# The sucrose-regulated Arabidopsis transcription factor bZIP11 reprograms metabolism and regulates trehalose metabolism

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## Summary

- The Arabidopsis basic region-leucine zipper transcription factor 11 (*bZIP11*) is known to be repressed by sucrose through a translational inhibition mechanism that requires the conserved sucrose control peptide encoded by the mRNA leader. The function of bZIP11 has been investigated in over-expression studies, and bZIP11 has been found to inhibit plant growth. The addition of sugar does not rescue the growth inhibition phenotype. Here, the function of the bZIP11 transcription factor was investigated.
- The mechanism by which bZIP11 regulates growth was studied using large-scale and dedicated metabolic analysis, biochemical assays and molecular studies.
- bZIP11 induction results in a reprogramming of metabolism and activation of genes involved in the metabolism of trehalose and other minor carbohydrates such as myo-inositol and raffinose. bZIP11 induction leads to reduced contents of the prominent growth regulatory molecule trehalose 6-phosphate (T6P).
- The metabolic changes detected mimic in part those observed in carbon-starved plants. It is proposed that bZIP11 is a powerful regulator of carbohydrate metabolism that functions in a growth regulatory network that includes T6P and the sucrose non-fermenting-1 related protein kinase 1 (SnRK1).

## Introduction

The Arabidopsis basic region-leucine zipper transcription factor 11 (*bZIP11*) gene is a member of the *bZIP* transcription factor gene family. *bZIP11* belongs to the S1 *bZIP* group that further consists of *bZIP1*, *2*, *44* and *53* (Jakoby *et al.*, 2002). *bZIP11* is expressed throughout the entire plant life cycle, especially in vascular tissues, such as the veins and their surrounding cells. Ectopic over-expression of *bZIP11* in Arabidopsis strongly inhibits plant growth in a bZIP11 concentration-dependent manner (Hanson *et al.*, 2008). Previous studies showed that bZIP11 has a function in regulating amino acid metabolism, but a broader function in metabolism was suggested by transcriptomic analysis. Increased bZIP11 nuclear activity leads to rapid changes in the levels of several amino acids, and two genes involved in amino acid metabolism, proline dehydrogenase 2 (*ProDH2*) and asparagine synthetase 1

(*ASN1*), were proposed as direct targets of bZIP11. In this paper we explore the wider regulatory potential of bZIP11 in metabolism.

bZIP11 is probably part of a molecular network that perceives the cellular sugar status and regulates metabolism and growth. The expression of *bZIP11* can be induced by sugars, such as sucrose. However, the translation of *bZIP11* and all other S1 *bZIP* transcription factors is repressed specifically by sucrose in a concentration-dependent manner. Transcripts of S1 group genes encode an evolutionarily conserved sucrose control (SC) peptide in their 5′-leader regions. Sucrose signaling represses the translation of *bZIP* main open reading frames (ORFs) and this repression requires the full-length SC peptide. It was postulated that in the presence of sucrose the SC peptide induces a stalling complex on *bZIP* mRNAs that prevents the translation of *bZIP* main ORFs (Wiese *et al.*, 2005; Hummel *et al.*, 2009; Rahmani *et al.*, 2009; Weltmeier *et al.*, 2009).

The regulation of metabolism is crucial for plant growth and development. In addition to bZIP11, several other regulatory systems have been identified that perceive and integrate metabolic signals to regulate growth (Smeekens *et al.*, 2010). These regulatory systems are often of evolutionarily ancient origin, such as the sucrose non-fermenting-1 related protein kinase 1 (SnRK1) which is a central regulator of metabolic stress responses and is found in all eukaryotic organisms (Halford & Hey, 2009). Another regulatory system that is particularly important in plants is the trehalose 6-phosphate (T6P) signaling system which links sugar status to growth (Paul, 2008; Smeekens *et al.*, 2010). The functioning and cross-talk of these and other identified regulatory systems must be resolved in molecular detail to understand the control of cellular metabolism and growth.

The SnRK1 protein kinase functions as a central regulator of metabolism that links energy status to growth. SnRK1 activation by nutrient depletion generally promotes catabolism and inhibits anabolism (Baena-Gonzalez *et al.*, 2007). Importantly, in protoplast co-transfection experiments, the SnRK1 catalytic subunits *KIN10* (At3g01090) and *KIN11* (At3g29160) were found to significantly augment the transcriptional potential of the S1 group bZIP transcription factors, including bZIP11 (Baena-Gonzalez *et al.*, 2007). Interestingly, it was shown that at physiological levels T6P inhibits the activity of SnRK1 (Zhang *et al.*, 2009), showing the interaction of these two regulatory systems.

T6P is a signaling molecule that has been shown to be a powerful growth regulator through the promotion of carbon utilization (Schluepmann *et al.*, 2003). T6P levels are positively correlated with the cellular sucrose status (Lunn *et al.*, 2006). In plants, T6P is synthesized from UDP-glucose and glucose 6-phosphate in a reaction catalyzed by trehalose 6-phosphate synthase (TPS). T6P is dephosphorylated by trehalose 6-phosphate phosphatase (TPP) to trehalose, which can be hydrolyzed to glucose by trehalase (TRE1). In Arabidopsis, 22 genes are annotated as associated with trehalose metabolism (Leyman *et al.*, 2001; Paul *et al.*, 2008). The expression patterns of various trehalose metabolism genes have been reported and enzymatic characterization of the proteins has been carried out (Muller *et al.*, 2001; Frison *et al.*, 2007; Ramon *et al.*, 2009; Vandesteene *et al.*, 2010). In Arabidopsis, TPS1 was proposed to be the single active T6P synthase (Vandesteene *et al.*, 2010). In the *tps1* mutant, the absence of T6P results in embryo lethality, growth inhibition of vegetative plants and repression of floral transition (Eastmond *et al.*, 2002; van Dijken *et al.*, 2004; Gomez *et al.*, 2006, 2010). Multiple stimuli affect the expression of the trehalose metabolism gene family. However, transcriptional regulators of these genes have not been reported to date.

In this study, the regulatory functions of bZIP11 in metabolism were explored. Large-scale metabolic analysis revealed that the induced nuclear presence of bZIP11 reprogrammed carbohydrate metabolic profiles dramatically. bZIP11 promoted a metabolic status that to some extent mimicked low-carbon conditions. Interestingly, bZIP11-mediated growth inhibition was not rescued by metabolizable sugars. The regulatory effect of bZIP11 on several metabolic pathways is presented, with emphasis on myo-inositol, raffinose and trehalose metabolism. bZIP11 regulated several trehalose metabolism genes, including *TRE1*, *TPP5* and *TPP6*, and induced trehalase enzymatic activity, with concomitant changes in trehalose and T6P levels. Thus, bZIP11 can act as a regulator of metabolism, is probably involved in carbon starvation adaptation responses, and is likely to function in an SnRK1–bZIP11–T6P regulatory circuit.

## Materials and Methods

### Plant material and growth conditions

*Arabidopsis thaliana* (var. Columbia (Col-0), CS60000) wild type (WT) and bZIP11 dexamethasone (dex)-inducible transgenic lines L and M in the Col-0 accession were created by Hanson *et al.* (2008). WT or transgenic line seeds were chlorine gas sterilized and stratified at 4°C in the dark for 2 d. For liquid medium culture, seedlings were grown in half-strength MS medium including vitamins and MES buffer (Duchefa; http://www.duchefa.com), supplemented with 100 mM sucrose for 7 d under continuous fluorescent light (100 μmol $m^{-2}$ $s^{-1}$) on a rotary shaker (60 rpm). Seedlings were treated with dexamethasone (10 μM) or mock treated for 6 h before being harvested in liquid nitrogen. For solid medium culture, the seedlings were grown on half-strength MS medium including vitamins and MES buffer (Duchefa) solidified using 0.8% (w/v) plant agar (Duchefa) supplemented with sugars as indicated and 0.2 μM dexamethasone, or mock treated,

and grown under a photoperiod of 16 h florescent light (100 μmol $m^{-2}$ $s^{-1}$) and 8 h dark for 7 d.

## GC-MS metabolic analysis

Seedlings cultured in liquid medium were carefully washed in deionized water and snap-frozen in liquid nitrogen. Ten biological replicates were used for each treatment per seedling line. Metabolites were extracted from the samples and analyzed according to the methods described by Gullberg *et al.* (2004), with some minor changes. Briefly, stable isotope reference compounds (15 ng $\mu l^{-1}$ each of [1,2,3-$^{13}C_3$]-myristic acid, [1,2,3,4-$^{13}C_4$]-hexadecanoic acid, [2,2,3,3-$^{2}H_4$]-succinic acid, [$^{13}C_5$, $^{15}N$]-glutamic acid, [25,26,26,26,27,27,27-$^{2}H_7$]-cholesterol, [$^{13}C_5$]-proline, [1,2,3,4-$^{13}C_4$]-disodium 2-oxoglutarate, [$^{13}C_{12}$]-sucrose, [2,2,3,3-$^{2}H_4$]-putrescine, [$^{2}H_6$]-salicylic acid and [$^{13}C_6$]-glucose) were added to an extraction mixture consisting of chloroform:MeOH:$H_2O$ (6 : 2 : 2). The samples (20 mg each) were then extracted in 1 ml of the extraction mixture, using a vibration mill set to a frequency of 30 Hz for 3 min, with 3-mm tungsten carbide beads added to each extraction tube to increase the extraction efficiency. The extracts were then centrifuged for 10 min at 16 100 ***g*** before 200 μl of each supernatant was transferred to a GC vial and evaporated to dryness. The samples were then derivatized by shaking them with 30 μl of methoxyamine hydrochloride (15 mg $ml^{-1}$) in pyridine for 10 min at 5°C before incubation for 16 h at room temperature. The samples were then trimethylsilylated by adding 30 μl of MSTFA (N-Methyltrimethylsilyltrifluoroacetamide) with 1% TMCS (Trimethylchlorosilane) to the samples and incubating them for 1 h at room temperature. After silylation, 30 μl of heptane was added.

Samples were analyzed, according to Gullberg *et al.* (2004), using GC-TOFMS (Gas Chromatography–Time of Flight Mass Spectrometer) together with blank control samples and a series of n-alkanes ($C_{12}$-$C_{40}$), which allowed retention indices to be calculated (Schauer *et al.*, 2005). One μl of each derivatized sample was injected splitless into a gas chromatograph equipped with a 10 m × 0.18 mm i.d. fused silica capillary column with a chemically bonded 0.18-μm DB 5-MS (www.home.agilent.com) stationary phase. The injector temperature was 270°C, the septum purge flow rate was 20 ml $min^{-1}$ and the purge was turned on after 60 s. The gas flow rate through the column was 1 ml $min^{-1}$; the column temperature was held at 70°C for 2 min, and then increased by 40°C $min^{-1}$ to 320°C, and held there for 2 min. The column effluent was introduced into the ion source of a Pegasus III GC-TOFMS (Leco, Michigan, USA). The transfer line and the ion source temperatures were 250°C and 200°C, respectively. Ions were generated by a 70-eV electron beam at an ionization current of 2.0 mA, and 30 spectra $s^{-1}$ were recorded in the mass range 50–800 m $z^{-1}$. The acceleration voltage was turned on after a solvent delay of 170 s. The detector voltage was 1660 V. All nonprocessed MS files from the metabolic analysis were exported into MATLAB (Math Works Natick, Massachusetts, USA), in which all data pretreatment procedures, such as baseline correction, chromatogram alignment, and hierarchical multivariate curve resolution (H-MCR) were performed using custom scripts (Jonsson *et al.*, 2005). All manual integrations were performed using CHROMATOF 2.32 software (Leco, Michigan, USA) or custom scripts. The data were normalized to the peak areas of the internal standards using the first scores vector (t1) from a principal component analysis model as a normalization vector.

## Construction of plasmid DNA

For transient expression of *bZIP* transcription factors in protoplasts, 35S driven 3× HA tagged *bZIP* constructs were made (Ehlert *et al.*, 2006). The *bZIP* transcription factor sequences were cloned into a modified version of the pHBT vector using Gateway® technology (Invitrogen; http://www.invitrogen.com). The modified pHBT, designated pHBTLDGFP, was created by Nco*I*/Not*I* digestion, Klenow fill-in and religation (T. Heinekamp, pers. comm.). The promoters of the *TRE1* (At4g24040), *TPP5* (At4g12430) and *TPP6* (At4g22590) genes were fused to the luciferase gene in pUGW35 (T. Nakagawa, pers. comm.) using Gateway™ technology (Invitrogen). The fragment of each promoter was amplified from Arabidopsis WT genomic DNA using primers listed in Supporting Information Table S1.

## Transient transcriptional activation test in protoplasts

Protoplasts were extracted from rosette leaves of 3–4-wk-old WT plants grown on soil under long-day (16 h light : 8 h dark) conditions. Protoplasts were co-transfected with the construct described above in the section Construction of plasmid DNA and plasmids containing either 35S-bZIP11 or 35S-ATH1. Luciferase activity was assayed for each co-transfection sample according to the Dual-Luciferase® Reporter (DLR™) Assay System protocol (Promega; http://www.promega.com) using a GloMax™ 20/20 Luminometer (Promega). This whole procedure followed the method described in Hanson *et al.* (2008), except for the adaptation of incubation time after co-transfection: 16 h incubation time for the construct with the *TRE1* promoter, and 6 h incubation time for the construct with the *TPP5* or *TPP6* promoter. Four or six biological replicates were used and three independent experiments were carried out for each promoter activation test. For details on protoplast isolation and transient expression procedures, see Methods S1.

### Trehalase activity assay

The procedures were adapted from Muller *et al.* (2001). Solid medium cultured seedling material (50 mg fresh weight per sample) was pulverized and suspended in the protein extraction buffer (50 mM morpholinoethane-sulfonic acid/$K^+$ (pH 6.3), 1 mM EDTA, 1 mM phenyl methyl-sulfonyl fluoride, 0.01% (v/v) Triton X-100 and 1% (w/v) polyvinylpyrrolidone). The suspensions were incubated on ice for 2 h, and following centrifugation the supernatants were used. The protein extracts were desalted using Amicon Ultra 0.5-ml 10K centrifugal filters (Millipore; http://www.millipore.com) before incubation with the substrate trehalose (Sigma; http://www.sigma.com) to a final concentration of 25 μM at 37°C. The reactions were terminated by boiling. The amount of glucose released from the reactions was determined using the D-glucose test kit (R-Biopharm; http://www.r-biopharm.com) according to the manufacturer's instructions. Three independent experiments were carried out and four biological replicates were used for each experiment.

### Trehalose 6-phosphate measurements

T6P was extracted from 50 mg fresh weight of seedlings grown for 7 d on solid medium and T6P contents were determined as in Delatte *et al.* (2009). Five biological replicates per line were used for each treatment.

### Root length measurements

Seedlings grown on solid medium were used for root length measurements as described above in the section plant material and growth conditions. Digital photos were taken of 7-d-old seedlings grown in the Petri dishes positioned vertically. Photographs were uploaded into the software ImageJ (http://rsbweb.nih.gov/ij/download.html). Root lengths were quantified and recorded for each seedling as indicated by the software. The root lengths were calculated to the actual lengths by taking the width of the Petri dish as a reference. Three or more independent experiments were carried out.

## Results

### The transcription factor bZIP11 changes the carbohydrate metabolic profile

The WT and the bZIP11 dex-inducible lines L and M were used in this study. In these transgenic lines, bZIP11 is fused at its carboxyl terminus to the hormone-binding domain of the rat glucocorticoid receptor, allowing the dex-induced nuclear presence of bZIP11 (termed 'induced bZIP11'). In these lines, sucrose does not repress *bZIP11* mRNA translation because of the absence of the sucrose-controlled uORF. Previous experiments showed that the L line displays a stronger response to dex addition than the M line (Hanson *et al.*, 2008). In these experiments, it was observed that bZIP11 induction promotes seedling growth arrest in a dex concentration-dependent way. To investigate whether this growth arrest could be relieved by the addition of metabolizable sugars, an experiment was performed where dex- and mock-induced WT and L and M lines were treated with 30 mM, glucose or sucrose, or sorbitol as a control treatment (Fig. 1). Dex addition had no effect on the growth of WT seedlings in any of the sugar treatments and dex inhibited growth of L and M line seedlings on sorbitol medium, as observed previously. This inhibition was more severe in the stronger overexpressing L line than in the weaker M line. Importantly, the addition of glucose or sucrose did not rescue the dex-induced growth inhibition phenotype (Fig. 1).

This bZIP11-mediated growth inhibition was further investigated in an unbiased large-scale metabolic study with 7-d-old seedlings of the WT and the L and M lines treated for 6 h with 10 μM dex. The impact of induced bZIP11

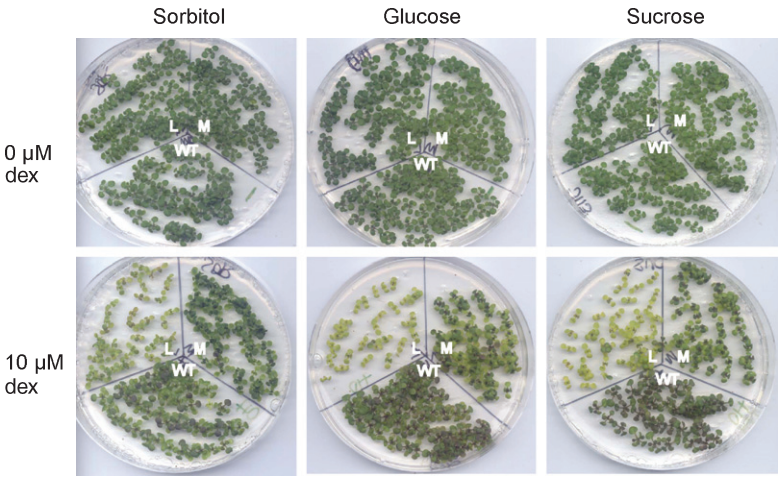


**Fig. 1** Induction of basic region–leucine zipper motif transcription factor 11 (bZIP11) inhibits Arabidopsis seedling growth, and this inhibition is not reversed by the addition of metabolizable sugars. Wild-type (WT), L line and M line seedlings, as indicated, were grown on 30 mM sorbitol, glucose or sucrose, respectively, for 7 d followed by addition of 5 ml of 10 μM dex to the plates. Photographs were taken 5 d after the dexamethasone (dex) treatment.

on metabolism was maximized by suppressing the translation of endogenous S1 group bZIP proteins, including bZIP11, by the addition of 100 mM sucrose to the medium, as sucrose efficiently suppresses S1 group mRNA translation (Wiese *et al.*, 2005; Rahmani *et al.*, 2009). Following dex or mock (solvent only) treatments, plant extracts were used for metabolomics analysis using a GC-MS platform, as described in the Materials and Methods. Ten biological replicates were used for each of the L and M lines. In total, 228 peaks were quantified for each GC-MS sample run and 60 metabolites were unambiguously identified (Table 1). Carbohydrates, amino acids and lipids, and their derivatives, were mainly identified (Table 1). The effect of dex vs mock treatment is expressed as the relative change ratio for each identified metabolite in each line (Table 1).

As shown in Table 1, for most of the metabolites in WT seedlings the relative change ratios of dex to mock treatment were *c.* 1.0, indicating that dex generally did not affect metabolism. These ratios differed from *c.* 0.2 to above 80 in the bZIP11-inducible L and M lines, indicating that changes were caused by induced bZIP11 nuclear migration upon dex treatment. Generally, the L and M lines displayed consistent metabolic changes but these were stronger in line L than in line M. Statistically significant changes were identified by applying a stringent statistic test (Wilcoxon test, Bonferroni-corrected) to the data set where the dex treatment was compared with the mock treatment in each line for each compound. Higher numbers of significant changes in metabolite levels were observed in line L than in line M (21 and six, respectively). None of the changes in the WT were found to be significant. In line L, 16 metabolites showed more than two-fold changes in the relative change ratio, of which 13 were statistically significant, whereas in line M seven metabolites showed more than twofold changes in this ratio, of which four were significant.

As shown in Fig. 2, induced bZIP11 increased the levels of metabolites which can serve as the entry metabolites to glycolysis and depleted the levels of intermediates in the tricarboxylic acid (TCA) cycle. Accordingly, the levels of sucrose, glucose, fructose, glucose 6-phosphate and fructose 6-phosphate were increased, while the levels of fumarate, succinate and citrate were decreased. Interestingly, it was also found that the levels of multiple amino acids changed significantly, such as proline and the aromatic amino acids phenylalanine, tryptophan and tyrosine.

In the bZIP11-induced lines, the levels of salicylic acid-glucopyranoside (SAG) was markedly increased compared with mock-treated transgenic lines. SAG is a storage form of the signaling molecule salicylic acid (SA) which functions in pathogen defense (Kawano *et al.*, 2004). Basal SAG levels were generally very low and a relatively small increase in the absolute amount of SAG resulted in a large increase in the ratio in the bZIP11-induced lines. The increased SAG levels upon bZIP11 induction might be related to the increased levels of its upstream precursors, such as aromatic amino acids.

## bZIP11 promotes raffinose biosynthesis and myo-inositol degradation

Substantial bZIP11-mediated changes in raffinose and myo-inositol were observed. Raffinose levels increased and myo-inositol levels decreased following bZIP11 activation (Table 1). Raffinose and myo-inositol are metabolites in the glucuronate biosynthetic pathway that requires galactinol and sucrose as substrates (Fig. 3a). In several species, raffinose has been proposed to function as a long-distance transportation sugar and as a carbon storage molecule (Sprenger & Keller, 2000). The function of raffinose in Arabidopsis is unclear, but it might be a reactive oxygen scavenger (Nishizawa *et al.*, 2008).

Protoplasts can be used as a rapid and convenient way to test the biological functions of the transfected genes, especially transcription factors (Baena-Gonzalez *et al.*, 2007; J. Ma *et al.*, unpublished observation). Isolated leaf protoplasts were transfected with a 35S-bZIP11 plasmid and incubated for 6 h, and RNA was then isolated. RNA from three independent samples was probed in triplicate using the Affymetrix ATH1(25K) microarray (Affymetrix Santa Clara, California, USA; experimental procedures in Methods S1). In this experiment, bZIP11 was found to induce the expression of several genes involved in raffinose biosynthesis and myo-inositol degradation significantly (Table S2).

Among the six Arabidopsis genes annotated as raffinose synthases, bZIP11 strongly induces expression of raffinose synthase 2 (At3g57520) and dark inducible 10 (*DIN10*; At5g20250) in protoplasts (Table S2). Raffinose synthesis from galactinol produces myo-inositol which is further oxidized to glucuronate (Fig. 3a). It is known that modulated expression levels of myo-inositol oxygenase-encoding genes, including *MIOX2* (At2g19800) and *MIOX4* (At4g26260), result in corresponding changes in myo-inositol contents (Kanter *et al.*, 2005; Endres & Tenhaken, 2008). In protoplasts, bZIP11 induces *MIOX2* and *MIOX4* gene expression (Table S2), probably explaining the reduced myo-inositol levels (Table 1). The endogenous factors that control *MIOX* gene expression are not known. However, it has been reported that an extended night induces the *MIOX2* and *MIOX4* transcript levels and lowers myo-inositol levels, while during the normal diurnal cycle myo-inositol levels are not affected (Gibon *et al.*, 2006).

## bZIP11 acts as a regulator of trehalose metabolism

Trehalose levels were decreased in the dex-treated L and M lines (Table 1) and the expression of *TRE1* was strongly

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**Table 1** Basic region–leucine zipper motif 11 (bZIP11) mediates reprogramming of metabolism in Arabidopsis seedlings

| | Relative change ratio of metabolite level in response to dex treatment (ratio ± SD) | | |
|---|---|---|---|
| Metabolite | Wild type | Transgene L | Transgene M |
| *Amino acids and derivatives* | | | |
| Alanine | 0.99 ± 0.18 | 1.33 ± 0.12* | 1.19 ± 0.09 |
| Beta-alanine | 1.07 ± 0.28 | 0.84 ± 0.15 | 0.74 ± 0.09 |
| 3-Cyanoalanine | 0.92 ± 0.14 | 0.83 ± 0.07 | 0.84 ± 0.10 |
| Ethanolamine | 0.85 ± 0.35 | 1.23 ± 0.25 | 1.12 ± 0.23 |
| 4-Aminobutyric acid | 1.07 ± 0.56 | 2.18 ± 1.12* | 1.50 ± 0.24 |
| Arginine | 1.02 ± 0.26 | 0.73 ± 0.13 | 1.02 ± 0.07 |
| Asparagine | 1.09 ± 0.13 | 0.90 ± 0.11 | 1.04 ± 0.09 |
| Aspartate | 0.99 ± 0.25 | 0.60 ± 0.13* | 0.73 ± 0.05 |
| Glutamate | 1.01 ± 0.42 | 0.49 ± 0.26 | 0.52 ± 0.08 |
| Glutamine | 0.95 ± 0.16 | 0.82 ± 0.18 | 0.96 ± 0.12 |
| Glycine | 1.15 ± 0.34 | 1.55 ± 0.19* | 1.34 ± 0.19 |
| Histidine | 1.02 ± 0.13 | 1.04 ± 0.12 | 0.96 ± 0.10 |
| Leucine | 1.53 ± 0.64 | 1.25 ± 0.28 | 1.10 ± 0.14 |
| Lysine | 1.20 ± 0.48 | 2.36 ± 0.41* | 1.50 ± 0.22 |
| Proline | 0.97 ± 0.27 | 0.21 ± 0.02* | 0.21 ± 0.02 |
| Phenylalanine | 1.14 ± 0.40 | 3.12 ± 0.76* | 1.84 ± 0.16 |
| Tryptophan | 1.14 ± 0.48 | 3.70 ± 0.77* | 2.61 ± 0.26* |
| Tyrosine | 1.38 ± 1.29 | 12.35 ± 3.86* | 2.56 ± 0.46 |
| Serine | 1.15 ± 0.14 | 1.33 ± 0.13* | 1.51 ± 0.15* |
| Threonic acid | 1.21 ± 0.21 | 0.82 ± 0.14 | 0.94 ± 0.05 |
| Threonine | 1.04 ± 0.23 | 1.33 ± 0.17* | 1.18 ± 0.08 |
| Homoserine | 1.06 ± 0.17 | 0.87 ± 0.13 | 1.00 ± 0.09 |
| O-Acetyl-L-serine | 1.11 ± 0.88 | 1.34 ± 0.31 | 1.12 ± 0.10 |
| Ornithine | 1.06 ± 0.27 | 0.81 ± 0.13 | 1.04 ± 0.11 |
| Valine | 1.07 ± 0.22 | 1.84 ± 0.18* | 1.51 ± 0.12 |
| *Carbohydrates* | | | |
| Fructose | 0.90 ± 0.21 | 2.51 ± 0.68* | 1.21 ± 0.08 |
| Glucose | 0.94 ± 0.12 | 1.87 ± 0.25* | 1.16 ± 0.05 |
| Sucrose | 1.07 ± 0.11 | 2.28 ± 0.26* | 1.63 ± 0.12 |
| Fructose 6-phosphate | 1.20 ± 0.25 | 2.00 ± 0.50 | 1.92 ± 0.25 |
| Glucose 6-phosphate | 1.13 ± 0.24 | 2.11 ± 0.53 | 2.03 ± 0.32 |
| Cellotriose | 0.68 ± 0.61 | 0.55 ± 0.50 | 0.43 ± 0.15* |
| Myo-inositol | 1.08 ± 0.11 | 0.46 ± 0.08* | 0.67 ± 0.07* |
| Maltose | 0.91 ± 0.58 | 0.62 ± 0.15 | 0.74 ± 0.13 |
| Raffinose | 1.02 ± 0.36 | 2.61 ± 0.97* | 1.46 ± 0.15 |
| Trehalose | 1.05 ± 0.46 | 0.36 ± 0.07* | 0.60 ± 0.14 |
| Xylose | 1.09 ± 0.29 | 1.70 ± 0.33 | 1.55 ± 0.22 |
| Citrate | 1.07 ± 0.30 | 0.64 ± 0.29* | 0.74 ± 0.11 |
| Fumarate | 0.8 ± 0.33 | 0.76 ± 0.25 | 0.44 ± 0.08 |
| Gluconate | 1.08 ± 0.15 | 1.05 ± 0.10 | 1.00 ± 0.09 |
| Glycerate | 1.04 ± 0.76 | 0.81 ± 0.29 | 0.45 ± 0.10* |
| Malate | 1.07 ± 0.55 | 0.88 ± 0.08 | 0.91 ± 0.20 |
| Succinate | 1.13 ± 0.39 | 0.49 ± 0.17* | 0.65 ± 0.13 |
| *Other metabolites* | | | |
| Adenosine 5-monophosphate | 1.23 ± 0.18 | 1.14 ± 0.10 | 1.15 ± 0.13 |
| 2-Linolenic acid | 1.02 ± 0.27 | 0.94 ± 0.14 | 0.89 ± 0.21 |
| Benzoic acid | 1.60 ± 0.89 | 1.25 ± 0.64 | 1.38 ± 0.79 |
| 3-Sitosterol | 1.04 ± 0.09 | 1.00 ± 0.09 | 1.05 ± 0.10 |
| Campesterol | 0.96 ± 0.09 | 0.84 ± 0.11 | 0.90 ± 0.16 |
| Dehydroascorbic acid dimer | 1.04 ± 0.10 | 1.03 ± 0.12 | 1.06 ± 0.09 |
| Linoleic acid | 1.15 ± 0.27 | 0.93 ± 0.21 | 1.09 ± 0.26 |
| Methyl stearate | 0.98 ± 0.14 | 0.90 ± 0.06 | 0.92 ± 0.09 |
| Monomethylphosphate | 0.79 ± 0.22 | 0.80 ± 0.22 | 1.02 ± 0.18 |
| Oleic acid | 1.23 ± 0.33 | 0.94 ± 0.23 | 0.93 ± 0.23 |
| Phosphoric acid | 1.19 ± 0.35 | 1.13 ± 0.12 | 1.27 ± 0.13 |

**Table 1** (Continued)

| Metabolite | Relative change ratio of metabolite level in response to dex treatment (ratio ± SD) | | |
|---|---|---|---|
| | Wild type | Transgene L | Transgene M |
| 3-Amino-2-piperidinone | 1.02 ± 0.26 | 0.75 ± 0.13 | 1.03 ± 0.09 |
| Pyroglutamic acid | 1.07 ± 0.05 | 1.03 ± 0.20 | 0.97 ± 0.08 |
| Salicylic acid-glucopyranoside | 1.31 ± 0.77 | 89.5 ± 29.59* | 26.85 ± 4.27* |
| Sinapinic acid | 0.93 ± 0.19 | 0.96 ± 0.12 | 0.80 ± 0.14 |
| Spermidine | 1.15 ± 0.25 | 1.28 ± 0.53 | 1.20 ± 0.24 |
| Stearic acid | 1.18 ± 0.23 | 0.93 ± 0.19 | 0.99 ± 0.20 |
| Threonic acid-1,4-lactone (trans) | 1.01 ± 0.11 | 0.82 ± 0.12 | 1.02 ± 0.14 |

Wild-type and *bZIP11* dexmethasone (dex)-inducible line L and line M seedlings were cultured in liquid half-strength MS medium for 7 d, followed by a 6-h treatment with either 10 μM dex or a mock treatment. GC-MS analysis was employed in this study. The relative change in the level upon dex treatment was normalized to the level in the mock treatment for each compound identified in each line. The relative change ratio ± SD is presented.

*Statistically significant changes ($P$-value < 0.05; Wilcoxon test, Bonferroni-corrected).

induced in the corresponding seedlings and protoplasts, as shown in Fig. 3(b) (Hanson *et al.*, 2008; Table S2). In addition, in seedlings the *TPP5* and *TPP6* genes were also significantly induced by bZIP11 (Hanson *et al.*, 2008; J. Ma *et al.*, unpublished observations). Previous studies on promoter sequences of genes regulated by bZIP11 identified ACGT as the core promoter element for transcriptional activation by bZIP11. Detailed studies of the *ASN1* promoter showed that in protoplasts the ACGT element is essential for bZIP11-mediated transcriptional activation of the *ASN1* promoter (Baena-Gonzalez *et al.*, 2007; Hanson *et al.*, 2008). The promoters of *TRE1*, *TPP5* and *TPP6* contain two or more such *cis* elements (Fig. S1). Thus, the function of bZIP11 in promoting the transcription of these trehalose metabolism genes was investigated by studying bZIP11-mediated transcriptional activity in protoplast co-transfection assays. Promoter elements of *c.* 1.5 kb of *TRE1*, *TPP5* and *TPP6* were fused to the luciferase reporter gene (Fig. S1) and constructs were co-transfected with a 35S-bZIP11 plasmid in Arabidopsis mesophyll protoplasts (Baena-Gonzalez *et al.*, 2007; Hanson *et al.*, 2008). The unrelated transcription factor *ATH1* (Arabidopsis thaliana homeodomain 1) (Quaedvlieg *et al.*, 1995) was used as a control in these experiments. bZIP11 efficiently induced transcription from *TRE1*, *TPP5* and *TPP6* promoters, whereas ATH1 essentially had no effect on these promoters (Fig. 4).

The transcriptional activation of these trehalose metabolism genes by bZIP11 probably contributes to the corresponding metabolic changes in trehalose and T6P metabolites. Trehalase enzymatic activity increased substantially in L and M seedlings upon dex induction of bZIP11 (Fig. 5a), with a concomitant decrease in trehalose levels (Table 1). Dex did not affect trehalase activity or trehalose levels in WT seedlings. Cellular T6P concentrations typically are in the low micromolar to nanomolar range, and sample pretreatment combined with sensitive LC-MS is needed for T6P identification and quantification (Lunn *et al.*, 2006; Delatte *et al.*, 2009). T6P contents were determined in L and M seedlings grown on trehalose medium in the presence or absence of dex, and it was found that bZIP11 induction resulted in a substantial reduction in T6P contents in both lines (Fig. 5b). *TPP5* and *TPP6* have been proposed to encode enzymatically active T6P phosphatases (Vandesteene *et al.*, 2010) that are probably responsible for T6P metabolism in dex-induced L and M seedlings.

Previous studies (Schluepmann *et al.*, 2004) showed that root growth in Arabidopsis seedlings grown on trehalose-containing medium is severely inhibited as a result of the accumulation of T6P. bZIP11 induction rescues root growth in L and M seedlings grown on trehalose medium, in accordance with the observed reduction in T6P contents (Fig 5c,d). At a concentration of 10 μM, validamycin A inhibits trehalase activity, without affecting plant growth (Muller *et al.*, 2001). In the presence of 10 μM validamycin A, root lengths of bZIP11-induced seedlings grown on trehalose medium were similar to those of the WT (Fig. 5c,d). Thus, bZIP11-induced trehalase activity is also important for root growth on trehalose medium. In conclusion, bZIP11 induction was found to activate *TRE1*, *TPP5* and *TPP6* expression, resulting in reduced T6P contents in L and M seedlings grown on trehalose medium and trehalose-resistant root growth.

## Discussion

The constitutive or dex-induced nuclear presence of bZIP11 inhibits plant growth in a concentration-dependent manner (Hanson *et al.*, 2008). In tobacco (*Nicotiana tabacum*), 35S promoter-driven *bZIP11* expression also severely inhibits growth (J. Ma *et al.*, unpublished observa-

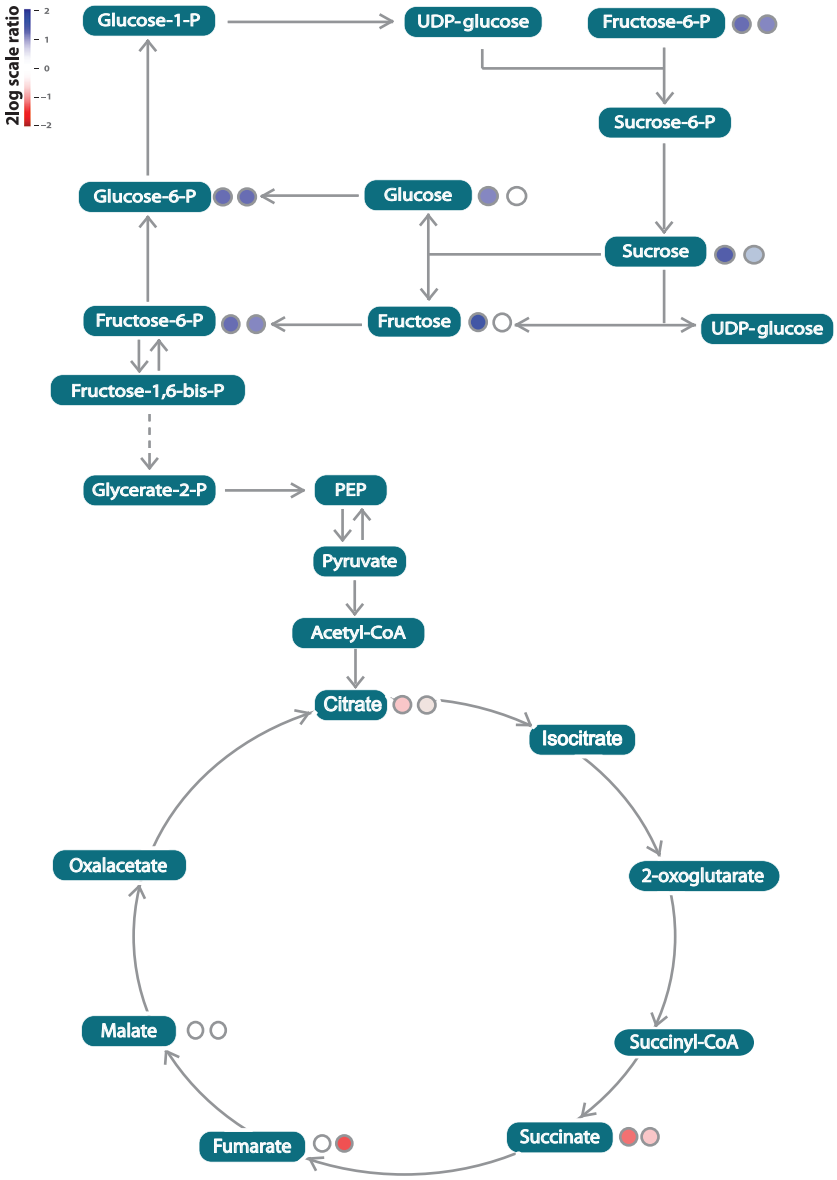


**Fig. 2** Basic region–leucine zipper motif transcription factor 11 (bZIP11)-induced metabolic changes in central sugar metabolism. The pathways presented are adapted from the MAPMAN program (Thimm *et al.*, 2004). Circles denote relative metabolite changes in L (left) and M (right) seedlings as identified in the metabolomics analysis, and the corresponding relative change ratio for metabolites in Table 1 was converted to a 2log scale and is shown on a red to blue color scale. Fructose-6-P, fructose-6-phosphate; Fructose-1,6-bis-P, fructose-1,6-bis-phosphate; Glucose-6-P, glucose-6-phosphate; Glucose-1-P, glucose-1-phosphate; Glycerate-2-P, glycerate-2-phosphate; PEP, phosphoenolpyruvate.

tions). The growth inhibition by bZIP11 induction in Arabidopsis was not reversed by the addition of metabolizable sugars such as glucose and sucrose (Fig. 1). In this study, large-scale metabolic, transcriptional and molecular analysis suggested that bZIP11 is a powerful regulator of metabolism. The controlled nuclear presence of bZIP11 was found to result in major metabolic reprogramming that is probably responsible for the growth inhibition phenotype.

## bZIP11 induction reprograms metabolism to mimic a low-carbon metabolic state

In plants, the cellular sugar status has a substantial impact on gene expression, as has been demonstrated under natural growth conditions, for example in plants grown under the natural diurnal cycle, and in conditions of sugar feeding (Gibon *et al.*, 2006). The expression of multiple genes is either repressed or induced by the addition of sugars.

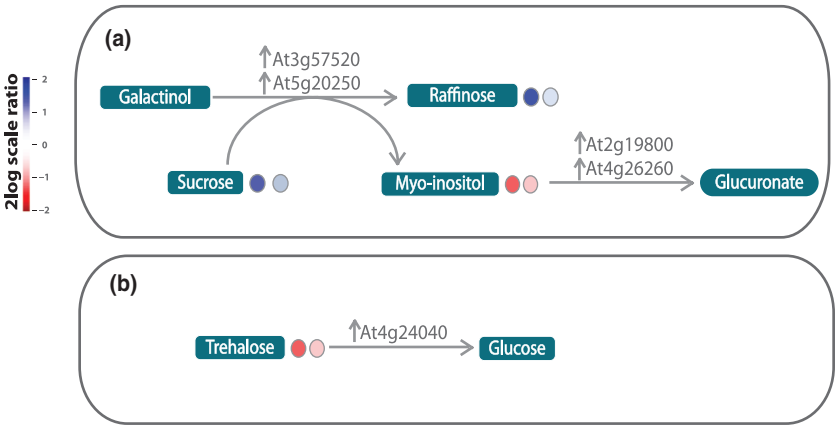


**Fig. 3** Pathways affected by basic region–leucine zipper motif transcription factor 11 (bZIP11) induction as indicated by changes in the levels of both metabolite and transcripts. The pathways presented are adapted from the MAPMAN program (Thimm *et al.*, 2004). Changes in metabolites are presented as in Fig. 1. Arrows represent gene expression changes as taken from Supporting Information Table S2. (a) Raffinose biosynthesis and myo-inositol degradation pathways. (b) Trehalose degradation pathway.

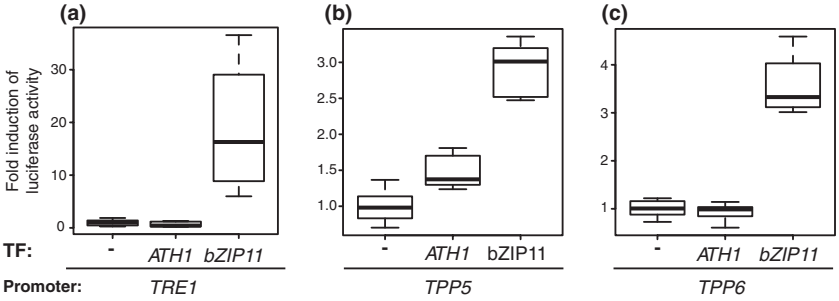


**Fig. 4** Basic region–leucine zipper motif transcription factor 11 (bZIP11) promotes transcription from trehalase 1 (*TRE1*) (a), trehalose 6-phosphate phosphatase 5 (*TPP5*) (b) and *TPP6* (c) promoters in transiently transfected Arabidopsis mesophyll protoplasts. Promoters were fused to the luciferase reporter gene and transfected either alone or in combination with a 35S-bZIP11 plasmid. Promoter fragments used were *c.* 1.5 kb in length and are presented in Supporting Information Fig. S2. A 35S-ATH1 construct, encoding the transcription factor ATH1 (Arabidopsis thaliana homeodomain 1), was used as an unrelated control. Presented is the fold-induction relative to the mean value of the transfections with the reporter construct only. The experiments were performed with four to six biological replicates and three independent repetitions. The box represents the interquartile range and the whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range from the box.

Interestingly, bZIP11 induces the expression of multiple genes previously described as being associated with a low-carbon state, including raffinose synthase 2, *DIN10*, *MIOX2*, *MIOX4* and *TRE1*. Accordingly, expression of these genes is induced by an extended night treatment leading to carbon depletion (Fig. S3; Gibon *et al.*, 2006).

Remarkably, the bZIP11-induced metabolic changes resembled those observed in plants grown under carbon limitation. An extended night leads to reduced levels of myo-inositol, proline, glutamate, fumarate, citrate and succinate and increased levels of aromatic amino acids (Hayashi *et al.*, 2000; Gibon *et al.*, 2006). bZIP11 induces the expression of two glutamate dehydrogenase encoding genes *GDH1* and *GDH2* (Hanson *et al.*, 2008), which are essential for the survival of Arabidopsis plants after an extended night treatment (Miyashita & Good, 2008). Carbon starvation conditions such as an extended night result in increased levels of aromatic amino acids, but the mechanism and function of this increase are unknown. In addition, sucrose starvation results in T6P depletion (Lunn *et al.*, 2006). Changes in metabolite content were consistent with changes in transcript levels of the corresponding metabolic genes responsive to bZIP11 (Table S2), which were shown to be both sucrose-repressed and extended night-induced (Blasing *et al.*, 2005; Gibon *et al.*, 2006). These bZIP11-mediated changes at both metabolite and transcript levels are remarkable, as 100 mM sucrose was added to the medium to repress endogenous S1 group *bZIP* genes. These findings suggest that bZIP11 is involved in adapting metabolism to low-carbon growth conditions.

Levels of sucrose, glucose, fructose, glucose 6-phosphate and fructose 6-phosphate increased following bZIP11 induction, while during an extended night these sugars decrease markedly (Gibon *et al.*, 2006). These sucrose-

derived metabolites might have been high as a result of the carbon-rich culture conditions in our study. bZIP11 induction might somehow prevent utilization or, perhaps, promote biogenesis of these sucrose-derived metabolites.

Other S1 group bZIP proteins have been implicated in the control of metabolism and plant growth (Weltmeier *et al.*, 2006; Alonso *et al.*, 2009; Kang *et al.*, 2010; Obertello *et al.*, 2010). The S1 group bZIP transcription factors preferentially heterodimerize with C group bZIP transcription factors bZIP9, bZIP10, bZIP25 and bZIP63 (Ehlert *et al.*, 2006). Selected heterodimers function synergistically as potent transcriptional activators on target promoters (Ehlert *et al.*, 2006; Rahmani *et al.*, 2009).

Genes encoding these S1 group bZIP proteins and their heterodimerizing C group partners are responsive to a variety of environmental signals, including light, nutrients and stressful growth conditions (Weltmeier *et al.*, 2009). Moreover, C/S1 *bZIP* genes show defined and overlapping expression patterns. What emerges is a C/S1 bZIP transcription factor regulatory network that controls distinct and overlapping genes involved in the regulation of metabolism and other processes (Alonso *et al.*, 2009). Such a network provides tremendous regulatory potential and allows plants to respond appropriately by adapting metabolism in a tissue-specific manner to a variety of growth conditions and nutrient concentrations (Hanson & Smeekens, 2009). Translation of all five S1 group *bZIP* mRNAs is sensitive to sucrose at physiological concentrations (Weltmeier *et al.*, 2009). This places the whole C/S1 bZIP transcription factor regulatory network under the control of the cellular sucrose concentration in a dynamic way with half-maximum inhibition of S1 mRNA translation in the range of 10–20 mM sucrose (Wiese *et al.*, 2005; Rahmani *et al.*, 2009).

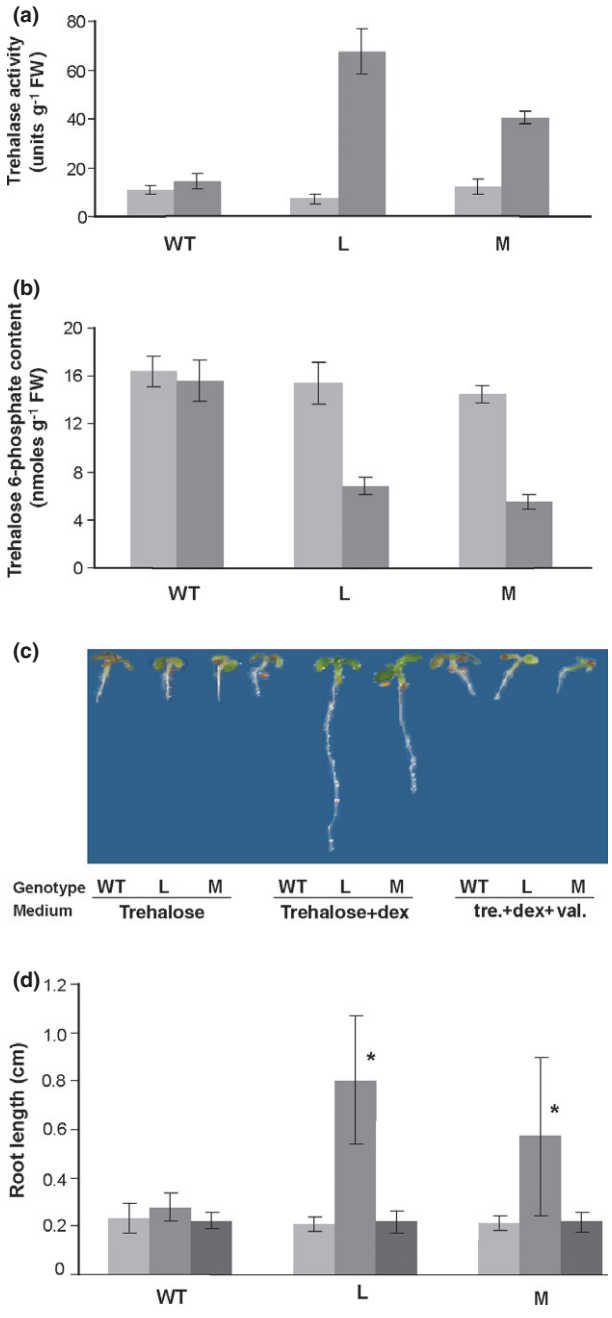


**Fig. 5** Basic region–leucine zipper motif transcription factor 11 (bZIP11) regulates trehalose metabolism. (a) Trehalase activity was assayed in 7-d-old wild-type (WT), L and M Arabidopsis seedlings grown on agar-solidified medium containing 100 mM sorbitol plus (dark-gray bars) or minus (light-gray bars) 0.2 μM dexamethasone (dex). (b) The trehalose 6-phosphate content was quantified in Seven-d-old WT, L and M seedlings grown on solid medium containing 100 mM trehalose plus (dark-gray bars) or minus (light-gray bars) 0.2 μM dex. (c) 7-d-old WT, L and M seedlings were grown on 100 mM trehalose in the presence of 0.2 μM dex and 10 μM validamycin A, as indicated. (d) Quantification of root lengths of seedlings presented in (c) (trehalose, light-gray bars; trehalose + dex, mid-gray bars; trehalose + dex + validamycin A, dark-gray bars). *Statistically significant ($P$-value < 0.05; Student's $t$-test) root length differences in comparisons of line L ($n$ = 219) or line M ($n$ = 166) to WT ($n$ = 75), respectively, for seedlings grown on medium containing trehalose and dex. Error bars indicate ± SD.

## bZIP11 is a regulator of trehalose metabolism

T6P is an essential growth regulator and promotes carbon utilization (Eastmond *et al.*, 2002; Schluepmann *et al.*, 2003). TPS, TPP and TRE enzymatic activities are involved in T6P metabolism in plants (Ramon *et al.*, 2009; Vandesteene *et al.*, 2010). The results of the present study suggest that bZIP11 induces expression of the *TRE1*, *TPP5* and *TPP6* genes. Induction of the expression of these genes corresponded to altered metabolite, transcript and, for trehalase, enzymatic activity levels, and partially restored root growth in bZIP11-induced seedlings grown on trehalose medium. The results of the protoplast transactivation assay suggested that the *TRE1*, *TPP5* and *TPP6* promoters

may be direct targets of bZIP11, but this requires further investigation.

bZIP11 activity is capable of reducing the T6P levels. Low T6P contents in bZIP11-induced lines may contribute to the reduced capacity to metabolize available sugar. The accumulation of sugar and sugar phosphates in bZIP11-induced lines may be attributable to a downstream block in sugar utilization. Interestingly, in the *tps1* null mutant, T6P levels are very low and sucrose, glucose, fructose and sugar phosphates also accumulate, indicative of a block in their utilization for growth, and a partial overlap in the expression patterns of *TPS1* and *bZIP11* has been observed (Weltmeier *et al.*, 2009; Gomez *et al.*, 2010). Thus, bZIP11 is a likely regulator of trehalose metabolism.

### bZIP11 functions in the SnRK1–T6P regulatory network

SnRK1 and T6P regulatory systems respond to cellular sugar status and coordinate metabolism and growth. These sugar-controlled regulatory systems must interact to either promote or inhibit growth. T6P inhibits SnRK1 enzymatic activity at physiological concentrations (Zhang *et al.*, 2009) and in protoplast transfection experiments the KIN10 catalytic subunit of SnRK1 activates the transcriptional activity of S1 group bZIP proteins, including bZIP11 (Baena-Gonzalez *et al.*, 2007). Our results suggest that bZIP11 functions in regulating trehalose metabolism and affects T6P levels. Moreover, bZIP11 increases the expression of the SnRK1 catalytic subunit *KIN11* two- to four-fold in seedlings and protoplasts (Hanson *et al.*, 2008; J. Ma *et al.*, unpublished observations). Thus, it appears that SnRK1, T6P and bZIP11 function in a regulatory circuit that links sucrose status to growth (Fig. S3).

*bZIP11* expression is associated with vascular tissues in different organs throughout plant development (Rook *et al.*, 1998; Weltmeier *et al.*, 2009). In seedlings, *bZIP11* is also expressed in several layers of mesophyll cells surrounding the veins. Also, in roots and generative tissues, *bZIP11* has a well-defined expression pattern (Weltmeier *et al.*, 2009). In these tissues, bZIP11 probably has a defined function in the control of metabolism during plant growth under normal diurnal conditions, in addition to its function during nutrient stress conditions. One function of bZIP11 in veins might be that of a sucrose-repressed brake on metabolism and thereby on nutrient translocation. In *bZIP11* over-expressing tobacco lines, carbon translocation is severely inhibited (S. Smeekens *et al.*, unpublished results). It is likely that the C/S1 bZIP transcription factor regulatory network has overlapping and distinct functions in controlling metabolism, and the challenge will be to unravel and understand the full potential of this regulatory network.

Our results suggest that bZIP11 reprograms metabolism to mimic a carbon limitation state. The finding that bZIP11 is a regulator of trehalose metabolism suggests that bZIP11 is part of a regulatory circuit that connects cellular carbon status to growth.

## Acknowledgements

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Graphic illustrations of the promoter regions used for the transcription activation study in protoplasts.

**Fig. S2** Expression patterns of basic region–leucine zipper motif transcription factor 11 (bZIP11)-regulated genes during the diurnal cycle and an extended night. Data are from Gibon *et al.* (2006).

**Fig. S3** Schematic illustration of the sucrose non-fermenting-1 related protein kinase 1 (SnRK1)–basic region–leucine zipper motif transcription factor 11 (bZIP11)–trehalose 6-phosphate (T6P) regulatory circuit.

**Table S1** Primer sequences used for constructs presented in Fig. S2

**Table S2** Genes induced by the expression of basic region–leucine zipper motif transcription factor 11 (bZIP11) in protoplasts that might explain the metabolic changes in raffinose, myo-inositol and trehalose metabolites quantified by GC-MS analysis as presented in Fig. 2

**Methods S1** Experimental procedures for the 35S-bZIP11 plasmid protoplast transfected microarray.

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