Reprogramming of metabolism by the *Arabidopsis thaliana* bZIP11 transcription factor

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Layout, cover & invitation design: Jingkun Ma
Printing: Offpage
ISBN: 978-94-6182-112-6

The study presented in this thesis was performed in the group of Molecular Plant Physiology, Utrecht University, Padualaan 8, 3584CH, Utrecht, The Netherlands. The presented work was supported by Centre for BioSystems Genomics (CBSG).

Reprogramming of metabolism by the *Arabidopsis thaliana* bZIP11 transcription factor

Herprogrammering van het metabolisme door de *Arabidopsis thaliana* bZIP11 transcriptie factor

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G. J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op vrijdag 11 mei 2012 des middags te 12.45 uur

door

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Chapter 1 Introduction

Sugar sensing and signaling networks

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Sugars have crucial signaling functions through the entire life cycle of plants. The details of sugar sensing and sugar signaling pathways are being uncovered but our understanding of the molecular interactions involved is still limited. Known regulatory components that have a function in linking cellular sugar availability with growth and development include the hexokinase glucose sensor protein, the trehalose 6-phosphate signaling molecule, the SnRK1 protein kinase that responds to carbon/energy status and the S1/C group bZIP transcription factors that function in the regulation of metabolism. In this Introduction our knowledge on the signaling function of sugars and the networks that link sugar status to growth is summarized and discussed.

Sugar signaling

In plants as in other organisms, sugars act both as intermediates in metabolism and as signaling molecules. Sugar addition to plants has profound regulatory effects. Sugars function as signaling molecules that regulate growth and development, and communicate with other signaling pathways, including phytohormone mediated signaling.

The main products of photosynthesis are glucose and sucrose that can be stored, transported or utilized as a metabolic substrate. Importantly, these sugars function as a signaling molecule (Ramon *et al.*, 2008). Sucrose is the major substance for long term transport. Following sucrose translocation by the vessel system to sink tissues, sucrose can be degraded by sucrose synthase or invertase activity, and such sucrose metabolism is important for growth (Barratt *et al.*, 2009; Wang *et al.*, 2010). For example, quantitative trait loci (QTL) analysis showed that vacuolar invertase activity is positively associated with root length in Arabidopsis (Sergeeva *et al.,* 2006). A comprehensive natural variation analysis in Arabidopsis demonstrated that high internal sugar and sugar phosphate levels are negatively correlated with biomass (Meyer *et al.*, 2007; Sulpice *et al.*, 2010). Thus, sugar availability by itself does not increase growth but other signals that promote utilization of available sugars are also important. Internal sucrose levels are positively correlated with the signaling molecule trehalose 6-phosphate, which promotes carbon utilization and, thereby, growth (Lunn *et al.*, 2006; Schluepmann *et al.*, 2003). Thus, trehalose 6-phosphate is among the signaling factors linking carbon availability to growth.

Many studies confirmed the regulatory function of sugars at the transcriptional and post-transcriptional level. Moreover, different sugars such as sucrose, glucose and fructose have overlapping as well as distinct effects on plants. Sugars stimulate α Amylase3 mRNA degradation via its 3' untranslated region (Chan *et al.*, 1998). Sucrose specifically represses the translation of S1 group *bZIP* transcription factors mRNAs (Wiese *et al.*, 2004; Wiese *et al.*, 2005; Weltmeier *et al.*,

2009). Sucrose is an effective inducer of transcription of many genes including genes encoding transcription factors (Usadel *et al.*, 2008). For example, sucrose promotes anthocyanin accumulation by inducing transcription of the *MYB75* transcription factor that regulates the expression of anthocyanin biosynthesis genes (Teng *et al.*, 2005; Loreti *et al.*, 2008). Sucrose induces lignin deposition in Arabidopsis seedlings more effectively than other sugars (Rogers *et al.*, 2005). Both glucose and sucrose promote activity of the key enzyme in starch biosynthesis, ADP-Glucose pyrophosphorylase (AGPase). In potato tubers AGPase induction by sucrose specifically requires the activity of the SnRK1 protein kinase that is a regulator of metabolism (Tiessen *et al.*, 2003). Sucrose and glucose differentially regulate the mitosis associated cyclin genes *CycD2* and *CycD3* (Riou-Khamlichi *et al.*, 2000). Other studies indicate that during seed development glucose availability stimulates cell division, and upon increasing sucrose level promotes cell differentiation and resource accumulation (Borisjuk 1998).

Sugars greatly affect metabolism of other nutrients, such as nitrogen and phosphate. Glucose addition to plants more prominently induces nitrogen assimilation associated genes than nitrogen does (Price *et al.*, 2004). Sucrose is considered a regulator of phosphate assimilation since increased internal sucrose levels induce phosphate transporter and phosphate metabolism genes (Lei *et al.*, 2011; Muller *et al.*, 2007).

Interaction between sugar and phytohormone signaling

Interactions between sugar and phytohormone signal transduction pathways have been abundantly observed. Sugars in combination with phytohormones regulate growth associated processes. GA and sucrose promote hypocotyl growth (Zhang *et al.*, 2010). Sucrose treatment increases the abundance of the PIF5 growth promoting protein, which results in hypocotyl elongation (Stewart *et al.*, 2011). Brassinosteroid (BR) signaling likely interacts with sugar signaling as well, since BR rescues the sucrose over sensitivity phenotype in a cell expansion defective mutant (Laxmi *et al.*, 2004). Salicylic acid (SA) is best known as a signaling molecule in plant defense but also impacts on carbon partitioning by differently inducing the activity of sugar metabolic enzymes in source and sink tissue, leading to increased soluble sugar levels in sink tissues (Dong *et al.*, 2011). Transcriptomics studies show that glucose and ABA signaling share many genes. The observed close interaction between sugar and ABA signaling is evident as well from the number of identified sugar insensitive mutants (see next section) that encode genes related to ABA metabolism and signal transduction. Multiple ABA associated mutants are insensitive to sugars, including sucrose, glucose and fructose. These findings imply that the ABA signaling pathway is a convergence point for sensing of different sugars.

Glucose signaling is antagonized by ethylene signaling and vice versa. The ethylene insensitive mutants *etr*, *ein2* and *ein3* are hypersensitive to glucose while the constitutive ethylene response mutant *ctr*1 is sugar insensitive (Moore *et al.*, 2003). Further research into the mechanism showed that glucose promotes the degradation of the important ethylene responsive transcription factor EIN3 (Yanagisawa *et al.*, 2003). Consistently, the overexpression of EIN3 results in insensitivity to glucose. In other plant species sucrose similarly inhibits the accumulation of an EIN3 like protein such as in carnation petals during early senescence (Hoeberichts *et al.*, 2007). In other studies, the interaction between glucose and auxin and cytokinin signaling was established. The auxin resistant mutants *axr1*, *axr2* and *tir1* are glucose insensitive, while cytokinin likely acts in an antagonistic way as the constitutive cytokinin response mutants are insensitive to glucose (Moore *et al.*, 2003). Thus, cross talk among different phytohormonal signaling systems and sugar signals appears to coordinate sugar signal mediated seedling establishment.

Sugar sensing mutants

Investigations into the molecular nature of sugar sensor and signaling systems were initiated in Arabidopsis by mutant identification procedures and the cloning of the genes responsible for altered sensitivity to sugars. This approach showed that different sugars can be sensed by different systems, and that there is extensive cross talk between sugars and phytohormone signaling pathways. As mentioned above, this is especially evident for ABA and ethylene, but also for auxin and cytokinin signaling. Thus, likely sugar signals regulate seedling establishment in coordination with phytohormonal signals. The sugar sensing mutants and their extensive cross talk with phytohormone signaling mutants has been well reviewed, such as in Ramon *et al.*, 2008. Below several mutant selection procedures and examples of resulting mutants are briefly presented.

High concentration (approximately 300 mM) of sugar such as sucrose, glucose or fructose inhibits germination and seedling establishment, including root and shoot growth inhibition, greening of cotyledons and initiation of true leaves. A most straightforward way to identify mutants in sugar responsiveness has been the use of germination and seedling establishment screens of mutated seed on growth media containing such high sugar concentrations. Many mutants were identified that overcome this growth inhibition. In addition, other selection procedures were developed that involve the use of sugar responsive promoters coupled to a reporter or selective marker gene. In this way the *sun* and *isi* mutants were identified. Selected mutants respond differently to added sugars. The major sugar insensitive mutant series identified include *gin* (*g*lucose *in*sensitive), *sun* (*s*ucrose *un*coupled), *isi* (*i*mpaired *s*ugar *i*nduction) and *sis* (*s*ugar *in*sensitive) mutants, and information on these mutants with known molecular defects is summarized

Sugar sensing mutants	Allelic mutant	Function	Glucose	Fructose	Sucrose	Reference
gin2/hxk1	-	Hexose kinase; HXK1	insensitive	sensitive	sensitive	Moore et al., 2003
gin5	aba3	ABA biosynthetic gene	insensitive	-	-	Arenas-Huertero et al., 2000
sis1/gin4	crt1	Calcium ion binding protein	insensitive	insensitive	insensitive	Gibson et al., 2001
sis3	-	Ring E3 ligase	-	-	insensitive	huang et al., 2010
gin1/isi4/sis4	aba2	ABA biosynthetic gene	insensitive	insensitive	insensitive	Rook et al., 2001
isi3/sis5/gin6/sun6	abi4	APETALA2 type transcription factor	insensitive	insensitive	insensitive	Arenas-Huertero et al., 2000; Rook *et al.*, 2001
sis7	-	ABA biosynthetic gene	insensitive	-	insensitive	huang et al., 2008
sis9	aba1	ABA biosynthetic gene	insensitive	-	-	huang et al., 2008
sis10	abi3	B3 domain transcription factor	insensitive	-	-	huang et al., 2008

Table 1-1. Well studied sugar insensitive mutants.

in Table 1-1. These mutants are mostly insensitive to multiple sugars and often encode genes whose products are involved in phytohormone metabolism or responses, especially in ABA related pathways. Mutants that appear to be independent of known hormone signaling have also been isolated, for example the *sis3* mutant that encodes a ring E3 ligase protein (Huang *et al.,* 2010).

Sugar sensing

The Arabidopsis HXK1 enzyme was the first glucose specific sensor protein identified in plants. HXK1 mediated glucose sensing does not require the catalytic activity of this enzyme as was shown by analysis of *hxk1* mutants that are catalytically inactive but still retain glucose signaling function. *hxk1* null mutants show an altered response to light and to phytohormones, indicating a function in coordinating sugar signals to growth (Moore *et al.*, 2003). Further biochemical and protein-protein interaction studies showed that HXK1 is present in a protein complex with HUP1 (vacuolar H^+ATPase B1) and HUP2 (Regulatory particle of proteasome; RPT5B) (Cho *et al.*, 2006). Both *hup1* and *hup2* mutants display a glucose insensitive phenotype and these mutants have lost the glucose mediated repression of photosynthesis genes (Cho *et al.*, 2007). This HXK1 protein complex is located in the nucleus and functions as a regulator of transcription. HXK1 belongs to the hexokinase/ hexokinase-like gene family. Other members of this family are expressed as well and can have enzymatic activity such as HXK2 (Karve *et al.*, 2008). Further investigation is needed to establish the function of members of this gene family in glucose sensing.

Glucose can be sensed independently from HXK1 as well. A small set of genes is regulated in response to glucose by the glucose mediated interaction between the membrane associated proteins RGS1 and GPA1, which are G protein signaling components (Grigston *et al.*, 2008). Further analysis showed that downstream of GPA1 the Thylakiod Formation 1 (THF1) protein is involved in mediating glucose signals.

Fructose specific signaling components have only recently been identified. Evidence for fructose specific signaling was obtained in a natural variation screen in Arabidopsis (Li, *et al.*, 2010). It appeared that the transcription factor ANAC089 is associated with fructose specific signaling, depending on the specific Arabidopsis accession. The ANAC089 variant in the Arabidopsis Cvi accession specifically suppresses fructose sensitivity. This variant lacks the C-terminal membrane bound domain that is present in ANAC089 variant of L*er* or Col accession, which does not show a fructose specific phenotype. Expression of the semi dominant truncated Cvi allele in the Col accession results in fructose insensitive root growth and cotyledon greening on high fructose containing medium. The *f*ructose *ins*ensitive 1 (*fins1*) mutant similarly provides insensitivity to high fructose con-

centration. The *fins1* gene encodes a putative fructose 1,6 bisphosphatase (FBP, At1g43670) and, similar to HXK1, FINS1 mediated fructose signaling does not require FBP catalytic activity (Cho and Yoo, 2011).

The functions of trehalose and trehalose 6-phosphate

Trehalose metabolism is evolutionary ancient and exists in organisms across Kingdoms. Multiple pathways exist for trehalose biosynthesis and degradation in the many prokaryotic and eukaryotic species that metabolize trehalose (Avonce *et al.*, 2006). In plants, trehalose synthesis occurs in two steps, involving trehalose 6-phosphate synthase (TPS) and trehalose 6-phosphate phosphatase (TPP). TPS converts UDP-glucose and glucose 6-phosphate into the intermediate trehalose 6-phosphate, which is dephosphorylated by TPP to trehalose. Trehalose can be hydrolysized by trehalase (TRE) into glucose (Figure 1-1).

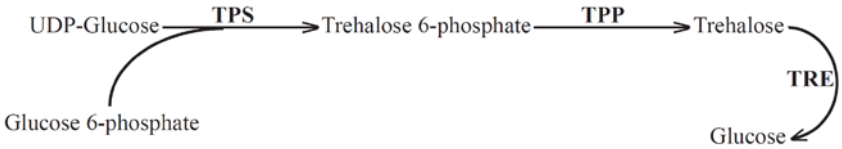

Figure 1-1. The plant trehalose metabolism pathway. The enzymes catalyzing the reactions are **TPS** (Trehalose 6-Phosphate Synthase), **TPP** (Trehalose 6-Phosphate Phosphatase) and **TRE** (Trehalase).

Trehalose is a versatile non reducing disaccharide. Due to its chemical properties, it promotes stress tolerance in bacteria or fungi and higher organisms. Also in plants trehalose plays a role in abiotic or biotic stress tolerance. However, it remains controversial whether trehalose in plants functions as a protective molecule or as a signal molecule that initiates the stress response. Trehalose content is very low in most plant species except for some resurrection plants (Fernandez *et al.*, 2010). Tolerance to abiotic stresses such as drought, cold or salt is conveyed by elevated trehalose levels. This effect of trehalose has been shown in several crop plants, without obvious detrimental morphological alterations in such plants. In tobacco, elevated levels of trehalose were obtained by introducing yeast TPS1 and/or TPS2 genes driven by the constitutive AtRbcS1A promoter or the stress response AtRAB18 promoter, which gave rise to drought tolerance without obvious morphological changes (Karim *et al.*, 2007). In maize, trehalose accumulation was obtained by infecting the plants with bacteria engineered to express a yeast TPS-TPP fusion protein. This TPS-TPP fusion protein produced substantial quantities of trehalose and greatly improved plant survival during drought stress (Rodriguez-Salazar *et al.*, 2009). In rice, increased levels of trehalose in transgen-

ic lines expressing bacterial *TPS* and *TPP* encoding genes gave rise to drought and salt tolerance (Ge *et al.*, 2008; Garg *et al.*, 2002; Jang *et al.*, 2003). Two of the endogenous rice TPPs, *OsTPP1* and *OsTPP2* are cold inducible genes, of which the *OsTPP1* over expression lead to enhanced cold tolerance (Shima *et al.*, 2007). In salt stressed Medicago nodules, the application of the trehalase activity inhibitor validamycin A increased trehalose levels and biomass (Lopez *et al.*, 2008a; Lopez *et al.*, 2008b; Lopez *et al.*, 2009). Trehalose metabolism associated biotic stress tolerance has been reported in Arabidopsis. Applying the trehalase activity inhibitor validamycin A to Arabidopsis results in trehalose accumulation, which relieves the clubroot disease infection by *Plasmodiophora brassicae* in the Col-0 accession to some extent (Gravot *et al.*, 2011). Also, in the Arabidopsis Col accession trehalose feeding and in vivo accumulation of trehalose due to the inactivation of the trehalose degrading gene *TRE1* or the over expression of *TPS11* results in increased resistance to aphid infestation by the phloem-feeding insect Myzus persicae (Singh *et al.*, 2011).

In most plants, trehalose 6-phosphate (T6P) levels are extremely low and near the detection limit of current analytical methodology (Delatte *et al.*, 2009; Delatte *et al.*, 2011). However, experiments suggest that trehalose 6-phosphate is an essential signaling molecule in plants that is indispensible for growth and development as reviewed in (Ponnu *et al.*, 2011). In Arabidopsis, lack of T6P leads to slower vegetative growth, failure of floral transition and embryo lethality (Gomez *et al.*, 2010; Schluepmann *et al.*, 2003; van Dijken *et al.*, 2004). These findings suggest that T6P plays an indispensible role in multiple developmental stages. Arabidopsis transgenic lines were created by over expressing bacterial enzymes OtsA or OtsB, required for T6P synthesis and degradation, respectively. By this means, T6P content could be manipulated. Transcriptomics experiments with these lines suggested that T6P induces genes that are associated with growth (Paul *et al.*, 2010). Arabidopsis lines with reduced T6P levels have a reduced capacity to utilize carbon resources. Such lines show a reduced growth with soluble sugars and starch accumulating (Gomez *et al.*, 2010). In transgenic potato tubers with depleted T6P levels, the accumulation of soluble sugars has been observed as well. These tubers displayed reduced yield (Debast *et al.*, 2011). These findings support the notion that T6P is involved in carbon utilization, thereby promotes growth (Schluepmann *et al.*, 2003).

Addition of trehalose to plants results in accumulation of T6P, which deregulates metabolism and promotes starch accumulation (Schluepmann *et al.*, 2004). Detailed biochemical studies showed that T6P promotes starch synthesis by post-translational redox activation of the key enzyme AGPase (Kolbe *et al.*, 2005; Lunn *et al.*, 2006). More study supports the link between trehalose metabolism and starch biosynthesis, as it has been demonstrated that trehalose feeding induc-

es *ADP-Glucose Pyrophosphorylase Large Subunit3* (*APL3)* starch biosynthetic gene and this can complement the starch deficient phenotype in *apl1* mutant (Fritzius *et al.*, 2001).

T6P levels are positively correlated to sugar availability (Paul, 2008). Carbon starvation depletes T6P level while sucrose feeding leads to rapid T6P accumulation in Arabidopsis seedlings (Lunn *et al.*, 2006). Similar observations have been reported during wheat grain filling, where T6P and sucrose levels are positively correlated (Martinez-Barajas *et al.*, 2011). Importantly, recent finding suggests that T6P is an inhibitor of the regulatory SnRK1 protein kinase activity. SnRK1 orchestrates reprogramming of gene expression and metabolism in response to carbon starvation or energy depletion (Baena-Gonzalez *et al.*, 2007). T6P inhibits SnRK1 activity at physiological levels in Arabidopsis extracts. Additionally, SnRK1 regulated marker genes in Arabidopsis seedlings are oppositely regulated by T6P (Zhang *et al.*, 2009). Similar findings were presented for transgenic potato tubers with altered T6P levels (Debast *et al.*, 2011). During natural senescence T6P levels are positively correlated with soluble sugar accumulation (Wingler *et al.,* 2012). Interestingly, Arabidopsis lines over expressing the bacterial TPP encoding gene *OtsB* have delayed senescence, mimicking the phenotype of *KIN10* overexpressors (Wingler *et al.,* 2012). These findings imply that T6P and SnRK1 respond differently to sugar availability in the same physiological contexts. Thus, T6P seems to connect the prevailing sugar status to various physiological and developmental processes but the underlying mechanisms need clarification.

So far, the trehalose metabolism associated genes are best characterized genetically and enzymatically in Arabidopsis. In this model plant genome, there are in total 22 genes annotated as associated with trehalose metabolism. Interestingly, there are eleven *TPS* genes and ten *TPP* genes, but only one gene annotated encoding a trehalase (*TRE1*) (Leyman *et al.*, 2001).

Trehalase enzymatic activity has been studied in Arabidopsis and in many other plant species (Muller *et al.*, 2001). Arabidopsis trehalase is an enzymatically active protein and the *AtTRE1* cDNA complements the yeast trehalase mutant. The same study shows that trehalase appears to be plasma membrane bound (Frison *et al.*, 2007). However, so far trehalase activity and trehalose contents were assayed in the soluble phase of plant extracts. The inhibition of trehalase activity in plants by validamycin A application results in increased trehalose content (Muller *et al.*, 2001). Similarly, Arabidopsis *tre1* knockout lines show accumulation of trehalose (Singh *et al.*, 2011). Thus, it is clear that the Arabidopsis *TRE1* encodes an in vivo active enzyme.

The trehalose 6-phosphate *TPS* and *TPP* metabolic genes have been classified into three classes according to the presence of protein functional domains. The

class I proteins contain a TPS domain only and comprise TPS1-4, class II TPS proteins contain both TPS and TPP domains and comprise TPS5-11, and class III proteins are TPPs that possess TPP domains only (Vandesteene *et al.* 2010).

The enzymatic activity characterization of TPSs and TPPs has been mainly performed heterologously by complementation of yeast mutant strains that lack TPS or TPP activity. In these studies TPS1, TPPA and TPPB were initially reported to be enzymatically active (Blazquez *et al.*, 1998;Vogel *et al.*, 1998). These results were confirmed in independent studies (Ramon *et al.*, 2009; Vandesteene *et al.* 2010). Moreover, probably all TPPs are enzymatically active (Vandesteene *et al.* 2010). Importantly, *tps1* knock out lines have much reduced T6P contents, indicating that TPS1 is enzymatically important in vivo (Gomez *et al.*, 2010). Initially it was considered that TPS1 is the only enzymatically active protein in plants as concluded from yeast complementation assays (Vandesteene *et al.*, 2010). However, independent complementation assays suggest that TPS6 and TPS11 have TPS activity as well (Chary *et al.*, 2008; Singh *et al.*, 2011). In addition, a TPP enzymatic activity was demonstrated for TPS11 and over expression of *TPS11* results in increased levels of trehalose, but its impact on T6P content was not determined (Singh *et al.*, 2011). Thus, the in vivo enzymatic activity of *TPSs* and *TPPs* needs further investigations. The expression patterns of class II TPS genes has been well studied and they are generally expressed both in seedlings and adult plants, especially in vigorously growing tissues, such as root tip, shoot meristem and seeds, suggesting that these genes have a role in growth (Ramon *et al.*, 2009). Coexpression analysis of the Arabidopsis TPP multigene family members also suggests the involvement of TPPs in a variety of metabolic and signaling functions in plants (Li *et al.*, 2008).

Forward and reverse genetics showed that TPS and TPP encoding genes play a role in plant architecture or defense. The maize TPP encoding gene *RAMOSA3* has an essential role in inflorescence architecture (Satoh-Nagasawa *et al.*, 2006). As mentioned before Arabidopsis *tps1* knock out lines display reduced vegetative growth, failure to proceed to floral transition and embryo lethality. Loss of function study reveals that *TPS6* is responsible for proper cell shape formation (Chary *et al.*, 2008) and *TPS11* plays a role in aphid resistance (Singh *et al.*, 2011).

Protein-protein interaction studies using the yeast two hybrid system identified the interaction between AtTPS1 and the cell cycle-dependent kinase CDKA, which is a kinase regulating the cell cycle (Geelen *et al.*, 2007). AtTPS5 was found to interact with the transcriptional coactivator MBF1c, a key regulator of thermotolerance establishment and the Arabidopsis *tps5* mutants are thermosensitive (Suzuki *et al.*, 2008). Accordingly, the expression of *TPS5* is substantially induced by heat, and also by sugars. Additionally, TPS5 is phosphorylated by SnRK1 which results in its binding to a 14-3-3 protein (Harthill *et al.*, 2006).

This suggests that TPS5 is involved in SnRK1 mediated metabolic adaptation. In rice, *OsTPS1* encodes the only known enzymatically active TPS enzyme as found by yeast complementation experiments. Interestingly, in these studies both yeast two hybrid and in planta split YFP experiments showed that OsTPS1 interacts with OsTPS5 and OsTPS8 (Zang *et al.*, 2011). These findings indicate that non-enzymatically active TPS proteins might function as regulators of active TPS protein members in controlling trehalose metabolism.

What emerges is a regulatory network centered around trehalose metabolism with the T6P metabolite at its core. Many other signaling systems interact with the trehalose metabolic system. The over expression of *TPS1* in Arabidopsis leads to altered glucose and ABA sensitivities (Avonce *et al.*, 2004). Similarly, in OtsA and OtsB transgenic potato tubers, altered ABA levels were observed. The ABA signaling mutant *abi4* appears to mediate the starch allocation effects caused by external trehalose feeding (Ramon *et al.*, 2007). The Arabidopsis *sweetie* mutant shows altered trehalose metabolism, deregulated carbon metabolism and growth, but the function of SWEETIE protein is unknown (Veyres *et al.*, 2008). Trehalose metabolism plays an important role in linking metabolism to multiple physiological events and likely is closely associated with the defined spatio-temporal expression patterns of trehalose metabolic genes.

Function of S1 group bZIP transcription factors in Arabidopsis

The Arabidopsis transcription factor (TF) bZIP11 and its close homologues (see below) appear to function as regulators of metabolism. These TFs are controlled by sugars at the transcriptional and/or translational level and play a role in the SnRK1 mediated metabolism reprogramming in response to low sugar/energy status. These TFs have a strong impact on metabolism and strongly affect plant growth when misexpressed. It is important to understand in detail how these TFs coordinate growth and metabolism under normal or stress related growth condition. The heterodimerizing capacity of these TFs provides an added layer of regulatory complexity that makes functional studies challenging.

bZIP11 belongs to the S1 group in the basic region-leucine zipper motif (bZIP) transcription factor gene family, which consists of over 70 members (Jakoby *et al.*, 2002). The S1 group transcription factors include bZIP1, 2, 11, 44 and 53. These TFs preferentially heterodimerize with C group bZIP transcription factors consisting of bZIP9, 10, 25 and 63. The heterodimerizing potential of S1 and C group TFs was shown by protein-protein interacting assays in yeast and leaf mesophyll protoplasts (Dietrich *et al.*, 2011; Ehlert *et al.*, 2006; Kang *et al.*, 2010; Weltmeier *et al.*, 2006). Such selective heterodimer formation augments transcription activating on target gene promoters and provides tremendous regulatory potential to these bZIP TFs. For the heterodimer of bZIP53 and bZIP10,

the synergistic activation effect was shown in protoplasts with the osmolarity response ACTCAT cis element present in the promoter of *Proline Dehydrogenase* (*ProDH*) (Weltmeier *et al.*, 2006). Additional studies supported the increased binding affinity of S1/C bZIP heterodimers to the ACGT core sequence containing promoter elements, including those present in the G-box and C-box, for example, in the case of the bZIP53 plus bZIP10 or bZIP53 plus bZIP25 combinations on G-box containing seed maturation genes (Alonso *et al.*, 2009). Also it is shown that bZIP1 and bZIP53 directly binds ACGT core sequence containing elements G-box or C-box (Dietrich *et al.*, 2011). This bZIP1 and bZIP53 in vivo binding directly induces expression of *ProDH* and results in proline depletion (Dietrich *et al.*, 2011). Independent studies also showed that bZIP1 to bind to G-box and C-box-containing promoters (Kang *et al.*, 2010). Other studies emphasize the importance of the ACGT core sequence in transcription activation of S1 group TFs. Hanson *et al.* identified ACGT core sequence as the over presented cis-element present in bZIP11 induced genes (Hanson *et al.*, 2008). Mutation of this ACGT core resulted in strongly decreased transcription activation of the *Asparagine Synthetase 1* (*ASN1*) promoter mediated by bZIP11.

S1 group bZIP TFs have a regulatory role in amino acid metabolism. System biology studies further revealed that S1 bZIPs function in metabolism and it was found that bZIP1 is a core regulator of gene expression that integrates light and nitrogen signals (Obertello *et al.*, 2010). Furthermore, for bZIP11 a function in regulating *ASN1* and *ProDH2* was observed but a much broader function in metabolism was suggested (Hanson *et al.*, 2008). bZIP1 and bZIP53 target the *ASN1* and *ProDH* promoter as well and modulate proline contents (Dietrich *et al.*, 2011). Substantial functional redundancy seems present among S1/C bZIP dimer TFs as observed by transcriptomics analysis. Other reports show that S1/C group bZIPs also function in response to environmental and physiological signals (Kaminaka *et al.*, 2006).

Interestingly, the effects on metabolism mediated by S1 bZIPs are in part dependent on SnRK1 activity that is induced by low carbon availability. The investigations in Arabidopsis protoplast of the SnRK1 catalytic subunit KIN10 show that the transcription activating activity of S1 group bZIPs is greatly enhanced by the KIN10 protein kinase (Baena-Gonzalez *et al.*, 2007). SnRK1 is a protein kinase orchestrating transcriptional reprogramming in response to low carbon availability by promoting catabolism and restricting anabolism. Importantly, sucrose translationally represses all five Arabidopsis S1 bZIPs through a conserved upstream open reading frame (uORF). In addition, glucose transcriptionally induces *bZIP11* but represses *bZIP1* and *bZIP63* (Kang *et al.*, 2010; Matiolli *et al.*, 2011). Thus, the availability of sugars greatly impacts on the transcription, translation of S1/C bZIP genes or proteins (Dietrich *et al.*, 2011; Kang *et al.*, 2010).

In all five Arabidopsis S1 group bZIP transcription factor genes a conserved uORF is present that is involved in the sucrose induced repression of translation (SIRT) of the downstream main open reading frame (mORF) (Rook *et al.*, 1998b). Rising sucrose levels gradually represses mORF protein translation in a process that requires the sucrose responsive uORFs (Rook *et al.*, 1998b). Remarkably, this effect is sucrose specific and conserved among all five S1 group Arabidopsis bZIP TFs (Wiese *et al.*, 2005; Weltmeier *et al.*, 2009). Detailed functional studies have been carried out on the uORFs of *bZIP11*. The S1 group bZIP sucrose regulated uORFs encode the conserved sucrose control peptide (SC peptide) that mediates SIRT. In the presence of physiological concentrations of sucrose the SC peptide likely functions as an attenuator peptide (Rahmani *et al.*, 2009). Increasing sucrose levels promote formation of the SC-peptide containing SIRT complex on the translating ribosome (Hummel *et al.*, 2009; Rahmani *et al.*, 2009). This complex blocks movement of upstream ribosomal complexes towards the main ORF, thereby preventing production of functional bZIP proteins. This translational regulation by sucrose of S1 group bZIPs thus functions in sucrose concentration dependent regulation of S1 bZIP target genes.

It was suggested that S1 group bZIP TFs are involved in a molecular network that is controlled by sugar availability (Wiese *et al.*, 2004). Elucidating the physiological function of the S1/C group bZIPs requires the establishment of their temporal and spatial expression patterns. Analysis showed that these bZIPs have defined and highly overlapping expression patterns in flower organs, siliques and seedlings (Weltmeier *et al.*, 2009). The generally overlapping expression patterns of S1 and C group bZIP TFs further points to their functional redundancy in vivo. The bZIP10 and other S1/C bZIPs are expressed in seeds and have a role in regulating seed maturation. The closest homologue of *bZIP10* and *bZIP25* in maize is the gene *Opaque2*, which similarly regulates seed storage protein expression (Vicente-Carbajosa *et al.*, 1997). Most S1/C group bZIPs are expressed highly in pollen and in floral organs. Interestingly, close homologues of the S1/C group proteins in tobacco have a function in pollen development (Iven *et al.*, 2010). *bZIP11* is expressed throughout the whole life cycle of Arabidopsis and its expression is associated most prominently with vascular tissues and their surrounding cells. Possibly, bZIP11 has an adaptor function in metabolism that signals the sugar availability for growth. Ectopic over expression of *bZIP11* severely inhibits growth (Hanson *et al.*, 2008) and results in massive reprogramming of the transcriptome and the metabolome. Taken together, the S1/C group TF network links carbon availability to amino acid and general metabolism, and is most likely linked to physiological and environmental conditions (Rook *et al.*, 1998a).

SnRK1 and its functions

The SnRK1 protein kinase represents the plant ortholog of the Snf1 (Sucrose non-

fermenting1) protein kinase in yeast and AMP-activated protein kinase (AMPK) in mammals (Ghillebert *et al.*, 2011). This family of protein kinases is responsive to the cellular energy status and adapts anabolic and catabolic processes to prevent cellular collapse. The plant SnRK1 is activated by stress conditions such as starvation or hypoxia that result in a low energy status. SnRK1 activation results in the altered expression or activation status of large numbers of genes and proteins, respectively, which are involved in metabolism (Baena-Gonzalez and Sheen, 2008). Moreover, SnRK1 appears to be involved in a broader context that includes biotic and abiotic stress, and plant development.

The SnRK1 is a serine-threonine kinase, which in Arabidopsis belongs to the SnRKs family consisting of 38 gene members (Hrabak *et al.*, 2003). SnRK1 is mainly involved in regulating metabolism whereas SnRK2 and SnRK3 mainly are involved in stress response pathways (Coello *et al.*, 2011). Genes classified as part of the SnRK1 family have a similar domain structure and high amino acid sequence similarity to SNF1 in yeast and AMPK in mammals. SnRK1 kinase is a heterotrimeric protein kinase. The protein complex consists of a catalytic subunit (α) and the β and γ regulatory subunits. The SnRK1 protein subunits are encoded by different genes and the SnRK1 complex therefore is heterogeneous, likely resulting in functional diversity (Polge and Thomas, 2007). These SnRK1 subunit isoforms can be differentially regulated. Two genes in Arabidopsis, *KIN10* and *KIN11* both encode catalytic subunits. Phosphate starvation condition differentially modulates the catalytic activity of KIN10 and KIN11 subunits by activating and reducing their activity, respectively (Fragoso *et al.*, 2009). Such differential regulation also occurs at the level of subcellular localization. In Arabidopsis, two of the three β subunits are targets of N-myristoyltransferase. N-myristoylation of β1 and β2 subunits promotes their membrane association, instead of the cytosolic or nuclear localization (Pierre *et al.*, 2007). In potato, two subunit isoforms can independently interact with cytosolic pyruvate kinase and significantly elevate pyruvate kinase enzymatic activity (Beczner *et al.*, 2010).

SnRK1 targets a range of enzymes at crucial regulatory points in metabolic pathways (Nozawa *et al.*, 2003; Halford and Hey 2009). SnRK1 inactivates most of these enzymes via phosphorylation. These enzymes include 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase (HMG CoAR), Nitrate Reductase (NR), Sucrose Phosphate Synthase (SPS), Trehalose-6-phosphate Synthase 5 (TPS5) and nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase (np-Ga3PDH) (Harthill *et al.*, 2006; Piattoni *et al.*, 2011). SnRK1 is also found to interact with proteins involved in diverse cellular processes. In Arabidopsis, KIN10 and KIN11 interact with Pleiotropic Regulatory Locus1 (PRL1) and Myoinositol Polyphosphate 5-Phosphatase 13 (5PTase13) (Ananieva *et al.*, 2008; Bhalerao *et al.*, 1999; Flores-Perez *et al.*, 2010). The subunit AKINβγ interacts with pathogen defense

related proteins (Gissot *et al.*, 2006). In barley, the seed specific SnRK1 interacts with the novel protein SnIP1 protein (SnRK1 interacting protein1) and also with the SnIP2 protein, a class I heat shock protein (Slocombe *et al.*, 2002; Slocombe *et al.*, 2004). These interactions position SnRK1 in a network regulated by sugars, phytohormones and developmental and environmental signals. SnRK1 itself is activated by the SnRK1 activating kinases (SnAK) SnAK1 and SnAK2 (Shen *et al.*, 2009; Crozet *et al.*, 2010).

In the moss *Physcomitrella patens*, SnRK1 is necessary to allow growth in the dark, whereas it is dispensable in the light (Thelander *et al.*, 2004). In potato leaves, SnRK1 is required for elevated pyruvate kinase activity during the night when sugar content is low (Beczner *et al.*, 2010). SnRK1 is probably involved in nutrient remobilization that supports rice survival under low energy condition such as during submergence (Lee *et al.*, 2009a). In Arabidopsis seedlings, SnRK1 activity is high under nutrient starvation conditions, while high sugar condition decreases SnRK1 activity and in this context 5PTase13 likely acts as a SnRK1 activity modulator through direct interaction (Ananieva *et al.*, 2008). Plant protoplast studies involving transfection of the catalytic subunit encoding *KIN10* and *KIN11* show that during starvation conditions the global transcriptome is reprogrammed in a way that inhibits anabolism and promotes catabolism (Baena-Gonzalez *et al.*, 2007). Moreover, it was found that SnRK1 mediates the expression of dark inducible genes in a HXK1 independent manner. In protoplast transfection experiments KIN10 enhances the transcription activity of S1 group bZIP transcription factors via phosphorylation. In Arabidopsis seedling and young leaves, the signaling molecule T6P functions as an inhibitor of SnRK1 activity. High T6P levels occur when sucrose is abundant and in such conditions SnRK1 must be inhibited to allow growth (Zhang *et al.*, 2009). Interestingly, *KIN10* knock out seedlings have improved efficiency in utilizing exogenous sugars to facilitate growth (Baena-Gonzalez *et al.*, 2007).

The regulation of metabolism by SnRK1 in response to sugars likely is cell autonomous. As discussed, SnRK1 is thought to be an evolutionary conserved safeguard of cellular functioning when energy levels are low. However, a role of SnRK1 in starch biosynthesis and sugar assimilation has been proposed as well. In endosperm tissues of sorghum and maize, SnRK1 expression is positively correlated with the starch deposition phase (Jain *et al.*, 2008) In potato tuber, SnRK1 is proposed to mediate sucrose induced AGPase activity (Tiessen *et al.*, 2003). The over expression of SnRK1 in potato resulted in high starch containing tubers (McKibbin *et al.*, 2006). In pea embryos with repressed SnRK1 activity, metabolic profiling suggests that SnRK1 plays a role in promoting sugar assimilation for nutrient storage (Radchuk *et al.*, 2006). Storage of reserves is important for subsequent seedling establishment and vigor. These SnRK1 repressed pea embry-

os have decreased levels of phytohormones including ABA, auxin and cytokinin, which likely results in poor seed quality and seedling establishment (Radchuk *et al.*, 2010). The seemingly opposite functions of SnRK1 in sugar metabolism might be regulated by the heterogeneity of the SnRK1 subunits in different organs and growth conditions, but this needs further exploration.

SnRK1 is involved in plant development and adaptation to biotic and abiotic conditions. The Arabidopsis γ subunit is important for reproductive development in Arabidopsis (Fang *et al.*, 2011). In barley, a SnRK1 reduced function mutant has impaired pollen development resulting in sterility (Zhang *et al.*, 2001). SnRK1 is important in plant pathogen defense as well. In Arabidopsis, the germinivirus proteins AL2 and L2 bind to SnRK1 giving rise to inactivation of its activity thereby enhancing susceptibility to viral infection (Hao *et al.*, 2003). In tomato, SnRK1 was found to interact with and inactivate viral pathogenesis protein, thereby suppressing viral gene transcripts (Shen *et al.*, 2011). SnRK1 functions as a prominent regulator of resource allocation. In *Nicotiana attenuata* herbivore feeding on leaves promotes the rapid allocation of carbon resources to roots and this reallocation response promotes subsequent reproductive capacity of the plant (Schwachtje *et al.*, 2006). This allocation response requires the rapid down regulation of the *N. attenuata* SnRK1 GAL83 (β) subunit. In conclusion, SnRK1 is a central regulator of metabolism and the molecular details of its function in diverse cellular processes are at dawn to be understood.

The TOR kinase signaling system

TOR (Target of Rapamycin) kinase is an evolutionarily conserved central regulatory kinase sensing nutrient input. Nowadays, its central position in regulating plant growth starts to attract attention (Xiong and Sheen, 2012). In eukaryotes, the perception of nutrients or growth factors signals typically leads to the activation of the TOR protein kinase signaling pathway that controls growth, development, and senescence. Components of the TOR signaling pathway are highly conserved in eukaryotes and have been particularly well studied in fungi and animals (Soulard *et al.*, 2009). TOR can be present in distinct complexes involved in the regulation of ribosome biogenesis, translation, and primary metabolism (Soulard *et al.*, 2009). In plants, TOR kinase signaling is a pivotal nutrient controlled regulatory system as well. Plants have a functional TOR kinase pathway, including the TOR–Raptor complex that activates the ribosomal protein S6 (rpS6) kinase (Agredano-Moreno *et al.*, 2007; Mahfouz *et al.*, 2006; Robaglia *et al.*, 2004). In animals, TOR activity is stimulated by the Rheb protein. Rheb is a Ras-type GTPase that is regulated by TCTP (translational controlled tumor protein), which activates Rheb GTPase activity and thereby TOR (Wullschleger *et al.*, 2006). TCTP is also present in plants and likely has a similar function in TOR activation (Berkowitz *et al.*, 2008). In Arabidopsis, reduced TCTP level leads to

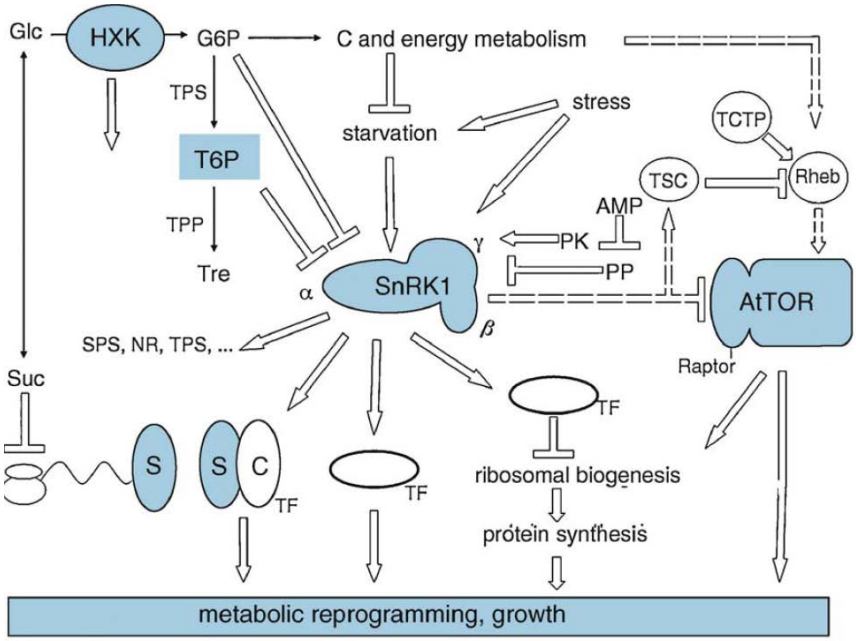

Figure 1-2. The identified growth regulators in sugar sensing and their interactions (Smeekens *et al.*, 2010).

dwarfed growth. Recent findings in mammalian cells suggest a function of the Rheb GTPase in sensing glycolytic flux, thereby directly linking glucose availability to TOR activity (Lee *et al.*, 2009b). In Arabidopsis, AtTOR and AtRaptor are required for normal seed development and growth (Anderson *et al.*, 2005; Deprost *et al.*, 2005; Deprost *et al.*, 2007; Menand *et al.*, 2002). AtTOR expression levels were correlated with vegetative organ size without visibly affecting plant morphology (Deprost *et al.*, 2007). A dwarfed phenotype was also obtained in Arabidopsis lines with reduced expression of the putative TOR target EBP1 (Horvath *et al.*, 2006). Overexpression of lily rpS6 kinase in Arabidopsis was linked to floral tissue development, probably through translational regulation of flower organ identity genes (Tzeng *et al.*, 2009). In addition, TOR likely controls autophagy-related processes in plants, as it does in other organisms (Diaz-Troya *et al.*, 2008). Plants seem to lack PKA and Akt/PKB protein kinase orthologs that regulate TOR in animal systems.

In animals, AMPK regulates TOR activity in different ways (Hardie, 2005; Hardie, 2007). Activated AMPK inhibits TOR kinase by phosphorylating the GAP (GTPase activating protein)TSC2. Phosphorylated TSC2 stimulates Rheb GTPase activity and thereby inhibits TOR, since Rheb only activates TOR in the GTP bound state. Moreover, AMPK phosphorylates and inhibits the TOR activator protein raptor (Gwinn *et al.*, 2008). It will be interesting to elucidate how plant SnRK1 and TOR signaling pathways interact in plants.

Conclusions and Thesis Outline

In plants, sensing systems for cellular carbon availability regulate growth and development. Central components in such systems are the hexokinase glucose sensor, the T6P signaling molecule and the TOR kinase system that signal sugar availability for growth. The absence of sugars is signaled by the SnRK1 protein kinase and the S1/C bZIP system. Current knowledge on sugar sensing networks is summarized in Figure 1-2. Major gaps remain in our understanding of the functioning of these systems.

The focus of this thesis is on the S1/C group of bZIP transcription factors. In **Chapter 2** the function of the S1 group bZIP11 transcription factor is investigated by analysis of bZIP11 downstream regulated genes and by bZIP11 mediated changes in the metabolome. From these experiments it is evident that bZIP11 is a powerful regulator of intermediary metabolism. One of the findings of these experiments was that bZIP11 impacts on trehalose metabolism and promotes degradation of T6P and trehalose via inducing genes *TPPs* and *TRE1*. bZIP transcription factors function as homo- or heterodimers and in **Chapter 3** the combinatorial effect of bZIP heterodimers on transcription has been studied using a leaf mesophyll protoplast system. Single and double transfections of different

S1 and C group members were performed and genome wide expression analyzed. Results showed that bZIP heterodimer combinations have a common set of regulated genes but that most combinations have a restricted set of regulated genes. Trehalose metabolism genes appeared as regulated by several different bZIP combinations, which stresses the fact that S1/C group bZIPs likely participate in T6P/trehalose degradation via inducing *TPPs*. In **Chapter 4** the function of trehalose and T6P is further explored by analyzing the effect of trehalose feeding to Arabidopsis seedlings on the transcriptome, which reveals the remarkable impact of trehalose on the expression of stress and signaling related genes, and identifies the possible interacting evidences of T6P, SnRK1 and S1/C bZIPs at transcriptomic levels. **Chapter 5** summarizes these findings and presents a model on the interaction of the major signaling systems investigated.

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Chapter 2

Transcription factor bZIP11 mediates metabolic reprogramming

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Part of this chapter has been published in *New Phytol. 2011 Aug;191 (3):733-45. as The sucrose-regulated Arabidopsis transcription factor bZIP11 reprograms metabolism and regulates trehalose metabolism*.

Summary

The Arabidopsis transcription factor *bZIP11* is known to be repressed by sucrose through a translational inhibition mechanism that requires the conserved mRNA leader encoded sucrose control peptide. The function of bZIP11 was investigated by overexpression studies and bZIP11 was found to inhibit plant growth. Sugar addition does not rescue the growth inhibition phenotype. The mechanism by which bZIP11 regulates growth was studied by large scale and dedicated metabolic analysis, biochemical assays and molecular studies. Besides the changes in amino acid metabolism, bZIP11 induction also results in changing of carbohydrate profile 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 levels 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 Snf1 related kinase SnRK1.

Introduction

The regulation of metabolism is crucial for plant growth and development, and multiple regulatory systems have been identified that perceive and integrate metabolic signals to regulate growth (Smeekens *et al.,* 2010). These regulatory systems often are of evolutionary ancient origins, such as the SNF1/AMPK/SnRK1 protein kinase that is a central regulator of metabolic stress responses and that is found in all eukaryotic organisms (Halford and Hey, 2009). Another regulatory system that is particularly important in plants is the trehalose 6-phosphate (T6P) signaling system that 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 details to understand the control of cellular metabolism and growth.

The Arabidopsis leucine zipper *bZIP11* transcription factor is a member of the S1 group *bZIP* genes that further consists of *bZIP1, 2, 44* and *53* (Jakoby *et al.,* 2002). Transcripts of these five genes encode in their 5' leader sequence an evolutionary conserved sucrose control (SC) peptide. Sucrose signaling represses translation of the bZIP main ORF and this repression requires the full length SC peptide. It was proposed that sucrose in a concentration dependent way induces a stalling complex on bZIP mRNAs that prevents translation of the bZIP main ORFs. (Wiese *et al.*, 2005; Hummel *et al.*, 2009; Rahmani *et al.*, 2009; Weltmeier *et al.*, 2009).

Previously, the bZIP11 protein was found to function as a regulator of amino acid metabolism. *Proline Dehydrogenase 2* (*ProDH2*) and *Asparagine Synthetase*

1 (*ASN1*) were proposed to be direct targets of bZIP11 (Hanson *et al.*, 2008). Ectopic expression of bZIP11 in Arabidopsis strongly inhibits plant growth in a bZIP11 concentration dependent manner (Hanson *et al.*, 2008). Transcriptomics analysis suggested this growth inhibition to be the consequence of the broad impact of bZIP11 on metabolism (Hanson *et al.*, 2008).

The S1 group bZIP transcription factors preferentially heterodimerize with C group transcription factors bZIP9, bZIP10, bZIP25, bZIP63 (Ehlert *et al.*, 2006). Selected heterodimers function synergistically as potent transcriptional activators on target promoters (Ehlert *et al.*, 2006; Rahmani *et al.*, 2009). For example, bZIP53 binds to the promoter and induces transcription of the *Proline Dehydrogenase1 (ProDH1)* gene but the presence of bZIP10 has a synergistic effect on expression (Weltmeier *et al.*, 2006; Alonso *et al.*, 2009). For S1-C bZIP heterodimers to be active they must be expressed in the same cells and in Arabidopsis leaves, seeds and anthers, the partially overlapping expression patterns of S1 and C group bZIP have been reported (Weltmeier *et al.*, 2009).

The SnRK1 protein kinase is 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 and KIN11 were found to significantly augment the transcriptional potential of the S1 group bZIP transcription factors, including bZIP11 (Baena-Gonzalez *et al.*, 2007).

Trehalose 6-phosphate (T6P) is a signaling molecule that was shown to be a dominant growth regulator that promotes carbon utilization (Schluepmann *et al.*, 2003). T6P levels are positively correlated to 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 different trehalose metabolism genes have been reported and the enzymatic characterization of the proteins has been studied (Muller *et al.*, 2001; Frison *et al.*, 2007; Ramon *et al.*, 2009; Vandesteene *et al.*, 2010). 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; Gomez *et al.*, 2010). Multiple stimuli affect the expression of the trehalose metabolism gene family. However, transcriptional regulators of these genes have not been reported so far. Recently, it was shown that at physiological levels T6P

inhibits the activity of SnRK1 (Zhang *et al.*, 2009), thereby connecting these two important metabolic control networks.

This chapter reports on bZIP11-mediated reprogramming of metabolism in Arabidopsis. Induced nuclear presence of bZIP11 strongly affects carbohydrate and amino acid content as determined by metabolomics analysis. Transcriptomics analysis supports the proposition that bZIP11 is a dominant controller of cellular metabolism. Interestingly bZIP11 mediated reprogramming of metabolism resembles metabolism in plants grown under starvation conditions. The regulatory effect of bZIP11 on several metabolic pathways is presented, especially on myo-inositol, raffinose and trehalose metabolism. bZIP11 regulates several trehalose metabolism genes, including *TRE1*, *TPP5* and *TPP6*, and induces the enzymatic activity of trehalase, with concomitant changes in trehalose and T6P levels. Thus, bZIP11 can act as a regulator of metabolism, probably involved in carbon starvation adaptation responses and might function in a T6P - SnRK1 - bZIP11 regulatory circuit.

Results

The transcription factor bZIP11 reprograms metabolism

Previously, the inducible bZIP11 overexpressing lines L and M were used to study the function of bZIP11 in amino acid metabolism (Hanson *et al.*, 2008). In these lines, bZIP11 is fused at its amino terminus to the hormone binding domain of the rat glucocorticoid receptor, allowing dexamethansone (dex) induced bZIP11 nuclear presence (named 'induced bZIP11'). In these lines sucrose does not repress the translation of bZIP11 due to the absence of its uORF. The L line displays a strong bZIP11 effect on gene expression, whereas the effect of the M line is weaker. 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, L and M lines were treated with 30 mM, glucose or sucrose, or sorbitol as control treatment (Figure 2-1). Dex addition has no effect on growth of WT Arabidopsis in any of the sugar treatments and dex inhibits growth on sorbitol medium as observed previously. This inhibition is more severe in the stronger overexpressing L line than in the weaker M line. Importantly, addition of glucose or sucrose does not rescue the dex induced growth inhibition phenotype (Figure 2-1).

This bZIP11 mediated growth inhibition was further investigated in an unbiased large-scale metabolic study with seven days old seedlings of WT, L and M lines treated for six hours with 10 µM dex. The impact of induced bZIP11 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,

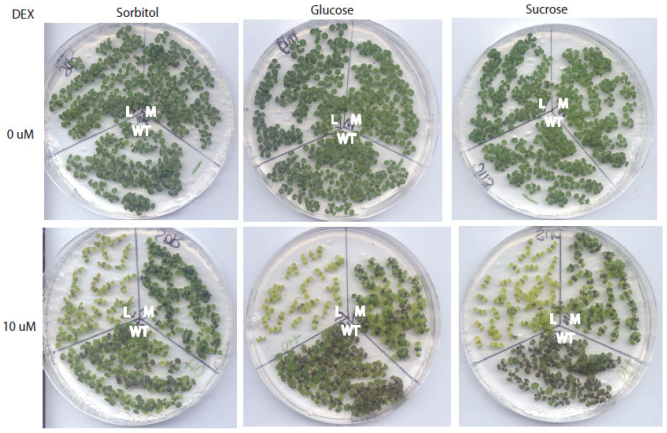

Figure 2-1. Induction of bZIP11 inhibits seedling growth and this inhibition is not reverted by addition of metabolizable sugars. WT, L and M line seedlings as indicated were grown on 30 mM sorbitol, glucose or sucrose, respectively for 7 days followed by addition of 5 ml 10 µM dex to the plates. Photographs were taken five days after the dex treatment.

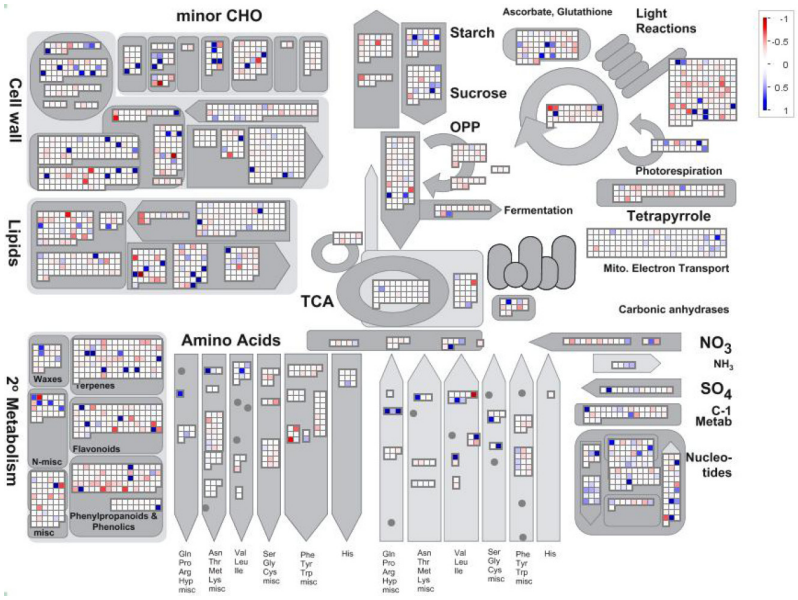

Figure 2-2. Mapman metabolism overview presents the effect of bZIP11 overexpression in Arabidopsis mesophyll protoplasts. The Affymetrix microarray data of bZIP11-transfected protoplast were loaded into the Mapman program. The cutoff of selection is above 2-fold change and p value < 0.05. Blue or red color intensity indicates expression change on a log2 scale.

since 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 Materials and Methods. Ten biological replicates were used for L and M lines each. In total 228 peaks were quantified for each GC-MS sample run and 60 metabolites were unambiguously identified (Table 2-1). Carbohydrates, amino acids and lipids, and their derivatives were mainly identified (Table 2-1). The effect of dex versus mock treatment is expressed as the relative change ratio for each identified metabolite in each line (Table 2-1).

As shown in Table 2-1, for most of the metabolites in wild type (WT) seedlings the relative change ratios between dex over mock treatment were around 1.0, indicating that dex generally did not affect metabolism. These ratios differed from approximately 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 compared to line M. A stringent statistic test (Wilcoxon test, Bonferroni corrected) was applied to the data set and statistically significant changes were identified. Higher numbers of significantly changed metabolite levels were observed in line L compared to line M (twenty-one and six, respectively). None of the changes in the WT were found to be significant. Seven metabolites changed more than two-fold without being statistically significant and eleven changes were found to be significant without a larger than two-fold change.

Previous transcriptomics experiments using dex induced L and M lines and the CATMA micro-arrays already suggested a broader impact of bZIP11 on metabolism (Hanson *et al.*, 2008). Here, transcriptomics experiments using the Affymetrix ATH1 array platform was used in combination with transfection of isolated mesophyll protoplasts to further access the transcriptional potential of bZIP11. ATH1 arrays provide a more sensitive assay and have a better coverage of the Arabidopsis transcriptome. Arabidopsis mesophyll protoplasts were isolated and transfected with a 35S-*bZIP11* construct. Following incubation for six hours, RNAs from three biological replicates were independently isolated and hybridized to the Affymetrix array and the data processed as described in the experimental procedures. Results show that in this system 336 genes were significantly (p-value<0.05) induced or repressed two-fold or more (Table 2-2, see page 67-74). Only twelve genes were repressed supporting the conclusion that bZIP11 primarily acts as a transcriptional activator (Table 2-2, see page 67-74) Many genes encode proteins associated with metabolism and these were displayed using the Mapman program (Thimm *et al.*, 2004) (Figure 2-2). The expression analysis in protoplasts confirms and extends the previously published

Metabolite	Relative change ratio of metabolite level in response to DEX treatment (Ratio ± SD)		
	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
Others			
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
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

Table 2-1. bZIP11 mediates reprogramming of metabolism in Arabidopsis seedlings. GC-MS analysis was employed in this study. Wild type and *bZIP11* dexmethasone inducible line L and M seedlings were cultured in liquid ½ MS medium for seven days, followed by a six hour treatment of either 10 mM dexmethasone or mock. The relative change level upon dexmethasone treatment was normalized to the mock treatment for each compound identified in each line. The relative change ratio ± stand deviation was presented. *statistically significant (p-value<0.05, Wilcoxon test, Bonferroni corrected) changes compared to the mock control.

analysis in seedlings and suggests that bZIP11 affects carbohydrate, amino acid and secondary metabolism.

bZIP11 affects amino acid metabolism

As reported previously (Hanson *et al.*, 2008), dex induction of L and M lines showed substantial amino acid level changes. The levels of amino acids derived from pyruvate, especially valine and alanine, increased in response to bZIP11 activation (Table 2-1). Proline levels decreased approximately five-fold (Table 2-1), which is consistent with the rapid induction of proline dehydrogenases *ProDH1* (At3g30775) and *ProDH2* (At5g38710) as well as *Pyrroline-5-carboxylate dehydrogenase* (*P5CDH,* At5g62530) in seedlings and protoplasts (Table 2-2 see page 67-74, Table 2-3 and Figure 2-3A). These three genes encode active enzymes (Deuschle *et al.*, 2004; Funck *et al.*, 2010). bZIP11 induces glutamate dehydrogenase encoding genes such as *GDH1* (At5g18170) and *GDH2* (At5g07440) (Table 2-3). GDH1 and GDH2 catalyze glutamate to oxoglutarate conversion, and reduced glutamate levels were detected in dex treated L and M seedlings (Table 2-1). The conversion of glutamate to oxoglutarate by GDH1 and GDH2 is critical for Arabidopsis survival during extended night (Miyashita and Good, 2008). However, oxoglutarate is not detected or identified in our metabolomics study.

Aromatic amino acids were strongly affected and levels of phenylalanine, tryptophan and tyrosine increased over two-fold. These amino acids are produced by the shikimate pathway and are precursors for the synthesis of important secondary metabolites. Genes associated with flavonoids and lignins were induced by bZIP11 (Table 2-4 see page 75-76 and Figure 2-2) but metabolites in these pathways were not identified. It is interesting to see the levels of salicylic acid-glucopyranoside (SAG) were strikingly increased. 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 the aromatic amino acids.

bZIP11 affects primary metabolism sugars

Dex treatment significantly increases the levels of several sugars and sugar phosphates in L and M lines but not in wild type. Most prominently, sucrose, glucose, fructose, fructose 6-phosphate and glucose 6-phosphate are increased. A

AGI	Gene name and annotated metabolic function	Protoplasts	Seedlings L	M	WT
At3g30775	*ProDH1*; proline dehydrogenase	2.7*	4.0*	3.2*	0.2
At5g38710	*ProDH2*; proline dehydrogenase	0.4*	1.6*	1.4*	0.2
At5g18170	*GDH1*; glutamate dehydrogenase	0.4	0.8*	0.4	0.0
At5g07440	*GDH2*; glutamate dehydrogenase	0.4*	2.3*	1.5*	0.0
At2g19800	*MIOX2*; myo-inositol oxygenase	2.9*	-	-	-
At4g26260	*MIOX4*; myo-inositol oxygenase	1.5*	-	-	-
At3g57520	*AtSIP2*; raffinose synthase	1.3*	1.2*	1.3*	0.0
At5g20250	*DIN10*; raffinose synthase	1.4*	-	-	-
At4g24040	*TRE1*; trehalase	1.7*	3.5*	3.1*	-0.1

Table 2-3. Genes induced by over expressing bZIP11 in protoplasts or seedlings that show concordant metabolite changes in the GC-MS analysis. Genes were selected from micro-array data sets obtained from 35S-*bZIP11* transfected protoplasts or bZIP11 induced line L and M seedlings (Hanson *et al.*, 2008). The expression of these genes is sugar repressed and extended night induced. Fold inductions were presented on a log2 scale. *statistically

Gene name	Primer name	Sequence
TRE1	TRE1-attB1	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTTGCTTAG CAGGGTGCAATC-3’
TRE1	TRE1-attB2	5’-GGGGACCACTTTGTACAAGAAAGCTGGGTTGAACAGA GAACGTTGCTGTTTAAG-3’
TPP5	TTP5-attB1	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTAGTCAGATC TGAGACTTATGTCTTCGTGACTC -3’
TPP5	TTP5-attB2	5’-GGGGACCACTTTGTACAAGAAAGCTGGGTGAATCTCAG AGATCGCAGAAGAAACCAAATCC-3’
TPP6	TTP6-attB1	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTACTGTCACC ACTGAATCCATGTTTGTCCTG-3’
TPP6	TTP6-attB2	5’-GGGGACCACTTTGTACAAGAAAGCTGGGTTTCTCGGAA TCTCAGGGATCTCGAAAGGAG-3’

Table 2-5. Primer sequences used for constructs illustrated in Figure 2-5. The sequences of primers used for the promoter and luciferase report gene fusion in pUGW35 vector were listed.

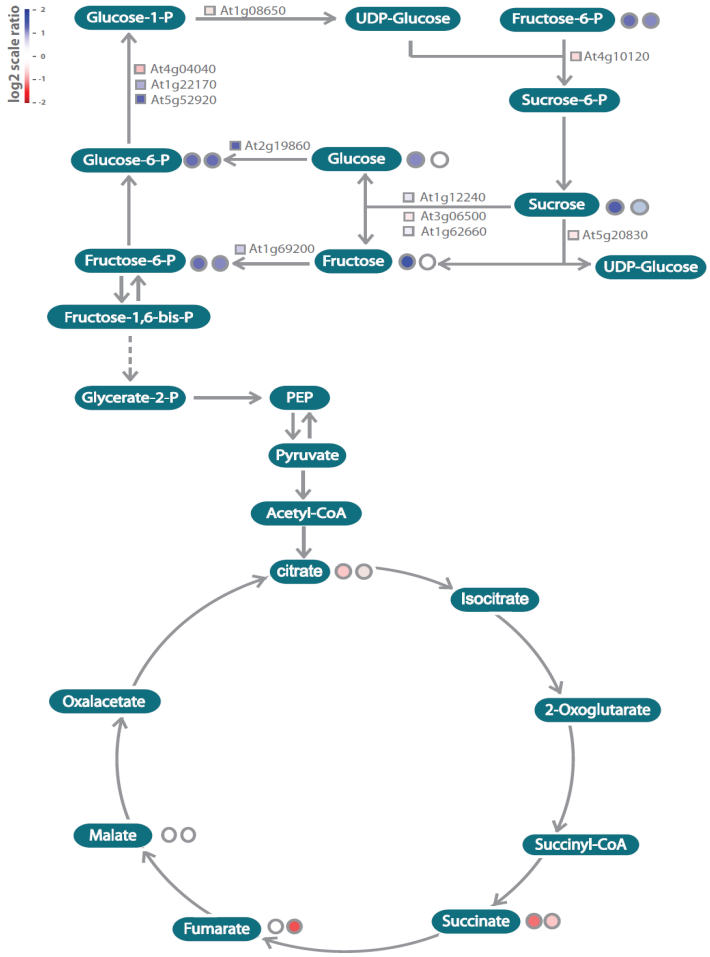

Figure 2-4. bZIP11 induced changes in metabolites and transcripts in central energy related metabolism. Pathways presented were adapted from the Mapman program. Circles denote relative metabolite changes in L (left) and M (right) seedlings as identified in the metabolomics analysis. The corresponding relative change ratio of metabolite in table 1 was converted into log2 scale and presented. The changes of gene expression are above 2-fold and p value < 0.05. Differentially expressed genes were positioned following colored BINs according to the Mapman program. The transcriptomics data set used here was from 35S-*bZIP11* transfected Arabidopsis mesophyll protoplasts. Abbreviations: 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).

group of genes annotated to be involved in the glycolytic pathway were over two-fold induced by bZIP11 in protoplasts. These include genes encoding hexokinase 2 (At2g19860, *AtHXK2*), glyceraldehyde 3-phosphate dehydrogenase A subunit 2 (At1g12900), phosphoglycerate mutase (At1g22170) and fructose biphosphate aldolase (At1g21330) (Table 2-2 and table 2-4, see page 67-76). However, glycolytic intermediates were not identified or detected in the analysis.

Interestingly, levels of TCA cycle intermediates decreased in dex treated L and M lines. Citrate, succinate and fumarate decreased two-fold in L and M lines while malate was not affected (Table 2-1). Thus, bZIP11 induces accumulation of metabolites on the top part of the glycolytic pathway, whereas several TCA cycle metabolites are depleted (Figure 2-4).

bZIP11 promotes raffinose biosynthesis and myo-inositol degradation

Substantial bZIP11 mediated changes in metabolites and genes involved in raffinose biosynthesis and myo-inositol degradation were observed (Figure 2-3B). Raffinose levels increase following bZIP11 activation (Table 2-1). In several species, raffinose is proposed to serve as long distance transportation sugar and as a carbon storage molecule (Sprenger and Keller, 2000). The function of raffinose in Arabidopsis is unclear but raffinose might be a reactive oxygen scavenger (Nishizawa *et al.*, 2008).

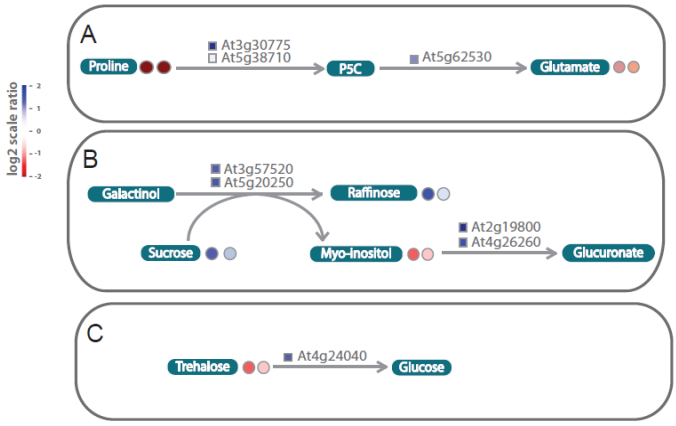

Figure 2-3. Pathways affected by bZIP11 induction as specified by both metabolites and transcripts. Pathways presented were adapted from the Mapman program. The presented changes are on a log2 scale, and the changes of gene expression are above 2-fold and p value < 0.05. The transcriptomics data was derived from 35S-*bZIP11* transfected Arabidopsis mesophyll protoplasts. (A) Proline degradation pathway. (B) The raffinose biosynthesis and myo-inositol degradation pathways. (C) Trehalose degradation pathway. Abbreviation: P5C (1-pyrroline-5-carboxylate).

Among the six Arabidopsis genes annotated as raffinose synthases, raffinose synthase 2 (At3g57520) is strongly induced by bZIP11 in both protoplasts and seedlings (Table 2-3).

Raffinose synthesis from galactinol produces myo-inositol that is further oxidized to glucuronate, a substrate for cell wall biogenesis. It is known that the modulated expression levels of myo-inositol oxygenase encoding genes including *MIOX2* (At2g19800) and *MIOX4* (At4g26260) lead to the altered levels of myo-inositol (Kanter *et al.*, 2005; Endres *et al.*, 2009). Two myoinositol dehydrogenase encoding genes, *MIOX2* (At2g19800) and *MIOX4* (At4g26260) were induced by bZIP11 and might contribute to the reduced myo-inositol levels (Table 2-1). Decreased myo-inositol levels and increased *MIOX2* and *MIOX4* transcript levels were also observed during extended night treatment of Arabidopsis, whereas during the normal diurnal cycle myo-inositol is not affected (Gibon *et al.*, 2006).

bZIP11 promotes degradation of trehalose and T6P

Trehalose levels were decreased in dex treated L and M lines (Table 2-1) and the expression of *TRE1* was induced in bZIP11 over expressing protoplasts and seedlings (Hanson *et al.,* 2008; Table 2-3 and figure 2-3C). In addition, *TPP5* and *TPP6* genes were significantly induced as well by bZIP11. In seedlings, bZIP11 induction activated *TPP5* expression two-fold (Hanson *et al.*, 2008) and in protoplasts bZIP11 induced *TPP6* expression four-fold (Table 2-2, see page 67-74). Previous studies on promoter sequences of genes regulated by bZIP11 identified the ACGT core promoter element recognized by bZIP11. Detailed studies of the *ASN1* promoter showed that in protoplasts the ACGT element is essential for bZIP11 on the promoter activation of *ASN1* (Baena-Gonzalez *et al.*, 2007; Hanson *et al.*, 2008). The promoters of *TRE1*, *TPP5* and *TPP6* contain two or more such cis-elements (Figure 2-5). 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 approximately 1.5 kb of *TRE1, TPP5* and *TPP6* were fused to the luciferase reporter gene (Figure 2-5) 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 *ATH1* homeodomain transcription factor (Quaedvlieg *et al.*, 1995) was used as a control in these experiments. bZIP11 efficiently induces transcription from *TRE1, TPP5* and *TPP6* promoters, whereas ATH1 essentially has no effect on these promoters (Figure 2-6).

The transcriptional activation of bZIP11 on these trehalose metabolism genes likely 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 (Figure 2-7A) with a concomitant decrease in trehalose levels (Table 2-1). Dex did not affect trehalase activity or trehalose levels in WT seedlings. Cellular T6P levels 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 levels were determined in L and M seedlings grown on trehalose medium in the presence or absence of dex and results show that bZIP11 induction efficiently reduces T6P levels in both lines (Figure 2-7B). *TPP5* and *TPP6* were proposed to encode enzymatically active T6P phosphatases (Vandesteene *et al.*, 2010) that might be 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 due to the accumulation of T6P. bZIP11 induction rescues root growth in L and M seedlings grown on trehalose medium in accordance with the observed lowered T6P levels (Figure 2-7C,D). Validamycin A inhibits trehalase activity, without affecting plant growth at the concentration of 10 μM (Muller *et al.*, 2001). In the presence of 10 μM validamycin A, root lengths of bZIP11 induced seedlings grown on trehalose medium are similar to WT (Figure 2-7C,D). Thus bZIP11 induced trehalase activity is important for root growth on trehalose medium as well. In conclusion, bZIP11 induction activates *TRE1*, *TPP5* and *TPP6* expression, resulting in lowered T6P levels in L and M seedlings grown on trehalose medium and trehalose resistant root growth.

Discussion

bZIP11 mediated changes in metabolism resemble carbon depletion condition

In Arabidopsis, constitutive or dex induced bZIP11 nuclear presence inhibits plant growth in a concentration dependent manner (Hanson *et al.*, 2008). Sugar addition only partially relieves this inhibition (Hanson *et al.*, 2008 and results not shown). In tobacco, 35S promoter driven *bZIP11* expression also severely inhibits growth (unpublished observations). The growth inhibition by bZIP11 induction in Arabidopsis is not relieved by the presence of metabolizable sugars such as glucose and sucrose in the growth medium (Figure 2-1). Previous analysis of L and M seedlings in Arabidopsis suggested that bZIP11 is a regulator of amino acid metabolism and *ASN1* and *PRODH2* were proposed to be direct targets of bZIP11 (Hanson *et al.*, 2008). In this chapter, metabolomics, transcriptomics and molecular analysis suggest that bZIP11 is a powerful regulator of metabolism. Controlled bZIP11 nuclear presence results in strong metabolic reprogramming that likely is responsible for the growth inhibition phenotype.

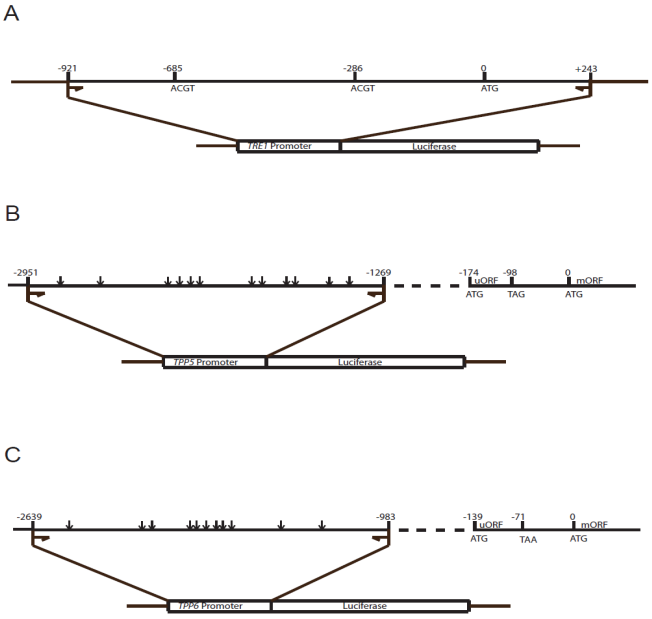

Figure 2-5. Graphical representation of *TRE1* (A), *TPP5* (B) and *TPP6* (C) promoter regions used for transcription activation studies in protoplasts. The ACGT core sequences are indicated by arrows. Nucleotide numbering is relative to starting codon.

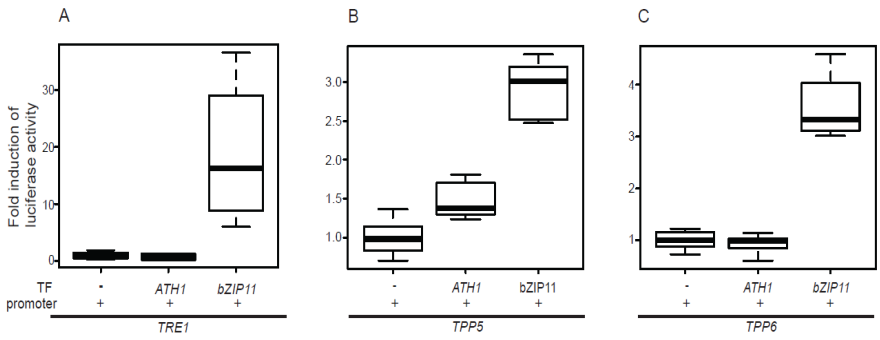

Figure 2-6. bZIP11 activates transcription from *TRE1* (A), *TPP5* (B) and *TPP6* (C) promoters in transiently transfected Arabidopsis mesophyll protoplasts. Promoters were fused to the luciferase reporter gene and transfected either alone or in combination with a 35S-*bZIP11* plasmid. Promoter fragments used are approx. 1.5 kb in length and are presented in figure 2-5. A 35S-*ATH1* construct, encoding the ATH1 transcription factor was used as an unrelated control and the results were compared to that of the transfections in which the transcription factor constructs was omitted. Three independent repetitions were performed with four to six biological replicates each.

Within six hours following dex treatment of L and M seedlings and of bZIP11 transfected protoplasts, massive changes in gene expression and metabolite content were observed when compared to control treatments. Remarkably, these bZIP11 induced changes resemble those observed for plants grown under carbon limitation. Extended night leads to reduced levels of myo-inositol, proline, glutamate, fumarate, citrate, succinate and increased levels of aromatic amino acids (Hayashi *et al.*, 2000; Gibon *et al.*, 2006). It is an interesting observation that extended night which is a carbon starvation condition raises the levels of aromatic acids, whereas the mechanism behind is not clear. In addition, sucrose

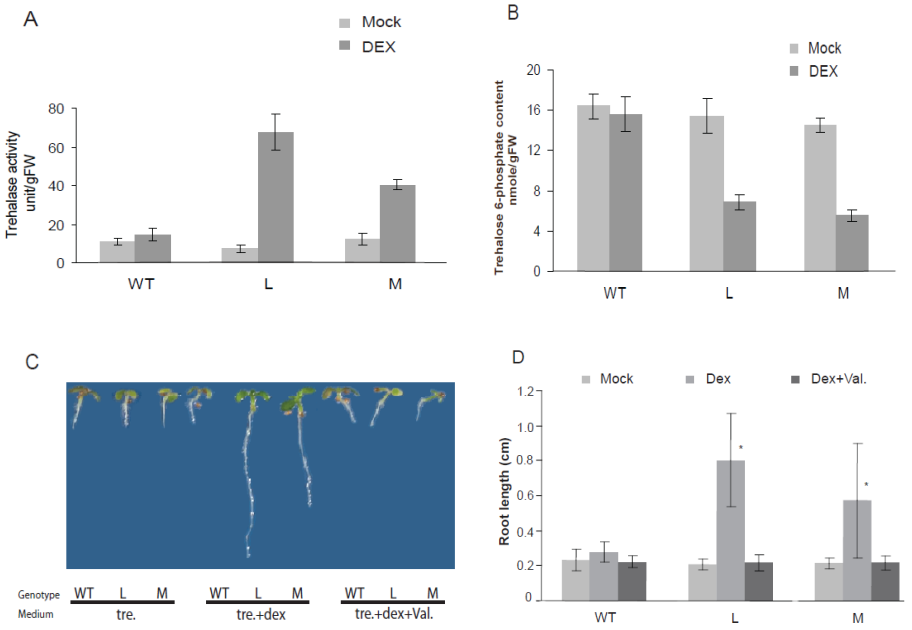

Figure 2-7. bZIP11 regulates trehalose metabolism. (A) Trehalase activity was assayed in seven days old WT, L and M seedlings grown on agar solid medium containing 100 mM sorbitol plus or minus 0.2 µM dex. (B) Trehalose 6-phosphate content was quantified in seven days old WT, L and M seedlings grown on agar solid medium containing 100 mM trehalose plus or minus 0.2 µM dex. (C) Seven days old WT, L and M seedlings grown on 100 mM trehalose in the presence of 0.2 µM dex and containing 10 µM validamycin A as indicated. (D) Quantification of root lengths of seedlings presented in (C). Abbreviations: tre. (trehalose), Val.(validamycin A). *statistically significant (p value<0.05, two sampled student t-test) root length difference between L (n=219) or M (n=166) line and WT (n=75) seedlings upon dex treatment on trehalose medium.

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 2-3), which were shown to be both sucrose repressed and extended night induced (Figure 2-8; Blasing *et al.*, 2005;

Gibon *et al.*, 2006). These bZIP11 mediated changes at both metabolite and transcript levels are remarkable since 100 mM sucrose was added to the medium to repress endogenous S1 group bZIP genes.

Levels of sucrose, glucose, fructose, glucose 6-phosphate, fructose 6-phosphate increase following bZIP11 induction, while during extended night these sugars decrease markedly (Gibon *et al.*, 2006). These sucrose-derived metabolites might increase due to the carbon rich culture condition 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). 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 (TF) regulatory network that controls distinct and overlapping genes involved in the regulation of metabolism and other processes as well (Alonso *et al.*, 2009). Such a C/S1 bZIP TF 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 levels (Hanson and 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 TF regulatory network under the control of the cellular sucrose concentration in a dynamic way with half maximum inhibition of S1 bZIPs mRNA translation in the range of 10-20 mM sucrose (Wiese *et al.*, 2005; Rahmani *et al.*, 2009).

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). Results presented in this manucript suggest that bZIP11 induces *TRE1*, *TPP5* and *TPP6* genes. Induction of these genes corresponds to altered metabolite, transcript and, for trehalase, enzymatic activity levels, and partially restored root growth in bZIP11 induced seedlings grown on trehalose medium. The protoplast transactivation assay suggests that *TRE1*, *TPP5* and *TPP6* promoters might be direct targets of bZIP11 but this requires further investigation.

bZIP11 activity is capable of lowering T6P levels. The lowered T6P levels might

contribute to the altered metabolizable sugar utilizing ability in the bZIP11 induced lines. The accumulation of sugar and sugar phosphates in bZIP11 induced lines might be due to the blockage of their utilization. Interestingly, also in the reduced function *tps1* mutants sucrose, glucose, fructose, sucrose 6-phosphate and hexose phosphates accumulate, likely due to a block in their utilization for

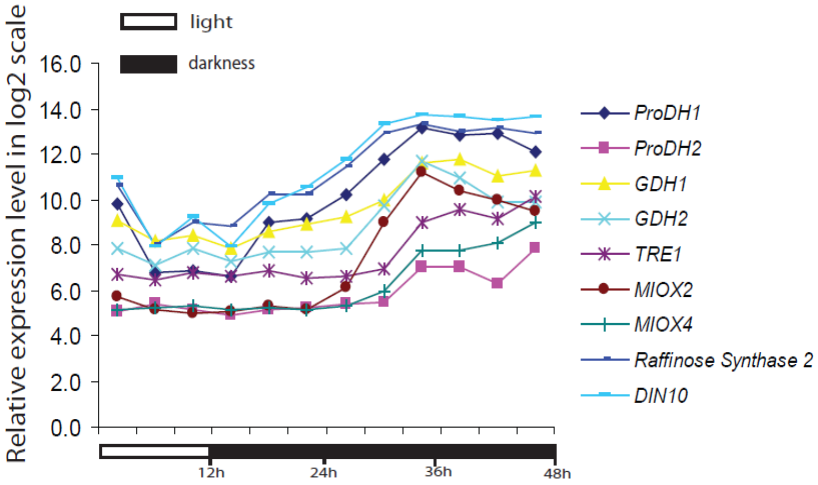

Figure 2-8. Expression pattern of selected bZIP11 regulated genes during the diurnal cycle and in extended night. Expression data are from Gibon *et al.*, (2006). The open bar (0-12 h) represents light treatment and the closed bar (12-48h) represents the dark treatment. Extended night is after 24h.

growth (Gomez *et al.*, 2010). The expression patterns of *TPS1* and *bZIP11* are partially overlapping. Therefore, it appears that bZIP11 acts as a regulator of trehalose metabolism.

bZIP11 in relation to the SnRK1 and T6P regulatory systems

As described in the introduction, SnRK1 and T6P regulatory systems somehow perceive 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 strongly activates the transcriptional activity of S1 group bZIP proteins, including bZIP11 (Baena-Gonzalez *et al.*, 2007). In this study, we show that bZIP11 regulates trehalose metabolism and reduces T6P levels. Thus, it appears that SnRK1, T6P and bZIP11 function in a regulatory circuit that links

sucrose status to growth. In Figure 2-9, the interactions of these regulatory systems is briefly presented .

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 expressed in several layers of mesophyll cells surrounding the veins as well. 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

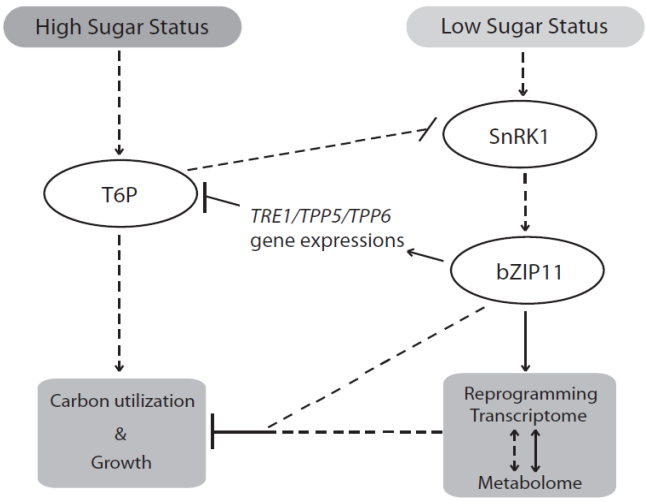

Figure 2-9. Model for the SnRK1 - bZIP11- T6P regulatory circuit. High cellular sugar condition activates the T6P signaling pathway to utilize carbon for growth. In these conditions SnRK1 activity is inhibited by high T6P levels. Low cellular sugar conditions promote SnRK1 activity and activate downstream signaling components, including bZIP11. Increased bZIP11 activity reprograms metabolism as evidenced by changes in the transcriptome and metabolome. The induction of *TRE1*, *TPP5* and *TPP6* reduces the levels of T6P, relieving T6P mediated inhibition of SnRK1. Solid lines indicate potential direct interactions. Stippled lines indicate potential intermediate steps.

metabolism and thereby on nutrient translocation. In *bZIP11* over expressing tobacco lines, carbon translocation is severely inhibited (unpublished results). Likely, the C/S1 bZIP TF 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.

Taken together, our findings suggest that bZIP11 reprograms metabolism to mimic a carbon starvation state, which is one step further in deciphering the elusive biological function of bZIP11. Furthermore, it is shown that bZIP11 transcriptionally regulates several trehalose metabolism genes. Thus, bZIP11 can function in a regulatory circuit with SnRK1 and T6P, connecting cellular carbon levels to growth.

Experimental procedures

Growth conditions

Arabidopsis thaliana seeds (var. Col-0, CS60000) were gas sterilized and stratified at 4°C in the dark for 2 days. For liquid medium culture, seedlings were grown in ½ MS media including vitamins and MES-buffer (Duchefa, www.duchefa.com), supplemented with 100 mM sucrose for 7 days under continuous fluorescent light (100 microE/m^2) on a rotary shaker (60 rpm). Seedlings were dexamethasone (10 μM) or mock treated for 6 h prior to the harvest in liquid nitrogen. For solid medium culture, the seedlings were grown on ½ MS media including vitamins and MES-buffer (Duchefa) solidified by 0.8% (w/v) Plant agar (Duchefa) supplemented with 100mM trehalose or sorbitol and 0.2 μM dexamethasone or mock treatment under a photoperiod of 16 h florescent light (100 microE/m^2) and 8 h dark for 7 days.

GC-MS metabolomic analysis

Seedlings cultured in liquid media were carefully washed in deionized water and snap frozen in liquid nitrogen. There were 10 biological replicates 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 μl^{-1} each of [$^{13}C_3$]-myristic acid, [$^{13}C_4$]-hexadecanoic acid, [$^{2}H_4$]-succinic acid, [$^{13}C_5$, ^{15}N]-glutamic acid, [$^{2}H_7$]-cholesterol, [$^{13}C_5$]-proline, [$^{13}C_4$]-disodium 2-oxoglutarate, [$^{13}C_{12}$]-sucrose, [$^{2}H_4$]-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 14,000 rpm 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 prior to incubation for 16 h at room temperature. The samples were then trimethylsilylated by adding 30 μl of MSTFA with 1% TMCS 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 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 x 0.18 mm i.d. fused silica capillary column with a chemically bonded 0.18 µm DB 5-MS 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, 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. 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 to 800 m z^{-1}. The acceleration voltage was turned on after a solvent delay of 170 s. The detector voltage was 1660 V. All non-processed MS-files from the metabolic analysis were exported into MATLAB, in which all data pre-treatment procedures, such as base-line 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 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 normalization vector.

Construction of plasmid DNA

For transient expression of bZIP11 transcription factor in protoplasts, 35S driven 3x HA tagged bZIP constructs were made (Ehlert *et al.*, 2006). The bZIP11 transcription factor sequence was 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 NcoI/NotI- digestion, Klenow fill in and religation (Thorsten Heinekamp, pers. com.). The promoters of the *TRE1* (At4g24040), *TPP5* (At4g12430) and *TPP6* (At4g22590) genes were fused to luciferase gene in pUGW35 (T. Nakagawa pers. com) using Gateway™ technology (Invitrogen). The fragment of each promoter was amplified from *Arabidopsis thaliana* wild type (var. Col-0, CS60000) genomic DNA using primers listed in table 2-5.

Transient expression of bZIP transcription factors in protoplasts

The protoplast preparation was adapted from (Yoo *et al.*, 2007). Arabidopsis mesophyll protoplasts were isolated from leaves (the second and/or third/fourth

pair) of 5 weeks old *Arabidopsis thaliana* (var. Col-0, CS60000) plants grown on soil under long day (16 h light / 8 h dark) conditions. Leaves were placed in enzyme solution for 8.5 h (1% cellulase R10, 0.3% macerozyme R10 (Yakult Honsha, http://www.yakult.co.jp/english/), 0.4 M mannitol, 20 mM KCl, 10 mM $CaCl_2$, 20 mM MES, 0.1% BSA (Sigma A-6793), pH 5.7). Protoplasts were collected and kept on ice in W5 medium (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 2 mM MES, pH 5.7) for 13h in the growth cabinet. Protoplasts were transferred to MMG solution (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7), and subjected to PEG transfection. To $2.12x10^6$ protoplasts, a total of 250 µg plasmid DNA was added followed by 30 min incubation in 1 volume of PEG solution (40% PEG 3500, 3 ml H_2O, 0.2 M mannitol, 0.1 M $CaCl_2$). After transfection the samples were diluted with 2 volumes of W5 solution and pelleted at 100 g for 2 min. Protoplasts were then resuspended in 4 ml WI medium (0.5 M mannitol, 20 mM KCl, 4 mM MES, pH 5.7), transferred to 5 cm Petri dishes pre-coated with 5% calf serum, and incubated for 6 h in the growth cabinet. After the incubation protoplasts were collected and 100-200 mg aliquots were flash frozen in liquid nitrogen for expression micro-array analysis.

Micro-array analysis

Total RNA was purified using the RNeasy kit (Qiagen, http://www.qiagen.com) and the RNA purity and integrity were confirmed by using a RNA 6000 Nano Assay (Agilent, http://www.home.agilent.com) and gel electrophoresis. cRNA labeling, hybridization, washing and scanning of Affymetrix Arabidopsis ATH1 GeneChips® (Affymetrix, http://affymetrix.com) was performed according to Affymetrix OneCycle Lab protocols. Data were analyzed statistically using the R language environment for statistical computing (http://www.r-project.org) version 2.11.1 and Bioconductor release 2.6 (Gentleman *et al.*, 2004). Data were normalized using the Robust Multichip Average (RMA) expression measure in the Affy package (Gautier *et al.*, 2004). Differentially expressed genes were identified using the LIMMA package (Smyth *et al.*, 2005). The probe set sequences were aligned to the Tair10 gene model database of transcripts (www.arabidopsis.org).

Transient transcriptional activation test in protoplasts

Protoplasts were extracted from rosette leaves of 3-4 weeks old *Arabidopsis thaliana* (var. Col-0, CS60000) plants grown on soil under long day (16 h light / 8 h dark) conditions. Protoplasts were co-transfected with the construct described above and either plasmids containing 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, 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 construct with *TRE1* promoter, and 6 h incubation time for the construct with *TPP5* or *TPP6* promoter.

Trehalase activity assay

Solid medium cultured seedling material (50 mg fresh weight per sample) was pulverized and suspended in the protein extraction buffer (50 mM morpholinoethanesulfonic acid /K^+ (pH 6.3), 1 mM EDTA, 1 mM phenyl methyl-sulfonyl fluoride, 0.01% (v/v) Triton X-100, 1% (w/v) polyvinylpyrrolidone). The suspensions were incubated on ice for 2 h, and following centrifugation at maximum speed for 10 minutes the supernatants were used. The protein extracts were desalted using Amicon Ultra 0.5ml 10K centrifugal filters (Millipore, www.millipore.com) prior to the incubation with the substrate trehalose (Sigma, www.sigma.com) to a final concentration of 25 μM at 37°C. The amount of glucose released from the reactions was determined by the D-glucose test kit (R-Biopharm, www.r-biopharm.com).

Trehalose 6-phosphate measurements

Trehalose 6-phosphate was extracted from 50 mg fresh weight solid medium cultured seedlings and the levels were determined as in Delatte et al., 2009. Five biological replicates per line were used for each treatment.

Root length measurements

Solid medium cultured seedlings as described above were used for root length measurements. Digital photos were taken from the seven days old seedlings grown on solid medium in the petri-dishes positioned vertically. Photos 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 absolute lengths by taking the width of petri-dish as a reference.

Acknowledgements

The 35S-*ATH1* plasmid was provided by Dr. Marcel Proveniers and Evelien van Eck-Stouten (Utrecht University). The pGWB vector series were kindly provided by Dr. Tsuyoshi Nakagawa (Shimane University, Izumo, Japan).

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AGI	Annotation	Fold Change
AT4G34590; AT4G34588	ARABIDOPSIS THALIANA BASIC LEUCINE-ZIPPER 11	8.27
AT1G03870	FLA9	7.28
AT3G45970	ATEXLA1 (ARABIDOPSIS THALIANA EXPANSIN-LIKE A1)	6.84
AT4G33420	peroxidase, putative	6.09
AT1G62480	vacuolar calcium-binding protein-related	5.45
AT3G09940	ATMDAR3/MDHAR (MONODEHYDROASCORBATE REDUCTASE)	5.29
AT5G08350	GRAM domain-containing protein / ABA-responsive protein-related	5.20
AT3G45860	receptor-like protein kinase, putative	4.87
AT2G40170	ATEM6/GEA6 (ARABIDOPSIS EARLY METHIONINE-LABELLED 6)	4.77
AT5G22580	Identical to Uncharacterized protein At5g22580	4.63
AT5G44130	FLA13 (FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 13 PRECURSOR)	4.60
AT5G66170	similar to unknown protein [Arabidopsis thaliana]	4.52
AT3G52500	aspartyl protease family protein	4.49
AT1G16850	unknown protein	4.28
AT2G44670	senescence-associated protein-related	4.26
AT5G56540	AGP14 (ARABINOGALACTAN PROTEIN 14)	4.12
AT1G27950	lipid transfer protein-related	4.05
AT4G33700	CBS domain-containing protein	3.98
AT5G15410	DND1 (DEFENSE NO DEATH 1)	3.94
AT1G15040	glutamine amidotransferase-related	3.89
AT2G46270	GBF3 (G-BOX BINDING FACTOR 3); transcription factor	3.81
AT3G20110	CYP705A20 (cytochrome P450, family 705, subfamily A, polypeptide 20); oxygen binding	3.78
AT2G35090; AT2G35070	[AT2G35090, similar to unknown protein	3.72
AT5G45690	similar to unknown protein	3.70
AT3G43430	zinc finger (C3HC4-type RING finger) family protein	3.61
AT5G62480	ATGSTU9 (GLUTATHIONE S-TRANSFERASE TAU 9); glutathione transferase	3.59
AT5G01050; AT5G01040	[AT5G01050, laccase family protein	3.56
AT1G73120	similar to hypothetical protein [Vitis vinifera] (GB:CAN69175.1)	3.55
AT5G45630	unknown protein	3.55
AT1G01620	PIP1C (PLASMA MEMBRANE INTRINSIC PROTEIN 1;3); water channel	3.55
AT3G55150	ATEXO70H1 (exocyst subunit EXO70 family protein H1); protein binding	3.51
AT5G39720	AIG2L (AVIRULENCE INDUCED GENE 2 LIKE PROTEIN)	3.47
AT5G43410	ethylene-responsive factor, putative	3.31
AT5G18450	AP2 domain-containing transcription factor, putative	3.23
AT1G66890	similar to 50S ribosomal protein-related hypothetical protein	3.22
AT1G17615	disease resistance protein (TIR-NBS class), putative	3.20
AT4G10510	subtilase family protein	3.16
AT5G24950	CYP71A15 cytochrome P450	3.14
AT2G40750	WRKY54 (WRKY DNA-binding protein 54); transcription factor	3.14
AT3G55730	MYB109 (myb domain protein 109); DNA binding / transcription factor	3.12
AT4G29600	cytidine deaminase, putative / cytidine aminohydrolase, putative	3.11

AT2G03730	ACR5 (ACT Domain Repeat 5)	3.06
AT3G55940	phosphoinositide-specific phospholipase C, putative	3.04
AT4G35150; AT4G35160	[AT4G35150, O-methyltransferase family 2 protein];[AT4G35160, O-methyltransferase family 2 protein]	3.00
AT5G04310	pectate lyase family protein	2.95
AT2G19800	MIOX2 (MYO-INOSITOL OXYGENASE 2)	2.86
AT3G56500	serine-rich protein-related	2.85
AT1G71980	protease-associated zinc finger (C3HC4-type RING finger) family protein	2.83
AT1G19450	integral membrane protein, putative / sugar transporter family protein	2.83
AT3G60280	UCC3 (UCLACYANIN 3); copper ion binding	2.82
AT2G47770	benzodiazepine receptor-related	2.77
AT1G15820	LHCB6 (LIGHT HARVESTING COMPLEX PSII); chlorophyll binding	2.76
AT1G75300	isoflavone reductase, putative	2.75
AT5G22460	esterase/lipase/thioesterase family protein	2.72
AT3G30775	ERD5 (EARLY RESPONSIVE TO DEHYDRATION 5); proline dehydrogenase	2.69
AT5G41080	glycerophosphoryl diester phosphodiesterase family protein	2.69
AT3G10720	pectinesterase, putative	2.67
AT1G75800	pathogenesis-related thaumatin family protein	2.65
AT2G06925	ATSPLA2-ALPHA/PLA2-ALPHA (PHOSPHOLIPASE A2-ALPHA); phospholipase A2	2.63
AT1G23060	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G70950.1)	2.62
AT3G17420	GPK1 (Glyoxysomal protein kinase 1); kinase	2.60
AT2G39130	amino acid transporter family protein	2.59
AT3G54940	cysteine proteinase, putative	2.58
AT3G23560	ALF5 (ABERRANT LATERAL ROOT FORMATION 5); antiporter/ transporter	2.57
AT3G09150	HY2 (ELONGATED HYPOCOTYL 2); phytochromobilin:ferredoxin oxidoreductase	2.55
AT4G28040	nodulin MtN21 family protein	2.54
AT1G03990	alcohol oxidase-related	2.45
AT1G52540	protein kinase, putative	2.45
AT3G11340	UDP-glucoronosyl/UDP-glucosyl transferase family protein	2.44
AT4G21830; AT4G21840	methionine sulfoxide reductase domain-containing protein	2.40
AT1G62510	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	2.40
AT5G46590	ANAC096 (Arabidopsis NAC domain containing protein 96); transcription factor	2.39
AT5G51680	hydroxyproline-rich glycoprotein family protein	2.38
AT3G28180	ATCSLC04 (CELLULOSE-SYNTHASE LIKE C 4); transferase	2.36
AT1G58440	XF1 (SQUALENE EPOXIDASE 1); oxidoreductase	2.35
AT4G01120	GBF2 (G-BOX BINDING FACTOR 2); DNA binding / transcription factor	2.34
AT1G30820	CTP synthase, putative / UTP--ammonia ligase, putative	2.30
AT2G35000	zinc finger (C3HC4-type RING finger) family protein	2.29
AT1G01570	fringe-related protein	2.29
AT2G01420	PIN4 (PIN-FORMED 4); auxin:hydrogen symporter/ transporter	2.28
AT2G38465	similar to unnamed protein product [Vitis vinifera] (GB:CAO61577.1)	2.27
AT3G56240	CCH (COPPER CHAPERONE)	2.26
AT2G21330	fructose-bisphosphate aldolase, putative	2.22
AT3G29160	AKIN11 (ARABIDOPSIS SNF1 KINASE HOMOLOG 11); protein kinase	2.20

AT3G18560	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G49000.1)	2.20
AT5G01670	aldose reductase, putative	2.20
AT5G04310	pectate lyase family protein	2.19
AT2G19320	unknown protein	2.19
AT4G22590; AT4G22592	trehalose-6-phosphate phosphatase	2.15
AT5G43150	similar to unnamed protein product [Vitis vinifera] (GB:CAO61459.1)	2.12
AT5G14500	aldose 1-epimerase family protein	2.11
AT1G23020	ATFRO3/FRO3 (FERRIC REDUCTION OXIDASE 3); ferric-chelate reductase	2.10
AT5G18840	sugar transporter, putative	2.10
AT4G23320	protein kinase family protein	2.08
AT4G17460	HAT1 (homeobox-leucine zipper protein 1); DNA binding / transcription factor	2.08
AT1G63950	heavy-metal-associated domain-containing protein	2.06
AT1G67480	kelch repeat-containing F-box family protein	2.06
AT1G02810	pectinesterase family protein	2.05
AT1G12900	GAPA-2; glyceraldehyde-3-phosphate dehydrogenase	2.04
AT4G29590	methyltransferase	2.03
AT2G21340	enhanced disease susceptibility protein, putative / salicylic acid induction deficient protein	2.02
AT4G38400	ATEXLA2 (ARABIDOPSIS THALIANA EXPANSIN-LIKE A2)	2.01
AT2G20370	KAM1/MUR3 (MURUS 3); catalytic/ transferase, transferring glycosyl groups	2.01
AT2G14170	ALDH6B2 (Aldehyde dehydrogenase 6B2); 3-chloroallyl aldehyde dehydrogenase	1.98
AT3G47340	ASN1 (DARK INDUCIBLE 6)	1.98
AT5G58650	PSY1 (PLANT PEPTIDE CONTAINING SULFATED TYROSINE 1)	1.98
AT5G07460	PMSR2 (PEPTIDEMETHIONINE SULFOXIDE REDUCTASE 2)	1.96
AT5G39020	protein kinase family protein	1.96
AT1G48960	universal stress protein (USP) family protein	1.96
AT4G21850	methionine sulfoxide reductase domain-containing protein / SeIR domain-containing protein	1.96
AT4G01430	nodulin MtN21 family protein	1.95
AT1G11440	similar to glycine-rich protein [Arabidopsis thaliana] (TAIR:AT3G29075.1)	1.95
AT3G26690	ATNUDT13 (ARABIDOPSIS THALIANA NUDIX HYDROLASE HOMOLOG 13)	1.94
AT2G26080	ATGLDP2 (ARABIDOPSIS THALIANA GLYCINE DECARBOXYLASE P-PROTEIN 2)	1.92
AT2G29730; AT2G29710	UDP-glucoronosyl/UDP-glucosyl transferase family protein	1.89
AT1G01540	protein kinase family protein	1.88
AT4G39320	microtubule-associated protein-related	1.88
AT1G73390	unknown protein	1.87
AT3G25010	disease resistance family protein	1.86
AT1G50890	binding	1.86
AT2G20670	unknown protein	1.85
AT5G67360	ARA12; subtilase	1.85
AT1G72500	inter-alpha-trypsin inhibitor heavy chain-related	1.84
AT1G55020	LOX1 (Lipoxygenase 1); lipoxygenase	1.84
AT1G51090	heavy-metal-associated domain-containing protein	1.84
AT4G36870	BLH2 (BEL1-LIKE HOMEODOMAIN 2, SAWTOOTH 1)	1.84
AT1G11530	C-TERMINAL CYSTEINE RESIDUE IS CHANGED TO A SERINE 1	1.82

AT4G12330	CYP706A7 (cytochrome P450, family 706, subfamily A, polypeptide 7); oxygen binding	1.82
AT1G16500	unknown protein	1.82
AT3G03180	Got1-like family protein	1.81
AT2G41830	cyclin-related	1.81
AT3G45850	kinesin motor protein-related	1.80
AT3G56880	VQ motif-containing protein	1.79
AT4G00080	UNE11 (unfertilized embryo sac 11); pectinesterase inhibitor	1.79
AT1G22810	AP2 domain-containing transcription factor, putative	1.79
AT3G12500	ATHCHIB (BASIC CHITINASE); chitinase	1.78
AT5G14700	cinnamoyl-CoA reductase-related	1.77
AT2G39710	aspartyl protease family protein	1.77
AT3G15357	unknown protein	1.76
AT2G45920	U-box domain-containing protein	1.76
AT3G55980	zinc finger (CCCH-type) family protein	1.76
AT2G35075	similar to unnamed protein product [Vitis vinifera] (GB:CAO47695.1)	1.75
AT3G19920	unknown protein	1.75
AT1G02660	lipase class 3 family protein	1.75
AT1G01630	SEC14 cytosolic factor, putative / phosphoglyceride transfer protein, putative	1.75
AT5G59680	leucine-rich repeat protein kinase, putative	1.75
AT3G03520	phosphoesterase family protein	1.75
AT2G38490	CIPK22 (CBL-INTERACTING PROTEIN KINASE 22); kinase	1.75
AT4G24040	ATTRE1/TRE1 (TREHALASE 1); alpha,alpha-trehalase/ trehalase	1.74
AT2G36310	inosine-uridine preferring nucleoside hydrolase family protein	1.74
AT3G16800	protein phosphatase 2C, putative / PP2C, putative	1.74
AT2G30600	BTB/POZ domain-containing protein	1.73
AT1G60950	FED A (FERREDOXIN 2); 2 iron, 2 sulfur cluster binding / electron carrier/ iron-sulfur cluster binding	1.71
AT4G36360	BGAL3 (beta-galactosidase 3); beta-galactosidase	1.70
AT2G30600	BTB/POZ domain-containing protein	1.69
AT5G44260	zinc finger (CCCH-type) family protein	1.69
AT5G58090	glycosyl hydrolase family 17 protein	1.68
AT3G61650; AT5G05620	TUBG1 (GAMMA-TUBULIN	1.67
AT5G48690	similar to ubiquitin-associated (UBA)/TS-N domain-containing protein	1.66
AT1G20840	TMT1 (TONOPLAST MONOSACCHARIDE TRANSPORTER1)	1.66
AT1G32150	bZIP transcription factor family protein	1.65
AT1G60680	AGD2 (ARF-GAP DOMAIN 2); aldo-keto reductase	1.64
AT4G38340	RWP-RK domain-containing protein	1.64
AT1G54740	similar to structural constituent of ribosome protein	1.62
AT1G74450	unknown protein	1.62
AT1G51850	leucine-rich repeat protein kinase, putative	1.61
AT3G57800	basic helix-loop-helix (bHLH) family protein	1.61
AT2G25200	unknown protein	1.61
AT1G73020	unknown protein	1.60
AT1G22570	proton-dependent oligopeptide transport (POT) family protein	1.60

AT3G53620	ATPPA4 (ARABIDOPSIS THALIANA PYROPHOSPHORYLASE 4)	1.60
AT4G33750	unknown protein	1.59
AT4G39660	AGT2 (ALANINE:GLYOXYLATE AMINOTRANSFERASE 2); alanine-glyoxylate transaminase	1.58
AT5G26731	unknown protein	1.58
AT3G45960	ATEXLA3 (ARABIDOPSIS THALIANA EXPANSIN-LIKE A3)	1.58
AT4G26690	MRH5/SHV3 (morphogenesis of root hair 5)	1.57
AT4G00430	TMP-C (PLASMA MEMBRANE INTRINSIC PROTEIN 1;4); water channel	1.57
AT3G28540	AAA-type ATPase family protein	1.56
AT1G58270	ZW9	1.56
AT1G63420	unknown protein	1.55
AT5G17010	sugar transporter family protein	1.54
AT1G53885; AT1G53903	senescence-associated protein-related	1.54
AT2G30500	kinase interacting family protein	1.54
AT1G16460	ATRDH2 (ARABIDOPSIS THALIANA RHODANESE HOMOLOGUE 2)	1.53
AT3G15450	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT4G27450.1)	1.53
AT5G22920	zinc finger (C3HC4-type RING finger) family protein	1.52
AT2G19460	unknown protein	1.52
AT3G48580	xyloglucan:xyloglucosyl transferase, putative	1.52
AT2G34600	JAZ7/TIFY5B (JASMONATE-ZIM-DOMAIN PROTEIN 7)	1.52
AT3G56930	zinc finger (DHHC type) family protein	1.51
AT1G22430	alcohol dehydrogenase, putative	1.51
AT1G64620	Dof-type zinc finger domain-containing protein	1.51
AT4G11820	MVA1 (HYDROXYMETHYLGLUTARYL-COA SYNTHASE)	1.49
AT2G39980	transferase family protein	1.49
AT1G13260	RAV1 (Related to ABI3/VP1 1); DNA binding / transcription factor	1.48
AT1G72430	auxin-responsive protein-related	1.48
AT1G04160	XIB (Myosin-like protein XIB)	1.48
AT4G37560	formamidase, putative / formamide amidohydrolase, putative	1.47
AT5G26040	HDA2 (histone deacetylase 2); histone deacetylase	1.46
AT2G36220	unknown protein	1.46
AT4G26260	MIOX4 (MYO-INOSITOL OXYGENASE 4)	1.45
AT4G17970	unknown protein	1.45
AT1G20330	SMT2 (STEROL METHYLTRANSFERASE 2)	1.45
AT5G05350	unknown protein	1.44
AT3G02460	plant adhesion molecule, putative	1.43
AT2G20290	XIG (Myosin-like protein XIG); motor/ protein binding	1.43
AT1G77590	LACS9 (LONG CHAIN ACYL-COA SYNTHETASE 9); long-chain-fatty-acid-CoA ligase	1.42
AT3G52840	BGAL2 (beta-galactosidase 2); beta-galactosidase	1.41
AT5G20250	DIN10 (DARK INDUCIBLE 10); hydrolase, hydrolyzing O-glycosyl compounds	1.41
AT1G03905	ABC transporter family protein	1.41
AT4G35770	SEN1 (DARK INDUCIBLE 1)	1.41
AT2G39450	ATMTP11/MTP11; cation transmembrane transporter	1.40
AT5G26740	unknown protein	1.40

AT3G61900	auxin-responsive family protein	1.40
AT1G76090	SMT3 (S-adenosyl-methionine-sterol-C-methyltransferase 3)	1.38
AT5G24910	CYP714A1 (cytochrome P450, family 714, subfamily A, polypeptide 1); oxygen binding	1.37
AT3G27880	unknown protein	1.37
AT3G23050	IAA7 (AUXIN RESISTANT 2); transcription factor	1.35
AT2G33060; AT2G33050	leucine-rich repeat family protein	1.35
AT1G05100	MAPKKK18 (Mitogen-activated protein kinase kinase kinase 18); kinase	1.35
AT3G57520	ATSIP2 (ARABIDOPSIS THALIANA SEED IMBIBITION 2)	1.34
AT1G13830	beta-1,3-glucanase-related	1.33
AT2G37180; AT2G37170	RD28 (plasma membrane intrinsic protein 2;3)	1.32
AT5G65160	tetratricopeptide repeat (TPR)-containing protein	1.31
AT2G39570	ACT domain-containing protein	1.30
AT2G37980	unknown protein	1.30
AT1G56660	unknown protein	1.30
AT5G36880	acetyl-CoA synthetase, putative / acetate-CoA ligase, putative	1.29
AT5G07010	sulfotransferase family protein	1.29
AT2G27040	AGO4 (ARGONAUTE 4); nucleic acid binding	1.29
AT1G69070	binding	1.28
AT5G48010	pentacyclic triterpene synthase, putative	1.28
AT5G17640	unknown protein	1.27
AT5G66650	unknown protein	1.26
AT5G53590; AT5G53588	auxin-responsive family protein	1.25
AT5G40510	unknown protein	1.25
AT1G75450	CKX5 (CYTOKININ OXIDASE 5); cytokinin dehydrogenase	1.25
AT4G09900	hydrolase, alpha/beta fold family protein	1.25
AT2G34070	unknown protein	1.24
AT1G21670	unknown protein	1.24
AT5G26920	calmodulin binding	1.23
AT2G39705	DVL11/RTFL8 (ROTUNDIFOLIA LIKE 8)	1.23
AT2G21410	VHA-A2 (VACUOLAR PROTON ATPASE A2); ATPase	1.23
AT5G11710	(EPSIN1); binding	1.23
AT1G62560	flavin-containing monooxygenase family protein / FMO family protein	1.22
AT4G05100	AtMYB74 (myb domain protein 74); DNA binding / transcription factor	1.22
AT2G26900	bile acid:sodium symporter family protein	1.22
AT2G27090	unknown protein	1.21
AT4G17090	CT-BMY (BETA-AMYLASE 3, BETA-AMYLASE 8); beta-amylase	1.21
AT2G04040	ATDTX1; antiporter/ multidrug efflux pump/ multidrug transporter/ transporter	1.21
AT1G17300	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G17285.1)	1.21
AT1G72790	hydroxyproline-rich glycoprotein family protein	1.20
AT1G22530	PATL2; transporter	1.20
AT3G50970	LTI30 (LOW TEMPERATURE-INDUCED 30)	1.20
AT3G27270	similar to DNA-binding storekeeper protein-related	1.19
AT5G17230	PSY (PHYTOENE SYNTHASE); geranylgeranyl-diphosphate geranylgeranyltransferase	1.19

AT4G23870	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT4G11020.1)	1.19
AT5G47250	disease resistance protein (CC-NBS-LRR class), putative	1.18
AT1G32410	vacuolar protein sorting 55 family protein / VPS55 family protein	1.18
AT5G53290	CRF3 (CYTOKININ RESPONSE FACTOR 3); DNA binding / transcription factor	1.18
AT2G26600	glycosyl hydrolase family 17 protein	1.17
AT5G18750	DNAJ heat shock N-terminal domain-containing protein	1.17
AT5G23760	heavy-metal-associated domain-containing protein	1.16
AT1G08600	ATRX/CHR20; ATP binding / DNA binding / helicase	1.16
AT3G51540	unknown protein	1.16
AT3G23750	leucine-rich repeat family protein / protein kinase family protein	1.16
AT1G71960	ABC transporter family protein	1.15
AT4G32360	NADP adrenodoxin-like ferredoxin reductase	1.14
AT4G15570	tRNA-splicing endonuclease positive effector-related	1.14
AT2G19860	ATHXK2 (HEXOKINASE 2); ATP binding / hexokinase	1.14
AT1G22170	phosphoglycerate/bisphosphoglycerate mutase family protein	1.14
AT3G05390	unknown protein	1.14
AT2G48030	endonuclease/exonuclease/phosphatase family protein	1.14
AT1G65840	ATPAO4 (POLYAMINE OXIDASE 4); amine oxidase	1.13
AT3G21650	serine/threonine protein phosphatase 2A (PP2A) regulatory subunit B', putative	1.13
AT5G18440	similar to hypothetical protein [Vitis vinifera] (GB:CAN73830.1)	1.13
AT1G61120	terpene synthase/cyclase family protein	1.13
AT1G27990	unknown protein	1.12
AT2G40420	amino acid transporter family protein	1.12
AT2G47550	pectinesterase family protein	1.12
AT5G50660; AT5G50560	unknown protein	1.12
AT1G67880	glycosyl transferase family 17 protein	1.11
AT1G73990	SPPA (signal peptide peptidase); protease IV/ serine-type endopeptidase	1.11
AT3G56980	BHLH039/ORG3 (OBP3-RESPONSIVE GENE 3); DNA binding / transcription factor	1.10
AT4G31980	unknown protein	1.10
AT3G45130	LAS1 (Lanosterol synthase 1); lanosterol synthase	1.10
AT4G23150	protein kinase family protein	1.10
AT1G05510	unknown protein	1.09
AT1G35910	trehalose-6-phosphate phosphatase, putative	1.09
AT5G62530	ALDH12A1 (Aldehyde dehydrogenase 12A1)	1.08
AT5G13120	peptidyl-prolyl cis-trans isomerase cyclophilin-type family protein	1.08
AT4G35570	HMGB5 (HIGH MOBILITY GROUP B 5); transcription factor	1.07
AT2G42060	CHP-rich zinc finger protein, putative	1.07
AT3G61260	DNA-binding family protein / remorin family protein	1.07
AT3G20100	CYP705A19 (cytochrome P450, family 705, subfamily A, polypeptide 19); oxygen binding	1.06
AT4G19140	similar to unnamed protein product [Vitis vinifera] (GB:CAO66715.1)	1.06
AT4G14680	APS3 (ATP sulfurylase 2); sulfate adenylyltransferase (ATP)	1.06
AT5G59310	LTP4 (LIPID TRANSFER PROTEIN 4); lipid binding	1.06
AT5G62490	ATHVA22B (Arabidopsis thaliana HVA22 homologue B)	1.06

AT5G58670	ATPLC1 (PHOSPHOLIPASE C 1); phospholipase C	1.06
AT4G01610	cathepsin B-like cysteine protease, putative	1.06
AT3G12110	ACT11 (ACTIN-11); structural constituent of cytoskeleton	1.05
AT1G30440	phototropic-responsive NPH3 family protein	1.04
AT4G37540	LBD39 (LOB DOMAIN-CONTAINING PROTEIN 39)	1.04
AT1G09155	ATPP2-B15 (Phloem protein 2-B15); carbohydrate binding	1.04
AT4G28703	unknown protein	1.04
AT3G49060	protein kinase family protein / U-box domain-containing protein	1.04
AT4G33905	peroxisomal membrane protein 22 kDa, putative	1.03
AT1G03090	MCCA (3-methylcrotonyl-CoA carboxylase 1)	1.03
AT5G16200	50S ribosomal protein-related	1.03
AT5G52250	transducin family protein / WD-40 repeat family protein	1.02
AT2G23110	unknown protein	1.02
AT5G04740	ACT domain-containing protein	1.01
AT5G48230	ACAT2/EMB1276 (ACETOACETYL-COA THIOLASE 2)	1.01
AT3G56300	tRNA synthetase class I (C) family protein	1.00
AT5G65630	GTE7 (GLOBAL TRANSCRIPTION FACTOR GROUP E 7); DNA binding	1.00
AT5G40450	unknown protein	1.00
AT4G15480	UGT84A1; UDP-glycosyltransferase/ sinapate 1-glucosyltransferase	1.00
AT1G80920	J8; heat shock protein binding / unfolded protein binding	-1.01
AT4G16860	RPP4 (RECOGNITION OF PERONOSPORA PARASITICA 4)	-1.03
AT5G14180	MPL1 (MYZUS PERSICAE-INDUCED LIPASE 1); catalytic	-1.04
AT1G48000	MYB112 (myb domain protein 112); DNA binding / transcription factor	-1.04
AT5G65640	BHLH093 (BETA HLH PROTEIN 93); DNA binding / transcription factor	-1.04
AT2G15890	MEE14 (maternal effect embryo arrest 14)	-1.04
AT5G66390	peroxidase 72 (PER72) (P72) (PRXR8)	-1.06
AT2G15080	disease resistance family protein	-1.06
AT2G46680	ATHB-7 (ARABIDOPSIS THALIANA HOMEOBOX 7); transcription factor	-1.08
AT3G59940	kelch repeat-containing F-box family protein	-1.09
AT3G54620	ARABIDOPSIS THALIANA BASIC LEUCINE ZIPPER 25	-1.20
AT5G46050	ATPTR3/PTR3 (PEPTIDE TRANSPORTER PROTEIN 3); transporter	-1.31

Table 2-2. Differentially expressed genes in Arabidopsis mesophyll protoplasts transfected with *bZIP11*. Transcriptomics (ATH1 array) experiments were performed in triplicate using mRNA isolated from Arabidopsis mesophyll protoplasts expressing the 35S-*bZIP11* constructs incubated for six hours. Indicated are significant changes in gene expression above one-fold (log2 scale and p value<0.05)

Probe	AGI and Annotation	Fold Change
248424_at	(at5g51680): hydroxyproline-rich glycoprotein family protein | chr5:20997320-20998907 FORWARD	2.38
247965_at	(at5g56540): Symbols: AGP14, ATAGP14 | AGP14 (ARABINOGALACTAN PROTEIN 14)	4.12
249037_at	(at5g44130): Symbols: FLA13 | FLA13 (FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 13 PRECURSOR)	4.60
265066_at	(at1g03870): Symbols: FLA9 | FLA9 (FASCICLIN-LIKE ARABINOOGALACTAN 9) | chr1:982506-983540 REVERSE	7.28
246114_at	(at5g20250): Symbols: DIN10 | DIN10 (DARK INDUCIBLE 10); hydrolase, hydrolyzing O-glycosyl compounds | chr5:6833678-6836788 FORWARD	1.41
251642_at	(at3g57520): Symbols: AtSIP2 | AtSIP2 (Arabidopsis thaliana seed imbibition 2); hydrolase, hydrolyzing O-glycosyl compounds | chr3:21288765-21293158 REVERSE	1.34
254321_at	MULTIPLE HITS: (at4g22590,at4g22592). at4g22590: trehalose-6-phosphate phosphatase	2.15
256319_at	(at1g35910): trehalose-6-phosphate phosphatase, putative | chr1:13363003-13365060 REVERSE	1.09
266693_at	(at2g19800): Symbols: MIOX2 | MIOX2 (MYO-INOSITOL OXYGENASE 2); inositol oxygenase | chr2:8530896-8533508 REVERSE	2.86
254001_at	(at4g26260): Symbols: MIOX4 | MIOX4; inositol oxygenase | chr4:13297698-13300387 FORWARD	1.45
250186_at	(at5g14500): aldose 1-epimerase family protein | chr5:4674233-4676765 REVERSE	2.11
251100_at	(at5g01670): aldose reductase, putative | chr5:251978-253960 FORWARD	2.20
265283_at	(at2g20370): Symbols: KAM1, MUR3 | MUR3 (MURUS 3);transferase	2.01
262105_at	(at1g02810): pectinesterase family protein | chr1:618265-620480 FORWARD	2.05
245151_at	(at2g47550): pectinesterase family protein | chr2:19508980-19511792 FORWARD	1.12
258764_at	(at3g10720): pectinesterase, putative | chr3:3354482-3357614 REVERSE	2.67
252320_at	(at3g48580): xyloglucan:xyloglucosyl transferase	1.52
252557_at	(at3g45960): Symbols: ATEXLA3, EXPL3, ATEXPL3, ATHEXP BETA 2.3 | ATEXLA3 (arabidopsis thaliana expansin-like a3)	1.58
252563_at	(at3g45970): Symbols: ATEXLA1, EXPL1, ATEXPL1, ATHEXP BETA 2.1 | ATEXLA1 (arabidopsis thaliana expansin-like a1)	6.84
252997_at	(at4g38400): Symbols: ATEXLA2, EXPL2, ATEXPL2, ATHEXP BETA 2.2 | ATEXLA2 (arabidopsis thaliana expansin-like a3)	2.01
257071_at	(at3g28180): Symbols: ATCSLC04, CSLC04, ATCSLC4, CSLC4 | ATCSLC04 (CELLULOSE-SYNTHASE LIKE C4)	2.36
251996_at	(at3g52840): Symbols: BGAL2 | BGAL2 (beta-galactosidase 2)	1.41
245706_at	(at5g04310): pectate lyase family protein | chr5:1203203-1207352 REVERSE	2.95
245708_at	(at5g04310): pectate lyase family protein | chr5:1203203-1207352 REVERSE	2.19
250662_at	(at5g07010): Symbols: ATST2A, ST2A | ST2A (SULFOTRANSFERASE 2A)	1.29
260915_at	(at1g02660): lipase class 3 family protein | chr1:571955-574981 REVERSE	1.75
250199_at	(at5g14180): Symbols: MPL1 | MPL1 (MYZUS PERSICAE-INDUCED LIPASE 1); catalytic | chr5:4571337-4574462 REVERSE	-1.04
253925_at	(at4g26690): Symbols: SHV3, MRH5, GPDL2 | SHV3 (SHAVEN 3)	1.57
249337_at	(at5g41080): glycerophosphoryl diester phosphodiesterase family protein | chr5:16441744-16443985 FORWARD	2.69
266500_at	(at2g06925): Symbols: ATSPLA2-ALPHA, PLA2-ALPHA | PLA2-ALPHA; phospholipase A2	2.63
259169_at	(at3g03520): phosphoesterase family protein | chr3:837877-840624 REVERSE	1.75
249638_at	(at5g36880): acetyl-CoA synthetase, putative / acetate-CoA ligase, putative | chr5:14534613-14540299 REVERSE	1.30
259761_at	(at1g77590): Symbols: LACS9 | LACS9 (LONG CHAIN ACYL-COA SYNTHETASE 9)	1.42
245346_at	(at4g17090): Symbols: CT-BMY, BAM3, BMY8 | CT-BMY (CHLOROPLAST BETA-AMYLASE)	1.21
266702_at	(at2g19860): Symbols: ATHXK2, HXK2 | HXK2 (HEXOKINASE 2)	1.14
255924_at	(at1g22170): phosphoglycerate/bisphosphoglycerate mutase family protein | chr1:7826289-7828326 FORWARD	1.14
258941_at	(at3g09940): Symbols: MDHAR, ATMDAR3 | MDHAR (MONODEHYDROASCORBATE REDUCTASE)REVERSE	5.29
261197_at	(at1g12900): Symbols: GAPA-2 | GAPA-2 (GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE A SUBUNIT 2)	2.04
263761_at	(at2g21330): fructose-bisphosphate aldolase, putative | chr2:9128151-9130240 REVERSE	2.23
266892_at	(at2g26080): Symbols: AtGLDP2 | AtGLDP2 (Arabidopsis thaliana glycine decarboxylase P-protein 2)	1.92
250662_at	(at5g07010): Symbols: ATST2A, ST2A | ST2A (SULFOTRANSFERASE 2A)	1.29

260915_at	(at1g02660): lipase class 3 family protein | chr1:571955-574981 REVERSE	1.75
250199_at	(at5g14180): Symbols: MPL1 | MPL1 (MYZUS PERSICAE-INDUCED LIPASE 1)	-1.04
253925_at	(at4g26690): Symbols: SHV3, MRH5, GPDL2 | SHV3 (SHAVEN 3)	1.57
249337_at	(at5g41080): glycerophosphoryl diester phosphodiesterase family protein | chr5:16441744-16443985 FORWARD	2.69
266500_at	(at2g06925): Symbols: ATSPLA2-ALPHA, PLA2-ALPHA | PLA2-ALPHA; phospholipase A2	2.63
259169_at	(at3g03520): phosphoesterase family protein | chr3:837877-840624 REVERSE	1.75
249638_at	(at5g36880): acetyl-CoA synthetase, putative / acetate-CoA ligase, putative | chr5:14534613-14540299 REVERSE	1.30
259761_at	(at1g77590): Symbols: LACS9 | LACS9 (LONG CHAIN ACYL-COA SYNTHETASE 9)	1.42
252855_at	(at4g39660): Symbols: AGT2 | AGT2 (ALANINE:GLYOXYLATE AMINOTRANSFERASE 2)	1.58
248690_at	(at5g48230): Symbols: EMB1276, ACAT2 | ACAT2 (ACETOACETYL-COA THIOLASE 2)	1.01
254845_at	(at4g11820): Symbols: MVA1 | MVA1; acetyl-CoA C-acetyltransferase/ hydroxymethylglutaryl-CoA synthase	1.49
250095_at	(at5g17230): phytoene synthase (PSY) / geranylgeranyl-diphosphate geranylgeranyl transferase	1.19
248729_at	(at5g48010): Symbols: THAS, THAS1 | THAS1 (THALIANOL SYNTHASE 1); catalytic/ thalianol synthase	1.28
252611_at	(at3g45130): Symbols: LAS1 | LAS1; lanosterol synthase | chr3:16512271-16517522 REVERSE	1.10
264886_at	(at1g61120): Symbols: TPS04, GES | TPS04 (TERPENE SYNTHASE 04); (E,E)-geranyllinalool synthase	1.13
267337_at	(at2g39980): transferase family protein | chr2:16687673-16689956 REVERSE	1.49
254836_at	(at4g12330): Symbols: CYP706A7 | CYP706A7; electron carrier	1.82
256509_at	(at1g75300): isoflavone reductase, putative | chr1:28255495-28257040 FORWARD	2.75
250149_at	(at5g14700): cinnamoyl-CoA reductase-related | chr5:4740252-4743446 REVERSE	1.77
265121_at	(at1g62560): Symbols: FMO GS-OX3 | FMO GS-OX3 (FLAVIN-MONOOXYGENASE GLUCOSINOLATE S-OXYGENASE 3)	1.23
252415_at	(at3g47340): Symbols: ASN1, DIN6, AT-ASN1 | ASN1 (GLUTAMINE-DEPENDENT ASPARAGINE SYNTHASE 1)	1.98
257315_at	(at3g30775): Symbols: ERD5, PRODH, AT-POX, ATPOX, ATPDH, PRO1 | ERD5 (EARLY RESPONSIVE TO DEHYDRATION 5)	2.69
247436_at	(at5g62530): Symbols: ALDH12A1, ATP5CDH, P5CDH	1.09
263275_at	(at2g14170): Symbols: ALDH6B2 | ALDH6B2; 3-chloroallyl aldehyde dehydrogenase	1.98
262712_at	(at1g16460): Symbols: ATRDH2, ATMST2, MST2 | ATRDH2 (ARABIDOPSIS THALIANA RHODANESE HOMOLOGUE 2)	1.53
257315_at	(at3g30775): Symbols: ERD5, PRODH, AT-POX, ATPOX, ATPDH, PRO1 | ERD5 (EARLY RESPONSIVE TO DEHYDRATION 5)	2.69
247436_at	(at5g62530): Symbols: ALDH12A1, ATP5CDH, P5CDH | ALDH12A1; 1-pyrroline-5-carboxylate dehydrogenase	1.09
245254_at	(at4g14680): Symbols: APS3 | APS3; sulfate adenylyltransferase (ATP) | chr4:8413288-8415391 REVERSE	1.06
266892_at	(at2g26080): Symbols: AtGLDP2 | AtGLDP2 (Arabidopsis thaliana glycine decarboxylase P-protein 2)	1.92
264529_at	(at1g30820): CTP synthase, putative / UTP--ammonia ligase, putative | chr1:10944913-10949458 REVERSE	2.30
251961_at	(at3g53620): Symbols: AtPPa4 | AtPPa4 (Arabidopsis thaliana pyrophosphorylase 4)	1.60
263929_at	(at2g36310): Symbols: URH1 | URH1 (URIDINE-RIBOHYDROLASE 1); adenosine nucleosidase	1.74
253678_at	(at4g29600): cytidine deaminase, putative / cytidine aminohydrolase, putative | chr4:14515578-14516501 FORWARD	3.11

Table 2-4. Differentially expressed genes associated with carbohydrate, amino acid and secondary metabolism sorted by Mapman program in *bZIP11* over expressed Arabidopsis mesophyll protoplasts. The micro-array data in table 2-2 was loaded into Mapman program, and genes associated with carbohydrate, amino acid and secondary metabolism according to the program were listed.

Chapter 3

Protoplast transcriptomics analysis reveals the overlapping and distinct functions of S1/C bZIP transcription factors in gene regulation

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Summary

S1 group bZIP transcription factors (TFs) function as regulators of metabolism and were shown to directly regulate genes encoding enzymes involved in amino acid metabolism. S1 group bZIPs preferentially interact with C group bZIP genes to form heterodimers that have synergistic effects on target gene transcriptional activation. In this paper, the transcriptional potential of selected S1/C group bZIPs has been investigated using a protoplast transient expression system. Genome wide transcriptomics analyses using Affymetrix ATH1 arrays were performed on protoplasts transfected by single S1/C bZIP genes or their combinations. The C group genes, *bZIP10*, and *bZIP63*, and the S1 group genes *bZIP1* and *bZIP11* were chosen for this study. bZIP11 emerged as the most potent transcriptional activator and induced the highest number of genes. Other individual *bZIP* genes minimally affected transcription on their own. *bZIP10* transfection by itself does not alter transcription but in combination with S1 group *bZIP1* or *bZIP11* strongly boosted gene expression compared to individual transfections. Importantly, the S1/C *bZIP* genes and their different combinations tested have overlapping and distinct effects in transcriptome. A set of 48 genes is regulated by all heterodimer combinations. These core overlapped genes by each combination of TFs are involved in metabolism or stress responses. The bZIP10 plus 11 heterodimer regulates the highest number of genes (approximately 900) and these genes are mainly involved in metabolism and signal transduction. Interestingly, minor carbohydrate metabolism related genes involved in raffinose, inositol and trehalose metabolism are regulated by different combinations. Furthermore, the bZIP combinations tested regulate the expression of other members of the bZIP family. Prominently, these bZIPs or their combinations tested induce the expression of *KIN11* which encodes the catalytic subunit of the SnRK1 energy sensing regulatory protein kinase. Thus, we conclude that these bZIPs have overlapping and specific functions in metabolic regulation, and they likely play in a broader bZIP TF network.

Introduction

Cellular signaling networks are complex and the regulation of gene expression can be operated by multiple signaling pathways. In these signaling pathways transcription factors are central regulators of gene expression. Individual transcription factors can interact with other transcription factors or regulatory components, binding to the cis elements in the promoter of genes thereby activating or suppressing transcription of downstream genes. Differentially regulated transcription factors that are part of functional complexes must be present simultaneously for an integrated response. In many cases, dimerization of transcription factors diversifies their specificities and increases affinity in gene regulation (Seo *et al.*, 2011).

The bZIP transcription factors are characterized by the adjacent positioning of a basic domain and a leucine zipper domain. The basic domain binds DNA whereas the zipper domain interacts mostly with other bZIP proteins. In Arabidopsis, over 70 bZIP transcription factors are annotated (Jakoby *et al.*, 2002). The functionality of bZIP transcription factor usually requires that the leucine zipper domains of two bZIP proteins interact. According to protein structural similarity and functionality, bZIPs are classified into 10 groups and some of these groups are further divided in subgroups (Jakoby *et al.*, 2002). bZIPs are involved in diverse functions including stress responses, adaptation to nutritional conditions and developmental processes.

The bZIP S1 subgroup consists of five genes, *bZIP1*, *2*, *11*, *44* and *53*. A defining feature of the S1 group bZIP genes is the presence of complex open reading frame configurations in the 5'-leader of the mRNA. One of these upstream open reading frames (uORFs) in S1 group bZIPs encodes the sucrose control (SC) peptide. This evolutionary conserved peptide regulates the translation of the main ORF of bZIP mRNA in response to sucrose concentrations. The SC peptide promotes the formation of a ribosome stalling complex in response to increasing sucrose levels, thereby represses the translation of the main ORF (Weltmeier *et al.*, 2009). S1 group bZIP proteins preferentially form heterodimers with C group bZIP proteins bZIP9, 10, 25 or 63 (Ehlert *et al.*, 2006). The specificity and affinity of protein-protein interactions among S1 and C group bZIP proteins have been extensively analyzed by two-hybrid interaction studies in both yeast and plant mesophyll protoplasts. In these studies, the preferential interaction between S1 and C group bZIP was shown, while the interaction between bZIPs within these groups is low. Among the C group bZIPs, bZIP10 and 25 are capable of interacting relatively strongly with each of the five S1 bZIPs (Ehlert *et al.*, 2006). Gene expression studies have shown that the expressing tissues of S1 and C group bZIP overlap, allowing for dimer formation and combinatorial regulation of downstream genes (Weltmeier *et al.,* 2009). The in vivo relevance of dimer formation was documented by the finding that overexpression of two dimerizing partners greatly enhances the transcriptional activation on target promoters (Alonso *et al.*, 2009; Dietrich *et al.*, 2011; Weltmeier *et al.*, 2006).

A number of studies have shown that S1 bZIPs are involved in the regulation of metabolism. In response to low energy conditions, bZIP1 and bZIP53 regulate asparagine and branched-chain amino acid metabolism (Dietrich *et al.*, 2011). Similarly, bZIP11 is a regulator of amino acid metabolism associated genes as well, likely through directly activating the transcription of genes involved in amino acid metabolism. For bZIP1 a function in sugar signaling and the integration of light and nitrogen metabolism was proposed (Kang *et al.*, 2010; Obertello *et al.*, 2010). The C group bZIP63 was proposed to be involved in sugar signaling

as well (Matiolli *et al.*, 2011).

Genes encoding enzymes involved in amino acid metabolism are proposed or identified as S1/C bZIP targets. *ASN1* encodes asparagine synthetase and *ProDH1* and *ProDH2* encode functional enzymes involved in the degradation of proline. S1 bZIPs transcriptionally activate the promoter of *ASN1* (Baena-Gonzalez *et al.*, 2007). bZIP1 and bZIP53 bind the promoters of *ASN1* and *ProDH1* in vivo (Dietrich *et al.*, 2011). *ProDH2* is proposed as a direct target of bZIP11 (Hanson *et al.*, 2008). Correspondingly, overexpression of bZIP1 or 53 and bZIP11 results in drastic depletion of proline content (Dietrich *et al.*, 2011; Hanson *et al.*, 2008).

SnRK1 (Snf1-related protein kinase1) is the plant member of a conserved family of eukaryotic protein kinases involved in the regulation of metabolism. A function of SnRK1 in starvation/low energy responses in plants was proposed (Baena-Gonzalez *et al.*, 2007). In Arabidopsis, *KIN10* and *KIN11* encode SnRK1 catalytic subunits. In protoplasts, the overexpression of transfected *KIN10* induces profound changes in the transcriptome (Baena-Gonzalez *et al.*, 2007). Prominently, many metabolism related genes are regulated by KIN10 as well. Moreover, S1/C bZIPs and KIN10 overexpression show significantly overlapping transcriptomic effects. For instance, the early response to KIN10 activity of *ASN1* gene expression is reminiscent to S1/C induced expression (Hanson *et al*, 2008). Interestingly, co-transfection experiments in protoplasts showed that the presence of KIN10 enhances the transcriptional activity of S1 group bZIPs, and about ten-fold induction of the corresponding signal has been observed. Thus, these findings imply a direct signaling route from SnRK1 to S1 bZIP proteins (Baena-Gonzalez *et al.*, 2007).

Likely, S1 bZIPs function in metabolic adaptation in response to low sugar conditions. Importantly, our previous study shows that in addition to amino acid metabolism, bZIP11 is a strong regulator of sugar metabolism as well (Ma *et al.,* 2011). In particular, we know that bZIP11 induces genes involved in metabolic pathways related to trehalose, raffinose and myoinositol metabolism, which are starvation inducible genes as well. Concordantly, bZIP11 induction of these metabolic genes results in corresponding changes in the levels of relevant metabolites. Previous findings mainly provide insight into the regulatory potential of individual bZIP proteins, but the effects of heterodimerization of S1 and C group bZIPs has not been explored. Leaf mesophyll protoplasts provide an efficient tool for transcription studies (Yoo *et al.*, 2007). Here, we use a protoplast system to address the role of S1/C group bZIP heterodimerization in transcription regulation.

Results

Probe	AGI	Annotation	1	10	11	63	1+10	1+63	10+63	10+11	11+63
255496_at	AT4G02640	bZIP10	-	6.85	-	-	7.18	-	7.19	6.62	-
245925_at	AT5G28770	bZIP63	-	-	-	8.45	-	8.10	8.32	-	8.09
248606_at	AT5G49450	bZIP1	3.69	-	-	-	3.56	3.49	-	-	-
253245_at	AT4G34590	bZIP11	-	-	8.27	-	-	-	-	8.34	8.24

Table 3-1. The induced expression of bZIP transcription factor upon the transfection of its expressing plasmid. Six hours after transfection, the expression of transfected genes are increased as presented. Fold change is on a log2 scale and p value < 0.02.

Probe	AGI	Annotation	bZIP1	bZIP10	bZIP11	bZIP63	bZIP1+bZIP10	bZIP1+bZIP63	bZIP10+bZIP63	bZIP10+bZIP11	bZIP11+bZIP63
256319_at	AT1G35910	TPP2	-	-	1.09	-	2.41	1.57	1.17	1,48	2,57
254806_at	AT4G12430;AT4G12432	TPP5	-	-	-	-	-	-	-	3,80	-
254321_at	AT4G22590;AT4G22592	TPP6	-	-	2.15	-	2.97	1.65	1.78	3,41	2,48
248404_at	AT5G51460	TPP9	-	-	-	-	-	-	-	1,28	-
264339_at	AT1G70290	TPS8	-	-	-	-	-	-	-	-1,19	-

Table 3-4. Trehalose metabolism related genes significantly regulated by bZIP individual genes or their combinations.The genes presented are significantly regulated with fold change above 1 (log2 scale) and p value < 0.02 among all the trehalose metabolism family genes.

Individual C and S1 group *bZIP* genes were cloned behind the 35S promoter and bZIP gene containing plasmids were purified for use in protoplast transfection. The bZIP transcription factors were immunotagged at the amino terminus by the addition of the HA peptide coding sequence to the constructs. Mesophyll protoplasts were isolated from the rosette leaves of four weeks old *Arabidopsis thaliana* Col-0 wild type plants grown under long day condition (16 h light/ 8 h dark). The protoplasts were transfected with either individual bZIP genes or combinations of different S1 and C group bZIP genes. In these experiments, C group bZIP10 (At4G02640) and bZIP63 (At5G28770), and S1 group bZIP1 (At5G49450) and bZIP11 (At4G34590) were used for individual and combinatorial transfections. Following transfection, protoplasts were incubated for 6 hours under continuous light. Following transfection and incubation, aliquots were processed for Western blotting by using HA specific antibodies that confirmed a high level expression of the *bZIP* constructs at the protein level (Micha Hanssen unpublished data).

As a control an empty vector construct was transfected. Transcriptomic changes were monitored using the Affymetrix ATH1 microarray. Transfections and microarray analyses were performed in triplicate. Genes responding above two-fold with p value <0.02 are considered as differentially expressed. Six hours after transfection, high levels of bZIP mRNA could be detected up to 256–fold in the microarray experiment (Table 3-1).

bZIP heterodimers regulate substantially more genes than homodimers.

Transfection of individual S1 or C group bZIPs delivers a minor response at the gene expression level, except for bZIP11 where 335 genes are more than two-fold changed in expression (Figure 3-1). In contrast, double transfections significantly increase the numbers of target genes. For example, bZIP10 and bZIP1 affect approximately 30 genes each, whereas the combination of bZIP10+1 modulates expression of 216 genes. Particularly, the bZIP10+11 combination is a powerful one with 872 genes regulated, whereas the bZIP63+11 combination shows slightly less regulated genes than bZIP11 alone.

Clearly, S1/C group bZIP heterodimers have much greater transcriptional potential than the individual bZIPs. In the bZIP1 combinatorial transfection with either bZIP10 or bZIP63 the number of genes regulated is approximately 6 times higher than with individual transfections (Figure 3-1). This suggests there is a functional dimerizing interaction between bZIP1 and bZIP63 (Kang *et al.*, 2010).

The bZIP10 plus 11 combination is a powerful regulator of gene expression

An important question is to address the specificity of the C plus S1 group gene combinations in regulating gene expressions. In the individual transfections,

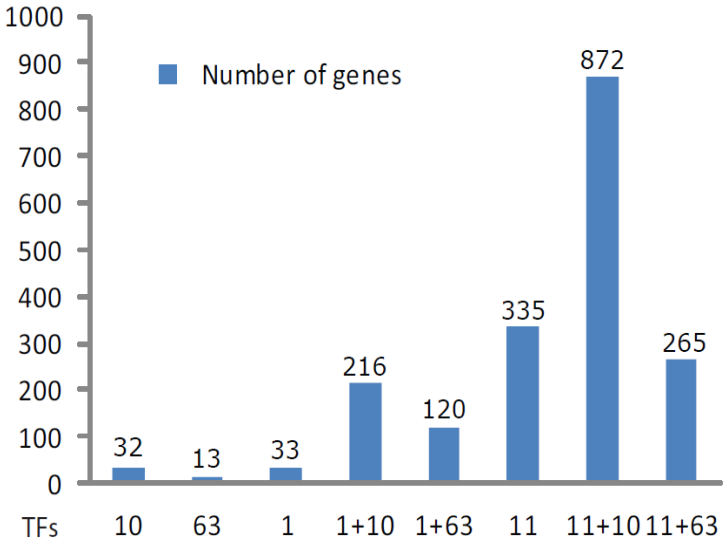

Figure 3-1. Numbers of genes regulated by bZIP transcription factors. Protoplast transfections with individual bZIP genes or the combinations are shown along the x axis. The numbers of differentially expressing genes are displayed on top of each column. Differential expressing gene selection standard is fold change above 1 (log2 scale) and p value < 0.02. Abbreviation: transcription factor (TF).

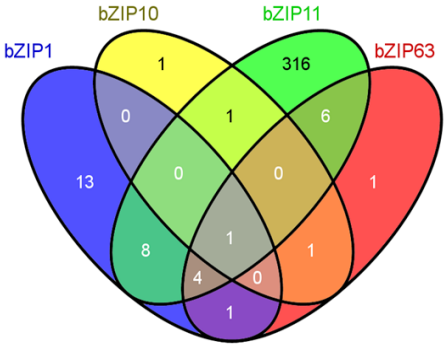

Figure 3-2. Venn diagram showing genes significantly regulated by the single bZIP gene transfections. Significance standard is fold change above 1 (log2 scale) and p value < 0.02.

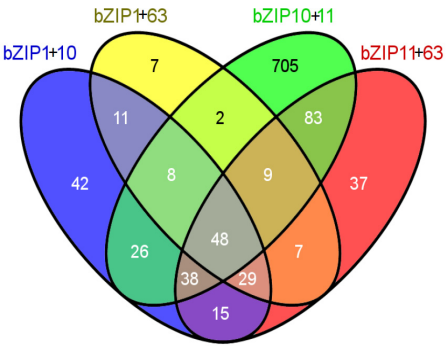

Figure 3-3. Venn diagram showing genes significantly regulated by double bZIP gene transfections. Significance standard is fold change above 1 (log2 scale) and p value < 0.02.

the number of regulated genes regulated by bZIP1, 10 and 63 is very low and the overlap is minor (Figure 3-2). However, the numbers of genes regulated are greatly increased with the combinatorial transfection of C and S1 bZIPs (Figure 3-3). Among the bZIP combinations tested, bZIP10 plus bZIP11 regulate the highest number of genes, 872 genes in total. Thus, bZIP10 itself has minor activity in transcription activation, but substantially enhances the regulatory potential of bZIP11. The most highly induced genes by the bZIP10+11 combination are presented in Table 3-2. Many of these highly induced genes are related to seed maturation such as seed storage albumin, oleosin and *LEA*s. Other genes may have a function in signal transduction, including *SKP1*-like genes that encode subunits of the SCF complex E3 ligases, which have been implicated in phytohormone signaling pathways through protein degradation. Also, the bZIP 1+10 and 11+63 combinations regulate a substantial number of genes, 216 and 265 genes, respectively. Thus, the C group bZIPs are involved in determining the specificity of regulated genes.

Genes regulated in common by four different bZIP combinations

bZIP transcription factors are G-box binding transcription factors that have the ACGT sequence at its core. Therefore, it is interesting to observe that the different bZIP combinations tested share substantial overlap in target genes (Figure 3-3). For example, bZIP10+11 and bZIP11+63 share 83 regulated genes. All four combinations of bZIP TFs (1+10, 1+63, 10+11 and 11+63) share regulation of 48 genes (Table 3-3). Among the induced genes, the bZIP transcription factor binding core sequence ACGT is overrepresented in the promoter sequences within approximately 500 bp upstream to the starting codon (Pscan program; http://159.149.109.9/pscan/). These overlapping regulated genes constitute a rather diverse set of genes that seems dominated by genes encoding components in signalling pathways, especially transcription factors. Two bZIP genes are among the list of commonly regulated genes, bZIP54 and bZIP55, implying that there is a hierarchical network among these bZIP TFs. This finding confirms that the G-box is important for bZIP transcription factor initiated transcription.

Starvation signature genes are induced by S1/C bZIP combinations

Our previous findings show that bZIP11 induces several genes involved in raffinose biosynthesis and myoinositol or trehalose degradation. In metabolomics experiments concordant changes in the levels of these metabolites were found. The changes in these minor carbohydrates are part of the signature for low energy situations such as those induced by extended night. Thus, the induction of bZIP11 initiates changes in these minor carbohydrates by regulating the corresponding genes, independent of the actual energy status. It is therefore of interest to know whether more bZIPs or combinations have a similar role in induc-

Probe	AGI	Annotation	Fold Induction (Log2 scale)
253904_at	AT4G27140	seed storage albumin 1	8.32
252944_at	AT4G39320	microtubule-associated protein-related	7.62
260950_s_at	AT1G06120; AT1G06090	Fatty acid desaturase family protein	7.31
245560_at	AT4G15480	UDP-Glycosyltransferase superfamily protein	7.27
258167_at	AT3G21560	UDP-Glycosyltransferase superfamily protein	7.27
265204_at	AT2G36650	unknown protein	6.84
260716_at	AT1G48130	1-cysteine peroxiredoxin 1	6.73
257944_at	AT3G21850	SKP1-like 9	6.34
264618_at	AT2G17680	Arabidopsis protein of unknown function (DUF241)	6.27
257942_at	AT3G21830	SKP1-like 8	6.23
253151_at	AT4G35670	Pectin lyase-like superfamily protein	6.08
252549_at	AT3G45860	cysteine-rich RLK (RECEPTOR-like protein kinase) 4	5.91
262205_at	AT2G01080	Late embryogenesis abundant hydroxyproline-rich glycoprotein	5.75
263948_at	AT2G35980	Late embryogenesis abundant hydroxyproline-rich glycoprotein	5.75
267354_at	AT2G39880	myb domain protein 25	5.53
253479_at	AT4G32360	Pyridine nucleotide-disulphide oxidoreductase family protein	5.43
254095_at	AT4G25140	oleosin 1	5.43
266544_at	AT2G35300	Late embryogenesis abundant protein, group 1 protein	5.20
251641_at	AT3G57470	Insulinase (Peptidase family M16) family protein	5.08
253676_at	AT4G29570	Cytidine/deoxycytidylate deaminase family protein	4.80
253678_at	AT4G29600	Cytidine/deoxycytidylate deaminase family protein	4.80
253682_at	AT4G29640	Cytidine/deoxycytidylate deaminase family protein	4.80
252557_at	AT3G45960	expansin-like A3	4.72
256839_at	AT3G22930	calmodulin-like 11	4.69
247314_at	AT5G64000	Inositol monophosphatase family protein	4.53
260837_at	AT1G43670	Inositol monophosphatase family protein	4.53
263828_at	AT2G40250	SGNH hydrolase-type esterase superfamily protein	4.49
251768_at	AT3G55940	Phosphoinositide-specific phospholipase C family protein	4.48
266542_at	AT2G35100	Exostosin family protein	4.47
257943_at	AT3G21840	SKP1-like 7	4.46
245161_at	AT2G33070	nitrile specifier protein 2	4.42
254697_at	AT4G17970	aluminum-activated, malate transporter 12	4.40
257100_at	AT3G25010	receptor like protein 41	4.39
247888_at	AT5G57920	early nodulin-like protein 10	4.38

Table 3-2. Highly induced genes that are specifically regulated by combination of bZIP10

plus 11 but are not significantly affected by the other bZIPs tested. The fold change is on a log2 scale and *p* value < 0.02.

Probe	AGI	1+10	1+63	10+11	11+63	Annotation
246034_at	AT5G08350	4.39	1.91	7.99	5.66	GRAM domain-containing protein
265066_at	AT1G03870	4.43	2.00	7.87	5.76	FASCICLIN-like arabinoogalactan 9
260713_at	AT1G17615	5.26	2.36	7.15	4.17	Disease resistance protein (TIR-NBS class)
252563_at	AT3G45970	2.85	2.79	7.13	5.03	expansin-like A1
248915_at	AT5G45690	4.05	1.66	6.21	4.28	Protein of unknown function (DUF1264)
265116_at	AT1G62480	5.35	2.63	6.17	4.91	Vacuolar calcium-binding protein-related
247136_at	AT5G66170	2.66	1.21	5.84	2.98	sulfurtransferase 18
263385_at	AT2G40170	4.45	3.53	5.59	4.36	Stress induced protein
264040_at	AT2G03730	1.35	1.47	5.40	3.49	ACT domain repeat 5
250662_at	AT5G07010	3.13	1.08	5.01	1.32	sulfotransferase 2A
247435_at	AT5G62480	1.33	1.10	4.80	1.99	glutathione S-transferase tau 9
252751_at	AT3G43430	2.87	1.29	4.69	3.97	RING/U-box superfamily protein
248959_at	AT5G45630	2.72	2.05	4.52	2.79	Protein of unknown function. DUF584
260741_at	AT1G15040	3.57	2.71	4.47	3.79	Class I glutamine amidotransferase-like superfamily protein
251763_at	AT3G55730	1.17	1.23	4.31	2.84	myb domain protein 109
253177_s_at	AT4G35150; AT4G35160	1.14	1.03	3.75	1.64	O-methyltransferase family protein
266693_at	AT2G19800	2.90	1.33	3.74	2.56	myo-inositol oxygenase 2
249337_at	AT5G41080	2.53	1.91	3.70	2.95	PLC-like phosphodiesterases superfamily protein
257994_at	AT3G19920	1.57	2.36	3.67	2.62	unknown protein
266555_at	AT2G46270	2.05	1.50	3.57	2.05	G-box binding factor 3
251689_at	AT3G56500	2.29	2.07	3.53	2.66	serine-rich protein-related
254321_at	AT4G22590; AT4G22592	2.97	1.65	3.41	2.48	Haloacid dehalogenase-like hydrolase (HAD) protein
257295_at	AT3G17420	1.09	1.26	3.23	2.10	glyoxysomal protein kinase 1
246209_at	AT4G36870	1.90	1.09	3.21	1.79	BEL1-like homeodomain 2
265111_at	AT1G62510	1.45	1.65	3.16	2.97	seed storage 2S albumin superfamily protein
255625_at	AT4G01120	2.74	2.04	3.12	2.37	G-box binding factor 2
263275_at	AT2G14170	1.60	1.77	3.00	1.60	aldehyde dehydrogenase 6B2
257315_at	AT3G30775	2.09	1.25	2.91	2.38	Methylenetetrahydrofolate reductase family protein
263830_at	AT2G40260	1.28	1.08	2.57	1.85	Homeodomain-like superfamily protein
258402_at	AT3G15450	1.91	1.52	2.42	1.86	Aluminium induced protein with YGL and LRDR motifs
256321_at	AT1G55020	3.00	2.86	2.36	2.87	lipoxygenase 1

267036_at	AT2G38465	1.42	1.42	2.32	2.17	unknown protein
249862_at	AT5G22920	1.49	1.22	2.20	2.07	CHY-type/CTCHY-type/RING-type Zinc finger protein
263827_at	AT2G40420	1.98	1.37	1.69	1.41	Transmembrane amino acid transporter family protein
257147_at	AT3G27270	1.65	1.90	1.68	1.67	TRAM. LAG1 and CLN8 (TLC) lipid-sensing domain protein
254385_s_at	AT4G21830; AT4G21840	1.61	1.05	1.66	1.69	methionine sulfoxide reductase B7/8
263378_at	AT2G40180	1.61	1.17	1.58	1.38	phosphatase 2C5
256319_at	AT1G35910	2.41	1.57	1.48	2.57	Haloacid dehalogenase-like hydrolase (HAD) protein
247264_at	AT5G64530	1.51	1.79	1.40	1.47	xylem NAC domain 1
257226_at	AT3G27880	1.35	1.28	1.39	1.86	Protein of unknown function (DUF1645)
258338_at	AT3G16150	1.53	1.23	1.36	1.50	N-terminal nucleophile aminohydrolases
260915_at	AT1G02660	1.38	1.47	1.28	1.61	alpha/beta-Hydrolases superfamily protein
247091_at	AT5G66390	-1.60	-1.14	-1.02	-1.38	Peroxidase superfamily protein
250199_at	AT5G14180	-1.48	-1.10	-1.23	-1.34	Myzus persicae-induced lipase 1
248932_at	AT5G46050	-2.05	-1.90	-1.29	-1.99	peptide transporter 3
247151_at	AT5G65640	-1.59	-1.08	-1.32	-1.14	beta HLH protein 93

Table 3-3. Genes highly induced or repressed by all four combinations of cotransfected C and S1 group bZIP transcription factors. Fold changes are on a log2 scale and *p* value < 0.02.

Transfected bZIPs	Induced	Repressed
bZIP10	-	-
bZIP63	bZIP11	-
bZIP10+bZIP63	-	-
bZIP1	-	-
bZIP1+bZIP10	bZIP54; bZIP55	-
bZIP1+bZIP63	bZIP54; bZIP55	-
bZIP11	bZIP54; bZIP55; bZIP68	bZIP25
bZIP11+bZIP10	bZIP55; bZIP59; bZIP68	bZIP25; bZIP1; bZIP56; bZIP2
bZIP11+bZIP63	bZIP54; bZIP55	-

Table 3-5. bZIP family genes significantly regulated by C/S1 bZIP single and double protoplast transfections as indicated. Selection standard is fold change above 1 (log2 scale) and *p* value < 0.02.

ing these minor carbohydrate metabolic pathways related genes. In Arabidopsis, there are six genes designated as raffinose synthase (RS), including At1g55740, At3g57520, At4g01265, At4g01970, At5g40390, and At5g20250, named *RS1* to *RS6*, respectively, and four genes characterized as functional myoinositol oxygenases (MIOX), AT1G14520, AT2G19800, AT4G26260, AT5G56640, named *MIOX1*, *MIOX2*, *MIOX4* and *MIOX5*, respectively. *MIOX3* is considered to be a pseudogene. One gene is annotated as trehalase (*TRE1,* At4G24040). Previous results showed that bZIP11 particularly induces *RS2, RS6, MIOX2, MIOX4* and *TRE1* among these genes listed above. Interestingly, in the current study we found that the other bZIPs and combinations tested also specifically regulate these five genes.

Protoplast expression analysis shows that significant induction of *TRE1* expression depends on the presence of bZIP11 and that the presence of C group bZIPs seems not to augment the induction of *TRE1* expression. The C group bZIP63 is more effective in inducing these genes compared to bZIP10. However, there is a large overlap in the induction of *RS* and *MIOX* genes. In single transfections, bZIP1, 11 and 63 induce *RS2, RS6* and *MIOX2*. bZIP10 does not induce transcription by itself (Figure 3-4), but significantly enhances the transcriptional activity of bZIP1 or bZIP11 on *RS2, RS6, MIOX2* and *MIOX4* (Figure 3-4). bZIP10 particularly shows a strong synergistic effect with bZIP1 or bZIP11 in inducing *MIOX4*.

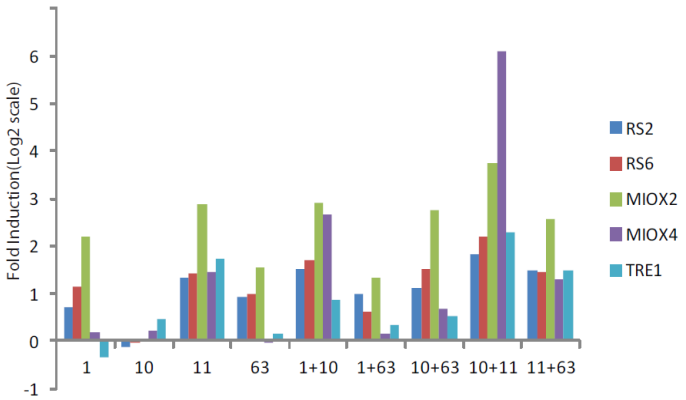

Figure 3-4. The differential effects of single and double bZIP gene transfections on the expression of genes involved in minor carbohydrate metabolic pathways. Along the x axis transfected bZIP genes and their combinations are shown. The fold change in gene expression is shown for each metabolic gene regardless of p values. Abbreviation: RS2 (Raffinose Synthase 2), RS6 (Raffinose Synthase 6), MIXO2 (Myoinositol Oxygenase 2), MIOX4 (Myoinositol Oxygenase 4) and

TRE1 (Trehalase 1).

Trehalose 6-phosphate phosphatase (TPP) dephosphorylates trehalose 6 phosphate (T6P), thereby producing trehalose. From our previous study, we know that bZIP11 induces *TPP5* and *TPP6* in protoplasts. Consistently, we observed that the over expression of *bZIP11* in seedlings results in a lowered T6P content (Ma *et al.,* 2011). We investigated which bZIP combinations are effective in inducing *TPP* genes in the protoplast system (Table 3-4). Again, bZIP11 is the most effective inducer of *TPP* genes, especially in combination with bZIP10 (Table 3-4). In total, 4 out of 10 *TPPs* are significantly induced by either one of the bZIP transfections tested. *TPP9* (*TPPA*) encodes an enzymatically active protein, and likely all other TPPs display enzymatic activity at least from in vitro enzymatic activity assays (Vandesteene *et al.* 2010 and unpublished data). None of the *TPSs* are induced by any bZIP combination tested but the combination of bZIP10 plus 11 represses *TPS8*. It appears that bZIPs promote the degradation of T6P and trehalose. T6P is a powerful signal molecule that promotes carbon utilization and growth. In contrast over expression of bZIP11 severely inhibits growth. Thus, it is interesting to investigate the interaction of the bZIP network and T6P signaling pathway in relation to growth control.

Cross regulation of SnRK1 and S1/C bZIP transcription factors

S1 group bZIPs likely participate in SnRK1 initiated signaling pathway in response to carbon starvation (Baena-Gonzalez *et al.*, 2007). In protoplasts the catalytic subunit KIN10 enhances transcriptional activity of S1 group bZIPs about ten-fold in inducing target genes (Baena-Gonzalez *et al.*, 2007). *KIN11* encodes SnRK1 catalytic subunit and it was found that the tested bZIPs induce the expression of *KIN11*, whereas the expression of *KIN10* is not significantly affected by any of the transfections tested. bZIP11 and the combination of bZIP10 plus 11 effectively induce the expression of *KIN11*. For instance, bZIP11 induces the expression of *KIN11* approx. four-fold while the combination of bZIP10 plus bZIP11 results in an eight-fold increase in induction . Further we know that p robably, SnRK1 activates S1 group bZIPs via phosphorylation (Baena-Gonzalez *et al.*, 2007). These findings indicate that SnRK1 and S1 bZIPs interact in a network harboring feedback regulation at multiple levels such as transcriptional and post-translational levels.

In this study, we investigated whether the S1 and C group bZIP transcription factors used regulate the expression of other bZIP transcription factors. Individual C and S1 bZIPs and their combinations induce or repress a number of *bZIP*s belonging to other groups (Table 3-5). Most often induced are the G group members *bZIP54, bZIP55* and *bZIP68*, which are induced by bZIP11 and several combinations of S1/C bZIPs. *bZIP54* and *bZIP55* are light inducible genes that likely par-

ticipate in seedling establishment. Interestingly, the combination of bZIP10 plus bZIP11 represses the C group *bZIP25* and S1 group *bZIP1* and *bZIP2*. Similarly, the bZIP10 plus bZIP11 combination represses H group *bZIP56* that encodes *ELONGATED HYPOCOTYL5* (*HY5*). *HY5* is a positive regulator of seedling establishment in the light by promoting photomorphogenic development, including inhibition of hypocotyl elongation.

Discussion

Previous studies have shown that S1 and C group bZIPs specifically interact to form heterodimers that have synergistic effects on gene transcription. Here, the effect of in vivo heterodimerization was studied on a genome wide scale. A leaf mesophyll protoplast transfection system was used to study changes in global gene expression upon single and double transfections of selected C and S1 group bZIP genes controlled by the 35S constitutive promoter. Our results suggest that S1/C bZIPs have distinct and overlapping functions in gene regulation.

S1/C group bZIP networks may function in response to carbon starvation

Asparagine and proline are functional metabolites under carbon starvation condition. Asparagine functions as nitrogen carrier instead of glutamine under carbon starvation conditions such as during the night, due to its low C/N ratio. Proline degradation under carbon starvation conditions releases carbon skeletons for energy generation. Consistently, the associated metabolic genes, *ASN1*, *ProDH1* and *ProDH2* are induced by dark or extended night. S1/C group bZIPs play a role in the induction of these genes in response to low energy status and likely this occurs in the SnRK1 mediated signaling network.

In this study we observed that S1/C group bZIPs induce several minor carbohydrate metabolic pathways related genes that previously were shown to be starvation inducible genes.

The S1/C group bZIPs regulated minor carbohydrate metabolic genes include *Raffinose synthase* (*RS*), *Myoinositol oxygenase* (*MIOX*), Trehalase (*TRE1*) and *Trehalose 6-phosphate phosphatase* (*TPP*). Except for the single *Trehalase* gene several different genes encode RS, MIOX and TPP enzymes. S1/C bZIPs particularly induce a set of genes among them, *RS2* and *RS6*, and *MIOX2* and *MIOX4*. *RS6* is also known as *DIN10*, originally selected as a dark induced marker gene. *RS2*, *MIOX2*, and *MIOX4* are highly induced during extended night conditions and this induction is repressed by metabolizable sugars (Gibon *et al.*, 2006). Increased gene expression of *RS* conveys oxidative stress tolerance in Arabidopsis (Nishizawa *et al.*, 2008). Enhanced *MIOX* activity was proposed to provide precursors for downstream metabolic pathways such as ascorbic acid biosynthesis

and for generating UDP-glucuronate for cell wall biosynthesis (Kanter *et al.*, 2005; Lorence *et al.*, 2004). *miox2* mutants incorporate nearly three times less inositol into cell wall polymers. *MIOX2* is expressed throughout the whole plants, while *MIOX4* is highly expressed in reproductive organs (Kanter *et al.*, 2005).

The overexpression of *KIN10* in protoplasts also induces *MIOX2*, *RS2* and *RS6* (Baena-Gonzalez *et al.*, 2007). Thus, it is likely that the coordinated effect of S1/C bZIPs on gene regulation for metabolism of specific amino acids and minor carbohydrate occur in response to carbon starvation conditions and probably involves SnRK1. The picture emerges that SnRK1 (KIN10/11) and S1/C bZIPs interact in a feedback regulated network in response to carbon starvation conditions.

S1/C bZIPs and T6P metabolism

The SnRK1 and T6P signaling components were proposed to have antagonistic effects in carbon metabolism (Zhang *et al.*, 2009). SnRK1 is important for adapting metabolism in response to low carbon status, while T6P promotes carbon utilization under carbon abundant conditions. T6P inhibits SnRK1 activity, and they appear to oppositely regulate marker genes (Zhang *et al.*, 2009). The over expression of KIN10 or bZIP11 overcomes the growth inhibition effects caused by accumulated T6P (Delatte *et al.*, 2011; Ma *et al.*, 2011). This implies a physiological interaction of KIN10 and S1/C bZIPs in balancing cellular metabolism.

Previous studies indicate that bZIP11 plays a role in the induction of *TRE1*, *TPP5* and *TPP6*, resulting in a lowering of T6P and trehalose levels (Ma *et al.*, 2011). In this study, other bZIPs and bZIP combinations have been found to induce T6P degradation associated genes, especially *TPP5* and *TPP6*. The combination of bZIP10 plus 11 appears the most powerful one in inducing T6P degradation associated genes. Besides *TPP5* and *TPP6*, the combination of bZIP10 plus 11 induces *TPP9* (*TPPA*) whose TPP activity has been confirmed (Vogel *et al.*, 1998). Thus, likely the S1/C bZIPs have a function in promoting T6P turnover. Surprisingly, the KIN10 transfections in protoplasts result in induction of trehalose metabolic genes *TPS9*, *10* and *11*, while the other *TPS* or *TPP* genes are not affected significantly (Baena-Gonzalez *et al.*, 2007). Therefore, it is interesting to know whether *KIN11* affects these *TPS* genes as well.

The transcriptome studies presented here suggest that S1/C bZIPs may function in a broader metabolic context such as seed development and maturation. The expression patterns of these transcription factors are consistent with such a broader function, as S1/C bZIPs show overlapping expression in reproductive organs and seeds. Previously, the S1/C genes *bZIP25* and *bZIP53* were shown to induce seed maturation associated genes (Alonso *et al.*, 2009). Here we found that the bZIP10

plus bZIP11 combination highly induces many genes normally expressed during the seed maturation stage, such as seed storage and LEA proteins. bZIP10 and 11 are co-expressed in the maturing seed and might function to induce seed storage genes. Prominently, the bZIP10 plus bZIP11 combination strongly induces many genes related to metabolism, growth and cellular signaling.

S1/C bZIPs affect many genes encoding molecules involved in signal transduction, including other *bZIP* genes. Moreover, it is evident that S1 and C group bZIPs and the upstream effector SnRK1 are powerful regulators of metabolism. The coordination of metabolism and development as mediated by S1/C bZIPs and the regulatory networks with which S1/C bZIPs interact requires detailed *in planta* investigations. Currently, single and multiple mutant combinations for different S1/C bZIPs are becoming available and are being studied. The response of the S1/C network to internal and external cues, especially under conditions of low energy stress might provide directions for crop yield improvement especially in suboptimal growth conditions.

Experimental procedures

Growth conditions

Arabidopsis thaliana Col-0 (var. Col-0, CS60000) wild type plants were grown under long day condition (16h light/ 8h dark) at 22ºC. Well expanded rosette leaves from 5 weeks old plants were selected and protoplasts were isolated from this material.

Plasmid construction

The mORF coding sequences of bZIP1, -10, -11 and -63 were N-terminally fused to the HA immunotag and cloned behind the 35S promoter in the modified vector of pHBT (Ehlert, et al., 2006).

Transient expression of bZIP transcription factors in protoplasts

The protoplast preparation procedure was adapted from Yoo *et al.*, 2007. Arabidopsis mesophyll protoplasts were isolated from four-week old rosette leaves of soil grown plants that were grown in 22°C under fluorescent lighting (18h light period, 120$\mu Em^{-2}s^{-1}$). Leaves were placed in enzyme solution for overnight digestion (1% (w/v) cellulase R10, 0.3% (w/v) macerozyme R10 (Yakult Honsha, http://www.yakult.co.jp/english/), 0.4 M mannitol, 20 mM KCl, 10 mM $CaCl_2$, 20 mM MES, 0.1% (w/v) BSA (Sigma A-6793), pH 5.7). Protoplasts were collected and kept on ice in W5 medium (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 2 mM MES, pH 5.7) for 13h in the growth cabinet. Protoplasts were transferred to MMG solution (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7),

and subjected to PEG-mediated transfection. To $2.12x10^6$ protoplasts, a total of 250 µg plasmid DNA was added followed by 30 min incubation in 1 volume of PEG solution (40% PEG 3500, 0.2 M mannitol, 0.1 M $CaCl_2$). After transfection the samples were diluted with 2 volumes of W5 medium and pelleted at 100 *g* for 2 min. Protoplasts were resuspended in 4 ml WI medium (0.5 M mannitol, 20 mM KCl, 4 mM MES, pH 5.7), transferred to 5 cm Petri dishes pre-coated with 5% calf serum, and incubated for 6 hours under continuous light in a growth cabinet (Snijders, Tilburg). After the incubation, protoplasts were pelleted at 100 *g,* and aliquots were snap frozen in liquid nitrogen for further analysis.

RNA extraction and quality control

Total RNA was extracted from pelleted protoplasts by using the RNeasy Plant Kit (Qiagen). RNA purity and integrity were confirmed by using a RNA 6000 Nano Assay (Agilent, http://www.home.agilent.com) and gel electrophoresis.

Western blotting

Western blotting was performed onto the protein extract from protoplast transfection aliquots according to procedures as published by Ehlert *et al.,* 2006.

Micro-array analysis

The RNA samples were labeled using the Affymetrix OneCycle kit and hybridized to Affymetrix ATH1 GeneChips according to the manufactures' instructions. Data were analyzed statistically using the R language environment for statistical computing (http://www.r-project.org) version 2.11.1 and Bioconductor release 2.6 (Gentleman *et al.*, 2004). Data were normalized using the Robust Multichip Average (RMA) expression measure in the Affy package (Gautier *et al.*, 2004) . Differentially expressed genes were identified using the LIMMA package (Smyth *et al.*, 2005). The probe IDs were matched to the TAIR10 gene annotations (www.arabidopsis.org).

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Chapter 4

Transcriptomics analysis of the interactions between the T6P signal, SnRK1 and S1/C bZIPs

Jingkun Ma, Johannes Hanson and Sjef Smeekens

Summary

Sugar availability is crucial for the survival and fitness of plant during its entire life cycle. The signaling molecule T6P reflects sugar status and plays a critical role in promoting growth, development and regulation of carbon metabolism. T6P inhibits SnRK1 and its targets S1/C bZIPs, both of which mediate the adaptation of metabolism under carbon and energy limiting conditions. Trehalose feeding results in T6P accumulation and has been used to understand the function of T6P. Here, Arabidopsis seedlings were treated with trehalose for 8 hours and the transcriptomic response was monitored by using Affymetrix ATH1 microarrays. The transcriptomic response to trehalose feeding was compared to that of overexpression of the SnRK1 catalytic subunit KIN10 and the overexpression of the bZIP10+11 heterodimer in protoplasts. Responding genes shared between KIN10 and bZIP10+11 overexpression are regulated in a similar way, but trehalose feeding regulates the shared genes in an opposite way especially those shared with KIN10. Additionally, genes co-regulated by both KIN10 and bZIP10+11 are involved particularly in carbohydrate and amino acid metabolism. These results provide molecular evidence that the T6P signal acts antagonistically to SnRK1 and S1/C bZIPs, while SnRK1 and S1/C bZIPs act synergistically in metabolic regulation. Moreover, a large number of stress responsive genes are affected by trehalose feeding.

Introduction

In plants, trehalose is a versatile molecule that can participate in various stress response processes, including cold, drought and pathogen defense (Fernandez *et al.* 2010). Trehalose is a disaccharide composed of two molecules of glucose linked in an α 1,1 configuration. In plants, trehalose 6-phosphate synthase (TPS) produces trehalose 6-phosphate (T6P) using UDP-glucose and glucose 6-phosphate (G6P) as substrates (Paul *et al.*, 2008). T6P is converted into trehalose by trehalose 6-phosphate phosphatase. Trehalose is hydrolyzed by trehalase into glucose.

A large number of trehalose metabolism associated genes are present in Arabidopsis and in other plant species as well. In total there are 22 such genes annotated in the Arabidopsis genome. The enzymatic characterization of these genes has been studied but it remains controversial which enzymes are enzymatically active in vivo. TPS1, TPPA and TPPB were first reported as enzymatically active (Vogel *et al.*, 1998). TPS1 seemed to be the only enzymatically active TPS among the eleven TPSs as suggested by complementation experiments in yeast (Vandesteene *et al.*, 2010). In Arabidopsis, reduced function of TPS1 causes decreased T6P levels, underpinning the in vivo activity of TPS1 (Avonce *et al.*, 2004). Controversially, other groups proposed TPS6 and TPS11 to have both TPS and TPP activity (Singh *et al.*, 2011). Overexpression of TPS11 results in trehalose accu-

mulation and gives rise to aphids resistance (Singh *et al.*, 2011). In Arabidopsis, a single annotated trehalase gene TRE1 is present that is enzymatically active (Muller *et al.*, 2001).

The TPS–TPP pathway produces T6P and trehalose but at very low levels, requiring advanced analytical methods for quantification (Delatte *et al.*, 2009). Trehalose and T6P are both considered as signaling molecules in plants but they function in distinct processes. Generally, accumulated trehalose induces stress resistance in many plant species as discovered through transgenic manipulation of trehalose levels, but very little is known about the mechanisms involved (Fernandez *et al.,*2010). The *TPS11* overexpressing lines show resistance to *Myzus persicae* in which the JA signaling pathway gene *PHYTOALEXIN DEFICIENT4* (*PAD4*) is involved (Singh *et al.*, 2011).

T6P function is crucial for plants, as reduced T6P results in slower vegetative growth, failure in floral transition and embryo lethality but the regulatory mechanisms are unknown (van Dijken *et al.*, 2004). T6P levels reflect availability of sugars and correlate especially with sucrose levels (Lunn *et al.*, 2006). T6P seems to license carbon utilization in plants, which is essential for growth (Schluepmann *et al.*, 2003). T6P promotes starch biosynthesis via post-translational activation of the key starch biosynthetic enzyme ADP-glucose pyrophosphorylase (AGPase) (Kolbe *et al.*, 2005; Lunn *et al.*, 2006). Intriguingly, T6P inhibits SnRK1 activity, which is a powerful regulator in signaling carbon limitation and energy depletion (Zhang *et al.*, 2009). Active SnRK1 and its downstream target S1/C bZIP transcription factors are involved in reprogramming metabolism to a starvation state (Baena-Gonzalez *et al.*, 2007).

Trehalose addition to the growth medium causes T6P accumulation in Arabidopsis seedlings and has been used previously to study the function of T6P in carbon metabolism and growth regulation (Ramon *et al.*, 2007; Schluepmann *et al.*, 2004). In this paper, Arabidopsis seedlings were supplied with trehalose and the response was monitored after eight hours treatment at the transcriptomic level using Affymetrix ATH1 microarrays. Moreover, available transcriptomic data from overexpression of the SnRK1 KIN10 subunit or S1/C bZIPs were compared to the trehalose induced transcriptomic effects.

Results

The trehalose induced effect on the Arabidopsis transcriptome was obtained by feeding 5 days old Arabidopsis Col-0 wild type seedlings with 100 mM trehalose or sorbitol for 8 hours. Sorbitol was used as an osmotic control to correct for osmotic effects of trehalose feeding. Both trehalose and sorbitol treatments were performed in triplicate and RNA samples processed independently using the Af-

fymetrix ATH1 microarray platform. Genes differentially expressed upon trehalose and sorbitol feeding were identified. In total, 7568 genes were differentially expressed with p value < 0.05. Results are presented in a volcano plot displaying gene number distribution of fold change versus p value (Figure 4-1). The changes with p value < 0.05 were considered significant. This results in 616 genes that are up or down regulated significantly more than 2-fold. Table 4-1 displays the 40 genes on the top of the list being either induced or repressed. Real time quantitative PCR analysis of 40 selected genes confirmed the expression changes as detected in the ATH1 Microarray (data not shown).

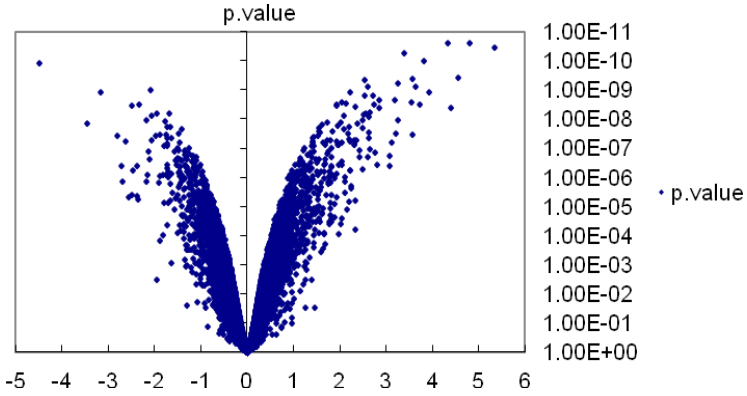

Figure 4-1. Volcano plot of gene number distribution to fold change and p value. Differentially expressed genes with p value < 0.05 were displayed. Fold change (x-axis) is on a log2 scale.

Effects of trehalose feeding on trehalose metabolic genes

Upon trehalose feeding, many genes involved in trehalose metabolism are affected (Table 4-2). Most of the *TPPs* and *TRE1* are significantly induced, while most of the class II TPSs were significantly repressed. Trehalose feeding results in in vivo accumulation of T6P and trehalose, and in response plants appear to induce T6P and trehalose turnover. TRE1 and all TPPs are enzymatically active (Muller *et al.*, 2001; Vandesteene *et al.*, 2010).

The effect of trehalose feeding on S1/C bZIP genes

The S1 group bZIPs and their heterodimerizing C group partners were found to induce *TPPs* and *TRE1* (Chapter 3). The effect of trehalose feeding on S1 and C group bZIPs is presented (Table 4-3) and generally expression of S1 and C bZIP transcription factors are not affected strongly. bZIP11 expression is two-fold induced by trehalose. Sucrose represses the translation of all S1 group bZIPs but trehalose does not have an inhibitory effect on the translation of these bZIPs. Possibly there is a feedback response to fine tune T6P levels. Thus, it is interesting

Intensity	Fold Change	AGI	Annotation
Up			
5.22	5.34	AT1G26380	FAD-binding domain-containing protein
5.26	4.81	AT2G30750	CYP71A12 (cytochrome P450, family 71, subfamily A, polypeptide 12)
5.83	4.57	AT5G57220	CYP81F2; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
7.47	4.40	AT2G43620	chitinase, putative
4.18	4.34	AT1G21120	O-methyltransferase, putative
4.56	3.92	AT1G14550	anionic peroxidase, putative
4.54	3.81	AT1G21110; AT1G21120	[AT1G21110, O-methyltransferase, putative];[AT1G21120, O-methyltransferase, putative]
4.97	3.74	AT2G15120; AT2G15220	pseudogene, disease-resistance family protein
4.36	3.65	AT1G26420	FAD-binding domain-containing protein
5.85	3.57	AT2G19190	FRK1 (FLG22-INDUCED RECEPTOR-LIKE KINASE 1); kinase
6.01	3.56	AT2G43570	chitinase, putative
3.79	3.55	AT1G66690; AT1G66700	S-adenosyl-L-methionine:carboxyl methyltransferase family protein
9.85	3.39	AT1G02930; AT1G02920	ATGSTF6; GSTF7 (GLUTATHIONE S-TRANSFERASE)
6.86	3.26	AT4G12500	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
6.00	3.25	AT4G39940	AKN2 (APS-kinase 2)
5.46	3.20	AT1G14540	anionic peroxidase, putative
4.22	3.19	AT5G64905	PROPEP3 (Elicitor peptide 3 precursor)
5.40	3.13	AT3G18250	unknown protein
5.05	3.08	AT2G17740	DC1 domain-containing protein
8.38	3.07	AT4G12490	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
Down			
3.91	-2.11	AT1G01750	ADF11 (ACTIN DEPOLYMERIZING FACTOR 11); actin binding
5.28	-2.12	AT5G62340	invertase/pectin methylesterase inhibitor family protein
5.53	-2.16	AT4G26010	peroxidase, putative
9.31	-2.17	AT1G75750	GASA1 (GAST1 PROTEIN HOMOLOG 1)
5.72	-2.18	AT4G21830; AT4G21840	methionine sulfoxide reductase domain-containing protein
8.93	-2.34	AT4G26530	fructose-bisphosphate aldolase, putative
5.61	-2.35	AT1G66100	thionin, putative
5.01	-2.37	AT3G62680	PRP3 (PROLINE-RICH PROTEIN 3); structural constituent of cell wall
5.05	-2.38	AT5G67400	peroxidase 73 (PER73) (P73) (PRXR11)
3.00	-2.47	AT5G35190	proline-rich extensin-like family protein
7.60	-2.50	AT5G65730	xyloglucan:xyloglucosyl transferase, putative
5.68	-2.50	AT1G05250; AT1G05240	[AT1G05250, peroxidase, putative];[AT1G05240, peroxidase, putative]
6.00	-2.56	AT4G02270	pollen Ole e 1 allergen and extensin family protein
5.22	-2.62	AT1G35140	PHI-1 (PHOSPHATE-INDUCED 1)
4.28	-2.72	AT2G33790	AGP30 (ARABINOGALACTAN PROTEIN30)

3.48	-2.80	AT2G01520	MLP328 (MLP-LIKE PROTEIN 328); copper ion binding
6.75	-3.17	AT5G57560	TCH4 (Touch 4); hydrolase, acting on glycosyl bonds / xyloglucan:xyloglucosyl transferase
6.66	-3.46	AT4G12510; AT4G12520	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
5.85	-4.48	AT5G46900; AT5G46890	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein

Table 4-1. Genes regulated by trehalose treatment with the highest induction or repression response. Fold change is on a log2 scale and p value < 0.05.

Probe	AGI	Gene name	Fold change
254806_at	AT4G12430;AT4G12432	*TPP5(TPPF)*	2.62
254321_at	AT4G22590;AT4G22592	*TPP6(TPPG)*	2.60
260059_at	AT1G78090	*TPP3(TPPB)*	1.94
252858_at	AT4G39770	*TPP7(TPPH)*	1.56
247228_at	AT5G65140	*TPP10(TPPJ)*	1.37
256319_at	AT1G35910	*TPP2(TPPD)*	0.93
250467_at	AT5G10100	*TPP8(TPPI)*	0.79
248404_at	AT5G51460	*TPP9(TPPA)*	0.76
264246_at	AT1G60140	*TPS10*	-0.25
260010_at	AT1G68020	*TPS6*	-0.49
263019_at	AT1G23870	*TPS9*	-0.70
264339_at	AT1G70290	*TPS8*	-0.98
266072_at	AT2G18700	*TPS11*	-1.18
254197_at	AT4G24040	*TRE1*	0.28

Table 4-2. Trehalose feeding significantly regulated trehalose metabolism associated genes. Fold induction is on a log2 scale and p value < 0.05.

Group	Gene Name	AGI	Fold change	p value
C	*bZIP9*	At5g24800	0.26	3.2E-01
	bZIP10	At4g02640	0.27	1.1E-01
	bZIP25	At3g54620	0.30	2.0E-02
	bZIP63	At5g28770	-0.55	1.5E-04
S1	*bZIP1*	At5g49450	-0.35	4.1E-02
	bZIP11	At4g34590	0.97	5.2E-04
	bZIP44	At1g75390	0.51	3.0E-03
	bZIP53	At3g62420	0.81	1.2E-05

Table 4-3. The effects of trehalose on C and S1 group bZIP transcription factors. Fold change is on a log2 scale.

to investigate the relation between bZIP11 and its dimerizing partners and genes involved in metabolizing T6P and trehalose. In the 8 hours treatment, trehalose feeding does not affect KIN10 or KIN11, which encode the SnRK1 catalytic subunit. However, the similar experiment has been performed in Schluepmann *et al.*, 2004, which analyzed gene expression changes in seedlings fed with trehalose for 24 hours on Affymetrix 8K microarray. There *KIN11* was found to be induced by trehalose. These findings indicate that the carbon sensing components such as T6P, bZIPs and SnRK1 probably have intensive interplay which guarantees the fine adjustment to rapidly changing sugar status.

SnRK1 induces TPS8 to 11 in a carbon starvation context and the expression of TPS8 to 11 has been considered to reflect carbon status (Baena-Gonzalez *et al.*, 2007; Zhang *et al.*, 2009). Interestingly, trehalose represses TPS8-11. Trehalose feeding results in T6P accumulation which signals carbon rich condition. Thus, trehalose feeding might adapt growth to a carbon rich condition independent of the actual carbon status.

Genes co-regulated by trehalose feeding, bZIP10+11 and KIN10

Previous findings suggest that SnRK1, S1/C bZIPs and T6P constitute a closely interacting network. In this regulatory network T6P likely acts antagonistically to SnRK1 and S1/C bZIPs in response to either high or low carbon conditions. It is interesting to know the possible interactions of SnRK1, S1/C bZIPs and T6P at the transcriptomic level. Therefore, the SnRK1 and S1/C bZIPs initiated transcriptomic effects were compared to that in response to trehalose feeding. The study from Baena-Gonzalez *et al.*, 2007 provides the transcriptomic changes initiated by over expressing the catalytically active SnRK1 KIN10 subunit in protoplasts. The combination of bZIP10+11 gives rise to a strong transcriptomic effect (Chapter 3) and it particularly induces genes participating in the degradation of T6P and trehalose. The transcriptomics data all represent relatively short time treatments, as the incubation of the protoplasts following transfection was 6 hours and the data presented here are from plants treated for 8 hours with trehalose. The Venn diagram (Figure 4-2) presents the shared genes regulated by KIN10, bZIP10+11 and trehalose. There are 18 genes regulated by all the three factors. Through Mapman ontology analysis, this set of 18 genes is represented in table 4-4 (see page 114). Among these 18 genes, 8 genes are regulated by trehalose feeding and both by KIN10 and bZIP10+11 in an opposite way. The KIN10 and trehalose feeding share 88 genes, of which 74 genes are regulated in an opposite way by these two initiators (Table 4-5, see page 115-118). Ontology analysis shows that these genes are involved in signaling, amino acid metabolism and cell wall modification. Among the 68 genes shared by trehalose and bZIP10+11, 39 genes are regulated in an opposite way (Table 4-6, see page 119-120). Thus, trehalose feeding has a strong opposite regulation of shared genes with KIN10 or

bZIP10+11, and especially KIN10. Among the 131 genes shared by bZIP10+11 and KIN10, 65 genes are regulated in a similar way (Table 4-7, see page 120-122). In this set many genes are involved in the metabolism of carbohydrates, lipids and amino acids. In this group, *MIOX2*, *RS2*, *RS6*, *ASN1* and *ProDH1* genes are found as studied in Chapters 2 and 3. These findings are consistent with the notion that T6P signals act antagonistically to SnRK1 and S1/C bZIPs, and that SnRK1 and S1/C bZIPs act synergistically in metabolic adaptation.

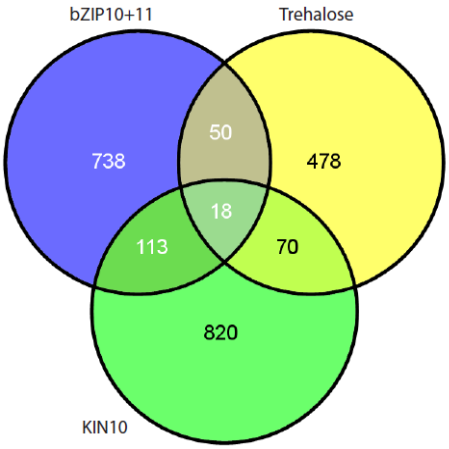

Figure 4-2. Venn diagram showing genes shared among trehalose feeding, overexpression of bZIP10+11 or KIN10 as detected by microarray analysis. Data sets are from Baena-Gonzalez et al., 2007 and Chapter 3. Presented are genes that are regulated over 2-fold with $p < 0.05$.

Trehalose regulates defense associated genes

Trehalose is a stress associated molecule and the ontology analysis of trehalose induced genes shows an overrepresentation of stress response genes, especially biotic stress associated genes (Figure 4-3). Prominently, trehalose feeding regulates several WRKY transcription factors. These WRKY TFs are involved in SA or JA initiated defense response signaling pathways, including the up regulated *WRKY18*, *40* and *60* (above two-fold). Interestingly, these three TFs interact and convey resistance or susceptibility to different pathogen types (Xu *et al.*, 2006).

Previous studies showed that in Arabidopsis trehalose accumulation results in resistance to *Myzus persicae* via JA signaling. Particularly, we found that the JA biosynthesis genes *LOX1* and *LOX2* are highly (four-fold) induced in response to trehalose. JA signaling components and responsive genes are induced as well. Among the JA signaling intermediate genes, *JAZ1* is induced approximately four-fold by trehalose. The defense associated *PHYTOALEXIN DEFICIENT3* (*PAD3*)

gene is induced highly, above four-fold, and is needed in the pathogen defense

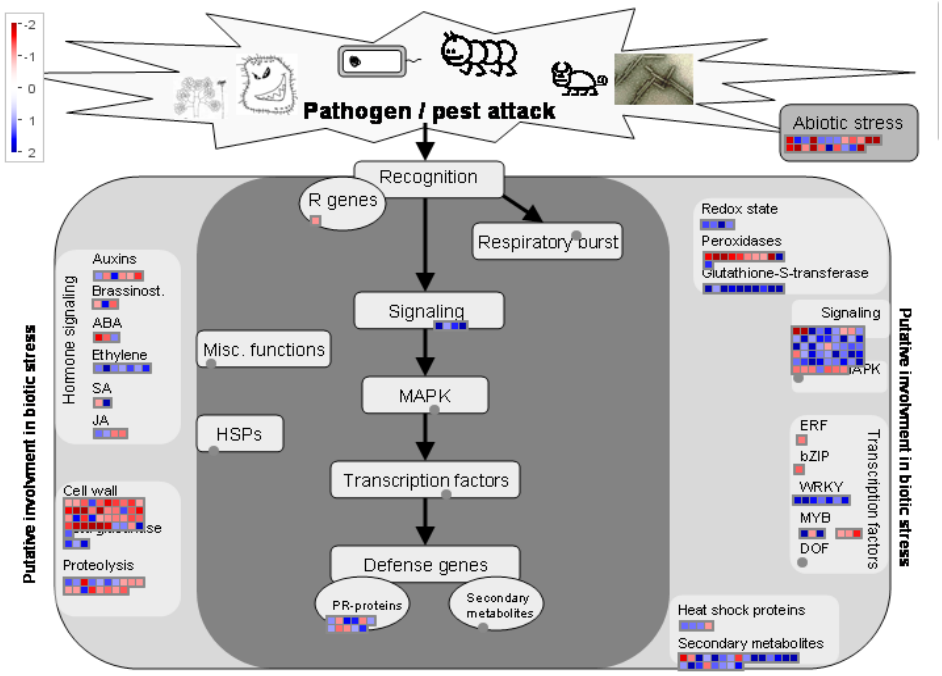

Figure 4-3. Mapman program presents genes responsive to trehalose feeding classified in stress response.

response. Trehalose also induces *AVIRULENCE INDUCED GENE2* (*AIG2*) approximately two-fold. Strikingly, with a longer treatment of 24 hours trehalose feeding, *AIG2* was highly induced up to 32-fold, while sucrose only induces its expression up to 2-fold (Schluepmann *et al.*, 2004). Thus, trehalose feeding not only induces defense responsive genes at early stage but also likely there is augmentation effect along time. Moreover, this effect seemingly is trehalose specific, thus, it sheds light on understanding trehalose initiated pathogen resistance.

The MYC2 transcription factor mediates JA responses. JAZ proteins antagonize JA signaling by suppressing the MYC2 transcription factor, thereby participate in the feedback regulation of the JA response (Chini *et al.*, 2007). Upon perception of the JA signal, JAZs are degraded to activate MYC2. Upon trehalose feeding, *JAZ1* is highly induced while *MYC2* is not affected during the eight hour incubation. Perhaps, the trehalose induced strong JA response initiates this feedback regulation.

Besides the defense associated phytohormone genes, also genes involved in growth associated phytohormone signaling are regulated. Investigating the effect

of trehalose feeding on growth related genes is interesting, since continuous trehalose feeding inhibits plant growth, while the T6P signal is essential for growth and development. Trehalose feeding regulates genes generally responsive to auxin and brassinosteriod as well as several other critical growth regulators. These regulators severely affect development when miss expressed. Trehalose feeding represses *Shoot apical meristem Arrest 1* (*SHA1*) up to two-fold. *SHA1* encodes a putative E3 ligase and has a function in shoot apical meristem maintenance during the adult vegetative stage. *SHA1* knock outs fail to initiate floral primordia thereby abolishing floral transition (Sonoda *et al.*, 2007). Trehalose represses several genes involved in hypocotyl elongation, them the transcription factor *TINY* (AT5G25810). Over expressing *TINY* in seedlings results in shorter hypocotyls and dwarf growth in the dark (Wilson *et al.*, 1996). *BAS1* and *SOB7* encode cytochrome P450 monooxygenases that metabolize and inactivate brassinosteroids, thereby inhibiting growth. Trehalose feeding strongly suppresses *BAS1* and its homologue *SOB7* is also significantly repressed. These two genes function redundantly in modulating photomorphogenic development, including promotion of cotyledon expansion and repression of hypocotyl elongation (Turk *et al.*, 2005). Puzzling, trehalose feeding results in reduced hypocotyl growth in the dark but represses growth inhibitors *BAS1* and *SOB7*.

Conclusions

Sugar sensing and signaling networks are important for adapting plant metabolism and growth to varying carbon availability conditions. T6P, SnRK1 and S1/C bZIPs participate in a network that responds to carbon availability (Smeekens *et al.*, 2010). However, the molecular details are not clear on the interaction among T6P, SnRK1 and S1/C bZIPs. T6P is primarily a carbon abundance signal involved in promoting growth and is expected to act antagonistically with SnRK1 and S1/C bZIPs systems that are inhibitory to growth. Here, trehalose feeding was used to manipulate T6P signals and to study its interaction at the transcriptomic level with SnRK1 and S1/C bZIPs.

Our analysis showed that trehalose feeding regulates the shared genes with KIN10 or bZIP10+11 in an opposite way, which supports the theory that T6P acts antagonistically with the SnRK1 and bZIP10+11 mediated signaling system. Furthermore, the KIN10 and bZIP10+11 shared genes confirm that they act synergistically in regulating metabolic adaptation. The co-regulated genes by KIN10 and bZIP10+11 are mainly involved in primary metabolism, covering the previous studied genes in this thesis. The transcriptomic analysis showed that the expression of genes responsive to both KIN10 and bZIP10+11 are mainly regulated in the same direction. Transcriptomic analysis of trehalose feeding, KIN10 and bZIP10+11 shows that trehalose mainly oppositely regulates responsive genes in the three datasets. These findings provide molecular underpinning of the antago-

nistic effects of T6P and the KIN10 and bZIP10+11 systems. Trehalose feeding induces a large number of stress associated genes consistent with previous findings on the function of trehalose in increasing stress tolerance. Trehalose feeding leads to in vivo raised levels of T6P and trehalose. Thus, the transcriptomic effects obtained by trehalose feeding resulted from both T6P and trehalose.

Experimental procedures

Growth conditions

Arabidopsis thaliana Col-0 wild type seeds were gas sterilized. After stratification in darkness at 4°C for two days, they were cultured in liquid half strength MS medium in sterilized 100 ml glass bottles. The liquid cultures were placed on a shaker with a rotation speed of 80 rpm under continuous fluorescent light in a growth cabinet for 5 days. Trehalose or sorbitol stocks were added to the cultures to a final concentration of 100 mM. After 8 hours, each sample was collected, rinsed, surface dried and snap frozen in liquid nitrogen.

RNA extraction

Total RNA was purified using the RNeasy kit (Qiagen, http://www.qiagen.com) and the RNA purity and integrity were confirmed by using a RNA 6000 Nano Assay (Agilent, http://www.home.agilent.com) and gel electrophoresis.

cDNA synthesis and Real time quantitative PCR

RNA samples were treated with DNase in the presence of RNase inhibitor at 37°C for 15min (www.fermentas.com) according to manufacturer's instruction. The reaction was terminated and enzymes were deactivated by adding EDTA to a final concentration of 2.5mM followed by incubation at 65°C for 10 min. Then oligo dT-primer and random primers were added and the reaction incubated at 75°C for 5min. The reaction was rapidly cooled on ice to prevent secondary structure formation. After a brief spin in a table top centrifuge, M-MLV reverse transcriptase (Fermentas) was added and the synthesis reaction was carried out at 42°C for 1 h. The reaction was diluted 4 times with Millipore water. Real time quantitative PCR was carried out using gene specific primers designed by using Clone Manager software (www.scied.com) with the sequence obtained from TAIR (www.arabidopsis.org) by using the 7900HT Fast Real-Time PCR system with Cybergreen® reaction mixture (Applied Biosystems). Gene expression was normalized to the expression of the house keeping gene *Phosphatase 2A* (At1g13320).

Micro-array procedures

RNA was used for probe synthesis and microarray hybridization using the Af-

fymetrix Arabidopsis ATH1 GeneChips® according to manufacturer's protocol (Affymetrix, http://affymetrix.com). Data were analyzed in the R language environment for statistical computing (http://www.r-project.org) version 2.11.1 and Bioconductor release 2.6 (Gentleman *et al.*, 2004), and were normalized using the Robust Multichip Average (RMA) expression measure in the Affy package (Gautier *et al.*, 2004). Differentially expressed genes were identified using the LIMMA package (Smyth *et al.*, 2005). The probe set sequences were aligned to AGI gene numbers and annotation correlated to the Tair10 gene model database (www.arabidopsis.org).

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Bin	Ontology	Probe	Annotation	bZIP10+11	KIN10	Trehalose
10.6.2	cell wall. degradation	248622_at	(at5g49360): BETA-XYLOSIDASE 1	3.22	5.06	-1.41
10.7	cell wall. modification	252563_at	(at3g45970): ATEXLA1	7.13	3.45	-1.21
33.99	development	253161_at	(at4g35770): SEN1	2.40	3.76	-1.31
34.19.1	transport	259431_at	(at1g01620): PIP1C. TMP-B. PIP1;3 | PIP1C	2.00	4.41	-1.04
34.19.2	transport	258054_at	(at3g16240): DELTA-TIP. TIP2;1	3.66	1.56	-1.73
35.1	unknown	265680_at	(at2g32150): haloacid dehalogenase-like hydrolase	1.67	2.75	-1.00
35.2	unknown	265387_at	(at2g20670): unknown protein	2.41	5.4	-1.13
26.10	misc.cyto-chrome P450	258277_at	(at3g26830): PAD3	-1.64	-2.41	2.64
33.99	development	248855_at	(at5g46590): anac096	4.76	-1.90	1.98
1.3	PS.calvin cyle	251218_at	(at3g62410): CP12-2. CP12	-1.40	2.41	-1.51
31.1	cell.organisa-tion	252411_at	(at3g47430): PEX11B	-1.03	1.53	-1.02
35.1	unknown	257615_at	(at3g26510): octicosapeptide	-1.18	2.71	-1.64
35.2	unknown	265478_at	(at2g15890): MEE14 | MEE14	-1.27	2.69	-1.04
35.2	unknown	262049_at	(at1g80180): unknown protein	-1.23	2.16	-1.03
24.2	Biodegrada-tion	262603_at	(at1g15380): lactoylglutathione lyase	1.26	4.98	1.23
26.8	misc.nitrilases	263216_s_ at	(at1g30720.at1g30730).FAD-binding domain-containing protein	1.18	4.62	1.51
35.1	unknown	260741_at	(at1g15040): glutamine amidotrans-ferase-related	4.47	9.12	1.53
20.2	stress.abiotic	254387_at	(at4g21850): ATMSRB9	3.80	-2.13	-1.78

Table 4-4. Mapman gene ontology classification of genes shared by trehalose feeding, overexpression of KIN10 or bZIP10+11 derived from the comparison presented in Figure 4-2. Genes above the line are co-regulated by bZIP10+11 and KIN10 but oppositely regulated by trehalose.

Bin	Ontology	Probe	Annotation	Trehalose	KIN10
10.4.4.1	cell wall.	246682_at	(at5g33290): XGD1 | XGD1 (XYLOGALACTURONAN DEFICIENT 1)	1.45	-1.8
10.7	cell wall.modification	261266_at	(at1g26770): ATEXPA10, AT-EXP10, ATEXP10	1.60	-3.27
10.8.99	cell wall. pectin*esterases.misc	255524_at	(at4g02330): ATPMEPCRB | ATPMEPCRB	1.88	-2.15
13.1.5.1.2	amino acid metabolism.	253162_at	(at4g35630): PSAT | PSAT	1.10	-1.41
13.1.6.5.5	amino acid metabolism.	249515_at	(at5g38530): tryptophan synthase-related	1.26	-2.01
16.5.1.1.3.1	secondary metabolism.	252827_at	(at4g39950): CYP79B2 | CYP79B2;	2.62	-1.59
16.5.1.1.4.1	secondary metabolism.	253534_at	(at4g31500): CYP83B1, SUR2, RNT1, RED1, ATR4	2.05	-1.69
21.1	redox.thioredoxin	259757_at	(at1g77510): ATPDIL1-2, PDI6, ATPDI6 | ATPDIL1-2 (PDI-LIKE 1-2)	1.43	-2.33
26.3	misc.gluco-, galacto- and mannosidases	262118_at	(at1g02850): BGLU11 | BGLU11 (BETA GLUCOSIDASE 11)	1.02	-1.49
26.9	misc.glutathione S transferases	266269_at	(at2g29480): ATGSTU2, GST20 | ATGSTU2	1.50	-1.62
26.10	misc.cytochrome P450	253534_at	(at4g31500): CYP83B1	2.05	-1.69
26.10	misc.cytochrome P450	258277_at	(at3g26830): PAD3	2.64	-2.41
26.10	misc.cytochrome P450	252827_at	(at4g39950): CYP79B2 | CYP79B2	2.62	-1.59
26.19	misc.plastocyanin-like	267472_at	(at2g02850): ARPN | ARPN (PLANTACYANIN)	1.27	-2.11
26.21	misc.protease inhibitor/seed storage	254819_at	(at4g12500): protease inhibitor/seed storage	3.26	-1.96
28.1	DNA.synthesis/chromatin structure	248036_at	(at5g55920): OLI2 | nucleolar protein	1.02	-2.23
29.3.4.1	protein.targeting.secretory pathway.ER	259852_at	(at1g72280): AERO1	1.07	-1.41
29.4	protein.postranslational modification	262507_at	(at1g11330): S-locus lectin protein kinase family protein	1.29	-2.25
29.5.9	protein.degradation. AAA type	250062_at	(at5g17760): AAA-type ATPase family protein	1.30	-1.98
30.2.1	signalling.receptor kinases.	267436_at	(at2g19190): FRK1	3.57	-1.55
30.2.99	signalling.receptor kinases.misc	246368_at	(at1g51890): leucine-rich repeat protein kinase	2.75	-1.47
33.99	development.unspecified	248855_at	(at5g46590): anac096	1.98	-1.9
33.99	development.unspecified	265414_at	(at2g16660): nodulin family protein	1.07	-2.15
34.2	transporter.sugars	257805_at	(at3g18830): ATPLT5	1.20	-1.8
34.13	transport.peptides and oligopeptides	254396_at	(at4g21680): proton-dependent oligopeptide transport (POT)	1.83	-2.89
34.16	transport.	251503_at	(at3g59140): ATMRP14 | ATMRP14	1.12	-1.93
34.99	transport.misc	258100_at	(at3g23550): MATE efflux family protein	1.82	-1.65
35.1	not assigned.no ontology	255278_at	(at4g04940): transducin family protein	1.19	-1.92
35.1	not assigned.no ontology	266967_at	(at2g39530): integral membrane protein	1.13	-1.48
35.2	unknown	253044_at	(at4g37290): unknown protein	2.50	-2.08

35.2	unknown	250307_at	(at5g12170): unknown protein	1.20	-1.78
35.2	unknown	253687_at	(at4g29520): unknown protein	1.18	-2.6
10.4.4.1	cell wall.	246682_at	(at5g33290): XGD1 | XGD1 (XYLOGALACTURONAN DEFICIENT 1)	1.45	-1.8
10.7	cell wall.modification	261266_at	(at1g26770): ATEXPA10, AT-EXP10	1.60	-3.27
10.8.99	cell wall. pectin*esterases.misc	255524_at	(at4g02330): ATPMEPCRB	1.88	-2.15
13.1.5.1.2	amino acid metabolism.	253162_at	(at4g35630): PSAT | PSAT	1.10	-1.41
13.1.6.5.5	amino acid metabolism.	249515_at	(at5g38530): tryptophan synthase-related	1.26	-2.01
16.5.1.1.3.1	secondary metabolism.	252827_at	(at4g39950): CYP79B2	2.62	-1.59
16.5.1.1.4.1	secondary metabolism.	253534_at	(at4g31500): CYP83B1, SUR2	2.05	-1.69
21.1	redox.thioredoxin	259757_at	(at1g77510): ATPDIL1-2, PDI6	1.43	-2.33
26.3	misc.gluco-, galacto- and mannosidases	262118_at	(at1g02850): BGLU11	1.02	-1.49
26.9	misc.glutathione S transferases	266269_at	(at2g29480): ATGSTU2	1.50	-1.62
26.10	misc.cytochrome P450	253534_at	(at4g31500): CYP83B1	2.05	-1.69
26.10	misc.cytochrome P450	258277_at	(at3g26830): PAD3	2.64	-2.41
26.10	misc.cytochrome P450	252827_at	(at4g39950): CYP79B2	2.62	-1.59
26.19	misc.plastocyanin-like	267472_at	(at2g02850): ARPN	1.27	-2.11
26.21	misc.protease inhibitor/seed storage	254819_at	(at4g12500): protease inhibitor	3.26	-1.96
28.1	DNA.synthesis/chromatin structure	248036_at	(at5g55920): OLI2	1.02	-2.23
29.3.4.1	protein.targeting.secretory pathway.ER	259852_at	(at1g72280): AERO1	1.07	-1.41
29.4	protein.postranslational modification	262507_at	(at1g11330): S-locus lectin protein kinase family protein	1.29	-2.25
29.5.9	protein.degradation. AAA type	250062_at	(at5g17760): AAA-type ATPase family protein	1.30	-1.98
30.2.1	signalling.receptor kinases.leucine rich repeat I	267436_at	(at2g19190): FRK1	3.57	-1.55
30.2.99	signalling.receptor kinases.misc	246368_at	(at1g51890): leucine-rich repeat protein kinase	2.75	-1.47
33.99	development.unspecified	248855_at	(at5g46590): anac096	1.98	-1.9
33.99	development.unspecified	265414_at	(at2g16660): nodulin family protein	1.07	-2.15
34.2	transporter.sugars	257805_at	(at3g18830): ATPLT5	1.20	-1.8
34.13	transport.peptides and oligopeptides	254396_at	(at4g21680): proton-dependent oligopeptide transport (POT)	1.83	-2.89
34.16	transport.	251503_at	(at3g59140): ATMRP14	1.12	-1.93
34.99	transport.misc	258100_at	(at3g23550): MATE efflux family protein	1.82	-1.65
35.1	unknown	255278_at	(at4g04940): transducin family protein	1.19	-1.92
35.1	unknown	266967_at	(at2g39530): integral membrane protein	1.13	-1.48
35.2	unknown	253044_at	(at4g37290): unknown protein	2.50	-2.08
35.2	unknown	250307_at	(at5g12170): unknown protein	1.20	-1.78

35.2	unknown	253687_at	(at4g29520): unknown protein	1.18	-2.6
1.1.1.2	PS.lightreaction.	262612_at	(at1g14150): oxygen evolving enhancer 3 (PsbQ) family protein	-1.15	2.33
1.1.5.2	PS.lightreaction.	258055_at	(at3g16250): NDF4	-1.03	1.85
1.3	PS.calvin cyle	251218_at	(at3g62410): CP12-2	-1.51	2.41
2.1.1.1	major CHO metabolism.synthesis.sucrose. SPS	255016_at	(at4g10120): ATSPS4F	-1.37	1.99
2.2.1.3.3	major CHO metabolism.degradation	260969_at	(at1g12240): ATBETAFRUCT4	-1.34	4.74
3.2.3	minor CHO metabolism.trehalose.potential TPS/TPP	266072_at	(at2g18700): ATTPS11	-1.19	2.21
10.1.2	cell wall.precursor synthesis.UGE	261211_at	(at1g12780): UGE1	-1.01	3.38
10.5.1	cell wall.cell wall proteins.AGPs	245318_at	(at4g16980): arabinogalactan-protein family	-1.46	1.53
10.6.2	cell wall.degradation. mannan-xylose-arabinose-fucose	248622_at	(at5g49360): BXL1	-1.41	5.06
10.6.2	cell wall.degradation. mannan-xylose-arabinose-fucose	253666_at	(at4g30270): MERI5B	-1.67	1.93
10.7	cell wall.modification	252563_at	(at3g45970): ATEXLA1	-1.21	3.45
10.7	cell wall.modification	247925_at	(at5g57560): TCH4	-3.17	4.24
11.4	lipid metabolism.TAG synthesis	248050_at	(at5g56100): oleosin	-1.22	4.38
15.1	metal handling. acquisition	248566_s_at	(at5g49740,at5g49730). at5g49740: ATFRO7; 5g49730: ATFRO6	-1.73	2.78
26.3.2	misc.gluco-, galacto- and mannosidases.	256772_at	(at3g13750): BGAL1	-1.17	1.96
26.3.2	misc.gluco-, galacto- and mannosidases.	247954_at	(at5g56870): BGAL4	-1.02	3.77
27.2	RNA.transcription	250255_at	(at5g13730): SIG4	-1.29	4.34
27.3.26	RNA.regulation of transcription.	250099_at	(at5g17300): myb family transcription factor	-1.56	2.89
29.2.1.99.99	protein.synthesis. ribosomal protein.	264238_at	(at1g54740): unknown	-1.75	3.31
29.4.1	protein.postranslational modification.kinase	250182_at	(at5g14470): GHMP kinase-related	-1.20	5.62
29.5.11.4.3.2	protein.degradation	267238_at	(at2g44130): kelch repeat-containing F-box family protein	-1.30	1.44
29.5.11.4.3.2	protein.degradation	260287_at	(at1g80440): kelch repeat-containing F-box family protein	-1.08	2.74
30.1.1	signalling.in sugar and nutrient physiology	245757_at	(at1g35140): PHI-1	-2.62	3.84
30.7	signalling.14-3-3 proteins	262412_at	(at1g34760): GRF11	-1.16	1.99
31.1	cell.organisation	252411_at	(at3g47430): PEX11B	-1.02	1.53
31.3	cell.cycle	256894_at	(at3g21870): CYCP2;1	-1.28	3.42
33.99	development	256548_at	(at3g14770): nodulin MtN3 family protein	-1.36	3.6
33.99	development	253161_at	(at4g35770): SEN1	-1.32	3.76
34.2	transporter.sugars	264482_at	(at1g77210): sugar transporter	-1.44	4.25

34.2	transporter.sugars	262456_at	(at1g11260): STP1	-1.13	1.36
34.19.1	transport.Major Intrinsic Proteins.PIP	259431_at	(at1g01620): PIP1C	-1.04	4.41
34.19.2	transport.Major Intrinsic Proteins.TIP	258054_at	(at3g16240): DELTA-TIP	-1.73	1.56
35.1	unknown	265680_at	(at2g32150): haloacid dehalogenase-like hydrolase	-1.00	2.75
35.1	unknown	257615_at	(at3g26510): octicosapeptide/Phox/Bem1p (PB1)	-1.64	2.71
35.1	unknown	246917_at	(at5g25280): serine-rich protein-related	-1.27	1.78
35.2	unknown	261567_at	(at1g33055): unknown protein	-1.42	5.09
35.2	unknown	252250_at	(at3g49790): ATP binding	-1.10	2.74
35.2	unknown	265478_at	(at2g15890): MEE14	-1.04	2.69
35.2	unknown	262049_at	(at1g80180): unknown protein	-1.03	2.16
35.2	unknown	253814_at	(at4g28290): unknown protein	-1.02	1.96
35.2	unknown	265387_at	(at2g20670): unknown protein	-1.13	5.4
35.2	unknown	254561_at	(at4g19160): unknown protein	-1.11	2.84

Table 4-5. Mapman gene ontology classification of genes shared by trehalose feeding and KIN10 overexpression. Data derived from the comparison presented in Figure 4-2.

Bin	Ontology	Probe	Annotation	Trehalose	bZIP10+11
16.5.1.1.3.2	secondary metabolism.	264052_at	(at2g22330): CYP79B3	1.27	-1.76
16.5.1.2.3	secondary metabolism.	255753_at	(at1g18570): MYB51	2.49	-1.01
17.5.2	hormone metabolism.	248794_at	(at5g47220): ATERF2	1.43	-1.08
20.2	stress.abiotic	258957_at	(at3g01420): ALPHA-DOX1	1.55	-1.89
26.7	misc.oxidases	254833_s_at	(at4g12280.at4g12290). at4g12280: copper amine oxidase at4g12290: amine oxidase	1.44	-1.27
26.8	misc.nitrilases. *nitrile lyases	261020_at	(at1g26390): FAD-binding domain-containing protein	1.63	-1.01
26.10	misc.cytochrome P450	258277_at	(at3g26830): PAD3	2.64	-1.64
26.10	misc.cytochrome P450	257623_at	(at3g26210): CYP71B23	1.35	-1.02
26.10	misc.cytochrome P450	264052_at	(at2g22330): CYP79B3	1.27	-1.76
27.3.25	RNA.regulation of transcription.	255753_at	(at1g18570): MYB51	2.49	-1.01
27.3.67	RNA.regulation of transcription.	255302_at	(at4g04830): ATMSRB5	1.01	-1.63
29.4	protein.postranslational modification	264223_s_at	(at3g16030): CES101	1.24	-1.05
30.2.12	signalling.receptor kinases.	248895_at	(at5g46330): FLS2	1.58	-1.27
33.99	development.unspecified	263948_at	(at2g35980): YLS9	1.30	-1.18
33.99	development.unspecified	264148_at	(at1g02220): ANAC003	1.97	-1.39
33.99	development.unspecified	265260_at	(at2g43000): anac042	2.64	-1.17
35.2	not assigned.unknown	264635_at	(at1g65500): unknown protein	2.21	-1.03
1.1.1.1	PS.lightreaction. photosystem II.	258239_at	(at3g27690): LHCB2.4. LHCB2.3	-1.19	6.07
10.5.1	cell wall.cell wall proteins.	247965_at	(at5g56540): AGP14. ATAGP14	-1.50	1.37
10.5.1	cell wall.cell wall proteins.	254785_at	(at4g12730): FLA2	-1.01	2.86
10.5.4	cell wall.cell wall proteins.	251843_x_at	(at3g54590): ATHRGP1	-1.24	1.62
10.6.2	cell wall.degradation.	248622_at	(at5g49360): BXL1. ATBXL1 | BXL1 (BETA-XYLOSIDASE 1)	-1.41	3.22
10.7	cell wall.modification	252563_at	(at3g45970): ATEXLA1, EXPL1, ATEXPL1, ATHEXP BETA 2.1	-1.21	7.13
10.7	cell wall.modification	247162_at	(at5g65730): xyloglucan:xyloglucosyl transferase	-2.50	2.47
11.2.1	lipid metabolism.	260950_s_at	(at1g06120,at1g06090).fatty acid desaturase	-1.13	7.31
17.1.3	hormone metabolism.	258498_at	(at3g02480): ABA-responsive protein-related	-1.33	1,71
20.2	stress.abiotic	254387_at	(at4g21850): ATMSRB9	-1.78	3,80
20.2	stress.abiotic	254385_s_at	(at4g21830,at4g21840). at4g21830: ATMSRB7; at4g21840: ATMSRB8	-2.17	1,66
26.12	misc.peroxidases	246991_at	(at5g67400): peroxidase 73 (PER73) (P73) (PRXR11)	-2.38	1,17
30.2.22	signalling.	251843_x_at	(at3g54590): ATHRGP1	-1.24	1.62

31.1	cell.organisation	256275_at	(at3g12110): ACT11 | ACT11 (actin-11)	-1.23	3.74
33.99	development.unspecified	253161_at	(at4g35770): SEN1, ATSEN1, DIN1 | SEN1 (SENESCENCE 1)	-1.31	2.40
34.19.1	transport	259431_at	(at1g01620): PIP1C, TMP-B, PIP1;3	-1.04	2.00
34.19.2	transport	258054_at	(at3g16240): DELTA-TIP, TIP2;1	-1.73	3.66
35.1	unknown	265680_at	(at2g32150): haloacid dehalogenase-like hydrolase	-1.00	1.67
35.1	unknown	254385_s_at	(at4g21830, at4g21840). at4g21830: ATM-SRB7; at4g21840: ATMSRB8	-2.18	1.66
35.2	unknown	262373_at	(at1g73120): unknown protein	-1.03	5.52
35.2	unknown	257041_at	no match	-1.37	4.89
35.2	unknown	265387_at	(at2g20670): unknown protein	-1.13	2.41

Table 4-6. Mapman gene ontology classification of genes shared by trehalose feeding and bZIP10+11 overexpression. Data derived from the comparison presented in Figure 4-2.

Bin	Ontology	Probe	Annotation	KIN10	bZIP 10+11
1.3.4	PS.calvin cyle.GAP	261197_at	(at1g12900): GAPA-2	2.06	1.95
2.1.1.3	major CHO metabolism. synthesis	260837_at	(at1g43670): fructose-1,6-bisphosphatase	2.55	1.16
2.2.2.1	major CHO metabolism	250007_at	(at5g18670): BMY3, BAM9	3.59	3.11
3.1.2.2	minor CHO metabolism	246114_at	(at5g20250): Raffinose biosynthase 6; DIN10	3.28	2.19
3.1.2.2	minor CHO metabolism	251642_at	(at3g57520): Raffinose biosynthase 2	2.37	1.83
3.4.4	minor CHO metabolism	266693_at	(at2g19800): MIOX2	3.56	3.74
9.3	mitochondrial electron transport	260536_at	(at2g43400): ETFQO	2.52	1.74
10.1.20	cell wall.precursor synthesis	255881_at	(at1g67070): DIN9, PMI2 | DIN9 (DARK INDUCIBLE 9)	3.04	2.19
10.6.2	cell wall.degradation	248622_at	(at5g49360): BXL1, ATBXL1 | BXL1 (BETA-XYLOSIDASE 1)	5.06	3.22
10.7	cell wall.modification	252563_at	(at3g45970): ATEXLA1, EXPL1, ATEXPL1, ATHEXP BETA 2.1	3.45	7.13
11.1.13	lipid metabolism	253840_at	(at4g27780): ACBP2 | ACBP2 (ACYL-COA BINDING PROTEIN ACBP 2	1.41	1.20
11.9.2.1	lipid metabolism.lipid degradation	261667_at	(at1g18460): lipase family protein	2.16	1.60
11.9.2.1	lipid metabolism.lipid degradation	260915_at	(at1g02660): lipase class 3 family protein	3.33	1.28
11.9.3.3	lipid metabolism.lipid degradation	249337_at	(at5g41080): glycerophosphoryl diester phosphodiesterase	3.13	3.70
11.9.4.3	lipid metabolism.lipid degradation	251421_at	(at3g60510): enoyl-CoA hydratase	1.87	2.13
12.3.1	N-metabolism	250032_at	(at5g18170): GDH1 | GDH1 (GLUTAMATE DEHYDROGENASE 1)	2.39	1.44
13.1.3.1.1	amino acid metabolism	252415_at	(at3g47340): ASN1, DIN6	5.22	3.82
13.1.4.1.4	amino acid metabolism. synthesis	264524_at	(at1g10070): ATBCAT-2	6.11	3.36
13.2.2.2	amino acid metabolism	257315_at	(at3g30775): ERD5, PRODH	4.55	2.91

13.2.3.1.1	amino acid metabolism	258338_at	(at3g16150): L-asparaginase	2.34	1.36
13.2.4.3	amino acid metabolism	263275_at	(at2g14170): ALDH6B2 | ALDH6B2; 3-chloroallyl aldehyde dehydrogenase	2.05	3.01
13.2.6.3	amino acid metabolism	251421_at	(at3g60510): enoyl-CoA hydratase/ isomerase family protein	1.87	2.13
13.99	amino acid metabolism.misc	264040_at	(at2g03730): ACR5 | ACR5; amino acid binding	2.67	5.40
17.1.3	hormone metabolism	266462_at	(at2g47770): benzodiazepine receptor-related	1.57	1.57
17.2.3	hormone metabolism	258402_at	(at3g15450): unknown protein	4.05	2.42
18.4.1	Co-factor and vitamine metabolism	264524_at	(at1g10070): ATBCAT-2	6.11	3.36
20.1.7	stress.biotic.PR-proteins	262366_at	(at1g72890): disease resistance protein (TIR-NBS class)	2.2	2.31
20.2	stress.abiotic	266462_at	(at2g47770): benzodiazepine receptor-related	1.57	1.57
21.1	redox.thioredoxin	261821_at	(at1g11530): ATCXXS1	1.74	1.42
23.3.1.3	nucleotide metabolism	264561_at	(at1g55810): uracil phosphoribosyltransferase	1.83	1.52
24.2	Biodegradation of Xenobiotics	262603_at	(at1g15380): lactoylglutathione lyase	4.98	1.26
26.8	misc.nitrilases, *nitrile lyases	263216_s_at	(at1g30720,at1g30730).FAD-binding domain-containing protein	4.62	1.18
26.21	misc.protease inhibitor/seed storage	265111_at	(at1g62510): protease inhibitor/seed storage	3.74	3.16
27.3.35	transcription	255625_at	(at4g01120): GBF2, ATBZIP54 | GBF2 (G-BOX BINDING FACTOR 2)	2.13	3.12
27.3.67	transcription	261324_at	(at1g44770): unknown protein	1.82	1.06
27.3.99	transcription	251710_at	(at3g56930): zinc finger (DHHC type) family protein	2.22	2.03
28.1.3	DNA.synthesis	263412_at	(at2g28720): histone H2B	2.1	1.75
29.2.1.1.3.2.99	protein.synthesis.ribosomal protein	245761_at	(at1g66890): unknown	2.05	1.73
29.4.1.59	protein.modification	252298_at	(at3g49060): protein kinase family protein / U-box domain-containing protein	1.31	1.47
29.5.11.4.2	protein.degradation.ubiquitin	255381_at	(at4g03510): RMA1	3.05	1.02
29.5.11.4.2	protein.degradation.ubiquitin	249862_at	(at5g22920): zinc finger (C3HC4-type RING finger) family protein	6.76	2.20
29.5.11.4.5.2	protein.degradation.ubiquitin	253061_at	(at4g37610): BT5	3.07	1.48
33.99	development.unspecified	247136_at	(at5g66170): unknown	2.05	5.84
33.99	development.unspecified	264758_at	(at1g61340): F-box family protein | chr1:22628265-22630009 FORWARD	1.85	1.49
33.99	development.unspecified	253829_at	(at4g28040): nodulin MtN21 family protein	4.6	1.05
33.99	development.unspecified	253161_at	(at4g35770): SEN1, ATSEN1, DIN1 | SEN1 (SENESCENCE 1)	3.76	2.40
34.3	transport.	263827_at	(at2g40420): amino acid transporter family protein	2.61	1.69
34.19.1	transport	259431_at	(at1g01620): PIP1C, TMP-B, PIP1;3	4.41	2.00
34.19.2	transport	258054_at	(at3g16240): DELTA-TIP, TIP2;1	1.56	3.66

35.1	unknown	261674_at	(at1g18270): ketose-bisphosphate aldolase	2.22	1.47
35.1	unknown	265680_at	(at2g32150): haloacid dehalogenase-like hydrolase	2.75	1.67
35.1	unknown	266984_at	(at2g39570): ACT domain-containing protein	1.98	1.87
35.1	unknown	260741_at	(at1g15040): glutamine amidotrans-ferase-related	9.12	4.47
35.1	unknown	252109_at	(at3g51540): unknown protein	2.89	1.56
35.1.9	unknown	267524_at	(at2g30600): BTB/POZ domain-containing protein	3.85	1.74
35.1.9	unknown	267523_at	(at2g30600): BTB/POZ domain-containing protein	4.47	1.98
35.1.26	unknown	267385_at	(at2g44380): DC1 domain-containing protein	5.44	1.31
35.2	unknown	260743_at	no match	3.87	1.77
35.2	unknown	262043_at	(at1g80190): PSF1 | PSF1 (PART-NER OF SLD FIVE 1)	3.29	2.04
35.2	unknown	266901_at	(at2g34600): JAZ7	2.18	1.74
35.2	unknown	247047_at	(at5g66650): unknown protein	3.35	1.31
35.2	unknown	264379_at	(at2g25200): unknown protein	2.2	2.13
35.2	unknown	258845_at	(at3g03150): unknown protein	1.75	1.58
35.2	unknown	266583_at	(at2g46220): unknown protein	1.92	1.66
35.2	unknown	265387_at	(at2g20670): unknown protein	5.40	2.41

Table 4-7. Mapman **g**ene ontology classification of genes shared by KIN10 and bZIP10+11 overexpression. Data derived from the comparison presented in Figure 4-2.

Chapter 5 Summarizing discussion

The S1/C bZIPs regulatory network in sugar sensing

Jingkun Ma, Johannes Hanson and Sjef Smeekens

Sugar availability and growth

Information on the prevailing nutrient status is crucial for fitness and productivity of plants. This is especially true for sugar availability, which varies during the diurnal cycle and under stress conditions. Sugar status is conveyed to signaling networks that integrate plant responses at the levels of tissue and organ. In recent years several systems have been uncovered that are involved in sugar dependent growth control. These include the trehalose 6-phosphate (T6P) regulatory molecule, the TOR kinase system, the C/S1 bZIP transcription factor group and the SnRK1 kinase (Smeekens *et al.* 2010). In this thesis, a contemporary overview of sugar signaling is provided in Chapter 1. In Chapter 2, the powerful regulatory function in metabolism of S1 group ZIP11 is investigated and the regulatory effects of bZIP11 in the metabolism of trehalose and T6P were discovered. In Chapter 3, investigations on the biological functions of C/S1 bZIPs are extended to include other *bZIP* genes, which are *bZIP1*, *10*, *11* and *63*. This reveals the overlapping and distinct functions of bZIP heterodimers. The overlapping function of C/S1 bZIP heterodimers has been observed in regulating several minor carbohydrate metabolic pathways, including trehalose metabolism at the transcriptome level. In Chapter 4, it is described that trehalose addition to seedlings regulates a large number of stress and signaling related genes, and identifies the S1 group bZIPs as trehalose responsive genes. The findings described in this thesis point to the central regulatory function in metabolism of the C/S1 group bZIP TFs, in particular bZIP11. The C/S1 group bZIP TFs mediated regulation of metabolism, as participants in the SnRK1-T6P regulatory network likely is prominent under carbon starvation conditions. However, C/S1 bZIPs also participate in a broader context, such as other stress conditions and development.

C/S1 group bZIPs mediate metabolic reprogramming in response to carbon starvation conditions

Previous findings indicate that C/S1 group bZIP proteins are targets of the SnRK1 protein kinase, which enhances the transcriptional activity of C/S1 bZIPs greatly in protoplasts (Baena-Gonzalez *et al.*, 2007). The signaling molecule T6P interacts with SnRK1 in response to carbon rich conditions, as an inhibitor of SnRK1 activity (Zhang *et al.*, 2009). Importantly, the findings presented in this thesis identify that the C/S1 bZIPs are regulators of the growth promoting signaling molecule T6P. These findings show that C/S1 bZIPs are part of a regulatory network that includes T6P and SnRK1 and that responds to the prevailing sugar status. SnRK1 and T6P signaling are activated by low and high carbon availability, respectively (Baena-Gonzalez *et al.*, 2007; Lunn *et al.*, 2006; Schluepmann *et al.*, 2003). Prominently, they are both central in regulating plant growth. The activation of C/S1 bZIPs by SnRK1 likely occurs under carbon starvation conditions. We observed that the C/S1 group bZIP transcription factors regulate T6P

and trehalose degradation related genes, including several *TPPs* and the single *Trehalase1*. Biochemical analysis in seedlings showed that trehalase enzymatic activity is increased upon bZIP11 activation, and that T6P and trehalose levels are reduced. Our findings suggest that the genes *TPP5*, *TPP6* and *TRE1* likely are direct targets of bZIP11. The response of these genes was first observed in large scale transcriptomics experiments with bZIP11 transfected leaf mesophyll protoplasts. The promoters of the *TPP5*, *TPP6* and *TRE1* genes contain multiple ACGT elements, generally required for bZIP family transcription factor binding (Hanson *et al.*, 2008). Consistently, bZIP11 is capable to activate transcription of the reporter gene fused to promoters of *TPP5*, *TPP6* and *TRE1*.

In addition to the trehalose metabolic genes discussed, bZIP11 induces minor carbohydrate metabolic genes involved in inositol and raffinose metabolism such as *RS2*, *RS6*, *MIOX2* and *MIOX4,* with concordant changes in metabolite levels. Characteristically the changes in these gene expressions and metabolites mimic a condition of carbon starvation in plants, such as during extended night. Thus bZIP11 and other C/S1 bZIPs (see below) enforce a regulatory metabolic state on plants that is similar to a starvation state. In bZIP11 activated plants growth is inhibited and this inhibition cannot be reversed by addition of sugars. The reduced glycolytic flux as observed at the metabolite level likely is part of this starvation signature. It is currently unknown why the inositol/raffinose pathway is induced and how these metabolites function in mitigating the starvation state. Carbon starvation influences amino acid content as well, such as depletion of proline and increase in aromatic amino acids (Usadel *et al.*, 2008). These starvation characteristics have been observed in bZIP11 mediated effects on amino acid metabolism. There are substantial changes in levels of amino acids and the expression of corresponding genes involved, such as *ASN1*, *ProDH1* or *ProDH2,* which are all proposed to be involved in metabolic adaptation to carbon depletion conditions. Levels of the three aromatic amino acids (phenylalanine, tryptophan, and tyrosine) rise quite substantially following bZIP11 activation and similarly during starvation. Also here the underlying molecular function of this rise in the low carbon adaptation response is unclear.

C/S1 bZIP heterodimerization

C/S1 bZIPs preferentially bind DNA as dimers and in previous experiments C and S1 group bZIPs were found to preferentially heterodimerize. Different C and S1 bZIPs interact with different affinities in protein two hybrid assays performed both in yeast and protoplast (Ehlert *et al.*, 2006; Weltmeier *et al.*, 2009). The dimerization of C and S1 bZIPs provides tremendous regulatory potential in controlling gene expression specificity. However, the dimerization effects of S1 and C group bZIPs in transcription has only been shown on individual genes, such as *ProDH* and *ASN1* (Baena-Gonzalez *et al.,* 2007*;* Weltmeier *et al.,* 2006). For the

C/S1 group bZIPs the function of heterodimerization in target gene specificity and in transcriptional activation strength has not been assessed in a broader way. In this thesis, we used leaf mesophyll protoplasts transfected with combinations of S1 and C group bZIP gene over expressing plasmid to investigate the specificity and function of bZIP heterodimers. In these experiments, the C group bZIP10 and 63 and the S1 group bZIP1 and 11 were used. The transcriptional potential of homodimers and heterodimers was investigated at the genome level by using Affymetrix ATH1 expression arrays.

In experiments with bZIP homodimers it was observed that only bZIP11 is very active in inducing target genes as it induces the largest number of genes compared to the other investigated bZIP genes. The other bZIP genes, especially C group bZIPs *bZIP10* and *bZIP63* have very minor effects on the transcriptome. The transfections with combinatorial bZIP genes confirms that bZIP10 heterodimerizes with S1 group bZIP proteins. In detail, the bZIP10+11 combination is highly active and regulates 872 genes, and bZIP10+1 also has a much higher number of regulated genes compared to its single transfections. All heterodimers have a number of regulated genes specific for the combination, but they share a substantial number of regulated genes. A set of 48 genes is regulated by all four heterodimers. Thus, this study shows that the S1 and C bZIPs have heterodimerizing capability and have overlapping, and distinct functions in regulating gene expression.

Furthermore, the C/S1 bZIPs have an overlapping function in regulating amino acid metabolic genes such as *ASN1* and *ProDH* and overlapping functions in regulating minor carbohydrate metabolism. The combinations of S1 and C group bZIPs tested induce *RSs*, *MIOXs*, *TPPs* and *TRE1* genes as mentioned before. Synergistic effects between C and S1 group bZIPs is evident in the regulation of the minor carbohydrate genes as well.

The interaction between C/S1 bZIPs and T6P

In addition to the impact of C/S1 bZIPs on trehalose/T6P metabolism as discussed before, the interaction between C/S1 bZIPs and trehalose metabolism is also observed in trehalose feeding experiments where *bZIP11* gene expression is induced (Chapter 4). However, trehalose feeding does not repress the translation of bZIP11 (Delatte *et al.* 2011). Remarkably, trehalose induces a large number of genes involved in defense response and signaling. However, trehalose feeding results in the accumulation of both T6P and trehalose in vivo. Therefore, the impact of trehalose feeding on the transcriptome does not discriminate between these two signals.

SnRK1 and T6P signal have tight connection in regulating multiple processes in

response to sugar availability (Delatte *et al.*, 2011; Martinez-Barajas *et al.*, 2011; Paul *et al.*, 2010; Wingler *et al.*, 2012; Zhang *et al.*, 2009). Trehalose feeding, SnRK1 and bZIP10+11 regulate a number of genes in an overlapping way. This strengthens the notion that these components function in a network that responds to carbon availability (Figure 5-1).

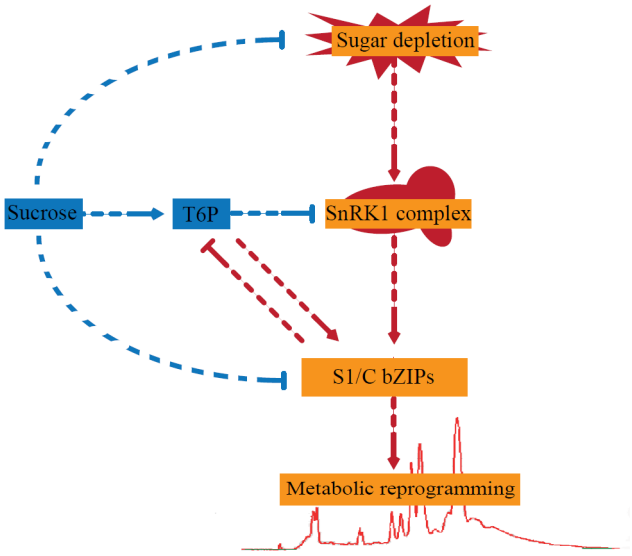

Figure 5-1. The C/S1 group bZIPs are part of the SnRK1 - T6P regulatory network that responds to sugar status. The blue lines indicate interactions under sugar rich conditions, while the red lines indicate interactions during sugar depletion. The bars and arrows at the end of the lines indicate inhibitory and stimulatory interactions, respectively. The outcome of interaction is the adaptation of metabolism to prevailing sugar conditions, resulting in stimulation or inhibiton of plant growth.

Rising sucrose levels in plants halt S1 group bZIP translation (Rahmani *et al.*, 2009; Wiese *et al.*, 2005). Sucrose thus reduces expression of S1 group bZIP target genes. Notably, *ASN1*, *RSs*, *MIOXs* and *TRE1* are sugar repressed genes. Carbon rich condition also increases the levels of G6P and T6P (Lunn *et al.*, 2006), thereby inhibiting the activity of SnRK1 (Halford and Hey, 2009; Zhang *et al.*, 2009). Thus, carbon rich conditions down regulate the SnRK1 and C/S1 bZIP network, while T6P will rise and promote growth.

At the start of the sugar depletion phase SnRK1 activity increases and consequently the C/S1 group bZIPs are activated, resulting in increased expression of downstream genes, including *TPPs* and *TRE1*. The induced TPP and TRE1 activity convert T6P and trehalose into glucose, thereby reducing T6P signaling. Furthermore, at the end phase of carbon rich conditions the accumulated T6P/tre-

halose promotes *bZIP11* expression as observed but at that stage high sucrose will inhibit bZIP11 activity until sucrose levels drop. This likely creates a sensitive regulatory switch that reprograms metabolism to carbon starvation conditions.

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Samenvatting in het Nederlands

Planten zetten door middel van fotosynthese CO2 om in koolhydraten. Via de Calvin-Benson cyclus wordt CO2 gebonden en omgezet in glyceraldehyde 3 fosfaat (GAP). Dit GAP wordt vervolgens via het intermediaire metabolisme omgezet in het niet-reducerende disaccharide sucrose. Sucrose is het belangrijkste transportsuiker in de plant en wordt naar de verschillende organen (sinks) getransporteerd en gebruikt voor groei en ontwikkeling. De optimale verdeling en benutting van suikers in de verschillende weefsels en cellen van de plant zijn van groot belang voor het (reproductieve) succes en biomassa productie van planten en tevens voor de opbrengst van gewassen in de landbouw.

Regelsystemen voor groei

Lang is onduidelijk geweest welke regelsystemen in planten de productie en het gebruik van suikers controleren maar in het laatste decennium zijn de onderliggende processen op moleculair niveau geïdentificeerd en bestudeerd. Enkele van deze systemen zijn algemeen aanwezig in alle bestudeerde eukaryoten en zijn evolutionair goed geconserveerd. Zo bezitten alle eukaryoten de belangrijke TOR (Target of Rapamycin) kinase en SNF1 (gist, sucrose non fermentable1)/AMPK (animaal, AMP activated protein kinase)/SnRK1 (plant, SNF1-related kinase1) systemen. TOR en SNF1/AMPK/SnRK1 zijn uiterst belangrijke centrale regelsystemen in de cel en bestaan uit Multi-eiwitcomplexen. Bij voldoende hoog nutriëntenniveau (suikers, aminozuren) wordt het TOR kinase regelsysteem geactiveerd en kan groei en ontwikkeling plaatsvinden. Daalt het suiker of energieniveau in de cel dan wordt TOR inactief en wordt het SNF1/AMPK/SnRK1 systeem geactiveerd en wordt het anabolisme geremd en het catabolisme gestimuleerd om de cel te beschermen tegen een te laag ATP niveau waardoor de cellulaire integriteit en overleving in gevaar komt.

In planten zijn naast de TOR en SnRK1 nog twee belangrijke systemen bekend die metabolisme aansturen. Trehalose 6-fosfaat (T6P) is een intermediair in de trehalose biosyntheseweg. T6P is in zeer lage concentraties aanwezig (micromolair niveau) maar is een essentieel signaalmolecuul voor groei. In mutanten met een sterk verlaagde T6P status komt de groei tot stilstand omdat planten de beschikbare suikers niet kunnen benutten. Dergelijke planten zijn niet in staat levensvatbaar zaad te produceren. T6P niveaus in de cel zijn gecorreleerd aan sucrose niveaus en het lijkt erop dat sucrose de T6P biosynthese stimuleert en op deze wijze groei bevordert. Andere suikers zoals glucose en fructose zijn veel minder in staat T6P niveaus te laten toenemen.

Naast T6P is in planten een ander voor groei belangrijk regelsysteem actief, de C/S1 groep van bZIP transcriptiefactoren. bZIP eiwitten zijn transcriptiefactoren

die cellulaire processen aansturen en de C/S1 bZIPs lijken vooral betrokken bij de regulatie van het metabolisme. Uit de C/S1 groep is vooral de Arabidopsis bZIP11 een bijzondere transcriptiefactor en regulator. Overexpressie van bZIP11 remt de groei van planten krachtig af, onafhankelijk van de suikerstatus. Net als bij een laag T6P niveau kan ook bij bZIP11 overexpressie de groei niet worden hersteld door toevoeging van suiker. bZIP11 vormt samen met de bZIPs 1, 2, 44 en 53 de S1 groep van bZIP transcriptiefactoren. Deze vijf S1 bZIPs delen een bijzonder expressieregulatie mechanisme. S1 bZIP mRNAs hebben in het 5' deel van de sequentie van hun mRNAs enkele overlappende korte leesramen voor peptiden. Een van deze peptiden van ~40 aminozuren lang is het Sucrose Control (SC) peptide. Dit SC peptide remt de translatie van het bZIP11 open leesraam (main ORF, mORF) af op een sucrose concentratie afhankelijke manier. Bij oplopende sucroseconcentraties in de cel zal het peptide de ribosoombeweging naar het mORF afremmen en daarmee de productie van S1 bZIP eiwitten remmen. Dit gebeurt bij fysiologische sucroseconcentraties in de cel en de half maximale remming van mORF translatie vindt plaats bij de relatief lage cellulaire sucrose concentratie van 10-20 mM. Dus sucrose stimuleert de aanmaak van T6P in de cel waardoor groei wordt gestimuleerd en sucrose remt de S1 bZIP translatie wat een remmer van groei is. In **Hoofdstuk 1** van dit proefschrift wordt een gedetailleerd literatuuroverzicht gegeven over onze kennis van de wijze waarop bovenstaande regelsystemen actief zijn in de cel. Tevens wordt daar kort ingegaan op de identificatie van enkele bekende sensoren die door de cel worden gebruikt voor de primaire waarneming van suikerniveaus.

De functie van C/S1 bZIPs

In dit proefschrift is onderzoek beschreven naar de rol van het C/S1 regulatiesysteem in de groei van planten met de nadruk op de bZIP11 transcriptiefactor. Eerder onderzoek aan bZIP11 liet zien dat het een krachtige regulator is van genen betrokken bij de aminozuurbiosynthese en dat bZIP11 overexpressie ook de cellulaire niveaus van enkele aminozuren wijzigt.

In **Hoofdstuk 2** wordt de functie van Arabidopsis bZIP11 als regulator van het metabolisme in detail onderzocht. Hiervoor zijn twee transgene lijnen gebruikt, L en M, waarin bZIP11 overexpressie zeer goed kan worden gereguleerd met dexamethason (dex) als inducerend agens. Dex toevoeging op ieder gewenst moment activeert de functie van bZIP11 en zo kunnen de effecten hiervan goed worden bestudeerd. Binnen enkele uren na bZIP activatie in Arabidopsis verandert het transcriptoom ingrijpend zoals waargenomen met Affymetrix Arabidopsis ATH1 microarray experimenten. Metabolomics studies van dit materiaal laten zien dat vele honderden metabolieten significant toenemen of afnemen. De meeste van deze metabolieten zijn niet geïdentificeerd maar de omstreeks zeventig geïdentificeerde en gekwantificeerde metabolieten laten zien dat vooral suikers (sucrose,

glucose, fructose), gefosforyleerde intermediairen in het suikermetabolisme, organische zuren en aminozuren in concentratie veranderen. Plaatsing van deze metabolieten op de biochemische routes laat zien dat in de top van de glycolyse ophoping van metabolieten plaatsvindt terwijl in de citroenzuurcyclus juist metabolieten dalen in niveau. Het lijkt erop dat door inductie van bZIP11 de glycolyse afgeremd wordt. Interessant is nu dat na bZIP11 inductie ook vele zogeheten 'minor' koolhydraten veranderen, zoals inositol en raffinose. Deze maken deel uit van een syntheseweg die ook wordt opgereguleerd wanneer planten te maken krijgen met ernstige suiker en energietekorten zoals bij het langdurig plaatsen in het donker (extended night). Ook werd waargenomen dat bZIP11 inductie het trehalose metabolisme stimuleert; de hoeveelheid trehalose alsook het regulatormolecuul T6P nemen in concentratie af. Het lijkt erop dat bZIP11 een staat van hongering induceert. Ook al is er voldoende suiker aanwezig of toegevoegd aan deze planten dan nog wordt dit niet herkend of gebruikt omdat het metabolisme door bZIP11 in een 'spaarstand' is gezet. Groei is dan niet mogelijk.

Het is karakteristiek voor bZIP eiwitten dat ze als dimeren binden aan DNA. De ZIPper domeinen van de twee bZIP partners binden elkaar en zo wordt een dimeer gevormd die vaak een veel krachtigere activator van genexpressie is dan de monomeren of homodimeren. In vorig onderzoek is vastgesteld dat de S1 groep bZIPs preferentieel dimeriseert met leden van de C groep bZIPs die wordt gevormd door bZIP9, 10, 25, en 63. In **Hoofdstuk 3** is het effect van dit dimerisatie proces op de genexpressie bestudeerd. Hiertoe zijn protoplasten geïsoleerd uit bladeren (mesophyllcellen) en getransfecteerd met bZIP coderend DNA. Enkele en dubbele transfecties zijn uitgevoerd met bZIPs waarvan uit voorgaand onderzoek al bekend was dat ze goede inter-acterende partners zijn. Na incubatie van de enkel of dubbel getransfecteerde protoplasten zijn transcriptomics experimenten uitgevoerd met de Affymetrix Arabidopsis ATH1 microarray. Interessant is nu dat alleen bZIP11 een krachtige reactie op de genexpressie in protoplasten laat zien. Andere geteste bZIPs hebben vrijwel geen effect op de genexpressie. Dit beeld verandert drastisch bij het testen van de heterodimeren. Door heterodimerisatie worden veel meer genen gereguleerd. Tevens werd gevonden dat iedere combinatie een unieke set van genen reguleert maar dat er tussen de combinaties ook grote overlap bestaat in de regulatie van genexpressie. Interessant is dat een set van ongeveer 48 genen door alle combinaties worden gereguleerd. Opvallend is nu dat diverse combinaties ook de genexpressie van genen betrokken bij het minor koolhydraatmetabolisme aansturen. Ook reguleren diverse bZIP combinaties een breder palet aan trehalose metabolisme genen dan eerder gevonden met transfectie van alleen bZIP11. Interessant is dat de geteste bZIPs ook diverse andere bZIP genen reguleren, waaronder ook andere C/S1 genen. Sommige van deze genen hebben een bekende en belangrijke biologische functies zoals HY5 (bZIP56) wat bij lichtafhankelijke ontwikkeling van de plant is betrokken.

Een terugkerende bevinding in het onderzoek aan de C/S1 bZIPs is de betrokkenheid bij het trehalose metabolisme en dan met name de regulering van T6P niveaus. De rol van trehalose en T6P is nader onderzocht in **Hoofdstuk 4.** Arabidopsis planten zijn hier op hoge concentraties trehalose gegroeid waardoor het T6P niveau aanmerkelijk wordt verhoogd. Op dit materiaal is een transcriptomics analyse gedaan en waaruit diverse conclusies kunnen worden getrokken. Zo induceert trehalose een groot aantal TPP genen en remt het TPS genen. Trehalose toevoeging activeert ook enkele C/S1 bZIP genen, met name bZIP11. Opmerkelijk is dat vele genen betrokken bij de afweer van planten tegen pathogene organismen worden geïnduceerd. Echter naast T6P is waarschijnlijk ook trehalose in planten een signaalmolecuul en er kan dus geen onderscheid worden gemaakt tussen effecten van T6P en trehalose.

De behaalde resultaten leiden nu tot een model waarin de C/S1 bZIPs onderdeel uitmaken van een regulatienetwerk samen met T6P en SnRK1 (zie Figure 5-1, Hoofdstuk 5). Bij voldoende suikers, waaronder sucrose, zal de T6P concentratie oplopen en daardoor de SnRK1 activiteit remmen. Bij voldoende sucrose zal specifiek de aanmaak van S1 bZIP eiwitten worden geremd, waardoor de C/S1 bZIPs het cellulaire metabolisme niet kunnen programmeren naar een hongerings/lage energie toestand. Het T6P signaal zal dan de groei van de plant bevorderen. Wanneer door bijvoorbeeld omgevingsfactoren suikerconcentraties teruglopen wordt de aanmaak van S1 bZIPs niet langer geremd en ook zal het T6P niveau dalen waardoor het SnRK1 kinase actief kan worden en de C/S1 bZIPs activeren. Hierdoor wordt het metabolisme naar een hongeringstoestand gebracht, gericht op het behoud van de cellulaire energie status en daarmee de cellulaire integriteit. Hierdoor wordt de groei van de plant geremd.

Acknowledgements

Firstly, I gratefully acknowledge the supportive, encouraging and open-minded supervision from my promoter Sjef Smeekens and co-promoter Johannes Hanson that ensured the presence of this book. In particular, thank you Sjef, thanks for accepting me as your lab member and guiding me through, and I appreciate the scientific writing skills that I have learned from you.

Doing a PhD is a long-term team work. I would like to thank the scientists that I had inspiring scientific communications or collaborated with, Marcel, Thierry, Chun-Ming, Jian, Sheng, Lazaro, Pieter and Henriette. For the financial support, thanks to KNAW, Utrecht University and CBSG enabled my scientific life in the Netherlands, and I am enjoying and impressed by the dynamic and nutritious atmosphere for researchers of plant sciences in the Netherlands.

For the last moment of being a PhD candidate, thank you Evelien and Leonie for being my paranimfen, and as colleagues thanks for the years of amiable and encouraging atmosphere you have brought to me.

Through years of presence in MPF at Utrecht University, including the supportive company from my colleagues, I appreciate the hospitality, enthusiasm and ration that I have experienced and learned from most of you. Especially, I like all the activities together, such as Eftling, Glowgolf, Madurodam, bowling, roller skating, cake break, excursions and many more. There is just so much to say that another book is needed, and luckily all the memory has been well installed in Jingkun. Thank you all my colleagues, former or present, including those mentioned above and more: Maureen, Micha, Anja, Jeroen, Jolanda, Dongping, Bas, Martijn, Julia, Jeba, Wouter, Nicole, Niek, Savani, Monika, Sylvia, Lennard, Magdalena, Prapti etc.

Generally, I think the Netherlands is a well organized and special country living humorous and friendly people. I enjoyed my life in Utrecht and also visiting to cities or places of The Hague, Maastricht, Nijmegen, Zeeland, Noord-holland etc. Life could have been colorless without the people I joined activities with and my spoken Chinese could have got worse, thank you Shouli, Baocheng, Shixi, Meng, Du, Li, Long, Hongtao, Hanzi, Fengfeng etc. Particularly, thank you Xin, Yun, Zhong and Shijie for the continent-independent friendship!

Now comes to the most important persons to me, my mother, my father and my sister. Thank you! Without the strength you have built in me, I couldn't have reached anywhere.

Finally, to all the people helped or care, mentioned or not mentioned, thanks for giving!

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谨以此与最亲爱的爸爸、妈妈和妹妹共勉。