnRK1 signaling pathway also involving bZIP11. Our work highlights the importance of bZIP11 and SnRK1 specifically in the growth response to trehalose and more generally in the regulation of growth by carbon availability to growing sinks.

Growth arrest is not due to excessive starch accumulation but to impaired utilization of sugar

The phenotype of Arabidopsis seedlings on trehalose is interesting because it involves a large change in carbon allocation within the seedling in addition to growth arrest. Starch accumulates in cotyledons, whilst growing sinks appear carbon limited. Starchless pgm 1 mutants are similarly impaired as wild type on trehalose, which rules out the possibility that sinks are carbon limited because starch accumulation sequesters available carbon. Feeding metabolisable sugar in combination with trehalose alleviates growth arrest, therefore, growth arrest is likely due to insufficient carbon in growing sinks (Schluepmann et al. 2004). Feeding trehalose could affect sucrose transport with high levels of trehalose displacing sucrose at the sucrose transporters and so reducing phloem loading and unloading processes. Growth arrest by trehalose, however, already occurs at trehalose concentrations of 25 mM when trehalose is fed in combination with the trehalase inhibitor Validamycin A (Wingler et al. 2000). At these concentrations, trehalose is unlikely having an effect on sucrose transport just by displacing sucrose. In contrast, T6P has previously been shown to cause the growth inhibition on trehalose and to have an important signaling function (Schluepmann et al. 2004; Kolbe et al. 2005; Zhang et al. 2009). One of these signaling functions is in plastid redox processes (Kolbe et al. 2005; Geigenberger, 2011). Starch accumulation in trehalose fed leaves of Arabidopsis is caused by T6P accumulation stimulating AGPase redox activation in plastids (Kolbe et al. 2005). This is consistent with the massive accumulation of starch seen in cotyledons of seedlings. The pgm1 mutants show that the effect of T6P on AGPase redox activation in cotyledons is not involved in the mechanism leading to growth arrest.
The transcription factor bZIP11 overcomes growth inhibition on trehalose

The FOX collection was utilized to screen for rescue of growth on trehalose. Strikingly we isolated three independent transgenic lines over-expressing bZIP11 a transcription factor shown to be important in the regulation of growth (Hanson et al., 2008). Translation of bZIP11 is repressed by sucrose (Wiese et al., 2004), however no such effect was produced by trehalose. A simple way of explaining rescue on trehalose is through breakdown of trehalose by trehalase (Schluepmann et al., 2004). However, trehalase activity was not elevated in seedlings over-expressing bZIP11 and neither did backcrossing bZIP11 into trehalase knockout compromise the rescue of seedlings by bZIP11, showing that bZIP11 rescues growth on trehalose without increasing the breakdown of the exogenously supplied trehalose. This implies that bZIP11 is part of a growth regulatory process that can be invoked to overcome the growth inhibition normally imposed by trehalose. The results obtained differ from those in Ma et al. 2011 where bZIP11 protein was fused to a nuclear targeting domain and where targeting of the fusion protein to the nucleus induced the expression and activity of TRE1. TRE1 was not detected as a target of native bZIP11 (Hanson et al. 2008), however, and this is consistent with results obtained with the FOX lines here. bZIP11 is expressed along the vasculature (Rook et al. 1998) and is proposed to regulate nitrogen metabolism by controlling the expression of ASN1 and PDH (Hanson et al., 2008). Sugar supply induces expression of bZIP11, therefore it was proposed that bZIP11 is important to relate nitrogen and carbon metabolism. Plants over-expressing KIN10 with active SnRK1 and primed to respond to starvation conditions consistently have reduced expression of bZIP11 (Baena-Gonzalez et al., 2007).

bZIP11 over-expression results in unprecedented levels of T6P recorded in Arabidopsis

T6P accumulation in plants grown on trehalose previously shown to be causally related to growth inhibition (Schluepmann et al., 2004). This was shown through specific reduction in T6P content through expression of a trehalose 6-phosphate hydrolase which rescued growth. Therefore it was surprising that bZIP11 overexpressors on trehalose exhibited huge accumulation of T6P to the highest levels so far reported for Arabidopsis (Fig. 4B). Sugars were also elevated in bZIP11 (Fig. 5E). A strong relationship between sucrose (Lunn et al., 2006) and sucrose and hexoses (Martinez-Barajas et al., 2011) and T6P has previously been found. It is thus possible that elevated T6P can be accounted for as a response to elevated sugar found in bZIP11 over-expressors compared with WT. Given the recent evidence that SnRK1 is a target of T6P (Paul et al., 2010; Zhang et al., 2009), that SnRK1 would be inhibited by T6P accumulation on trehalose (Fig. 4C) and that bZIP11 over-
expression increases the impact of endogenous SnRK1 activity (Baena-Gonzalez et al., 2007) we hypothesised that high SnRK1 activity could also be a mechanism of rescue of growth on trehalose.

**KIN10 over-expression rescues growth on trehalose**

SnRK1 is essential for carbon utilization in growth. KIN10 over-expression increases seedling growth compared to WT when carbon availability is limiting, whereas seedlings with low SnRK1 activity through KIN10 antisense thrive when carbon availability is high (Baena-Gonzalez et al., 2007). We found that as with bZIP11, over-expression of SnRK1 also rescues seedling growth on trehalose (Fig. 5A). Like seedlings expressing bZIP11, KIN10 expressors also accumulate free sugars compared with either WT or treF when they are grown in osmoticum control conditions (Fig. 5E). This would imply that SnRK1 too is part of a mechanism that regulates growth in the presence of trehalose and that T6P through inhibition of SnRK1 prevents growth on trehalose. This conclusion is supported by previous work which shows the inhibition of SnRK1 by T6P in Arabidopsis seedlings, by direct *in vitro* assay and indirectly by profiling known targets of SnRK1 in seedlings with altered T6P steady state (Zhang et al., 2009).

**Interaction of bZIP11 and SnRK1**

A subset of S-class bZIP transcription factors are proposed to mediate some of the transcriptional re-programming in response to altered SnRK1 activity (Baena-Gonzalez et al., 2007). The mechanism linking kinase activity signaling and transcriptional control by S-Class bZIP proteins is not known, but possibly involves phosphorylation of the bZIP protein which would explain potentiation of the SnRK1 response when KIN10 and bZIP11 are over-expressed simultaneously in protoplasts (Baena-Gonzalez et al., 2007). Recombinant bZIP EEL/DPBF4 and ABI5 were substrates of SnRK1 after immunoprecipitation of KIN10GFP protein fusion (Bitrián et al. 2011). We confirm KIN10 and bZIP11 interaction when seedlings are on trehalose: 1) over-expression of bZIP11 has the effect of overcoming the effects of low SnRK1 activity on trehalose, 2) the majority of genes tested that are jointly controlled by KIN10 and bZIP11 are expressed in a manner consistent with low activity of either KIN10 or bZIP11 in WT seedlings on trehalose (Table I; Fig. 6A), 3) under differing conditions, KIN10 and bZIP11 permit growth of sinks in seedlings on trehalose and 4) T6P accumulates in bZIP11 over-expressing seedlings grown on sorbitol. Comparison of data from Baena-Gonzalez et al. (2007) and Hanson et al. (2008) shows that 44 genes may be jointly regulated by bZIP11 and KIN10 (Table I). Constitutive and high expression of bZIP11 may therefore counteract T6P inhibition of SnRK1 activity on trehalose and impact at least a subset of the targets that are commonly regulated by bZIP11 and KIN10. These targets of bZIP11 are likely important for the control of carbon utilization for growth.
S1-Class bZIP transcription factors are thought to act redundantly but have a differential expression responsiveness and pattern (Hanson et al., 2008; Weltmeier et al., 2006; Weltmeier et al., 2009). Seedlings over-expressing or with antisense to bZIP1 (Kang et al., 2010) were sensitive to trehalose however (Fig. 2E), suggesting that bZIP11 has a different function from bZIP1. Control of the bZIP11 gene-expression is opposite to that of the other S-class bZIP capable of potentiating the effect of KIN10 expression: bZIP11 is repressed under carbon starvation conditions or by high SnRK1 activity (Baena-Gonzalez et al., 2007 and Fig. S1). bZIP11 expression is furthermore induced by increased carbon availability and this is consistent with results presented here where bZIP11 is required for carbon utilization for growth.

The arrest of hypocotyl growth by trehalose in seedlings in the dark was not reported previously. Seedlings on the combination of trehalose and Validamycin in the dark were reported not to accumulate starch and therefore it was concluded that carbon accumulating as starch in light grown seedlings on trehalose was of photosynthetic origin (Wingler et al., 2000). Inhibition of hypocotyl growth in dark grown seedlings on trehalose, and suppression thereof by over-expression of either KIN10 or bZIP11 indicates that the pathway of growth inhibition by trehalose in the dark and in the light likely involves the same mechanism. In the dark, gluconeogenesis from lipids stored in both the endosperm and the cotyledons fuels hypocotyl growth (Penfield et al., 2004). In light or dark therefore, KIN10/bZIP11 activities are limiting growth of sink tissues in seedlings as T6P accumulates on trehalose. Inhibition of the growth of sink tissues when SnRK1 was reduced by antisense inhibition has previously been reported in barley pollen grains and in potatoes (Purcell et al., 1998; Zhang et al., 2001). Importantly the work with segregating pollen grains by Zhang et al. 2001 shows that the effect is in the cells where SnRK1 is low and is not due to low SnRK1 in surrounding tissue.

A possible model of interactions that control carbon availability in growing sinks

T6P inhibition of SnRK1 could be part of a regulatory loop that relates SnRK1 activity with the amount of sucrose (Fig. 7A). In this regulatory loop sucrose-induced T6P increase inhibits SnRK1 when sucrose is plenty. As sucrose decreases T6P decreases and active SnRK1 signals nutrient stress such that more carbon is then made available to the heterotrophic growing cells. This regulatory loop could explain why both too little and too much of (either SnRK1 or) T6P are growth inhibitory (Schluempmann et al. 2003; Schluempmann et al. 2004; Debast et al., 2011). It also would explain the reported correlation between levels of sucrose and T6P (Lunn et al., 2006; Martinez-Barajas et al., 2011).
Glucose (Glc) and sucrose (Suc) feeding cause AGPase redox activation and thus increased starch synthesis by two differing pathways that are likely also relevant for the growth responses to these sugars (Tiessen et al., 2003; Michalska et al., 2009; Geigenberger, 2011). When feeding Glc, T6P does not accumulate (Delatte et al., unpublished) and glucose-6-phosphate (G6P) is shunted through the plastidic oxidative part of pentose phosphate pathway (OPP) generating NADPH for NADPH Thioredoxin Reductase C (NTRC) dependent reduction of AGPase and thus activation (Michalska et al., 2009). In contrast, feeding sucrose or trehalose leads to T6P increase (Schluepmann et al., 2004; Lunn et al., 2006). T6P increase may result from the increased amount of substrate uridine diphosphate glucose (UDPG) when sucrose is cleaved by sucrose synthase. Alternatively T6P increase in response to sucrose feeding may result from a sensing/signaling system affecting either T6P synthesis or degradation. The sucrose pathway to AGPase redox activation was shown to depend upon SnRK1 activity (Thiessen et al., 2003) but we have yet to know at which step SnRK1 is required. SnRK1 may not be required when trehalose grown seedlings convert the carbon fixed by chloroplasts into starch, alternatively Factor I may be absent in cotyledons. A number of Class II T6P synthases (TPS), including TPS5 are likely targets of SnRK1 but until now these enzymes were not shown to synthesize T6P (Harthill et al., 2006). When feeding sucrose or trehalose, T6P also causes NTRC dependent AGPase redox activation in the chloroplasts (Kolbe et al., 2005; Michalska et al., 2009). Although the interaction with redox-signaling may be an important aspect of T6P signaling, we have shown here that T6P accumulation on trehalose and consequent accumulation of starch in cotyledons does not cause growth arrest.

When feeding trehalose, T6P accumulation inhibits SnRK1. Inhibition of SnRK1 depends on and intermediary factor (Factor I) present in seedling extracts but not in leaf extracts (Zhang et al., 2009). Possibly, SnRK1 activates bZIP11 transfer to the nucleus or complexing of the transcription factor in such a way that bZIP11 controls a part of the SnRK1 output that is required for growth. Thus when T6P accumulates and inhibits SnRK1, over-expression of bZIP11 may act as a surrogate for SnRK1 in the growing zones. Antisense SnRK1 restricted to individual pollen of barley in particular (Zhang et al., 2001) but also work in developing potato tubers (Purcell et al., 1998) suggest that SnRK1 is required in individual cells of growing sinks for growth and starch accumulation.

In short-term trehalose feeding experiments of potato slices AGPase redox activation was also found and it was dependent on the presence of SnRK1 (Kolbe et al., 2005). SnRK1 may therefore coordinate substrate availability for starch synthesis and AGPase redox activation in sink tissues. In this respect, it is important to take into account that the subcellular localization of SnRK1’s catalytic subunit KIN10 was shown to differ in the different tissues of the seedlings (Bitrián et al., 2011): this
may also change SnRK1 susceptibility to T6P. In growing zones of root and shoot KIN10 was reported in the nucleus, whilst in the hypocotyls it was not. It is thus possible that in growing sinks SnRK1 is required to signal nutrient stress so as to activate processes that will make carbon available to growing cells. We conclude that SnRK1 inhibition (by artificially increasing T6P when feeding trehalose or by antisense SnRK1) may uncouple carbon starvation from growth responses leading to the swollen cells observed in the growing zones of roots of Arabidopsis seedlings on trehalose (tre) compared to sorbitol (sorb) (Fig. 7B). More research is needed to understand the precise role of bZIP11 in the carbon allocation responses and to understand where and how SnRK1 as well as bZIP11 over-expression lead to the accumulation of free sugars in seedlings.
METHODS

Plant materials and growth conditions

Lines in Col.0 accession are: Tref line 42 (Schuepmann et al., 2003), tre1-1 is Salk 147073c (http://signal.salk.edu Alonso et al., 2003), FOX lines (Ichikawa et al., 2006), the bZIP1 over and antisense lines (Kang et al., 2010) and finally the line to test translational repression of bZIP11 that contains the 5 prime untranslated leader of bZIP11 mRNA fused to GUS/GFP under the control of the UBIQUITIN10 promoter (UBQ10:5'UTR-GUS/GFP, (Wiese et al., 2004)). Lines in Ler accession: tre1-2 (Vandesteene, 2009), KIN10 O1 and KIN10 O2 (Baena-Gonzalez et al., 2007). Seed were generally vapor-phase sterilized (Clough and Bent, 1998), plated on medium and cold treated at 4°C for 72 h in darkness. Medium used for growth of seedlings was generally agar-solidified (0.8 % w/v) half-strength MS (Murashigue and Skoog, 1962) with 100 mM of filter-sterilized sorbitol or trehalose. The plates were then transferred for growth at 22°C in a long-day light cycle (16 h light/8 h dark) regime with 100 μM m⁻² s⁻¹ light intensity and 80% humidity. When grown in the dark, seedlings were first exposed to 6 h light at 22°C to promote germination before transfer to darkness at 22°C.

Seedling screening for trehalose resistance

Seed from the Arabidopsis FOX collection (Ichikawa et al., 2006; T1 generation) was collected using a bamboo skewer with a small pinhole at one end serving as a measure for 20-30 seed; collection was at the Plant Science Center, RIKEN Yokohama (Japan). Twenty to thirty T1 seed from 100 differing transgenic lines were pooled in one single pool; 141 pools covered the entire collection. Seedlings from differing pools are therefore necessarily from a differing transgenic event. During the primary screen, 1000 seed per pool of 100 independent transgenic lines were screened twice on plates containing half-strength MS salts and vitamins supplemented with 100 mM trehalose. The germination frequency varied between 100% and 30%. Seedlings were chosen over a period of 1 to 3 weeks of growth that displayed longer roots and more growth of leaf primordia than wild type (Fig. 1a 93-1 tre). For seven of the pools, more than one seedling of the pool (1-6 seedlings) was identified to be resistant. The seedlings were transferred to soil and grown for T2 seed set; a total of 157 seedlings were grown resulting in seed of 121 lines for a secondary screen. Seedlings recovered from the pool 70 mostly died upon transfer to soil, after repeated screening only one plant survived to set seed and was dealt with later in a separate secondary screening. Secondary screening was carried out on the T2 generation seed for each line by testing seedling growth after 14 d on 100 mM trehalose and 100 mM sorbitol to test specificity of the resistance to trehalose, and on medium with
12 μg L⁻¹ hygromycin B to evaluate expression of the marker gene associated with the presence of the T-DNA insertion. Lines from 6 different pools were retained after the secondary screening with average root length unchanged on sorbitol control but at least 3-fold longer than WT on trehalose medium. Lines obtained from differing pools necessarily are of independent transgenic origin, whilst lines obtained from the same pool likely are not. FOX Lines from the T2 generation were subsequently grown to generate T3 and T4 seed stock with 100 % trehalose resistance and homozygous for the presence of bZIP11 cDNA.

**Lugol and GUS staining**

Seedlings were grown for 14 d in long-day light cycle. Seedlings were harvested at midday, then washed in 70% ethanol before starch staining with 43.4 mM KI/5.7 mM I₂ (Lugol solution from Sigma, Wingler et al., 2000) and mounted in a mixture of chloral hydrate/glycerol/water (vol 8:1:2). To test the inhibition of bZIP11 translation, seedlings were grown in long-day light cycle for 7 d on MS medium, then transferred for 48 h to MS supplemented with either Suc or trehalose at 0, 20, 100 mM concentration. GUS staining was in GUS buffer (0.1 M NaH₂PO₄, 0.1 M Na₂HPO₄, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, 10 mM EDTA, 0.1% (v/v) Triton X-100, and 1 mg mL⁻¹ of 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid) according to (Wiese et al., 2004). The experiment was repeated with seedlings grown for 14 d on 100 mM of either sucrose or trehalose with results similar to those obtained after only 48 h incubation.

**Propidium iodide staining of roots and confocal microscopy**

Seedlings were grown for 5 d on MS supplemented with 100 mM of either sorbitol or trehalose. Seedlings were mounted with the root only between the cover slips and in 10 μM propidium iodide, mounting was immediately before visualization under the confocal microscope (63· NA 1.4 Plan apochromat water immersion objective, Leica SP2 inverted laser confocal microscope with an Ar 488-nm laser excitation, dichroic 488/543/633 and emission settings 562-588 nm).

**DNA extractions, PCR and sequencing**

To genotype the F2 generation seedlings obtained from the crosses of WT with FOX lines, seedlings were grown for 14 d on MS with 100 mM trehalose, then DNA was extracted from a single seedling with long root; the DNA extraction was as previously described (Cheung et al., 1993). Primers GS4 and GS6 (Supplemental Table SI) were used for PCR-amplification of the cDNA in the FOX lines as described in Ichikawa et al., 2006. Genotyping of tre1-1 was as recommended.
using primers LBB1, LP1tre1, RP1tre1, LP2tre1, RP2tre1 listed in Supplemental Table I.

Assays for trehalase activity

Trehalase activity was assayed as previously described with some modifications (Brodmann et al., 2002). Seedlings (T2) were grown on trehalose medium, short root WT seedlings or seedlings with long roots from the FOX lines were used. Seedlings were pooled as 70 mg FW and ground in 50 µl Buffer (0.1 M MES/KOH (pH 6.3), 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 mM DTT, 0.01% (vol/vol) Triton-X-100) at 4°C. Subsequently, 100 ul Buffer was added, the crude extract mixed then centrifuged 5 min, 13000 rpm at 4°C to remove the insoluble fraction. Sugars in the soluble supernatant were then removed by repeated dilution then concentration of the proteins in the extract using regenerated cellulose membrane (Amicon Ultra- 0.5 ml 10K Ultracel, Millipore), dilution was with 3 consecutive additions of 300 µl 20 mM MES/KOH (pH 6.3). The final volume of extract was adjusted to 50 µl. To assay trehalase, extract (10 µl) was incubated in triplicate with 15 mM trehalose for 1 h at 37°C, then boiled for 10 min. Alternatively control assays were boiled immediately. Glc released from trehalose was quantified (Enzytec D-Glucose kit, scil Diagnostics, Viemheim, Germany) as the difference between boiled controls and samples which were assayed for 1 h. Values were averaged from 3 biological replicates with standard deviations.

T6P determinations

Seedlings were grown under long-day growth conditions for 14 d. Five replicate samples of 50 mg each were harvested at midday by snap-freezing. Lactose-6-phosphate (5 nmol) was added as an internal standard. Materials were then ground frozen, extracted and analysed as described in (Delatte et al., 2009). Briefly, seedling extracts obtained by the subsequent liquid–liquid and solid-phase extractions were reconstituted in water and analysed by anion-exchange chromatography combined with electrospray ionization mass spectrometry. The method provided baseline resolution of T6P and allowed its specific detection at m/z 421 with good linearity. T6P concentrations were inferred from a five-point calibration curve using the signal obtained for the internal standard to correct for potential recovery losses.

Assays for SnRK1 activity

Seedlings were grown for 14 d in 100 mM sorbitol or trehalose, snap frozen at midday as 100 mg FW replicates. Three biological replicates for each data point were each ground frozen then extracted and T6P as well as sugars removed by the desalting procedure previously described (Zhang et al., 2009). SnRK1 activity determinations were using the AMARA peptide as substrate.
**Sugardeterminations**

Soluble sugars were extracted from samples of 14 d old seedlings; samples were 3 biological replicates for each data point. The extraction procedure was as previously described (Schluepmann et al., 2003). Sucrose, glucose and fructose were assayed enzymatically (Enzytec D-Glucose/D-Fructose/Sucrose kit, scil Diagnostics, Viemheim, Germany).

**RNA extraction and Q-PCR**

RNA was extracted from flash frozen seedlings collected at midday (50 mg FW). Samples were in triplicate biological replicates for each data point and the extraction protocol was according to the instruction manual (Spectrum Plant Total RNA Kit, Sigma-Aldrich). DNase treatment, reverse transcription and Q-PCR were as described previously (Hanson et al., 2008). Primers used for the Q-PCR reactions are described in Supplemental Table SII. Primers from the genes jointly targeted by KIN10 and bZIP11 were taken from the CATMA site http://www.catma.org/.
ACKNOWLEDGMENTS

We wish to thank Dr. Jyan-Chyun Jang from the Department of Horticulture and Crop Science and Plant Biotechnology Center at The Ohio State University, U.S., for providing the bZIP1 over-expressing and anti-sense lines. We thank Prof. Patrick van Dijck and Dr. Lies Vandesteene from K.U.-Leuven, Belgium, for providing the tre1-2 line in the Ler background, Dr. Elena Baena-Gonzalez from the Instituto Gulbenkian de Ciência in Oeiras, Portugal, for providing the KIN10 over-expressing lines. We thank Fritz Kind and Ronald Leito from the department of Biology at Utrecht University for support in photography. In addition, we would like to thank two anonymous reviewers for their pertinent comments.
Table I. Genes jointly controlled by SnRK1 and bZIP11

Two hundred and sixty one differentially expressed genes 2 h after nuclear transfer of bZIP11 (Hanson et al., 2008) were compared with the 1021 genes altered by 6 h transient KIN10 expression in protoplasts (Baena-Gonzalez et al., 2007), the commonly regulated genes are listed with TAIR annotation (May 2011).

<table>
<thead>
<tr>
<th>Induced</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>At1g02660</td>
<td>alpha/beta-Hydrolases superfamily protein; putative triglyceride lipase activity</td>
</tr>
<tr>
<td>At1g10070</td>
<td>BCAT-2 chloroplast branched-chain amino acid aminotransferase</td>
</tr>
<tr>
<td>At1g18460</td>
<td>alpha/beta-Hydrolases superfamily putative lipase family</td>
</tr>
<tr>
<td>At1g32170</td>
<td>XTR4 xyloglucan endotransglycosylase-related protein</td>
</tr>
<tr>
<td>At1g62510</td>
<td>Bilfunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein</td>
</tr>
<tr>
<td>At1g64660</td>
<td>MGL cytosolic methionine gamma lyase</td>
</tr>
<tr>
<td>At2g25200</td>
<td>Plant protein of unknown function (DUF868)</td>
</tr>
<tr>
<td>At2g30600</td>
<td>BTB/POZ domain-containing protein; involved in cell adhesion</td>
</tr>
<tr>
<td>At2g32150</td>
<td>Haloacid dehalogenase-like hydrolase (HAD) superfamily protein; nitrate responsive</td>
</tr>
<tr>
<td>At2g33380</td>
<td>CALEOSIN 3 calcium binding, induced by NaCl, ABA and dessication</td>
</tr>
<tr>
<td>At2g36220</td>
<td>induced protein</td>
</tr>
<tr>
<td>At2g38400</td>
<td>AGT3 alanine:glyoxylate aminotransferase 2 homolog</td>
</tr>
<tr>
<td>At2g39570</td>
<td>ACT domain-containing protein; functions in amino acid binding</td>
</tr>
<tr>
<td>At2g47770</td>
<td>TSPO(outerior membrane tryptophan-rich sensory protein)-related</td>
</tr>
<tr>
<td>At3g13450</td>
<td>DIN4 branched chain alpha-keto acid dehydrogenase E1 beta</td>
</tr>
<tr>
<td>At3g26510</td>
<td>Oticosapese/Phox/Bem1p family protein</td>
</tr>
<tr>
<td>At3g30775</td>
<td>ERD5, PRO1, PRODH, proline dehydrogenase</td>
</tr>
<tr>
<td>At3g47340</td>
<td>ASN1 glutamine-dependent asparagine synthetase</td>
</tr>
<tr>
<td>At3g48360</td>
<td>BT2 (AIBT-2) component of the TAC1-mediated telomerase activation pathway</td>
</tr>
<tr>
<td>At3g57520</td>
<td>RS2, SIP2 raffinose-specific alpha-galactosidase</td>
</tr>
<tr>
<td>At3g61060</td>
<td>PP2-A13, Phloem protein 2-A13</td>
</tr>
<tr>
<td>At3g61890</td>
<td>HB-12, homeobox-leucine zipper protein HB-12</td>
</tr>
<tr>
<td>At4g15530</td>
<td>PPDK pyruvate,orthophosphate dikinase</td>
</tr>
<tr>
<td>At4g28040</td>
<td>nodulin MtN21 /EamA-like transporter family protein (Drug/Metabolite Transporter)</td>
</tr>
<tr>
<td>At4g35770</td>
<td>SEN1 senescence-associated protein</td>
</tr>
<tr>
<td>At5g04040</td>
<td>SDP1, triacylglycerol lipase that is involved in storage lipid breakdown</td>
</tr>
<tr>
<td>At5g18670</td>
<td>BMY3, BAM9 glycosyl hydrolase family 14 (beta-amylose)</td>
</tr>
<tr>
<td>At5g22290</td>
<td>CHY-type/CTCHY-type/RING-type Zinc finger protein</td>
</tr>
<tr>
<td>At5g49360</td>
<td>BXL1, bifunctional iD-xylolidase/x-L-arabinofuranosidase</td>
</tr>
<tr>
<td>At5g53590</td>
<td>SAUR-like auxin-responsive protein family</td>
</tr>
<tr>
<td>At5g66170</td>
<td>STR18, Encodes a thiosulfate sulfurtransferase/rhodanase</td>
</tr>
<tr>
<td>At5g66650</td>
<td>Protein of unknown function DUF607</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Repressed</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>At1g26770</td>
<td>EXP10 (Expansins(Alpha-Expansin Gene Family)) : expansin 10</td>
</tr>
<tr>
<td>At1g64060</td>
<td>RboH(Respiratory burst oxidase family, Cytochrome b558 - H⁺-channel)</td>
</tr>
<tr>
<td>At1g69530</td>
<td>EXP1 (Alpha-Expansin Gene Family)</td>
</tr>
<tr>
<td>At1g70230</td>
<td>TBL27, trichome birefringence-like, plant-specific DUF231</td>
</tr>
<tr>
<td>At1g76790</td>
<td>IGMT5, indole glucosinolate O-methyl transferase 5</td>
</tr>
<tr>
<td>At2g18660</td>
<td>Major facilitator superfamily protein, endomembrane system</td>
</tr>
<tr>
<td>At2g38170</td>
<td>CAX1, high affinity vacuolar calcium antipporter</td>
</tr>
<tr>
<td>At2g38940</td>
<td>PHT1:4 member of the Pht1 family of phosphate transporters</td>
</tr>
<tr>
<td>At3g09270</td>
<td>GSTU8 glutathione transferase belonging to the tau class of GSTs</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oppositely regulated</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>At2g14170</td>
<td>ALDH6B2, methylmalonate-semialdehyde dehydrogenase</td>
</tr>
<tr>
<td>At3g57040</td>
<td>ARR9 Response Regulator A- Type, cytokinin signalling</td>
</tr>
<tr>
<td>At5g17760</td>
<td>P-loop containing nucleoside triphosphate hydrolases superfamily protein</td>
</tr>
</tbody>
</table>

*a also in the list of 7 genes which are induced by bZIP11 under the control of its own promoter.*
**FIGURE LEGENDS**

**Figure 1.** Carbon allocation, growth and trehalase activity of seedlings on 100 mM trehalose. Seedlings were grown under long-day conditions for 14 d on medium with 100 mM sorbitol osmoticum control (sorb) or trehalose (tre). A, Starch staining. Seedlings were harvested at midday, stained with lugol and mounted in chloral hydrate. WT, wild type Col.0 seedlings; pgm1, seedlings lacking plastidic phosphoglucomutase 1 (Caspar et al., 1985); 93-1, seedlings from the FOX line 93-1. The bar represents 3 mm. B, Root lengths. Average root-lengths from n> 20 seedlings of the differing genotypes with standard deviation. WT, wild type; treF, seedlings over-expressing *E. coli* trehalase treF; lines from the FOX collection of FOX pools 128, 89, 33 and 93 were line 128, 89-1, 89-3, 33-1G, 33-1, 93-1 and 93-32. C, Trehalase activity in extracts of seedlings grown on trehalose for 14 d; the data are averages with standard deviations of 3 independent extracts. *ANOVA P< 0.050.

**Figure 2.** Characterization of the independent FOX lines expressing bZIP11 cDNA from the pools 33, 93 and 70. A, The trehalose resistance is a dominant trait as shown for lines 33-1 and 93-1. P, seedlings from the parental lines: WT, wild type; 33-1, FOX line 33-1; 93-1, FOX line 93-1. F1, the first generation from FOX line crosses with WT. B, PCR amplification of the FOX cDNA using DNA template from plants of the lines 70, 128, 89, 93-1, 33-1; MW, molecular weight marker lambda Pst1. C, Expression of bZIP11 in 14-d-old seedlings from FOX lines 70, 93, the line expressing the *E. coli* trehalase treF (treF) and WT Col.0. D, Expression of the TRE1 trehalase in the genotypes from C. E, Seedlings with altered expression of bZIP1 do not grow on medium with 100 M trehalose (tre) compared to sorbitol (sorb): WT, wild type; bZIP1oe and bZIP1as are the bZIP1 over-expressing and antisense lines described in Kang et al. (2010). F, Expression of bZIP1 in seedlings of the FOX lines 70 and 93, the line expressing treF and WT grown on medium with sorbitol (sorb) or trehalose (tre). Expression was determined by QPCR and is given relative to PP2A (At1g13320). Error bars represent the standard deviation of 3 replicates.

**Figure 3.** The roles of trehalase (TRE1) and of uORF2 in the FOX lines growing on trehalose. Error bars represent the standard deviation of 3 replicates. A, Expression of TRE1 in wild type Col.0 (WT) and seedlings from two different plants of the tre1-1 line (Salk 147073c). B, Trehalase activity in flowers from wild type (WT) and several plants from the tre1-1 line (P1-4). C, tre1-1 and tre1-2 seedling growth compared to their respective WT. Growth was on MS medium without (MS) or with 25 and 50 mM trehalose (25 mM T, 50 mM T). D, Genotype analysis of long root seedlings in the F2 generation of the cross 93-1 with wild type. DNA from wild type (WT) and 11 differing seedlings (1-10) was used as template. PCR was carried out to amplify the wild type sequence of TRE1 (WT TRE1) or the T-DNA insertion at the TRE1 locus (KO TRE1) in the above agarose gel (tre1). PCR was also
carried out to amplify the FOX cDNA in the gel below (FOX cDNA). E, Unlike on sucrose, translational repression of bZIP11 does not occur on trehalose. Seedlings expressing the 5 prime mRNA uORFs of the bZIP11 mRNA fused to the GUS gene were grown for 7 d on MS medium, transferred for 48 h to medium with sucrose (SUC) or trehalose (TRE) at 0, 20 or 100 mM (0, 20, 100), then stained for GUS activity.

**Figure 4.** T6P accumulation and *in vitro* T6P inhibition of SnRK1 in seedlings grown on trehalose. WT, wild type Col.0; TreF, seedlings expressing *E. coli* trehalase treF; 93, 70 and 33 seedlings from the FOX lines 93, 70 and 33 respectively. A, Seedlings grown on osmoticum control for 14 d (100 mM sorbitol). B, Seedlings grown on 100 mM trehalose for 14 d. C, SnRK1 activity assayed using the AMARA peptide in 14-d-old seedlings grown on 100 mM trehalose from wild type accession Col.0 (WT Col.0), Fox lines 93 and 33 and from wild type accession Ler (WT Ler). Error bars represent SEM of 3 biological replicates.

**Figure 5.** KIN10 over-expressing seedlings grow on trehalose without increased trehalase or bZIP11 expression. Seedlings were grown on trehalose (tre) compared to osmoticum control (sorb) for 14 d. A, Phenotype of Ler wild type (WT) and KIN10 over-expression line O1. B, trehalase activity in extracts from Ler WT and the lines over-expressing KIN10 O1 and O2. C, *TRE1* expression determined relative to *PP2A* by Q-PCR in the genotypes from B. D, *bZIP11* expression. The levels are averages of three biological replicates and error bars are standard deviation.

**Figure 6.** Do KIN10 and bZIP11 act in the same pathway? A, Expression of targets common to KIN10 and bZIP11. Seedlings of WT were grown for 14 d on 100 mM of either sorbitol (sorb) or trehalose (tre), collected at midday. Expression was determined by Q-PCR relative to PP2A, then normalized to the level of expression on sorbitol. B, Soluble sugars Suc, Glc and Fru in the seedlings with the genotype WT (Col.0), treF expressors (treF), bZIP11 expressors from line 93 (93) and 33 (33), wild type Ler (ler), KIN10 overexpressing lines O1 and O2. In A and B levels are averages of three biological replicates and error bars are standard deviation. C, Phenotype of bZIP11-expressing seedlings on trehalose in continuous darkness. After 78 h at 4°C, seed were exposed to light and 22°C for 6 h then grown for 14 d in continuous darkness on medium with 100 mM of either sorbitol (sorb) or trehalose (tre). WT, wild type Col.0; 33-1-1 and 33-1-2 seed from two plants of the FOX-line 33-1; 93-3-2, seed from the FOX line 93. D, Phenotype of KIN10-expressing seedlings on trehalose in continuous darkness. Seed were treated as in C; WT, wild type Ler; KIN10oe, seed from the O2 line (Baena-Gonzalez et al., 2007).
Figure 7. A) Model of interactions affecting growth and starch accumulation on trehalose when T6P accumulates. Glucose (Glc) and sucrose (Suc) feeding cause AGPase redox activation and thus starch synthesis by differing pathways that are likely also relevant for the growth responses to these sugars (Tiessen et al., 2003; Michalska et al., 2009; Geigenberger, 2011). When feeding Glc, T6P does not accumulate (Delatte et al., unpublished) and glucose-6-phosphate (G6P) in plastids is shunted through the oxidative part of the pentose phosphate pathway (OPP) generating NADPH for NADPH-Thioredoxin Reductase C (NTRC) dependent reduction of AGPase and thus activation. In contrast, feeding sucrose or trehalose leads to T6P increase which acts upon AGPase redox by an unknown mechanism (Schluempmann et al., 2004; Kolbe et al., 2005; Lunn et al., 2006; Michalska et al., 2009). Sucrose inhibits translation of bZIP11 by way of uORF2 (Wiese et al., 2004), but trehalose does not. When feeding trehalose, T6P accumulates. T6P accumulation inhibits SnRK1; this inhibition of SnRK1 depends on an intermediary Factor I present in young tissues (Zhang et al. 2009). Possibly, SnRK1 phosphorylation activates bZIP11 transfer to the nucleus or complexing of the transcription factor in such a way that bZIP11 controls a part of the SnRK1 output that is required for growth. Thus when T6P accumulates and inhibits SnRK1 in young tissues, over-expression of bZIP11 may act as a surrogate for SnRK1. B) Antisense SnRK1 restricted to individual pollen of barley in particular (Zhang et al., 2001) but also work in developing potato tubers (Purcell et al., 1998) show that SnRK1 is required in growing heterotrophic cells for growth and starch accumulation. It therefore is possible that SnRK1 is needed to respond to nutrient stress so as to make carbon available in growing sinks. SnRK1 inhibition (by artificially increasing T6P when feeding trehalose or by antisense SnRK1) would hence uncouple growth from carbon starvation responses leading to the swollen cells observed in the growing zones of roots of Arabidopsis seedlings on trehalose (tre) compared to sorbitol (sorb). Root tips were stained with propidium iodide in water immediately prior to visualization under the confocal microscope.
LITERATURE CITED


Halford NG, Hey SJ (2009) Snf1-related protein kinases (SnRKs) act within an intricate network that links metabolic and stress signalling in plants. Biochemical Journal 419: 247-259


www.plantphysiol.org on December 30, 2017 - Published by Downloaded from Copyright © 2011 American Society of Plant Biologists. All rights reserved.


Figure 1. Carbon allocation, growth and trehalase activity of seedlings on 100 mM trehalose. Seedlings were grown under long-day conditions for 14 d on medium with 100 mM sorbitol osmoticum control (sorb) or trehalose (tre). A, Starch staining. Seedlings were harvested at midday, stained with lugol and mounted in chlora hydrate. WT, wild type Col.0 seedlings; pgm1, seedlings lacking plastidic phosphoglucomutase 1 (Caspar et al., 1985); 93-1, seedlings from the FOX line 93-1. The bar represents 3 mm. B, Root lengths. Average root-lengths from n > 20 seedlings of the differing genotypes with standard deviation. WT, wild type; treF, seedlings over-expressing E.coli trehalase treF; lines from the FOX collection of FOX pools 128, 89, 33 and 93 were line 128, 89-1, 89-3, 33-1G, 33-1, 93-1 and 93-32. C, Trehalase activity in extracts of seedlings grown on trehalose for 14 d; the data are averages with standard deviations of 3 independent extracts. *ANOVA P< 0.050.
Figure 2. Characterization of the independent FOX lines expressing bZIP11 cDNA from the pools 33, 93 and 70. A, The trehalose resistance is a dominant trait as shown for lines 33-1 and 93-1. P, seedlings from the parental lines: WT, wild type; 33-1, FOX line 33-1; 93-1, FOX line 93-1. F1, the first generation from FOX line crosses with WT. B, PCR amplification of the FOX cDNA using DNA template from plants of the lines 70, 128, 89, 93-1, 33-1; MW, molecular weight marker lambda Pst1. C, Expression of bZIP11 in 14-d-old seedlings from FOX lines 70, 93, the line expressing the E.coli trehalose treF (treF) and WT Col.0. D, Expression of the TRE1 trehalase in the genotypes from C. E, Seedlings with altered expression of bZIP1 do not grow on medium with 100 M trehalose (tre) compared to sorbitol (sorb): WT, wild type; bZIP1oe and bZIP1as are the bZIP1 over-expressing and antisense lines described in Kang et al. (2010). F, Expression of bZIP1 in seedlings of the FOX lines 70 and 93, the line expressing treF and WT grown on medium with sorbitol (sorb) or trehalose (tre). Expression was determined by QPCR and is given relative to TUB2 (At1g13320). Error bars represent the standard deviation of 3 replicates.
Figure 3. The roles of trehalase (TRE1) and of uORF2 in the FOX lines growing on trehalose. Error bars represent the standard deviation of 3 replicates. A, Expression of TRE1 in wild type Col.0 (WT) and seedlings from two different plants of the tre1-1 line (Salk 147073c). B, trehalase activity in flowers from wild type (WT) and several plants from the tre1-1 line (P1-4). C, tre1-1 and tre1-2  seedling growth compared to their respective WT. Growth was on MS medium without (MS) or with 25 and 50 mM trehalose (25 mM T, 50 mM T). D, Genotype analysis of long root seedlings in the F2 generation of the cross 93-1 with wild type. DNA from wild type (WT) and 11 differing seedlings (1-10) was used as template. PCR was carried out to amplify the wild type sequence of TRE1 (WT TRE1) or the T-DNA insertion at the TRE1 locus (KO TRE1) in the above agarose gel (tre1). PCR was also carried out to amplify the FOX cDNA in the gel below (FOX cDNA). E, Unlike on sucrose, translational repression of bZIP11 does not occur on trehalose. Seedlings expressing the GUS transgene under the control of the bZIP11 mRNA fused to the GUS gene were grown for 7 d on MS medium, transferred for 48 h to medium with sucrose (SUC) or trehalose (TRE) at 0, 20 or 100 mM (0, 20, 100), then stained for GUS activity.
Figure 4. T6P accumulation and in vitro T6P inhibition of SnRK1 in seedlings grown on trehalose. WT, wild type Col.0; treF, seedlings expressing E.coli trehalase treF; 93, 70 and 33 seedlings from the FOX lines 93, 70 and 33 respectively. A, Seedlings grown on osmoticum control for 14 d (100 mM sorbitol). B, Seedlings grown on 100 mM trehalose for 14 d. C, SnRK1 activity assessed using the AMARA assay in 14 d-old seedlings grown on 100 mM trehalose from wild type accession Col.0 (WT Col.0), Fox lines 93 and 33 and from wild type accession Ler (WT Ler). Error bars represent SEM of 3 biological replicates.
Figure 5. KIN10 over-expressing seedlings grow on trehalose without increased trehalase or bZIP11 expression. Seedlings were grown on trehalose (tre) compared to osmoticum control (sorb) for 14 d. A, Phenotype of Ler wild type (WT) and KIN10 over-expression line O1. B, trehalase activity in extracts from Ler WT and the lines over-expressing KIN10 O1 and O2. C, TRE1 expression determined relative to PP2A by Q-PCR in the genotypes from B. D, bZIP11 expression. The levels are averages of three biological replicates and error bars are standard deviation.
Figure 6. Do KIN10 and bZIP11 act in the same pathway? A, Expression of targets common to KIN10 and bZIP11. Seedlings of WT were grown for 14 d on 100 mM of either sorbitol (sorb) or trehalose (tre), collected at midday. Expression was determined by Q-PCR relative to PP2A, then normalized to the level of expression on sorbitol. B, Soluble sugars Suc Glc and Fru in the seedlings with the genotype WT (Col.0), treF expressors (treF), bZIP11 expressors from line 93 (93) and 33 (33), wild type Ler (ler), KIN10 overexpressing lines O1 and O2. In A and B levels are averages of three biological replicates and error bars are standard deviation. C, Phenotype of bZIP11-expressing seedlings on trehalose in continuous darkness. After 78 h at 4°C, seed were exposed to light and 22°C for 6 h then grown for 14 d in continuous darkness on medium with 100 mM of either sorbitol (sorb) or trehalose (tre), WT, wild type Col.0; 33-1-1 and 33-1-2 seed from two plants of the FOX-line 33-1; 93-3-2, seed from the FOX line 93. D, Phenotype of KIN10-expressing seedlings on trehalose in continuous darkness. After 78 h at 4°C, seeds were exposed to light and 22°C for 6 h then grown for 14 d in continuous darkness on medium with 100 mM of either sorbitol (sorb) or trehalose (tre), WT, wild type Col.0; KIN10oe, seed from the O2 line (Baena-Gonzalez et al., 2007).
Figure 7. A) Model of interactions affecting growth and starch accumulation on trehalose when T6P accumulates. Glucose (Glc) and sucrose (Suc) feeding cause AGPase redox activation and thus starch synthesis by differing pathways that are likely also relevant for the growth responses to these sugars (Tiessen et al., 2003; Michalska et al., 2009; Geigenberger, 2011). When feeding Glc, T6P does not accumulate (Delatte et al., unpublished) and glucose-6-phosphate (G6P) in plastids is shunted through the oxidative part of the pentose phosphate pathway (OPP) generating NADPH for NADPH-Thioredoxin Reductase C (NTRC) dependent reduction of AGPase and thus activation. In contrast, feeding sucrose or trehalose leads to T6P increase which acts upon AGPase redox by an unknown mechanism (Schluepmann et al., 2004; Kolbe et al., 2005; Lunn et al., 2006; Michalska et al., 2009). Sucrose inhibits translation of bZIP11 by way of uORF2 (Wiese et al., 2004), but trehalose does not. When feeding trehalose, T6P accumulates. T6P accumulation inhibits SnRK1; this inhibition of SnRK1 depends on an intermediary Factor I present in young tissues (Zhang et al. 2009). Possibly, SnRK1 phosphorylation activates bZIP11 transfer to the nucleus or complexing of the transcription factor in such a way that bZIP11 controls a part of the SnRK1 output that is required for growth. Thus when T6P accumulates and inhibits SnRK1 in young tissues, over-expression of bZIP11 may act as a surrogate for SnRK1. B) Antisense SnRK1 restricted to individual pollen of barley in particular (Zhang et al., 2001) but also work in developing potato tubers (Purcell et al., 1998) show that SnRK1 is required in growing heterotrophic cells for growth and starch accumulation. It therefore is possible that SnRK1 is needed to respond to nutrient stress so as to make carbon available in growing sinks. SnRK1 inhibition (by artificially increasing T6P when feeding trehalose or by antisense SnRK1) would hence uncouple growth from carbon starvation responses leading to the swollen cells observed in the growing zone or roots of potato tubers on trehalose. T6P compared to sorbitol (sorb). Root tips were stained with propidium iodide in water immediately prior to visualization under the confocal microscope.