Sweet connections, bZIP transcription factors and the regulation of metabolism in Arabidopsis

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bZIP transcriptie factoren en de regulatie van metabolisme in Arabidopsis

(met een samenvatting in het Nederlands)

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List of abbreviations used:

ABA	Abscisic acid
ABI	*ABA INSENSITIVE*
ADP	Adenosine diphosphate
AGT	*ALANINE:GLYOXYLATE AMINOTRANSFERASE*
AMP	Adenosine monophosphate
AMPK	*ADENOSINE MONOPHOSPHATE-ACTIVATED PROTEIN KINASE*
ANOVA	Analysis of variance
APR1	*APS REDUCTASE 1*
ASN1	*ASPARAGINE SYNTHETASE1*
ASP3	*ASPARTATE AMINOTRANSFERASE 3*
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BLS1	*BRASSINOSTEROID, LIGHT AND SUGAR 1*
bZIP	Basic region/leucine zipper
C	Carbon
CATMA	Complete Arabidopsis Transcriptome MicroArray
CCA1	*CIRCADIAN CLOCK ASSOCIATED 1*
CHOP	C/EBP homologous protein
chx	cycloheximide
CO_2	Carbon dioxide
CYCD	D-type cyclin
dex	Dexamethasone
DIN1	DARK INDUCIBLE1
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EIN3	*ETHYLENE INSENSITIVE 3*
FOS	Fructo-oligosaccharides
GA	Gibberellic acid
GC-MS	Gas-phase chromatography coupled mass spectrometry
GCN	General Control Nondepressible
GFP	Green Fluorescent Protein
GIN2	*GLUCOSE INSENSITIVE 2*
GLN2	*GLUTAMINE SYNTHETASE 2*
GO	Gene ontology
GPA1	*G PROTEIN ALPHA SUBUNIT 1*
GS2	*GLUTAMINE SYNTHETASE 2*

GST	Gene-specific tag
HBD	Hormone binding domain
HSI2	*HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENE 2*
HSL1	*HAESA-LIKE 1*
HSP	*HEAT SHOCK PROTEIN*
HSR8	*HIGH SUGAR RESPONSE 8*
HT	Hexose transporter
HXK1	*HEXOKINASE 1*
LIMMA	Linear Models for Microarray Data
Log2FC	Base 2 logarithmic fold change
LUC	Luciferase
mRNA	Messenger RNA
MS	Murashige and Skoog
N	Nitrogen
NASC	Nottingham Arabidopsis Stock Centre
PAL4	*PHENYLALANINE AMMONIA-LYASE 4*
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PPDK	*PYRUVATE ORTHOPHOSPHATE DIKINASE*
PRL1	*PLEIOTROPIC REGULATORY LOCUS 1*
ProDH	*PROLINE DEHYDROGENASE*
QPCR	Real time quantitative PCR
REGIA	REgulatory Gene Initiative in Arabidopsis
RGS1	REGULATOR OF G-PROTEIN SIGNALING 1
RILs	Recombinant inbred lines
RNA	Ribonucleic acid
SNF1	Sucrose non-Fermenting 1
SnRK1	SNF1 Related kinase 1
SULTR2	*SULPHATE TRANSPORTER 2*
SUT2	*SUCROSE TRANSPORTER 2*
SWI/SNF	Switch/sucrose nonfermentable
T6P	Trehalose 6-phosphate
TCA	Tricarboxylic acid
TPP	Trehalose phosphate phosphatase
TPS	Trehalose phosphate synthase
TRE1	TREHALASE 1

UDP	Uracil diphosphate
uORF	Upstream open reading frame
VSN	Variance Stabilization and Normalization

Note that in the main text:

Arabidopsis thaliana gene names are printed in capitalized italics (*e.g.* *ANS1*)

Arabidopsis thaliana mutant names are printed in italics (*e.g. gin2*)

Arabidopsis thaliana protein names are printed in capitals (*e.g.* ASN1)

“Doubt is the father of invention.”
-Galileo Galilei

Chapter 1: General introduction, Sweet connections: Metabolism and stress

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Sugar signaling

Carbohydrates or saccharides (from the Greek word "sákcharon", meaning sugar) are the most abundant group of organic molecules on our planet. Not surprisingly, sugars have many important functions in all living organisms. They form the basis for structural components (*e.g.* cellulose in plants, chitin and cartilage in animals) and are responsible for the storage and transport of energy (*e.g.* sucrose, starch, glycogen). Carbohydrates are usually classified into three distinct groups. Monosaccharides, such as glucose or fructose, are three to seven carbon atoms long, linear or circular molecules, which contain a ketone or aldehyde functional group and hydroxyl groups on most or all of the non-carbonyl carbon atoms. Disaccharides consist of two monosaccharides (*e.g.* sucrose, which consists of a glucose and a fructose moiety). When more than two monosaccharides are linked together, sugars are referred to as oligosaccharides (up to 9) or polysaccharides (10 or more). Oligosaccharides can be found as parts of glycoproteins or glycolipids. For instance, blood types A and B in humans are discerned by two different oligosaccharide glycolipids embedded in the cell membranes of red blood cells, AB-type blood has both, while type O has neither. In some plants, oligomers of fructose (Fructooligosaccharides or FOS) are present, that are commercially exploited as non-caloric sweetener and soluble fiber (Ritsema and Smeekens, 2003). Well-known examples of polysaccharides in plants are starch and cellulose, which are both polymers of glucose. Carbohydrates are synthesized from carbon dioxide by photosynthesis in photoautotrophic organisms such as plants. Energy harvested from light is used in a series of chemical reactions to convert carbon dioxide into sugars. A side-product of this process is oxygen, which is released into the atmosphere. Most life forms on earth, including humans, therefore, depend on plants for their survival. For normal growth and development of plants it is of vital importance to achieve a balance between the fixation of carbon into carbohydrates and its utilization and storage. In most plants sugars are mainly transported as sucrose (Figure 1.1). Sucrose is synthesized in mesophyll cells in leaves and subsequently loaded into the phloem by sucrose-H^+ symporters. Phloem loading is driven by a proton gradient that is actively maintained by H^+-ATPases. Subsequent import into sink tissues can either be symplastic, driven by the sucrose gradient established by sucrose utilization in sink tissues, or apoplastic by an active transport step. In sink tissues sucrose and its hydrolytic products are used as an energy source and for growth or storage.

In plants, sugars have long been thought of as simple metabolic substrates that as such determine growth-rate. In this view more available sugar would lead to more growth. Interestingly, in a natural variation study on plant growth it was observed that the opposite

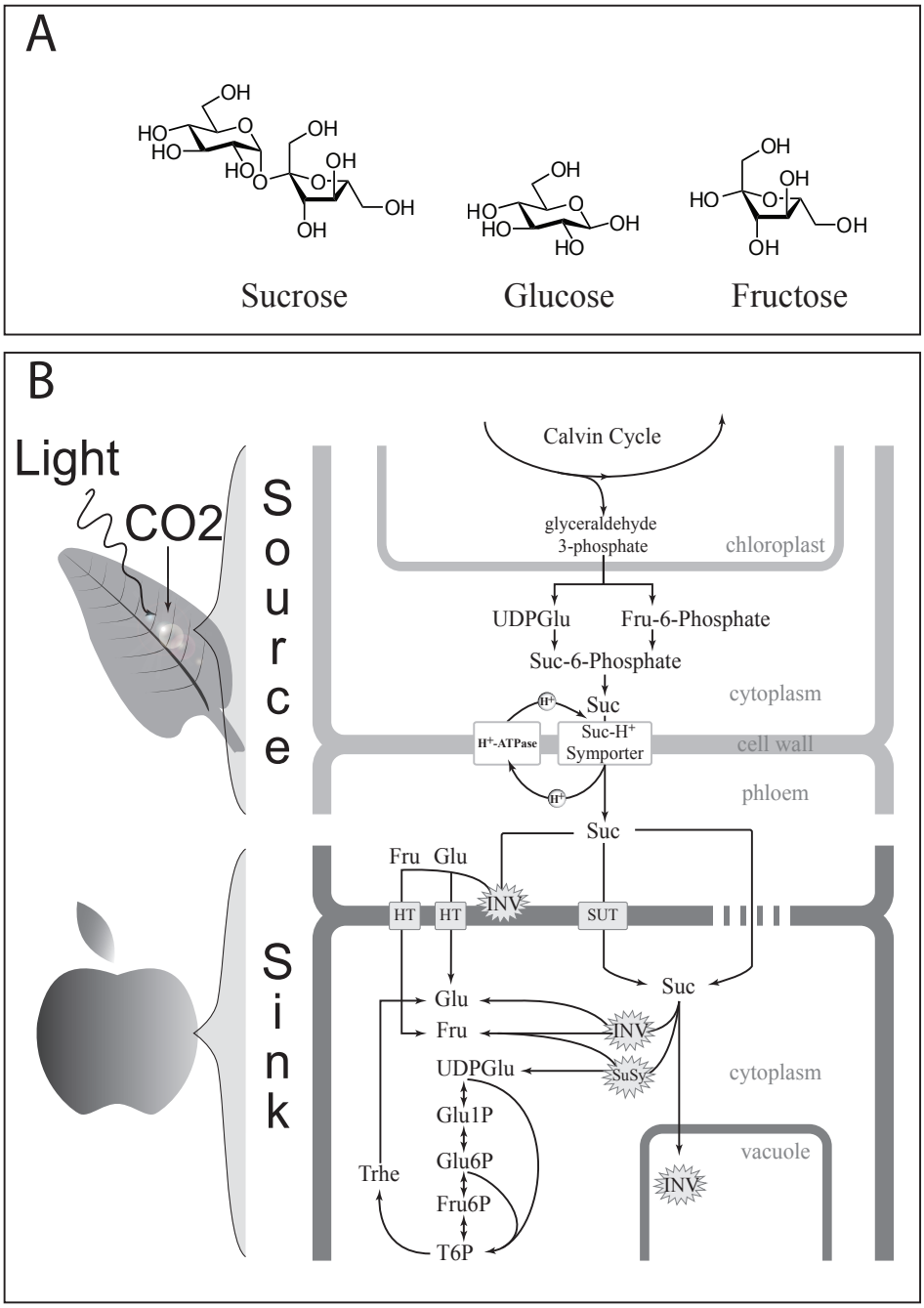

Figure 1.1 Sugars and their deployment in plants.

A) The chemical structures of sucrose (the main transport sugar in plants) and its hydrolytic products, glucose and fructose.

B) Sugars are actively transported from source to sink tissues in plants. After formation in photosynthesis, sucrose is actively loaded into the phloem. In sink tissues where a net consumption of sugars occurs, sucrose is imported directly or following cleavage into glucose and fructose by extracellular invertases. Storage and utilization of sucrose in sink tissue is preceded by sucrose breakdown through either invertases or sucrose synthases.

occurs (Meyer *et al.*, 2007). Slow growing plants seem to accumulate sugars, while fast-growing plants invest their carbon in growth and subsequently have low sugar levels. Hence, the balance between carbon fixation, carbon storage and growth seems to be adjustable, depending on genetic and environmental factors. Signaling pathways with various inputs such as nutrients, redox status, light, the circadian clock and the developmental status must be linked to growth control. Importantly, sugars are a major input into this complex growth-signaling network. The notion that highly abundant metabolic intermediates such as sugars can also possess signaling capabilities has long been questioned but is now generally accepted. Many mutants in sugar signaling have been identified in Arabidopsis (Table 1.1). Generally, these mutants are characterized by altered gene expression patterns that result in either insensitivity or oversensitivity to sugars. Well-known examples of sugar-regulated genes are genes involved in photosynthesis, and genes involved in sugar metabolism and transport, such as invertases, sucrose synthases and sucrose-H^+ symporters (Vaughn *et al.*, 2002; Koch, 2004). Sugars are potent signaling molecules and it has been demonstrated, that sugars can alter the transcript levels of thousands of genes (Price *et al.*, 2004; Thimm *et al.*, 2004; Thum *et al.*, 2004; Bläsing *et al.*, 2005; Li *et al.*, 2006; Osuna *et al.*, 2007). Molecular details of the sugar-sensing mechanisms involved, however, remain largely unknown.

So far, the best-documented sugar sensor in plants is the Arabidopsis HXK1. HXK1 phosphorylates glucose and fructose on the carbon-6 position into hexose-6-phosphate, which is the first step in glycolysis. Aside from its metabolic function, HXK1 is a glucose sensor that initiates glucose signaling. Sugar-signaling pathways that depend on HXK1 do not necessarily require its catalytic activity, as demonstrated by work on the *HXK1* mutant *gin2* (Moore *et al.*, 2003). HXK1 dependant signaling in the *gin2* mutant could be restored by introducing a catalytically non-functional HKX1, indicating a signaling function for the recognition of the hexose by HXK1. Direct introduction of glucose-6-phosphate in protoplasts by electroporation failed to elicit the same signaling effects as glucose-6-phosphate produced by hexokinases (Jang and Sheen, 1994). Unlike in yeast, no evidence exists for a signaling function of the phosphorylation step of glucose by HXK1. Also,

Table 1.1. Sugar signaling mutants

Diverse genetic screens have lead to the identification of many mutants that show altered responses to sugars.

Mutant	Phenotype	Reference
cai, carbohydrate insensitive	Development is arrested on low nitrogen & 100 mM sucrose	(Boxall, Martin et al. 1996)
gin, glucose insensitive	Seedling establishment on 330 mM glucose	(Zhou, Jang et al. 1998)
glo, glucose oversensitive	Seedling development arrest on 220 mM glucose	(Rolland, Moore et al. 2002)
gss, glucose super sensitive	Developmental arrest on 56 mM glucose	(Pego, Kortstee et al. 2000)
hba, high-level beta amylase	High amylase activity on 175 mM sucrose	(Mita, Hirano et al. 1997)
hsi2, high sucrose induction	Increased sporamin expression on low sugar levels	(Tsukagoshi, Saijo et al. 2005)
hsr, high sugar response	Increased activity of APL3 on 34 mM sucrose	(Baier, Hemmann et al. 2004)
isi, impaired sucrose induction	Impaired APL3 induction on 100 mM sucrose	(Rook, Corke et al. 2001)
lba, low-level beta amylase	Low amylase activity on 175 mM sucrose	(Mita, Murano et al. 1997)
mig, mannose insensitive	*germination* Seed germination on 7.5 mM mannose	(Pego, Kortstee et al. 2000)
prl1, pleiotropic regulatory locus1	Reduced seedling growth on 175 mM sucrose	(Nemeth, Salchert et al. 1998)
ram, reduced sucrose response	Reduced amylase activity in pgm 1 background	(Laby, Kim et al. 2001)
rsr, reduced sucrose response	Reduced expression of patatin on 90mM sucrose	(Martin, Hellmann et al. 1997)
sig, sucrose insensitive growth	Seedling development on 350 mM sucrose	(Pego, Kortstee et al. 2000)
sis, sugar insensitive	Seedling development on 300 mM sucrose	(Laby, Kincaid et al. 2000)
sss, sucrose super sensitive	No germination on 350 mM sucrose	(Pego, Kortstee et al. 2000)
sun, sucrose uncoupled	Reduced expression of PC on 88 mM sucrose	(Dijkwel, Huijser et al. 1997)
uns, unusual sugar response	Early flowering / low chlorophyll on 146 mM sucrose	(Ohto, Onai et al. 2001)

HXK-independent sugar-signaling pathways exist, as revealed by the signaling properties of the glucose analogue 6-deoxyglucose that is not recognized by HXK1 (Roitsch *et al.*, 1995). Aside from its involvement in HXK-dependent signaling, glucose has also been proposed to be involved in G-protein-coupled signaling. In agreement with the role of G-protein signaling in the modulation of cell division (Ullah *et al.*, 2001; Chen *et al.*, 2003; Ullah *et al.*, 2003; Jones and Assmann, 2004), a role for the Arabidopsis G-protein GPA1 has been suggested in the glucose mediated root cell division/expansion (Huang *et al.*, 2006). Furthermore, a regulator of G signaling protein (RGS1) has been suggested to be part of a glucose-G-protein signaling complex at the plasma membrane. It was demonstrated that RGS1, but not its intrinsic GTPase activity, was needed for glucose mediated gene activation (Grigston *et al.*, 2008). In *Atrgs1*-null seedlings, however, only 10 out of the ca. 2000 genes known to be responsive to glucose showed an altered response compared to wild-type seedlings. Considering the pleiotropic effects of mutants in G-protein coupled receptors in other organisms, this places some uncertainty on the importance of RGS1 as a receptor.

Recently, the signaling capacity of trehalose 6-phosphate (T6P) was established (Zhang *et al.*, 2009). T6P is an intermediate in trehalose biosynthesis. Trehalose occurs only in trace amounts in most plants, yet more putative metabolic enzymes for trehalose are found in the Arabidopsis genome than for any other carbohydrate (Avonce *et al.*, 2006). Mutants in trehalose phosphate synthase (TPS) and trehalose phosphate phosphatase (TPP) enzymes show phenotypes that indicate signaling functions of these enzymes or trehalose-related metabolites. For example, TPS1 is required during embryo development (Eastmond *et al.*, 2002), vegetative growth and the transition to flowering (van Dijken *et*

al., 2004). TPP is of importance during inflorescence architecture development in maize (Satoh-Nagasawa *et al.*, 2006). In various starvation and sugar feeding treatments it was observed that T6P levels correlated to sucrose levels much better than to those of glucose or fructose (Lunn *et al.*, 2006; Paul, 2008). T6P is therefore proposed to be an indicator of the sucrose status of cells, and to direct processes such as starch synthesis, accordingly (Kolbe *et al.*, 2005). More recently, T6P was shown to inhibit the activity of SnRK1 (Zhang *et al.*, 2009), which is a central regulator of energy metabolism in plants.
Another well-known example of a phosphorylated sugar with signaling capacities is fructose 2,6-bisphosphate (Stitt, 1990). Involved in a feedback mechanism that regulates partitioning of fixed carbon between starch and sucrose, Fructose 2,6-bisphosphate is regulated through modulation of the activities of enzymes involved in its synthesis and degradation.

Sucrose utilization in sink tissues requires cleavage by either invertases or sucrose synthases. Invertases cleave sucrose into glucose and fructose, while sucrose synthases cleave sucrose into fructose and UDPglucose. Sucrose synthase is only found in the cytoplasm, but invertases can be located in the cytoplasm, vacuole or cell wall. In the cell wall invertases are associated with hexose transporters (Ehness and Roitsch, 1997; Fotopoulos *et al.*, 2003). Sucrose hydrolysis outside the cell and subsequent import of hexoses maintains the sucrose gradient in the phloem, needed for carbon transport to sink tissues. In tissues in which an apoplastic phloem-unloading step is involved sucrose import is usually preceded by hydrolysis (Patrick and Offler, 2001). However, in carrot roots and potato tubers a role for invertases was suggested even though continuous plasmodesmatal connections are present there, and thus no apoplastic step seems involved (Sturm and Tang, 1999) (Cheng *et al.*, 1996). Sucrose synthase, localized in sieve-tube elements and companions cells (Wachter *et al.*, 2003), plays a role in storage and maturation stages of organ development and symbiotic nitrogen fixation (Gordon *et al.*, 1999). Another key function for sucrose synthase lies in generating UDPglucose for cell wall synthesis, as demonstrated in maize mutants (Cheng *et al.*, 1996; Winter and Huber, 2000) and transgenic carrot (Sturm and Tang, 1999) and cotton (Ruan *et al.*, 2003). Sucrose hydrolysis by sucrose synthases also generates UDPglucose needed for callose formation (Subbaiah and Sachs, 2001; Salnikov *et al.*, 2003) and several other cell wall polysaccharides (Doblin *et al.*, 2002; Albrecht and Mustroph, 2003). At the regulatory level, the production of UDPglucose instead of glucose offers the possibility to bypass glucose signaling (Wobus and Weber, 1999). Sucrose synthases operate more efficiently than invertases under low oxygen conditions (Zeng *et al.*, 1999). Under such

hypoxic conditions the production of UDPglucose from sucrose leads to the conservation of adenylates, as glucose produced by invertases require an additional ATP for entry into glycolysis. Thus, both sucrose synthases and invertases play important regulatory roles in sugar signaling, as they can affect the levels of the individual signaling components (*i.e.* sucrose, fructose and (UDP-)glucose). As of yet, however, there is no evidence supporting a sugar sensory function for either group of enzymes.

Many of the signaling effects of sucrose can be attributed to its hydrolytic products (UDP-)glucose and fructose, but sucrose-specific transcriptional and translational regulation has been observed as well. This sucrose specificity is supported by the signaling capacities of non-metabolizable sucrose analogs such as palatinose and turanose in the regulation of metabolism and gene expression (Loreti *et al.*, 2000; Fernie *et al.*, 2001; Atanassova *et al.*, 2003; Tiessen *et al.*, 2003). One proposed, yet debated, sucrose sensor is SUT2/SUC3, one of the nine sucrose transporters in the Arabidopsis genome (Barker *et al.*, 2000; Eckardt, 2003). The main argumentation for SUT2/SUC3 being a sensor for sucrose, rather than a transporter, is based on its low sucrose transporting capacity when expressed in yeast, its structural similarities to yeast sugar sensors and its low translatability. Arabidopsis *sut2/suc3* mutant plants, however, show no phenotypes under normal growth conditions (Barth *et al.*, 2003). *SUT2/SUC3* has a function in the metabolic control of pollen tube development in tomato (Hackel *et al.*, 2006), but its sucrose-sensing function remains to be established.

Taken together, little is known on exactly how different sugars are sensed, but the importance of the signaling capacities of sugars is broadly accepted.

Signal integration

Sugar signaling is complicated by its close interaction with other signaling systems such as the circadian clock, light, nutrients, hormones and the developmental status of plants (Rolland *et al.*, 2006; Smith and Stitt, 2007). One example of the complexity of signal integration in sugar signaling is provided by the regulation of asparagine and glutamine synthesizing enzymes. The genes encoding these enzymes are regulated by sugars as well as by light, the circadian clock and nitrogen. Organic nitrogen assimilates, such as glutamate and glutamine, are metabolically active compounds that may also serve as regulators of gene expression (Oliveira and Coruzzi, 1999; Rawat *et al.*, 1999). Asparagine is the main transport form of nitrogen (Pate, 1980), probably due to its relative inertness and its high nitrogen to carbon ratio. During the night, when sucrose levels are low,

asparagine levels are high. Light is able to override the sugar-repression of *ASN1* and *GLN2* in etiolated seedlings. By contrast, sugar represses the induction of *GLN2* and *ASN2* in light-grown plants (Thum *et al.*, 2003). The circadian clock master control gene CCA1 reduces asparagine levels in favor of glutamine. CCA1 directly down-regulates the transcription of a bZIP transcription factor (bZIP1), proposed to induce ASN1 expression. In addition, CCA1 directly induces the expression of a glutamine synthetase (GLN1.3) (Gutierrez *et al.*, 2008). Glutamine and glutamate in turn feed back to the circadian clock, as they can shift the phase of CCA1 expression (Gutierrez *et al.*, 2008).

Sugar and hormone connections are also well established. Sugar-metabolic enzymes such as invertases and sucrose synthases are regulated, both by sugars and by hormones (Sonnewald *et al.*, 1995; Harada *et al.*, 2005; Hartig and Beck, 2005; Proels and Roitsch, 2009), and hormone biosynthesis is under the control of sugars (Arenas-Huertero *et al.*, 2000; Gazzarrini and McCourt, 2001; Rolland *et al.*, 2006). Glucose delays seed germination in Arabidopsis, both through ethylene and abscisic acid (ABA) signaling (Price *et al.*, 2003; Dekkers *et al.*, 2004). The glucose-induced delay in germination is reduced in mutants deficient in ABA (Dekkers *et al.*, 2004), whereas glucose can suppress the germination arrest caused by ABA. Ethylene signaling is involved because glucose, in a HXK dependent manner, inhibits germination by promoting the degradation of the EIN3 protein (Yanagisawa *et al.*, 2003). Interestingly, the glucose effect on ethylene signaling partly involves ABA (Leon and Sheen, 2003), but he mechanism behind this, is not yet understood. Furthermore, ethylene reduces hypocotyl elongation. Sugar can inhibit hypocotyl elongation through increased levels of ethylene by the induction of ethylene biosynthetic genes (Jang *et al.*, 1997).

Both sugars and gibberellic acid (GA) regulate the expression of alpha-amylase in the aleurone cells of barley (Perata *et al.*, 1997). As one of the central enzymes in the process of starch mobilization, alpha-amylase is important for germination and the positive effect of GA on germination has been known for decades.

The HLS1 gene connects auxin and sugar signaling (Ohto *et al.*, 2006). Mutations in this gene cause altered auxin distributions, resulting in hypersensitivity to sucrose. Cytokinins have also been linked to sugar signaling (Moore *et al.*, 2003; Rolland and Sheen, 2005). Elevated sucrose levels caused by cytokinins can induce the transcript levels of two sulfur–responsive genes (*APR1* and *SULTR2-2*) (Ohkama *et al.*, 2002). Together, sugars and the phytohormones auxin and cytokinin are also important regulators of cell division (Hartig and Beck, 2006). In shoot tissue of tobacco it was found that auxin and monosaccharides up-regulate the expression of the D cyclin genes *CYCD2;1* and *CYCD3;1*,

but that cytokinins act negatively on their expression. In root tissue from Arabidopsis, however, it was found that sucrose and cytokinins act synergistically on the induction of *CYCD2;1* expression. Interestingly, in accordance with this differential regulation of *CYCD2;1* in shoot and root tissue, cytokinins were found to affect extracellular invertases in an opposite manner in these tissues, providing comparable feedback regulation in root and shoot tissue. Cytokinins cause a decrease in extracellular invertase activity and the associated uptake of monosaccharides by tobacco shoot tissue (Hartig and Beck, 2005), whereas cytokinins up-regulate extracellular invertase in *Chenopodium rubrum* root tissue (Ehness and Roitsch, 1997). This differential regulation of cyclins and invertases illustrates the complexity of the crosstalk and integration of sugar- and hormone signaling pathways.

The involvement of brassinosteroids (BR) in sugar signaling has also been observed. Null mutants of the *BLS1* gene show a short hypocotyl phenotype in dark, a short root phenotype in light and sugar hypersensitivity. These phenotypes could be rescued with BR application, indicating a possible role for BR in the integration of light, hormone and sugar signaling (Laxmi *et al.*, 2004). Taken together, the connections between sugar and hormone signaling are many, as shown by both genetic and molecular methods. The integration of these signaling pathways in plants awaits further study.

SNF1 related kinases in sugar mediated stress signaling

The SNF1/AMPK/SnRK1 family of protein kinases is required for energy homeostasis in species ranging form fungi to plants and mammals. SNF1/AMPK/SnRK1 kinases are highly conserved, as demonstrated by experiments showing that Arabidopsis SnRK1 can be activated by the mammalian upstream kinase responsible for AMPK activation (Mackintosh *et al.*, 1992) and that upstream regulators of SnRK1 also function as upstream regulators of Snf1 in yeast (Hey *et al.*, 2007).

In mammals, increased cellular AMP/ATP ratios activate AMPK to preserve energy levels by inhibiting anabolic (energy consuming) pathways and stimulating, catabolic (energy producing) pathways. Therefore, AMPK is considered a key enzyme in energy metabolism. Its activation has various effects in multiple tissues including increased fatty acid oxidation, glucose uptake and glycolysis, stimulation of mitochondrial biogenesis as well as inhibition of fatty acid and glycogen synthesis and gluconeogenesis (Kola, 2008). Recently, it has been shown that AMPK activity is essential for cell survival during prolonged hypoxia; a condition with dramatic effects on AMP/ATP ratios (Borger

et al., 2008). Activation of AMPK by AMP is antagonized by high (mM) concentrations of ATP. A high AMP/ATP ratio is symptomatic of low cellular energy levels. For this reason, AMPK has been likened to a cellular fuel gauge (Hardie and Carling, 1997; Hardie, 2007). Ultimately, activation of the AMPK pathway results in the activation of catabolic pathways, limitation of ATP consumption and inhibition of cell growth and division. In animals, AMPK was shown to be a regulator of appetite, showing it is not only involved in metabolism at the cellular level, but also at the organism level (Andersson *et al.*, 2004). The yeast AMPK ortholog SNF1 is similarly involved in nutrient responsive cellular processes. Best known is the adaptation to glucose limitation. In yeast, glucose limitation leads to substantially increased AMP/ATP ratios (Wilson *et al.*, 1996), but activation of the SNF1 complex by AMP has not been shown so far.

A connection between SnRKs and sugar signaling in plants was found in the *PRL1* gene. Mutations in *PRL1* result in hypersensitivity to glucose and sucrose (Nemeth *et al.*, 1998). Interestingly, PRL1 negatively regulates the SNF1 related kinases KIN10 and KIN11 (Bhalerao *et al.*, 1999) by impairing their interaction with a conserved SCF ubiquitin ligase (SKP1/ASK1) (Farras *et al.*, 2001). This suggests a role for SnRKs in phosphorylation of SCF substrates. More recently, PRL1 was shown to be required in a sugar-signaling pathway triggered by cell wall changes caused by mutations in *HSR8/MUR4* that lead to altered gene expression and cell division/expansion (Li *et al.*, 2007).

In plants, different stresses activate conserved SnRK1s, the plant orthologs of the SNF1/AMP kinases (Baena-Gonzalez *et al.*, 2007). More specifically, KIN10 and KIN11 are able to activate a broad range of genes promoting catabolism and suppressing anabolism. In yeast, SNF1 is activated in response to low cellular glucose levels and is required for the de-repression of genes that are repressed by glucose (Celenza and Carlson, 1986; Ronne, 1995; Gancedo, 1998). Halford *et al.* (Halford and Dickinson, 2001) previously suggested that SnRK1 is activated by high sucrose. However, more recently it was shown that, as in yeast, high sucrose or glucose levels repress SnRK1 signaling (Baena-Gonzalez *et al.*, 2007). The SnRK1 kinase KIN10 was found to induce the expression of *ASPARAGINE SYNTHETASE1* (*ASN1*). In agreement with the sugar-repression of SnRK1 activity, *ASN1* is known to be sugar-repressed. Furthermore, *ASN1* is a known target gene of an S1-class bZIP transcription factor (bZIP11) (Hanson *et al.*, 2008, this thesis). This class of transcription factors is repressed by sucrose at the translational level (Wiese *et al.*, 2004). When simultaneously induced in protoplasts, KIN10 and S1-class bZIPs were shown to induce ASN1 synergistically, indicating signaling of KIN10 through bZIPs.

bZIP transcription factors

bZIPs form a superfamily of transcription factors found in all eukaryotes that are named after their conserved bZIP domain. The bZIP domain consists of a basic region responsible for DNA binding, and a leucine zipper region responsible for dimerization of bZIPs. Dimerization through the amphipatic coiled-coil leucine zipper domain is required for DNA binding (Figure 1.2). Plant bZIP transcription factors are not as well studied as yeast or human bZIPs, even though the model plant Arabidopsis has about four times more *bZIP* genes than yeast and roughly one and a half times more than man (Amoutzias *et al.*, 2008). The Arabidopsis genome holds 75 *bZIP* genes that can be divided into 10 classes based on phylogeny and shared domains (Jakoby *et al.*, 2002)(Figure 1.2). In spite of their numbers, few Arabidopsis bZIPs have been functionally described. Members of the C-class are characterized by an extended leucine zipper with up to nine heptad repeats. C-class bZIPs contain conserved domains that form potential target sites for protein modifications, such as phosphorylation, which can regulate nuclear translocation and DNA-binding properties (Ciceri *et al.*, 1997). Two of the C-class bZIPs (*AtbZIP10* and *AtbZIP25*) are the closest homologues of the maize *Opaque2* gene, which is known to regulate storage protein expression (Vicente-Carbajosa *et al.*; Vicente-Carbajosa *et al.*, 1998; Onate *et al.*, 1999; Onodera *et al.*, 2001). Furthermore, bZIP10 was shown to be a positive regulator of plant defense and hypersensitive cell death (Kaminaka *et al.*, 2006). This function of bZIP10 is antagonized by LSD1 (LESIONS SIMULATING DISEASE RESISTANCE1), which retains bZIP10 outside of the nucleus. The interaction of LSD1 with the C-class of bZIP transcription factors is limited to bZIP10, probably, since the interaction is facilitated by a region in the C-terminal part of the bZIP protein that is the least conserved part of the C-class of bZIP transcriptions factors.

The S-class is the largest group of bZIPs and consists of low molecular weight proteins that lack other known domains but, like the C-class bZIPs, harbor an unusually long zipper domain of 8-9 leucines. The S-class contains a sub-class of 5 bZIPs (bZIP1, -2, -11, -44 and -53) called the S1-class. These bZIPs can be repressed at the translational level by sucrose, through a highly conserved upstream open reading frame (uORF) in the 5' region of their messengers (Rook *et al.*, 1998c; Wiese *et al.*, 2004; Weltmeier *et al.*, 2009) (Figure 1.3).

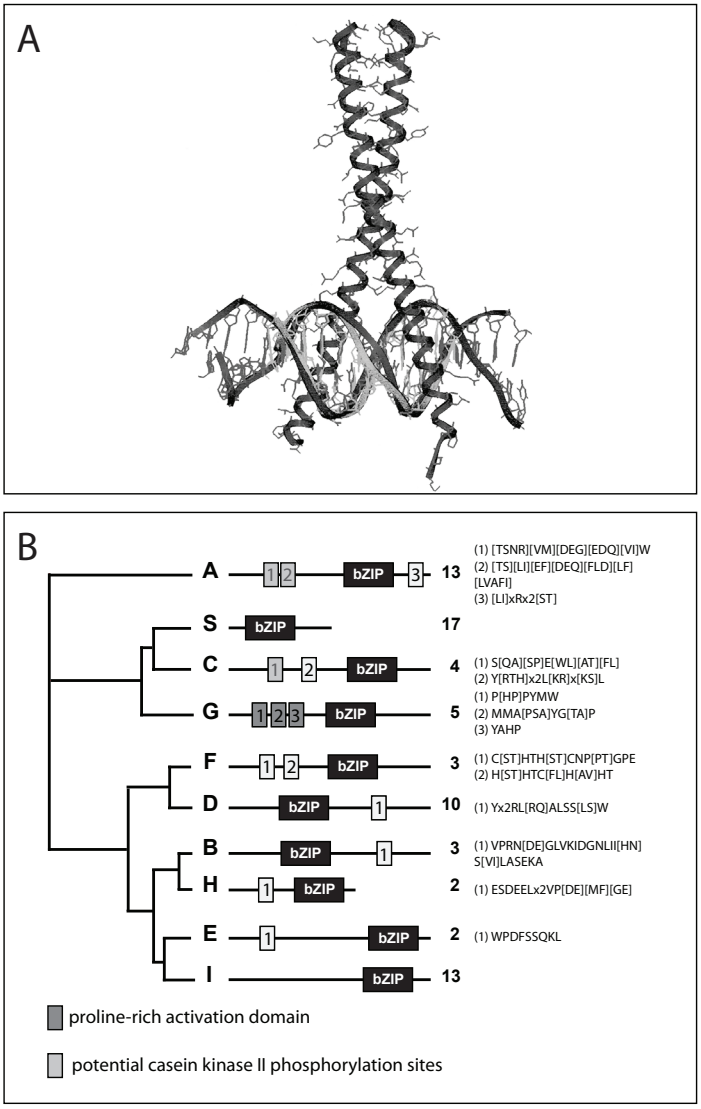

Figure 1.2. bZIP transcription factors

A) bZIP transcription factors bind DNA as dimers. The structure of the GCN4-bZIP protein bound to DNA has been established by X-ray crystallography (Ellenberger *et al.*, 1992; Keller *et al.*, 1995). More recently, NMR studies revealed a 12 bp long GCN4-binding, self-complementary duplex DNA d(CATGACGTCATG)$_2$ (light grey) to be curved in the bound and free state as well (Khandelwal *et al.*, 2001). This was shown to significantly aid the recognition by the bZIP transcription factor. Figure A shows the superposition of the solution structure of GCN4 cognate DNA (light grey) over the DNA in the ATF/CREB–GCN4 complex (Khandelwal *et al.*, 2001).

B) Phylogenetic relation of the bZIP domain of the bZIP protein family in Arabidopsis. bZIP transcription factors in the Arabidopsis genome sorted into classes according to common domains (Jakoby *et al.*,

2002). Shown are 10 major groups of bZIPs (A-I and S). The overall protein structure is also shared within the groups as indicated by the schematic drawings. The sequences of the group-specific domains are shown on the right of the number of proteins within each group.

Regulation of gene expression by transcription factor dimerization

The existence of families of transcription factors with specific dimerizing and DNA binding properties allows dynamic and complex gene regulation (Klemm *et al.*, 1998). The dimerization of transcription factors provides extensive potential for differential regulation of targets genes, *e.g.* via specific DNA binding capacities of dimers. The Jun and ATF2 bZIP transcription factors are examples of this (Hai and Curran, 1991). Jun-ATF2 heterodimers show distinct DNA-recognition abilities from the Jun or ATF2 homodimers. Consequently, the heterodimers and the homodimers have distinct effects on gene expression. Further potential for differential gene regulation through transcription factor dimerization comes from the regulation of the formation of functional dimers. The availability of each monomer, their affinity for each other, as well as post-translational modifications, such as phosphorylation, will determine which functional dimers are formed, and thus which target genes will be regulated. For example, the Myc-Max and Mad-Max heterodimers both affect a myriad of genes in an opposite manner (Palena *et al.*, 1999; Grandori *et al.*, 2000). The Myc-Max helix-loop-helix leucine-zipper dimer will recruit either the SWI/SNF nucleosome remodeling complex or histone acetyl transferases at the promoter of target genes, leading to gene activation. The Mad-Max dimer, however, will lead to gene silencing, by recruitment of histone deacetylases.

Phylogenetic analysis has revealed duplication events that created the topology of the metazoan bZIP dimer network at their origin, roughly one billion years ago (Amoutzias *et al.*, 2007). This suggests the establishment of bZIP transcription factor networks is an important factor in the evolution of all eukaryotes. In Arabidopsis the homo- and heterodimerization capacities of bZIPs have been characterized using yeast- and plant-two hybrid systems (Ehlert *et al.*, 2006a). S1-class bZIPs preferably dimerize with members of the C-class (Figure 1.4), although it also has been shown that S1-class bZIPs, in particular bZIP11, are able to form functional homodimers. C-class bZIPs in contrast, do not tend to form homodimers. Signaling through C/S1-class dimers, putative phosphorylation sites in C-class bZIPs offer possibilities for regulation of activity. S1-class bZIPs and SnRK1s have been shown to act synergistically on the expression mutual target genes, likely trough activation of S1-bZIPs by KIN10/KIN11 (Baena-Gonzalez *et al.*, 2007). Possibly, KIN10/KIN11 can also activate C/S1-class dimers through

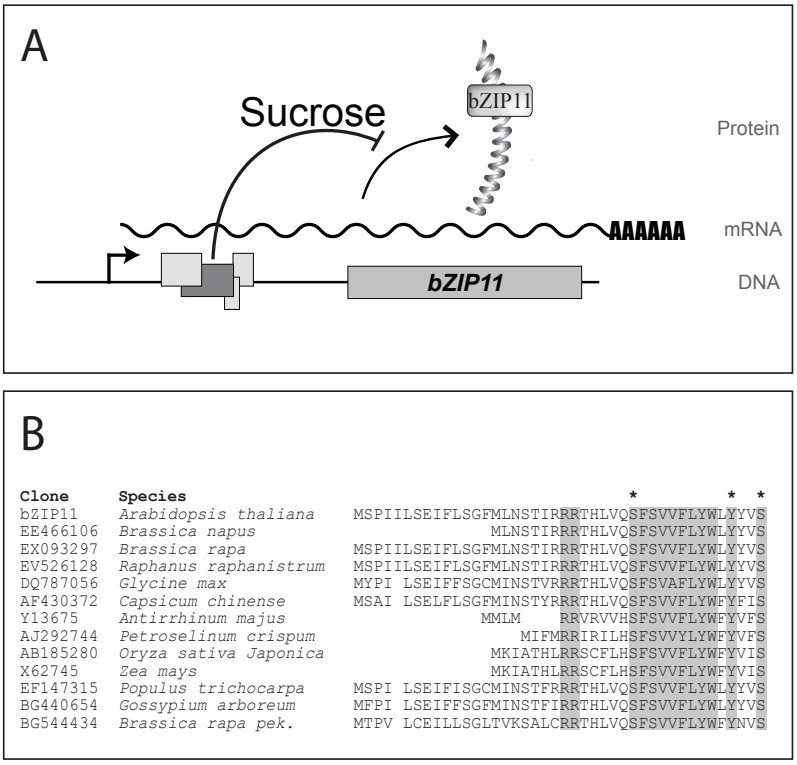

Figure 1.3. A conserved sucrose-control peptide mediates translational inhibition of S1-class bZIP transcription factors

A) Model of translational repression of S1- class bZIPs. The 5' region of S1- class bZIP transcription factor bZIP11 harbors four upstream open reading frames (uORFs, indicated by gray boxes on the DNA). The second uORF (indicated by a dark grey box) codes a highly conserved peptide that mediates repression of translation of the main ORF in the presence of sucrose, independent of mRNA levels.

B) Conservation of the sucrose-control peptide in plant bZIPs.

The 5'-leader of the mRNA of Arabidopsis S1-class bZIP transcription factors (only bZIP11 is shown) harbor an upstream open reading frame, responsible for repression of translation in response to sucrose. (Rook *et al.*, 1998; Wiese *et al.*, 2004; Weltmeier *et al.*, 2009). The sucrose-control peptide is highly conserved, and only present in plant orthologs of bZIPs. Conservation is more prominent in the C-terminal part of the peptide (grey boxes). Stars indicate amino acids essential for translational inhibition (Rahmani *et al.*, 2009).

phosphorylation of C-class bZIPs at evolutionary conserved sites that are absent in S1-class bZIPs. Ultimately however, the control of C/S1-class signaling lies with sugars, as both SnRK1 activity and S1-class bZIP translation are sugar-repressed.

Genes regulated by C- and S1-class bZIP transcription factors

The connection between carbon and nitrogen signaling is well established (Gutierrez *et al.*, 2007) and of importance for the coordination of growth and development (Weigelt *et al.*, 2008). bZIPs form such a connection between C and N metabolism as they affect the

AD:bZIP		BD:bZIP	C				S1				
			bZIP9	bZIP10	bZIP25	bZIP63	bZIP1	bZIP2	bZIP11	bZIP44	bZIP53
C		bZIP9	5	4	12	10	33	55	134	16	102
		bZIP10	28	2	8	71	271	63	345	49	179
		bZIP25	19	4	11	10	74	207	500	189	150
		bZIP63	18	7	11	11	13	50	172	15	33
S1		bZIP1	2	10	30	8	2	13	21	3	8
		bZIP2	6	9	50	11	4	16	68	3	17
		bZIP11	5	6	25	13	9	10	112	2	13
		bZIP44	5	6	25	13	9	10	12	2	13
		bZIP53	5	15	28	10	7	56	36	4	6

Figure 1.4. Dimerization properties of C- and S1-class bZIP transcription factors in Arabidopsis protoplasts

The interaction pattern of C- and S1-Class bZIPs, as obtained by plant two hybrid analysis is shown (adopted from Ehlert *et al.*, (2006)). Activation of a GAL-UAS$_4$:GUS reporter was quantified after co-expression of BD-bZIP and AD-bZIP fusion proteins. Results were normalized by co-transfection of a 35S:NAN plasmid. Mean values of GAL4:GUS

reporter gene activity (in relative GUS/NAN units) are given. Interaction-strength of bZIP fusion proteins is indicated by background shading as well.

expression of genes involved in N metabolism and are controlled by sugars, either directly (transcriptionally and translationally) or indirectly through SnRK1. Most notably, *ASN1* (At3g47340) has been found to be a target of the bZIP11 transcription factor (Hanson *et al.*, 2008 and chapter 2 of this thesis). Asparagine was the first amino acid isolated from plants over 200 years ago (Vauquelin and Robiquet, 1806), possibly due to its high abundance in plants, as it serves as one of the main carriers of fixed nitrogen (Pate, 1980). bZIP transcription factors, however, control the metabolism of more amino acids next to asparagine. Proline plays several different roles in metabolism, *e.g.* as a source of energy (Blum and Ebercon, 1976), a source of carbon and nitrogen compounds (Ahmad and Hellebust, 1988), hydroxyl radical scavenger (Smirnoff and Cumbes, 1989) as well as osmotic protectant (Nakashima *et al.*, 1998). Osmotic stress leads to accumulation of proline. The accumulated proline will be degraded by proline dehydrogenase (ProDH) after cessation of the osmotic stress (*i.e.* during re-hydration). S1-class bZIPs were reported to bind *ProDH* in a study searching for transcription factors capable of binding the ACTCAT consensus sequence in the promoter of *ProDH* and many other re-hydration responsive genes (Oono *et al.*, 2003). Moreover, it was found that *ProDH* was activated by dimers of bZIP53 in combination with any of the C-class bZIPs, but to varying extends (Weltmeier *et al.*, 2006). Thus, an important question is to what extend the activation of target genes by bZIP transcription factors is redundant or specific. In this thesis the target gene specificity of bZIP dimers will be addressed.

Conservation of ASN1 regulation by bZIP transcription factors

Interestingly, *ASN* genes in other eukaryotes are also regulated by nutrient availability through the action of bZIP transcription factors, GCN4 in yeast, ATF5, ATF4 and CHOP in mammals (Siu *et al.*, 2001; Al Sarraj *et al.*, 2005; Hinnebusch, 2005). Similar to bZIP11, these transcription factors are translationally regulated by nutrient availability through important uORFs in the 5' leader of the mRNAs (Lu *et al.*, 2004; Vattem and Wek, 2004; Hinnebusch, 2005). In mammals, synthesis of GCN4 is increased by the transcription factor GCN2, due to translation initiating from an initiation codon that is not used under normal conditions (Hinnebusch, 1994). This process requires activation of the eukaryotic

initiation factor 2 alpha (eIF2α) through phosphorylation. Phosphorylation of eIF2α by the Arabidopsis homologue of GCN2 (AtGCN2) has been confirmed (Zhang *et al.*, 2008), yet no obvious candidate for a GCN4 homologue in Arabidopsis has been identified (Halford *et al.*, 2004), likely due to the larger range of bZIP transcription factors in plants. In humans, glucose deprivation induced the expression of asparagine synthetases through activating transcription factor-4 in an AMP-activated protein kinase–independent manner (Cui *et al.*, 2007). Taken together, these findings suggests that the regulation of asparagine synthesis through bZIP transcription factors is of ancient origin and that different groups of organisms regulate the bZIP transcription factors involved in accordance with their nutritional needs. It also suggests that other functions of bZIPs presented here, need not be restricted to plants.

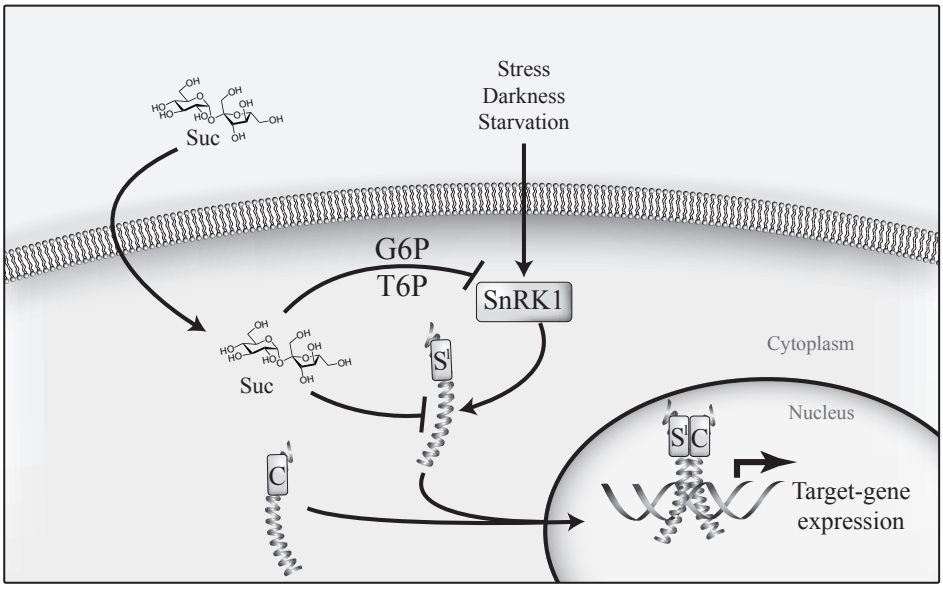

Figure 1.5. A model of sugar signaling through bZIP/SnRK1 signaling

Differential regulation of the transcription of bZIPs allows control over which monomers are present for the subsequent formation of specific dimers. S1-class bZIPs are translationally repressed by a uORF in their mRNA in the presence of sucrose. SnRK1 kinases activate S1-class bZIPs (Baena-Gonzalez et al., 2007). SnRK1 kinases are induced by various stresses, such as darkness and starvation that result in energy deficit. The phosphorylated sugars glucose-6-phosphate (G6P) and trehalose-6-phosphate (T6P) inhibit SnRK1 activity. The picture shows that the regulation of gene expression through bZIP/SnRK1 signaling is under the control of sugars at various levels.

Outline of this thesis

bZIP transcription factors are part of an intricate signaling network connecting sugar signaling to metabolism (Figure 1.5). Many inputs and components of this system are known, but little is known on the actual function of bZIP transcription factors. In this thesis the function of bZIPs within this vital signaling network is studied. The model organism *Arabidopsis thaliana* was used to identify targets genes and metabolic effects of bZIP transcription factors.

In chapter 2, target genes of bZIP11 are presented. Using transgenic plants in which the *in vivo* amount of the bZIP11 protein can be induced, in combination with global transcriptional profiling and further experimentation, bZIP11 is established as an inducer of the expression of hundreds of genes. Among the target genes of bZIP11 are *ASN1* and the *ProDH2* (At5g38710), linking bZIP, and thus sugar signaling, to amino acid metabolism. Many of the targets identified are previously known sugar regulated genes.

In chapter 3, the effect of induced bZIP11 activity on metabolite levels is investigated using the same transgenic plant lines used to identify bZIP11 target genes. The effect on metabolite levels is studied using both targeted and unbiased approaches. It is concluded that bZIP11 induction causes metabolic reprogramming, as its over-expression has profound effects on amino acid levels as well as other metabolites.

In chapter 4, the translational effects of specific bZIP dimers are characterized. The dimerization preferences of a number of bZIP transcription factors in planta are known (Ehlert *et al.*, 2006a) Therefore, specific bZIPs were co-expressed in protoplasts, which were subjected to mRNA profiling experiments. From these experiments it can be concluded that different bZIP dimers have differential effects on target gene expression.

Finally in chapter 5, the findings presented in this thesis will be briefly summarized and their implications discussed.

“I love fools’ experiments. I am always making them.”

-Charles Darwin

Chapter 2: Identification of bZIP11 target genes

Micha Hanssen, Johannes Hanson, Anika Wiese and Sjef Smeekens

Based on: “The sucrose-regulated transcription factor bZIP11 affects amino acid metabolism by regulating the expression of *ASPARAGINE SYNTHETASE1* and *PROLINE DEHYDROGENASE2*” The Plant Journal (2008) 53, 935–949

Summary

Translation of the transcription factor bZIP11 is repressed by sucrose in a process that involves a highly conserved peptide encoded by the 5' leaders of *bZIP11* and other plant *bZIP* genes. Likely, a specific signaling pathway operating at physiological sucrose concentrations exerts control via a feedback mechanism. In this chapter bZIP11 targets are identified using transiently increased nuclear bZIP11 levels and genome-wide expression analysis. bZIP11 affects the expression of hundreds of genes with proposed functions in both biochemical pathways and signal transduction. The expression levels of approximately 20% of the genes identified are affected by *bZIP11* promoter-mediated overexpression of *bZIP11*. This suggests that these genes are likely bZIP11 specific targets. The remaining genes, which only appear affected by ectopic expression of bZIP11, likely are targets of closely related bZIPs. *ASN1* and *ProDH2* are among the rapidly activated bZIP11 targets, whose induction is independent of protein translation. Transient expression experiments in Arabidopsis protoplasts show that the bZIP11-dependent activation of the *ASN1*gene is dependent on a G-box element present in the promoter. A model is proposed in which sugar signals control amino acid metabolic genes via the bZIP11 transcription factor.

Introduction

Sugars serve as a source for energy and carbon but are also potent signaling molecules that regulate physiology and development. Plants sense the levels of available sugars via several independent pathways (Rolland *et al.*, 2006). Massive effects on the transcriptome are detected in response to sugar treatments as expected due to the central role of sugars in plant metabolism (Price *et al.*, 2004; Blasing *et al.*, 2005; Gonzali *et al.*, 2006; Gutierrez *et al.*, 2007). These changes in gene expression depend on the activity of a manifold of transcription factors, and consequently several different cis-elements have been identified as present in sugar-responsive promoters (Grierson *et al.*, 1994; Yokoyama *et al.*, 1994; Thum *et al.*, 2004; Geisler *et al.*, 2006). Several transcription factors responsible for sugar-regulated transcriptional control have been identified, including MYB75/PAP1 that regulates anthocyanin biosynthesis (Teng *et al.*, 2005; Tohge *et al.*, 2005), HSI2, HSL1, HSL2, ABI4, ABI5, and ABI8 that are involved in both abscisic acid and sugar signaling during germination (Huijser *et al.*, 2000; Laby *et al.*, 2000; Brocard-Gifford *et al.*, 2004; Acevedo-Hernandez *et al.*, 2005; Tsukagoshi *et al.*, 2005; Tsukagoshi *et al.*, 2007), EIN3 that is regulated by both ethylene and glucose (Yanagisawa *et al.*, 2003) and *bZIP11* (previously described as *ATB2*) (Wiese *et al.*, 2005). The translation of *bZIP11* mRNA is specifically inhibited by sucrose through an upstream open reading frame (uORF) in the 5' leader of the mRNA (Rook *et al.*, 1998c; Wiese *et al.*, 2004). This uORF encodes a conserved peptide that is essential for sucrose-regulated translation of the main bZIP11 encoding ORF.

One of the genes most affected by sugar treatments is *ASPARAGINE SYNTHETASE1* (*ASN1*) (Lam *et al.*, 1998; Price *et al.*, 2004; Bläsing *et al.*, 2005). The ASN1 enzyme synthesizes asparagine and glutamate from aspartate and glutamine. Asparagine serves as an important nitrogen storage and transport compound and it is synthesized at night and during low-sugar conditions (Lam *et al.*, 1994). When sugar levels are high the expression of the *ASN1* gene is repressed, leading to reduced levels of asparagine (Lam *et al.*, 1994). Glutamine then serves as the major transported form of nitrogen. Thus, the ASN1 enzyme has a key function in regulating diurnal and sugar-dependent nitrogen transport in plants, confirmed by overexpression of the gene in planta (Lam *et al.*, 2003). The metabolism of the amino acid proline is likewise regulated. Proline levels vary in response to sugar levels (Hayashi *et al.*, 2000). This is achieved through

transcriptional regulation of the genes encoding delta1-pyrroline-5-carboxylate synthetase (P5CS) (Hellmann *et al.*, 2000), which synthesizes proline and proline dehydrogenase (ProDH), which degrades proline (Hayashi *et al.*, 2000). In plants, bZIP factors regulate ProDH gene expression in the form of heterodimeric complexes consisting of one C-class and one S1-class bZIP protein (Satoh *et al.*, 2004; Weltmeier *et al.*, 2006). bZIP proteins of the S1-class, like bZIP11, are all translationally regulated by sucrose, which probably mediates the sugar-dependent regulation of the *ProDH* gene. Amino acids are central in nitrogen metabolism and translocation but little is known about sugar-dependent regulation of amino acid metabolism. The regulatory interaction between the sugar- and nitrogen-dependent transcriptional responses is well documented (Gutierrez *et al.*, 2007). However, the mechanistic details are poorly understood.

In this chapter, bZIP11 is proposed to be involved in the regulation of metabolism of specific amino acids. Induced *in vivo* activity of the bZIP11 protein, combined with global transcriptional profiling and further experimentation establishes the *ASN1* and the *ProDH2* (*At5g38710*) genes as regulated by bZIP11 in Arabidopsis. This is in agreement with these genes being regulated by sugars and establishes a role of bZIP11 as a link between sugar signaling and the regulation of genes essential for the metabolism of amino acids central for transport and utilization of nitrogen in plants.

Results

bZIP11 is a potent regulator of gene expression

Constitutive expression of *bZIP11* causes severe alterations of plant growth and development and reduced seed set and viability (Figure 2.1). To study the immediate effects of bZIP11, transgenic plants were generated in which bZIP11 nuclear localization was controlled by a dexamethasone (dex)-inducible system. The intrinsic efficient nuclear exclusion of non-ligand bound glucocorticoid receptor (Picard *et al.*, 1988), enables specific control of transcription factor activity via dex-induced nuclear migration (Sakai *et al.*, 2001; Hay *et al.*, 2003). Transgenic Arabidopsis plants constitutively expressing *bZIP11* C-terminally fused to the glucocorticoid-binding domain of the rat glucocorticoid receptor (HBD-domain) under the control of the 35S promoter were generated. Twenty nine of these bZIP11:HBD transgenic lines with single integration loci of the marker gene were tested for the presence of the transgenic transcript. These lines showed levels of the transgene expression ranging from two to 100-fold higher than that of the endogenous

Figure 2.1. bZIP transcription factors affect the growth and development of Arabidopsis plants.
A soil grown plant overexpressing bZIP11 (right) compared to a wild type plant (left). The dramatic phenotype of the overexpressor hints the important functions of these transcription factors.

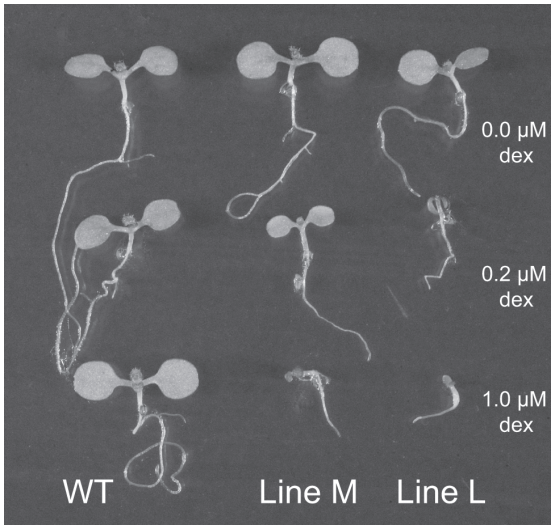

Figure 2.2 bZIP11 affects the growth and development of Arabidopsis.
Seven day old transgenic *bZIP11:HBD* (lines L and M) and wt seedlings grown on media supplemented with different concentrations of dexamethasone (dex).

bZIP11 mRNA, both in the presence and absence of dex (data not shown). These transcript levels agreed well with the severity of the growth inhibition phenotype of the transgenic seedlings continuously grown on dex-containing media (Figure 2.2 and data not shown). Two lines (M and L) were chosen for further analysis. These lines have stable, high level of *bZIP11* expression and display a growth inhibition phenotype on dex-containing media, but normal growth on media without dex (Figure 2.2). For mRNA profiling 7-day-old M and L seedlings grown on 100 mM sucrose-containing media were treated for 2-h with 10 µM dex or mock treated with solvent. This sucrose concentration was chosen as it was previously shown to efficiently repress translation of the endogenous *bZIP11* gene (Wiese *et al.*, 2004). Two independent transgenic lines were used in the experiment, so the effects of bZIP11 could be separated from those of T-DNA integration and other transformation related artifacts. Details on the profiling procedure are presented in the materials and methods section. Signals representing 561 genes changed significantly in the L transgenic line compared to the response in the wt with an absolute change in the transgene of more than two-fold. In the M transgenic line 399 genes were similarly identified. In both L and M transgenic lines 261 genes were independently identified as being differentially expressed. On average the changes in signal strength were bigger in the L transgene than in the M transgene for the genes identified as differentially expressed. This agrees well with the observation that the L line expresses the bZIP11:HBD fusion transcript to a higher level than the M line and shows a stronger growth phenotype compared to the M

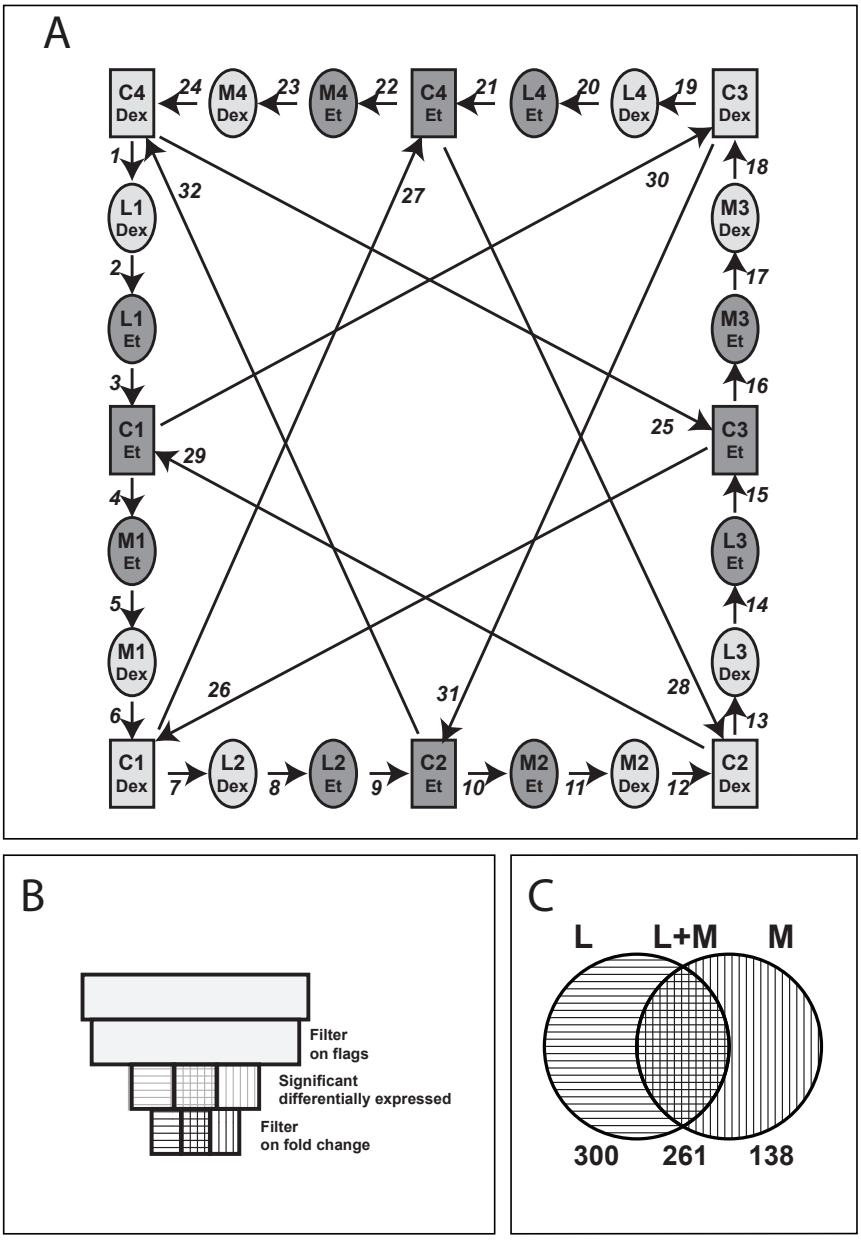

Figure 2.3. Design and result of the mRNA profiling experiment.

(A) The figure shows the experimental design of slide hybridizations. Four independent replicates were labeled with both Cy3 and Cy 5 dyes and hybridized according to a mixed loop design. Arrows represent individual two color micro array slides, indicated with numbers. Ellipses represent transgenic RNA from transgenic seedlings and squares represent wt samples. Light grey symbols represent RNA samples form dexamethasone treated plants and dark grey symbols represent mock control

treated RNA samples. Every independent RNA is labeled within the symbol with: Et, Ethanol (mock) treated; Dex, dexamethasone treated; L, transgene line L; M, transgene M; C, wt. Numbers indicate the biological replicate used (B) General methodology used to identify genes that are differentially expressed. Signal from spots not flagged bad were fitted to a linear model (for details, see materials and methods) to identify spots differentially expressed ($q<0.05$). The resulting spot values were filtered on fold change in the transgene and only spots that showed an average change in signal over twofold were considered for further analysis. The procedure was used independently for the L and M transgenic lines indicated with horizontal stripes and vertical stripes, respectively. Shared changes are indicated in medium grey both horizontal and vertical stripes. Note the size of the rectangles is not indicative of the number of genes. (C) Venn diagram of differentially expressed genes in the two independent transgenic lines, M and L. Numbers represent gene models (TAIR6). Shared changes are indicated in both horizontal and vertical stripes and changes gene expression that could only be identified in one of the lines is indicated in either horizontal or vertical stripes, respectively. Note that the size of the circles is not indicative of the number of genes.

line (Figure 2.2 and data not shown). The results are shown in supplementary table S1 and the experimental scheme is summarized in Figures 2.3 and 2.4. Of the 261 genes identified as differentially expressed, 163 genes were induced and 98 were repressed by the dex treatment. On average, changes were greater for genes that were induced by dex treatment than for those that were repressed (Table S1).

The 2-h dex treatment left the wt Arabidopsis plants unchanged as expression of only a single gene was significantly and more than two-fold changed by dexamethasone treatment in the wt (*At1g73120*, repressed). Five other genes showed minor but statistically significant changes (*At5g42590*, *At5g13170*, *At4g35770*, *At1g75380* and *At1g62290*). Twelve genes were shown to be differentially expressed between the untreated transgenes and the wt seedlings using the same criteria (data not shown). This indicates that the presence of the bZIP11:HBD transgene had a minimal effect on the transcriptome under the conditions used for the experiment. Results obtained in the array experiment were confirmed using real-time quantitative PCR. The expression levels of 65 genes were tested. The results from 61 out of these 65 (94 %) genes confirmed the results from the array experiment (Table 2.1). Moreover, the mRNA profiling results were independently confirmed by a profiling experiment using the same system but with fewer replicates and only 1-h dex treatment (data not shown) and an array experiment using two Affymetrix ATH1 slides and RNA from plants harboring inducible *bZIP11* mRNA expression constructs (data not shown).

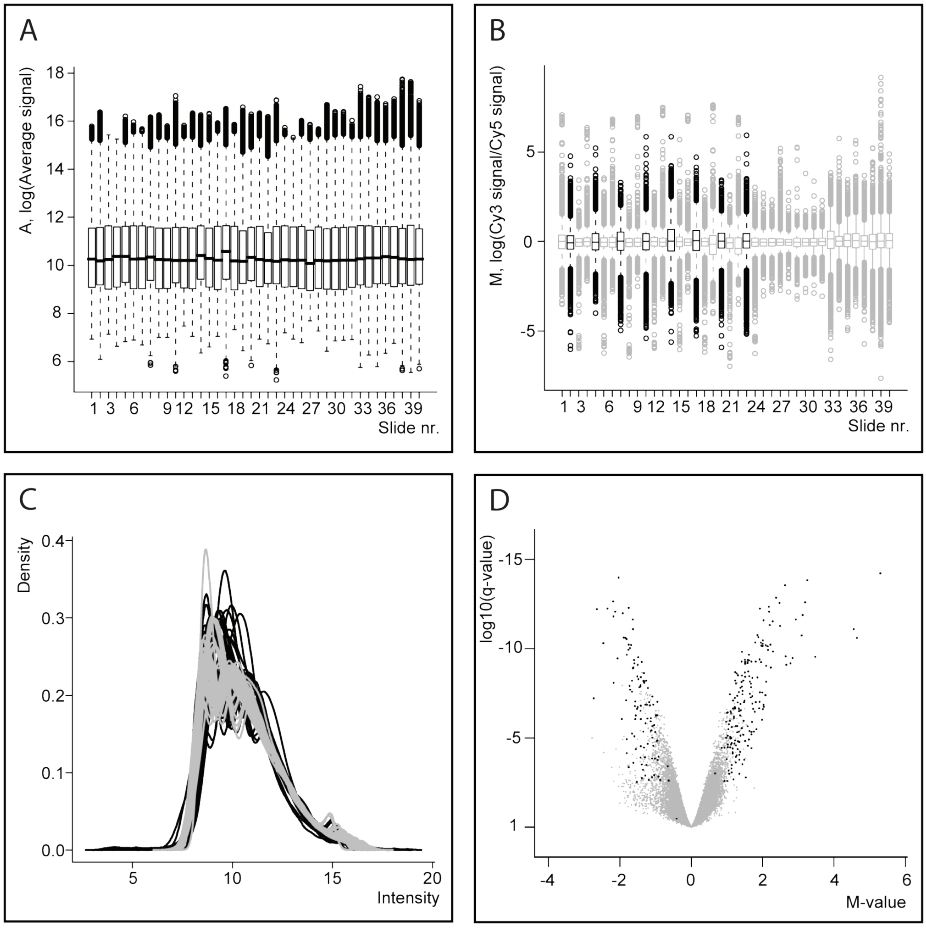

Figure 2.4. Summary of the mRNA profiling results.

(A)The figure shows normalized average signal intensities over the slides used. (B) The figure shows the normalized ratios between the two different signal intensities of the slides used. Results from slides in which dexamethasone and ethanol treated transgenic lines are compared are depicted in black. The interquartile range is depicted as a box and the 95% confidence interval is represented by dashed lines. Median levels are represented by horizontal bars and outlying data points are depicted by open circles. Numbers correspond to the hybridization numbers of the slides in figure 2.2 (C) Normalized signal distributions of the individual sides in the experiment. Black lines represent the Cy5 signal and grey lines represent the Cy3 signal. (D) The figure shows the normalized relative signal changes in the M transgene in response to dexamethasone in relation to the corresponding change in the WT plotted against the q-value of that change. The q-value for the change is plotted against the logged change of the signal. The spots with an average signal change of at least twofold and q-value less than 0.05 in the independent L transgene are depicted in black.

Table 2.1. Q-PCR confirmation of Genes induced by bZIP11 in the micro array experiment

	Induction, array[A]		Induction, Q-PCR[B]				
AGI[1]	L transgene	M transgene	L transgene	M transgene	p value L[2]	p value M[2]	Description[3]
At1g02660	3.9	4.5	8.9	11.6	2.36E-09	9.65E-10	lipase class 3 family protein
At1g09460	3.5	3.7	5.9	5.8	1.25E-08	1.72E-08	glucan endo-1,3-beta-glucosidase-related
At1g10070	5.2	3.2	12.9	11.3	6.61E-06	4.72E-04	similar to branched-chain amino acid aminotransferase 5
At1g15040	11.4	8.5	28.2	29.0	1.13E-09	1.40E-08	glutamine amidotransferase-related
At1g19450	4.4	3.0	4.9	6.1	6.61E-07	1.94E-04	integral membrane protein
At1g21680	4.3	3.4	3.2	3.3	5.21E-05	3.49E-04	expressed protein, similar to TolB protein precursor
At1g52800	4.2	4.9	33.6	38.6	1.90E-07	6.81E-08	oxidoreductase, 2OG-Fe(II) oxygenase family protein
At1g73120	18.4	8.7	2.5	4.0	9.21E-12	1.65E-09	expressed protein
At1g75000	3.5	4.0	Induction*	Induction*	6.87E-08	2.29E-08	GNS1
At2g25200	2.4	2.5	9.3	7.5	3.21E-04	3.49E-04	expressed protein
At2g30600	2.4	2.5	8.7	9.0	1.51E-05	1.50E-05	similar to BTB
At2g32150	3.2	2.9	Induction*	Induction*	2.51E-08	1.89E-07	haloacid dehalogenase-like hydrolase family protein
At2g38400	4.9	4.9	9.0	6.5	1.80E-09	2.84E-09	alanine--glyoxylate aminotransferase
At2g39130	2.9	1.9	1.0	1.0	5.16E-04	3.86E-02	amino acid transporter family protein,
At2g39570	5.0	5.6	Induction*	Induction*	3.69E-10	6.40E-10	ACT domain-containing protein
At2g47770	44.6	39.2	22.8	32.9	9.21E-12	4.68E-11	benzodiazepine receptor-related
At2g48020	2.0	2.6	2.1	1.8	7.62E-04	3.39E-05	sugar transporter, putative, similar to ERD6 protein
At2g48080	2.4	3.6	Induction*	Induction*	1.44E-06	1.40E-08	oxidoreductase, 2OG-Fe(II) oxygenase family protein
At3g13450	4.9	2.6	16.1	12.2	4.22E-06	2.70E-03	2-oxoisovalerate dehydrogenase
At3g29160	4.9	2.0	Induction*	Induction*	1.05E-05	3.83E-02	Snf1-related protein kinase (KIN11)
At3g30775	13.4	7.6	5.6	4.5	3.47E-11	2.52E-09	proline oxidase, mitochondrial (proDH)
At3g47340	2.1	1.5	7.2	13.0	4.80E-05	3.69E-02	Glutamine-dependent asparagine synthetase 1 (ASN1)
At3g48360	5.9	7.1	12.5	9.3	3.97E-07	1.39E-07	speckle-type POZ protein-related
At3g61060	5.1	3.2	12.5	9.3	6.30E-09	1.95E-06	F-box family protein
At4g18650	12.4	25.0	413.0	433.5	1.85E-07	1.74E-08	transcription factor-related
At4g24040	12.5	9.1	18.1	14.8	3.23E-11	6.40E-10	glycosyl hydrolase family protein 37
At4g27657	14.5	6.2	19.4	13.8	1.74E-13	1.07E-10	expressed protein
At4g33700	7.6	5.6	7.6	5.3	3.05E-10	4.82E-09	CBS domain-containing protein
At4g35770	25.1	12.9	9.3	8.3	9.02E-17	1.58E-14	senescence-associated protein (SEN1)
At4g39660	4.5	3.1	20.5	0.0	5.84E-08	5.88E-06	alanine--glyoxylate aminotransferase, putative
At5g04310	5.2	4.5	7.4	6.9	1.99E-10	1.17E-09	pectate lyase family protein
At5g15410	5.7	4.1	4.6	4.8	4.00E-11	2.64E-09	cyclic nucleotide-regulated ion channel (CNGC2)
At5g18670	6.5	4.6	4.4	5.4	3.05E-10	4.54E-09	beta-amylase, putative (BMY3)
At5g18840	4.8	3.5	Induction*	Induction*	3.38E-09	1.89E-07	sugar transporter, putative
At5g22920	5.8	4.7	5.6	4.5	3.19E-08	3.40E-07	zinc finger (C3HC4-type RING finger) family protein
At5g26340	3.9	2.5	5.8	4.7	6.15E-08	2.93E-05	hexose transporter, putative
At5g47240	3.0	2.6	5.1	2.7	4.30E-06	6.05E-05	MutT / nudix family protein
At5g49360	5.3	3.9	4.3	4.3	8.85E-10	3.54E-08	glycosyl hydrolase family 3 protein
At5g66170	7.1	5.2	9.8	8.2	9.21E-12	4.62E-10	similar to senescence-associated family protein

Notes
A) Relative differental induction of the gene upon dexametasone tratment in respective transgenic line compared to wt in the array experiment
B) Relative differental induction of the gene upon dexametasone tratment in respective transgenec line in Q-PCR experiment. No value; note determined
1) AGI gene model assosiated with GST sequence. If GST is covering two gene models both are indicated. Annotation Tair6 is used.
2) Q values associated with the relative change in signal in the arrray experiment (P value corrected for multiple testing)
3) Annotation, Tair6. Within brackets, Arabidopsis gene name (if included in annotation)
*) Two products generated in Q-PCR experiment, signal more than twofold induced in response to dexamethasone

bZIP11 controlled genes are sugar-regulated

The translation of *bZIP11* is repressed by sucrose (Wiese *et al.*, 2004). The genes that were affected by bZIP11 were compared to other genes regulated by sugars in publicly available micro-array experiments. An analysis similar to the one successfully used by Bläsing *et al.* (2005) was performed. Different treatments affect different numbers of genes. The comparisons were therefore restricted to the 150 genes most affected by each treatment. A high proportion of the genes affected by bZIP11 are also regulated by sugars in three independent experiments (Table 2.2). Of 163 genes induced by bZIP11, 25 (15.3%) are among the 150 most repressed by glucose treatment, 16 (9.8%) are among the 150 most repressed by sucrose treatment and 24 (14.7%) are among the 150 most repressed after increased carbon assimilation in three independent experiments. The treatments that caused expression changes most significantly similar to the changes caused by bZIP11, were all affecting cellular sugar levels in the plant (sugar treatments and treatments affecting carbon assimilation). The responses to the other treatments were less correlated

Table 2.2. Summary of genes responsive to bZIP11 and other treatments

The 150 most affected genes from each experiment were compared with the genes shown to be regulated by bZIP11. The numbers of genes affected by both the treatment and bZIP11 are presented in the table. Numbers within brackets indicate the percentage of the total number of genes affected by bZIP11. P-values indicate the probability of getting the indicated number of genes (or more) regulated by the treatment and bZIP11 by random sampling, assuming random distribution among 12000 expressed genes. The P-values are corrected for multiple testing using the Bonferroni correction. P-values higher than 0.05 are omitted. The gene lists for the different treatments are adopted from Bläsing et al. (2005) and references therein. Note that several genes are affected by more than one treatment.

	bZIP11-induced (total 163 genes)		bZIP11-repressed (total 98 genes)	
Treatment	**Number**	**P-value**	**Number**	**P-value**
Glucose-repressed	**25** (15.3%)	$1.49 * 10^{-18}$	**2** (2.0%)	
Glucose-induced	**0**		**16** (16.3%)	$1.92 * 10^{-12}$
Sucrose-repressed	**16** (9.8%)	$5.59 * 10^{-9}$	**2** (2.0%)	
Sucrose-induced	**1** (0.6%)		**3** (3.1%)	
Light-repressed (etiolated seedlings)	**11** (6.7%)	$1.25 * 10^{-4}$	**3** (3.1%)	
Light-induced (etiolated seedlings)	**0**		**6** (6.1%)	$2.08 * 10^{-2}$
NO_3-repressed (30 min treatment)	**5** (3.1%)		**3** (3.1%)	
NO_3-induced (30 min treatment)	**7** (4.3%)		**0**	
NO_3-repressed (3 h treatment)	**4** (2.5%)		**2** (2.0%)	
NO_3-induced (3 h treatment)	**6** (3.7%)		**5** (5.1%)	
Mannitol-repressed (3 h treatment)	**10** (6.1%)	$7.24 * 10^{-4}$	**6** (6.1%)	$2.08 * 10^{-2}$
Mannitol-induced (3 h treatment)	**6** (3.7%)		**3** (3.1%)	
Carbon assimilation-repressed	**24** (14.7%)	$2.13 * 10^{-17}$	**1** (1.0%)	
Carbon assimilation-induced	**0**		**6** (6.1%)	$2.08 * 10^{-2}$
Light-repressed (4h treatment)	**5** (3.1%)		**9** (9.2%)	$6.13 * 10^{-5}$
Light-induced (4h treatment)	**2** (1.2%)		**8** (8.2%)	$2.22 * 10^{-4}$
Total	**163** (100%)		**98** (100%)	

to bZIP11-mediated changes (Table 2.2). It is striking that genes induced by bZIP11 are generally repressed by sugar treatments. Reciprocally, genes repressed by bZIP11 are often induced by sugar treatments. *bZIP11* is translationally repressed by high sucrose levels in wt plants (Wiese *et al.*, 2004). The profiling experiment thus identifies likely *in vivo* bZIP11 target genes, indicating the power of the analysis. The bZIP11 transcription factor possibly affects some biochemical processes more than others. This was tested by a gene ontology (GO) analysis of the 261 genes identified as differentially expressed. Several GO terms are present at higher frequencies than expected from a random distribution among the induced genes, exemplified by the high number of genes involved in amino acid catabolism (3; 0,2 expected), having proline dehydrogenase activity (2; 0,02 expected) or pyridoxal phosphate binding activity (2; 0,1 expected). Among the genes repressed by induced bZIP11 activity were genes involved in shoot morphogenesis (2; 0,2 expected), cell wall modification (5; 0,3 expected), or genes encoding proteins with pectate lyase activity (3; 0,05 expected). However, none of the GO-terms were enriched to statistical significant levels. The data are summarized in Table 2.3.

Table 2.3. Gene ontology terms associated with differential gene expression in response to differential bZIP11 activity.

The table indicates all non-redundant gene ontology GOterms associated with the differentially expressed genes that are shared with at least two gene models and present to more than threefold higher numbers than expected based on random distribution of GO terms among the genes considered to be expressed (logged average level of normalized signal strength higher than ten).

	Expressed[a]	Induced			Repressed		
	Number[b]	Number[b]	expect.[c]	ratio[d]	Number[b]	expect.[c]	ratio[d]
Biological process							
Morphogenesis	116 (2.1%)				6 (13.3%)	0.9	**6.5**
Response to hormone stimulus	217(4.1%)	5 (6.0%)	3.4	1.5	5 (12.5%)	1.6	**3.1**
Response to temperature stimulus	64 (1.2%)	2 (2.4%)	1.0	2.0	2 (5.0%)	0.5	**4.2**
Response to water	35 (0.7%)	3 (3.6%)	0.5	**5.5**		0.3	0.0
Shoot morphogenesis	24 (0.5%)				2 (5.0%)	0.2	**11.1**
Response to pathogen	154 (3.0%)	2 (2.5%)	2.4	0.8	5 (12.5%)	1.2	**4.2**
Programmed cell death	59 (1.1%)	2 (2.5%)	0.9	2.2	2 (5.0%)	0.5	**4.4**
Response to salicylic acid stimulus	49 (1.0%)				2 (5.0%)	0.4	**5.3**
Carbohydrate transport	36 (0.7%)	4 (5.0%)	0.6	**7.1**	1 (2.5%)	0.3	**3.6**
Response to water deprivation	32 (0.7%)	2 (2.5%)	0.5	**4.0**		0.2	0.0
Two-component signal transduction system	27 (0.6%)	1 (1.3%)	0.4	2.4	2 (5.0%)	0.2	**9.6**
Response to cold	26(0.5%)	2 (2.5%)	0.4	**5.0**	1 (2.5%)	0.2	**5.0**
Negative regulation of signal transduction	16 (0.4%)	1 (1.3%)	0.2	**4.0**		0.1	0.0
Cation transport	152 (3.4%)	81 (4.8%)	1.8	**4.4**	1 (3.3%)	1.0	1.0
Defense response to pathogen	127 (2.9%)	2 (3.7%)	1.5	1.3	3 (10.0%)	0.9	**3.5**
Defense response to pathogen, incompatible interaction	90 (2.1%)	2 (3.7%)	1.1	1.8	2 (6.7%)	0.6	**3.3**
Hormone-mediated signaling	83 (1.9%)	2 (3.7%)	1.0	2.0	3 (10.0%)	0.6	**5.4**
Oligopeptide transport	26 (0.6%)	1 (1.9%)	0.3	**3.2**	1 (3.3%)	0.2	**5.7**
Nitrogen compound catabolism	16 (0.4%)	3 (5.6%)	0.2	**15.4**			
Amine catabolism	15 (0.4%)	3 (5.6%)	0.2	**16.4**			
Response to pathogenic bacteria	14 (0.4%)	1 (1.9%)	0.2	**6.0**	2 (6.7%)	0.1	**21.5**
Regulation of programmed cell death	13 (0.3%)	1 (1.9%)	0.2	**6.4**	1 (3.3%)	0.1	**11.5**
Metal ion transport	93 (2.6%)	4 (9.5%)	1.1	**3.8**	1 (4.2%)	0.6	1.6
Cell wall modification	45 (1.3%)				5 (20.8%)	0.3	**17.1**
Inorganic anion transport	22 (0.6%)				2 (8.3%)	0.1	**13.9**
Ethylene mediated signaling pathway	22 (0.6%)	1 (2.4%)	0.3	**4.0**	1 (4.2%)	0.1	**7.0**
Amino acid catabolism	15 (0.5%)	3 (7.1%)	0.2	**17.4**			
Phenylpropanoid biosynthesis	37 (1.7%)		0.3	0.0	21 (1.8%)	0.3	**7.3**

Defense response to pathogenic bacteria, incompatible interaction	6 (0.3%)	1 (5.0%)	0.1	**19.2**	1 (5.9%)	0.0	**22.6**
Protein ubiquitination	32 (3.4%)	2 (25.0%)	0.3	**7.5**			
Terpenoid metabolism	26 (2.8%)				22 (2.2%)	0.2	**8.2**
Cell wall loosening (sensu Magnoliophyta)	23 (2.4%)				4 (44.4%)	0.2	**18.5**
Polyisoprenoid biosynthesis	19 (2.0%)				2 (22.2%)	0.2	**11.2**
Cell wall modification during multidimensional cell growth	16 (1.7%)				3 (33.3%)	0.2	**20.0**
Proline catabolism	2 (0.3%)	2 (25.0%)	0.0	**119.0**			
Glutamate biosynthesis	2 (0.3%)	2 (25.0%)	0.0	**119.0**			
Molecular function							
Ligase activity, forming carbon-nitrogen bonds	126 (2.2%)	6 (7.1%)	1.9	**3.2**	1 (2.6%)	0.8	1.2
Monooxygenase activity	89 (1.6%)	1 (1.2%)	1.3	0.8	2 (5.3%)	0.6	**3.4**
Carbon-oxygen lyase activity	76 (1.4%)	1 (1.2%)	1.1	0.9	3 (7.9%)	0.5	**6.0**
Heme binding	53 (1.0%)	1 (1.2%)	0.8	1.3	2 (5.3%)	0.3	**5.7**
Sugar transporter activity	52 (0.9%)	4 (4.7%)	0.8	**5.2**	2 (5.3%)	0.3	**5.8**
Pyridoxal phosphate binding	7 (0.2%)	2 (2.4%)	0.1	**19.6**			
Hydrolase activity, hydrolyzing O-glycosyl compounds	161 (3.2%)	10 (13.0%)	2.4	**4.1**	3 (7.9%)	1.2	2.5
Porter activity	158 (3.2%)	8 (10.4%)	2.4	**3.3**	4 (10.5%)	1.2	**3.4**
Carboxylic ester hydrolase activity	127 (2.6%)	2 (2.6%)	1.9	1.0	5 (13.2%)	1.0	**5.2**
UDP-glycosyltransferase activity	56 (1.2%)	1 (1.3%)	0.9	1.2	2 (5.3%)	0.4	**4.7**
Thiol-disulfide exchange intermediate activity	41 (0.9%)	2 (2.6%)	0.6	**3.2**			
Transaminase activity	34 (0.7%)	3 (3.9%)	0.5	**5.8**			
Inorganic anion transporter activity	27 (0.6%)				2 (5.3%)	0.2	**9.9**
Water channel activity	15 (0.3%)	1 (1.3%)	0.2	**4.3**	1 (2.6%)	0.1	**8.8**
Proline dehydrogenase activity	1 (0.1%)	2 (2.6%)	0.0	**130.0**			
Ubiquitin-protein ligase activity	91 (2.6%)	4 (8.2%)	1.1	**3.2**	1 (4.0%)	0.4	1.6
Hydrogen ion transporter activity	67 (1.9%)	3 (6.1%)	0.8	**3.2**			
Lipase activity	47 (1.4%)	2 (4.1%)	0.5	**3.1**	2 (8.0%)	0.2	**6.0**
Sugar porter activity	46 (1.3%)	4 (8.2%)	0.5	**6.3**	1 (4.0%)	0.2	**3.1**
Pectinesterase activity	38 (1.1%)				2 (8.0%)	0.2	**7.5**
Pectate lyase activity	12 (0.4%)	1 (2.0%)	0.1	**6.0**	3 (12.0%)	0.1	**35.3**
Alanine-glyoxylate transaminase activity	4 (0.2%)	2 (4.1%)	0.0	**37.1**			
Cation:cation antiporter activity	20 (1.4%)	3 (18.8%)	0.2	**13.8**			
Monovalent cation:proton antiporter activity	17 (1.2%)	3 (18.8%)	0.2	**16.3**			

Cellular compartment							
Ubiquitin ligase complex	43 (0.7%)	3 (3.3%)	0.5	**5.5**			
Integral to membrane	293 (5.5%)	10 (17.2%)	3.2	**3.2**	2 (7.1%)	1.5	1.3
Anchored to membrane	124 (2.3%)	6 (10.3%)	1.3	**4.5**			
Cell wall (sensu Magnoliophyta)	99 (1.9%)	2 (3.5%)	1.1	1.9	3 (10.7%)	0.5	**5.8**

[a] Gene ontology termes assosiated to the gene models considdered to be expressed in the mRNA profiling experiment (A level cutof)

[b] The number of gene models associated with the gene ontology term

[c] The expected number of genes associated with the gene ontology term, based on a random distribution of termes among the gene models detectable in the mRNA profiling system

[d] The ratio between the numer of gene models associated with the GO term and the expected number. Values over three are depicted in boldface letters.

Effects of non-ectopic and constitutive overexpression of bZIP11 partially overlap

The transgenic plants that were used for array analysis expressed the *bZIP11:HBD* transgene constitutively. In wt plants *bZIP11* expression is mostly associated with vascular tissue (Rook *et al.*, 1998a). Therefore, some of the genes identified as regulated by bZIP11:HBD might not be targets of the factor in the wt, even though the fusion protein binds to the promoters of these genes and activates expression in transgenic plants. To identify bZIP11 specific target genes, transgenic lines were created in which *bZIP11* expression was controlled by its own promoter. These lines express a truncated *bZIP11* mRNA lacking the 5'UTR. Thereby the translation of the protein is no longer repressed by sucrose (Rook *et al.*, 1998c). The expression pattern of the transgene lacking the 5'-leader of the mRNA resembles the pattern of the endogene (Rook *et al.*, 1998c). Grown on high sucrose, such leaderless transgenic plants will express bZIP11 protein spatially directed by the endogenous promoter. At this high sucrose concentration the endogenous *bZIP11* as well as the other four sucrose-controlled bZIPs (*bZIP1*, *2*, *44* and *53*) are not translated due to sucrose repression (Figure 2.5). Fifteen single-loci transgenic lines were generated. *bZIP11* was only moderately overexpressed in the leaderless lines (Figure 2.5). Three lines with consistent high levels of the transgenic transcript were selected for further experimentation (line 19.2.4, 34.3.1 and 45.4). These three lines were analyzed for expression of 35 genes identified as regulated by bZIP11 in the mRNA profiling experiment. Seven genes, including the previously characterized *ASN1*, showed higher levels of expression in the leaderless lines compared to wt when grown on media

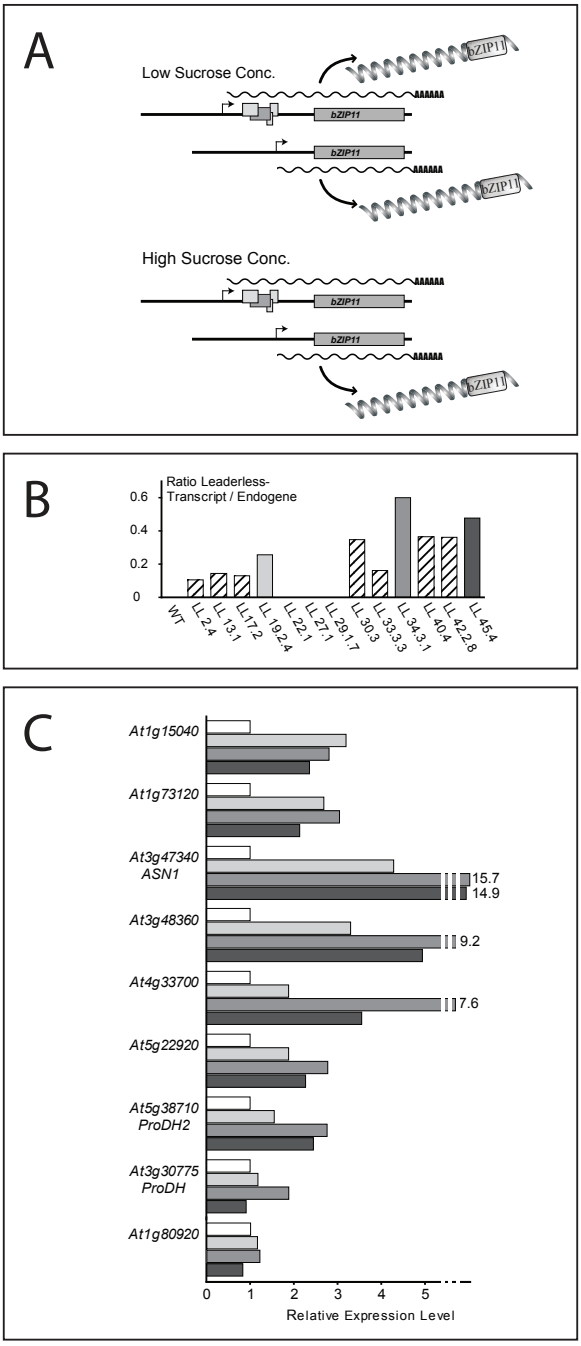

Figure 2.5. Altered gene expression directed by a bZIP11 leaderless gene

(A) Scheme of the specific effect of the leaderless *bZIP11* transcript. The endogene with the 5'leader

including uORFs is depicted on top of and in comparison with the transgene. The transgene consists of the *bZIP11* promoter fused directly to the *bZIP11* coding sequence. When transgenic seedlings are grown on high sucrose (lower panel) the endogenous *bZIP11* gene is translationally repressed, while the translation of the transgene is not repressed due to the absence of the leader sequence (Wiese *et al.*, 2004). (B) Relative level of the *bZIP11* leaderless transcript in ten day old seedlings grown on 200 mM sucrose in relation to the endogenous *bZIP11* transcript determined by real-time quantitative PCR analysis in several independent transgenic lines. The three lines depicted in shades of gray were selected for further experimentation. The levels represent averages of two independent mRNA quantifications. (C) The relative expression levels of seven genes that are expressed at higher levels in the leaderless lines grown on 100 mM sucrose than in wt plants, compared with the relative expression level of *ProDH* and *At1g80920* for comparison, in wt (open bars) and three leaderless lines (shades of gray). The relative expression levels in three independent leaderless lines are shown (light gray, line 19.2.4; medium gray, line 34.3.1 and dark gray, line 45.4). Expression levels were normalized to the levels of *ACTIN2* (*At3g18780*).

containing high levels of sucrose (Figure 2.5). The expression of the *ProDH* gene was not affected in this experiment. This indicates that although bZIP11 is able to bind to the *ProDH* promoter it is not a physiologically relevant bZIP11 target gene, as previously indicated (Satoh *et al.*, 2004; Weltmeier *et al.*, 2006). *ASN1* expression was determined in all fifteen leaderless lines generated and a correlation was observed between the levels of the transgenic *bZIP11* transcript and the *ASN1* transcript (Figure 2.5 and data not shown). The annotations of the seven genes controlled by specific tissue specific overexpression of bZIP11 are summarized in Table 2.4.

The expression of genes directly regulated by bZIP11 is most likely immediately responsive to changed bZIP11 activity. Therefore, the kinetics of induction were determined using the bZIP11:HBD lines. Expression levels were assayed from ten minutes to 2-h after dex addition for the seven genes shown to be regulated by the tissue-specific overexpression of bZIP11 in the leaderless lines. The *ProDH* gene, previously identified as a direct target of ectopically expressed bZIP11 was also tested (Satoh *et al.*, 2004; Weltmeier *et al.*, 2006). For four genes tested, *At1g15040*, *ProDH2* (*At5g38710*), *ASN1* and *ProDH,* more than two-fold increased expression levels were detectable after forty minutes and the expression continued to rise over time (Figure 2.6). Four genes responded slower and to a lesser extent to dex treatments, *At1g73120*, *At3g48360*, *At4g33700* and *At5g229020* (Figure 2.6). In summary, three genes (*ASN1*, *At1g15040* and *ProDH2* (*At5g38710*)), are expressed to higher levels in the leaderless lines and rapidly induced by bZIP11:HBD.

Table 2.4. Seven of 35 genes tested are differentially expressed in leaderless transgenic lines treated with sucrose

AGI	Gene Name	Annotation [A]	bZIP11 induction[B]	CHX inhibition[C]	Sugar repression[D]
AT1G15040		Glutamine amidotransferase-related	Fast	Strong	44%
AT1G73120		unknown protein	Slow	weak	31%
AT3G47340	*ASN1, DIN6*	glutamine-dependent asparagine synthetase	Intermediate	no	86%
AT3G48360	*BT2*	Component of the TAC1-mediated telomerase activation pathway	Intermediate	weak	57%
AT4G33700		CBS domain-containing protein	Slow	no	9%
AT5G22920		Zinc finger (C3HC4-type RING finger) protein	Slow	weak	67%
AT5G38710	*ProDH2*	Proline dehydrogenase, putative	Fast	no	67%
AT3G30775	*ProDH, EDR5*	Proline dehydrogenase	Fast	no	59%

[A] According to the Tair7 annotation
[B] Figure 3. this manuscript
[C] Figure 4. this manuscript
[D] Genevestigator, sucrose dataset. Values represent percent reduction of mRNA level upon sucrose treatment

The *bZIP11* gene and its four closest homologs are all translationally repressed by high sucrose levels (Wiese *et al.*, 2005 and our unpublished observations). To increase the possibility of identifying target genes by mRNA profiling the seedlings were grown in sucrose-containing media to limit synthesis of these endogenous bZIP proteins. To investigate whether the identified genes were controlled by bZIP11 in a situation where the homologous proteins are present, the seedlings were grown in media containing low sugar levels (4.0 mM) and dex-dependent changes of gene expression were determined. Under low-sucrose conditions most genes tested were induced in response to nuclear translocation of bZIP11 and only two genes (At3g4836 and At5g22920) did not respond (Supplementary table S1). Thus, high sucrose levels are not a prerequisite for bZIP11-dependent control of transcription.

bZIP11 controls gene expression directly

The translational inhibitor cycloheximide (chx) was used to test if bZIP11-dependent effects on the target genes require translation. Transgenic seedlings expressing the bZIP11:HBD fusion protein were treated with chx, dex or both, before the relative expression of putative bZIP11 target genes was probed. Dex-induction is independent of translation, as dex affects the cellular localization of the pre-existing protein (Picard *et al.*, 1988). Gene expression levels were quantified using quantitative PCR following 1-h long chx and dex treatments (Figure 2.7), as described previously for similar experiments (Sakai *et al.*, 2001). The endogenous *bZIP11* gene is unaffected by these treatments (data not shown). Importantly, all genes tested were induced by the 1-h dex treatment. Several of the genes were also induced by the chx treatment. Cycloheximide treatments did not inhibit the dex-mediated induction of *ASN1*, *At4g37700*, *ProDH2* (*At5g38710*) and

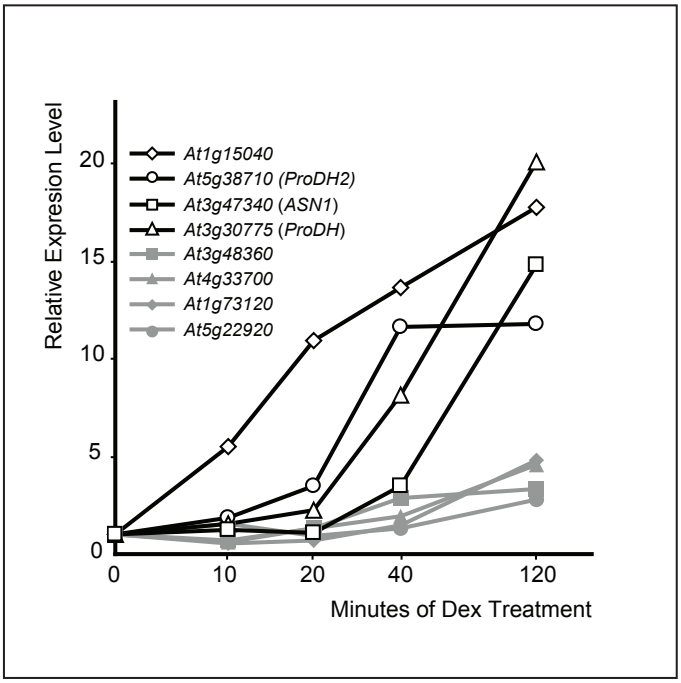

Figure 2.6. bZIP11 affects gene expression rapidly

Relative expression levels of putative bZIP11 target genes in response to different durations of 10 μM dexamethasone treatment of bZIP11:HBD seedlings. The expression levels are depicted relative to the expression level at time zero. Values represent means of two independent biological replicates. The time series are represented in a logarithmic scale for clarity. The figure shows results obtained using the M transgenic line. None of the genes tested responded to the treatment in the wt control seedlings and the results were confirmed in the independent transgenic line L (data not shown). The relative expression levels were determined by real-time quantitative PCR normalized to the levels of *ACTIN2* (*At3g18780*).

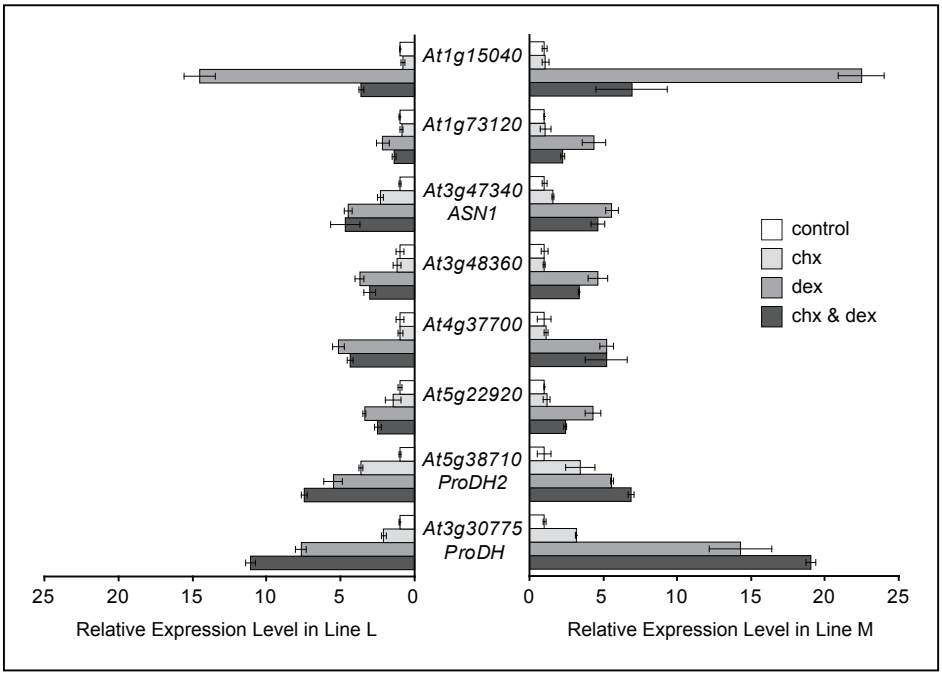

Figure 2.7. Requirement for protein synthesis for bZIP11-mediated induction of expression

Relative expression levels of putative bZIP11 target genes in bZIP11:HBD transgenic seedlings. Seedlings were control treated (open bars), treated with 35 µM of the protein synthesis inhibitor cycloheximide (light gray bars), 10 µM dexamethasone (medium gray bars) or a combination of the two treatments (dark gray bars) for 1 h. Relative expression levels of eight genes were determined by real-time quantitative PCR. Expression levels were normalized to the levels of *ACTIN2* (*At3g18780*). The data represents averages of two independent experiments. Error bars represent standard deviations from the mean. The experiment was performed independently with two transgenic lines (L line left and M line right). Similar observations were made in independent experiments with longer treatments (data not shown).

ProDH. The *ProDH* gene has previously been shown to be directly induced by bZIP11 and closely related S1-class bZIP proteins (Satoh *et al.*, 2004; Weltmeier *et al.*, 2006), which was confirmed by this analysis. Treatments with chx reduced the induction of the genes *At1g15040*, *At1g73120, At3g48360* and *At5g22920* to different extents. This indicates an indirect mechanism of bZIP11-dependent activation of these genes. The genes that most likely represent physiologically relevant target genes are *ASN1* and *ProDH2*. These genes are deregulated in the leaderless lines and rapidly induced by bZIP11 independently of chx treatments.

bZIP11 modulates gene expression by promoter binding

Genes directly regulated by bZIP11 must contain a bZIP11 binding element. Such putative sites will be enriched in the differentially expressed genes. This was tested using sequence element finding algorithms. Several sites were statistically enriched among upstream sequences of the differentially expressed genes compared to similar sequences in all Arabidopsis genes (Table 2.5). As apparent from Table 2.5, all algorithms identified sites containing the ACGT core sequence as significantly enriched among the promoters of genes induced by bZIP11. Most algorithms identified sites related to the G-box element (CACGTG) (Jakoby *et al.*, 2002). No conclusive sites were detected among the bZIP11-repressed genes. The different algorithms identified different sequences with lower associated significance among the promoters of the repressed genes. This indicates that the mechanistic nature of bZIP11 repression is different from bZIP11 activation. It involves less conserved binding sites and possibly bZIP11 is not directly repressing these genes by binding to the promoters.

To test if bZIP11 binds to the promoters of the physiologically relevant target genes the promoters were fused to the reporter gene luciferase and bZIP11-dependent expression was investigated by transient transformation. Arabidopsis protoplasts were transfected with three plasmids: a plasmid containing the promoter:LUC construct, a plasmid containing bZIP11 or an unrelated transcription factor expressed under the control of the constitutive 35S promoter and a plasmid used for normalization. Both the *ASN1* and the *ProDH2* promoter were tested for bZIP11-dependent activation. Co-expression of *bZIP11* increased the LUC activity of transformed protoplast several fold compared to protoplasts transformed with only the promoter constructs (Figure 2.8). To test whether the elevated LUC activity levels were specific to bZIP11 the unrelated transcription factor ATH1 (Quaedvlieg *et al.*, 1995) was co-expressed as well with the promoter:LUC

Table 2.5. bZIP11 preferably activates genes with promoters containing ACGT elements

The table summarizes results from the use of five algorithms identifying significantly enriched cis-elements within the promoters of the genes shown to be regulated by bZIP11. The promoter sequences of the induced or the repressed genes, respectively, were compared to the promoter sequences of all identified genes of the Arabidopsis genome.

Algorithm	Induced genes			Repressed genes		
	Site [A]	P value [B]	ACGT core[C]	Site [A]	P value [B]	ACGT core[C]
Motiffinder[1]	AC<u>ACGT</u>	3.8×10^{-11}	8	GCCCAA	1.6×10^{-6}	*
WeederWeb[2]	CC<u>ACGT</u>	<0.001	5	GGACCC	<0.001	None
MotifSampler[3]	knn<u>ACGT</u>n	8.3×10^{-5}	N.A.	ATTAAT	1.3×10^{-4}	N.A
BioProspector[4]	A/C<u>CACGT</u>	4.4×10^{-4}	N.A		No site	
MEME[5]	C<u>ACGT</u>GT/GCA	2.8×10^{-10}	N.A	G/AAGAAA	2.6×10^{-8}	N.A

[1] http://www.arabidopsis.org/tools/bulk/motiffinder/
[2] WeederWeb (Pavesi, 2004)
[3] MotifSampler (Thijs, 2002)
[4] BioProspector (Liu, 2001 and Jensen, 2004)
[5] (Bailey, 1994)
[A] Most significantly enriched site identified, ACGT motif underlined
[B] Computed P-value for most significant site. NOTE P-values can not be compared between algorithms
[C] Number of the most significant sites found that contained the ACGT core
N.A. Not applicable
* None, among the one hundred most significantly enriched sites identified.

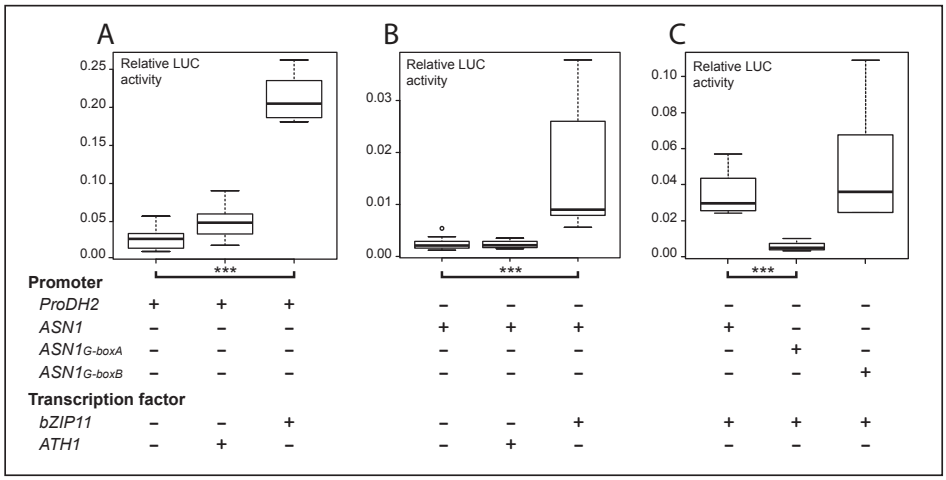

Figure 2.8. bZIP11 activates the ProDH2 and ASN1 promoters in transiently transformed Arabidopsis protoplasts

Promoters of *ProDH2* and *ASN1* were fused to reporter gene Luciferase (*LUC*). LUC activity was assayed in transiently transformed Arabidopsis protoplasts. (A) Relative LUC activity levels of Arabidopsis protoplasts transformed with the *ProDH2* reporter plasmid alone or in combination with constructs constitutively expressing the *ATH1* or the *bZIP11* gene. (B) Relative LUC activity levels of Arabidopsis protoplasts transformed with the *ASN1* reporter plasmid alone or in combination with constructs constitutively expressing the *ATH1* or the *bZIP11* gene. (C) Relative LUC activity levels of Arabidopsis protoplasts transformed with the wt *ASN1* reporter plasmid or mutants in which either G-boxA ($ASN1_{G\text{-}boxA}$) or G-boxB ($ASN1_{G\text{-}boxB}$) is changed (core ACGT changed to AAAA).

Boxes represent the interquartile range and the thick line represents the median of the values. The dashed lines represent values within 1.5 times the box width and circles represent outliers outside this range. Each box represents data of four to six independent experiments. The experiments have been replicated independently yielding similar results. Stars indicate statistical significance ($P<0.001$, Wilcoxon rank sum test).

constructs. *ATH1* expression did not affect the LUC activity levels (Figure 2.8). The *ASN1* promoter used in the experiments includes two G-boxes. G-boxA and G-boxB, positioned 352 and 555 bp before the transcriptional start site, respectively. The G-boxes were specifically removed by mutagenesis and the mutated promoters were tested for bZIP11-dependent transcriptional activation. Mutating the G-boxA in the *ASN1* promoter abolished the bZIP11-dependent increase in LUC activity (Figure 2.8). Mutations in G-boxB did not influence the LUC activity levels in the experiment.

Discussion

The transcription factor bZIP11 is translationally repressed by sucrose signals in Arabidopsis (Rook *et al.*, 1998b). Transcriptional profiling experiments performed to identify genes putatively regulated by bZIP11 yielded statistically highly significant data. Within 2-h of ectopic induction, hundreds of genes responded to bZIP11. Further experimentation, using *bZIP11* directed by its own promoter led to the conclusion that bZIP11 is directly regulating *ASN1* (*At3g47340*) and *ProDH2* (*At5g38710*). The bZIP11-dependent induction of *ASN1* expression was further shown to require a G-box element in the *ASN1* promoter, in agreement with our model of bZIP11 binding to the ASN1 promoter. The *ASN1* and *ProDH2* genes encode enzymes involved in amino acid metabolism. Therefore, bZIP11 is a direct regulatory link between sucrose-mediated signaling and amino acid metabolism. In this way the prevailing sucrose concentration controls amino acid metabolism.

bZIP11 is a potent regulator of gene expression

The mRNA profiling experiment shows a large bZIP11-induced effect on gene expression. Two hours of increased nuclear bZIP11 levels results in 261 differentially expressed genes. The use of two independent transgenic lines will prevent genes to be

identified as being differentially expressed due to artifactual differences in mRNA levels, *e.g.* caused by integration of the transgene in the genome. Stringent statistical criteria for differential expression were used and profiling results were confirmed to over 90% by real-time quantitative PCR. The number of genes identified (261) most likely is an underestimate of the actual number of genes affected by bZIP11. Similar studies in plants using other transcription factors have resulted in fewer differentially expressed genes (Maruyama *et al.*, 2004; Tohge *et al.*, 2005). The unusually high number of differentially expressed genes, might be a consequence of the sensitive experimental approach, using at least eight replications of each sample type, but most likely also reflects the intrinsic transcriptional activity of the bZIP11 protein. However, to bind DNA and affect target gene expression bZIPs need to form dimers. Even though bZIP11 is capable of forming homodimers (Ehlert *et al.*, 2006a), the so-called S1-class bZIPs, to which bZIP11 belongs (Jakoby *et al.*, 2002), preferentially form heterodimers with another class of bZIPs (C-class bZIPs) (Ehlert *et al.*, 2006a). It must thus be noted that the observed effects of overexpressing bZIP11 in a constitutive manner are those of bZIP dimers for the formation of which, the prevailing bZIP11 monomer concentration was rate-limiting. Furthermore, secondary effect caused by the depletion of bZIP11 binding-partners from the pool of bZIP monomers cannot be excluded. In nature, the temporal and spatial expression of bZIPs will determine which bZIP dimers are available for target-gene activation. *bZIP11* is expressed in cells surrounding the vasculature in the aerial tissues and its transcription is controlled by factors such as light and sugars (Rook *et al.*, 1998c; Rook *et al.*, 1998a). For most of the bZIP11 target genes presented here spatial expression patterns are not described. However, the suggested direct target of bZIP11, *ASN1*, is expressed in the cells surrounding the vasculature (Nakano *et al.*, 2000), similar to the *bZIP11* homolog of rice (*Oryza sativa*), further supporting the direct interaction between the bZIP11 protein and the *ASN1* promoter in wt plants. It is likely that some of the genes affected by 35S promoter directed ectopic *bZIP11* expression in the mRNA profiling experiments, are not bZIP11 specific targets. For example, the *ProDH* gene (*At3g30775*) was shown to be activated by bZIP53, bZIP2, bZIP11 and bZIP44 in transient assays (Satoh *et al.*, 2004) and by ectopic overexpression of bZIP11 (this thesis). However, *bZIP11* and *bZIP44,* are not co-expressed with *ProDH* (Satoh *et al.*, 2004) and when *bZIP11* is controlled by its own promoter, *ProDH* expression is not changed. Thus, although regulated by bZIP11 *in vivo* when ectopically expressed, *ProDH* is not a bZIP11 specific target in wt plants. Seven out of 35 genes tested are regulated by increased bZIP11 activity in the leaderless lines, where *bZIP11* expression is directed by its own promoter. Assuming representative

sampling, this suggests that approximately one fifth of the genes controlled by ectopic bZIP11 are specifically controlled by bZIP11 in planta.

bZIP11 target-gene activation is specific

The promoters of the genes that are induced by ectopic expression of bZIP11 share a consensus ACGT-element and most enriched sites are similar to the G-box motif (CACGTG) (Table 2.5). bZIP transcription factors are known to bind G-box elements in plants (Jakoby *et al.*, 2002; Hartmann *et al.*, 2005; Kaminaka *et al.*, 2006). The importance of this G-box element is also confirmed by mutation analysis of the *ASN1* promoter. This element has also been shown to be important for stress-dependent regulation of *ASN1*, through the activity of bZIP11 or a closely related protein (Baena-Gonzalez *et al.*, 2007). The bZIP11 protein binds to a related element in the promoter of the *ProDH* gene, the ProDH element, when ectopically expressed (Satoh *et al.*, 2004; Ehlert *et al.*, 2006a; Weltmeier *et al.*, 2006). The ProDH element is not enriched among the promoters of the genes induced by bZIP11 and absent from the parts of the *ASN1* and *ProDH2* promoters used for transient expression studies. Possibly, the bZIP11 protein can bind to different types of elements. The bZIP11 protein binds DNA as a dimer (Ehlert *et al.*, 2006a; Weltmeier *et al.*, 2006). The bZIP11 protein is capable of forming both homodimers and heterodimers with other bZIP proteins in Arabidopsis protoplasts and yeast cells (Ehlert *et al.*, 2006a). Different bZIP11-containing dimers activate *ProDH* expression differently but the bZIP11 homo-dimer only poorly activates *ProDH* expression (Weltmeier *et al.*, 2006). Herein lies a possible role for the C-class bZIPs. Overall S1-class bZIPs show a high degree of sequence similarity, leaving little room for promoter sequence specific regulation of gene expression. Likewise, C-class bZIPs all share very similar basic (DNA-binding) domains. Unlike the S1-class however, C-class bZIPs contain non-bZIP domains that differ from each other (Jakoby *et al.*, 2002), potentially allowing for differential activation. Differential activities of different bZIP dimers are a documented way of regulating transcription in both animals and plants (Halazonetis *et al.*, 1988; Shimizu *et al.*, 2005; Weltmeier *et al.*, 2006 and chapter 4 of this thesis).

No consensus sequences were found to be enriched among the promoters of genes that are repressed by ectopic bZIP11 activity. This indicates that the mechanism of gene repression by bZIP11 is different from that used for gene induction. Possibly, bZIP11 does not directly repress these genes. This assumption is supported by the facts that fewer genes are repressed by bZIP11 than are activated and that the average changes in

expression levels are lower for the repressed genes than for the activated ones. Several of the genes activated by bZIP11 encode regulatory proteins that might be responsible for repression of gene expression. Most likely, bZIP11 itself participates in a complex that activates transcription upon DNA binding.

Biological relevance of identified bZIP11 targets

Direct target-genes of transcriptions factors will be fast responding and independent of protein translation. Therefore, the effect of translational inhibitor cycloheximide (chx) was tested to determine which of the seven genes deregulated in the bZIP11 overexpressing lines are direct targets of the bZIP11 transcription factor. In the presence of chx four of the genes were shown to be induced by bZIP11, thus representing genes directly affected by the bZIP11 fusion protein in the transgenes. Furthermore, a direct target is likely to be rapidly induced in the bZIP11:HBD lines. Three of the genes with chx insensitive bZIP11-induced expression are rapidly induced *ASN1*, *ProDH2* and *ProDH*. The *ProDH* gene was previously shown to be a direct target of bZIP11 only when ectopically expressed (Satoh *et al.*, 2004). However, data presented here and by Satoh *et al*. (2004) show that the gene is not controlled by bZIP11 in wt Arabidopsis plants. Unfortunately, no suitable bZIP11 loss of function mutant is currently available, precluding the analysis of target gene expression in such material (our unpublished data). Nonetheless, evidence on the target genes presented here indicates that these genes are indeed direct and thus biologically relevant targets of bZIP11. Furthermore, bZIP11-containing dimers most likely regulate gene expression in response to sucrose signals, as translation of S1-class bZIPs, such as bZIP11, is efficiently repressed by sucrose (Rook *et al.*, 1998c). Not surprisingly, significantly higher than random numbers of the genes shown to be induced by bZIP11 are repressed by sucrose, glucose or CO_2 treatments. These treatments raise cellular sugar levels either directly or indirectly by increased carbon assimilation. Similarly, genes repressed by bZIP11 are significantly more frequently induced by these treatments. Therefore, results presented here validate the hypothesis that bZIP11 acts in a signaling pathway affecting gene expression in response to changed sucrose levels in Arabidopsis. Taken together, it is shown that by ectopic overexpression of bZIP transcription factors, biologically relevant target genes can be identified. The genes regulated by bZIP11 encode diverse proteins and no specific gene ontology term is significantly enriched. This is expected as sugar signaling controls expression of up to a third of the Arabidopsis genes (Price *et al.*, 2004; Blasing *et al.*, 2005).

Materials and methods

Cloning and DNA manipulation

The glucocorticoid fusion vector was constructed by amplifying the cDNA encoding rat glucocorticoid receptor hormone binding domain (Primers: 5'-GCGAGCTCAAAGGGATTCAGCAAGCCACT, 5'-GCCAATTGTCATTTTTGATGAAACAGAAGC) and fusing it into the 35S promoter/PolyA cassette of p35S-2 (http://www.pgreen.ac.uk/JIT/JIT_fr.html) using *Sac*I and *Mun*I restriction digests and ligation. Gateway compatible recombination sites were added using the Gateway® Vector Conversion System according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA, USA). The coding sequence of *bZIP11* was transferred using Gateway recombination (Invitrogen, Carlsbad, CA, USA). The resulting fusion was moved to the binary vector pCAMBIA1300 by means of restriction digestion and ligation. The construct used for the expression of the leaderless *bZIP11* transcript was assembled by replacing the available promoter in pGWB18 with the 2763 bp *Hin*DIII/*Nhe*I fragment (Rook *et al.*, 1998a) using standard methods (Sambrook *et al.*, 1989) followed by recombination of the *bZIP11* coding sequence into the Gateway site. Dexamethasone controlled plant expression of the *bZIP11* cDNA was achieved using the GVG system (Aoyama and Chua, 1997). The *ASN1* promoter luciferase and *ProDH2* promoter luciferase constructs were created by amplifying the promoters (ASNF:5'-GGGGACAAGTTTGTACAAAAAAGCAG GCTTTTCTAGCCGTAGCTCGTAG-3' ASNR:5'-GGGGACCACTTTGTACAAG AAAGCTGGGTTCTGATATAAGCCACTCCAGTC-3', ProDH2F:5'-GGGGACA AGTTTGTACAAAAAAGCAGGCTTGTCTCTGACTCGACTTTCTTAATAG-3', ProDH2R:5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTACGCCGTACAC TAACATACC-3') and transferring the fragments to the firefly luciferase-containing pUGW35 vector using Gateway technology (Invitrogen, Carlsblad, Ca, USA.). Mutations in the promoters were introduced by PCR amplification (ASN1 G-boxA R:5'-GTTACCGGATAACTCTGACCAGCTGTTTCAAAAGCTTAATTAACCTT CTTACGACGTCCGATGGGTTC-3', ASN1 G-boxA F: 5'-GGACGTCGTAA GAAGGTTAATTAACAAAAGGAAACAGCTGGTCAGAGTTATC-3', ASN1 G-boxB R: 5'-GATTGTTCTGATTGCTTTAGAGCCGTACAAAA GTTATCACGACAATGGTGTATTTGATTG-3',

ASN1 G-boxB F: 5'-TCAAATACACCATTGTCGTGATAACAAAAGTACGGCTCTA AAGCAATCAGAAC-3',ProDH2 G-box F: 5'-CGTCAAGTCAACATCAAAAAGCCA AAAGATATTTGTTATGCAAATTATGAAGATAAGC-3'
and ProDH2 G-box R: 5'-CTTATCTTCATAATTTGCATAACAAATATCAAAAGGC TTTTTGATGTTGACTTGAC-3'). The integrity of all vectors used was confirmed by sequencing using the ABIprism 377 sequencer (ABI, Foster City, CA, USA). Binary vectors were electroporated to *Agrobacterium tumefaciens* cells, strain C58C1 pMP90 (Koncz and Schell, 1986) and used for transformation.

Plant material and transformation

In all experiments Arabidopsis plants (accession Col-0, Ohio stock nr CS60000) were grown in liquid culture in half strength MS media (Duchefa, Haarlem, the Netherlands) supplemented with 0.5 g/l MES, pH 5.8 and sugars as indicated. Seedlings were grown in constant fluorescent light (100 microE/m^2) in a Microclima cabinet (Snijders, Tilburg the Netherlands), on a rotary shaker (60 rpm). Gas sterilized seeds were stratified for three to four days.
The floral dip transformation method was used to stably transform soil grown Arabidopsis plants (Bechtold *et al.*, 1993). Transgenes were selected on solid MS media supplemented with 0.8% plant agar and 1% sucrose (Duchefa) and the appropriate antibiotics (Duchefa) and transferred to soil. T2 selfed progeny were tested for segregation of the selectable marker on solid MS media. Only seedlings of lines showing a three to one segregation pattern were considered for further analysis. Homozygous plants were generated by selfing and used for further experimentation. Several lines of independently transformed plants (originating from different transformation experiments) were generated and studied. For the glucocorticoid fusion 42 lines were generated. 17 lines representing at least nine independent transformation events showed a three to one segregation and were assayed for *bZIP11* expression and the absence of a detectable phenotypic deviation from wt when grown without dexamethasone. Two lines were selected and used in further experimentation. Similarly, 49 lines expressing leaderless *bZIP11* construct were generated. 15 lines showed a three to one segregation and were assayed for the presence of leaderless *bZIP11* mRNA. Three lines with high leaderless mRNA levels were used in further experimentation.

Insertional mutants were delivered from NASC (http://arabidopsis.info/NASC ID: N423483) or acquired from INRA (http://dbsgap.versailles.inra.fr/publiclines/, INRA-fst line E 035E09).

For transcriptional profiling, mRNA expression and amino acid analysis Arabidopsis seedlings were grown in liquid media containing 100 mM sucrose for seven days in continuous light in separate flasks. Dexamethasone, 10 μM (Sigma-Aldrich, Spruce St, St. Louis, USA) or 0.1% ethanol (mock treatment) was added and the plants were incubated for indicated times before harvesting.

Transient expression and reporter activity quantification

Arabidopsis mesophyll protoplasts were created and PEG transfected according to the protocol presented by Sheen (http://genetics.mgh.harvard.edu/sheenweb/).

For each assay, 35.000 protoplasts were transfected with 2.5 μg of the normalizing plasmid encoding Renilla luciferase and 10 μg of the plasmids either containing *ASN1* or *ProDH2* promoter driven firefly luciferase. To study the interaction of bZIP11 with the *ASN1* and *ProDH2* promoters, protoplasts were co-transfected with 10 μg of plasmids either containing *bZIP11* or *ATH1* directed by the 35S promoter. Protoplasts were transfected during 30 minutes, after which they were collected and transferred to 1 ml of W5 Solution. After 16-h of incubation at 22^{o}C under continuous light, protoplasts were harvested and luciferase was measured according to the Dual-Luciferase® Reporter (DLR™) Assay System protocol (Promega Corporation, Madison, WI, USA) on a GloMax™ 20/20 Luminometer (Promega Corporation, Madison, WI, USA).

RNA and real-time PCR analysis

Plant material was snap frozen in liquid nitrogen and ground using glass beads in a Micro-Dismembrator S™ (B. Brown Biotech Int., Melsungen, Germany). RNA was purified using the RNeasy kit (Qiagen, Hilden, Germany) and the RNA purity and integrity were confirmed by using a RNA 6000 Nano Assay (Agilent, Pala Alto CA, USA) or gel electrophoresis. For real-time quantitative PCR analysis genomic DNA was removed using pretreatment of the total RNA with DNase (Fermentas, GmBH, St. Leon-Roth, Germany) and cDNA was synthesized using anchored oligo-T primers (Biolegio, Nijmegen, The Netherlands). cDNA was synthesized using MLV reverse transcriptase

(Promega, Madison, WI, USA) according to the instructions of the manufacturer. Real-time PCR was performed using ABIprism7700 Sequence detector and either Taqman™ or Cybergreen™ chemistry (ABI, Foster City, CA, USA). Expression levels were calculated relative to *ACTIN2* (*At3g18780*) levels using the Q-gene method that takes the relative efficiencies of the different primer pairs into account (Muller *et al.*, 2002). Primers were designed according the recommendations of the PCR master-mix manufacturer (ABI) or designed to be gene-specific by the CATMA consortium (http://www.CATMA.org). Full lists of primer sequences can be obtained from the authors.

Array analysis

Total RNA (1.25 µg) from four independent replicates of each treatment and seedling type was amplified using the Message Amp II kit (ABI). Five µg of amplified RNA, with an average length of at least 1000 bases, as determined by a RNA 6000 Nano Assay (Agilent, Palo Alto, CA, USA), were used as template in a cDNA synthesis reaction including 5-(3-aminoallyl)-dUTP (Ambion) and Superscript III (Invitrogen. Carlsbad, CA, USA) (ratio dUTP/dTPP of 2.33). RNA template was removed by hydrolysis with 3 µl 2.5 M NaOH per 30 µl for 15 min at 65°C. After neutralization by equimolar amounts of HCl the cDNA was purified with the MINELUTE PCR purification kit (Qiagen, Venlo, NL) and coupled with Cy3 or Cy5 mono-reactive dye (GE Healthcare Bio-Sciences AB, Sweden), respectively. The reaction was quenched after 60 minutes with 4.5 µl 4 M hydroxylamine (Sigma-Aldrich, St. Louis, MO, USA) and incubated in the dark for 15 minutes. The labeled cDNA was purified as described above and incorporation of Cy3 or Cy5 was determined by the UVMINI-1240 spectrophotometer (Shimadzu, Kyoto, Japan) at 550 nm or 650 nm, respectively. For all samples, 1 µg of labeled, heat denatured, cDNA with specific labeling of at least 30 pmol incorporated label per ng probe for each dye was used in 90 µl hybridization solution (25% formamide (Sigma-Aldrich), 5 x SSC, 0.2% SDS (Sigma-Aldrich), 20 mg/ml denatured herring sperm DNA (Sigma-Aldrich). Hybridization of Cy3 and Cy5 labeled samples was performed in a mixed loop design, Figure 2.3, resulting in at least eight independent assays of each group of RNA. Hybridization was performed with CATMA version 2 arrays, Complete Arabidopsis Transcriptome Micro-array , (Hilson *et al.*, 2003; Allemeersch *et al.*, 2005) containing 24 576 gene-specific tags (GSTs). The GSTs are between 150 and 500 bp in length and show no more than 70% identity with any other sequence in the genome. Detailed information about CATMA and database access can be found at http://www.catma.org/ (Crowe *et al.*, 2003) and http://genomics.

bio.uu.nl/. Slides were spotted using GAPSII glass slides (Corning, Corning, NY, USA), a BIOROBOTICS MICROGRID II TAS spotter (Genomic Solutions, Ann Arbor, MI, USA) and cross-linked for 4-h at 80°C. Before use, the GSTs were denatured by boiling of the slides in demineralized water for three minutes followed by water and ethanol washes. The slides were pre-hybridized at 55°C for at least 30 minutes in 5x SSC, 25% formamide (Sigma-Aldrich, St. Louis, MO, USA), 1% BSA fraction V (Sigma-Aldrich), 0,1% SDS (Sigma-Aldrich). 100 µl pre-hybridization solution was used and reactions were performed under a LifterSlip™ (Erie Scientific Company, Portsmouth, NH, USA) in a hybridization chamber (Corning, Corning, NY, USA) containing two drops of 20 µl water, covered by tinfoil and hybridized for 1-h at 42°C in a water bath. After pre-hybridization, slides were washed in water, spun dry and used for hybridization. Hybridization was performed at 42°C over night (at least 16 h) as for pre-hybridization. Washings of slides was performed using gradually lowered SSC concentrations in 0.2% SDS to stringent conditions at 55°C, followed by quick washes in washing solution without SDS at room temperature. The slides were thereafter dipped five times in demineralized water, spun dry and immediately scanned using SCANARRAY EXPRESS HT scanner (PerkinElmer,Wellesley, MA, USA) manually adjusted to uniform signal strengths according to the manufacturers instructions. Spot intensity was determined using Imagene™ ver. 6.5.1 (BioDiscovery, El Segundo, CA, USA) in accordance with the documentation of the software.

Slides were hybridized according to a mixed loop design, Figure 2.3, resulting in four biological replications with two technical replicates (dye-swapped) for each transgene and four times for the wt samples, resulting in at least eight replicates of each group in every comparison. Some of the slides were rescanned due to spatial bias based on visual examination of the scanned images. Data from all slides was later used in the statistical analysis.

Statistical analysis and Bioinformatics

Data from the mRNA profiling experiment was analyzed statistically using the R language environment for statistical computing (http://www.r-project.org) version 2.2 and Bioconductor release 1.7 (http://www.bioconductor.org). Differentially expressed genes were identified using the LIMMA package (Smyth, 2004). Data was primarily filtered to remove data from spots automatically flagged as bad by Imagene™. Data was normalized using the VSN package (Huber *et al.*, 2003) and linear fitted using the

recommendations of the LIMMA vignette according to a fractional model that included the dye-swaps as technical replicates and the interesting contrasts (differential expression in response to dex in the transgenes compared to the differential expression in the wt control plants). The data was also normalized using the loess method which gave similar results (data not shown), however as the variances were not equal across hybridizations (dex had a limited effect in the wt plants in the experiment), VSN normalization will reflect the in planta situation better (Freudenberg *et al.*, 2004). Visual examination of raw and normalized distributions by diagnostic plots confirmed that conclusion. A posterior residual standard deviation was employed (Smyth, 2004) independently for each treatment and the interesting contrasts (change in the transgene compared to the change in the wt). The obtained P-values were corrected for multiple testing errors using the BH procedure (Benjamini and Hochberg, 1995), yielding q-values. Lists of q-values were transferred to Microsoft Excel™ and sorted. The GST sequences were aligned to the Tair6 gene model database of transcripts. Gene models aligned to more than one GST were identified and one of the duplicated GSTs was used based on its corresponding q value. Genes were classified as differentially expressed if they were significantly changed ($q \leq 0.05$) in both transgenic lines independently compared to the wt situation and affected more than two-fold by the treatment in both transgenic lines, Figure 2.3. Genes with lower than two-fold changes were not considered differentially expressed. To compare the list of differentially expressed genes with other gene lists Microsoft Excel™ was used.

For gene ontology data, several publicly available tools that produced very similar results were used, FATIGO (Al-Shahrour *et al.*, 2005), GOstat (Beissbarth and Speed, 2004), Tair (http://www.arabidopsis.org/tools/bulk/go/), MAPMAN (Thimm *et al.*, 2004) and BINGO (Maere *et al.*, 2005). For the analysis of binding sites in the promoters of the differentially expressed genes, a 500bp upstream sequence of each promoter was searched for sites and compared to the background site file consisting of the 500bp promoter sequences of the all Arabidopsis genes (BLAST set, 500bp upstream, Tair6). Several programs were used: motiffinder (http://www.arabidopsis.org/tools/bulk/motiffinder/), Motif Sampler (Thijs *et al.*, 2002), MEME (Bailey and Elkan, 1994) and WeederWeb (Pavesi *et al.*, 2004). The programs were run using default settings accepting more than one site in each promoter and using both strands. The programs were also run using sites of different lengths and using longer promoter sequences with similar results (data not shown). More elaborate algorithms were also employed: BioProspector (Liu *et al.*, 2001), BioOptimizer (Jensen and Liu, 2004). Due to the lack of processing power, only the twenty-five most significantly affected genes were used in this later case, using

1000 bp upstream sequences.

Data from all other experiments were analyzed using the R language environment for statistical computing (http://www.r-project.org) version 2.5. ANOVA analysis, Wilcoxon rank sum tests and T-test were performed using standard packages (stats).

“A man should look for what is, and not for what he thinks should be.”
-Albert Einstein

Chapter 3: bZIP11 is a potent regulator of metabolism

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Summary

The transcription factor bZIP11 was previously shown to be a potent inducer of gene-expression. It was shown that many of bZIP11 target genes are involved in metabolism. No specific metabolic pathway is targeted, but rather many aspects of metabolism are affected. Here it is shown that the documented gene expression changes are biologically relevant. The levels of many central components of glycolysis, amino acid and trehalose metabolism are changed in response to changed bZIP11 activity. A model is proposed in which bZIP11 acts as an integrator of sugar signaling and metabolism.

Introduction

Plants are autotrophic organisms that require their growth rate and metabolism to be tightly regulated. This regulation has been demonstrated by reduction of growth following the down-regulation of key-metabolic processes such as sucrose synthesis. (Fernie *et al.*, 2002; Chen *et al.*, 2005). Sugars, such as sucrose and glucose, have long been known to be important factors in the regulation of growth and development of plants (Gibson, 2005). More recently, it was shown that the levels of many metabolites correlate to growth rate, and that there is no single metabolite that can explain variation in biomass on its own (Meyer *et al.*, 2007). Using Arabidopsis recombinant inbred lines (RILs), it was shown that a highly significant correlation between biomass and metabolic composition exists. One explanation for this correlation lies in the fact that these metabolites serve as substrates for metabolic processes that direct growth. Another, more likely explanation is that many of these metabolites are part of the input of signaling networks determining growth.

Due to their sessile nature, plants need to adjust growth to various stresses they encounter. Energy deprivation is a symptom often associated with stress. Therefore, it has been suggested that energy-deficiency signals, such as low sugar levels, trigger adaptation responses independent of the origin of the stress (Baena-Gonzalez *et al.*, 2007). For instance, sucrose is not only the main transported form of sugar and thus energy, but also has known signaling functions (Smeekens, 2000). Low sucrose will activate SnRK1 kinases, which are important integrators of sugar and stress signaling (Baena-Gonzalez *et al.*, 2007). Another well-studied example of the signaling function of sucrose is the

translational regulation of the S1-class of bZIP transcription factors. Elevated levels of sucrose inhibit translation of these transcription factors through a highly conserved upstream open reading frame in the 5' region of their mRNAs (Rook *et al.*, 1998c; Wiese *et al.*, 2004; Rahmani *et al.*, 2009; Weltmeier *et al.*, 2009). Besides the regulation at the translational level, bZIP mRNA expression is differentially regulated by various metabolic and environmental signals, including sugars (Figure 3.1). This differential regulation allows internal and external conditions to determine which bZIPs are being expressed. Furthermore, evidence has been presented that S1-class bZIPs induce gene expression after activation by the sucrose repressed SnRK1 kinases (Baena-Gonzalez *et al.*, 2007), indicating another role for sucrose in SnRK1/bZIP mediated stress signaling. bZIPs require dimerization in order to bind DNA and activate gene expression. Although some bZIPs like bZIP11 can form funtional homodimers, S1-class bZIPs preferentilly dimerize with C-class bZIPs. Because C-class bZIPs do not form homodimers, sucrose inhibits their activity by preventing formation of C/S1 dimers due to translational repression of S1-class bZIPs. Functionally, bZIPs are important in growth and development of plants, as exemplified by the severe phenotype of plants over-expressing bZIP11 (Figure 2.1). Not surprisingly, bZIP11 has been found to have a broad range of target-genes (Chapter 2). By overexpressing a dex- inducible bZIP11 fusion protein (bZIP11:HBD), targets of bZIP dimers for which the bZIP11 monomer pool was rate limiting were identified. A substantial subset of these targets are involved in metabolic processes.

Results

bZIP11 changes the expression of genes involved in metabolism

In chapter two of this thesis, it was found that the bZIP11 transcription factor strongly affects gene expression levels. The nature of the genes affected by bZIP11 is manifold. Manual examination of the genes that are directly or indirectly affected by bZIP11, revealed that many of the genes are involved in metabolism. Gene ontology (GO) analysis of the target genes, however did not reveal any GO annotations to be over-represented to statistically significant levels (Table 2.2). In Tables 3.1 to 3.3 selected summaries are given of genes involved in amino acid metabolism, glycolysis and trehalose metabolism, respectivly.

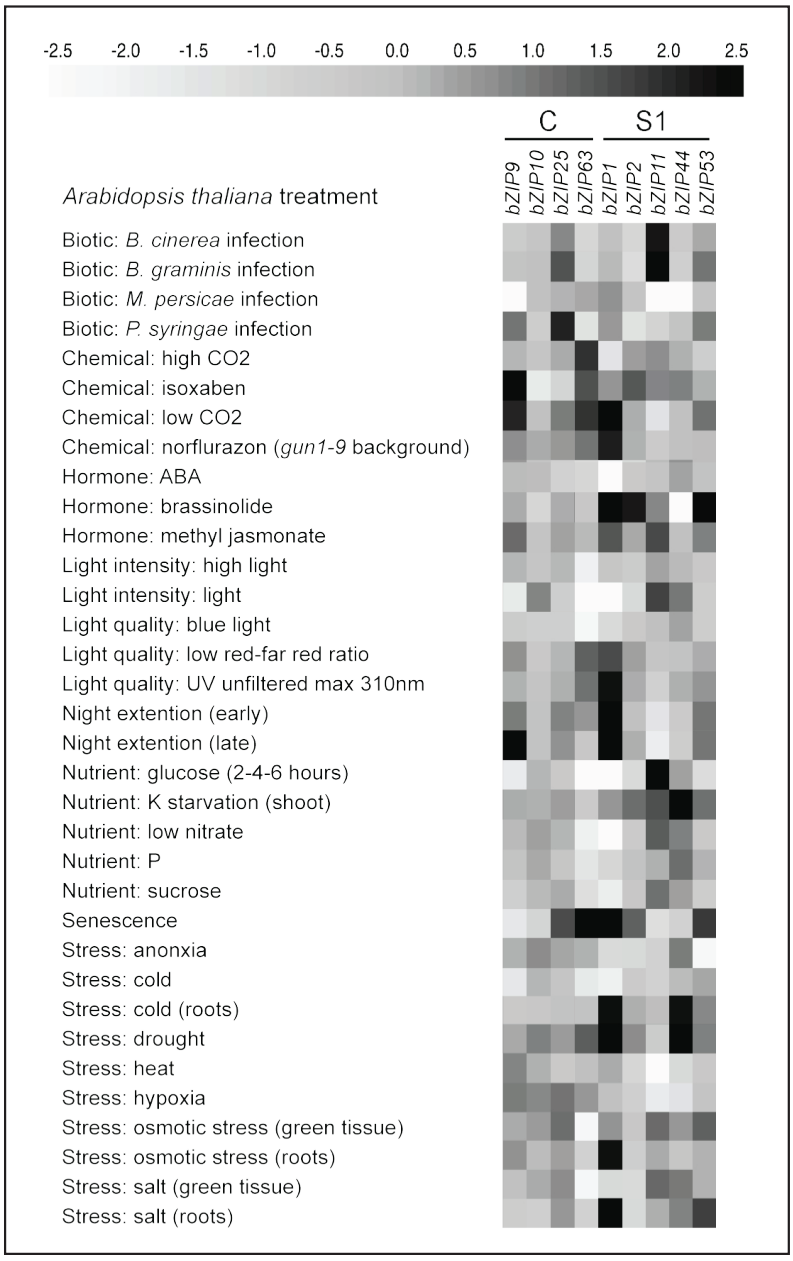

Figure 3.1. Differential transcriptional regulation of bZIP transcription factors.
The responses of bZIP transcription factor genes to various exogenous stimuli are shown (data from Genevestigator®). Shades of grey indicate levels of changed gene expression in response to the treatments.

Table 3.1. bZIP11-regulated genes affecting amino acid levels

The table shows a list of selected bZIP11 target genes involved in amino acid metabolism or other processes affecting amino acid levels. Differential expression compared to wt is given as log2 values.

AGI	Annotation	Diff. exp.	p value
AT3G30775	ProDH1, proline oxidase, / osmotic stress-responsive proline dehydrogenase	3.7	3.5E-11
AT1G15040	glutamine amidotransferase-related	3.51	1.1E-09
AT2G38400	AGT3 (ALANINE:GLYOXYLATE AMINOTRANSFERASE 3)	2.28	1.8E-09
AT4G39660	AGT2 (ALANINE:GLYOXYLATE AMINOTRANSFERASE 2)	2.16	5.8E-08
AT5G38710	ProDH2, proline oxidase / osmotic stress-responsive proline dehydrogenase	1.47	1.5E-05
AT1G77670	aminotransferase class I and II family protein	1.18	3.3E-07
AT3G47340	ASN1 (DARK INDUCIBLE 6)	1.1	4.8E-05
AT5G11520	ASP3 (ASPARTATE AMINOTRANSFERASE 3)	0.90	3.2E-03
AT5G35630	GS2 (GLUTAMINE SYNTHETASE 2)	0.70	5.8E-03
AT5G36160	aminotransferase-related	0.56	9.9E-03
AT5G05270	chalcone-flavanone isomerase family protein	-0.48	2.1E-02
AT3G10340	PAL4 (PHENYLALANINE AMMONIA-LYASE 4)	-0.6	4.2E-02
AT5G63590	FLS (Flavonol synthase)	-0.90	1.8E-03
AT5G13930	ATCHS/CHS/TT4 (CHALCONE SYNTHASE)	-1.01	1.0E-03

Table 3.2. bZIP11-regulated genes involved in general sugar metabolism

The table shows a list of selected bZIP11 target genes involved in general sugar metabolism. Differential expression compared to wt is given as log2 values.

AGI	Annotation	Diff. exp.	p value
AT4G15530	PPDK (PYRUVATE ORTHOPHOSPHATE DIKINASE)	2.26	1.2E-09
AT5G26340	MSS1 (SUGAR TRANSPORT PROTEIN 13)	1.97	6.2E-08
AT3G53620	ATPPA4 (ARABIDOPSIS THALIANA PYROPHOSPHORYLASE 4)	1.48	1.5E-03
AT5G57655	xylose isomerase family protein	1.33	9.2E-07
AT2G21330	fructose-bisphosphate aldolase, putative	1.30	1.0E-03
AT3G49160	pyruvate kinase family protein	0.94	1.4E-04
AT1G12850	phosphoglycerate/bisphosphoglycerate mutase family protein	0.93	3.7E-03
AT1G09420	G6PD4 (GLUCOSE-6-PHOSPHATE DEHYDROGENASE 4)	0.74	1.5E-03
AT3G06483	PDK (PYRUVATE DEHYDROGENASE KINASE)	0.74	4.3E-02
AT1G12780	UGE1 (UDP-D-GLUCOSE/UDP-D-GALACTOSE 4-EPIMERASE 1)	0.73	2.1E-02
AT1G55120	ATFRUCT5 (BETA-FRUCTOFURANOSIDASE 5)	0.68	2.2E-02
AT3G62620	sucrose-phosphatase-related	0.65	1.9E-02
AT5G48300	ADG1 (ADP GLUCOSE PYROPHOSPHORYLASE SMALL SUBUNIT 1)	0.64	3.2E-02
AT5G64380	fructose-1,6-bisphosphatase family protein	0.51	4.5E-02
AT4G26270	phosphofructokinase family protein	0.50	4.7E-02
AT3G43190	SUS4	0.47	3.4E-02
AT4G17260	L-lactate dehydrogenase, putative	-0.43	2.1E-02
AT2G30130	ASL5 (phosphoenolpyruvate carboxykinase1)	-0.56	4.1E-02
AT5G52560	ATUSP (ARABIDOPSIS THALIANA UDP-SUGAR PYROPHOSPHORYLASE)	-0.80	2.7E-02
AT3G19480	D-3-phosphoglycerate dehydrogenase, putative / 3-PGDH, putative	-1.03	3.1E-02
AT5G04120	phosphoglycerate/bisphosphoglycerate mutase family protein	-1.27	1.3E-04
AT1G61800	GPT2 (glucose-6-phosphate/phosphate translocator 2)	-1.67	1.3E-08

Table 3.3. bZIP11-regulated genes involved in trehalose metabolism

The table shows a list of selected bZIP11 target genes involved in trehalose metabolism. Differential expression compared to wt is given as log2 values.

AGI	Annotation	Diff. exp.	p value
AT4G24040	ATTRE1/TRE1 (TREHALASE 1)	3.64	3.2E-11
AT1G60140	ATTPS10 (TREHALOSE PHOSPHATE SYNTHASE)	1.11	4.7E-05
AT5G10100	trehalose-6-phosphate phosphatase, putative	0.85	2.3E-02
AT2G18700	ATTPS11 (Arabidopsis thaliana trehalose phosphatase/synthase 11)	0.80	3.6E-02
AT1G06410	ATTPS7 (Arabidopsis thaliana trehalose-phosphatase/synthase 7)	0.52	1.4E-02
AT5G51460	ATTPPA (Arabidopsis thaliana trehalose-6-phosphate phosphatase)	0.12	8.0E-01

ASN1 (*At3g47340*), *GS2* (*At5g35630*) and *ProDH2* (*At5g38710*) are all bZIP11-induced genes, the products of which are directly involved in the synthesis or degradation of amino acids. *ASN1* and *GS2* encode asparagine and glutamine synthetic genes, respectively, while *ProDH2* encodes a proline degrading enzyme. Among the bZIP11-induced targets are also several aminotransferase encoding genes: *At1g77670* (aminotransferase class I and II family protein), *AGT3* (*At2g38400*), *AGT2* (*At4g39660*), *ASP3* (*At5g11520*) and *At5g36160* (aminotransferase-related).

More than ten genes encoding enzymes involved or putatively involved in glycolysis were found to be up or down regulated upon bZIP11 induction (Figure 3.2). Most interestingly among this set, *PPDK* (*At4g15530*), usually associated with C4 metabolism was found to be induced by bZIP11. Other putative bZIP11 target-genes involved in sugar metabolism included trehalose metabolism genes. Several trehalose phosphate synthase encoding genes (*TPS7*, *TPS10* and *TPS11*), as well as the only known trehalase-encoding gene in Arabidopsis (*TRE1*) were induced by bZIP11.

Taken together, the expression of many genes involved in different aspects of metabolism was affected by bZIP11. The products of all of these genes bear the potential to alter metabolite levels in the plant.

Nuclear bZIP11 changes the levels of hundreds of metabolites

Many of the bZIP11-regulated genes identified previously are involved in metabolic processes. Therefore, the possible effect of bZIP11 on the metabolome was investigated. Using GC-MS, the general metabolic status of seedlings overexpressing the bZIP11:HBD construct was analyzed following incubation in the presence or absence of dex. The results were compared to those obtained from wt seedlings in the presence or absence of dex. Seedlings in which bZIP11:HBD was localized to the nucleus by dex showed a dramatically altered overall metabolic status (Figure 3.3). In both independent transgenic lines, the levels of over 1000 mass peaks, likely originating from hundreds of metabolites was altered significantly. Wt seedlings showed little metabolic response to dex, in agreement with the small effects of dex on the transcriptome in wt plants.

Principal component analysis was performed on the raw GC-MS peak data after alignment with Metalign to determine the global nature of the changes in metabolite levels caused by dex in both transgenic lines and the wt. From Figure 3.3 it is clear that, mock (ethanol) treated, as well as dex treated wt seedlings group together. This indicates that dex treatment had little effect on wt seedlings compared to the effects of dex on the

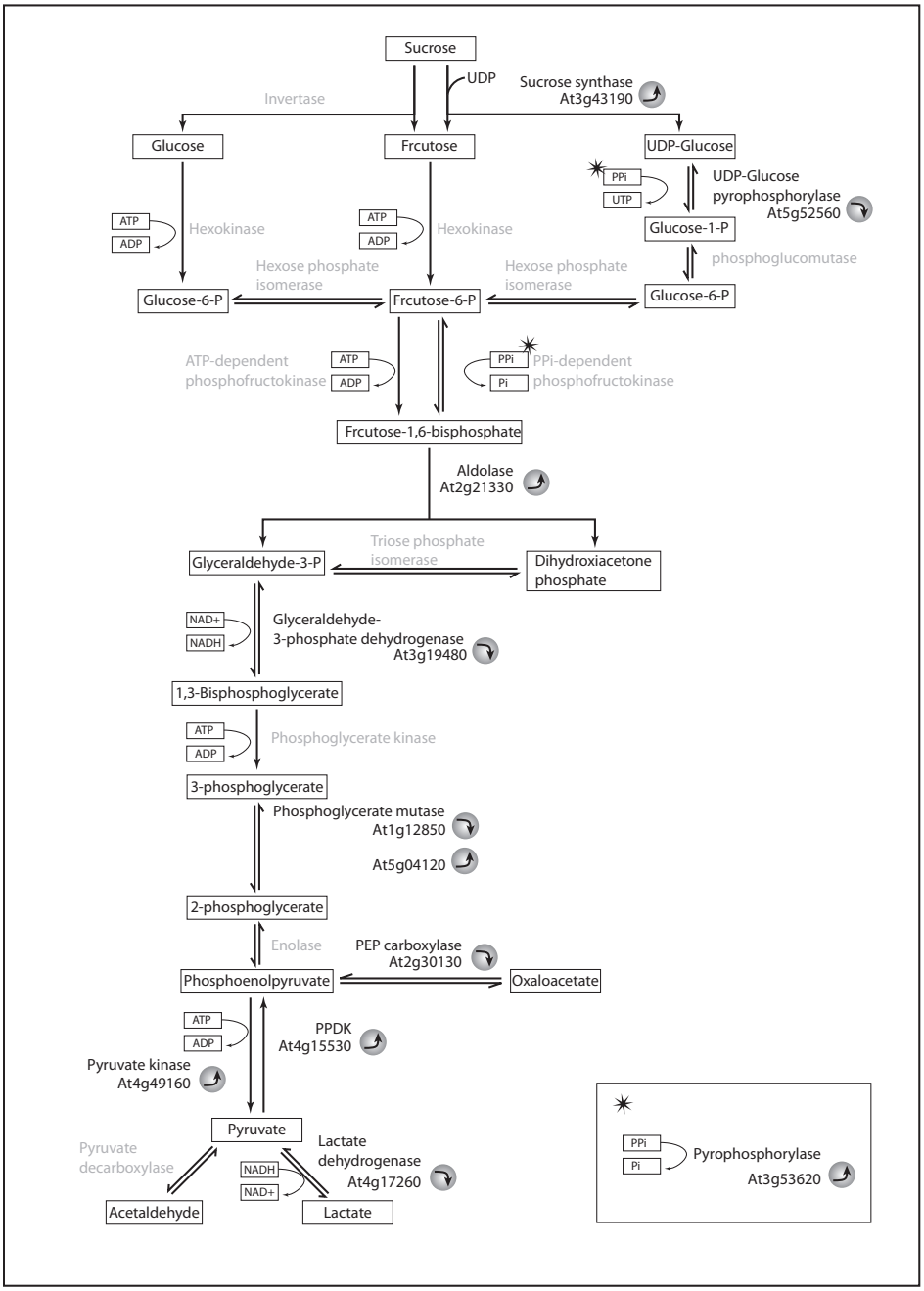

Figure 3.2. bZIP11 target genes involved in glycolysis.

The figure shows a schematic representation of glycolysis. Genes enconding proteins that are annotated to be involved in metabolism are indicated. Upward and downward pointing arrows indicate induction and repression of the genes by bZIP11. Putatively affected proteins are shown in black. The

square shows an irreversible reaction facilitated by PYROPHOSPHORYLASE4 (AT3G53620) that will pull the reactions indicated by a star toward sucrose synthesis, by limiting pyrophosphate (PPi).

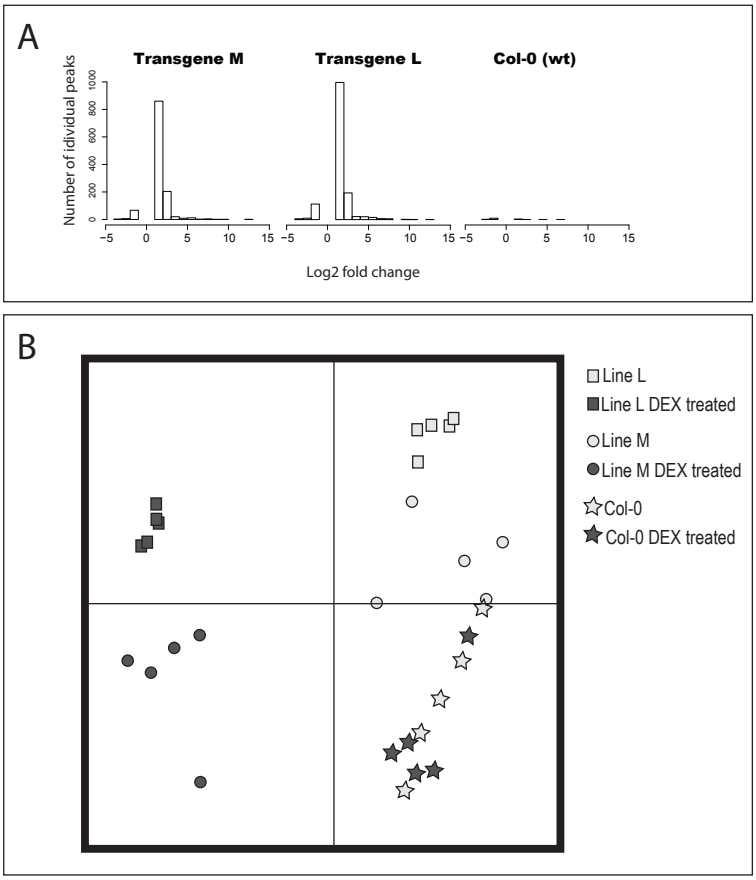

Figure 3.3. bZIP11 reprograms metabolism, as determined by GC-MS analysis

A) Histogram of the peak intensities showing more than two-fold change in intensity in response to dex. Intensity changes are grouped according to base 2 log fold changes. Peaks showing less than two-fold changes are removed for clarity. In the individual transgenic lines L and M, the bZIP11:HBD fusion protein was induced by dex, leading to significant changes in the expression of over 1000 GC/MS peaks in both lines. In wt seedlings dex had very little effect. B) PCA plot of GC-MS data of mock (ethanol) or dex treated wt and bZIP11:HBD transgenic lines L and M. Mock and dex treated wt seedlings group together, indicating that dex treatment had little effect on wt seedlings. Ethanol treated transgenic seedlings differed from wt seedlings, but in a similar way as both L and M seedlings grouped in the same plot quadrant. The shift of both L and M seedlings along one component axis upon dex treatment indicates the dramatic effect of nuclear localization of the bZIP11:HBD construct on metabolism.

transgenic seedlings. Ethanol treated transgenic seedlings differed from wt seedlings, but only according to one component. It might be expected that L and M seedlings behave in a similar way, as they express the same construct. Upon dex treatment, both L and M seedlings showed a similar dramatic shift along one component axis, indicating the dramatic effect of nuclear localization of the bZIP11:HBD construct on metabolism.

Further analysis of the GC-MS data revealed different classes of metabolites to be affected. Nuclear bZIP was able to alter levels of free amino acids, as well as intermediates of the citric acid cycle and sugars (Figure 3.4). Among the amino acids, most notably, serine and glutamic acid levels decreased, whereas phenylalanine levels rose upon dex induction of bZIP11. Levels of malic acid and succinic acid, both intermediates in the citric acid cycle, were lowered upon bZIP11 induction, suggesting a general decrease in citric acid cycle activity. The levels of sucrose, glucose and fructose increased in the bZIP11:HBD transgenic lines after induction with dex. Changes in amino acid and sugar levels were confirmed by independent measurements (see next section).

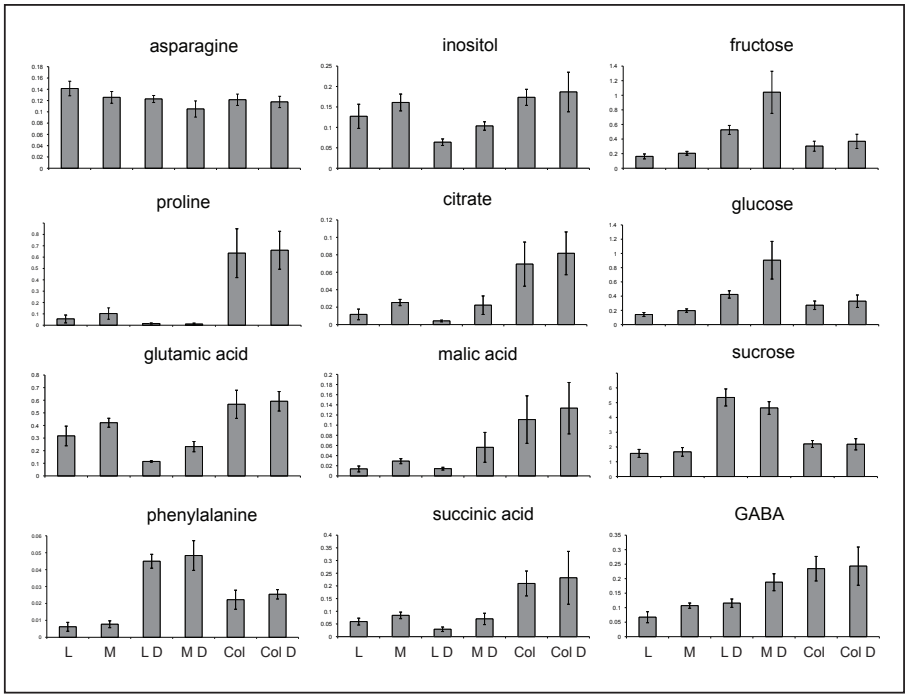

Figure 3.4. Metabolite levels in bZIP11:HBD transgenic seedlings compared to wt.
The graphs show the levels of selected metabolites as determined by GC-MS. Metabolite levels in wild type (Col) and two independent transgenic lines (L and M) expressing a dex inducible bZIP11 construct are shown before and after dex (Col D, L D and M D) treatment.

Nuclear bZIP11 modulates the levels of free amino acids

ASN1 and *ProDH2* (*At5g38710*) are induced rapidly after nuclear entry of bZIP11:HBD and were also expressed to higher levels in the leaderless lines in which *bZIP11* expression is directed by its own promoter (Chapter 2). These genes encode enzymes involved in biosynthesis or breakdown of amino acids. *ASN1* encodes an asparagine synthetase, and *ProDH2* (*At5g38710*) encodes a proline dehydrogenase. Therefore, the amino acid levels of seven-day-old transgenic and wt seedlings grown on high sucrose concentrations (100 mM), treated with 10 μM dex for six hours were analyzed (Table 3.4). Dex treatment affected the levels of several amino acids in the transgenic lines. Proline levels were reduced over five-fold. In the same samples phenylalanine levels were increased approximately five-fold. Less prominent changes in amino acid levels were also observed. Alanine levels were slightly increased and leucine and isoleucine levels decreased. Dex treatment did not affect amino acid levels in the wt seedlings. Taken together, these result confirmed the notion that the effects of bZIP11 on transcription of putative targets genes results in significant changes in the amino acid levels, as shown above by the un-biased approach of GC-MS based metabolic profiling.

Interestingly, amino acid measurements of dex-induced transgenic seedlings grown on media containing low sucrose concentrations showed different results. While proline levels were still severely lowered, the earlier observed effects on alanine and isoleucine were less prominent while the effect on phenylalanine was absent (data not shown).

Nuclear bZIP11 modulates sugar levels

Messenger RNA profiling of bZIP11:HBD overexpressing seedlings showed the expression of genes involved in glycolysis and the citric acid cycle to be affected after induction with dex (Chapter 2). Both glycolysis and the citric acid cycle potentially alter sugar levels in plants. Global metabolic profiling indeed revealed levels of intermediates of the citric acid cycle as well as sugars levels to be altered. Therefore, sugar levels were confirmed independently, using transgenic seedlings in which bZIP11 transcription was under the control of a dex inducible promoter. As observed in the GC-MS based profiling experiment, enzymatic analysis of sugar levels revealed bZIP11 to increase sugar levels in seedlings. Sucrose as well as glucose and fructose levels increased after induction of bZIP11 with dex, while the overall ratio of these sugars within seedlings remained basically unaltered (Figure 3.5).

Table 3.4. Amino acid levels of dexamethasone treated wt and *bZIP11* transgenic seedlings

Seven-day-old seedlings were grown in liquid media containing 100mM sucrose supplemented with 10 mM dexamethasone (dex) or mock control treatment by the solvent for 6-h. The levels are presented as µmol per g fresh weight of the seedling ± standard deviation. Methionine, cysteine and tryptophan levels were not detected. All measurements were performed in five independent replicates, except for dex treated M seedlings, with four independent replicates. Statistical significant ($p < 0.05$, ANOVA) differences from wt levels are indicated with an asterisk.

Amino acid	Wild type		Transgene L		Transgene M	
	control	dex	control	dex	control	dex
Alanine	7.49 ±1.73	7.37 ±1.72	8.46 ±1.46	12.47 ±1.87	6.65 ±0.92	9.38 ±0.70
Arginine	3.25 ±0.32	3.34 ±0.38	4.07 ±0.66	4.03 ±0.48	3.36 ±0.35	4.40 ±0.66
Asparagine	7.19 ±0.48	7.84 ±0.37	6.49 ±0.51	6.38 ±0.59	5.99 ±0.98	6.43 ±0.69
Aspartate	1.19 ±0.08	1.32 ±0.22	1.48 ±0.27	1.18 ±0.14	1.23 ±0.15	1.33 ±0.11
Glutamine	43.0 ±7.8	51.3 ±10.1	49.8 ±14.8	52.7 ±20.8	32.7 ±3.4	38.4 ±3.3
Glutamate	5.83 ±0.65	6.42 ±0.48	6.61 ±2.00	4.70 ±1.11	5.55 ±0.83	5.09 ±1.13
Glycine	6.25 ±2.77	6.68 ±3.37	8.41 ±5.44	7.79 ±4.51	4.54 ±2.57	4.83 ±0.98
Histidine	0.59 ±0.10	0.64 ±0.11	0.63 ±0.18	0.66 ±0.22	0.47 ±0.04	0.55 ±0.04
Leucine	0.28 ±0.04	0.23 ±0.10	0.24 ±0.05	0.18 ±0.08	0.27 ±0.07	0.11 ±0.01
Lysine	0.32 ±0.05	0.26 ±0.10	0.28 ±0.07	0.27 ±0.04	0.35 ±0.08	0.23 ±0.03
Isoleucine	0.19 ±0.05	0.19 ±0.05	0.24 ±0.04	0.19 ±0.09	0.21 ±0.06	0.13 ±0.03
Phenylalanine	0.24 ±0.04	0.23 ±0.04	0.22 ±0.08	**1.27 ±0.15***	0.17 ±0.04	**0.90 ±0.16***
Proline	3.78 ±0.52	3.48 ±1.19	3.16 ±0.82	**0.45 ±0.14***	3.75 ±0.65	**0.56 ±0.12***
Serine	4.01 ±0.54	4.06 ±0.33	4.02 ±0.47	4.34 ±0.36	3.66 ±0.46	4.58 ±0.35
Threonine	2.32 ±0.19	2.34 ±0.10	3.11 ±0.73	3.04 ±0.74	2.51 ±0.38	2.97 ±0.22
Tyrosine	0.36 ±0.08	0.28 ±0.13	0.34 ±0.15	0.52 ±0.06	0.42 ±0.07	0.56 ±0.08
Valine	0.52 ±0.03	0.50 ±0.08	0.59 ±0.05	0.80 ±0.31	0.51 ±0.09	0.52 ±0.03
Total	87.4 ±12.3	97.2 ±13.4	98.7 ±25.3	101.4 ±29.9	72.7 ±7.7	81.3 ±4.6

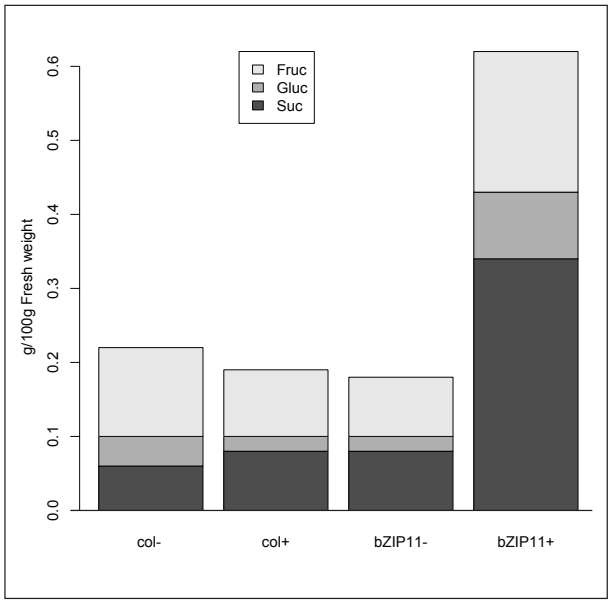

Figure 3.5. Induced bZIP11 expression results in increased sugar levels

Sugar levels of seven-day-old transgenic and wt seedlings were analyzed before and after dex induction. Seedlings expression dex induced *bZIP11* showed a significant increase in sucrose, glucose and fructose levels. The overall ratio of sugars remained unaltered.

Trehalase activity can be induced by bZIP11

Independent experiments have shown that bZIP11 affects the expression of genes involved in metabolism as well as the levels of many metabolites. Many bZIP11 target genes are involved in metabolic processes. Metabolites involved in these processes are also affected by bZIP11. These data suggest that altered gene expression caused by bZIP11 results in an altered metabolic status in transgenic plants. Trehalase activity in bZIP11 over-expressing seedlings was measured to confirm the direct link between increased gene epression and increased enzymatic activity. The trehalase gene *TRE1* is induced upon induction of the bZIP11:HBD construct. *TRE1* encodes the only known enzyme in Arabidopsis converting trehalose into glucose. Figure 3.6 shows that dex treatment of bZIP11:HDB transgenic seedlings, but not wt seedlings, increased trehalase activity. This indicates that increased mRNA levels of the *TRE1* gene coincide with elevated enzyme activity. Interestingly, bZIP11:HBD transgenic seedlings showed a trehalose resistant phenotype when induced with dex. In wt seedlings elevated levels of trehalose in the medium cause growth arrest. bZIP11:HBD over-expressing seedlings, however, were able to overcome this growth arrest upon induction with dex (Figure 3.6).

Discussion

bZIP11 possibly affects growth by directing metabolism

The metabolic profile of plants has been shown to be a predictive feature for biomass accumulation (Meyer *et al.*, 2007). Interestingly, many target genes of bZIP11 are involved in metabolic processes. This might indicate a central role of bZIPs in the regulation of growth. *bZIPs* of the S1- and C-class may be important signal integrators as their expression is differentially affected by many internal and external signals. In the study by Meyer *et al.* sucrose, glutamine and phenylalanine were among compounds most significantly negatively correlating to biomass. The levels of these metabolites were increased in bZIP11 overexpressing plants, correlation to the impaired growth phenotype of bZIP11 overexpressors. Intermediates of the TCA cycle, such as succinate, citrate and malate also have a negative correlation to biomass. Here it was found that the levels of these compounds were also affected in bZIP11:HBD transgenic plants. Dex-induction did not lead to an increase in the levels of these compounds, in contrast with the effects observed on other metabolites that have a negative correlation to growth. However, these

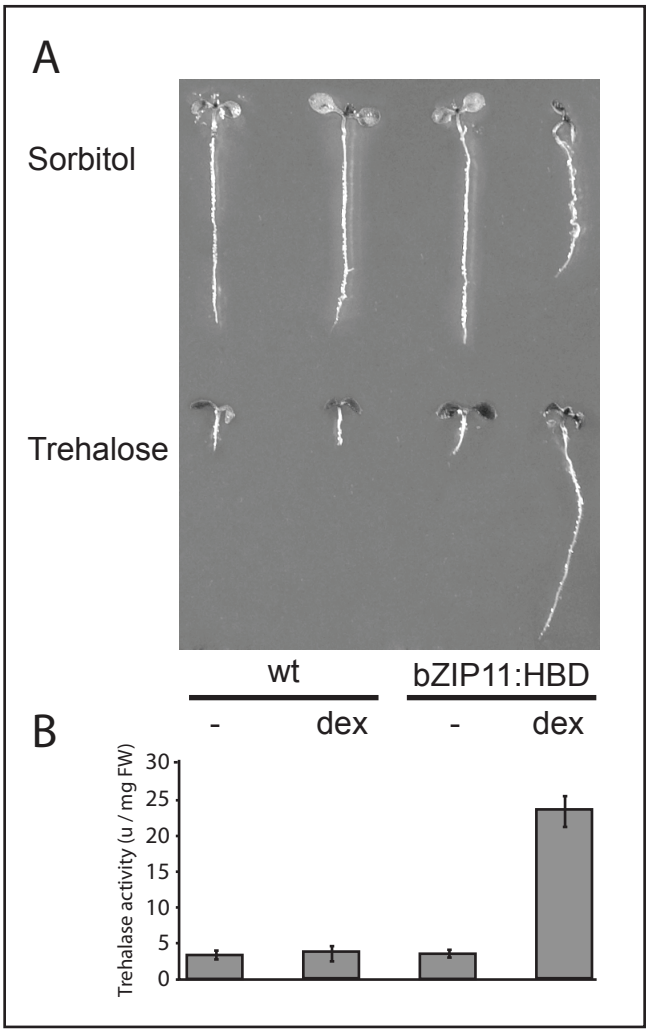

Figure 3.6. Increased bZIP11 activity confers resistance to trehalose

(A) Trehalose (100 mM) in the medium causes growth arrest in Arabidopsis seedlings. In transgenic seedlings expressing a bZIP11:HBD construct growth arrest is overcome by induction of bZIP11 by dex. Control treatment with 100 mM sorbitol did not lead to growth arrest. (B) Trehalase activity of seedlings grown on sorbitol containing medium was determined. Trehalase activity in bZIP11:HBD expressing seedlings is strongly increased upon dex induction. Likely, increased trehalase activity is responsible for the observed growth on trehalose containing medium.

compounds were present at lower levels in bZIP11:HBD overexpressing plants compared to wild type plants even without dex-induction. This suggests an indirect effect of the bZIP11:HBD contruct on the levels of these TCA intermediates. Possibly, this effect explains the lack of correlation to the observations made by Meyer *et al*. (2007)

Effects on amino acid metabolism

Amino acids are synthesized from ammonium in a number of reactions. The first step in this process is the fixation of ammonia into glutamine by cytoplasmic or plastidic glutamine synthetases. In chloroplasts glutamine synthetase mainly reassimilates photorespiratory ammonium. The chloroplastic *GS2* (*At5g35630*) was up regulated by induction of bZIP11. In accordance with this finding, glutamine levels were increased by bZIP11 induction. Following fixation of ammonia into glutamine and glutamate, nitrogen is transferred to other amino acids by aminotransferases, several of which were up regulated by bZIP11 (Table 3.1). Glutamine is used for asparagine synthesis by *ASN1* (*At3g47340*), which was also up regulated by bZIP11. Asparagine levels however, were unaffected by bZIP11, likely because seedlings in these experiments already contain high concentrations of nitrogen rich amino acids (Table 3.4) due to abundance of nitrogen in the growth medium. In nature low sugar availability will lead to reallocation of nitrogen into asparagine, which has a higher C/N ratio than glutamine and glutamate. This process can be facilitated through induction of *ASN1* by bZIP11, which is no longer translationally repressed under such low sugar conditions. Proline was the most significantly affected amino acid by induction of bZIP11. Dex treatment of transgenic seedlings caused proline levels to decrease seven-fold. Likely, this dramatic decrease is caused by the induction of both known proline-degrading enzymes in Arabidopsis. *ProDH* (*At3g30775*) as well as *ProDH2* (*At5g38710*) were both up regulated by bZIP11.

Induction of bZIP11 caused a five-fold increase in phenylalanine levels. A possible reason for higher phenylalanine levels could be the increased levels of a gene encoding a prephenate aminotransferase enzyme (*AGT3*, *At2g38400*) (Ehlting *et al.*, 2005) that synthesizes arogenate, the proposed precursor of both tyrosine and phenylalanine. *PAL4* (*At3g10340*) is down regulated. PALs are essential for the synthesis of secondary phenolic compounds in plants from phenylalanine. Furthermore, a gene involved in the synthesis of one of the largest groups of phenolics (flavonoids) is down regulated. *CHS*/*TT4* (*At5g13930*) encodes chalcone synthase, a key enzyme involved in the biosynthesis

of flavonoids, required for the accumulation of purple anthocyanins in leaves and stems.

Interestingly, the effect on phenylalanine levels was absent when seedlings were grown in low sucrose conditions. Potentially, this is a result of sugar signaling through differential bZIP dimer formation. Under high sucrose conditions, all S1-class bZIPs will be translationally repressed (Rook *et al.*, 1998b; Wiese *et al.*, 2004; Weltmeier *et al.*, 2009). This will result in the presence of more C-class monomers that would otherwise bind to the now absent S1-class monomers. Vice-versa, under low sucrose conditions different effects of bZIP11 overexpression might be observed due to the deficiency of specific C-class monomers.

Effects on sugar metabolism

Induction of bZIP11 caused an increase in glucose, fructose and sucrose levels. Interestingly, the expression of several enzymes (putatively) involved in glycolysis was affected (Figure 3.2). The induction of bZIP signaling by low sugar levels thus suggests a role for bZIP11 in the preservation of sugars in low energy conditions. The increase of sucrose levels provides a possible feedback mechanism in sugar signaling, due to the repression of bZIP translation by sucrose. Moreover, the conservation of sugars by bZIP11 is in agreement with the energy conserving effects of bZIP-activating SnRK1 kinases during stress (Baena-Gonzalez and Sheen, 2008). The SnRK1 kinases will also be affected by the feedback mechanism caused by the increase of sucrose levels, as sucrose represses SnRK1 activity (Baena-Gonzalez *et al.*, 2007).

Trehalose metabolism was also affected by bZIP11. The trehalose-6-phosphate (T6P) synthase encoding genes *TPS7*, *TPS10* and *TPS11* (*At1g06410*, *At1g60140* and *At2g18700*, respectively) as well as the T6P phosphatase enconding genes *TPPA* and *TPPB* (*At5g51460* and *At2g18700,* respectively) were up regulated by bZIP11. Interestingly, TPS11 was previously shown to be a SnRK1 target gene (Baena-Gonzalez and Sheen, 2008). All these enzymes potentially affect cellular T6P levels, but T6P levels in bZIP11 overexpressing seedlings, still await determination. Recently, T6P has been shown to be an inhibitor of SnRK1 (KIN10/KIN11) protein kinases (Zhang *et al.*, 2009). Together with the proposed regulation of bZIP transcriptional activity by KIN10/KIN11 (Baena-Gonzalez *et al.*, 2007), this represents another possible feedback mechanism in sugar signaling. Next to T6P metabolic enzymes, the only known gene in the Arabidopsis genome encoding a trehalose-degrading enzyme (*TRE1*, *At4g24040*) was

induced by bZIP11. In agreement with the increased levels of *TRE1* mRNA, trehalase activity was induced by bZIP11. Moreover, seedlings overexpressing the dex inducible bZIP11 construct showed resistance to high concentrations of trehalose in the medium when treated with dex. Treahlose causes growth arrest in plants by increasing T6P levels (Schluepmann *et al.*, 2003). T6P is believed to signal high sugar availability to stimulate growth. Overstimulation by high T6P levels in absence of sugars needed for growth will therefore hinder growth. Possibly, bZIP11 overexpressing seedlings are able to overcome growth arrest by converting trehalose into glucose that can be utilized for growth.

Taken together, *bZIP11* expression strongly affects metabolism. The data presented here suggest several possible feedback mechanisms in the crosstalk between sugar signaling and metabolism. Furthermore, the conservation of bZIP transcription factors, the translational repression of S1-class bZIPs through a conserved uORF and the conservation of bZIP target genes suggest that these regulatory pathways are conserved among plants.

Materials and Methods

Plant material and transformation

In all experiments Arabidopsis plants (accession Col-0, Ohio stock nr CS60000) were grown in liquid culture in half strength MS media (Duchefa, Haarlem, the Netherlands) supplemented with 0.5 g/l MES, pH 5.8 and sugars as indicated. Seedlings were grown in constant fluorescent light (100 microE/m^2) in a Microclima cabinet (Snijders, Tilburg the Netherlands), on a rotary shaker (60 rpm). Gas sterilized seeds were stratified for three to four days.

For amino acid analysis, Arabidopsis seedlings were grown in liquid media for seven days in continuous light in separate flasks. Dexamethasone, 10 μM in 0.1% ethanol (Sigma-Aldrich, Spruce St, St. Louis, USA) or 0.1% ethanol (mock treatment) was added and the plants were incubated for indicated times before harvesting.

Amino acid analysis

Plant material was flash-frozen in liquid nitrogen and ground. Amino acids were extracted using 80% methanol at room temperature followed by a re-extraction using 20% methanol.

The two extracts were pooled; freeze dried and stored at -80ºC until redissolved for analysis. Analysis of amino acids was performed by cation exchange chromatography on a Jeol JLC-500/V amino acid analyzer. Separation was achieved by using buffers with different pH and ion concentrations. After separation, amino acids were visualized by reaction with ninhydrin and detected by spectrophotometry at 570/440 nm. Quantification of amino acids was achieved with the Empower software (Waters, MA, USA).

Trehalase activity measurements

Seeds were sterilized and plated onto ½ MS solid medium with either 100 mM sorbitol or 100 mM trehalose and stratified at 4ºC in the dark for two days. Plates were transferred to a growth chamber and grown for 7 days in long day conditions. For protein extraction, 50 mg fresh weight of seedlings per sample was lyophilized, powdered, and vortex suspended in 500 ml of extraction buffer containing morpholinoethane sulfonic acid/K^+ (50 mM, pH 6.3), EDTA (1 mM), phenylmethyl-sulfonylfluoride (1 mM), Triton X-100 (0.01%, w/v), and insoluble polyvinylpyrrolidone (1%, w/v; Polyclar AT).

The suspension was incubated for at least 2 h at 0°C and centrifuged (13,000 rpm, 15min) and the supernatant was isolated. A total volume of 2.5 ml was passed through PD10 Columns (GE Healthcare), equilibrated with 5 mM phosphate buffer, pH 6.3, and eluted with 3.2 ml 5 mM phosphate buffer, pH 6.3. Next, 350 µl 250 mM trehalose was added to 3.15 ml elute, mixed and incubated at 37ºC for two hours. Glucose released from this enzymatic reaction was measured by D-Glucose test kit (Boehringer Mannheim / R-Biopharm).

Sugar measurements

Seven-day-old seedlings expressing a dex RNA-inducible *bZIP11* construct and wt seedlings were grown in liquid medium (1/2 MS). After 6-h dex treatment, sugar levels were measured using the Sucrose/D-Glucose/D-Fructose test kit (Boehringer Mannheim / R-Biopharm) according to the manufacturors instructions.

GC-MS analysis of metabolism

Gas chromatography mass spectrometry-based metabolite profiling was performed as described by Fu *et al.* (Fu *et al.*, 2009). Frozen and ground samples of approximately 50 mg fresh weight were weighed accurately in a 2 ml Eppendorf vial with punctured

lid, and 1.4 ml cold methanol and ribitol as internal standard were added. Samples were extracted for 20 min in a shaking water bath at 70°C. After centrifuging at 21000 g for 5 min, 500 µL of the supernatant was transferred to a new 2 ml Eppendorf vial. A two-phase extraction method was used to separate polar and apolar compounds by adding 500 µl water and 700 µl chloroform (Lisec *et al.*, 2006). After vortexing and centrifugation at 21000 g for 5 min, 200 µL of the polar phase was dried under vacuum. The dried extracts were derivatized by methoximation and trimethylsilylation essentially as described by Lisec *et al.* (2006) using a CombiPal robot for on-line derivatization. Octadecane was added to the o-methylhydroxiaminehydrochloride in pyridine to check for accuracy of the pipetting of the robot. Samples were injected with an Optic3 injector (ATAS) at 70°C with a gradient of 6°C/sec to 240°C using a split flow of 10 ml and a column flow of 2 ml in a GC6890N gas chromatograph (Agilent Technologies) on a ZB50 capillary column (30 m x 0.32 mm i.d., 0.25 µm DF; Phenomenex) with a column temperature of 70°C for 2 minutes and a gradient of 10°C/min to 310°C and a final time of 3 min. The GC was coupled to a Pegasus III time-of-flight mass spectrometer (LECO) and compounds were detected at a scanning rate of 20 spectra per second (mass 50-600). Metalign™ software (www.metalign.nl) was used to extract all mass signals detected and to align these signals across the samples (Tikunov *et al.*, 2005).

“In nature we never see anything isolated, but everything in connection with something else which is before it, beside it, under it and over it.”

-Johann Wolfgang von Goethe

Chapter 4: Gene expression analysis reveals differential functions of bZIP dimers in protoplasts

Micha Hanssen, Andrea Ehlert, Sjef Smeekens, Wolfgang Dröge-Laser and Johannes Hanson

Summary

Basic region/leucine zipper motif (bZIP) proteins comprise a large family of plant transcription factors. bZIP transcription factors affect gene expression by binding DNA as specific homo- or heterodimeric complexes. The specific transcriptional activity of different related dimer species has not been studied in great detail in plants. Unraveling the functions of the complex network of different bZIP dimers in plants demands determination of the target genes of each dimer species. For this purpose, *Arabidopsis thaliana* mesophyll protoplasts were transfected with plasmid DNA encoding two different bZIP transcription factors: bZIP10 (At4g02640) and bZIP11 (At4g34590). High bZIP10 protein levels hardly affected gene expression, as revealed by global gene expression analysis. Increased expression of bZIP11 strongly affected gene expression, but when bZIP11 was transfected together with bZIP10 a major synergistic effect on gene expression was observed. These results provide solid evidence that different bZIP dimers have differential effects on gene expression.

Introduction

Basic region/leucine zipper motif (bZIP) proteins form a large family of transcription factors defined by a basic region that interacts in a sequence specific manner with the major groove of DNA through hydrogen bonding, and an amphipatic leucine zipper region that is responsible for dimerization. The Arabidopsis genome holds a total of 75 bZIP genes, only a handful of which have been functionally described in the literature. Jakoby *et. al.* (Jakoby *et al.*, 2002) divided all bZIPs in Arabidopsis into classes, based on shared domains in their sequence. One particular class, the S-class consists of low molecular weight proteins that lack other known domains but harbor an unusually long zipper domain of 8-9 leucines. An S-subclass (S1) of bZIPs is characterized by the presence of a highly conserved uORF in the 5' leader of its messenger RNA that allows translational control by sucrose (Rook *et al.*, 1998c; Wiese *et al.*, 2004; Weltmeier *et al.*, 2009). The best-studied member of this class, bZIP11, is involved in the regulation of amino acid metabolism, by affecting the expression of *ASN1* and *ProDH2* (Hanson *et al.*, 2008).

In order to bind DNA and affect gene expression, bZIP transcription factors must form dimers. S1-class bZIPs preferably dimerize with members of the C-class,

although it has been shown that S1-class bZIPs, in particular bZIP11, are also able to form homodimers (Ehlert *et al.*, 2006a). C-class bZIPs in contrast, do not tend to form homodimers. The formation of bZIP homo- and heterodimers potentially offers tremendous combinatorial flexibility to regulate gene transcription. For instance, *ProDH* gene expression is known to be regulated by several heterodimeric complexes consisting of a C-class and an S1-class bZIP protein (Satoh *et al.*, 2004; Weltmeier *et al.*, 2006). Other genes however, seem to be regulated by specific bZIP proteins, as was determined by micro-array analysis (Hanson *et al.*, 2008). So far, the possibility of different gene-regulatory functions of different dimers, consisting of related bZIP proteins has not been investigated. For this purpose, 5'HA-tagged bZIP10 and 5'HA-tagged bZIP11 enconding plasmids were transfected into *Arabidopsis thaliana* mesophyll protoplasts, either alone or together. Overexpression of only bZIP11 would result in effects on gene expression by mostly homodimers, even though effects of heterodimers involving endogenously present bZIPs cannot be excluded. Changes in gene expression after overexpression of both *bZIP10* and *bZIP11* in the same protoplasts likely represent bZIP10+11 dimer effects. In this manuscript we demonstrate the differential gene-regulatory activity of different bZIP dimers, and thereby show the importance of dimerization specificity in the regulation of gene expression by bZIP transcription factors.

Results

mRNA profiling of mesophyll protoplasts is a suitable system for gene expression analysis

Gene expression in *Arabidopsis* mesophyll protoplasts, transiently overexpressing bZIPs was studied to identify the targets of bZIP transcription factors. Overexpression of *bZIP* genes was achieved by transfecting *Arabidopsis* mesophyll protoplasts with, plasmids encoding 5'HA-tagged bZIP10 or bZIP11, combinations thereof or an empty vector as control. Sixteen hours following transfection, the mRNA expression levels of *bZIP10* and *bZIP11* were 120 and 400-fold higher, respectively, than levels in protoplasts transfected with the empty vector. The bZIP10 and bZIP11 proteins were demonstrated to be abundantly expressed and fully translated by Western analysis, using antibodies directed against the HA-tag (Figure 4.1). After transfection, global gene expression in protoplasts

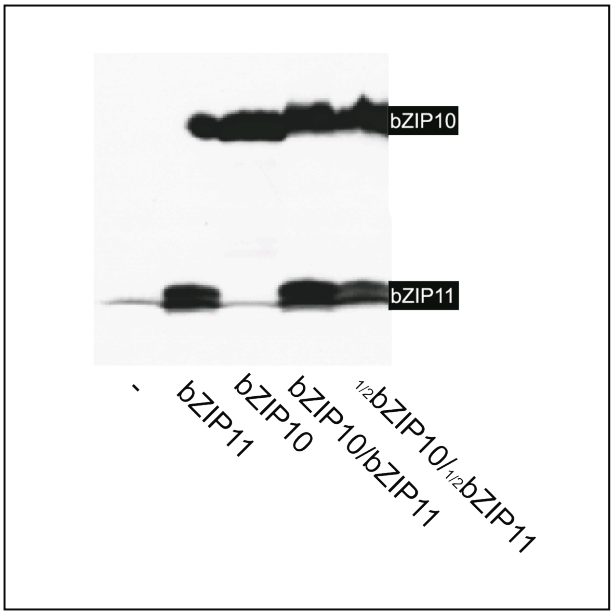

Figure 4.1. bZIPs are highly expressed in transfected protoplasts.
Western analysis after immunoprecipitation with an HA antibody shows that the full-length bZIP fusion proteins are highly abundant in transected protoplasts. Protoplasts were transfected with an empty control vector (-), HA-tagged *35S:bZIP10*, HA-tagged *35S:bZIP11* or both *bZIP10* and *bZIP11* fusion constructs. In the right-most lane results are shown for an experiment where both bZIP fusion constructs were transfected at half the concentration of the plasmids. The boxes right of the blot represent the mobility of bZIP10 and bZIP11 HA-fusion proteins as determined by previous experiments and molecular weitght markers.

was compared using the ATH1 GeneChip®. Next, the expression for each probeset was calculated, a linear model was fit to the data and expression levels of genes were compared (Figure 4.2). Results obtained from the array data were confirmed by quantitative real-time PCR (Table 4.1). In all protoplast preparations, a representative set of about 9000 genes was expressed to levels statistically significant above background. Previous experiments have shown around 11000 genes to be expressed in 7-day-old seedlings (Allemeersch *et al.*, 2005; Hanson *et al.*, 2008). A MAPMAN representation of the 9000 genes expressed in protoplasts shows that genes of all GO terms in the map are well represented (Figure 4.3). Inherent to the protoplast system some classes are underrepresented because of tissue specificity. Root or cell wall specific genes are underepresented in mesophyll protoplasts.

Nonetheless, these findings indicate that the protoplast system is representative of the whole-plant system and suitable for gene expression studies, as demonstrated before (Birnbaum *et al.*, 2005; Baena-Gonzalez *et al.*, 2007).

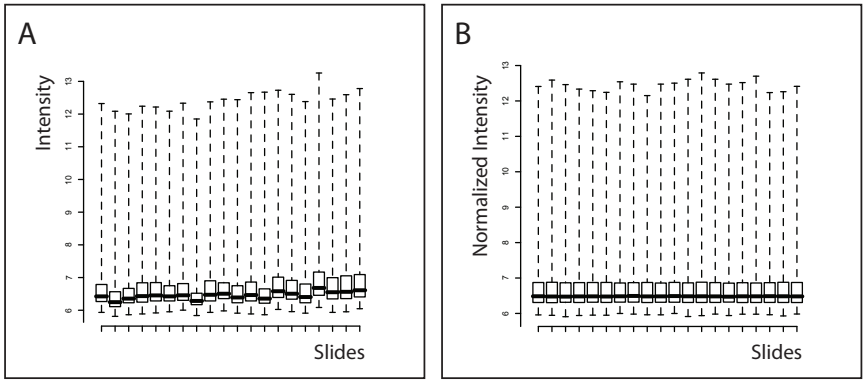

Figure 4.2. Gene expression in transfected protoplasts

The boxplots show average signal intensities over the slides used before (A) and after (B) normalization. The interquartile ranges are depicted as boxes, the dashed lines represent the 95% confidence interval and horizontal bars represent median levels.

Table 4.1. Gene expression levels determined by micro-array are comparable to corresponding levels determined by quantitative real-time PCR

			bZIP10		***bZIP11***		***bZIP10* and *bZIP11***	
AGI	**Gene name**	**Expr level[a]**	**Array**	**Q-PCR**	**Array**	**Q-PCR**	**Array**	**Q-PCR**
AT3G47340	*ASN1*	4.4	0.0	-0.2	1.3	1.3	4.3	3.0
AT5G38710	*ProDH2*	10.0	-0.3	-0.4	0.4	0.6	1.0	0.9
AT1G10070	*ATBCAT-2*	4.7	-0.2	0.0	1.0	1.6	3.4	3.6
AT4G34590	*bZIP11*	8.6	-0.1	-0.3	8.7	13.3	8.7	13.0
AT4G33700		7.4	0.3	-0.2	4.3	4.5	4.4	4.0
AT3G30775	*ProDH*	9.5	0.5	0.5	3.2	4.1	3.6	4.3
AT3G18780	*ACT2*	1.0	-0.1	-0.2	0.0	0.1	2.0	1.4
AT1G15040		9.4	0.2	0.0	4.3	4.3	4.9	4.6
AT4G35770	*SEN1*	10.1	-0.1	-0.3	1.5	2.1	2.8	3.2

[a] Average expression level over all microarrays

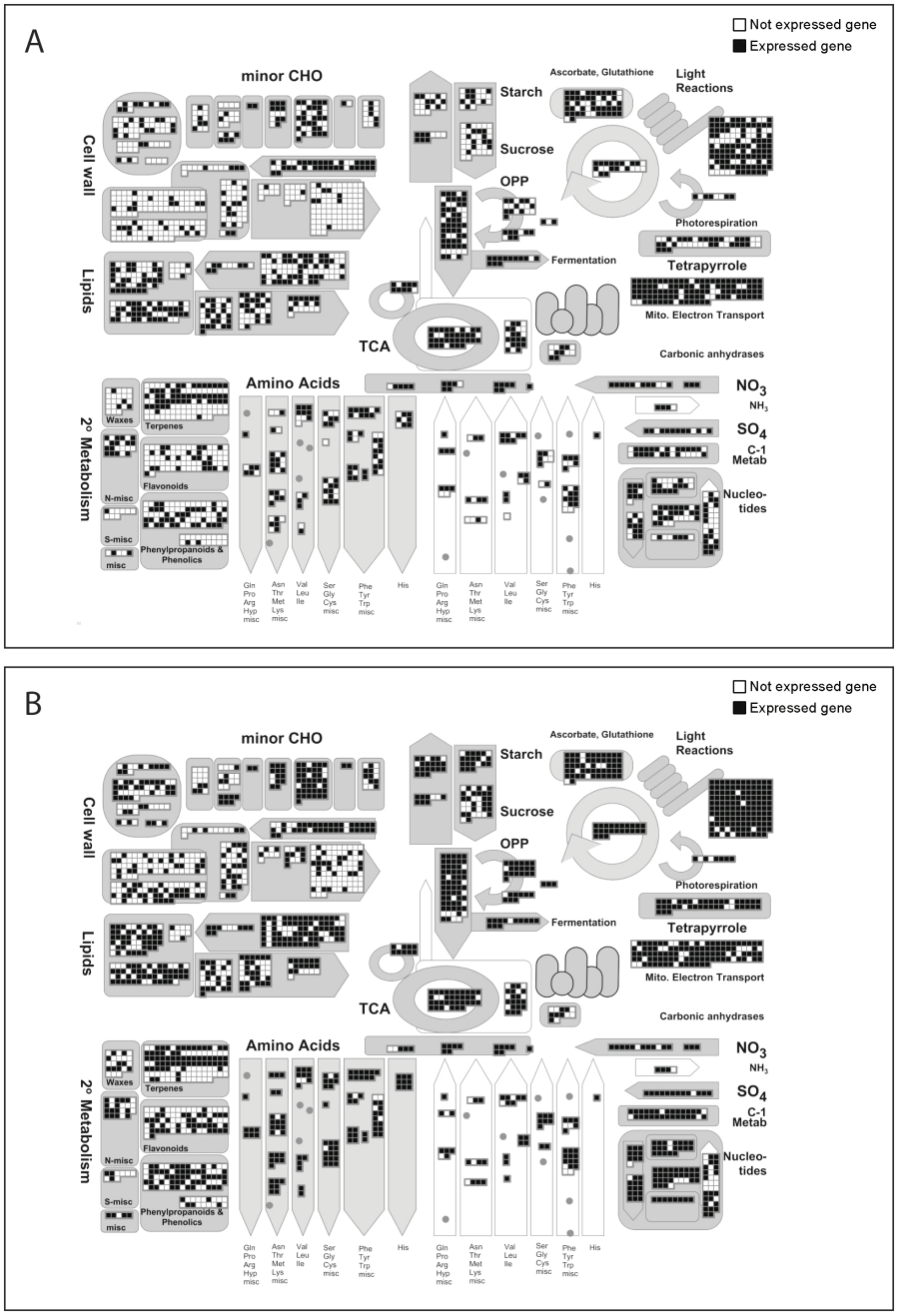

Figure 4.3. Gene expression in protoplasts is comparable to gene expression in seedlings

The figure shows MAPMAN representations of all expressed genes in protoplasts (A) compared to

7-day-old seedlings (B). Grey squares represent genes expressed at levels that were statistically significant above background levels in all slides of the respective experiments.

Effects of bZIP11 overexpression in seedlings can be reproduced in protoplasts

A total of 383 genes were expressed at statistically significantly ($q \leq 0.02$) higher levels (two-fold) after protoplasts were transfected with plasmid DNA encoding constitutively expressed HA-tagged bZIP11. In an earlier mRNA profiling experiment the effect of elevated levels of bZIP11 on the transcriptome in seedlings was studied (Hanson *et al.*, 2008). It was found that bZIP11 is a potent activator of gene expression. Upon increasing nuclear bZIP11 levels, a total of 163 genes were expressed at statistically significantly higher levels. Out of these 163 genes, 44 were up regulated in protoplasts overexpressing *bZIP11*, compared to expression levels in protoplasts transfected with the empty vector (Table 4.2). Given the set of 11000 expressed genes in the previous study and assuming complete randomness, the binominal probability of picking up 44 out of the 163 up regulated genes from that study would be 2.6×10^{-25}. This confirms that the experimental system used here is of biological relevance. Interestingly, among the up regulated genes several bZIP transcription factors were identified. The C-class bZIP transcription factor *bZIP25* was down regulated more than 3 fold, which made it the single most down regulated gene in this study. The S1-class bZIP transcription factor bZIP1 was down regulated more than twofold. Members of the G class of bZIP transcription factors were up regulated. Out of the five bZIPs in this class, *bZIP68*, *bZIP54* and *bZIP55* were up regulated 4, 6, and 20-fold, respectively. Analysis of the ATH1 probeset sequences of these genes revealed no significant similarities to the bZIP11 sequence, ruling out non-specific binding of the abundant bZIP11 transcript to these probesets.

Overexpression of bZIP10 causes minor changes in gene expression

To date little is known about the biological functions of C-class bZIPs. mRNA levels in protoplasts transfected with plasmid DNA encoding HA-tagged bZIP10, show that bZIP10 by itself has little capability to activate gene expression (Supplementary table S2 and Figure 4.4). Only seven genes (besides *bZIP10* itself) were induced at least two-fold upon induction of bZIP10 levels. None of the induced genes showed a significant expression level increase above four-fold. Among the group of seven up-regulated

Table 4.2. Genes regulated by bZIP11 in protoplasts as well as seedlings

A list of genes with increased mRNA levels in protoplasts expressing a constitutive HA-tagged bZIP11, which were also induced in seedlings after bZIP11 induction (Hanson *et al.,* 2008). Changes in mRNA levels are shown logarithmically.

		Seedlings		Protoplasts	
AGI	**Description**	**Log2FC**	**adj.P.Val**	**Log2FC**	**adj.P.Val**
AT1G02660	lipase class 3 family protein	3.89	2.36E-09	2.02	1.28E-09
AT1G10070	ATBCAT-2; branched-chain-amino-acid transaminase/ catal…	5.21	6.61E-06	1.03	2.55E-03
AT1G15040	glutamine amidotransferase-related	11.41	1.13E-09	4.33	3.98E-14
AT1G16850	unknown protein	2.79	2.04E-08	4.62	2.37E-14
AT1G18460	lipase family protein	6.06	3.47E-09	1.57	1.48E-06
AT1G19450	integral membrane protein, putative / sugar transporter…	4.41	6.61E-07	3.18	6.35E-06
AT1G30820	CTP synthase, putative / UTP--ammonia ligase, putative	4.40	6.70E-06	2.28	2.48E-06
AT1G51090	heavy-metal-associated domain-containing protein	6.16	7.81E-07	2.27	7.74E-07
AT1G56660	unknown protein	3.57	3.29E-08	1.65	1.59E-06
AT1G60190	armadillo/beta-catenin repeat family protein / U-box do…	8.93	4.98E-07	1.19	1.21E-04
AT1G62510	protease inhibitor/seed storage/lipid transfer protein…	2.20	5.17E-05	2.55	1.40E-10
AT1G64620	Dof-type zinc finger domain-containing protein	6.12	7.33E-10	1.67	3.31E-06
AT1G71980	protease-associated zinc finger (C3HC4-type RING finger…	8.44	2.02E-07	2.98	2.82E-08
AT1G73120	similar to hypothetical protein [Vitis vinifera] (GB:CA…	18.37	9.21E-12	3.66	2.39E-09
AT2G25200	similar to unknown protein [Arabidopsis thaliana] (TAIR…	2.44	3.21E-04	1.88	7.60E-07
AT2G26600	glycosyl hydrolase family 17 protein	3.33	7.81E-07	1.18	6.85E-06
AT2G30600	BTB/POZ domain-containing protein	2.36	1.51E-05	2.03	5.13E-07
AT2G36310	inosine-uridine preferring nucleoside hydrolase family…	2.91	1.16E-07	1.87	2.09E-06
AT2G39130	amino acid transporter family protein	2.88	5.16E-04	2.72	1.03E-07
AT2G39570	ACT domain-containing protein	5.00	3.69E-10	1.45	3.41E-08
AT2G41830	cyclin-related	2.32	7.22E-03	2.12	2.84E-08
AT2G47770	benzodiazepine receptor-related	44.60	9.21E-12	2.81	1.40E-10
AT3G29160	AKIN11 (ARABIDOPSIS SNF1 KINASE HOMOLOG 11); protein ki…	4.92	1.05E-05	2.39	8.54E-11
AT3G30775	ERD5 (EARLY RESPONSIVE TO DEHYDRATION 5); proline dehyd…	13.39	3.47E-11	3.18	5.30E-10
AT3G43430	zinc finger (C3HC4-type RING finger) family protein	3.42	4.21E-06	3.24	5.77E-10
AT3G47340	ASN1 (DARK INDUCIBLE 6)	2.11	4.80E-05	1.34	2.66E-06
AT3G53620	ATPPA4 (ARABIDOPSIS THALIANA PYROPHOSPHORYLASE 4); inor…	2.79	1.47E-03	1.80	1.52E-07
AT3G57520	ATSIP2 (ARABIDOPSIS THALIANA SEED IMBIBITION 2); hydrol…	2.28	1.39E-06	1.56	4.66E-08
AT4G23870	similar to unknown protein [Arabidopsis thaliana] (TAIR…	2.49	5.45E-04	1.30	2.85E-06
AT4G24040	ATTRE1/TRE1 (TREHALASE 1); alpha,alpha-trehalase/ treha…	12.49	3.23E-11	1.55	8.46E-10
AT4G28040	nodulin MtN21 family protein	5.37	3.87E-07	2.66	2.02E-07
AT4G33420	peroxidase, putative	2.35	1.73E-05	5.69	1.67E-13
AT4G33700	CBS domain-containing protein	7.59	3.05E-10	4.26	3.30E-11
AT4G33750	unknown protein	2.90	1.31E-07	1.50	1.68E-07
AT4G35770	SEN1 (DARK INDUCIBLE 1)	25.14	9.02E-17	1.52	4.51E-08
AT4G39660	AGT2 (ALANINE:GLYOXYLATE AMINOTRANSFERASE 2); alanine-g…	4.47	5.84E-08	1.99	1.69E-05
AT5G04310	pectate lyase family protein	5.19	1.99E-10	3.71	4.19E-11
AT5G08350	GRAM domain-containing protein / ABA-responsive protein…	5.98	1.29E-08	5.03	1.20E-12
AT5G15410	DND1 (DEFENSE NO DEATH 1); calcium channel/ calmodulin…	5.75	4.00E-11	4.21	1.31E-12
AT5G22460	esterase/lipase/thioesterase family protein	3.74	9.26E-09	2.19	2.85E-09
AT5G22920	zinc finger (C3HC4-type RING finger) family protein	5.76	3.19E-08	1.79	1.13E-05
AT5G58650	PSY1 (PLANT PEPTIDE CONTAINING SULFATED TYROSINE 1)	7.34	3.32E-10	1.90	6.22E-04
AT5G66170	similar to unknown protein [Arabidopsis thaliana] (TAIR…	7.08	9.21E-12	4.91	1.13E-14
AT5G66650	similar to unknown protein [Arabidopsis thaliana] (TAIR…	4.81	3.44E-09	1.27	1.04E-03

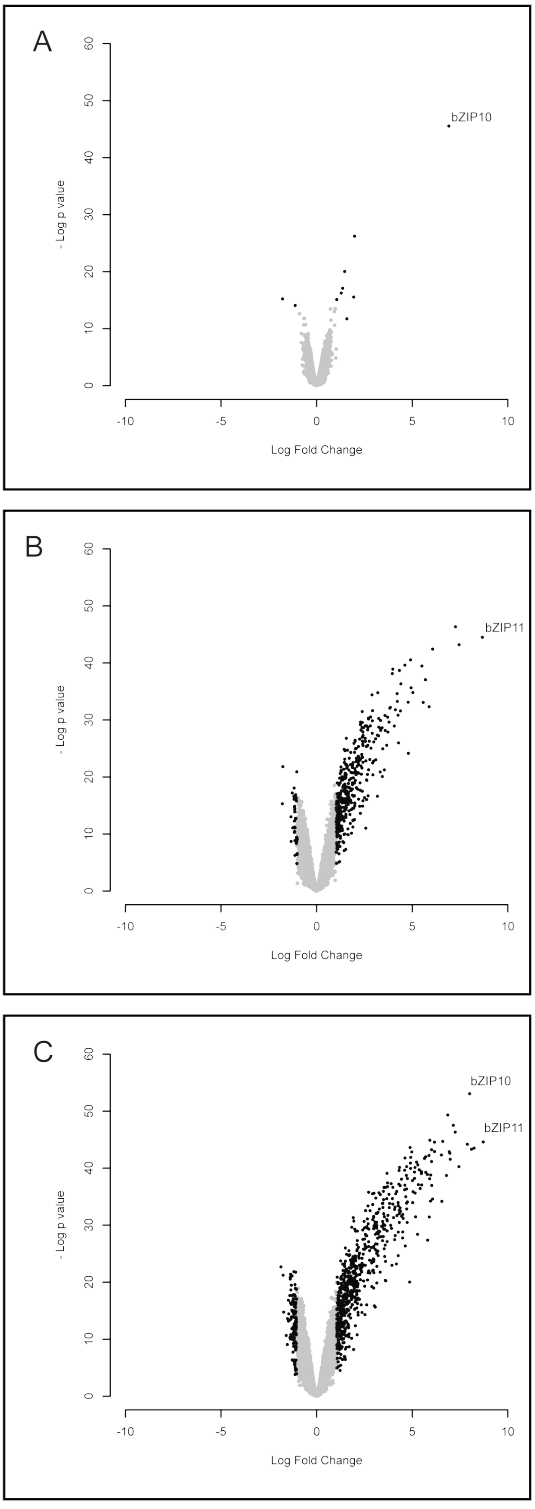

Figure 4.4. Differential gene expression in transfected protoplasts

The volcanoplots show the normalized relative signal changes in the protoplasts transfected with *bZIP10* (A), *bZIP11* (B) or *bZIP10* and *bZIP11* (C), in relation to the corresponding change in the protoplasts transfected with the empty vector. Logged signal changes are plotted against the p-value of that change. Dots with an average signal change of at least twofold are depicted in black.

genes was *SUC7*, a proposed sucrose-proton symporter. Remarkably, *bZIP25* expression was down regulated more than three-fold by bZIP10 transfection, as was the case after induction of bZIP11. The only other repressed gene was *HSP18.2*, mRNA levels of which dropped slightly below half of those in the control treatment.

bZIPs have differential effects on gene expression

The effects of induced levels of both bZIP10 and bZIP11 together were examined to explore possible synergistic effect of bZIP dimers. It was found that when bZIP10 was added in combination with bZIP11, the effect on gene expression levels was much larger than the combined effect of both individual transcription factors. In total, 645 genes were statistically significantly upregulated more than two-fold (Supplementary table S3 and Figure 4.5), whereas only 7 or 383 genes were upregulated by the single bZIP10 or bZIP11 transfections, respectively. Out of the 645 bZIP10+11 responsive genes 269 also responded to bZIP11 alone. Some of these 269 overlapping genes responded stronger when bZIP11 was complemented with bZIP10, others responded in the same way or weaker. The *2S SEED STORAGE PROTEIN 1* gene (*At4g27140*) showed the strongest combinatorial expression effect of bZIP10+11. It's expression was unaffected by bZIP11, but increased over 500-fold when bZIP11 was complemented with bZIP10. In an essentially identical experimental setup both bZIP plasmids were transfected into protoplasts at half DNA concentrations (results not shown). Effects on gene expression of these transfections were similar to the ones described here.

Different bZIP dimers likely recognize different promoters. Promoters of genes affected by bZIP10+11 dimers were compared to promoters of genes affected by bZIP11 alone, probably forming bZIP11 homodimers (Figure 4.6). The one Kb upstream regions of the top-100 genes most significantly affected by either type of dimer were subjected to motif analysis. It was found that the different dimers affect genes with promoters that show different motif enrichment. In agreement with the data obtained for experiments on seedlings (Chapter 2), an ACGT motif was found to be highly enriched in promoters of genes affect by dimers containing bZIP11.

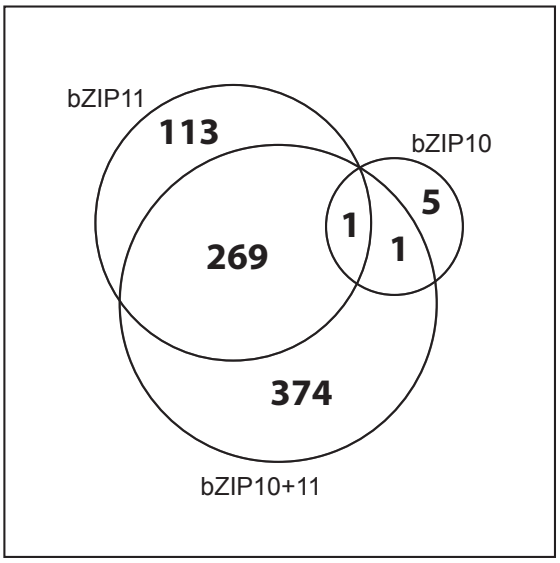

Figure 4.5. Differential effects of bZIP dimers

The Venn diagram shows the number of induced genes in protoplasts following transfection with *35S:bZIP* constructs. bZIP11 expression led to activation of 113 genes that were not induced after co-expression of bZIP10 and bZIP11, indicating a response to dimers containing bZIP11 but not bZIP10. bZIP10 expression led to activation of 5 genes that were not induced after co-expression of bZIP10 and bZIP11, indicating a response to dimers containing bZIP10 but not bZIP11. Co-expression of bZIP10+11 led to activation of 374 genes that were not induced after induction of either bZIP10 or bZIP11 alone, indicating bZIP10+11 dimer specificity for the induction of these genes.

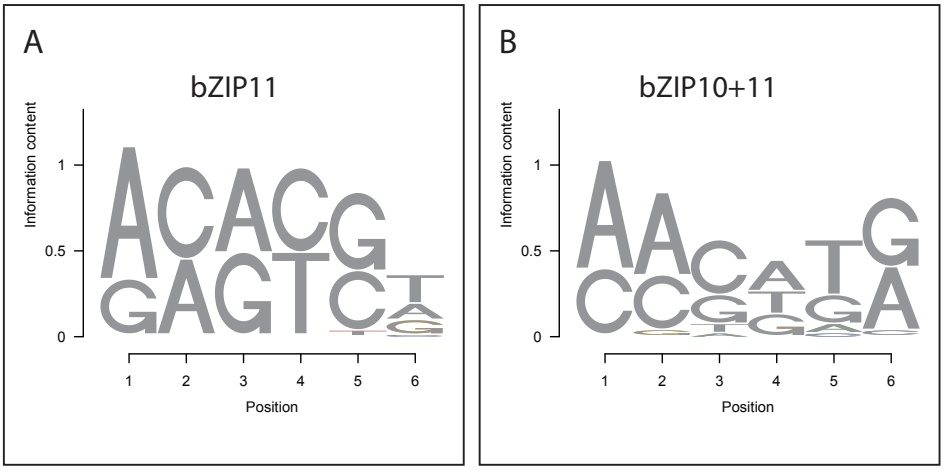

Figure 4.6. Motif analysis of promoters of bZIP targets.

The position weight matrixes of the 5 most significantly enriched motifs in the promoters of the 100 genes most significantly affected by dimers of bZIP11 (A) or bZIP10+11 dimers (B).

bZIPs have differential effects on biological processes

The notion that different bZIP dimers affect different sets of genes and thereby different biological processes, was substantiated by gene ontology (GO) analysis. bZIP10 and bZIP11 were found to have a much stronger inducing than repressing effect on gene expression levels. Therefore, the most significantly up regulated genes (fold change ≥ 2) were subjected to GO analysis. The topGO software package (Alexa and Rahnenfuhrer, 2006) was used to identify GO categories whose member genes showed a statistically different response after induction of bZIP levels. bZIP11 effects were compared to those of the empty vector. The effect of the bZIP10+11 combination was compared to the effect bZIP11 alone (Table 4.3). In agreement with the observation that different bZIPs have differential effects on gene expression, they also affect biological processes differentially. Some GO terms are induced after both transfections but the majority of the 20 most affected GO categories are unique for either bZIP10+11 or bZIP11 treatment.

Table 4.3A. Differentially enriched GO terms based on molecular function among genes affected by bZIPs

The table indicates statistically enriched gene ontology (GO) terms associated with the differentially expressed genes after different protoplast transfections. Effects of bZIP11 are compared to effects specific to the bZIP10+11 dimer. Statistical significance is indicated by shading.

			bZIP11		bZIP10+11 compared to bZIP11	
GO.ID	Term	Total in genome	Changed	p value	Changed	p value
GO:0000254	C-4 methylsterol oxidase activity	2	1	0.046	-	-
GO:0000293	ferric-chelate reductase activity	1	1	0.048	-	-
GO:0003700	transcription factor activity	447	19	0.007	36	0.002
GO:0003842	1-pyrroline-5-carboxylate dehydrogenase ...	1	1	0.048	-	-
GO:0003910	DNA ligase (ATP) activity	2	1	0.046	-	-
GO:0004066	asparagine synthase (glutamine-hydrolyzi...	1	1	0.023	1	0.048
GO:0004105	choline-phosphate cytidylyltransferase a...	2	1	0.046	-	-
GO:0004194	pepsin A activity	15	3	0.033	-	-
GO:0004312	fatty-acid synthase activity	6	2	0.007	2	0.031
GO:0004349	glutamate 5-kinase activity	1	1	0.048	-	-
GO:0004888	transmembrane receptor activity	39	4	0.012	6	0.010
GO:0005275	amine transmembrane transporter activity	28	4	0.044	-	-
GO:0008081	phosphoric diester hydrolase activity	28	3	0.026	5	0.010
GO:0008113	peptide-methionine-(S)-S-oxide reductase...	11	3	0.014	-	-
GO:0008379	thioredoxin peroxidase activity	1	1	0.023	-	-
GO:0008453	alanine-glyoxylate transaminase activity	4	2	0.013	-	-
GO:0008483	transaminase activity	34	3	0.043	-	-
GO:0009054	electron acceptor activity	1	1	0.023	-	-
GO:0009673	low affinity phosphate transmembrane tra...	1	1	0.023	-	-
GO:0015200	methylammonium transmembrane transporter...	1	1	0.023	-	-
GO:0015250	water channel activity	14	3	0.004	5	0.000
GO:0015385	sodium:hydrogen antiporter activity	10	2	0.021	-	-
GO:0016597	amino acid binding	13	2	0.035	3	0.022
GO:0016620	oxidoreductase activity, acting on the a...	36	6	0.007	-	-
GO:0019203	carbohydrate phosphatase activity	8	2	0.014	3	0.005
GO:0030570	pectate lyase activity	3	2	0.002	2	0.007
GO:0030599	pectinesterase activity	5	2	0.021	-	-
GO:0043565	sequence-specific DNA binding	13	3	0.022	-	-
GO:0050113	inositol oxygenase activity	2	2	0.001	2	0.002
GO:0051739	ammonia transporter activity	1	1	0.023	-	-

Table 4.3B. Differentially enriched GO terms based on biological process among genes affected by bZIPs

The table indicates statistically enriched gene ontology (GO) terms associated with the differentially expressed genes after different protoplast transfections. Effects of bZIP11 are compared to effects specific to the bZIP10+11 dimer. Statistical significance is indicated by shading.

			bZIP11		bZIP10+11 compared to bZIP11	
GO.ID	Term	Total in genome	Changed	p value	Changed	p value
GO:0000038	very-long-chain fatty acid metabolic pro...	4	-	-	2	0.013
GO:0000304	response to singlet oxygen	5	-	-	2	0.021
GO:0000914	phragmoplast formation	1	1	0.022	1	0.049
GO:0002240	response to molecule of oomycetes origin	2	1	0.044	-	-
GO:0005513	detection of calcium ion	6	-	-	2	0.031
GO:0005991	trehalose metabolic process	8	-	-	3	0.005
GO:0006000	fructose metabolic process	2	1	0.044	-	-
GO:0006560	proline metabolic process	7	-	-	3	0.003
GO:0006809	nitric oxide biosynthetic process	2	1	0.044	-	-
GO:0006833	water transport	4	-	-	2	0.013
GO:0006869	lipid transport	19	3	0.008	5	0.002
GO:0006875	cellular metal ion homeostasis	16	2	0.049	-	-
GO:0007020	microtubule nucleation	1	1	0.022	-	-
GO:0007155	cell adhesion	11	-	-	4	0.001
GO:0009269	response to desiccation	16	-	-	3	0.040
GO:0009744	response to sucrose stimulus	26	3	0.020	6	0.001
GO:0009826	unidimensional cell growth	34	5	0.006	6	0.020
GO:0009828	cellulose and pectin-containing cell wal...	4	2	0.003	3	0.000
GO:0009972	cytidine deamination	2	1	0.044	-	-
GO:0010103	stomatal complex morphogenesis	1	1	0.022	-	-
GO:0010165	response to X-ray	1	1	0.022	-	-
GO:0010231	maintenance of seed dormancy	1	1	0.022	-	-
GO:0010256	endomembrane organization	1	1	0.022	-	-
GO:0016126	sterol biosynthetic process	15	-	-	3	0.033
GO:0016567	protein ubiquitination	48	-	-	6	0.028
GO:0030397	membrane disassembly	1	1	0.022	-	-
GO:0030497	fatty acid elongation	2	1	0.044	-	-
GO:0042335	cuticle development	5	-	-	2	0.021
GO:0042545	cell wall modification	11	-	-	5	0.041
GO:0045449	regulation of transcription	445	-	-	30	0.041
GO:0046351	disaccharide biosynthetic process	15	-	-	3	0.033
GO:0046470	phosphatidylcholine metabolic process	1	1	0.022	-	-
GO:0046785	microtubule polymerization	1	1	0.022	-	-
GO:0048765	root hair cell differentiation	7	-	-	3	0.003
GO:0048767	root hair elongation	3	2	0.002	-	-

Discussion

The transcription factor bZIP11 had significant effects on gene expression when overexpressed in *Arabidopsis thaliana* mesophyll protoplasts. Elevated levels of bZIP11 caused a total of 383 genes to show increased mRNA levels. Comparatively, few genes (36) were down regulated. This finding is in agreement with results obtained earlier. Also in seedlings, elevated levels of nuclear bZIP11 induced the expression of significantly more genes than it repressed (Hanson *et al.*, 2008). In contrast to the situation in stably transformed seedlings, not all cells in the protoplast system overexpressed the bZIP proteins, due to the limit of transfection efficiency. This implies that the effects on genes that are repressed are limited to the cells that have been transfected. The drop in mRNA in those cells is potentially overshadowed by the mRNA still present in the untransfected cells. However, for *bZIP25* it was shown that its expression was lowered 3-fold. Assuming total repression of *bZIP25* in transfected protoplasts, this indicates a

transformation efficiency of 66%.

Among the set of confirmed bZIP11 target genes were *KIN11*, and two dark inducible (DIN) genes, *DIN1* and *DIN6*/*ASN1*. Of the 163 genes upregulated in the seedling-based microarray experiment, 44 genes were upregulated in protoplasts. Even though statistically speaking this number is highly significant, from a biological standpoint one might ask why not more genes were confirmed in the protoplast system. The main reason may be found in the different tissue specificities inherent to the systems used. When studying gene expression in mesophyll protoplasts, mRNAs of genes specifically expressed in other tissues (*e.g.* roots or flowers) will not be present. Target genes identified in 7 day old seedlings that were not confirmed in protoplasts were expressed in other tissues than mesophyll cells. Data from the AtGenExpress project (Schmid *et al.*, 2005) revealed that in seedlings, 85% of the missing genes were expressed in roots (51), cotyledons (18), hypocotyls (3), or the shoot apex (1). Interestingly, among the newly identified targets of bZIP11 were other bZIPs. The S1-class bZIP transcription factor *bZIP1* as well as the C-class bZIP transcription factor bZIP25 were down regulated more than twofold. Three out of five members of the G class of bZIP transcription factors were up regulated (*bZIP68*, *bZIP54* and *bZIP55*). These results suggest a network in which bZIPs affect each others expression.

Compared to bZIP11, bZIP10 showed minimal effects on gene expression in protoplasts. Only 7 genes were upregulated, and 2 were downregulated by bZIP10. One possible reason for the smaller effect bZIP10 may be found in the fact that it was overexpressed to lower levels then bZIP10: 120-fold compared to over 400-fold. However, we expect both bZIPs to be saturated in terms of gene expression and protein translation. Furthermore, bZIP10+11 exerted quite dramatic effects on gene expression, which also argues against this explanation. So far, bZIP10 is known to be involved in defense and cell death in Arabidopsis following infection with the pathogen *Hyaloperonospora parasitica* (Kaminaka *et al.*, 2006). It is also known to be an ortholog of maize *OPAQUE2,* which participates in the regulation of seed storage protein genes (Lara *et al.*, 2003). Accordingly, the expression of the *2S SEED STORAGE PROTEIN 1* increased over 500-fold in bZIP10+11 transfections. That effect dwarfs even the 35S constitutive promoter driven expression increase of bZIP10. This confirms the involvement of bZIP10 in regulation of seeds storage protein, however it also implies that bZIP10 does not regulate the expression of this gene by itself, as the expression of the *2S SEED STORAGE PROTEIN 1* remained unchanged in protoplasts only overexpressing bZIP10.

In order to bind DNA with their basic region, bZIP transcription factors need to

form dimers with their ZIP domain. The eukaryotic bZIP transcription factors Jun and Fos are well-studied examples. Sedimentation equilibrium studies and thermal unfolding experiments have revealed that dimer stability decreases in the order Jun-Fos > Jun-Jun > Fos-Fos (O'Shea *et al.*, 1989). This has implications for the function of the dimers. While neither the Jun nor Fos monomer will bind the Jun/Fos DNA binding site (Turner and Tjian, 1989), the Jun, but not the Fos, homodimer will bind the DNA binding site (Neuberg *et al.*, 1989). In analogy to this system, it has been shown that in Arabidopsis, C-class bZIPs predominantly dimerize with S1-class bZIPs, but are unable to form homodimers (Ehlert *et al.*, 2006a). S1-class bZIPs in contrast, can form homodimers, even though they prefer dimerization with the C-class. Induction of either bZIP10 (C-class) or bZIP11 (S1-class) levels in protoplasts resulted in 7 or 383 genes being statistically significantly up regulated more than 2 fold, respectively. If however both bZIP10 and bZIP11 levels were increased, 645 genes were statistically significantly up regulated more than 2 fold. This results in the distinction of three groups of genes: a group of 5 genes specifically responding to bZIP10 dimerized with a non-bZIP11 protein, a group of 113 genes specifically responding to bZIP11 homodimers or bZIP11 dimerized with a non-bZIP10 protein, and a group of 374 genes specifically responding to bZIP10 dimerized with bZIP11. The synergistic effect caused by both transcription factors is not due to a mere increase in the total amount of transcription factor protein present. In an independent experiment in which only half the amount of bZIP plasmid DNA was used, highly similar results to the ones presented here were obtained, and protein levels in both cases were found to be similar by western analysis (Figure 4.1). In this situation protoplasts would have a comparable amount of bZIP protein to that after a single plasmid transfection.

The synergistic effects of bZIP10 and bZIP11 provide evidence for the fact that different bZIP dimers have different functions. Considering the number of S1- and C-class bZIPs and the fact that they can be differentially regulated, a complex network of bZIP dimers emerges. Additionally, the regulation of the SNF1-related protein kinase *KIN11* (*At3g29160*) by the bZIP10+11 dimer is intriguing as S1-class bZIPs have been claimed to be involved in stress mediated SNF1-related protein kinase 1 (SnRK1) signaling (Baena-Gonzalez *et al.*, 2007). SnRK1 kinases play an important role in the regulation of transcription, metabolism and development in response to energy limitation, *e.g.* due to carbon starvation (Hardie, 2007., Baena-Gonzalez *et al.*, 2007). The increased *KIN11* expression in response to bZIP11 thus presents a possible positive feedback mechanism. Recently, T6P has been shown to be a regulator in SnRK1 signaling (Zhang *et al.*, 2009). Interestingly, 6 genes involved in trehalose metabolism are affected in a way that

might result in reduced T6P levels. The expression levels of four trehalose-6-phosphate phosphatase genes (*TPP2*, *TPP5*, *TPP6* and *TPP9*), as well as that of the single trehalase gene (*TRE1*) were increased by the bZIP10/11 dimer. Furthermore, expression levels of a trehalose phosphatase/synthase (*TPS8*), were reduced. Likely, bZIP11 thus affects T6P levels, but this has not been tested. This effect of bZIP11 would be in agreement with the finding that sucrose can lead to a large (30-fold) and fast increase in T6P levels (Lunn *et al.*, 2006), as sucrose will inhibit the production of S1-class of bZIPs. bZIP11 effects on trehalose metabolism could, therefore, represent another possible feedback mechanism in the sugar/SnRK1/bZIP pathway. Moreover, the effect of bZIP11 on trehalose metabolism was shown to be biologically relevant (Chapter 3). Induction of bZIP11 abolishes growth arrest of seedlings caused by high concentrations of trehalose in the medium. Taken together, these results suggest an intricate signaling network involving SnRKs and bZIPs is regulating growth and development by directing gene transcription, and metabolism in accordance with the nutritional status. In this complex system sugars have an impact at multiple levels.

Materials and Methods

Construction of plasmid DNA

For transient expression of bZIP transcription factors in protoplasts, 35S driven 3x 5' HA tagged bZIP constructs were made (Ehlert *et al.*, 2006a). The bZIP transcription factors were made available through the REGIA project, and were cloned into a modified version of the pHBT vector using Gateway® technology (Invitrogen, http://www.invitrogen.com). The modified pHBT, designated pHBTLΔGFP was created by NcoI/NotI- digestion, Klenow fill in and religation (Thorsten Heinekamp, pers. com.).

Transient expression of bZIP transcription factors in protoplasts

The protoplast preparation was adapted from Sheen (Sheen, 2001). Arabidopsis mesophyl protoplasts were isolated from leaves (the second and/or third/fourth pair) of 5 weeks old Col-0 (CS60000) plants grown on soil under long day (16h light / 8h dark) conditions. Leaves were placed in enzyme solution for 8.5h (1 % cellulase R10, 0.3% macerozyme

R10 (Yakult Honsha, Tokyo, Japan), 0.4 M mannitol, 20 mM KCl, 10 mM CaCl, 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 MgCl2, 4 mM MES pH 5.7), and subjected to PEG transfection. To 2.12x106 protoplasts, a total of 250µg plasmid DNA was added followed by a 30 min. incubation in 1 volume of PEG solution (40% PEG 3500, 3 ml H2O, 0.2M mannitol, 0.1 M CaCl). After transfection the samples were diluted with 2 volumes of W5 solution and pelleted at 100g 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 5cm Petri dishes pre-coated with 5% calf serum, and incubated for 6h in the growth cabinet. After the incubation protoplasts were collected and 100-200 mg aliquots were flash frozen in liquid nitrogen.

Western analysis

For Western analysis, 100 mg of plant material was ground to a fine powder in liquid nitrogen, and denatured in 300 µl of buffer containing 4 M urea, 16.6% glycerol, 5% -mercaptoethanol and 5% SDS. Aliquots (10 µl) were then fractionated on a 10% polyacrylamide gel. Blotting onto PVDF membrane (Millipore, Braunschweig, Germany) and immunodetection were carried out as described by Harlow and Lane (Harlow and Lane, 1988).

RNA extraction and cDNA synthesis

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. Genomic DNA was removed using pretreatment of the total RNA with DNase (Fermentas, http://www.fermentas.com) and cDNA was synthesized using anchored oligo-T primers (Biolegio, http://www.biolegio.com), and MLV reverse transcriptase (Promega, http://www.promega.com) according to the manufacturer's instructions.

Micro-array analysis

cRNA labeling, hybridization, washing and scanning of Affymetrix Arabidopsis ATH1

GeneChips® (Affymetrix, http://affymetrix.com) was performed by ServiceXS (http://www.servicexs.com), according to Affymetrix OneCycle Lab protocols. Data from the micro-array were analyzed statistically using the R language environment for statistical computing (http://www.r-project.org) version 2.7.1 and Bioconductor release 2.1 (Gentleman *et al.*, 2004). Data were normalized using the robust multi-arrayexpression measure (RMA) in the Affy package (Gautier L., 2004). Differentially expressed genes were identified using the LIMMA package (Smyth *et al.*, 2005). The obtained P-values were corrected for multiple testing errors using the BH procedure (Benjamini, 1995), yielding q-values. Lists of q-values were transferred to Microsoft Excel™ and sorted. The GST sequences were aligned to the Tair7 gene model database of transcripts. Genes were classified as being differentially expressed if expression significantly ($q \leq 0.02$) changed. Genes with lower than two-fold expression level changes, even if statistically significant, were not considered to be differentially expressed. To compare the list of differentially expressed genes with other gene lists Microsoft Excel™ was used.

For gene ontology data genes were organized based on both biological process and molecular function annotations and several publicly available tools were used, topGO (Alexa and Rahnenfuhrer, 2006), Tair (http://www.arabidopsis.org/tools/bulk/go/) and MAPMAN (Thimm *et al.*, 2004).

QPCR

Real-time PCR was performed using the 7900HT Fast Real-Time PCR System and Cybergreen® chemistry (Applied Biosystems). Expression levels were calculated relative to the phosphatase 2A (PP2A) regulatory subunit (At1g13320) (Czechowski *et al.*, 2005) levels using the Q-gene method that takes the relative efficiencies of the different primer pairs into account (Muller *et al.*, 2002). Primers were designed according to the recommendations of the PCR master-mix manufacturer (Applied Biosystems) or designed to be gene-specific by the CATMA consortium (http://www.catma.org). Full lists of primer sequences can be obtained from the authors.

“Oh, people can come up with statistics to prove anything, 14% of people know that.”

-Homer Simpson

Chapter 5: General Discussion, bZIP transcription factors and the regulation of metabolism

Micha Hanssen

In this thesis effects on metabolism of specific *Arabidopsis thaliana* bZIP transcription factors are characterized and their targets genes are identified. Gene expression and metabolic analyses were performed following overexpresion of bZIPs in stably transformed plants and in transient expression in protoplasts. It was found that bZIP activity had profound effects on both gene expression and metabolism.

bZIP transcription factors are regulated at various levels. A multitude of biotic and abiotic signals differentially regulate the transcription of bZIPs (Figure 3.1). Transcriptional regulation is not connected to the class to which a bZIP belongs. Rather, bZIPs are individually regulated in time and space. For instance, the S1-class members *bZIP1* and *bZIP11* are oppositely regulated in response to carbon availability. Increasing available carbon by glucose addition leads to increased *bZIP11* transcript levels and decreased *bZIP1* transcripts. Similarly prolonged night treatment results in increased *bZIP1* transcripts and decreased *bZIP11* transcripts. In response to drought the C-class members *bZIP9* and *bZIP63* are transcriptionally up and down regulated, respectively. This differential regulation allows for control over which monomers are available for translation in certain conditions and which specific dimers are subsequently formed. Another control system for the formation of functional bZIP dimers operates at the translational level. Translation of S1-class bZIPs is repressed in the presence of sucrose through a uORF in the 5' leader sequence of their mRNAs (Weltmeier *et al.*, 2009). This uORF encodes a sucrose control peptide that is evolutionarily conserved among all S1-class bZIP genes in different plant species (Wiese *et al.*, 2005; Hummel *et al.*, 2009). Transcription factors must bind to DNA in the nucleus in order to exert their function. Therefore, yet another possibility for bZIP regulation lies in their localization. For example, it was found that the LSD1 protein retains bZIP10 outside of the nucleus, thereby antagonizing the function of bZIP10 in plant defense and hypersensitive cell death (Kaminaka *et al.*, 2006).

The Arabidopsis SnRK1 family kinases KIN10 and KIN11 regulate transcription of their downstream target genes in part through bZIPs (Baena-Gonzalez *et al.*, 2007). It was found that co-expression of SnRK1 kinases and bZIPs led to a marked increase in the transcriptional activion capacity of bZIPs, likely due to phosphorylation by the SnRK1 kinases. Furthermore, bZIP and SnRK1 activity share regulation by various factors such as light and sugar. Taken together, the combined regulation at the transcriptional, translational and transcription factor activation level permit tight regulation of bZIP expression and their downstream targets (Figure 5.1).

Differential regulation of bZIP monomer expression and functional dimer formation is only biologically relevant if different bZIP dimers have different effects on gene expression.

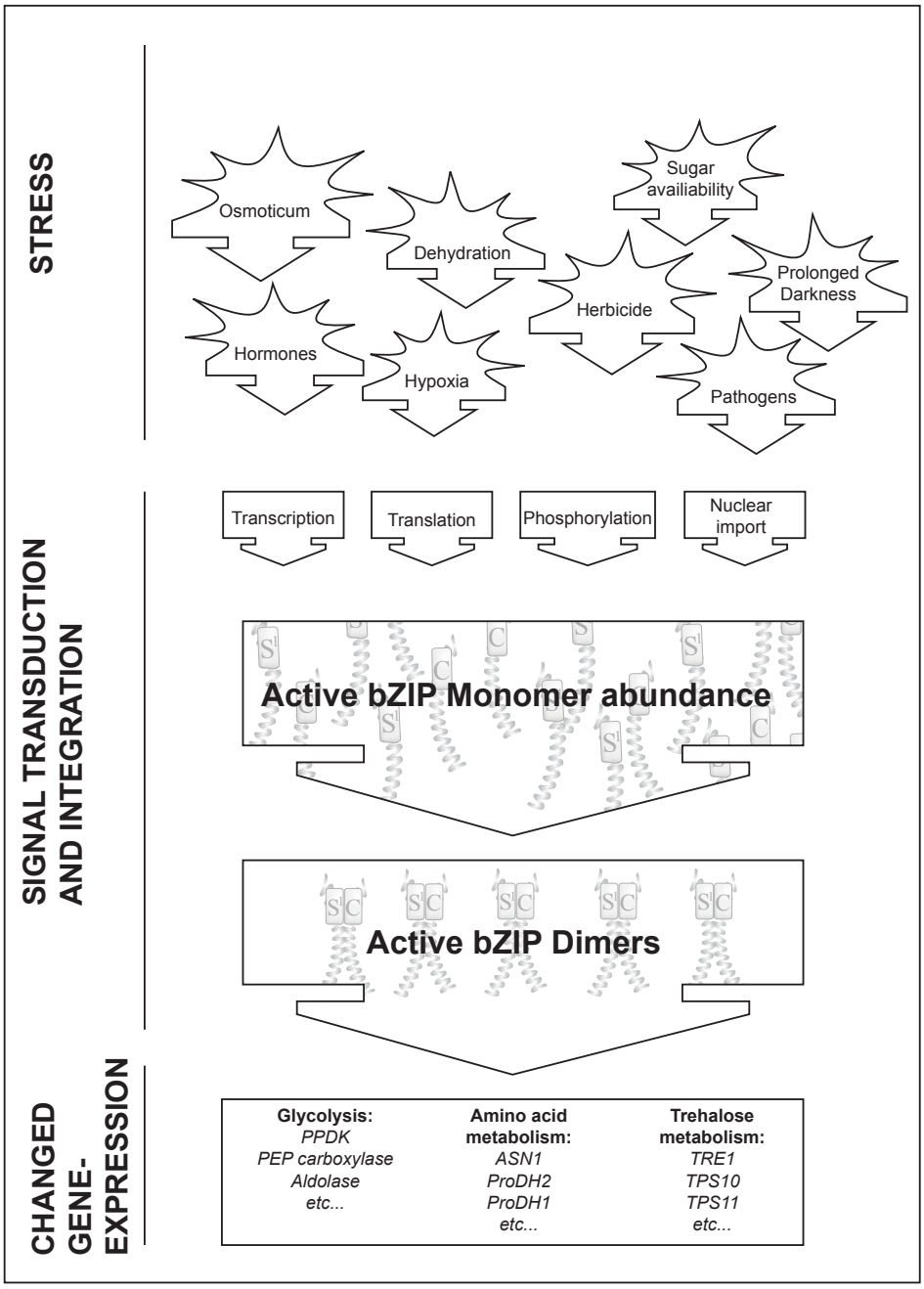

Figure 5.1. Adaptation of metabolism to stress through bZIP transcription factors.
Many different stresses affect the abundance of active bZIP monomers. Stress signals may also affect internal factors such as sugar availability. The levels of active bZIP monomers in the nucleus are modified by several signal transduction pathways by the above-mentioned stresses. Active monomer abundance is regulated through changed transcription, translation, phosphorylation and nuclear

import of individual bZIP genes and proteins. Dimer formation depends on the relative abundance of monomers as well as the dimerization characteristics of the monomers. After formation of active dimers, bZIPs trigger an integrated response by affecting gene expression of genes involved in various aspects of metabolism.

This thesis presents evidence that different bZIP dimer species have differential effects on gene expression (Chapter 4). Control of gene expression by dimerizing transcription factors provides extensive opportuneties for regulation. So far the function of few bZIP transcription factor has been studied in plants. One well-known bZIP target gene is *ProDH* (Satoh *et al.*, 2002; Weltmeier *et al.*, 2006). It was found that *ProDH* was activated by dimers of the S1-class member bZIP53 in combination with any of the four C-class bZIPs, but to varying extends (Weltmeier *et al.*, 2006). This thesis addresses the differential activities of different dimer species on a global level. In the transcriptome studies presented here, it was found that bZIP dimers have distinct effects on gene expression. The results indicate that targets genes of bZIP11 containing dimers overlap to some degree, but that many target genes are regulated by specific dimers. Therefore, it will be highly interesting to extend this finding to other C/S1 dimers in future research.

Among the target genes of C/S1 class bZIP proteins were many genes associated with various aspects of metabolism. bZIP induced changes in gene expression result in according changes of metabolite levels, as determined by targeted and un-targeted metabolic analyses (Chapter 3). Induced nuclear activity of bZIP11 led to changed levels of amino acids and sugars, amongst others. This may be part of an energy conserving mechanism invoked by SnRK1/bZIP dependent stress signaling. Likely, increased levels of sucrose glucose and fructose are the result of the regulation of various genes encoding enzymes involved in glycolysis, such as PPDK, PEP carboxylase and aldolase (Chapter 3). Thus, the bZIP dimers studied in this thesis may inhibit growth and other energy consuming processes in plants by regulating gene expression to adapt to the potentially detrimental effects of stress.

Several of the C/S1 transcription factor dimer target genes are also known target genes of SnRK1 kinases that were previously shown to regulate starvation responses through activation S1-class bZIP factors (Baena-Gonzalez *et al.*, 2007). SnRK1 kinases are key regulators in starvation and stress responses and respond to several different stimuli (Baena-Gonzalez and Sheen, 2008). Therefore, a function of bZIP signaling in adaptation of metabolism to stress is proposed. Interestingly, the regulation of both SnRK1 activity and bZIP translation by sugars provides opportunities for feedback regulation. Increased

activity of bZIP11 results in increased sugar levels, which represents a negative feedback mechanism as increased sucrose levels inhibit the translation of S1-class bZIP proteins (Wiese *et al.*, 2004).

Seedlings overexpressing bZIP11 are resistant to high levels of trehalose in the growth medium. These results link bZIP signaling to trehalose metabolism. Likely, this is the consequence of increased trehalase activity. The regulation of trehalose metabolism by bZIP11 provides possible feedback signaling mechanism, as T6P, an intermediate in trehalose biosynthesis, was recently established as a negative regulator of SnRK1 activity (Zhang *et al.*, 2009) and thereby negatively regulate the expression of targets of S1-class bZIP proteins.

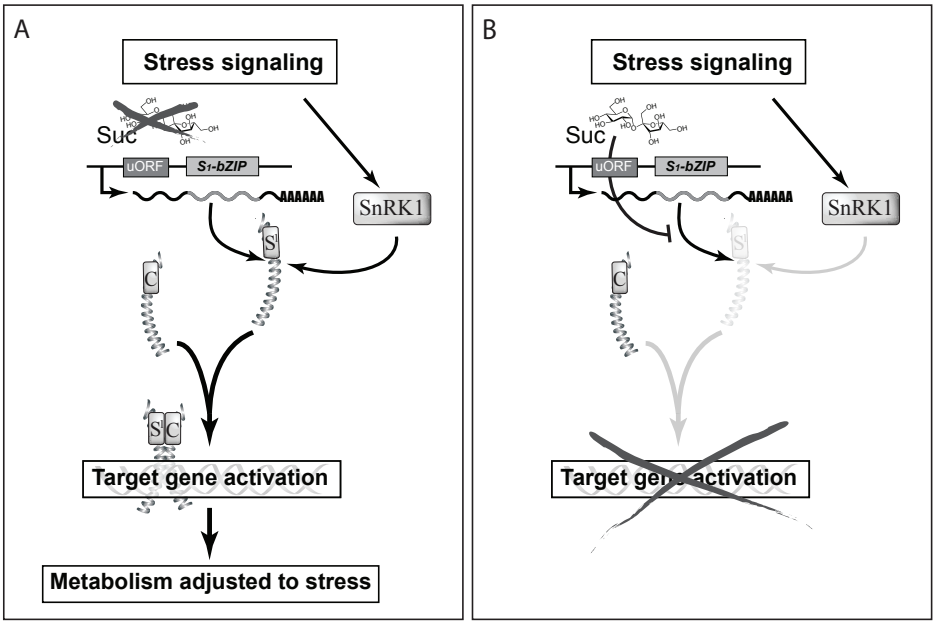

Figure 5.2. Sucrose availability determines stress signaling through bZIPs.

A) Different stresses will lead to activation gene expression through SnRK1/bZIP signaling. A common consequence of stress is energy limitation *i.e.* low sugar levels. In response, the activation of SnRK1 kinases leads to activation of bZIP transcription factors that alter the expression of genes involved in metabolism. As a result, metabolism will be adjusted and energy will be conserved. (B). In conditions where energy is not limiting, however, energy-conserving responses are undesirable. In these conditions sucrose will stop SnRK1/bZIP signaling by inhibiting translation of S1-class bZIPs.

Under laboratory conditions plants usually encounter few or single stress factors. In nature, however, plants can encounter several different stresses simultaneously, such as pathogens, drought, heat and mechanical injury. Plants can respond to different types of stress in order to act on the most serious factor. This thesis describes one possible mechanism of such signal integration in response to stress. bZIP monomers form dimers based on their relative abundance and mutual affinity. As documented in this thesis, different bZIP dimers have both overlapping, but also distinct sets of target genes. A picture emerges in which signaling pathways in response to different stresses can form an integrated response in the form of altered gene expression by controlling the relative abundance of bZIP monomers. It is easy to envisage the selective advantage of this complicated network, as proper responses to environmental stresses will directly determine evolutionary fitness.

Interestingly, the SnRK/bZIP network regulates responses to stresses involving cellular energy limitation. This positions the sucrose induced translational repression of translation of S1-class bZIP proteins in a logical context. When plants are exposed to energy-limiting stress, they respond by restricting growth to save resources and avoid a damaging energy deficit. However, in the presence of sufficient sucrose this response would be counterproductive and should be avoided. The translational inhibition of S1-class bZIP transcription factors in the presence of sucrose will counteract the energy conserving response through the SnRK/bZIP network (Figure 5.2).

Future crops must to be able to produce high yields under several different types of stresses simultaneously. It is therefore of great importance to investigate how plants are able to rate different stresses and come the most optimal response. Understanding the ‘decision making’ processes of cellular systems is thus not only of interest in the context of basic science but likely impacts on agriculture in the perspective of a world with deteriorating topsoils, changing climates and growing populations.

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Supplementary table S1. Genes induced or repressed by bZIP11 in transgenic lines after induction with dex

		L transgenic		M transgenic		Confirmed in low
AGI	Description	Log2FC[A]	p value	Log2FC[A]	p value	sucrose[B]
AT1G01190	cytochrome P450, putative, similar to cytochrome P450...	3.4	1.17E-09	2.6	1.13E-07	
AT1G01430	expressed protein, similar to hypothetical protein GB:...	-1.1	1.00E-05	-1.3	1.52E-06	
AT1G02660	lipase class 3 family protein, contains Pfam profile P...	2.0	2.36E-09	2.2	9.65E-10	
AT1G03800	encodes a member of the ERF (ethylene response factor)...	1.6	2.69E-07	1.4	2.69E-06	
AT1G04120	ABC transporter family protein, Strong similarity to M...	1.5	1.07E-06	1.2	2.82E-05	
AT1G04880	high mobility group (HMG1/2) family protein / ARID/BRI...	2.1	8.85E-10	1.6	9.56E-08	
AT1G06060	RanBPM-related, similar to RANBPM {GI:13194576)(Homo s...	-0.8	1.85E-04	-1.6	6.78E-09	
AT1G07090	expressed protein, contains Pfam profile PF04852: Prot...	-1.1	4.51E-02	-1.4	2.95E-02	
AT1G09460	glucan endo-1,3-beta-glucosidase-related, similar to g...	1.8	1.25E-08	1.9	1.72E-08	
AT1G10070	similar to branched-chain amino acid aminotransferase...	2.4	6.61E-06	1.7	4.72E-04	Yes
AT1G11080	serine carboxypeptidase S10 family protein, similar to...	1.6	1.67E-05	1.0	5.29E-03	
AT1G11545	xyloglucan:xyloglucosyl transferase, putative / xylogl...	-1.4	6.33E-05	-1.5	2.94E-05	
AT1G12080	expressed protein	1.7	2.00E-04	1.5	8.76E-04	
AT1G12110	nitrate/chlorate transporter (NRT1.1) (CHL1), identica...	-1.6	3.20E-07	-1.5	2.87E-06	
AT1G14610	valyl-tRNA synthetase / valine--tRNA ligase (VALRS), n...	-0.6	3.98E-03	-1.8	3.40E-08	
AT1G15040	glutamine amidotransferase-related	3.5	1.13E-09	3.1	1.40E-08	
AT1G16850	expressed protein	1.5	2.04E-08	1.2	5.92E-07	
AT1G18460	lipase family protein, similar to triacylglycerol lipa...	2.6	3.47E-09	1.9	1.04E-06	
AT1G19330	expressed protein	-0.9	2.03E-02	-0.9	3.52E-02	
AT1G19450	integral membrane protein, putative / sugar transporte...	2.1	6.61E-07	1.6	1.94E-04	Yes
AT1G20330	S-adenosyl-methionine-sterol-C-methyltransferase, iden...	0.8	2.04E-03	1.0	6.53E-04	
AT1G20440	dehydrin (COR47), identical to dehydrin COR47 (Cold-in...	1.1	1.61E-02	1.6	1.39E-03	
AT1G21460	nodulin MtN3 family protein, contains similarity to MT...	-1.1	2.26E-03	-1.3	3.49E-04	
AT1G21680	expressed protein, similar to TolB protein precursor (...	2.1	5.21E-05	1.8	3.49E-04	Yes
AT1G22190	AP2 domain-containing transcription factor, putative,...	1.1	3.01E-04	1.1	2.11E-04	
AT1G23070	expressed protein, contains Pfam profile PF03619: Doma...	3.4	4.34E-09	2.7	2.45E-07	
AT1G23550	Encodes a protein with similarity to RCD1 but without...	1.9	2.55E-06	1.8	1.45E-05	
AT1G24625	zinc finger (C2H2 type) family protein (ZFP7), identic...	-1.0	3.61E-04	-1.1	1.92E-04	
AT1G26450	beta-1,3-glucanase-related, similar to beta-1,3-glucan...	1.7	1.51E-04	1.0	1.57E-02	
AT1G26770	expansin, putative (EXP10), similar to expansin At-EXP...	-0.9	4.47E-02	-1.1	2.74E-02	
AT1G26930	kelch repeat-containing F-box family protein, contains...	1.3	5.12E-07	1.3	6.53E-07	
AT1G26960	homeobox-leucine zipper protein, putative / HD-ZIP tra...	3.5	9.21E-12	3.2	6.73E-11	
AT1G27080	proton-dependent oligopeptide transport (POT) family p...	1.9	1.20E-08	1.6	1.89E-07	
AT1G29660	GDSL-motif lipase/hydrolase family protein, low simila...	-2.6	7.18E-09	-2.5	2.93E-08	
AT1G30530	UDP-glucoronosyl/UDP-glucosyl transferase family prote...	-1.6	1.80E-02	-1.8	1.12E-02	
AT1G30820	CTP synthase, putative / UTP--ammonia ligase, putative...	2.1	6.70E-06	1.2	7.25E-03	
AT1G32170	xyloglucan:xyloglucosyl transferase, putative / xylogl...	1.1	9.92E-05	0.8	1.74E-03	
AT1G35870	gypsy-like retrotransposon family, has a 1.4e-152 P-va...	2.2	2.55E-06	1.9	2.78E-05	
AT1G49960	xanthine/uracil permease family protein Note: annotate...	1.0	3.13E-05	1.0	4.33E-05	
AT1G49980	similar to UMUC-like DNA repair family protein [Arabid...	2.1	3.06E-05	1.8	2.61E-04	
AT1G51090	heavy-metal-associated domain-containing protein, con...	2.6	7.81E-07	1.6	7.48E-04	
AT1G52190	proton-dependent oligopeptide transport (POT) family p...	-1.2	2.03E-02	-1.2	3.51E-02	
AT1G52250	dynein light chain type 1 family protein, similar to S...	2.7	1.65E-08	2.1	1.59E-06	
AT1G52800	oxidoreductase, 2OG-Fe(II) oxygenase family protein, s...	2.1	1.90E-07	2.3	6.81E-08	Yes
AT1G54095	expressed protein	2.1	1.52E-08	2.1	3.45E-08	
AT1G56600	galactinol synthase, putative, similar to galactinol s...	1.1	2.23E-03	1.2	1.10E-03	
AT1G56660	expressed protein	1.8	3.29E-08	1.1	1.05E-04	
AT1G60190	armadillo/beta-catenin repeat family protein / U-box d...	3.2	4.98E-07	3.5	1.21E-07	
AT1G60680	aldo/keto reductase family protein, contains Pfam prof...	1.9	1.83E-04	1.7	1.81E-03	
AT1G61800	glucose-6-phosphate/phosphate translocator, putative,...	-1.7	1.25E-08	-2.2	6.40E-10	
AT1G62510	protease inhibitor/seed storage/lipid transfer protein...	1.1	5.17E-05	1.0	2.65E-04	
AT1G63840	zinc finger (C3HC4-type RING finger) family protein, s...	1.1	5.44E-05	1.2	9.34E-06	
AT1G64060	respiratory burst oxidase protein F (RbohF) (RbohAp108...	-1.4	1.02E-05	-1.2	8.36E-05	
AT1G64390	endo-1,4-beta-glucanase, putative / cellulase, putativ...	-1.8	6.32E-08	-1.6	3.40E-07	
AT1G64620	Dof-type zinc finger domain-containing protein, simila...	2.6	7.33E-10	1.9	1.54E-07	
AT1G64660	Cys/Met metabolism pyridoxal-phosphate-dependent enzym...	1.5	8.21E-05	1.2	8.25E-04	
AT1G66190	expressed protein, ; expression supported by MPSS	0.9	1.65E-02	1.6	3.27E-04	
AT1G67290	glyoxal oxidase-related, contains similarity to glyoxa...	1.4	4.26E-07	1.5	1.58E-07	
AT1G67720	leucine-rich repeat family protein / protein kinase fa...	2.1	6.16E-07	1.7	1.75E-05	
AT1G69530	expansin, putative (EXP1), identical to expansin (At-E...	-2.2	1.80E-04	-2.7	9.59E-06	Yes
AT1G70230	expressed protein	-1.2	3.58E-03	-1.3	1.72E-03	
AT1G71960	ABC transporter family protein, similar to breast canc...	1.3	5.21E-05	1.7	1.06E-06	
AT1G71980	protease-associated zinc finger (C3HC4-type RING finge...	3.1	2.02E-07	2.0	3.06E-05	
AT1G72450	expressed protein	-2.4	1.23E-08	-1.4	3.66E-05	
AT1G73120	expressed protein	4.2	9.21E-12	3.1	1.65E-09	
AT1G74055	expressed protein	1.8	3.32E-07	1.6	1.59E-06	
AT1G74670	gibberellin-responsive protein, putative, similar to S...	-2.1	1.73E-07	-1.5	2.46E-05	
AT1G74940	senescence-associated protein-related, similar to sene...	-2.3	2.04E-08	-1.7	4.91E-06	Yes
AT1G75000	GNS1/SUR4 membrane family protein, contains Pfam profi...	1.8	6.87E-08	2.0	2.29E-08	
AT1G75900	family II extracellular lipase 3 (EXL3), EXL3 (PMID:11...	-1.1	3.19E-05	-1.2	2.00E-05	
AT1G76790	O-methyltransferase family 2 protein, similar to caffe...	-1.5	4.26E-03	-1.3	2.43E-02	
AT1G77400	expressed protein	1.3	2.35E-05	1.4	6.26E-06	
AT1G77885	expressed protein	-2.0	1.49E-08	-1.2	1.96E-05	
AT1G78780	pathogenesis-related family protein, contains similari...	1.7	1.48E-07	1.5	9.55E-07	
AT1G79160	expressed protein	-1.7	2.82E-08	-1.4	1.01E-06	
AT1G79400	cation/proton exchanger, putative (CHX2), monovalent c...	-1.3	1.93E-05	-1.0	8.81E-04	

AT1G79570	protein kinase family protein, low similarity to EDR1...	1.7	2.48E-08	1.8	2.87E-08	
AT2G01150	zinc finger (C3HC4-type RING finger) family protein, c...	1.6	2.89E-06	1.3	3.67E-05	
AT2G03090	expansin, putative (EXP15), identical to SWISS-PROT:O8...	-1.7	4.97E-04	-1.3	4.46E-03	
AT2G04240	zinc finger (C3HC4-type RING finger) family protein, c...	1.2	3.61E-03	1.7	1.17E-04	
AT2G04660	E3 ubiquitin ligase, putative, E3, ubiquitin ligase; c...	1.6	2.11E-04	1.3	3.63E-03	
AT2G10130	pseudogene, similar to putative AP endonuclease/revers...	-1.7	5.81E-07	-1.7	1.49E-06	
AT2G12462	similar to expressed protein [Arabidopsis thaliana] (T...	-2.1	5.24E-07	-1.4	8.81E-05	
AT2G14170	methylmalonate-semialdehyde dehydrogenase, putative, s...	-2.4	2.53E-10	-2.4	9.65E-10	
AT2G14835	zinc finger (C3HC4-type RING finger) family protein, c...	1.7	4.40E-05	1.3	9.64E-04	
AT2G15750	expressed protein Note annotated by JH	-1.5	1.14E-06	-1.4	2.38E-06	
AT2G16660	nodulin family protein, similar to nodulin-like protei...	-1.9	7.93E-09	-1.8	1.99E-08	
AT2G17680	expressed protein, contains Pfam profile PF03087: Arab...	2.1	4.78E-08	1.5	1.30E-05	
AT2G20670	expressed protein, contains Pfam profile PF04720: Prot...	2.3	3.62E-11	1.9	9.65E-10	
AT2G20825	expressed protein	1.4	7.58E-05	1.7	8.75E-06	
AT2G22122	expressed protein	-1.9	8.28E-04	-1.6	7.34E-03	
AT2G25000	WRKY family transcription factor, contains Pfam profil...	1.5	2.14E-04	1.3	9.49E-04	
AT2G25200	expressed protein	1.3	3.21E-04	1.3	3.49E-04	Yes
AT2G26170	thromboxane-A synthase, putative / cytochrome P450 fam...	-1.1	3.90E-06	-1.0	5.81E-05	
AT2G26600	glycosyl hydrolase family 17 protein	1.7	7.81E-07	1.4	1.76E-05	
AT2G26690	nitrate transporter (NTP2), identical to nitrate trans...	-1.5	1.64E-03	-1.4	4.63E-03	
AT2G28120	nodulin family protein, similar to nodulin-like protei...	1.7	2.04E-07	1.2	2.66E-05	
AT2G28180	cation/hydrogen exchanger, putative (CHX8), monovalent...	1.4	2.65E-05	1.0	1.65E-03	
AT2G29670	expressed protein	-1.3	3.05E-07	-1.9	1.35E-09	
AT2G30010	expressed protein	-1.7	1.91E-07	-1.0	3.56E-04	
AT2G30360	CBL-interacting protein kinase 11 (CIPK11), identical...	1.5	5.13E-08	1.4	1.89E-07	
AT2G30600	similar to BTB/POZ domain-containing protein [Arabidop...	1.2	1.51E-05	1.3	1.50E-05	
AT2G32150	haloacid dehalogenase-like hydrolase family protein, c...	1.7	2.51E-08	1.5	1.89E-07	
AT2G33380	similar to Ca+2-binding EF hand family protein [Arabid...	1.9	9.74E-06	1.3	1.96E-03	
AT2G35760	integral membrane family protein, contains TIGRFAM TIG...	-1.1	2.54E-05	-1.3	1.56E-06	
AT2G36220	expressed protein	1.7	3.92E-06	1.3	1.88E-04	
AT2G36310	inosine-uridine preferring nucleoside hydrolase family...	1.5	1.16E-07	1.0	1.15E-04	
AT2G37640	expansin, putative (EXP3), identical to Alpha-expansin...	-1.2	8.89E-03	-1.3	9.19E-03	Yes
AT2G38170	calcium exchanger (CAX1), identical to high affinity c...	-1.4	1.39E-07	-1.5	1.24E-07	
AT2G38310	expressed protein, low similarity to early flowering p...	-1.1	1.02E-02	-1.0	2.35E-02	
AT2G38400	alanine--glyoxylate aminotransferase, putative / beta-...	2.3	1.80E-09	2.3	2.84E-09	
AT2G38940	phosphate transporter (PT2), identical to phosphate tr...	-1.7	2.58E-04	-1.8	7.34E-04	
AT2G39130	amino acid transporter family protein, belongs to INTE...	1.5	5.16E-04	0.9	3.86E-02	
AT2G39570	ACT domain-containing protein, contains Pfam ACT domai...	2.3	3.69E-10	2.5	6.40E-10	
AT2G40330	Bet v I allergen family protein, contains Pfam profile...	-1.1	2.45E-02	-1.6	1.99E-03	
AT2G41180	sigA-binding protein-related, low similarity to SigA b...	-1.5	6.73E-04	-1.7	4.21E-04	
AT2G41830	cyclin-related, contains Pfam profile PF02984: Cyclin,...	1.2	7.22E-03	2.0	9.31E-05	
AT2G42440	LOB domain protein 17 / lateral organ boundaries domai...	-0.9	1.99E-03	-0.6	3.69E-02	
AT2G47220	3' exoribonuclease family domain 1 protein-related, si...	1.1	1.65E-04	1.3	2.82E-05	
AT2G47770	benzodiazepine receptor-related, contains weak similar...	5.5	9.21E-12	5.3	4.68E-11	Yes
AT2G48020	sugar transporter, putative, similar to ERD6 protein {...	1.0	7.62E-04	1.4	3.39E-05	
AT2G48080	oxidoreductase, 2OG-Fe(II) oxygenase family protein, c...	1.2	1.44E-06	1.8	1.40E-08	
AT3G01470	homeobox-leucine zipper protein 5 (HAT5) / HD-ZIP prot...	-1.1	9.30E-03	-0.9	2.65E-02	
AT3G04010	glycosyl hydrolase family 17 protein, similar to beta-...	1.0	6.21E-04	1.1	7.11E-04	
AT3G04070	no apical meristem (NAM) family protein, contains Pfam...	1.3	1.46E-05	1.2	2.46E-05	
AT3G04210	disease resistance protein (TIR-NBS class), putative,...	-1.0	9.44E-04	-1.1	6.50E-04	
AT3G06145	expressed protein	-1.2	1.35E-03	-1.1	5.02E-03	
AT3G06770	glycoside hydrolase family 28 protein / polygalacturon...	-1.4	7.81E-07	-1.6	2.12E-07	
AT3G07010	pectate lyase family protein, similar to pectate lyase...	-1.3	3.64E-06	-1.4	2.67E-06	
AT3G08040	MATE efflux family protein, low similarity to enhanced...	-1.0	8.45E-04	-1.1	3.10E-04	
AT3G09270	glutathione S-transferase, putative, similar to glutat...	-2.2	1.71E-09	-1.8	5.60E-08	
AT3G11410	protein phosphatase 2C, putative / PP2C, putative, ide...	1.3	2.36E-06	1.1	4.54E-05	
AT3G12580	heat shock protein 70, putative / HSP70, putative, str...	-1.6	1.42E-07	-1.6	1.64E-07	
AT3G13450	2-oxoisovalerate dehydrogenase / 3-methyl-2-oxobutanoa...	2.3	4.22E-06	1.4	2.70E-03	Yes
AT3G13650	disease resistance response protein-related/ dirigent...	-1.6	5.13E-08	-1.3	1.59E-06	
AT3G15170	cup-shaped cotyledon1 protein / CUC1 protein (CUC1), i...	1.7	2.86E-06	1.1	6.83E-04	
AT3G15510	no apical meristem (NAM) family protein (NAC2), identi...	2.0	2.36E-09	1.4	8.39E-07	
AT3G16520	UDP-glucoronosyl/UDP-glucosyl transferase family prote...	3.0	3.05E-10	2.2	5.68E-08	
AT3G17110	pseudogene, glycine-rich protein	1.0	2.98E-04	1.0	1.52E-04	
AT3G17660	human Rev interacting-like family protein / hRIP famil...	1.7	4.81E-08	1.5	2.19E-07	
AT3G19370	expressed protein	-1.4	1.76E-07	-1.4	1.89E-07	
AT3G19390	cysteine proteinase, putative / thiol protease, putati...	1.2	3.03E-05	0.7	1.99E-02	
AT3G19850	phototropic-responsive NPH3 family protein, contains N...	-0.9	9.01E-05	-1.1	7.93E-06	
AT3G21560	UDP-glucosyltransferase, putative, similar to UDP-gluc...	-1.4	3.23E-07	-1.5	2.42E-07	
AT3G21770	peroxidase 30 (PER30) (P30) (PRXR9), identical to SP:Q...	-2.1	3.47E-11	-1.6	2.52E-09	
AT3G24300	ammonium transporter 1, member 3 (AMT1.3), nearly iden...	-2.7	1.59E-05	-1.8	2.98E-03	
AT3G25700	chloroplast nucleoid DNA-binding protein-related, cont...	-1.1	1.23E-05	-1.3	1.79E-06	
AT3G26510	octicosapeptide/Phox/Bem1p (PB1) domain-containing pro...	2.1	3.65E-09	1.6	5.41E-07	
AT3G29160	Snf1-related protein kinase (KIN11), identical to prot...	2.3	1.05E-05	1.0	3.83E-02	Yes
AT3G30775	proline oxidase, mitochondrial / osmotic stress-respon...	3.7	3.47E-11	2.9	2.52E-09	Yes
AT3G43060	pseudogene, hypothetical protein	1.3	3.91E-04	1.2	2.72E-03	
AT3G43430	zinc finger (C3HC4-type RING finger) family protein, c...	1.8	4.21E-06	1.3	3.70E-04	
AT3G43950	expressed protein	-0.6	2.23E-03	-1.7	3.45E-08	
AT3G44450	expressed protein	-0.9	8.03E-04	-0.9	8.28E-04	
AT3G47000	glycosyl hydrolase family 3 protein, beta-D-glucan exo...	1.6	1.15E-06	1.1	5.93E-04	
AT3G48240	octicosapeptide/Phox/Bem1p (PB1) domain-containing pro...	1.9	4.97E-08	2.0	3.11E-08	

AT3G48360	speckle-type POZ protein-related, contains Pfam PF006...	2.6	3.97E-07	2.8	1.39E-07	No
AT3G48520	cytochrome P450 family protein, similar to Cytochrome...	1.5	3.53E-05	1.3	1.36E-03	
AT3G48720	transferase family protein, similar to hypersensitivit...	-3.2	3.55E-11	-2.6	9.67E-10	
AT3G48920	myb family transcription factor (MYB45), similar to My...	-1.4	3.84E-05	-1.7	2.51E-06	
AT3G50800	expressed protein	2.0	3.20E-09	1.6	1.61E-07	
AT3G51630	protein kinase family protein, contains Pfam profile:...	1.2	3.93E-04	1.1	1.62E-03	
AT3G52370	beta-lg-H3 domain-containing protein / fasciclin domai...	-1.6	4.49E-05	-1.6	8.81E-05	
AT3G52430	phytoalexin-deficient 4 protein (PAD4), identical to p...	-1.3	9.58E-06	-1.3	1.94E-05	
AT3G53620	inorganic pyrophosphatase, putative (soluble) / pyroph...	1.5	1.47E-03	1.1	1.86E-02	
AT3G53720	cation/hydrogen exchanger, putative (CHX20), monovalen...	1.4	4.00E-04	1.2	3.05E-03	
AT3G54500	expressed protein	1.5	2.99E-06	1.2	7.79E-05	
AT3G56710	sigA-binding protein, identical to SigA binding protei...	-1.2	2.54E-04	-1.6	1.39E-05	
AT3G57040	two-component responsive regulator / response reactor...	-1.5	8.73E-07	-1.6	3.66E-07	
AT3G57520	alkaline alpha galactosidase, putative, similar to alk...	1.2	1.39E-06	1.2	1.49E-06	
AT3G58040	seven in absentia (SINA) family protein, similar to si...	2.0	1.91E-05	1.1	9.16E-03	
AT3G59620	expressed protein	1.5	2.22E-06	0.9	1.74E-03	
AT3G61060	F-box family protein / lectin-related, low similarity...	2.4	6.30E-09	1.7	1.95E-06	
AT3G61890	homeobox-leucine zipper protein 12 (HB-12) / HD-ZIP tr...	1.9	8.57E-09	1.5	6.04E-07	
AT3G62930	glutaredoxin family protein, contains glutaredoxin dom...	1.7	1.19E-05	1.5	1.05E-04	
AT4G00940	Dof-type zinc finger domain-containing protein, simila...	2.4	4.60E-08	2.1	2.84E-07	
AT4G00980	zinc knuckle (CCHC-type) family protein, contains Pfam...	-1.1	3.53E-05	-1.1	8.15E-05	
AT4G01870	tolB protein-related, contains weak similarity to TolB...	1.2	4.07E-04	1.1	1.26E-03	
AT4G03400	auxin-responsive GH3 family protein, similar to auxin-...	-1.2	1.79E-06	-1.1	1.89E-05	
AT4G10380	major intrinsic family protein / MIP family protein, c...	-1.3	4.90E-07	-1.3	1.49E-06	
AT4G13870	Werner Syndrome-like exonuclease (WEX), contains Pfam...	-1.3	1.69E-02	-1.4	1.43E-02	
AT4G14400	ankyrin repeat family protein, contains ankyrin repeat...	-2.1	1.57E-09	-1.7	4.55E-08	
AT4G15340	pentacyclic triterpene synthase (04C11), identical to...	-1.6	1.93E-07	-1.5	9.45E-07	
AT4G15530	similar to pyruvate,orthophosphate dikinase [Flaveria...	2.3	1.24E-09	1.7	1.89E-07	
AT4G16515	expressed protein	-1.3	6.27E-06	-1.0	1.73E-04	
AT4G18340	glycosyl hydrolase family 17 protein, similar to elici...	1.4	1.63E-06	1.4	1.64E-06	
AT4G18650	transcription factor-related, contains weak similarity...	3.6	1.85E-07	4.6	1.74E-08	
AT4G19140	expressed protein	1.5	9.17E-04	1.1	2.06E-02	
AT4G19450	nodulin-related, weak similarity to nodule-specific pr...	-1.1	2.05E-05	-1.2	1.45E-05	
AT4G20820	FAD-binding domain-containing protein, similar to SP:P...	2.6	3.87E-07	2.0	1.91E-05	
AT4G21870	26.5 kDa class P-related heat shock protein (HSP26.5-P...	-1.8	1.03E-04	-1.6	5.71E-04	
AT4G23190	protein kinase family protein, contains Pfam PF00069:...	1.4	1.29E-04	1.2	1.83E-03	
AT4G23700	cation/hydrogen exchanger, putative (CHX17), similar t...	1.5	2.53E-08	1.1	6.30E-06	
AT4G23870	expressed protein, predicted proteins, Arabidopsis tha...	1.3	5.45E-04	2.0	5.18E-06	
AT4G24040	glycosyl hydrolase family protein 37 / trehalase, puta...	3.6	3.23E-11	3.2	6.40E-10	
AT4G24350	phosphorylase family protein, contains Pfam PF01048: P...	-1.6	1.43E-03	-1.4	8.55E-03	
AT4G24780	pectate lyase family protein, similar to pectate lyase...	-1.5	1.69E-05	-1.3	1.36E-04	
AT4G25433	peptidoglycan-binding LysM domain-containing protein,...	1.7	9.90E-07	2.1	3.65E-08	
AT4G26950	expressed protein, contains Pfam profile PF04520: Prot...	-1.6	2.22E-05	-1.2	1.16E-03	
AT4G27590	copper-binding protein-related, low similarity to copp...	2.4	7.18E-09	1.5	1.49E-05	
AT4G27657	expressed protein	3.9	1.74E-13	2.6	1.07E-10	Yes
AT4G28040	similar to nodulin MtN21 family protein [Arabidopsis t...	2.4	3.87E-07	2.1	6.96E-06	
AT4G30490	AFG1-like ATPase family protein, contains Pfam profile...	2.8	1.31E-07	2.0	2.38E-05	
AT4G31140	glycosyl hydrolase family 17 protein, similar to elici...	1.2	6.56E-03	1.7	3.51E-04	
AT4G33220	pectinesterase family protein, contains Pfam profile:...	-1.2	4.38E-04	-0.9	5.76E-03	
AT4G33420	peroxidase, putative, identical to class III peroxidas...	1.2	1.73E-05	1.2	2.86E-05	
AT4G33700	CBS domain-containing protein, contains Pfam profiles...	2.9	3.05E-10	2.5	4.82E-09	
AT4G33750	expressed protein	1.5	1.31E-07	1.2	1.23E-05	
AT4G34950	nodulin family protein, similar to nodulin-like protei...	-1.7	4.44E-04	-1.9	8.76E-05	
AT4G35770	senescence-associated protein (SEN1), identical to sen...	4.7	9.02E-17	3.7	1.58E-14	
AT4G36640	SEC14 cytosolic factor family protein / phosphoglyceri...	-1.8	5.17E-09	-1.3	6.30E-07	
AT4G38840	auxin-responsive protein, putative, auxin-inducible SA...	-1.0	3.23E-05	-1.2	3.21E-06	
AT4G39100	PHD finger family protein / bromo-adjacent homology (B...	1.5	2.33E-06	1.2	6.67E-05	
AT4G39660	alanine--glyoxylate aminotransferase, putative / beta-...	2.2	5.84E-08	1.6	5.88E-06	
AT4G40060	homeobox-leucine zipper protein 16 (HB-16) / HD-ZIP tr...	-1.8	1.85E-06	-1.7	3.91E-06	
AT5G02890	transferase family protein, contains Pfam profile PF02...	-2.7	3.23E-11	-2.2	1.84E-09	Yes
AT5G03380	similar to heavy-metal-associated domain-containing pr...	1.0	2.35E-05	1.0	4.58E-05	
AT5G03670	expressed protein	-1.5	5.17E-09	-1.8	9.65E-10	
AT5G03760	glycosyl transferase family 2 protein, similar to beta...	-2.3	5.10E-07	-2.2	1.79E-06	
AT5G04040	patatin-related, contains Patatin domain PF01734	1.3	8.16E-07	1.6	1.68E-07	
AT5G04310	pectate lyase family protein, similar to pectate lyase...	2.4	1.99E-10	2.2	1.17E-09	
AT5G05220	expressed protein	3.3	9.66E-09	2.8	2.12E-07	
AT5G07440	glutamate dehydrogenase 2 (GDH2), identical to glutama...	2.3	9.78E-07	1.5	4.73E-04	
AT5G07580	encodes a member of the ERF (ethylene response factor)...	-2.2	8.75E-12	-2.0	6.10E-11	Yes
AT5G08330	TCP family transcription factor, putative, similar to...	-1.1	1.22E-03	-1.6	6.07E-05	
AT5G08350	GRAM domain-containing protein / ABA-responsive protei...	2.6	1.29E-08	1.7	7.64E-06	
AT5G11070	expressed protein	1.0	2.72E-04	1.0	5.32E-04	
AT5G11090	serine-rich protein-related, contains some similarity...	1.9	1.41E-08	1.3	5.08E-06	
AT5G15410	cyclic nucleotide-regulated ion channel / cyclic nucle...	2.5	4.00E-11	2.0	2.64E-09	Yes
AT5G15740	expressed protein, contains Pfam PF03138: Plant protei...	-1.2	5.30E-04	-1.6	2.23E-05	
AT5G16120	hydrolase, alpha/beta fold family protein, similar to...	1.1	5.97E-03	1.1	1.27E-02	
AT5G17760	AAA-type ATPase family protein, contains Pfam profile:...	1.5	1.02E-04	1.9	5.26E-06	
AT5G18470	curculin-like (mannose-binding) lectin family protein,...	1.2	6.55E-07	1.4	6.83E-08	
AT5G18670	beta-amylase, putative (BMY3) / 1,4-alpha-D-glucan mal...	2.7	3.05E-10	2.2	4.54E-09	
AT5G18840	sugar transporter, putative, similar to ERD6 protein {...	2.3	3.38E-09	1.8	1.89E-07	
AT5G20190	expressed protein	-1.4	1.09E-06	-1.8	9.51E-08	

AT5G22460	esterase/lipase/thioesterase family protein, low simil...	1.9	9.26E-09	1.6	2.12E-07	
AT5G22920	zinc finger (C3HC4-type RING finger) family protein, c...	2.5	3.19E-08	2.2	3.40E-07	No
AT5G23100	expressed protein, contains Pfam profile PF04759: Prot...	1.1	1.41E-03	1.4	2.40E-04	
AT5G23660	nodulin MtN3 family protein, similar to MtN3 GI:161960...	-0.6	8.57E-03	-0.7	1.02E-02	
AT5G24570	expressed protein	-1.5	1.07E-06	-1.5	1.30E-06	
AT5G24660	expressed protein	-1.1	1.31E-03	-2.1	1.39E-07	
AT5G25840	expressed protein	-1.0	5.05E-03	-1.0	5.01E-03	
AT5G26340	hexose transporter, putative, strong similarity to hex...	2.0	6.15E-08	1.3	2.93E-05	
AT5G34830	expressed protein, ; expression supported by MPSS	1.8	5.13E-08	1.5	1.49E-06	
AT5G38710	proline oxidase, putative / osmotic stress-responsive...	1.5	1.47E-05	1.2	1.91E-04	
AT5G39610	no apical meristem (NAM) family protein, contains Pfam...	-1.1	1.56E-06	-1.0	1.44E-05	
AT5G41080	glycerophosphoryl diester phosphodiesterase family pro...	3.1	5.57E-13	3.3	1.83E-13	
AT5G42590	cytochrome P450 71A16, putative (CYP71A16), Identical...	-3.4	3.52E-13	-2.1	1.17E-09	
AT5G47130	Bax inhibitor-1 family / BI-1 family, similar to SP:Q9...	1.7	1.41E-04	1.2	4.37E-03	
AT5G47240	MutT/nudix family protein, similar to SP:P53370 Nucleo...	1.6	4.30E-06	1.4	6.05E-05	Yes
AT5G47370	homeobox-leucine zipper protein 2 (HAT2) / HD-ZIP prot...	2.0	1.53E-04	1.4	8.52E-03	
AT5G48850	male sterility MS5 family protein, similar to male ste...	-0.9	3.38E-04	-1.9	1.74E-08	
AT5G49330	myb family transcription factor, contains Pfam profile...	-1.6	1.18E-05	-1.8	1.23E-05	
AT5G49360	glycosyl hydrolase family 3 protein	2.4	8.85E-10	2.0	3.54E-08	Yes
AT5G51780	basix helix-loop-helix (bHLH) family protein, contains...	1.2	1.15E-04	1.0	1.22E-03	
AT5G51790	basix helix-loop-helix (bHLH) family protein, contains...	1.3	1.93E-03	1.3	3.46E-03	
AT5G51800	expressed protein	2.0	1.94E-04	1.5	5.59E-03	
AT5G52680	heavy-metal-associated domain-containing protein, low...	1.7	8.36E-03	1.5	2.81E-02	
AT5G53590	auxin-responsive family protein, similar to indole-3-a...	1.6	4.78E-07	1.9	2.87E-08	
AT5G54750	transport protein particle (TRAPP) component Bet3, put...	1.7	1.57E-07	1.4	4.38E-06	
AT5G57710	heat shock protein-related, contains similarity to 101...	-1.3	1.61E-04	-1.1	1.40E-03	
AT5G58650	expressed protein	2.9	3.32E-10	2.2	3.40E-08	
AT5G60710	zinc finger (C3HC4-type RING finger) family protein, l...	-1.0	5.44E-05	-1.0	5.99E-05	
AT5G61440	thioredoxin family protein, low similarity to thioredo...	1.3	8.62E-04	1.4	5.40E-04	
AT5G62280	expressed protein	-1.3	1.20E-05	-1.3	1.89E-05	
AT5G62350	invertase/pectin methylesterase inhibitor family prote...	-1.3	2.37E-05	-1.1	3.07E-04	
AT5G62550	expressed protein	-0.8	7.72E-04	-1.8	1.74E-08	
AT5G63180	pectate lyase family protein, similar to pectate lyase...	-1.9	6.30E-05	-1.4	1.49E-03	
AT5G64080	protease inhibitor/seed storage/lipid transfer protein...	1.6	3.04E-03	1.2	3.33E-02	
AT5G65110	similar to acyl-CoA oxidase, putative [Arabidopsis tha...	1.2	9.90E-06	1.2	2.86E-05	
AT5G65500	protein kinase family protein, contains Pfam profile:...	1.9	6.84E-04	1.3	2.04E-02	
AT5G66170	similar to senescence-associated family protein [Arabi...	2.8	9.21E-12	2.4	4.62E-10	
AT5G66580	expressed protein	1.8	1.47E-05	1.3	9.15E-04	
AT5G66650	expressed protein, contains Pfam domain, PF04678: Prot...	2.3	3.44E-09	2.0	3.64E-08	
AT5G66985	expressed protein	1.5	6.46E-07	1.4	3.06E-06	
AT5G67160	transferase family protein, similar to anthranilate N-...	1.6	7.59E-07	1.6	9.27E-07	
AT5G67440	phototropic-responsive NPH3 family protein, contains N...	1.3	4.13E-07	1.2	1.86E-06	

Notes

A) Relative differental induction (base 2 logarhitmic) of the gene upon dexametasone tratment in respective transgenic line compared to wt in the array experiment

B) Indication of whether gene activation / repression by bZIP11 was confirmed by Q-PCR under low sucrose conditions. No value; not determined

Supplementary table S2. Induced genes after bZIP10 transfection compared to bZIP11 or bZIP10 and bZIP11 (co-)transfection

mRNA levels in protoplasts transfected with bZIP10, bZIP11 or both were compared to mRNA levels in protoplasts transfected with the empty vector. Changes in mRNA levels are shown logarithmically. Statistically significant changes in gene expression of two-fold of larger are indicated by gray shading.

		bZIP10		bZIP11		bZIP10 & bZIP11	
AGI	Description	Log2FC	adj.P.Val	Log2FC	adj.P.Val	Log2FC	adj.P.Val
AT5G51060	RHD2 (ROOT HAIR DEFECTIVE 2)	1.98	4.65E-08	-0.39	7.01E-02	-0.52	6.92E-03
AT5G48720	similar to unknown protein [Arabidopsis thaliana] (TAIR…	1.29	4.01E-04	-0.33	2.68E-01	0.03	9.53E-01
AT4G02640	BZO2H1 (ARABIDOPSIS THALIANA BASIC LEUCINE ZIPPER 10);…	6.92	3.80E-16	-0.07	9.07E-01	6.60	1.27E-16
AT1G66570	ATSUC7 (SUCROSE-PROTON SYMPORTER 7); carbohydrate trans…	1.94	6.71E-04	-0.91	2.35E-02	2.17	1.76E-06
AT1G64930	CYP89A7 (cytochrome P450, family 87, subfamily A, polyp…	1.36	2.15E-04	-0.31	3.30E-01	-0.37	1.41E-01
AT1G62480	vacuolar calcium-binding protein-related	1.58	1.16E-02	5.89	9.97E-12	6.55	3.81E-13
AT2G29440	ATGSTU6 (GLUTATHIONE S-TRANSFERASE 24); glutathione tra…	1.47	1.52E-05	-0.10	8.33E-01	-0.32	1.49E-01
AT2G29410	MTPB1; efflux transmembrane transporter/ zinc ion trans…	1.05	7.84E-04	-0.11	8.12E-01	-0.07	8.54E-01

Supplementary table S3. Induced genes after bZIP10 and bZIP11 co-transfection compared to bZIP11 or bZIP11 transfection

mRNA levels in protoplasts transfected with bZIP10, bZIP11 or both were compared to mRNA levels in protoplasts transfected with the empty vector. Changes in mRNA levels are shown logarithmically. Statistically significant changes in gene expression of two-fold of larger are indicated by gray shading.

		bZIP10		bZIP11		bZIP10 & bZIP11	
AGI	Description	Log2FC	adj.P.Val	Log2FC	adj.P.Val	Log2FC	adj.P.Val
AT1G01120	KCS1 (3-KETOACYL-COA SYNTHASE 1); acyltransferase	-0.22	9.99E-01	0.00	9.98E-01	2.53	2.29E-05
AT1G01540	protein kinase family protein	0.13	9.99E-01	2.20	9.75E-06	2.10	7.97E-06
AT1G01620	PIP1C (PLASMA MEMBRANE INTRINSIC PROTEIN 1;3); water ch…	-0.06	9.99E-01	3.58	2.51E-10	1.88	1.18E-06
AT1G02660	lipase class 3 family protein	0.01	9.99E-01	2.02	1.28E-09	1.42	8.60E-08
AT1G02700	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.09	9.99E-01	0.16	7.39E-01	1.77	8.60E-08
AT1G02810	pectinesterase family protein	0.13	9.99E-01	2.75	1.28E-09	1.07	1.79E-04
AT1G03870	FLA9	-0.01	9.99E-01	7.45	1.32E-15	7.88	1.62E-16
AT1G04160	XIB (Myosin-like protein XIB)	-0.27	8.37E-01	1.47	9.30E-07	1.69	5.88E-08
AT1G04430	dehydration-responsive protein-related	0.16	9.91E-01	0.39	2.01E-01	1.07	8.42E-05
AT1G05100	MAPKKK18 (Mitogen-activated protein kinase kinase kinas…	-0.11	9.99E-01	1.40	1.15E-06	3.21	2.09E-12
AT1G06110	SKIP16 (SKP1/ASK-INTERACTING PROTEIN 16); protein bindi…	0.30	7.96E-01	0.92	4.76E-04	3.00	1.83E-11
AT1G06120	[AT1G06120, fatty acid desaturase family protein];[AT1G…	-0.07	9.99E-01	0.21	6.73E-01	6.99	8.55E-16
AT1G07430	protein phosphatase 2C, putative / PP2C, putative	-0.21	9.79E-01	0.44	2.33E-01	1.09	3.98E-04
AT1G07570	APK1A (Arabidopsis protein kinase 1A); kinase	-0.13	9.99E-01	0.06	9.29E-01	2.45	7.06E-10
AT1G07910	ATRNL/RNL (ARABIDOPSIS THALIANA RNA LIGASE); 2',3'-cycl…	-0.21	9.31E-01	0.07	9.08E-01	1.45	3.89E-07
AT1G08600	ATRX/CHR20; ATP binding / DNA binding / helicase	0.48	8.88E-01	1.37	5.08E-03	4.26	2.06E-09
AT1G08630	THA1 (THREONINE ALDOLASE 1); aldehyde-lyase/ threonine…	-0.08	9.99E-01	0.84	3.98E-03	1.58	1.81E-06
AT1G09155	ATPP2-B15 (Phloem protein 2-B15); carbohydrate binding	0.08	9.99E-01	0.98	2.16E-05	1.32	2.12E-07
AT1G09600	protein kinase family protein	-0.10	9.99E-01	-0.20	6.43E-01	1.31	7.95E-06
AT1G10070	ATBCAT-2; branched-chain-amino-acid transaminase/ catal…	-0.17	9.99E-01	1.03	2.55E-03	3.45	1.76E-10
AT1G10530	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.07	9.99E-01	-0.02	9.80E-01	1.71	3.19E-06
AT1G10560	armadillo/beta-catenin repeat family protein / U-box do…	-0.24	8.77E-01	1.05	4.77E-05	3.67	2.53E-13
AT1G10940	ASK1 (ARABIDOPSIS SERINE/THREONINE KINASE 1); kinase	0.20	9.80E-01	0.28	5.23E-01	1.95	2.12E-07
AT1G11440	similar to glycine-rich protein [Arabidopsis thaliana]…	-0.14	9.99E-01	2.00	1.93E-05	1.99	1.00E-05
AT1G11530	ATCXXS1 (C-TERMINAL CYSTEINE RESIDUE IS CHANGED TO A SE…	0.09	9.99E-01	1.70	1.60E-06	1.07	2.13E-04
AT1G12080	contains domain PTHR22683 (PTHR22683)	0.03	9.99E-01	0.50	2.50E-01	2.73	2.30E-08
AT1G12450	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.19	9.99E-01	0.01	9.92E-01	1.38	2.96E-04
AT1G12900	GAPA-2; glyceraldehyde-3-phosphate dehydrogenase	-0.07	9.99E-01	2.18	1.35E-08	2.04	1.35E-08
AT1G13260	RAV1 (Related to ABI3/VP1 1); DNA binding / transcripti…	-0.02	9.99E-01	1.37	4.86E-07	1.34	2.95E-07
AT1G14910	epsin N-terminal homology (ENTH) domain-containing prot…	0.02	9.99E-01	0.78	4.52E-02	1.67	2.74E-05
AT1G15040	glutamine amidotransferase-related	0.15	9.74E-01	4.33	3.98E-14	4.90	1.47E-15
AT1G15380	lactoylglutathione lyase family protein / glyoxalase I…	0.10	9.99E-01	0.95	7.24E-06	1.69	9.05E-10
AT1G15640	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.27	8.69E-01	-0.14	7.69E-01	1.90	2.59E-08
AT1G15820	LHCB6 (LIGHT HARVESTING COMPLEX PSII); chlorophyll bind…	-0.27	9.77E-01	2.93	3.65E-08	1.02	4.36E-03
AT1G16170	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.08	9.99E-01	0.45	3.76E-01	4.08	1.50E-10
AT1G16310	cation efflux family protein	0.03	9.99E-01	0.52	2.71E-02	4.92	3.09E-15
AT1G16500	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.03	9.99E-01	2.25	2.81E-05	3.15	1.55E-07
AT1G16850	unknown protein	0.22	8.77E-01	4.62	2.37E-14	2.62	1.92E-11
AT1G17240	leucine-rich repeat family protein	0.57	2.83E-01	0.56	2.71E-02	1.68	9.60E-08
AT1G17590	CCAAT-binding transcription factor (CBF-B/NF-YA) family…	-0.13	9.99E-01	-0.06	9.29E-01	1.31	2.75E-06
AT1G17600	disease resistance protein (TIR-NBS-LRR class), putativ…	-0.07	9.99E-01	-0.02	9.80E-01	1.99	2.30E-08
AT1G17615	disease resistance protein (TIR-NBS class), putative	-0.21	9.56E-01	2.91	3.85E-10	6.94	3.49E-16
AT1G17810	BETA-TIP (BETA-TONOPLAST INTRINSIC PROTEIN); water chan…	0.12	9.99E-01	-0.02	9.85E-01	3.92	2.49E-11
AT1G18270	ketose-bisphosphate aldolase class-II family protein	0.02	9.99E-01	0.52	1.49E-02	1.48	6.10E-08
AT1G18460	lipase family protein	0.03	9.99E-01	1.57	1.48E-06	1.78	1.18E-07
AT1G19050	ARR7 (RESPONSE REGULATOR 7); transcription regulator/ t…	-0.02	9.99E-01	0.21	6.49E-01	1.18	6.87E-05
AT1G19110	inter-alpha-trypsin inhibitor heavy chain-related	0.18	8.27E-01	0.92	1.15E-06	1.07	6.16E-08
AT1G19440	very-long-chain fatty acid condensing enzyme, putative	0.05	9.99E-01	1.07	5.72E-05	1.35	1.40E-06
AT1G19450	integral membrane protein, putative / sugar transporter…	0.08	9.99E-01	3.18	6.35E-06	3.02	5.68E-06
AT1G19570	[AT1G19570, DHAR1 (DEHYDROASCORBATE REDUCTASE); glutath…	-0.10	9.99E-01	0.52	2.10E-02	1.06	9.91E-06
AT1G19960	similar to transmembrane receptor [Arabidopsis thaliana…	0.06	9.99E-01	0.55	1.63E-01	2.88	6.58E-09
AT1G20300	pentatricopeptide (PPR) repeat-containing protein	-0.24	9.99E-01	0.64	4.46E-01	3.07	7.06E-06
AT1G20330	SMT2 (STEROL METHYLTRANSFERASE 2)	-0.19	9.68E-01	1.34	8.23E-06	1.41	1.86E-06
AT1G20840	TMT1 (TONOPLAST MONOSACCHARIDE TRANSPORTER1); carbohydr..	0.07	9.99E-01	1.65	2.12E-05	1.92	1.43E-06
AT1G20880	RNA recognition motif (RRM)-containing protein	-0.12	9.99E-01	0.93	5.98E-03	1.05	1.10E-03
AT1G21070	transporter-related	0.14	9.99E-01	0.34	3.33E-01	1.18	4.29E-05
AT1G21080	DNAJ heat shock N-terminal domain-containing protein	0.03	9.99E-01	0.50	7.54E-02	1.41	2.88E-06
AT1G21370	similar to unnamed protein product [Vitis vinifera] (GB…	0.12	9.99E-01	0.22	4.92E-01	1.58	5.83E-08
AT1G21910	AP2 domain-containing transcription factor family prote…	-0.21	9.68E-01	0.11	8.59E-01	1.27	3.14E-05
AT1G22190	AP2 domain-containing transcription factor, putative	0.10	9.99E-01	0.78	1.68E-03	1.26	4.15E-06
AT1G22370	ATUGT85A5 (UDP-GLUCOSYL TRANSFERASE 85A5); transferase,…	-0.12	9.99E-01	0.06	9.32E-01	1.10	2.67E-05
AT1G22430	alcohol dehydrogenase, putative	-0.06	9.99E-01	1.87	5.83E-08	3.05	1.40E-11
AT1G22440	alcohol dehydrogenase, putative	-0.25	8.70E-01	0.59	1.42E-02	1.07	2.45E-05
AT1G22810	AP2 domain-containing transcription factor, putative	0.01	9.99E-01	2.08	1.66E-06	3.00	3.89E-09
AT1G23020	ATFRO3/FRO3 (FERRIC REDUCTION OXIDASE 3); ferric-chelat…	0.18	9.74E-01	2.54	8.46E-10	2.52	3.20E-10
AT1G23030	armadillo/beta-catenin repeat family protein / U-box do…	0.02	9.99E-01	0.15	6.92E-01	1.86	3.53E-09
AT1G23060	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.04	9.99E-01	2.60	2.85E-09	1.10	1.31E-04
AT1G24360	3-oxoacyl-(acyl-carrier protein) reductase, chloroplast…	0.02	9.99E-01	0.11	8.22E-01	2.03	2.55E-09
AT1G24575	unknown protein	0.13	9.99E-01	0.37	1.86E-01	1.63	1.17E-07
AT1G26190	phosphoribulokinase/uridine kinase family protein	0.13	9.99E-01	0.87	1.01E-02	1.30	1.28E-04
AT1G26450	beta-1,3-glucanase-related	0.09	9.99E-01	0.13	7.11E-01	2.65	5.70E-12
AT1G26970	protein kinase, putative	-0.05	9.99E-01	0.12	7.39E-01	1.73	1.78E-09
AT1G27950	lipid transfer protein-related	-0.32	9.72E-01	4.28	1.65E-09	5.89	4.25E-12
AT1G27960	ECT9 (evolutionarily conserved C-terminal region 9)	-0.05	9.99E-01	0.00	9.98E-01	1.92	1.65E-07
AT1G27990	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.16	9.79E-01	0.87	5.68E-04	5.88	5.48E-16
AT1G28070	protein binding	0.20	9.93E-01	-0.13	8.53E-01	1.47	2.62E-05
AT1G28250	similar to hypothetical protein [Oryza sativa (japonica…	-0.15	9.99E-01	0.53	1.42E-01	1.09	6.15E-04
AT1G30720	[AT1G30720, FAD-binding domain-containing protein];[AT1…	0.26	9.99E-01	0.19	8.84E-01	1.50	3.93E-03
AT1G30820	CTP synthase, putative / UTP--ammonia ligase, putative	0.78	4.15E-01	2.28	2.48E-06	4.50	4.29E-11
AT1G31290	PAZ domain-containing protein / piwi domain-containing…	0.21	9.93E-01	1.41	1.70E-04	2.08	6.49E-07
AT1G32150	bZIP transcription factor family protein	-0.03	9.99E-01	1.89	2.31E-04	2.17	2.47E-05
AT1G32200	ATS1 (ACYLTRANSFERASE 1)	-0.10	9.99E-01	0.95	1.47E-03	1.18	7.99E-05
AT1G32410	vacuolar protein sorting 55 family protein / VPS55 fami…	0.05	9.99E-01	1.20	9.57E-06	1.73	3.03E-08
AT1G32540	LOL1 (LSD ONE LIKE 1)	-0.10	9.99E-01	0.83	7.52E-04	2.58	7.70E-11
AT1G32920	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.15	9.99E-01	0.42	3.12E-01	1.15	4.68E-04
AT1G35910	trehalose-6-phosphate phosphatase, putative	-0.11	9.99E-01	0.64	4.91E-03	2.12	7.55E-10

AT1G38065	[AT1G38065, similar to unknown protein [Arabidopsis tha…	-0.19	9.79E-01	0.32	3.88E-01	1.91	9.08E-08
AT1G42470	patched family protein	0.01	9.99E-01	0.25	3.55E-01	1.15	2.55E-06
AT1G43670	fructose-1,6-bisphosphatase, putative / D-fructose-1,6-…	0.00	9.99E-01	0.19	7.44E-01	1.70	2.75E-06
AT1G44770	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.09	9.99E-01	0.66	4.19E-01	1.35	1.80E-02
AT1G48130	ATPER1 (Arabidopsis thaliana 1-cysteine peroxiredoxin 1…	0.01	9.99E-01	0.60	1.04E-01	6.79	7.83E-15
AT1G48960	universal stress protein (USP) family protein	0.06	9.99E-01	1.92	1.60E-07	2.03	3.19E-08
AT1G48970	GTP binding / translation initiation factor	0.08	9.99E-01	0.12	7.00E-01	1.51	4.91E-09
AT1G48980	similar to oxidoreductase, 2OG-Fe(II) oxygenase family…	-0.06	9.99E-01	-0.05	9.46E-01	5.37	3.79E-15
AT1G50890	binding	0.09	9.99E-01	2.03	1.32E-07	3.44	1.92E-11
AT1G50900	similar to hypothetical protein [Vitis vinifera] (GB:CA…	0.07	9.99E-01	0.53	4.69E-02	1.22	1.05E-05
AT1G51070	basic helix-loop-helix (bHLH) family protein	-0.22	9.99E-01	-0.22	8.14E-01	2.89	2.76E-07
AT1G51090	heavy-metal-associated domain-containing protein	0.06	9.99E-01	2.27	7.74E-07	2.46	1.14E-07
AT1G51140	basic helix-loop-helix (bHLH) family protein	0.03	9.99E-01	0.01	9.90E-01	1.54	1.02E-06
AT1G52260	ATPDIL1-5 (PDI-LIKE 1-5); thiol-disulfide exchange inte…	0.37	8.91E-01	0.75	5.70E-02	1.13	1.72E-03
AT1G52540	protein kinase, putative	0.27	9.99E-01	2.69	5.91E-06	1.68	6.55E-04
AT1G54740	similar to structural constituent of ribosome [Arabidop…	-0.66	5.26E-01	1.82	1.64E-05	1.03	3.40E-03
AT1G55020	LOX1 (Lipoxygenase 1); lipoxygenase	-0.29	8.74E-01	2.36	1.02E-08	3.73	3.35E-12
AT1G55450	embryo-abundant protein-related	0.37	9.78E-01	0.37	6.83E-01	1.23	1.44E-02
AT1G55810	uracil phosphoribosyltransferase, putative / UMP pyroph…	0.17	9.99E-01	0.84	5.18E-03	1.54	4.16E-06
AT1G56210	copper chaperone (CCH)-related	0.18	9.57E-01	0.81	6.76E-04	3.78	1.27E-13
AT1G56660	unknown protein	0.14	9.99E-01	1.65	1.59E-06	2.72	4.63E-10
AT1G57680	similar to unnamed protein product [Vitis vinifera] (GB…	0.17	9.99E-01	0.62	2.46E-01	1.53	5.80E-04
AT1G58440	XF1 (SQUALENE EPOXIDASE 1); oxidoreductase	-0.19	9.53E-01	2.43	5.48E-10	2.73	2.79E-11
AT1G60190	armadillo/beta-catenin repeat family protein / U-box do…	0.02	9.99E-01	1.19	1.21E-04	2.01	6.24E-08
AT1G60670	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.11	9.99E-01	0.50	1.16E-01	1.00	4.50E-04
AT1G60950	FED A (FERREDOXIN 2); 2 iron, 2 sulfur cluster binding…	-0.09	9.99E-01	1.87	5.37E-06	3.14	1.44E-09
AT1G61170	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.24	8.94E-01	0.13	7.74E-01	1.05	5.41E-05
AT1G61340	F-box family protein	-0.18	9.91E-01	0.50	1.28E-01	1.77	7.19E-07
AT1G61460	S-locus protein kinase, putative	-0.04	9.99E-01	-0.05	9.20E-01	3.52	4.56E-14
AT1G62480	vacuolar calcium-binding protein-related	1.58	1.16E-02	5.89	9.97E-12	6.55	3.81E-13
AT1G62510	protease inhibitor/seed storage/lipid transfer protein…	-0.18	9.53E-01	2.55	1.40E-10	3.50	2.91E-13
AT1G62560	flavin-containing monooxygenase family protein / FMO fa…	-0.08	9.99E-01	1.39	2.70E-06	1.55	2.51E-07
AT1G62660	beta-fructosidase (BFRUCT3) / beta-fructofuranosidase /…	-0.35	7.41E-01	0.48	9.47E-02	1.03	1.33E-04
AT1G63090	ATPP2-A11 (Phloem protein 2-A11); carbohydrate binding	-0.32	6.61E-01	0.74	7.89E-04	1.20	1.25E-06
AT1G63120	ATRBL2 (ARABIDOPSIS THALIANA RHOMBOID-LIKE 2); serine-t…	0.09	9.99E-01	0.39	3.90E-01	2.96	3.46E-09
AT1G63950	heavy-metal-associated domain-containing protein	-0.04	9.99E-01	1.69	5.72E-05	4.73	9.79E-12
AT1G64620	Dof-type zinc finger domain-containing protein	-0.13	9.99E-01	1.67	3.31E-06	2.87	5.47E-10
AT1G66230	MYB20 (myb domain protein 20); DNA binding / transcript…	-0.40	8.51E-01	0.36	4.87E-01	1.45	9.62E-05
AT1G66570	ATSUC7 (SUCROSE-PROTON SYMPORTER 7); carbohydrate trans…	1.94	6.71E-04	-0.91	2.35E-02	2.17	1.76E-06
AT1G66890	similar to 50S ribosomal protein-related [Arabidopsis t…	-0.25	7.34E-01	2.90	1.54E-12	1.46	1.12E-08
AT1G67070	DIN9 (DARK INDUCIBLE 9); mannose-6-phosphate isomerase	-0.25	9.28E-01	0.54	5.51E-02	2.59	5.79E-10
AT1G67480	kelch repeat-containing F-box family protein	-0.32	8.45E-01	2.29	2.22E-08	3.49	1.29E-11
AT1G67880	glycosyl transferase family 17 protein	0.42	8.47E-01	1.53	1.52E-04	1.56	6.51E-05
AT1G68410	protein phosphatase 2C-related / PP2C-related	-0.01	9.99E-01	1.17	3.95E-06	2.11	3.41E-10
AT1G68795	CLE12 (CLAVATA3/ESR-RELATED 12); receptor binding	-0.42	6.11E-01	0.42	1.43E-01	1.73	1.14E-07
AT1G69040	ACR4 (ACT REPEAT 4); amino acid binding	-0.03	9.99E-01	-0.03	9.67E-01	1.25	6.48E-06
AT1G69070	binding	0.03	9.99E-01	1.39	3.35E-05	1.08	2.95E-04
AT1G69270	RPK1 (RECEPTOR-LIKE PROTEIN KINASE 1); kinase	-0.10	9.99E-01	0.21	5.96E-01	1.07	4.46E-05
AT1G70410	carbonic anhydrase, putative / carbonate dehydratase, p…	-0.04	9.99E-01	1.13	9.35E-05	1.31	7.36E-06
AT1G70590	F-box family protein	-0.09	9.84E-01	0.26	9.40E-02	1.91	4.65E-12
AT1G71020	armadillo/beta-catenin repeat family protein / U-box do…	0.07	9.99E-01	0.92	1.27E-03	1.60	9.79E-07
AT1G71420	pentatricopeptide (PPR) repeat-containing protein	-0.24	9.03E-01	0.05	9.43E-01	3.14	9.70E-12
AT1G71960	ABC transporter family protein	0.13	9.99E-01	1.00	6.23E-03	3.69	2.06E-10
AT1G71980	protease-associated zinc finger (C3HC4-type RING finger…	0.39	8.77E-01	2.98	2.82E-08	4.11	7.70E-11
AT1G71990	FUT13 (fucosyltransferase 13); fucosyltransferase/ tran…	-0.30	8.00E-01	0.56	2.76E-02	1.42	1.12E-06
AT1G72650	TRFL6 (TRF-LIKE 6); DNA binding / transcription factor	0.02	9.99E-01	0.40	1.58E-01	1.47	9.07E-07
AT1G72730	eukaryotic translation initiation factor 4A, putative /…	-0.27	8.91E-01	-0.51	7.54E-02	1.85	7.66E-08
AT1G72790	hydroxyproline-rich glycoprotein family protein	0.14	9.99E-01	1.17	3.66E-04	4.75	4.15E-13
AT1G72800	nuM1-related	0.08	9.99E-01	0.08	9.24E-01	2.27	4.07E-08
AT1G72890	disease resistance protein (TIR-NBS class), putative	-0.13	9.99E-01	1.01	6.71E-03	2.70	3.06E-08
AT1G73120	similar to hypothetical protein [Vitis vinifera] (GB:CA…	0.43	8.60E-01	3.66	2.39E-09	5.18	4.15E-12
AT1G73190	ALPHA-TIP/TIP3;1 (ALPHA-TONOPLAST INTRINSIC PROTEIN); w…	-0.11	9.99E-01	0.07	9.34E-01	1.46	1.65E-05
AT1G73340	oxygen binding	-0.03	9.99E-01	0.62	3.95E-02	2.37	7.05E-09
AT1G73990	SPPA (signal peptide peptidase); protease IV/ serine-ty…	-0.02	9.99E-01	1.32	7.40E-04	1.14	1.64E-03
AT1G74450	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.06	9.99E-01	1.88	3.84E-05	2.24	2.08E-06
AT1G74800	galactosyltransferase family protein	0.03	9.99E-01	0.24	6.42E-01	1.20	1.83E-04
AT1G75160	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.27	9.40E-01	0.54	1.03E-01	1.93	2.42E-07
AT1G75210	5' nucleotidase family protein	-0.01	9.99E-01	0.28	1.63E-01	1.26	7.19E-08
AT1G75300	isoflavone reductase, putative	0.49	7.07E-01	2.78	2.21E-08	3.21	9.70E-10
AT1G75310	AUL1 (auxin-like 1 protein); heat shock protein binding	0.57	6.74E-01	1.34	8.05E-04	1.28	6.58E-04
AT1G75450	CKX5 (CYTOKININ OXIDASE 5); cytokinin dehydrogenase	-0.07	9.99E-01	1.43	5.84E-05	1.85	1.11E-06
AT1G75730	similar to hypothetical protein [Vitis vinifera] (GB:CA…	-0.10	9.99E-01	0.64	1.28E-01	2.79	3.21E-08
AT1G75800	pathogenesis-related thaumatin family protein	-0.16	9.68E-01	2.71	5.25E-11	1.92	2.87E-09
AT1G76460	RNA recognition motif (RRM)-containing protein	0.16	9.99E-01	0.87	2.80E-03	1.03	2.96E-04
AT1G76950	PRAF1; Ran GTPase binding / chromatin binding / zinc io…	0.29	9.80E-01	1.10	1.32E-02	1.42	8.52E-04
AT1G77250	PHD finger family protein	0.08	9.99E-01	0.25	7.19E-01	1.04	5.99E-03
AT1G77640	AP2 domain-containing transcription factor, putative	-0.08	9.99E-01	0.05	9.46E-01	1.00	1.30E-04
AT1G77760	NIA1 (NITRATE REDUCTASE 1)	-0.09	9.99E-01	0.61	4.43E-03	1.77	4.20E-09
AT1G78050	phosphoglycerate/bisphosphoglycerate mutase family prot…	-0.39	7.07E-01	0.20	6.55E-01	1.06	1.66E-04
AT1G78270	ATUGT85A4 (UDP-GLUCOSYL TRANSFERASE 85A4); UDP-glycosyl…	-0.04	9.99E-01	1.01	1.15E-04	1.02	5.20E-05
AT1G79340	ATMC4 (METACASPASE 4); caspase/ cysteine-type peptidase	-0.04	9.99E-01	0.50	1.51E-01	2.10	1.17E-07
AT1G79530	[AT1G79530, GAPCP-1; glyceraldehyde-3-phosphate dehydro…	0.03	9.99E-01	0.38	6.77E-01	1.54	3.03E-03
AT1G79760	DTA4 (DOWNSTREAM TARGET OF AGL15-4)	0.11	9.99E-01	0.04	9.60E-01	3.30	4.10E-11
AT1G80190	PSF1	0.00	1.00E+00	-0.29	3.40E-01	1.95	8.54E-09
AT2G01080	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.27	8.26E-01	0.95	2.00E-04	5.64	7.61E-16
AT2G01090	ubiquinol-cytochrome C reductase complex 7.8 kDa protei…	0.13	9.99E-01	0.17	8.40E-01	2.18	1.93E-06
AT2G01420	PIN4 (PIN-FORMED 4); auxin:hydrogen symporter/ transpor…	-0.07	9.99E-01	3.09	9.04E-10	1.02	7.12E-04
AT2G02170	remorin family protein	0.24	9.32E-01	0.54	5.53E-02	2.06	1.61E-08
AT2G02180	TOM3 (tobamovirus multiplication protein 3)	0.02	9.99E-01	0.36	3.81E-01	1.23	1.16E-04
AT2G03240	EXS family protein / ERD1/XPR1/SYG1 family protein	-0.42	4.89E-01	0.66	4.74E-03	1.10	1.00E-05
AT2G03730	ACR5 (ACT Domain Repeat 5)	0.19	9.79E-01	3.34	6.66E-11	5.71	5.17E-15
AT2G04795	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.07	9.99E-01	0.26	7.95E-01	2.62	4.88E-06
AT2G05070	[AT2G05070, LHCB2.2 (Photosystem II light harvesting co…	0.02	9.99E-01	-0.21	5.24E-01	1.67	3.20E-08

AT2G06925	ATSPLA2-ALPHA/PLA2-ALPHA (PHOSPHOLIPASE A2-ALPHA); phos…	0.12	9.99E-01	2.82	4.19E-11	2.10	9.12E-10
AT2G07050	CAS1 (CYCLOARTENOL SYNTHASE 1); cycloartenol synthase	0.24	9.68E-01	1.02	1.92E-03	1.89	6.83E-07
AT2G14170	ALDH6B2 (Aldehyde dehydrogenase 6B2); 3-chloroallyl ald…	-0.04	9.99E-01	2.22	2.82E-08	3.15	4.80E-11
AT2G17480	MLO8 (MILDEW RESISTANCE LOCUS O 8); calmodulin binding	-0.15	9.95E-01	0.08	9.06E-01	1.73	1.38E-07
AT2G17680	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.05	9.99E-01	0.76	5.56E-04	5.92	1.27E-16
AT2G19460	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.02	9.99E-01	1.88	1.04E-06	3.08	3.05E-10
AT2G19670	protein arginine N-methyltransferase, putative	0.15	9.79E-01	0.69	2.80E-03	1.68	2.47E-08
AT2G19800	MIOX2 (MYO-INOSITOL OXYGENASE 2)	0.71	2.55E-01	3.36	1.93E-10	4.37	1.03E-12
AT2G20370	KAM1/MUR3 (MURUS 3); catalytic/ transferase, transferri…	0.50	3.50E-01	2.39	7.06E-10	4.69	7.76E-15
AT2G20670	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.10	9.99E-01	2.37	7.78E-08	3.06	7.31E-10
AT2G20680	glycosyl hydrolase family 5 protein / cellulase family…	-0.09	9.99E-01	0.88	2.89E-04	3.28	1.23E-12
AT2G20770	GCL2 (GCR2-LIKE 2); catalytic	-0.46	7.49E-01	0.33	5.18E-01	2.95	4.16E-09
AT2G20840	secretory carrier membrane protein (SCAMP) family prote…	0.19	9.88E-01	0.24	6.22E-01	1.36	2.65E-05
AT2G21330	fructose-bisphosphate aldolase, putative	0.06	9.99E-01	2.32	2.45E-10	1.43	1.07E-07
AT2G21340	enhanced disease susceptibility protein, putative / sal…	-0.19	8.99E-01	2.30	8.18E-11	3.26	1.04E-13
AT2G21410	VHA-A2 (VACUOLAR PROTON ATPASE A2); ATPase	-0.08	9.99E-01	1.53	4.82E-08	1.27	3.08E-07
AT2G22170	lipid-associated family protein	-0.01	9.99E-01	0.86	3.33E-02	2.66	9.77E-08
AT2G22760	basic helix-loop-helix (bHLH) family protein	-0.04	9.99E-01	0.26	5.23E-01	2.24	9.18E-09
AT2G23110	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.16	9.91E-01	1.09	1.09E-04	1.48	1.19E-06
AT2G23640	reticulon family protein (RTNLB13)	-0.14	9.99E-01	-0.02	9.87E-01	2.17	1.59E-05
AT2G24150	HHP3 (heptahelical protein 3); receptor	0.09	9.99E-01	1.09	1.27E-03	2.08	2.64E-07
AT2G25200	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.11	9.99E-01	1.88	7.60E-07	2.42	8.74E-09
AT2G25440	leucine-rich repeat family protein	-0.24	8.77E-01	-0.67	4.58E-03	2.55	6.32E-11
AT2G26080	ATGLDP2 (ARABIDOPSIS THALIANA GLYCINE DECARBOXYLASE P-P…	0.28	7.82E-01	2.03	4.02E-09	2.98	3.89E-12
AT2G26190	calmodulin-binding family protein	0.19	9.84E-01	1.25	9.27E-05	1.68	1.11E-06
AT2G26600	glycosyl hydrolase family 17 protein	0.43	4.36E-01	1.18	6.85E-06	1.81	7.88E-09
AT2G27040	AGO4 (ARGONAUTE 4); nucleic acid binding	0.16	9.99E-01	1.63	6.13E-06	1.77	9.27E-07
AT2G27090	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.15	9.43E-01	1.23	1.73E-07	2.04	3.73E-11
AT2G28720	histone H2B, putative	-0.28	8.70E-01	0.50	7.53E-02	1.63	4.07E-07
AT2G28830	armadillo/beta-catenin repeat family protein / U-box do…	0.38	5.77E-01	0.37	1.37E-01	1.80	8.74E-09
AT2G29390	SMO2-1 (sterol 4-alpha-methyl-oxidase 1); C-4 methylste…	-0.17	9.81E-01	0.10	8.62E-01	1.80	5.20E-08
AT2G29730	[AT2G29730, UDP-glucoronosyl/UDP-glucosyl transferase f…	0.04	9.99E-01	2.22	1.60E-08	4.70	5.77E-14
AT2G29980	FAD3 (FATTY ACID DESATURASE 3); omega-3 fatty acid desa…	-0.09	9.99E-01	0.30	3.52E-01	3.01	1.67E-11
AT2G30550	lipase class 3 family protein	-0.02	9.99E-01	0.92	3.02E-02	1.48	3.16E-04
AT2G30600	BTB/POZ domain-containing protein	0.11	9.99E-01	2.03	5.13E-07	2.36	2.70E-08
AT2G30600	BTB/POZ domain-containing protein	-0.24	9.48E-01	1.75	1.86E-06	2.33	1.47E-08
AT2G30830	2-oxoglutarate-dependent dioxygenase, putative	-0.12	9.99E-01	0.76	2.17E-02	1.95	5.19E-07
AT2G31040	ATP synthase protein I -related	0.00	1.00E+00	0.88	2.45E-04	1.04	1.78E-05
AT2G31130	similar to unnamed protein product [Vitis vinifera] (GB…	-0.05	9.99E-01	0.14	6.54E-01	1.01	2.42E-06
AT2G31300	[AT2G31300, ARPC1b (actin-related protein C1b); nucleot…	-0.03	9.99E-01	1.01	2.00E-04	1.45	1.06E-06
AT2G31370	bZIP transcription factor (POSF21)	-0.04	9.99E-01	0.95	2.33E-01	1.50	1.78E-02
AT2G32000	DNA topoisomerase family protein	0.33	5.56E-01	0.50	1.08E-02	1.21	3.35E-07
AT2G32140	transmembrane receptor	0.07	9.99E-01	0.49	5.65E-01	1.83	8.09E-04
AT2G32150	haloacid dehalogenase-like hydrolase family protein	-0.16	8.85E-01	0.77	1.32E-05	1.95	8.13E-12
AT2G32660	disease resistance family protein / LRR family protein	-0.02	9.99E-01	0.98	4.45E-06	1.46	7.64E-09
AT2G33070	jacalin lectin family protein	-0.05	9.99E-01	-0.01	9.79E-01	4.62	7.41E-16
AT2G33570	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.06	9.99E-01	0.16	7.70E-01	1.55	3.49E-06
AT2G34430	[AT2G34430, LHB1B1 (Photosystem II light harvesting com…	-0.33	7.19E-01	0.72	2.95E-03	2.50	1.36E-10
AT2G34600	JAZ7/TIFY5B (JASMONATE-ZIM-DOMAIN PROTEIN 7)	-0.50	5.01E-01	1.80	3.76E-07	2.38	3.15E-09
AT2G34740	protein phosphatase 2C, putative / PP2C, putative	-0.03	9.99E-01	0.62	1.99E-02	3.80	9.96E-13
AT2G35000	zinc finger (C3HC4-type RING finger) family protein	0.13	9.99E-01	2.58	2.73E-08	1.02	1.20E-03
AT2G35075	similar to unnamed protein product [Vitis vinifera] (GB…	0.05	9.99E-01	2.46	1.69E-10	4.60	3.99E-15
AT2G35090	[AT2G35090, similar to unknown protein [Arabidopsis tha…	-0.14	9.99E-01	3.25	1.68E-10	4.27	7.15E-13
AT2G35100	ARAD1 (ARABINAN DEFICIENT 1); catalytic/ transferase, t…	0.13	9.99E-01	0.61	2.02E-01	1.43	4.62E-04
AT2G35300	late embryogenesis abundant group 1 domain-containing p…	0.09	9.99E-01	0.13	6.73E-01	4.89	2.59E-16
AT2G36310	inosine-uridine preferring nucleoside hydrolase family…	-0.04	9.99E-01	1.87	2.09E-06	3.49	8.99E-11
AT2G36430	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.18	9.79E-01	0.64	1.75E-02	2.72	2.11E-10
AT2G36470	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.13	9.99E-01	-0.07	9.18E-01	1.25	1.45E-05
AT2G36640	ATECP63 (EMBRYONIC CELL PROTEIN 63)	0.06	9.99E-01	0.21	4.80E-01	3.71	4.18E-14
AT2G36650	similar to CHUP1 (CHLOROPLAST UNUSUAL POSITIONING 1) [A…	0.10	9.99E-01	-0.01	9.79E-01	6.86	4.32E-18
AT2G37180	[AT2G37180, RD28 (plasma membrane intrinsic protein 2;3…	-0.21	9.99E-01	1.44	1.83E-03	3.32	2.79E-08
AT2G37980	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.10	9.99E-01	1.24	2.34E-03	3.15	1.12E-08
AT2G38400	AGT3 (ALANINE:GLYOXYLATE AMINOTRANSFERASE 3); alanine-g…	0.10	9.99E-01	0.56	9.84E-02	1.22	1.26E-04
AT2G38465	similar to unnamed protein product [Vitis vinifera] (GB…	0.69	6.04E-02	2.92	1.67E-11	2.70	1.23E-11
AT2G38490	CIPK22 (CBL-INTERACTING PROTEIN KINASE 22); kinase	0.07	9.99E-01	1.81	2.61E-06	3.17	3.13E-10
AT2G39130	amino acid transporter family protein	0.35	9.28E-01	2.72	1.03E-07	4.27	4.37E-11
AT2G39400	hydrolase, alpha/beta fold family protein	0.34	7.84E-01	0.24	5.73E-01	1.27	1.92E-05
AT2G39435	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.26	9.43E-01	0.24	6.13E-01	2.30	1.79E-08
AT2G39450	ATMTP11/MTP11; cation transmembrane transporter/ mangan…	-0.17	9.15E-01	1.29	1.36E-07	1.38	2.17E-08
AT2G39570	ACT domain-containing protein	0.43	2.78E-01	1.45	3.41E-08	2.13	3.73E-11
AT2G39705	DVL11/RTFL8 (ROTUNDIFOLIA LIKE 8)	0.15	9.99E-01	1.21	1.15E-03	1.79	7.36E-06
AT2G39710	aspartyl protease family protein	-0.58	8.42E-01	1.65	1.93E-03	2.02	1.24E-04
AT2G39800	[AT2G39800, P5CS1 (DELTA1-PYRROLINE-5-CARBOXYLATE SYNTH…	0.19	9.83E-01	0.92	2.52E-03	1.05	4.06E-04
AT2G39880	MYB25 (myb domain protein 25); DNA binding / transcript…	0.07	9.99E-01	0.50	4.48E-02	5.14	2.69E-15
AT2G40170	ATEM6/GEA6 (ARABIDOPSIS EARLY METHIONINE-LABELLED 6)	0.20	9.99E-01	5.58	4.92E-12	5.95	3.71E-13
AT2G40180	ATHPP2C5; protein serine/threonine phosphatase	-0.09	9.99E-01	0.65	1.14E-01	2.05	2.03E-06
AT2G40250	GDSL-motif lipase/hydrolase family protein	-0.02	9.99E-01	0.26	3.87E-01	4.44	1.36E-14
AT2G40260	myb family transcription factor	-0.16	9.86E-01	0.79	1.70E-03	3.05	9.28E-12
AT2G40420	amino acid transporter family protein	0.07	9.99E-01	1.47	1.59E-05	2.31	1.39E-08
AT2G40470	LBD15 (LOB DOMAIN-CONTAINING PROTEIN 15)	0.13	9.99E-01	0.46	2.74E-01	4.32	9.79E-12
AT2G40490	HEME2; uroporphyrinogen decarboxylase	0.18	9.99E-01	0.75	6.70E-02	2.93	2.22E-08
AT2G41830	cyclin-related	-0.01	9.99E-01	2.12	2.84E-08	3.54	4.13E-12
AT2G42490	copper amine oxidase, putative	0.01	9.99E-01	0.96	2.41E-03	1.28	6.91E-05
AT2G43280	far-red impaired responsive family protein / FAR1 famil…	0.93	4.24E-03	0.89	2.11E-04	1.00	2.92E-05
AT2G43400	ETFQO (ELECTRON-TRANSFER FLAVOPROTEIN:UBIQUINONE OXIDOI	0.22	9.81E-01	1.08	1.74E-03	2.15	2.33E-07
AT2G44110	MLO15 (MILDEW RESISTANCE LOCUS O 15); calmodulin bindin…	-0.31	7.33E-01	-0.24	4.41E-01	1.23	2.64E-06
AT2G44670	senescence-associated protein-related	0.55	4.55E-01	4.22	4.58E-12	4.66	1.89E-13
AT2G45280	ATRAD51C (Arabidopsis thaliana Ras Associated with Diab…	0.31	9.33E-01	0.99	4.67E-03	1.39	8.98E-05
AT2G45910	protein kinase family protein / U-box domain-containing…	-0.11	9.91E-01	0.18	4.59E-01	1.99	2.79E-11
AT2G45920	U-box domain-containing protein	-0.14	9.99E-01	2.12	5.96E-08	3.24	3.73E-11
AT2G46220	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.05	9.99E-01	-0.24	4.51E-01	1.91	6.28E-09
AT2G46250	myosin heavy chain-related	-0.05	9.99E-01	0.43	3.50E-02	3.71	2.50E-14
AT2G46270	GBF3 (G-BOX BINDING FACTOR 3); transcription factor	0.18	9.72E-01	4.41	3.15E-13	3.82	5.16E-13

AT2G47520	AP2 domain-containing transcription factor, putative	-0.17	9.99E-01	-0.19	7.82E-01	1.52	5.99E-05
AT2G47550	pectinesterase family protein	0.01	9.99E-01	1.24	4.58E-05	1.32	9.50E-06
AT2G47770	benzodiazepine receptor-related	-0.08	9.99E-01	2.81	1.40E-10	1.32	2.59E-06
AT3G01300	[AT3G01300, protein kinase, putative];[AT5G15080, prote…	0.41	8.43E-01	0.25	6.78E-01	1.06	2.16E-03
AT3G01500	CA1 (CARBONIC ANHYDRASE 1); carbonate dehydratase/ zinc…	-0.13	9.68E-01	0.45	1.15E-02	2.92	1.17E-13
AT3G02460	plant adhesion molecule, putative	0.12	9.99E-01	1.57	1.09E-05	2.00	1.83E-07
AT3G02480	ABA-responsive protein-related	0.20	9.99E-01	0.66	9.13E-02	1.30	3.30E-04
AT3G03150	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.15	9.99E-01	0.46	2.06E-01	1.62	4.16E-06
AT3G03180	Got1-like family protein	0.31	9.08E-01	1.46	5.53E-05	2.77	4.20E-09
AT3G03520	phosphoesterase family protein	0.16	9.95E-01	1.57	2.83E-06	3.07	6.32E-11
AT3G04110	GLR1 (GLUTAMATE RECEPTOR 1)	-0.11	9.99E-01	0.18	6.37E-01	2.42	1.45E-10
AT3G04220	disease resistance protein (TIR-NBS-LRR class), putativ…	-0.15	9.96E-01	1.09	1.13E-04	3.15	1.65E-11
AT3G04230	40S ribosomal protein S16 (RPS16B)	0.08	9.99E-01	0.54	8.41E-02	1.34	1.98E-05
AT3G05240	pentatricopeptide (PPR) repeat-containing protein	0.14	9.68E-01	0.12	7.33E-01	1.15	6.90E-07
AT3G05260	short-chain dehydrogenase/reductase (SDR) family protei…	-0.09	9.99E-01	0.04	9.58E-01	1.05	4.72E-05
AT3G05390	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.10	9.99E-01	1.25	3.15E-03	1.33	9.44E-04
AT3G09150	HY2 (ELONGATED HYPOCOTYL 2); phytochromobilin:ferredoxi…	0.11	9.99E-01	2.41	2.76E-05	2.37	1.62E-05
AT3G09940	ATMDAR3/MDHAR (MONODEHYDROASCORBATE REDUCTASE); monoc	-0.49	9.40E-01	4.80	8.27E-09	5.81	1.63E-10
AT3G10720	pectinesterase, putative	-0.07	9.99E-01	3.09	2.30E-08	2.09	2.34E-06
AT3G11340	UDP-glucoronosyl/UDP-glucosyl transferase family protei…	0.03	9.99E-01	2.23	2.21E-08	1.52	1.93E-06
AT3G12110	ACT11 (ACTIN-11); structural constituent of cytoskeleto…	-0.11	9.99E-01	0.77	7.18E-03	3.40	1.83E-11
AT3G12500	ATHCHIB (BASIC CHITINASE); chitinase	-0.26	7.34E-01	2.29	1.21E-10	1.13	1.18E-06
AT3G13430	zinc finger (C3HC4-type RING finger) family protein	0.21	8.79E-01	0.72	9.70E-04	1.16	2.03E-06
AT3G13965	pseudogene, hypothetical protein	0.07	9.99E-01	0.52	1.56E-01	1.21	2.16E-04
AT3G14280	similar to hypothetical protein [Vitis vinifera] (GB:CA…	-0.09	9.99E-01	0.45	2.58E-01	1.85	1.67E-06
AT3G15450	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.02	9.99E-01	2.06	2.06E-07	3.51	2.79E-11
AT3G16120	dynein light chain, putative	-0.19	8.70E-01	0.22	3.33E-01	3.00	1.09E-13
AT3G16150	L-asparaginase, putative / L-asparagine amidohydrolase,…	-0.30	7.59E-01	0.93	2.05E-04	1.66	5.29E-08
AT3G16240	DELTA-TIP (delta tonoplast integral protein); water cha…	0.04	9.99E-01	0.28	4.03E-01	4.14	1.17E-13
AT3G16350	myb family transcription factor	-0.11	9.99E-01	0.85	4.99E-03	1.58	3.12E-06
AT3G16560	protein phosphatase 2C-related / PP2C-related	-0.20	8.91E-01	0.34	1.24E-01	1.30	1.75E-07
AT3G16857	ARR1 (ARABIDOPSIS RESPONSE REGULATOR 1); transcription…	0.15	9.93E-01	0.90	5.54E-04	1.15	1.87E-05
AT3G17420	GPK1 (Glyoxysomal protein kinase 1); kinase	-0.09	9.99E-01	2.94	2.12E-07	3.60	5.15E-09
AT3G17640	leucine-rich repeat family protein	-0.11	9.99E-01	0.00	1.00E+00	1.79	1.73E-07
AT3G18490	aspartyl protease family protein	-0.08	9.99E-01	0.92	3.65E-05	1.21	5.19E-07
AT3G18560	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.29	9.79E-01	1.99	2.66E-05	1.04	9.20E-03
AT3G18780	ACT2 (ACTIN 2); structural constituent of cytoskeleton	-0.13	9.99E-01	0.05	9.45E-01	2.04	4.73E-09
AT3G19920	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.17	9.79E-01	2.40	1.28E-09	4.19	8.58E-14
AT3G20100	CYP705A19 (cytochrome P450, family 705, subfamily A, po…	-0.28	9.43E-01	1.69	7.52E-06	3.69	3.71E-11
AT3G20110	CYP705A20 (cytochrome P450, family 705, subfamily A, po…	0.52	4.81E-01	3.86	1.07E-11	6.97	4.19E-16
AT3G21810	zinc finger (CCCH-type) family protein	0.31	9.74E-01	0.17	8.57E-01	1.10	7.21E-03
AT3G21830	ASK8 (ARABIDOPSIS SKP1-LIKE 8); ubiquitin-protein ligas…	-0.14	9.99E-01	-0.05	9.52E-01	5.91	1.30E-14
AT3G21840	ASK7 (ARABIDOPSIS SKP1-LIKE 7); ubiquitin-protein ligas…	-0.21	9.79E-01	-0.09	9.05E-01	4.20	3.47E-12
AT3G21850	ASK9 (ARABIDOPSIS SKP1-LIKE 9); ubiquitin-protein ligas…	-0.02	9.99E-01	-0.07	8.98E-01	6.01	2.93E-16
AT3G22150	pentatricopeptide (PPR) repeat-containing protein	-0.10	9.99E-01	0.03	9.70E-01	1.21	5.39E-06
AT3G22740	HMT3 (Homocysteine S-methyltransferase 3); homocysteine…	0.01	9.99E-01	0.34	2.97E-01	3.99	5.67E-13
AT3G22930	calmodulin, putative	-0.15	9.79E-01	0.65	3.03E-03	4.71	2.48E-15
AT3G23050	IAA7 (AUXIN RESISTANT 2); transcription factor	-0.20	9.91E-01	1.34	1.77E-04	2.17	1.71E-07
AT3G23560	ALF5 (ABERRANT LATERAL ROOT FORMATION 5); antiporter/ t…	-0.14	9.99E-01	2.61	4.79E-07	2.99	3.03E-08
AT3G23630	ATIPT7 (Arabidopsis thaliana isopentenyltransferase 7);…	-0.19	9.68E-01	-0.04	9.61E-01	2.22	3.09E-09
AT3G23750	leucine-rich repeat family protein / protein kinase fam…	0.15	9.99E-01	1.18	2.25E-04	1.82	5.14E-07
AT3G24180	catalytic	0.01	9.99E-01	0.47	3.45E-03	1.50	5.84E-10
AT3G24503	ALDH2C4 (REDUCED EPIDERMAL FLUORESCENCE1); 3-chloroally…	-0.16	9.99E-01	0.95	2.70E-03	1.10	3.47E-04
AT3G25010	disease resistance family protein	-0.11	9.99E-01	1.78	7.13E-09	4.31	4.05E-15
AT3G25780	AOC3 (ALLENE OXIDE CYCLASE 3)	0.10	9.99E-01	1.17	6.05E-05	1.41	2.82E-06
AT3G26080	[AT3G26080, plastid-lipid associated protein PAP / fibr…	-0.03	9.99E-01	0.32	7.80E-01	1.45	1.16E-02
AT3G26280	CYP71B4 (cytochrome P450, family 71, subfamily B, polyp…	-0.18	9.78E-01	0.01	9.89E-01	1.31	4.31E-06
AT3G26300	CYP71B34 (cytochrome P450, family 71, subfamily B, poly…	0.16	9.43E-01	0.86	6.14E-05	3.10	2.34E-13
AT3G26460	major latex protein-related / MLP-related	-0.17	9.99E-01	-0.15	8.39E-01	2.09	5.25E-07
AT3G26570	PHT2;1 (phosphate transporter 2;1)	-0.08	9.99E-01	0.67	1.55E-02	1.84	1.11E-07
AT3G26670	similar to permease-related [Arabidopsis thaliana] (TAI…	0.02	9.99E-01	0.64	9.51E-03	1.72	6.10E-08
AT3G26680	SNM1 (SENSITIVE TO NITROGEN MUSTARD 1)	0.17	9.99E-01	0.88	4.58E-03	1.27	6.07E-05
AT3G26980	MUB4 (MEMBRANE-ANCHORED UBIQUITIN-FOLD PROTEIN 4 PRECUR	0.11	9.99E-01	1.07	5.38E-04	1.19	8.61E-05
AT3G27270	similar to DNA-binding storekeeper protein-related [Ara…	-0.08	9.99E-01	1.08	8.05E-04	1.59	4.54E-06
AT3G27540	glycosyl transferase family 17 protein	0.14	9.99E-01	1.07	1.28E-02	1.67	1.18E-04
AT3G27690	LHCB2:4 (Photosystem II light harvesting complex gene 2…	-0.02	9.99E-01	0.31	4.19E-01	5.79	7.03E-15
AT3G27880	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.33	8.74E-01	1.27	2.12E-04	1.84	1.02E-06
AT3G28180	ATCSLC04 (CELLULOSE-SYNTHASE LIKE C 4); transferase, tr…	-0.04	9.99E-01	2.84	3.74E-09	3.38	9.18E-11
AT3G28540	AAA-type ATPase family protein	-0.13	9.99E-01	1.65	1.43E-05	1.61	9.56E-06
AT3G28720	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.18	9.99E-01	0.06	9.64E-01	1.20	4.13E-03
AT3G29160	AKIN11 (ARABIDOPSIS SNF1 KINASE HOMOLOG 11); protein ki…	0.41	3.90E-01	2.39	8.54E-11	3.33	1.32E-13
AT3G30340	nodulin MtN21 family protein	-0.18	9.99E-01	-0.19	7.69E-01	1.46	6.64E-05
AT3G30390	amino acid transporter family protein	0.13	9.99E-01	0.72	1.01E-01	1.49	2.21E-04
AT3G30775	ERD5 (EARLY RESPONSIVE TO DEHYDRATION 5); proline dehyd…	0.47	6.67E-01	3.18	5.30E-10	3.56	2.79E-11
AT3G43430	zinc finger (C3HC4-type RING finger) family protein	-0.28	9.33E-01	3.24	5.77E-10	4.39	1.75E-12
AT3G43510	transposable element gene	0.43	3.30E-01	0.98	1.66E-05	1.50	2.26E-08
AT3G44400	disease resistance protein (TIR-NBS-LRR class), putativ…	0.09	9.99E-01	0.45	2.48E-01	1.53	1.54E-05
AT3G45530	DC1 domain-containing protein	0.11	9.99E-01	0.45	1.54E-01	1.22	3.63E-05
AT3G45730	unknown protein	0.26	9.58E-01	0.79	1.97E-02	2.13	2.33E-07
AT3G45860	receptor-like protein kinase, putative	-0.24	9.28E-01	5.51	2.37E-14	5.48	4.83E-15
AT3G45960	ATEXLA3 (ARABIDOPSIS THALIANA EXPANSIN-LIKE A3)	0.21	9.68E-01	2.40	1.60E-08	5.32	3.22E-14
AT3G45970	ATEXLA1 (ARABIDOPSIS THALIANA EXPANSIN-LIKE A1)	-0.12	9.99E-01	7.26	1.70E-16	7.25	4.37E-17
AT3G47340	ASN1 (DARK INDUCIBLE 6)	0.00	9.99E-01	1.34	2.66E-06	4.30	3.02E-14
AT3G47510	unknown protein	0.14	9.96E-01	0.04	9.56E-01	5.12	5.03E-15
AT3G48580	xyloglucan:xyloglucosyl transferase, putative / xyloglu…	-0.05	9.99E-01	1.72	1.13E-05	2.13	2.90E-07
AT3G48640	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.25	9.31E-01	0.26	5.34E-01	1.35	1.03E-05
AT3G49060	protein kinase family protein / U-box domain-containing…	-0.08	9.99E-01	1.20	1.28E-05	1.57	1.60E-07
AT3G50650	scarecrow-like transcription factor 7 (SCL7)	-0.38	5.43E-01	0.31	2.22E-01	1.69	1.39E-08
AT3G50660	DWF4 (DWARF 4)	0.02	9.99E-01	0.79	4.28E-03	1.15	5.05E-05
AT3G51120	zinc finger (CCCH-type) family protein	0.37	7.26E-01	0.18	6.94E-01	1.07	1.17E-04
AT3G51180	zinc finger (CCCH-type) family protein	0.48	6.55E-01	0.36	3.69E-01	1.08	4.62E-04
AT3G51540	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.01	9.99E-01	1.22	9.94E-03	1.60	5.20E-04
AT3G51680	short-chain dehydrogenase/reductase (SDR) family protei…	-0.17	9.94E-01	0.39	2.45E-01	1.12	1.02E-04

AT3G51730	saposin B domain-containing protein	-0.06	9.99E-01	0.68	1.28E-02	1.08	9.21E-05
AT3G51920	CAM9 (CALMODULIN 9); calcium ion binding	-0.05	9.99E-01	0.88	3.35E-05	1.01	2.75E-06
AT3G52500	aspartyl protease family protein	0.51	5.43E-01	4.94	5.85E-13	3.57	1.56E-11
AT3G52840	BGAL2 (beta-galactosidase 2); beta-galactosidase	-0.03	9.99E-01	1.12	2.16E-05	1.81	1.47E-08
AT3G53320	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.09	9.99E-01	0.30	3.24E-01	1.91	1.13E-08
AT3G53610	ATRAB8; GTP binding	-0.01	9.99E-01	0.22	3.33E-01	1.03	9.37E-07
AT3G53620	ATPPA4 (ARABIDOPSIS THALIANA PYROPHOSPHORYLASE 4); inor…	0.00	1.00E+00	1.80	1.52E-07	3.51	2.57E-12
AT3G53720	ATCHX20 (CATION/H+ EXCHANGER 20); monovalent cation:pro…	-0.28	6.43E-01	0.78	9.17E-05	2.69	7.62E-13
AT3G54270	sucrose-phosphatase 3 (SPP3)	-0.29	7.33E-01	-0.25	3.93E-01	1.07	9.18E-06
AT3G54280	ATP binding / DNA binding / helicase	0.10	9.99E-01	0.24	7.09E-01	1.41	1.55E-04
AT3G54590	ATHRGP1; structural constituent of cell wall	-0.11	9.99E-01	1.53	1.47E-02	2.12	4.84E-04
AT3G54690	sugar isomerase (SIS) domain-containing protein / CBS d…	0.03	9.99E-01	0.69	3.23E-03	1.45	2.59E-07
AT3G54940	cysteine proteinase, putative	-0.15	9.99E-01	2.99	1.42E-09	5.42	5.47E-14
AT3G55150	ATEXO70H1 (exocyst subunit EXO70 family protein H1); pr…	-0.45	6.33E-01	3.58	3.35E-11	4.10	9.23E-13
AT3G55450	protein kinase, putative	-0.14	9.54E-01	0.40	2.35E-02	1.57	1.66E-09
AT3G55730	MYB109 (myb domain protein 109); DNA binding / transcri…	0.14	9.78E-01	3.20	1.20E-12	4.34	2.69E-15
AT3G55790	unknown protein	-0.06	9.99E-01	0.25	7.74E-01	1.28	3.28E-03
AT3G55940	phosphoinositide-specific phospholipase C, putative	0.08	9.99E-01	2.91	6.83E-11	4.37	3.60E-14
AT3G55950	protein kinase family protein	0.40	9.43E-01	0.27	7.54E-01	1.08	1.51E-02
AT3G55980	zinc finger (CCCH-type) family protein	0.48	3.50E-01	1.99	7.13E-09	3.46	5.10E-13
AT3G56050	protein kinase family protein	-0.12	9.57E-01	0.18	3.65E-01	1.26	4.73E-09
AT3G56240	CCH (COPPER CHAPERONE)	0.20	9.53E-01	2.07	1.89E-08	2.98	2.65E-11
AT3G56300	tRNA synthetase class I (C) family protein	0.05	9.99E-01	1.53	1.06E-04	2.11	9.27E-07
AT3G56410	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.09	9.99E-01	0.01	9.91E-01	1.36	7.94E-05
AT3G56500	serine-rich protein-related	0.00	1.00E+00	3.44	2.81E-07	4.17	7.78E-09
AT3G56830	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.07	9.99E-01	0.13	7.82E-01	1.53	3.01E-07
AT3G56930	zinc finger (DHHC type) family protein	-0.02	9.99E-01	1.62	6.13E-08	1.90	2.42E-09
AT3G57320	similar to hypothetical protein [Vitis vinifera] (GB:CA…	0.11	9.99E-01	0.08	8.87E-01	1.37	1.22E-06
AT3G57470	peptidase M16 family protein / insulinase family protei…	0.30	6.98E-01	0.58	5.44E-03	4.92	6.91E-16
AT3G57520	ATSIP2 (ARABIDOPSIS THALIANA SEED IMBIBITION 2); hydrol…	0.29	7.12E-01	1.56	4.66E-08	2.13	1.75E-10
AT3G57800	basic helix-loop-helix (bHLH) family protein	-0.20	9.43E-01	1.49	3.87E-07	2.02	2.13E-09
AT3G58060	cation efflux family protein / metal tolerance protein,…	0.08	9.99E-01	0.22	7.35E-01	2.01	1.53E-06
AT3G59630	diphthamide synthesis DPH2 family protein	-0.11	9.99E-01	0.16	6.73E-01	1.13	5.20E-06
AT3G60260	phagocytosis and cell motility protein ELMO1-related	0.04	9.99E-01	1.13	6.80E-03	1.21	2.13E-03
AT3G60280	UCC3 (UCLACYANIN 3); copper ion binding	-0.09	9.99E-01	2.57	7.39E-04	4.87	1.34E-07
AT3G60290	oxidoreductase, acting on paired donors, with incorpora…	0.02	9.99E-01	0.22	4.24E-01	1.12	1.72E-06
AT3G60510	enoyl-CoA hydratase/isomerase family protein	0.12	9.99E-01	0.43	2.03E-01	2.35	8.70E-09
AT3G60520	zinc ion binding	0.02	9.99E-01	0.91	9.27E-03	1.93	1.39E-06
AT3G61260	DNA-binding family protein / remorin family protein	0.12	9.79E-01	1.27	1.14E-07	1.44	8.30E-09
AT3G61310	DNA-binding family protein	-0.40	9.08E-01	-0.05	9.69E-01	1.20	2.67E-03
AT3G61340	F-box family protein	-0.07	9.99E-01	0.14	7.18E-01	1.01	2.66E-05
AT3G61650	[AT3G61650, TUBG1 (GAMMA-TUBULIN); structural molecule]…	0.70	2.16E-01	2.18	4.54E-08	3.67	6.20E-12
AT3G61740	DNA binding	0.10	9.99E-01	0.27	5.59E-01	1.17	1.46E-04
AT4G00050	UNE10 (unfertilized embryo sac 10); DNA binding / trans…	0.19	9.99E-01	0.26	6.78E-01	3.53	6.70E-10
AT4G00080	UNE11 (unfertilized embryo sac 11); pectinesterase inhi…	0.28	7.86E-01	2.07	3.87E-09	5.23	1.29E-15
AT4G00430	TMP-C (PLASMA MEMBRANE INTRINSIC PROTEIN 1;4); water ch…	-0.22	9.04E-01	1.52	2.75E-07	1.93	3.82E-09
AT4G00440	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.04	9.99E-01	0.98	1.11E-02	1.74	2.10E-05
AT4G00750	dehydration-responsive family protein	0.01	9.99E-01	0.38	2.50E-01	1.16	5.98E-05
AT4G01120	GBF2 (G-BOX BINDING FACTOR 2); DNA binding / transcript…	0.12	9.99E-01	2.60	1.40E-10	3.44	5.16E-13
AT4G01430	nodulin MtN21 family protein	-0.05	9.99E-01	2.68	2.39E-09	4.08	1.30E-12
AT4G02640	BZO2H1 (ARABIDOPSIS THALIANA BASIC LEUCINE ZIPPER 10);…	6.92	3.80E-16	-0.07	9.07E-01	6.60	1.27E-16
AT4G03510	RMA1 (Ring finger protein with Membrane Anchor 1); prot…	-0.30	9.44E-01	0.22	7.35E-01	1.26	4.69E-04
AT4G04990	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.21	9.28E-01	0.36	1.49E-01	3.99	4.56E-14
AT4G05190	ATK5 (Arabidopsis thaliana kinesin 5); microtubule moto…	-0.21	9.91E-01	-0.07	9.41E-01	1.90	1.39E-06
AT4G09110	zinc finger (C3HC4-type RING finger) family protein	-0.25	9.43E-01	0.26	5.69E-01	2.17	4.35E-08
AT4G09900	hydrolase, alpha/beta fold family protein	0.37	6.01E-01	1.31	2.66E-06	1.23	2.88E-06
AT4G10510	subtilase family protein	0.04	9.99E-01	3.43	1.70E-10	1.44	1.37E-05
AT4G11800	protein serine/threonine phosphatase	0.18	9.36E-01	0.92	5.26E-05	3.49	8.49E-14
AT4G11840	PLDGAMMA3 (phospholipase D gamma 3); phospholipase D	0.06	9.99E-01	0.66	4.60E-02	1.97	3.48E-07
AT4G12230	esterase/lipase/thioesterase family protein	-0.02	9.99E-01	0.49	5.70E-02	1.17	9.96E-06
AT4G12430	[AT4G12430, trehalose-6-phosphate phosphatase, putative…	-0.15	9.74E-01	0.13	7.18E-01	3.61	4.56E-14
AT4G12470	protease inhibitor/seed storage/lipid transfer protein…	0.13	9.95E-01	0.61	7.28E-03	1.75	1.47E-08
AT4G12700	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.24	9.61E-01	0.83	9.77E-03	1.07	6.21E-04
AT4G12730	FLA2	-0.02	9.99E-01	0.82	1.01E-03	2.62	7.93E-11
AT4G14680	APS3 (ATP sulfurylase 2); sulfate adenylyltransferase (…	-0.56	5.28E-01	1.17	4.53E-04	1.26	1.12E-04
AT4G14900	hydroxyproline-rich glycoprotein family protein	0.15	9.99E-01	0.67	1.16E-01	1.09	3.31E-03
AT4G15130	cholinephosphate cytidylyltransferase, putative / phosp…	0.01	9.99E-01	0.22	5.37E-01	1.49	4.72E-07
AT4G15480	UGT84A1; UDP-glycosyltransferase/ sinapate 1-glucosyltr…	-0.24	8.54E-01	1.42	3.86E-07	7.15	1.74E-17
AT4G15570	tRNA-splicing endonuclease positive effector-related	0.34	6.75E-01	1.36	1.55E-06	1.87	7.64E-09
AT4G15800	RALFL33 (RALF-LIKE 33)	0.02	9.99E-01	0.77	1.66E-05	1.04	1.42E-07
AT4G16110	ARR2 (ARABIDOPSIS RESPONSE REGULATOR 2); transcription…	0.19	9.99E-01	0.67	1.28E-01	1.30	8.64E-04
AT4G16170	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.05	9.99E-01	0.28	3.90E-01	1.70	9.95E-08
AT4G16260	glycosyl hydrolase family 17 protein	-0.19	9.53E-01	0.19	6.23E-01	1.01	5.42E-05
AT4G16430	basic helix-loop-helix (bHLH) family protein	0.09	9.99E-01	0.20	7.64E-01	1.15	1.18E-03
AT4G16780	ATHB-2 (ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 2); DNA b…	0.02	9.99E-01	0.73	1.92E-02	2.97	5.34E-10
AT4G16790	hydroxyproline-rich glycoprotein family protein	-0.04	9.99E-01	0.16	7.83E-01	1.07	4.92E-04
AT4G17090	CT-BMY (BETA-AMYLASE 3, BETA-AMYLASE 8); beta-amylase	0.14	9.99E-01	1.40	2.00E-04	1.28	2.76E-04
AT4G17180	glycosyl hydrolase family 17 protein	0.17	9.89E-01	0.30	3.94E-01	1.05	1.22E-04
AT4G17460	HAT1 (homeobox-leucine zipper protein 1); DNA binding /…	-0.17	9.99E-01	2.19	1.70E-07	4.32	2.48E-12
AT4G17970	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.11	9.99E-01	1.59	8.87E-06	4.02	4.65E-12
AT4G18220	[AT4G18220, purine permease family protein];[AT4G18210,…	-0.36	9.05E-01	-0.03	9.79E-01	2.01	3.10E-06
AT4G20500	[AT4G20500, transposable element gene];[AT4G20730, tran…	-0.13	9.99E-01	-0.03	9.74E-01	2.77	8.97E-10
AT4G20820	FAD-binding domain-containing protein	0.24	9.54E-01	0.41	2.61E-01	3.30	9.59E-11
AT4G21830	[AT4G21830, methionine sulfoxide reductase domain-conta…	0.11	9.99E-01	2.63	5.09E-07	2.14	4.01E-06
AT4G21850	methionine sulfoxide reductase domain-containing protei…	0.03	9.99E-01	2.38	1.69E-10	3.90	2.80E-14
AT4G22590	[AT4G22590, trehalose-6-phosphate phosphatase, putative…	-0.04	9.99E-01	2.38	1.80E-11	3.68	5.99E-15
AT4G22620	auxin-responsive family protein	-0.34	7.33E-01	0.21	5.83E-01	1.71	1.01E-07
AT4G23320	protein kinase family protein	0.00	1.00E+00	2.42	3.34E-08	4.70	5.06E-13
AT4G23870	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.06	9.99E-01	1.30	2.85E-06	3.15	2.45E-12
AT4G24040	ATTRE1/TRE1 (TREHALASE 1); alpha,alpha-trehalase/ treha…	-0.09	9.99E-01	1.55	8.46E-10	1.83	2.09E-11
AT4G24060	Dof-type zinc finger domain-containing protein	-0.15	9.99E-01	0.28	4.55E-01	1.60	5.86E-07
AT4G24390	F-box family protein (FBX14)	0.13	9.99E-01	0.07	9.39E-01	1.04	4.93E-04
AT4G24400	CIPK8 (CBL-INTERACTING PROTEIN KINASE 8); kinase	-0.21	9.78E-01	0.14	8.26E-01	1.77	7.22E-07

AT4G24810	ABC1 family protein	0.49	5.22E-01	0.40	2.09E-01	1.29	1.38E-05
AT4G25140	OLEO1 (OLEOSIN1)	-0.05	9.99E-01	0.64	1.62E-02	5.12	1.26E-14
AT4G25450	ATNAP8 (Arabidopsis thaliana non-intrinsic ABC protein…	-0.05	9.99E-01	0.11	8.72E-01	2.38	1.19E-08
AT4G26260	MIOX4 (MYO-INOSITOL OXYGENASE 4)	0.24	9.74E-01	0.54	1.48E-01	6.02	3.50E-14
AT4G26480	KH domain-containing protein	0.10	9.99E-01	0.49	3.64E-01	1.35	9.10E-04
AT4G26570	ATCBL3 (CALCINEURIN B-LIKE 3)	0.15	9.74E-01	1.12	6.84E-06	1.59	2.31E-08
AT4G26600	nucleolar protein, putative	0.44	8.25E-01	1.00	1.01E-02	1.05	3.75E-03
AT4G26690	MRH5/SHV3 (morphogenesis of root hair 5); glycerophosph…	0.07	9.99E-01	1.90	2.47E-04	1.70	4.26E-04
AT4G26700	ATFIM1 (Arabidopsis thaliana fimbrin 1); actin binding	0.04	9.99E-01	0.24	3.63E-01	1.19	1.16E-06
AT4G27140	2S seed storage protein 1 / 2S albumin storage protein…	-0.13	9.69E-01	0.04	9.35E-01	8.00	2.06E-19
AT4G27590	copper-binding protein-related	-0.13	9.99E-01	-0.18	6.07E-01	3.11	2.45E-12
AT4G27780	ACBP2 (ACYL-COA BINDING PROTEIN ACBP 2)	-0.16	9.97E-01	0.64	2.46E-02	1.42	5.10E-06
AT4G27790	calcium-binding EF hand family protein	-0.18	9.99E-01	0.06	9.53E-01	1.68	1.16E-05
AT4G28040	nodulin MtN21 family protein	-0.35	9.32E-01	2.66	2.02E-07	1.08	3.60E-03
AT4G29080	PAP2 (PHYTOCHROME-ASSOCIATED PROTEIN 2); transcription…	-0.10	9.99E-01	-0.03	9.78E-01	1.16	1.48E-04
AT4G29140	MATE efflux protein-related	0.06	9.99E-01	0.63	1.05E-02	1.24	5.21E-06
AT4G29570	cytidine deaminase, putative / cytidine aminohydrolase,…	0.05	9.99E-01	0.99	4.22E-04	4.60	6.63E-14
AT4G29590	methyltransferase	-0.06	9.99E-01	2.42	3.17E-10	2.66	2.37E-11
AT4G29600	cytidine deaminase, putative / cytidine aminohydrolase,…	0.07	9.99E-01	2.67	2.39E-10	6.17	3.49E-16
AT4G29640	cytidine deaminase, putative / cytidine aminohydrolase,…	-0.14	9.99E-01	0.04	9.67E-01	1.56	1.64E-05
AT4G29720	ATPAO5 (POLYAMINE OXIDASE 5); amine oxidase	0.19	9.93E-01	0.61	6.18E-02	2.33	2.26E-08
AT4G31250	leucine-rich repeat transmembrane protein kinase, putat…	0.05	9.99E-01	1.28	6.55E-05	3.70	7.76E-12
AT4G31750	protein phosphatase 2C, putative / PP2C, putative	0.06	9.99E-01	0.49	1.50E-02	1.31	1.62E-07
AT4G31980	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.09	9.99E-01	1.04	2.10E-05	1.28	7.21E-07
AT4G32360	NADP adrenodoxin-like ferredoxin reductase	0.16	9.99E-01	1.54	1.13E-05	5.49	3.36E-14
AT4G33420	peroxidase, putative	-0.11	9.99E-01	5.69	1.67E-13	8.10	2.93E-16
AT4G33630	EX1 (EXECUTER1)	0.30	7.10E-01	0.56	1.06E-02	1.53	5.60E-08
AT4G33700	CBS domain-containing protein	0.33	8.92E-01	4.26	3.30E-11	4.44	3.80E-12
AT4G33750	unknown protein	-0.20	9.09E-01	1.50	1.68E-07	1.01	1.62E-05
AT4G33905	peroxisomal membrane protein 22 kDa, putative	0.16	9.73E-01	1.23	5.91E-06	2.94	6.34E-12
AT4G33920	protein phosphatase 2C family protein / PP2C family pro…	-0.18	9.99E-01	0.81	5.89E-02	1.64	9.19E-05
AT4G34590	[AT4G34590, GBF6 (ARABIDOPSIS THALIANA BASIC LEUCINE-ZI…	-0.08	9.99E-01	8.67	5.38E-16	8.71	1.27E-16
AT4G35150	[AT4G35150, O-methyltransferase family 2 protein];[AT4G…	0.25	9.99E-01	3.54	1.04E-07	3.94	9.37E-09
AT4G35510	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.10	9.99E-01	1.06	4.22E-04	1.00	3.97E-04
AT4G35570	HMGB5 (HIGH MOBILITY GROUP B 5); transcription factor	0.42	9.79E-01	1.18	8.06E-02	1.48	1.25E-02
AT4G35670	glycoside hydrolase family 28 protein / polygalacturona…	-0.02	9.99E-01	0.21	7.06E-01	2.54	9.37E-09
AT4G35770	SEN1 (DARK INDUCIBLE 1)	-0.10	9.99E-01	1.52	4.51E-08	2.79	1.59E-12
AT4G36500	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.11	9.90E-01	0.26	1.98E-01	1.13	1.73E-07
AT4G36870	BLH2 (BEL1-LIKE HOMEODOMAIN 2, SAWTOOTH 1); DNA binding…	-0.05	9.99E-01	1.93	6.35E-06	3.22	2.05E-09
AT4G37540	LBD39 (LOB DOMAIN-CONTAINING PROTEIN 39)	-0.32	7.07E-01	0.55	1.47E-02	1.16	3.85E-06
AT4G37560	formamidase, putative / formamide amidohydrolase, putat…	0.20	9.57E-01	1.85	1.59E-07	1.94	3.35E-08
AT4G37610	BT5 (BTB and TAZ domain protein 5); protein binding / t…	0.14	9.79E-01	0.75	5.99E-04	2.41	3.14E-11
AT4G37640	ACA2 (CALCIUM ATPASE 2); calmodulin binding	-0.09	9.99E-01	0.17	6.79E-01	1.06	3.48E-05
AT4G38340	RWP-RK domain-containing protein	0.05	9.99E-01	1.76	3.75E-04	1.33	2.79E-03
AT4G38400	ATEXLA2 (ARABIDOPSIS THALIANA EXPANSIN-LIKE A2)	-0.20	9.57E-01	2.16	1.60E-08	6.01	1.21E-15
AT4G38420	SKS9 (SKU5 Similar 9); copper ion binding / oxidoreduct…	0.02	9.99E-01	0.11	8.73E-01	2.27	1.09E-08
AT4G38570	PIS2 (PROBABLE CDP-DIACYLGLYCEROL--INOSITOL 3-PHOSPHATI…	0.17	9.78E-01	0.42	1.21E-01	2.20	2.07E-09
AT4G39320	microtubule-associated protein-related	-0.13	9.99E-01	2.34	1.95E-07	7.44	2.48E-15
AT4G39660	AGT2 (ALANINE:GLYOXYLATE AMINOTRANSFERASE 2); alanine-g…	0.25	9.91E-01	1.99	1.69E-05	3.56	2.37E-09
AT5G01080	beta-galactosidase	0.24	9.99E-01	0.16	8.72E-01	1.67	1.69E-04
AT5G01520	zinc finger (C3HC4-type RING finger) family protein	-0.15	9.95E-01	-0.12	8.15E-01	1.63	2.12E-07
AT5G01610	Identical to Uncharacterized protein At5g01610 [Arabido…	-0.02	9.99E-01	0.60	8.97E-03	1.39	3.98E-07
AT5G01670	aldose reductase, putative	-0.12	9.99E-01	2.55	1.30E-09	1.62	3.67E-07
AT5G01790	unknown protein	0.02	9.99E-01	0.14	8.49E-01	4.51	2.88E-12
AT5G03520	AtRABE1d/AtRab8C; GTP binding	0.10	9.99E-01	0.69	7.05E-04	1.43	3.92E-08
AT5G03550	similar to meprin and TRAF homology domain-containing p…	-0.15	9.99E-01	0.05	9.58E-01	1.38	9.82E-06
AT5G04310	pectate lyase family protein	-0.11	9.99E-01	3.71	4.19E-11	5.97	7.50E-15
AT5G04310	pectate lyase family protein	0.07	9.99E-01	2.68	1.69E-10	5.29	1.95E-15
AT5G04740	ACT domain-containing protein	-0.04	9.99E-01	1.08	4.33E-06	2.48	8.39E-12
AT5G05250	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.22	9.52E-01	0.86	2.05E-03	1.64	5.82E-07
AT5G05350	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.16	9.99E-01	1.87	4.10E-05	2.00	9.30E-06
AT5G07010	sulfotransferase family protein	-0.27	9.33E-01	1.54	8.93E-06	5.04	8.85E-14
AT5G07070	CIPK2 (CBL-INTERACTING PROTEIN KINASE 2); kinase	0.07	9.99E-01	0.33	3.05E-01	1.20	1.70E-05
AT5G07460	PMSR2 (PEPTIDEMETHIONINE SULFOXIDE REDUCTASE 2); peptid…	-0.31	9.91E-01	2.61	1.05E-05	1.27	8.57E-03
AT5G08170	ATAIH/EMB1873 (AGMATINE IMINOHYDROLASE); agmatine deimi…	0.00	9.99E-01	1.18	1.15E-04	1.00	3.60E-04
AT5G08350	GRAM domain-containing protein / ABA-responsive protein	0.36	8.37E-01	5.03	1.20E-12	8.23	2.62E-16
AT5G08360	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.32	8.08E-01	-0.31	3.73E-01	2.44	1.60E-09
AT5G09420	ATTOC64-V/MTOM64 (ARABIDOPSIS THALIANA TRANSLOCON AT TH…	0.34	6.35E-01	1.06	1.34E-05	1.13	2.88E-06
AT5G11320	YUC4 (YUCCA4); monooxygenase	-0.11	9.99E-01	0.46	1.56E-01	3.22	5.86E-11
AT5G11700	glycine-rich protein	-0.02	9.99E-01	0.52	1.69E-01	1.31	1.26E-04
AT5G11710	(EPSIN1); binding	0.05	9.99E-01	1.42	9.36E-08	2.94	6.11E-13
AT5G12340	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.13	9.99E-01	1.03	2.89E-04	1.05	1.29E-04
AT5G12850	zinc finger (CCCH-type) family protein	-0.03	9.99E-01	0.16	7.16E-01	3.96	2.53E-13
AT5G14130	peroxidase, putative	0.03	9.99E-01	0.06	9.31E-01	2.04	3.96E-09
AT5G15410	DND1 (DEFENSE NO DEATH 1); calcium channel/ calmodulin…	0.38	7.07E-01	4.21	1.31E-12	3.14	2.35E-11
AT5G17640	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.15	9.34E-01	1.49	5.44E-09	1.36	7.94E-09
AT5G17790	VAR3 (VARIEGATED 3); binding	0.30	9.08E-01	0.75	2.11E-02	1.22	1.55E-04
AT5G18150	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.25	9.91E-01	1.07	8.11E-03	1.11	3.56E-03
AT5G18170	GDH1 (GLUTAMATE DEHYDROGENASE 1); oxidoreductase	-0.34	4.10E-01	0.34	6.52E-02	1.14	1.59E-07
AT5G18270	ANAC087; transcription factor	-0.01	9.99E-01	-0.33	4.06E-01	1.28	4.48E-05
AT5G18440	similar to hypothetical protein [Vitis vinifera] (GB:CA…	0.53	1.02E-01	1.20	3.61E-07	1.29	5.68E-08
AT5G18450	AP2 domain-containing transcription factor, putative	-0.33	8.64E-01	3.35	2.51E-10	5.15	1.07E-13
AT5G18600	glutaredoxin family protein	0.08	9.99E-01	0.04	9.56E-01	2.98	4.35E-12
AT5G18670	BMY3 (BETA-AMYLASE 9); beta-amylase	0.54	8.85E-01	0.79	2.03E-01	3.62	1.11E-07
AT5G19580	glyoxal oxidase-related	-0.01	9.99E-01	0.04	9.62E-01	3.19	1.18E-10
AT5G20250	DIN10 (DARK INDUCIBLE 10); hydrolase, hydrolyzing O-gly…	0.40	3.20E-01	1.62	4.98E-09	2.56	1.58E-12
AT5G20885	zinc finger (C3HC4-type RING finger) family protein	-0.05	9.99E-01	0.43	1.92E-01	1.63	1.25E-06
AT5G22460	esterase/lipase/thioesterase family protein	-0.28	8.08E-01	2.19	2.85E-09	5.70	6.63E-16
AT5G22580	Identical to Uncharacterized protein At5g22580 [Arabido…	0.02	9.99E-01	6.07	2.12E-15	1.22	7.36E-06
AT5G22920	zinc finger (C3HC4-type RING finger) family protein	0.08	9.99E-01	1.79	1.13E-05	2.92	5.27E-09
AT5G23350	[AT5G23350, GRAM domain-containing protein / ABA-respon…	0.15	9.99E-01	1.05	2.57E-04	5.48	6.05E-15
AT5G23760	heavy-metal-associated domain-containing protein	0.05	9.99E-01	1.44	1.75E-02	1.70	2.78E-03
AT5G24910	CYP714A1 (cytochrome P450, family 714, subfamily A, pol…	0.19	9.99E-01	1.53	9.34E-05	2.99	5.21E-09

AT5G24950	[AT5G24950, CYP71A15 (cytochrome P450, family 71, subfa…	-0.21	9.65E-01	3.82	1.17E-11	4.26	4.66E-13
AT5G25110	CIPK25 (CBL-INTERACTING PROTEIN KINASE 25); kinase	-0.20	9.99E-01	-0.29	6.00E-01	1.14	9.67E-04
AT5G25280	serine-rich protein-related	-0.16	9.68E-01	-0.04	9.52E-01	1.09	3.74E-06
AT5G25890	IAA28 (IAA-ALANINE RESISTANT 2); transcription factor	-0.40	9.42E-01	0.68	1.91E-01	1.38	1.57E-03
AT5G26040	HDA2 (histone deacetylase 2); histone deacetylase	-0.22	9.79E-01	1.55	3.55E-05	2.29	9.45E-08
AT5G26240	CLC-D (chloride channel D); anion channel/ voltage-gate…	0.12	9.91E-01	0.33	1.40E-01	1.48	3.39E-08
AT5G26731	unknown protein	-0.46	2.80E-01	1.53	4.81E-08	1.54	1.76E-08
AT5G26740	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.11	9.79E-01	1.44	4.42E-09	2.72	9.53E-14
AT5G26920	calmodulin binding	-0.33	9.23E-01	1.31	5.47E-04	1.86	4.60E-06
AT5G27740	EMB2775 (EMBRYO DEFECTIVE 2775); DNA binding / nucleosi…	0.37	8.60E-01	1.07	2.09E-03	1.32	1.39E-04
AT5G36880	acetyl-CoA synthetase, putative / acetate-CoA ligase, p…	0.00	1.00E+00	1.52	1.73E-07	1.82	5.42E-09
AT5G37550	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.11	9.99E-01	-0.03	9.70E-01	4.75	1.27E-14
AT5G37580	protein binding	0.19	8.84E-01	0.40	4.60E-02	1.69	2.08E-09
AT5G38910	germin-like protein, putative	-0.02	9.99E-01	0.34	5.31E-01	2.23	5.40E-07
AT5G39720	AIG2L (AVIRULENCE INDUCED GENE 2 LIKE PROTEIN)	-0.01	9.99E-01	4.12	1.49E-11	2.99	4.60E-10
AT5G40010	AATP1 (AAA-ATPASE 1); ATP binding / ATPase	0.05	9.99E-01	-0.12	9.10E-01	1.78	3.86E-05
AT5G40470	similar to F-box family protein (FBL4) [Arabidopsis tha…	0.29	9.99E-01	0.97	2.56E-01	1.93	4.17E-03
AT5G40510	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.08	9.99E-01	1.49	1.68E-05	2.00	1.51E-07
AT5G41080	glycerophosphoryl diester phosphodiesterase family prot…	-0.01	9.99E-01	3.32	1.41E-07	4.64	4.26E-10
AT5G41440	zinc finger (C3HC4-type RING finger) family protein	-0.16	9.68E-01	0.01	9.88E-01	2.51	2.29E-11
AT5G41610	ATCHX18 (cation/hydrogen exchanger 18); monovalent cati…	-0.23	9.79E-01	-0.26	6.41E-01	1.34	1.05E-04
AT5G43150	similar to unnamed protein product [Vitis vinifera] (GB…	-0.33	8.26E-01	1.92	3.46E-07	1.97	1.10E-07
AT5G43410	ethylene-responsive factor, putative	-0.15	9.99E-01	3.25	4.97E-11	2.08	1.12E-08
AT5G43620	[AT5G43620, S-locus protein-related];[AT1G66500, zinc f…	0.46	8.87E-01	0.63	2.63E-01	1.29	3.53E-03
AT5G43840	AT-HSFA6A (Arabidopsis thaliana heat shock transcriptio…	0.00	9.99E-01	-0.02	9.78E-01	1.19	4.51E-05
AT5G44060	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.37	8.08E-01	0.13	8.48E-01	1.86	6.89E-07
AT5G44130	FLA13 (FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 13 PRECUR…	0.48	6.92E-01	4.79	4.92E-12	4.71	1.23E-12
AT5G45630	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.16	9.99E-01	4.39	1.68E-11	5.02	4.66E-13
AT5G45690	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.04	9.99E-01	3.46	4.02E-09	6.06	2.65E-13
AT5G46590	ANAC096 (Arabidopsis NAC domain containing protein 96);…	-0.39	9.21E-01	2.39	2.86E-06	4.50	1.14E-10
AT5G47250	disease resistance protein (CC-NBS-LRR class), putative	0.00	1.00E+00	1.34	1.71E-05	3.45	8.39E-12
AT5G47870	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.22	9.43E-01	0.86	1.26E-03	1.34	3.90E-06
AT5G48010	pentacyclic triterpene synthase, putative	-0.02	9.99E-01	1.00	1.19E-04	3.47	9.75E-13
AT5G49360	BXL1 (BETA-XYLOSIDASE 1); hydrolase, hydrolyzing O-glyc…	0.19	9.99E-01	0.24	6.63E-01	3.13	9.11E-10
AT5G49710	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.23	8.85E-01	0.49	4.08E-02	1.49	1.82E-07
AT5G49750	leucine-rich repeat family protein	0.03	9.99E-01	-0.02	9.82E-01	2.55	4.26E-10
AT5G49760	leucine-rich repeat family protein / protein kinase fam…	0.02	9.99E-01	0.78	1.16E-01	1.15	6.99E-03
AT5G51460	ATTPPA (Arabidopsis thaliana trehalose-6-phosphate phos…	-0.17	9.99E-01	0.71	1.97E-02	1.44	1.01E-05
AT5G51630	disease resistance protein (TIR-NBS-LRR class), putativ…	0.09	9.99E-01	0.32	2.53E-01	1.03	2.71E-05
AT5G51680	hydroxyproline-rich glycoprotein family protein	-0.30	9.91E-01	2.50	7.77E-06	5.28	6.32E-11
AT5G52540	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.10	9.99E-01	0.78	2.02E-03	2.24	1.39E-09
AT5G52920	PKP-BETA1/PKP1/PKP2 (PLASTIDIC PYRUVATE KINASE 1); pyru…	0.19	9.78E-01	0.86	2.10E-03	2.12	1.47E-08
AT5G53530	vacuolar protein sorting-associated protein 26, putativ…	0.01	9.99E-01	0.45	3.82E-01	2.51	2.59E-07
AT5G53830	VQ motif-containing protein	-0.21	9.99E-01	0.87	1.93E-02	2.10	1.02E-06
AT5G54040	DC1 domain-containing protein	0.08	9.99E-01	0.14	8.25E-01	1.12	3.04E-04
AT5G56540	AGP14 (ARABINOGALACTAN PROTEIN 14)	0.15	9.99E-01	4.06	1.40E-10	1.20	5.96E-04
AT5G56800	similar to F-box family protein [Arabidopsis thaliana]…	-0.15	9.83E-01	0.03	9.59E-01	2.19	5.33E-10
AT5G57000	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.16	9.43E-01	0.43	2.28E-02	1.27	8.66E-08
AT5G57160	ATLIG4 (ARABIDOPSIS THALIANA DNA LIGASE IV)	0.58	5.22E-01	0.49	1.86E-01	1.81	1.55E-06
AT5G57420	IAA33 (indoleacetic acid-induced protein 33); transcrip…	-0.08	9.99E-01	-0.29	6.72E-01	1.59	1.52E-04
AT5G57785	unknown protein	0.01	9.99E-01	0.21	5.79E-01	1.97	9.18E-09
AT5G57910	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.09	9.99E-01	0.27	2.30E-01	1.39	3.84E-08
AT5G57920	plastocyanin-like domain-containing protein	-0.16	9.99E-01	-0.11	8.86E-01	3.87	1.56E-11
AT5G58090	glycosyl hydrolase family 17 protein	-0.07	9.99E-01	1.71	4.88E-07	1.02	1.60E-04
AT5G58650	PSY1 (PLANT PEPTIDE CONTAINING SULFATED TYROSINE 1)	0.10	9.99E-01	1.90	6.22E-04	3.59	1.04E-07
AT5G58670	ATPLC1 (PHOSPHOLIPASE C 1); phospholipase C	-0.05	9.99E-01	1.28	7.63E-04	1.87	4.83E-06
AT5G58940	CRCK1 (CALMODULIN-BINDING RECEPTOR-LIKE CYTOPLASMIC KIN..	-0.01	9.99E-01	0.92	2.38E-03	2.00	1.03E-07
AT5G59310	LTP4 (LIPID TRANSFER PROTEIN 4); lipid binding	0.12	9.99E-01	0.84	2.15E-03	6.53	5.32E-16
AT5G59360	unknown protein	0.09	9.99E-01	0.15	8.59E-01	1.31	4.84E-04
AT5G59680	leucine-rich repeat protein kinase, putative	-0.05	9.99E-01	2.42	1.24E-05	2.99	3.51E-07
AT5G59730	ATEXO70H7 (EXOCYST SUBUNIT EXO70 FAMILY PROTEIN H7); pr…	-0.02	9.99E-01	1.12	1.27E-03	1.83	2.13E-06
AT5G61290	flavin-containing monooxygenase family protein / FMO fa…	-0.11	9.99E-01	-0.08	9.42E-01	2.96	2.05E-08
AT5G61440	thioredoxin family protein	0.05	9.99E-01	0.38	2.05E-01	1.81	7.99E-08
AT5G62480	ATGSTU9 (GLUTATHIONE S-TRANSFERASE TAU 9); glutathione…	0.14	9.74E-01	3.99	3.56E-14	4.98	3.49E-16
AT5G62490	ATHVA22B (Arabidopsis thaliana HVA22 homologue B)	-0.12	9.99E-01	1.25	1.32E-05	3.96	2.53E-13
AT5G62530	ALDH12A1 (Aldehyde dehydrogenase 12A1); 1-pyrroline-5-c…	0.06	9.99E-01	1.03	2.29E-05	1.43	1.28E-07
AT5G62540	UBC3 (UBIQUITIN-CONJUGATING ENZYME 3); ubiquitin-protei…	0.06	9.99E-01	0.73	8.22E-05	1.12	1.42E-07
AT5G62610	basic helix-loop-helix (bHLH) family protein	0.44	6.61E-01	0.59	5.08E-02	1.15	9.87E-05
AT5G62960	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.20	9.84E-01	1.00	1.36E-03	1.60	3.43E-06
AT5G64000	SAL2; 3'(2'),5'-bisphosphate nucleotidase/ inositol or…	0.28	8.54E-01	0.14	7.59E-01	4.61	3.40E-14
AT5G64530	ANAC104/XND1 (Arabidopsis NAC domain containing protein…	-0.42	7.09E-01	0.35	3.69E-01	1.38	2.09E-05
AT5G64970	mitochondrial substrate carrier family protein	0.39	7.84E-01	0.60	7.13E-02	1.09	4.20E-04
AT5G65130	AP2 domain-containing transcription factor, putative	-0.20	9.72E-01	0.38	2.51E-01	1.81	1.90E-07
AT5G65160	tetratricopeptide repeat (TPR)-containing protein	-0.03	9.99E-01	1.24	1.71E-04	4.32	1.51E-12
AT5G65430	GRF8 (GENERAL REGULATORY FACTOR 8); protein phosphoryla…	0.01	9.99E-01	1.02	7.42E-04	1.27	3.60E-05
AT5G65510	AIL7 (AINTEGUMENTA-LIKE 7); DNA binding / transcription…	-0.03	9.99E-01	0.14	7.08E-01	1.30	3.99E-07
AT5G65630	GTE7 (GLOBAL TRANSCRIPTION FACTOR GROUP E 7); DNA bindi…	0.25	9.43E-01	1.21	1.33E-04	1.42	1.03E-05
AT5G65730	xyloglucan:xyloglucosyl transferase, putative / xyloglu…	-0.12	9.99E-01	0.09	9.47E-01	2.33	4.89E-06
AT5G65790	MYB68 (myb domain protein 68); DNA binding / transcript…	0.01	9.99E-01	0.56	6.90E-02	2.07	4.93E-08
AT5G65930	ZWI (ZWICHEL); calmodulin binding / microtubule motor	0.06	9.99E-01	0.84	1.76E-04	1.14	1.95E-06
AT5G66170	similar to unknown protein [Arabidopsis thaliana] (TAIR…	0.14	9.83E-01	4.91	1.13E-14	6.16	1.27E-16
AT5G66610	zinc ion binding	0.02	9.99E-01	0.75	2.26E-02	1.28	1.07E-04
AT5G66640	LIM domain-containing protein-related	0.20	9.99E-01	0.03	9.79E-01	1.17	1.68E-03
AT5G66650	similar to unknown protein [Arabidopsis thaliana] (TAIR…	-0.34	9.32E-01	1.27	1.04E-03	1.15	1.44E-03
AT5G66985	unknown protein	-0.03	9.99E-01	0.31	3.76E-01	2.50	7.35E-10
AT5G67160	transferase family protein	-0.04	9.99E-01	0.21	5.20E-01	1.92	3.83E-09
AT5G67360	ARA12; subtilase	0.51	3.76E-01	1.91	3.50E-08	1.31	2.87E-06

Samenvatting

Suikers zijn van groot belang voor planten. Ze dienen niet alleen als bron van energie en bouwsteen voor de groei van de plant, maar ook als signalerings moleculen. Suikers kunnen dienen als signalen die de groei en ontwikkeling van planten sturen door o.a. de expressie van genen te reguleren.
In dit proefschrift is de rol van bZIP eiwitten in de model plant Arabidopsis thaliana (zandraket) onderzocht. Deze specifieke groep eiwitten speelt namelijk een belangrijke rol in suikersignalering in planten. Een specifieke subklasse van de bZIP eiwitten (de S1 klasse) kan alleen functioneren als er weinig suiker (sucrose) aanwezig is in de cel. De bZIP eiwitten zijn transcriptie factoren die genen kunnen aanschakelen die vervolgens een effect hebben op het metabolisme van de plant.

In het eerste hoofdstuk word een overzicht gegeven van reeds bekende relaties tussen suikersignalering, stress en de groei en ontwikkeling van planten. In de volgende drie hoofdstukken wordt met behulp van experimenten steeds meer inzicht verkregen in de rol van bZIP transcriptie factoren in Arabidopsis. Met behulp van onder meer micro array experimenten wordt in hoofdstuk twee onderzocht welke genen door de transcriptie factor bZIP11 kunnen worden geactiveerd. Uit de verkregen data, die overeenstemmen met resultaten uit andere laboratoria, blijkt dat bZIP11 een krachtige activator van gen expressie is. Onder meer worden meerdere genen die betrokken zijn bij (aminozuur) metabolisme geïdentificeerd als zijnde bZIP11 induceerbaar.
In hoofdstuk drie wordt vervolgens aangetoond dat bZIP11 niet alleen genen induceert die betrokken zijn bij metabolisme, maar dat dit ook daadwerkelijk gevolgen heeft voor het metabolisme van de plant. Er wordt aangetoond dat onder meer de concentratie van bepaalde suikers en aminozuren in planten veranderd als bZIP11 in deze planten wordt geactiveerd. De fysiologische relevantie hiervan wordt verder versterkt door het feit dat planten met een verhoogde bZIP11 activiteit resistent zijn tegen verhoogde concentraties van de suiker trehalose in het groei medium. Arabidopsis plantjes die geconfronteerd worden met kunstmatig verhoogde concentraties van deze suiker zullen normaal gesproken geremd worden in hun groei. Wanneer we echter de transcriptie factor bZIP11 activeren kunnen planten door een nog onbekend mechanisme toch doorgroeien.

Omdat bZIP transcriptie factoren genen activeren in de vorm van dimeren (een combinatie van twee bZIPs die samen een actief complex vormen) wordt in hoofdstuk vier gekeken naar de functie van verschillende dimeren. Zo wordt er (wederom met behulp van micro array experimenten) aangetoond dat verschillende dimeren verschillende groepen

genen activeren. Uit de resultaten blijkt verder dat verschillende combinaties van bZIPs ook zeer verschillende hoeveelheden genen activeren. Transcriptie factor complexen bestaande uit twee bZIP10 eenheden konden in de experimenten slechts 7 genen activeren. De eerder gebruikte complexen bestaande uit twee bZIP11 eenheden konden 383 genen activeren. Combinatie van bZIP10 en bZIP11 in een complex leidde echter tot activering van 645 genen. Deze differentiële regulatie van gen expressie door bZIP transcriptie factoren heeft belangrijke biologische implicaties en dient dan ook verder onderzocht te worden. Daarbij zal ook moeten worden gekeken naar andere dan de hier beschreven bZIPs. Ook de integratie van bZIP suikersignalering met andere signaleringsnetwerken zal moeten worden onderzocht om hun functie in de groei en ontwikkeling van planten verder te kunnen begrijpen.

Curriculum Vitae

Micha Hanssen was born in Heerlen, the Netherlands on January 24, 1981. He completed his pre-university education (VWO) at the Eijkhagen College in Landgraaf in 1999 before starting his biology study at the University of Utrecht the same year. He completed the master program Biomolecular Sciences, including internships at the CBS Fungal Biodiversity Center and in the NMR spectroscopy research group in 2004. In May 2005 he started his PhD studies in the Molecular Plant Physiology group at Utrecht University, where the work presented in this thesis was performed.

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