Translational control of the Arabidopsis bZIP transcription factor gene *ATB2* by a conserved uORF

Translationele regulatie van de Arabidopsis bZIP transcriptiefactor *ATB2* door een geconserveerd uORF

Proefschrift

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Chapter 1

General introduction: Sugar sensing and post-transcriptional regulation of gene expression

Sugar mediated modulation of gene expression in plants

All parts of the plant need sugars for metabolic processes whereas only chlorophyll containing cells can produce sugars via photosynthesis. Sucrose and its derivates represent the major forms of transported carbon. Through symplasmic- and apoplasmic transport sugars are loaded into the phloem and delivered to heterotropic tissues (Lalonde, S. et al. 1999). Sugar supply throughout time is not constant and tissues respond differentially via metabolic and developmental changes. Next to the well known metabolic and transport functions, sugars also act as hormones in the control of metabolic and developmental changes. The effective concentrations lie within the millimollar range instead of the micromolar range, as is the case with hormones (Sheen, J. et al. 1999).
Changes in sugar levels can affect the expression of many genes (Koch, K. E. 1996). Examples have been presented for regulation at the transcriptional or post-transcriptional level, including the stability of mRNA's and proteins (Smeekens, S. 2000). Elaborate signaling networks have evolved to direct these, often complex, changes. Much of our understanding of sugar signaling networks comes from experiments with yeast as research has progressed furthest there. The molecular details of how plants perceive sugars is mostly incomplete but experiments have shown that plants have different sensing pathways for different sugars. The current knowledge of sugar sensing and signaling mechanisms will be discussed together with relevant information on yeast sugar sensing.

Hexose induced signaling

Hexoses can be sensed by at least two independent systems in plants (Smeekens,S. 2000, Xiao, W. et al. 2000). The difference between these systems is the requirement for phosphorylation by hexokinase. Glucose analogs, which cannot be phosphorylated by hexokinase (3-O-methylglucose and 6-deoxyglucose), initiate signaling and alter gene expression. For example, the sugar and amino acid-responsive patatin class I pat(B33) promoter is induced by the glucose analog 3-O-mGlc in transgenic Arabidopsis plants harboring the Pat(B33)-iudA construct (Martin, T. et al. 1997). Recently a B-box, involved in sugar regulation, has been identified in the patatin promoter from potato. The B-box contains a conserved 10 base pair motif and is recognized by the plant-specific DNA-binding protein Storekeeper (STK) (Zourelidou, M. et al. 2002). STK defines a new family of plant-specific DNA binding proteins of unknown function. Another example is the induction by glucose and 6-dGlc of the expression of extracellular invertase and sucrose synthase in a cell suspension culture of *Chenopodium rubrum* while in *Chlorella kessleri* these sugar induce several genes, including

a glucose transporter (Hilgarth, C. et al. 1991, Roitsch, T. et al. 1995). The molecular details on hexokinase independent sensing and signaling remain unclear in plants.
Hexokinase dependent sugar sensing requires glucose or other phosphorylatable sugars whereas 6-deoxyglucose and 3-O-methyl-glucose are ineffective. Information on hexokinase dependent sugar sensing and signaling in plants is very limited. Recently the signaling function of hexokinase was further defined by the separation of the signaling and enzymatic activity (Moore, B. et al. 2003).

The yeast *Saccharomyces cerevisiae* has provided detailed understanding of glucose sensing because it serves as an excellent system for studying sugar regulated gene expression. It can adapt its cellular metabolism and growth to varying external conditions such that the preferred carbon source glucose is used. Research has shown that hexokinase regulates the expression of many genes (Vincent, O. et al. 2001, Xiao, W. et al. 2000). Glucose dependent translocation of hexokinase Hxk2p to the nucleus is required for regulation of these genes (Rodriguez, A. et al. 2001). Hxk2p is found in both the nucleus and cytoplasm and possibly the phosphorylation status of hexokinase plays a role in nuclear translocation. Furthermore, the dimeric-monomeric equilibrium is affected by phosphorylation (Moreno, F. and Herrero, P. 2002, Randez-Gil, F. et al. 1998).
Six HXK and HXK-like (HXKL) genes have been identified in the genome of *Arabidopsis thaliana* (2000). Photosynthesis genes are down regulated via a hexokinase dependent signaling pathway and overexpression of the *Arabidopsis* HXK1 in tomato resulted in reduced chlorophyll content in leaves, reduced photosynthesis rates and reduced photochemical quantum efficiency of photosystem II reaction centers (Dai, N. et al. 1999). In addition, the transgenic tomato plants underwent rapid senescence. Repression of *Arabidopsis HXK1* expression delays the senescence process suggesting that hexokinase must be involved in senescence regulation in *Arabidopsis thaliana* (Dai, N. et al. 1999a, Xiao, W. et al. 2000). AtHXK1 null mutants (*gin2-1* and *gin2-2*) display a Glc insensitivity phenotype while overexpression of the AtHXK1 gene, by the 35S-promoter, restores sensitivity to glucose in the *gin2* background (Brandon Moore et al. 2003, Rolland, F. et al. 2002). Overexpression of the yeast HXK2 protein in *Arabidopsis* caused a dominant negative effect on HXK signaling since elevated yeast HXK activity lowers hexose concentrations, leading to reduced signaling (Jang, J. C. et al. 1997).

An important step towards understanding the function of HXK-mediated signaling in plants was the separation of the enzymatic and regulatory function as has been presented for yeast. (Mayordomo, I. and Sanz, P. 2001, Moore, B. et al. 2003). Mannose represses germination of *Arabidopsis thaliana* seeds by a mechanism that involves hexokinase (Pego, J. V. et al. 1999). Addition of the hexokinase

inhibitor mannoheptulose or metabolizable sugars restores germination on mannose. It was suggested that the phosphorylation of mannose by HXK triggers a signaling cascade leading to repression of germination.

Yeast contains a vast array of integral membrane proteins, which appear to be involved in transmembrane transport. Over 20 proteins have been identified as sugar permeases (Andre, B. 1995, Boles, E. and Hollenberg, C. P. 1997). Interestingly some of these sugar transporters are involved in sensing and signal initiation (Andre, B. 1995a, Nelissen, B. et al. 1997). One of these transporters (Snf3) was found to encode a high-affinity glucose transporter like protein (Bisson, L. F. et al. 1987). The SNF3 gene does not encode a functional glucose transporter since it cannot restore glucose transport in mutants with deleted transporters (Reifenberger, E. et al. 1995). Moreover, a dominant mutation in a suppressor of the *snf3* mutant, *rgt2*, results in constitutive expression of several *HXT* genes. The *RGT2* gene encodes a transporter like protein, similar to the *SNF3* gene (Ozcan, S. et al. 1996). Introducing the same mutation, as found in *rgt2*, into the glucose sensor Snf3, results in constitutive expression of a different set of HXT genes (Ozcan, S. et al. 1996). When *RGT2* is deleted, a five fold reduction of low affinity glucose transporters is observed when grown on high levels of glucose. The unusually long C-terminal domains of both sensor-proteins interact with the proteins Std1 and Mth1, which show no homology to other known proteins, (Ganster, R. W. et al. 1998, Schmidt, M. C. et al. 1999) to relay the glucose signal. Transplantation of the unusually long C-terminal domain of Snf3 onto the glucose transporters HXT1p and HXT2p converts them into glucose sensors that generate a signal for glucose induced HXT expression (Ozcan, S. et al. 1998).

The yeast galactokinase Gal1p, and the closely related but catalytically inactive Gal3p, have been shown to function as ligand sensors (Bhat, P. J. and Hopper, J. E. 1992, Bhat, P. J. and Murthy, T. V. 2001). This finding, together with the glucose sensing data, makes the idea of separate sensors for different sugar molecules appealing. Moreover, several other yeast nutrient sensors (e.g amino acids and ammonium) appear to operate in a similar way as the glucose sensors (Forsberg, H. and Ljungdahl, P. O. 2001).

Signal transduction

Glucose is transported into the cell by hexose transporters and phosphorylated by hexokinase in yeast. Next to phosphorylation, a G-protein-coupled receptor system is present in yeast that senses glucose directly and both processes are required for glucose induced cAMP signaling (Rolland, F. et al. 2000). Changes in the ATP-cAMP ratio result in activation of cAMP-dependent protein kinases through binding

to its regulatory subunits. Members of this protein kinase subfamily are central components of highly conserved protein kinase cascades that are present in most, if not all, eukaryotic cells. In animal cells, the AMP-activated protein kinase acts as a metabolic sensor or "fuel gauge" that monitors the AMP and ATP ratio (Hardie, D. G. and CARLING, D. 1997). In yeast, the homologous SNF1 complex is activated in response to glucose deprivation (Carlson, M. 1999). In plants, involvement of SNF-like kinases in responses to environmental and/or nutritional stress seems most likely (Hardie, D. G. et al. 1998). Plants homologs are named Snf1 related protein kinase (SnRK1) and are thought to be hetero-trimeric complexes. SnRK1 phosphorylates a number of key regulatory enzymes such as 3-hydroxy-3-methylglutaryl-Coenzyme A reductase, nitrate reductase and sucrose phosphate synthase (Sugden, C. et al. 1999). Moreover SnRK1 is required for expression of sucrose synthase in potato tubers and excised leaves (Slocombe, S. P. et al. 2002). One of the subunits of the SnRK1, named StubGAL83 from potato, has recently been shown to be involved in metabolic processes (Lovas, A. et al. 2003). Moreover, antisense repression of StubGAL83 affects root and tuber development. One of the proteins that interact with the Arabidopsis SnRK1 protein is the PRL1 (pleiotropic regulatory locus 1) protein. PRL1 encodes 54kDa protein containing seven WD-40 repeats. The *prl1* mutant is hypersensitive sucrose. Using PRL1 as bait in a yeast two-hybrid approach identified two SnRK1 subunits, AKINb1 and AKINb2. In the presence of glucose, the activity of the AKIN kinase is enhanced when expressed in yeast, which is also observed in the Arabidopsis *prl1* mutant. It appears that the PRL1 protein functions as a negative regulatory factor repressing AKIN activity under normal circumstances. The PRL1 protein interacts with the C-terminal domain of the SnRK protein. This C-terminal domain also interacts with a conserved SCF (Skp1-cullin-F-box) ubiquitine ligase subunit (Farras, R. et al. 2001). SnRK activity is enhanced by the *prl1* mutation which results in sensitized growth responses to auxin and other hormones (Nemeth, K. et al. 1998) whereas mutations affecting the SCF subunits reduce auxin sensitivity (Gray, W. M. et al. 1999).
In barley an SnRK1 interacting protein (SnIP1) was identified by a two-hybrid assay using barley SnRK1b, a seed-specific form of SnRK1 (Slocombe, S. P. et al. 2002). It appears that the SnIP1 is plant specific because database searches identified no homologs outside the plant kingdom. Nevertheless, weak similarity was found with the yeast SNF4 protein and mammalian AMPKg protein. The yeast SNF4 gene encodes the g-subunit of the Snf1 kinase and it is thought to be involved in regulating SNF1 kinase activity in response to glucose levels (Jiang, R. and Carlson, M. 1996, McCartney, R. R. and Schmidt, M. C. 2001, Slocombe, S. P. et al. 2002).

Sucrose specific signaling

The disaccharide sucrose consists of one glucose and one fructose molecule. Sucrose can be hydrolyzed by invertase and sucrose synthase. The expression of many genes appears to be affected by addition of sucrose to plants, but for most of these, the effect is not specific for sucrose. The cleavage products of sucrose, glucose and fructose, can often exert the same effect as sucrose suggesting that sucrose, as a molecule, does not play signaling a role. However, genes have been identified that are regulated specifically by sucrose. The effect of sucrose on gene expression has been shown for the phloem specific *rolC* promoter, the *Arabidopsis thaliana* basic leucine zipper transcription factor *ATB2* and the *Beta vulgaris* sucrose transporter *BtSUC* (Chiou, T. J. and Bush, D. R. 1998, Rook, F. et al. 1998, Weise, A. et al. 2000, Wenzler, H. C. et al. 1989, Yokoyama, R. et al. 1994). The sucrose transporter of *Beta vulgaris* is regulated at the transcriptional level while the basic leucine zipper transcription factor ATB2 is regulated at the post-transcriptional level by sucrose (Chiou, T. J. and Bush, D. R. 1998, Rook, F. et al. 1998). Transgenic Arabidopsis plants, harboring the *GUS* or *LUC* genes under the control of the *ATB2* promoter and leader sequences, are translationally repressed when high levels of exogenous sucrose are applied (Rook, Elzinga unpublished results). Although it is not known how the sucrose signal is perceived, an important sucrose regulatory element of the *ATB2* gene is located in the unusually long 5' leader sequence. Deletion of the 5' leader sequence abolishes the post-transcriptional sucrose regulation, leaving the gene under transcriptional regulation only. Originally, the *ATB2* gene was isolated in a screen for light regulated transcription factors. Thus, transcription of *ATB2* is induced by light whereas translation is repressed by sucrose (figure 1).

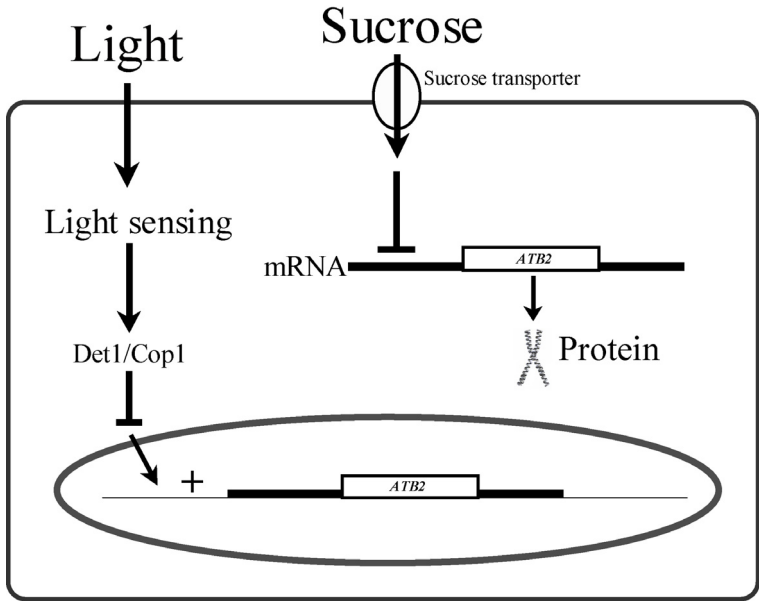


Figure 1: Schematic representation of the transcriptional and post-transcriptional regulatory pathways of the *ATB2* gene. Light activates *ATB2* transcription via the DET1/COP1 proteins (Rook, F. et al. 1998). High concentrations of sucrose repress translation via the 5'UTR (chapter 1).

Genetic experiments have shown that plants sense hexose and sucrose via different sensing pathways. In analogy to monosaccharide sensing in yeast, sucrose transporters have been identified that contain extended domains. SUT2, from tomato and Arabidopsis, and AtSUC3 both contain an extended cytoplasmic loop of about 50 amino acids (Barker, L. et al. 2000, Meyer, S. et al. 2000). Deletion of the loop from AtSUC3 does not change the kinetic properties of the transporter suggesting that the function of the loop is independent of transporter function. At this time, it has not been shown that the loop has a function in signaling. It has been shown that SUT4 from potato and tomato localizes to the enucleate sieve elements of minor veins and functions as a low-affinity/high capacity transporter (Weise, A. et al. 2000). Recently it was indicated that SUT1, SUT2 and SUT4 transporters from potato have the potential to interact and it was shown that SUT1 and SUT2 can form homooligomers (Reinders, A. et al. 2002). Next to regulation by interaction, it was observed that transporter phosphorylation appears to inhibit activity (Roblin, G. et al. 1998). Such regulation by phosphorylation and transporter interaction could be a fast way to balance sucrose transport according to supply and demand. It is an appealing idea to suggest that sucrose sensing is mediated by membrane sensors in a manner similar to glucose sensing in yeast.

Sugars and hormones

Sugar signaling research has been greatly aided by the identification of mutants, which show an altered response to sugars. These mutants have been most useful in obtaining information on the sugar-induced signal transduction pathways. Different studies revealed a tight link between sugar and hormonal signaling pathways. Screens for sugar response mutants identified mutants that also showed altered responses to hormones. Glucose-insensitive mutants (*gin*-mutants) can germinate and grow on 6% of glucose. The *gin*-phenotype can be mimicked by applying 1-aminocyclopropane-1 carboxylic acid (ACC) to WT-seeds, allowing germination and growth on 6% of glucose. ACC is the immediate precursor of ethylene and mutants, such as *eto1*, which constitutively synthesize ethylene, display a glucose insensitive (*gin*) phenotype. Alternatively, many, but not all, ethylene insensitive mutants display a hypersensitivity to glucose (Zhou, L. et al. 1998). Therefore, the ethylene pathway appears to negatively interact with sugar signaling pathways.
The hexokinase dependent sensing pathway requires an intact ABA signaling pathway, which was shown by the isolation of the *sun6*, *gin6*, *sis5* and *is3i* mutants (Arenas-Huertero, F. et al. 2000, Huijser, C. et al. 2000, Laby, R. J. et al. 2000, Rook, F. et al. 2001). The *sun6* mutant was isolated by screening an EMS population for reduced ability of the plastocyanin promoter to respond to elevated levels of sucrose (3%). The *SUN6* gene proved identical to the *ABI4* gene and is disturbed in the ABA signaling pathway. *Sun6* is able to germinate on medium containing ABA while also displaying glucose insensitive (gin), mannose insensitive (mig) and sucrose-uncoupled phenotype (sun) phenotypes. All *aba* (ABA biosynthesis mutants) and several *abi* mutants (ABA insensitive mutants) are to varying degrees sugar sensing mutants but it is unclear if ABA levels or ABA sensitivity, or both maybe modulated by sugars. Recently it was shown that the *Zea mays* ABI4 protein binds to the CE-1 like sequence, which, together with the ABRE element, is involved in promoting ABA-regulated gene expression (Niu, X. et al. 2002). The ZmABI4 protein is able to bind to the promoter of the ABA and sugar-responsive *ADH1* gene thereby providing further support for interacting ABA-and sugar-regulated signal transduction pathways. The ABA signaling pathway also interacts with the ethylene signaling pathway because the *gin1* mutant was found to be allelic to *aba2* (Laby, R. J. et al. 2000, Rook, F. et al. 2001). Recent isolation of alleles of *ctr1* and *ein2* as enhancer and suppressor mutations of *abi1*, respectively, illustrate the antagonistic relation between the ethylene and ABA signaling pathway during germination and seedling development (Beaudoin, N. et al. 2000, Ghassemian, M. et al. 2000). During germination in rice, gibberellic acid induces synthesis and secretion of a-amylases and other hydrolases. It was shown that the expression of the a-amylase genes are under the control of sugar and gibberelin (Perata, P. et al. 1997, Sheu, J. J. et al. 1996). The mRNAs

of several a-amylase genes in rice are differentially up regulated by sucrose starvation and down regulated by sucrose provision. Regulation occurs through transcription rate and mRNA stability control through the 3' UTR. In barley the sugar signal overrides the gibberelin response (Chan, M. T. and Yu, S. M. 1998, Perata, P. et al. 1997, Yu, S. M. et al. 1991) . Recently a link between auxin and cytokinin was suggested based on the analysis of the *gin2* mutant (Rolland, F. et al. 2002).

Translational regulation of gene expression by uORF's

The induction or repression of genes, as a response to the availability of metabolites, is tightly regulated for many genes. Regulatory mechanisms for important genes have evolved which involve both transcription and translation. Controlling translation provides an efficient way to balance protein production from transcription. The existence of numerous different regulatory mechanisms at the level of translation is suggested by many examples ((figure 2)Dean, K. A. et al. 2002, Gaba, A. et al. 2001, Klaff, P. et al. 1996, Messenguy, F. et al. 2002, Proud, C. G. 2001, Wang, L. and Wessler, S. R. 2001). One of these regulatory mechanisms involves the presence of uORF(s) in the leader sequence. Some of these uORF encode small peptides that can be essential for the regulation (Diba, F. et al. 2001, Fang, P. et al. 2000, Jousse, C. et al. 2001, Lincoln, A. J. et al. 1998, Raney, A. et al. 2002). The peptides encoded by the uORF's are able to interact with the translating ribosome, or other components of the translation machinery such as the eukaryotic release factor 1 (Harrod, R. and Lovett, P. S. 1995, Janzen, D. M. et al. 2002, Law, G. L. et al. 2001). Upon translation and/or release of the uORF encoded peptide an interaction with the translation machinery renders the complex either inactive or enhances translation of the main ORF. Interaction of the uORF peptide with the translating ribosome seems the most direct and rapid method of regulating translation but interaction with other components is also possible. The uORF2 peptide from the glucocorticoid receptor (GR) transcript 1A has been shown, by indirect fluorescent antibody staining, to be located both in the interior of the cell and at the plasma membrane (Diba, F. et al. 2001). The localization of the uORF2 peptide illustrates that interaction with other components is possible thus increasing the regulatory possibilities.
The conserved amino acid sequence of uORF peptides is not always essential for the translational regulation. It has been shown that the context of the startcodon of the uORF is important for translational regulation of the p27 gene, which contains an conserved uORF of unknown function (Gopfert, U. et al. 2003). There are several examples known in which the peptide sequences encoded by the uORF are of major importance (Fang, P. et al. 2000, Mize, G.J. et al. 1998, Reynolds, K. et al. 1996). Translational control by these peptides is influenced by

the amino acids composition. Modifying the amino acid sequences of these peptides changes the translational control properties of the uORF peptide.
Another mechanism, involving uORF's, is the stalling of ribosomes independently of the amino acid sequence of the uORF's (Meijer, H. A. and Thomas, A. A. 2003). Multiple uORF's, strongly affect the translation rate of the main ORF. According to the ribosome scanning model (Kozak, M. 1989) few ribosomes get past multiple uORF's to translate the main ORF. Point-mutating the startcodon of the uORF1, of the connexin41 gene from *Xenopus laevis* which contains a total of three uORF, results in a hundred fold increase in translation rate (Meijer, H. A. et al. 2000). These results are similar for the GCN4 gene of *Saccharomyces cerevisiae* which contains 4 uORF's (Hinnebusch, A.G. 1993).

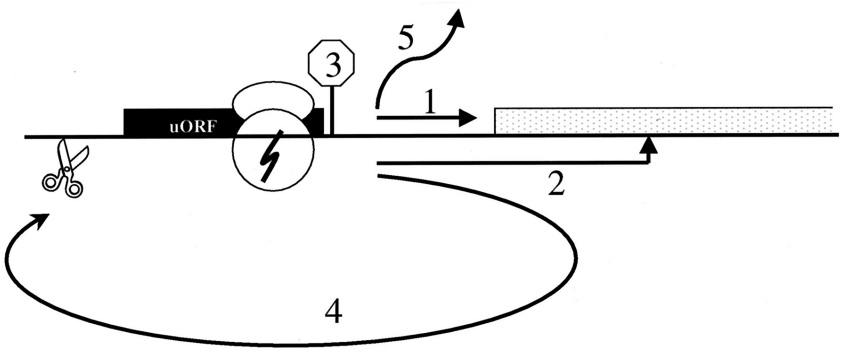


Figure 2: The different possible fates of a ribosome after translating a uORF. 1) the ribosome stays associated with the mRNA, 2) the ribosome is shunted, 3) the ribosome is stalled, inhibiting further translation by other ribosomes, 4) uORF translation meditated mRNA decay, 5) dissociation of the ribosome. (David R.Morris and Adam P.Geballe 2000).

Although uORF's regulate translation, through mechanisms that have not been fully elucidated yet, additional mechanisms also exist. For instance, translational control can be exerted via internal ribosome entry sites (IRES) located in the 5'UTR. IRES mediated translation is cap-independent and the ribosome lands close to the initiator AUG and may be dependent on secondary and tertiary structure and transacting factors (Le, S. Y. and Maizel, J. V., Jr. 1997, Pilipenko, E. V. et al. 2000). Secondary RNA structure is also important for the binding of iron regulatory proteins to the 5'-UTR of ferritin, eALAS, aconitase and SDHb mRNAs (Mikulits, W. et al. 1999). A small 5'UTR hairpin is bound by iron regulatory proteins (IRP1 and IRP2) when iron levels are high, preventing translation. Combinations of these regulatory elements are found within the family of viruses. A hairpin preceded by a uORF shunts the ribosome to the main ORF whereby the hairpin-stem is important

but not its sequence (Hemmings-Mieszczak, M. and Hohn, T. 1999, Pooggin, M. M. et al. 1999). Another combination of translatory elements is that of an IRES and uORF. The CAT-1 gene encodes an arginine/lysine transporter in which translation is controlled via an IRES element within the leader sequence (Yaman, I. et al. 2003). A small uORF is located within the IRES element and both translation of the uORF and phosphorylation of the translation initiation factor eIF2alpha are required for regulation. Translation of the uORF unfolds the secondary structure of an inhibitory structure present in the leader sequence resulting in an active IRES. The RBM3 mRNA also contains an IRES sequence which has been shown to be highly modular with at least nine discrete cis-acting-sequences (Chappell, S. A. and Mauro, V. P. 2003). One of these cis-acting elements is contained within a uORF and its activity is masked by translation of this uORF. Association studies have revealed the possibility of specific binding to cytoplasmic proteins by some of the cis-acting elements. Such studies have revealed that all four cis-acting sequences could bind specifically to distinct cytoplasmic proteins.

Outline of this thesis

Elaborate regulatory systems have evolved to guide sugar-controlled processes in the plants. The *ATB2* gene encodes a basic leucine zipper domain (bZIP) transcription factor. It is know that the 5'UTR of the *ATB2* gene is important for regulation by sucrose because deletion of the 5'UTR abolishes this regulation. In Chapter 2, it was investigated whether the 5'UTR is necessary and sufficient for sucrose-mediated repression. Cloning the 5'UTR out of context with a constitutive promoter resulted in a sucrose regulated reporter gene.

To identify mutants in the sucrose regulatory system, a non-destructive mutant screen was developed. The screen is based upon isolating mutants with high expression by measuring luminescence levels. A dual reporter system, comprising the *GUS* and *LUC* genes, was used for this screen.

In chapter 3, the previously developed mutant screen was used to identify mutants in an EMS mutagenized seeds population. Regulatory mutants will be able to provide greater understanding of the underlying sucrose regulatory mechanism. Putative regulatory mutants were isolated and analyzed in detail. The activity levels of both reporter genes were determined and the RNA levels were measured to exclude promoter-up mutants. Interestingly, no mutants were found that were disturbed in the sucrose regulation of both reporter genes. Possibly, mutants in the sucrose regulatory pathway are lethal and can therefore not be isolated.

The leader sequence of the *ATB2* gene is unusually long and contains several open reading frames (uORF). The longest reading frame encodes a peptide that is conserved among almost all plant species. To

determine its role in the proposed sucrose regulation, deletion and point mutation constructs of the leader sequence were made. The results indicate that the uORF is an important component of the sucrose regulatory pathway. In chapter 4, the sucrose regulatory mechanism of conserved uORF is discussed in relation to other genes that also contain the conserved uORF.

Chapter 2

Optimalization of a non-destructive luminescence assay for monitoring repression of the *Arabidopsis thaliana ATB2* bZIP transcription factor gene by sucrose

with Mies Borrias, Johannes Hanson, Anika Wiese, Peter Weisbeek and Sjef Smeekens

Abstract

Transcription of the Arabidopsis thaliana bZIP transcription factor *ATB2* is induced by light and sugars. Elevated concentrations of sucrose repress gene expression though a posttranscriptional mechanism. Thus, sucrose induces steady state mRNA levels and represses translation. It has been shown that deletion of the *5'UTR* of the ATB2 gene abolishes the translational repression by sucrose. To investigate if the leader sequence is sufficient to confer sucrose repression it was assayed alone in a reporter gene assay. These experiments confirmed the necessity and sufficiency of the leader sequence for sucrose repression. To gain further insight into the regulatory mechanism of sucrose regulation a non-destructive assay has been developed. This non-destructive assay utilizes a dual reporter construct (*GUS* and *LUC* genes) and enables the identification of regulatory mutants. Several lines, expressing the dual reporter construct, have been isolated and tested for their response to physiological concentrations of sucrose, a prerequisite for future use in mutagenesis experiments.

Introduction

Sugars are well known as metabolic intermediates and as respiratory substrates. Moreover, sugars serve as structural and storage components (Wobus, U. and Weber, H. 1999). In addition, sugars, most interestingly, also have a signaling function (Sheen, J. et al. 1999). The effect of sugars as signaling molecules lies in the millimollar range as opposed to the micromolar range in which the classical plant hormones are functional. To exert their effect as signaling molecules sugars must be sensed. At least three pathways have been proposed for sugar signaling in plants (Rook, F. et al. 1998, Xiao, W. et al. 2000). Hexokinase dependent- and independent sensing pathways can sense hexoses while a third pathway senses sucrose. Sucrose is readily degraded by invertases and sucrose-synthases, which makes it difficult to study sucrose-specific effects. However, sucrose specific effects can be observed for the *BtSUC* and the *ATB2* genes. The sucrose transporter from *Beta vulgaris* is regulated at the transcriptional level by a sucrose specific pathway (Chiou, T. J. and Bush, D. R. 1998) while the *ATB2* gene, which encodes a basic domain leucine zipper transcription factor from *Arabidopsis thaliana*, is transcriptionally regulated by light and translationally by sucrose (Rook, F. et al. 1998). Light induces transcription of the *ATB2* gene while sucrose concentrations exceeding 25 mM repress translation.

The *ATB2* gene was originally identified in a screen for light regulated transcription factors. The gene contains no introns and is induced by light through the DET/COP system (Rook, F. et al. 1998).The ATB2 protein consists of a highly conserved basic domain region and a helical leucine zipper domain containing a periodic repetition of leucine residues at every seventh position. The protein consists of only 160 amino acids (18 kDa). Analysis of the mRNA sequence reveals an unusually long leader sequence of 383 nt. An important element essential for sucrose mediated repression is located in the 5'UTR since deletion of the 5'UTR abolishes sucrose mediated translational repression. Detailed analysis reveals multiple uORF's present in the leader sequence of which the largest is remarkably conserved. The conservation encompasses homologs from mono- and dicotyledonous plant species, which are all bZIP-type transcription factors.

We are interested in the sucrose specific regulatory system and are taking a genetic approach to identify the mechanism involved. The role of the leader sequence, independently from other endogenous regulatory sequences, in sucrose specific regulation was investigated. In order to isolate mutants in the sucrose repression pathway it is essential to monitor expression non-destructively. Therefore, the previously used *GUS* construct (Rook, F. et al. 1998) was modified A double reporter construct was created with the *GUS* and *LUC* genes (Jefferson, R. A. et al. 1987, Luehrsen KR et al. 1992). The double reporter construct enables non-destructive detection of aberrant expression next to real-time expression analysis. The ability to detect

aberrant expression levels can be used to screen for mutants. The double reporter gene-construct was transformed to Arabidopsis plants and several lines were isolated which expressed both the *GUS* and *LUC* transgenes. The optimal line, which displays a wt-like regulation of the reporter genes, was selected.

Results

A dual reporter system was created by using the endogenous ATB2 sequences whereby only the coding region of the ATB2 gene was replaced by the coding region from a reporter gene (figure 1). This was done for both of the used reporter genes thus creating two independently regulated reporter genes. Using this cloning strategy all of the sequences with potential regulatory elements outside the coding sequence are conserved. Two reporter genes were used to prevent isolation of promoter-up mutants. Mutants with aberrant luminescence levels will be identified with the optimized screen (see below). However, only true mutants will also display aberrant GUS expression. True mutants will thus display aberrant expression for both reporter genes.

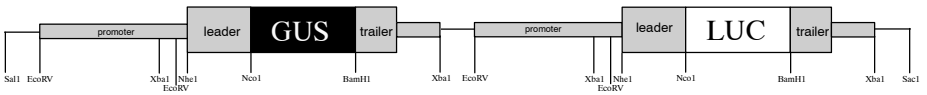


Fig 1: Schematic representation of the double reporter construct in which the *GUS* and *LUC* genes are driven separately by *ATB2* regulatory sequences. The grey boxes represent endogenous *ATB2* sequences. The black box represents the â-glucuronidase coding sequence and the white box represents the Luciferase coding sequence.

The dual reporter system was transformed by root-transformation of Arabidopsis thaliana ecotype C24 (Valvekens, D. et al. 1988). Kanamycin selection enabled us to isolate transgenic regenerants, which were propagated. Several individual lines were tested for expression of the *LUC* and *GUS* reporter genes. Six individual homozygous lines, expressing sufficient levels of *LUC* and *GUS*, were assayed for their response to sucrose (data not shown). Genomic DNA was isolated from these lines and the copy-number was determined with Southern blotting. Line 5-4 had only one T-DNA copy and showed an average luminescence level compared to the other tested lines (data not shown). Moreover, the response to sucrose was as reported previously (Rook, F. et al. 1998). Repression of *GUS* translation is observed when seedlings containing the dual reporter construct are grown in 100 mM sucrose in liquid medium (figure 2).
To determine if the LUC reporter gene of this line also displays sufficient repression by sucrose, the growth conditions and luminescence measurement conditions were optimized. Different sowing options

were tested, e.g. seeds imbedded in agar, seeds imbedded in agar with liquid medium on top, seeds sown on top of solid agar. All of the tested sowing methods were tested with different concentrations of sucrose (data not shown).
The luminescence measurements did not differ greatly between the different sowing methods. Seeds sown on the medium, without liquid medium on top, was chosen. This sowing method is the least susceptible to infections from microorganisms and provides the best conditions for luminescence measurements. Luminescence levels were measured with a light sensitive camera. Line 5-4 displays repression when grown with 4% sucrose and was chosen for future experiments (figure 3). In addition, GUS activity was determined to confer repression of the *GUS* gene when line GL 5-4 is grown on solid medium.

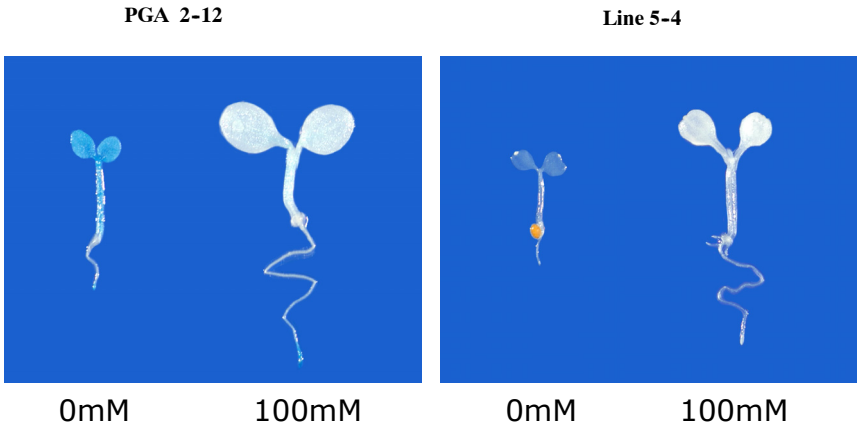


Figure 2: Gus stained seedlings grown in different sucrose concentrations. The *ATB2* transgenic plants harboring different constructs were tested for the response of the reporter gene to sucrose: line PGA 2-12 (4A) (Rook, F. et al. 1998) and line GL 5-4 (4B). Line GL 5-4 contains a double reporter construct. Seedlings were grown for 5 days in constant light with or without sucrose before being harvested and stained.

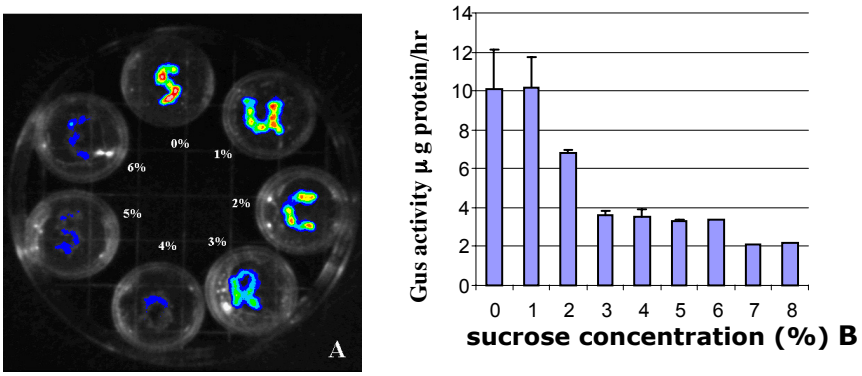


Figure 3: ATB2 repression by sucrose on solid medium. Line GL5-4 (*Arabidopsis thaliana* ecotype C24), containing the dual reporter system, was grown on plates containing 0,5 MS medium with increasing concentrations of sucrose (0-6%). Luciferase (figure 3A) and Glucuronidase activity (figure 3B) were determined by luminescence and enzymatic assays, respectively.

The optimized method for detecting luciferase activity can be used to detect aberrant luminescence levels. Mutants for line GL 5-4 i.e., a mutant emitting much more luminescence than the general population of plants, must be isolated from a dense population of plants, and therefore needs to be unambiguously located on the plate. To facilitate easy detection and isolation the optimal seed-density was determined for the wt GL 5-4 line (figure 4). Approximately 16 seeds per square (500 seeds per plate) was chosen as a sufficient density for easy identification, isolation of potential mutants. Also, the chosen density will facilitate equable application of the substrate, luciferine, which is needed for luminescence detection.

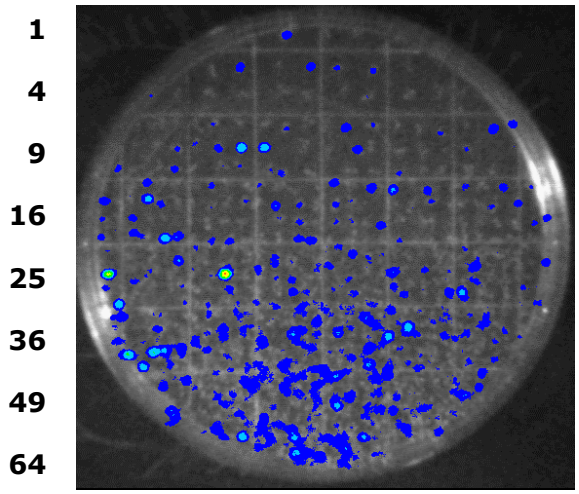


Figure 4: Luminescence levels of seedlings sown in a low to high-density order. The top of the plate contains few seedling and the number of seedlings increases towards the bottom of the plate. The numbers indicated left of the plate indicate the number of seeds present in a square. Seeds were grown on solid medium with 4% sucrose for 5 days under constant light before luminescence levels were determined.

Previously it has been shown that high sucrose concentrations repress translation but induce steady state mRNA levels (Rook, F. et al. 1998). To confer wt regulation of the dual reporter construct, RNA levels were measured in response to sucrose (figure 5). The RNA levels of the *LUC* reporter gene resembles wt. Also, an increase in the overal RNA levels from the *GUS* reporter gene is observed. However, the difference in RNA levels between 0 mM and 100 mM sucrose is lower for the *GUS* reporter gene then the wt.

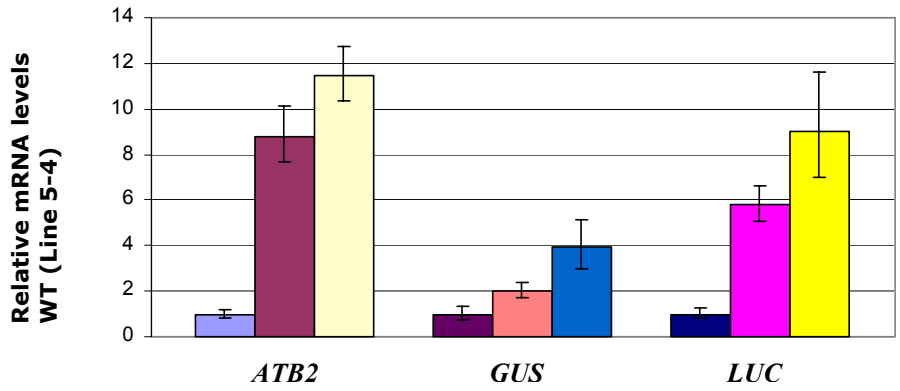


Figure 5: Relative expression levels of the *ATB2*, *GUS* and *LUC* genes. Seeds from wt (line 5-4) were grown in liquid medium containing 0,5 MS and 0, 20 or 100 mM sucrose. After 5 days of growth under constant light, the seedlings were harvested. RNA was isolated and used for quantitive analysis by qPCR. Left bar, middle bar, right bar: 0,20 100 mM sucrose.

The 5'UTR is sufficient for repressing translation in a sucrose dependent manner

Previously it has been shown that the 5'UTR is necessary for sucrose specific regulation (Rook, F. et al. 1998). This was illustrated by the fact that deletion of the 5'UTR abolishes sucrose specific regulation. We here tested the requirement of other endogenous regulatory sequences from the *ATB2* gene. Two constructs were made whereby only the 5 'UTR sequence is used from the *ATB2* gene. The 5'UTR was cloned between the *35S* or ubiqiutine promoter, *GUS/GFP* coding sequence and terminator sequence from the *NOS* gene (figure 6). Transgenic lines were generated by floral dip (Clough, S. J. and Bent, A. F. 1998) and homozygous independent lines were selected for further analysis. These constructs were used to investigate whether the 5'UTR is sufficient for sucrose specific regulation. The construct was transformed to *Arabidopsis thaliana* and Gus staining revealed that the expression of the *GUS/GFP* transgene is observed in the whole seedling. When grown in repressing concentrations of sucrose, significant lower GUS activity was detected. However, repression of the transgene is only observed in the shoot (figure 7).

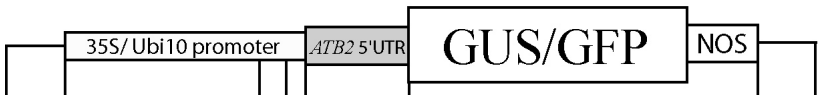


Figure 6: Schematic representation of the construct used to investigate the sufficiency of the 5'UTR to confer sucrose repression. The grey bar represents endogenous *ATB2* sequences. The *ATB2* 5'UTR-GUS/GFP sequence was cloned between the CaMV35S or ubiquitine10 promoter (35S/ubi10) and nopaline synthase terminator (NOS).

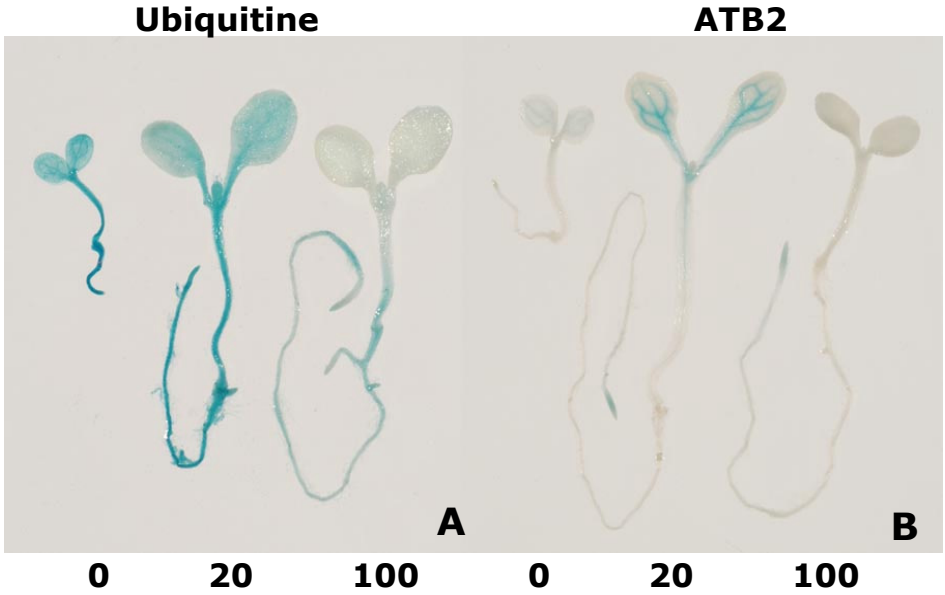


Figure 7: Histochemical staining of seedlings transformed with a construct where the *ATB2* leader sequence was separated from endogenous *ATB2* sequences. The *ATB2* leader sequence, driven driven by the ubiquitine10 promoter (Sun, C. W. and Callis, J. 1997) (7A), is used to confer repressability of the *GUS/GFP* reporter gene, line ubi 6-3 was used. 7B: control line 7-1 which contains PGA construct (Rook, F. et al. 1998). Seedlings were grown 5 days in liquid medium with different concentrations of sucrose, the seedlings were stained for activity. From left to right 0, 20 and 100 mM sucrose.

Discussion

In order to investigate whether the *ATB2* leader sequence by itself is sufficient for repressing translation, the *ATB2* leader sequence was cloned in front of a chimaeric *GUS/GFP* coding sequence and transcription was driven by either the 35S or ubiquitine10 promoter. The ubiquitine promoter and 35S promoter confer expression throughout the whole seedling. When grown under repressing conditions, repression is only observed in the shoot tissues. *ATB2* expression is induced by light (Rook, F. et al. 1998) and possibly additional factors needed for sucrose repression share a similar expression pattern. A light-induced phosphorylated novel plant protein that is subsequently released from thylakoid membranes is an example of such a light dependent regulatory system (Carlberg, I. et al. 2003). However, the expression pattern of the constitutive promoter confers expression throughout the whole seedling. When grown with repressing concentrations of sucrose, repression is observed in the shoot tissue. This excludes the possibility of addition factors having an identical expression pattern. Also, it clearly shows that the repression mechanism is operational all over the shoot. The additional factors that are expressed in tissues, which normally do not express the ATB2 gene, could constitute part of a general regulatory mechanism. To identify additional factors in this regulatory system, a mutant screen was devised. Non-destructive analysis of ATB2 expression enables the detection of aberrant expression levels, which can be used for the isolation of mutants. (Chinnusamy, V. et al. 2002, Greer, L. F., III and Szalay, A. A. 2002). A high throughput method for determining luciferase expression was developed by measuring luminescence levels. Several different assay conditions were tested and it was found that a concentration of 4 % sucrose (117 mM) strongly represses LUC and GUS activity. This concentration is optimal for simultaneously repressing both reporter genes. Each reporter construct is driven separately by identical regulatory sequences and the two-reporter gene system was developed so that false positive mutants, e.g. promoter-up mutants, can be identified and discarded. Line GL5-4 was chosen for further analysis because it displays simultaneous repression of both reporter genes in response to high concentrations of sucrose. High concentrations of sucrose induce *ATB2* steady state mRNA levels (Rook, F. et al. 1998). Analyzing the steady state mRNA levels of the reporter genes from line GL 5-4 shows a similar response to elevated sucrose levels. Although the overall increase in expression of the *GUS* gene is lower then wt it can be concluded that both reporter genes are regulated like wt.

Future analysis of *ATB2* regulation is greatly aided by the construction of the double reporter construct and the knowledge that the leader sequence is sufficient for gene specific sucrose regulation. Future experiments will focus on identifying mutants with the non-destructive assay method. An EMS mutegenized seed collection is ideal for identifying mutants with the recently developed high throughput luminescence based mutant screen. Non-destructive screening should facilitate the isolation of mutants that are disturbed in the response to high levels of sucrose. These mutants can provide insight into the sucrose specific regulatory system and help identify additional components or regulatory mechanisms.

Material and Methods

Assembly of the dual reporter system The firefly-luciferase gene was cloned as a Nco1-BamH1 fragment from pRO17 (provided by Rob Oosterling) into the PGA vector (Rook, F. et al. 1998), whereby the GUS coding sequence was replaced by the luciferase coding sequence creating the vector PGA-*LUC*. PGA was digested with SacI and the overhang was made blunt with T4-DNA polymerase (MBI Fermentas). The PGA-*LUC* vector was digested with SalI and made blunt with T4-DNA polymerase. The blunted constructs were ligated with T4-DNA ligase. The fragment containing the *GUS* and *LUC* genes was cloned into pBIN19 the using Sal1- Sac1 sites.

Construction of a construct to test sufficiency of the 5'UTR for sucrose regulation The GUS coding sequence from the pbPGA vector ((Rook, F. et al. 1998)) was exchanged by the GUS-GFP coding sequence from pCambia 1304 . pbPGA was digested with BamH1, made blunt with T4-polymase (promega, Leiden, the Netherlands) and subsequently digested with NcoI. pGreen 1304 (Hellens, R. P. et al. 2000) was digested with BstEII and made blunt with T4-polymerase and digested with NcoI. The GUS-GFP fragment was ligated into the digested pbPGA vector creating pbPGGAThe vector pGreen 1304 (Hellens, R. P. et al. 2000), which contains the 35S promoter was digested with XhoI and the ends were filled in with T4-DNA polymerase. Subsequently, the promotor was cut out with EcoR1 and ligated into an EcoR1- SmaI digested binary vector pGreen 229 creating the vector pGreen 229-35S. The PGA vector in which the Gus gene has been replaced by the GFP-GUS fusion-gene (PGA-GG) was digested with Xba1 and the fragment containing the leader-GFP-Gus fragment was cloned XbaI into the pGreen 229-35S construct

The PGA-GG vector was digested with XbaI and ligated into an XbaI digested binary vector pGreen 229. The Callis vector (Sun, C. W. and Callis, J. 1997) containing the ubiquitine-10 promoter was digested HindIII-BamH1 and cloned HindIII-BamH1 into pGreen 229 vector containing the GFP-Gus fusion gene.

Luminescence assay Seeds from transgenic Arabidopsis plants were sterilized by the chlorine gas method for 4 hrs. The seeds were transferred to a downflow cabinet and allowed to air dry for 30 min. For easy dispersal, 0.1% of agar was added and the seeds were allowed to hydrate for 5-10 minutes. The seeds were

dispersed on 0,5 MS medium containing 0.7% plant agar and varying concentrations of sucrose. After drying, the plates were sealed with parafilm and aluminum foil and incubated at 4ºC for 2 days. Next, the plates were transferred to the growth chamber were the aluminum foil and parafilm were removed. The plates were incubated 4 days with constant light at 22ºC after which the plates were transferred to the downflow cabinet and sprayed with a 5 mM filter sterile luciferine solution (5 mM luciferine, X mM Tris-HCl pH 8.0, 0.01% Trition X-100). After spraying the plates were placed back in the growth chamber, under constant light, for one day. The plates were sprayed with a 1 mM filter sterile luciferine solution and incubated 1 min at room temperature and luminescence was immediately determined for 10 minutes. Luminescence was measured with a Hamamatsu Argus 20 image processor and c2400-47 VIM camera.

Glucuronidase assay Glucuronidase activity was determined with the GUS-light kit from Tropix, (Bedford, MA). Three times ten seedlings were homogenized by crushing the seedlings in isolation-buffer as provided by the manufacturer. The cell-extract was centrifuged 2 minutes at 12000 rpm and the supernatant was transferred to a fresh eppendorf tube.The protein extract was incubated with substrate as described by the manufacturer. Luminescence was determined in a BioOrbit 1253 luminometer for 5 seconds in triplo. Protein content was measured using the Bradford method (BradFord, B. B. 1976).

Southern Blot Genomic DNA was isolated using the Dellaporte protocol (Dellaporte, S. L et al. 1983). The genomic DNA was subsequently digested with EcoRV. *GUS* and *LUC* probes (prepared by Rob Oosterling) were digested with Xba1-SacI and isolated from gel for probe generation. Random hexamer fragments (MBI fermentas) were used to generate a radioactive probe from the *GUS* and *LUC* fragments.

Quantative PCR RNA was isolated using the Purescript, RNA islation kit from Gentra Systems (Minneapolis) according to the manufactures instructions. RNA was made DNA-free with the DNA-free^{TM} kit from Ambion Ltd (Cambridgeshire). The DNA free RNA was checked for DNA by PCR with a primerpair for *ATB2* (forward AGACGATCTAACGGCTCAGGTT reverse TGCGTTGTGATGCTGACACTT) and *TUB4*, (forward TGGACAATGAGGCT CTCTACG, reverseCAGGGAAACGAAGACA GCAAG)cDNA synthesis was performed with M-MLV Reverse Transcriptase from Promega (Madison, USA), according to the manufactures instructions. The qPCR reactions were performed with a FAM/Tamra labeled probe obtained from Isogen (Maarssen, Holland). Taqman Universal Master Mix, No AmpErase UNG, (Applied Biosystems, Roche Molecular Systems) was used in the qPCR reaction. All primers and probes were designed using the Primer Express^{TM} v1.0 software of Applied Biosystems. Q-PCR results were analysed with SDS v1.7 software from Applied Biosystems. Results for *AtbZIP2* were calculated using equation $R=(E_{target})^{\Delta Ct_{target}\ (control\text{-}sample)}/\ (E_{ref})^{\Delta Ct_{ref}\ (control\text{-}sample)}$ as described by (Pfaffl, M. W. 2001). Relative quantification for all other primer-probe combinations was sufficiently similar to *Actin2* to use the $\Delta\Delta$Ct method (User bulletin #2, ABI Prism 7700 Sequence Detection System, ABI, 2001). Primer-probe efficiencies for all primer-probe sets were determined according to equation $E=10^{(-1/slope)}$ as described by (Rasmussen, R. 2001). See table 1 for sequences of primers and probes, which were used for the qPCR reaction.

Table 1: Sequences of primers and probes used in the quantative RT-PCR reaction to measure the relative RNA content of seedlings grown with different sucrose concentrations. Probes and primers for the *GUS*, *LUC*, *ATB2* and *ACT* genes are depicted.

primers	**probes**
GUS forward 5'AACCCCAACCCGTGAAATC 3'	*GUS* 5'ACTCGACGGCCTGTGGGCATTC 3'
GUS reverse 5'CACAGTTTTCGCGATCCAGAC 3'	
LUC forward 5'TCCATGGTCACCGACGC 3'	*LUC* 5'AAGAAAGGCCCGGCGCCATTCT 3'
LUC reverse 5'GGTTCCATCTTCCAGCGGA 3'	
ACT forward 5'GCTGAGAGATTCAGACTGCCCA3'	*ACT* 5'AAGTCTTGTTCCAGCCCTCGTTTGTGG3'
ACT Reverse 5' CACAGTTTTCGCGATCCAGAC 3'	
*ATB*2 forward 5'TCGTCAGGATCGGAGGAGAGT3'	*ATB2* 5'AACGTAAACAGGAGCTCTCAAACCGTGAA3'
ATB2 Reverse 5'GATCGTCTAGGAGCTTTTGTTTCTTC3'	

Chapter 3

Isolation of ATB2 regulatory mutants using a non-destructive assay

with Johannes Hanson and Sjef Smeekens

Abstract

The Arabidopsis *thaliana* bZIP transcription factor gene *ATB2* was identified in a screen for light regulated transcription factors. This gene is regulated by two independent regulatory systems. Transcriptionally by light and translationally by sucrose. Regulation by sucrose is specific. Other mono- and disaccharides don't exert the same effect. It has been shown that the sequence of the 5' leader is necessary and sufficient for sucrose-mediated regulation (chapter 1). Concentrations exceeding 25 mM sucrose repress translation. The leader sequence contains several open reading frames. The largest uORF is highly conserved and is 129 nt long. In order to gain further insight into the sucrose regulation system a screen for regulatory mutants was devised. A *ATB2 GUS/LUC* double reporter containing transgenic line was used in a EMS-screen. Putative mutants, that do not display the repression phenotype, have been identified and analyzed in detail. After extensive analysis, it was concluded that only false positive mutants were isolated. Possibly the sucrose regulatory system consists of few components. Sucrose sensing plays a central role, is important for the metabolism and mutants thereof are lethal and have therefore interestingly not been identified.

Introduction

Expression of the *ATB2* gene is controlled by light and sucrose. Light induces transcription and sucrose represses translation (Rook, F. et al. 1998). Transcription is repressed in darkness in a COP1 and DET1-dependent manner (Rook, F. et al. 1998). Repression by sucrose is specific and the leader sequence is necessary and sufficient for sucrose specific regulation. The *ATB2* is the first known gene being translationally repressed by sucrose. *ATB2* is prominently expressed in the vascular tissues of seedlings and young vegetative tissues. It is highly expressed in funiculi upon fertilization of ovules (Rook, F. et al. 1998). This expression pattern and the translational regulation by sucrose suggests that *ATB2* might coordinate metabolism-associated processes in newly established sinks (Rook, F. et al. 1998).
Detailed analysis of the leader sequence of *ATB2* showed the presence of several upstream open reading frames (uORF). The upstream ORF2 encodes a peptide of 42 amino acids. Interestingly this peptide is highly conserved in uORFs in some other bZIP 5'-untranslated regions in Arabidopsis as well as in other dicotyledonous and monocotyledonous plants. Upstream open reading frames occur in fewer than 10% of vertebrate mRNAs-at-large; a notable exception are oncogene transcripts, two-thirds contain uORFs (Kozak, M. 1987). Apparently, genes at key positions in regulatory pathways often have an extra level of regulation via uORFs. Although the precise regulatory mechanisms are not known, the list of genes regulated by uORF's is growing. Only a minority of the genes present in databases have annotations that identify the precise start of the transcript-leader. Erroneous annotations often predict transcripts that are in fact too short. Correcting these annotations will undoubtedly identified additional uORF regulated genes. Nevertheless, genes with uORF have already been identified and analyzed in more detail. For instance, the S-adenosylmethionine decarboxylase is a mammalian gene that contains a highly conserved uORF that confers polyamine-regulated suppression (Hill, J. R. and Morris, D. R. 1992). Using a primer extension inhibition assay and in vitro protein synthesis reactions it was shown that ribosome's seem to pause at the termination codon of the uORF (Law, G. L. et al. 2001). Furthermore, inhibition of translation correlated with the stability of the ribosome pause at the uORF, e.g. elevated polyamine levels increased the stability of the paused ribosome. Altered uORF sequences, which abolished regulation, greatly influenced the pause of the ribosomes. In fact, the forth and fifth residues of the uORF peptide are important because altering these aa results in elimination of the stabilization of ribosome's in response to elevated polyamine levels (Raney, A. et al. 2002). In yeast the *CPA1* gene, which encodes a subunit of the carbamoylphosphate synthetase (CSPaseA) complex, is under translational control of arginine and this control is mediated by a conserved uORF. When no, or low levels of arginine are present, ribosome's reach the *CPA1* ORF by scanning past

the uORF. High levels of arginine repress the synthesis of Cpa1. An 25 aa peptide, encoded by the uORF located in the 250 bp long leader is responsible and sufficient for regulation because inserting the mRNA sequence encoding the leader peptide in the leader sequence of another gene, places this gene under arginine repression (Delbecq, P. et al. 1994). The region between amino acids 6-21 is well conserved between similar peptides present upstream of *CPA1*-homologous genes in other fungi. The leader peptide appears to destabilize the 5' end of the mRNA because transcript levels go down when arginine is present while this is not the case when a non-functional uORF is present (Delbecq, P. et al. 2000). Several uORF's are also involved in the regulation of the GCN4 gene in yeast (Miller, P. F. and Hinnebusch, A. G. 1990). Translation of the first uORF enables reinitiation and the place of reinitiation is determined by amino acid limitation. Under repressing conditions, the level of eIF2 is high and the ribosome's initiate at the downstream uORF's while under derepression, the eIF2 levels are low and the ribosome's reinitiate at the main ORF. The phosphorylation status of the eIF2 determines where reinitiation occurs (Hinnebusch, A. G. 1993). Reinitiation at uORFs 2-4 results from a reduction in the GTP-bound form of eIF-2 that delivers charged initiator tRNA (iMet) to the ribosome while phosphorylation of eIF-2 by the protein kinase GCN2 decreases the concentration of eIF-2.GTP.Met-tRNA(iMet) complexes by inhibiting the guanine nucleotide exchange factor for eIF-2 so that reinitiation occurs at the main ORF.

It appears that different regulatory mechanisms exist involving uORF's. The ATB2 gene is regulated by a mechanism mediated by a conserved uORF. This mechanism differs from the other described systems because translation can be inhibited while the mRNA level doesn't change dramatically. Both the AdoMetC and CPA1 genes are regulated via repression, which causes the mRNA of CPA1 to become destabilized and degraded. The GCN4 is regulated via the phosphorylation status of eIF2, which determines if the uORF or ORF is going to be translated. It appears that several mechanisms exist for regulation via uORF's (Gaba, A. et al. 2001) and is not clear if there are common factors present for these different regulatory systems. A well excepted method for the identification of mutants is the use of an EMS based screen (Redei, G. P. and Koncz, C. 1992). Several EMS screens, in which the LUC gene is used as a reporter gene, have been successful in identification of regulatory mutants(Chinnusamy, V. et al. 2002, Eckardt, N. A. 2001, Meier, C. et al. 2001). The luciferase gene allows non-destructive high throughput analysis of mutegenized seedlings. In this paper, we show the application of a previously optimized screen for the detection of aberrant expression of sucrose-induced repression (chapter1). Mutants have been identified and have been characterized in greater detail.

Results

Approximately 50.000 seeds from line GL5-4 (chapter1) were treated with EMS. The M1 seeds were divided into 42 batches and propagated. The M1 population contained 7% chlorophyll deficient seedlings whereas the M2 population contained 2% chlorophyll deficient seedlings, which falls in the expected range (Redei, G. P. and Koncz, C. 1992). The M2 population was sown on medium containing 4% sucrose and incubated under continues light for 4 days after which the seedlings were sprayed with 5 mM Luciferine. The next day, the seedlings were sprayed with 1 mM of luciferine and after a 1-minute incubation period at room temperature, the seedlings were measured 10 minutes to determine luminescence levels. 82 primary mutants (0,2%) were selected from a screen of 40000 M2 seeds (figure 1). The primary mutants were propagated and picked for a quantative Gus-analysis after conformation of an enhanced expression profile. Approximately 50 offspring seeds per EMS-line were re-analyzed for enhanced expression (figure 2).

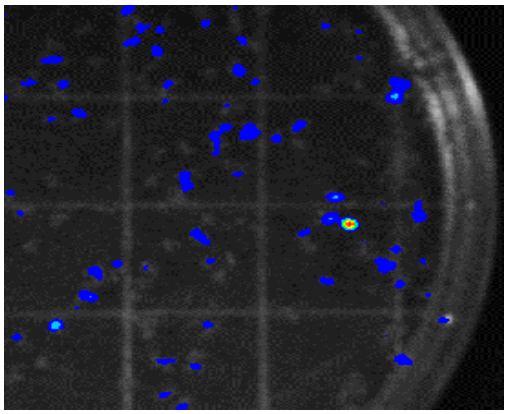


Figure 1: CCD image of an EMS-mutant displaying enhanced luminescence levels. EMS treated seeds were sterilized and sown on solid MS-medium containing 4% sucrose. After incubation of 5 days in the growth chamber, the seeds were assayed for luciferase expression by measuring luminescence.

An observation made during the EMS-screen is that sometimes completely white seedlings with very high luminescence levels observed. These seedlings were isolated and grown on medium containing 20 mM sucrose. All of these isolated seedlings were not viable although one plant flowered but did not set seed.

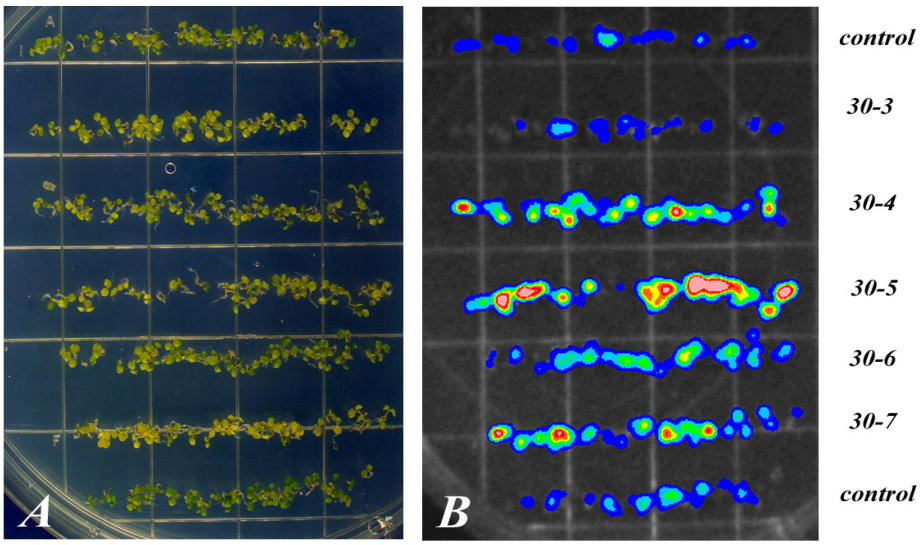


Figure 2A: Picture displaying offspring seeds of primary EMS-mutants that were sown on solid medium containing 0,5 MS-salts and 4% sucrose. Approximately 50 seeds per line were incubated 5 days in the growth chamber under constant light. Figure 2B: CCD image of propagated primary EMS-mutants. Luminescence levels were measured, mutant lines display high luminescence levels.

A repression assay, using GUS as a reporter gene, was performed to confirm the response of the luciferase gene from the EMS-mutants. Seeds were incubated in liquid medium containing 0, 20 or 100 mM sucrose under continues light. After 5 days, seedlings were harvested and GUS activity was determined quantatively (figure 3). The GUS activity levels from the EMS mutants responded like wild type except for line 30-5. This line has a reduced response to 20 mM sucrose but the levels from 100 mM sucrose resembled the wild type levels. Interestingly, the GUS activity levels results did not reflect the LUC levels.

To gain further insight into the differential response of the reporter genes the sucrose mediated repression of the luciferase gene was determined quantatively. Seeds were grown on solid medium, containing increasing concentrations of sucrose, and incubated 5 days under continues light. The seedlings were harvested and luciferase activity was quantatively determined (figure 4). In the absence of sucrose, luminescence levels are low in comparison to luciferase levels when grown on 100 mM sucrose. The luminescence levels of plants grown with 20 mM sucrose were significantly lower when compared to luminescence levels of plants grown in 100 mM sucrose. Luciferase

repression in the wt is observed when seedlings grown with 20 mM sucrose are compared to seedlings grown with 100 mM sucrose. Two mutant lines, 24-3 and 33-4, display an altered LUC response to 20 mM sucrose when compared to the wt response of the same concentration. The LUC activity levels at 0 mM and 100 mM sucrose reflect the wt levels.

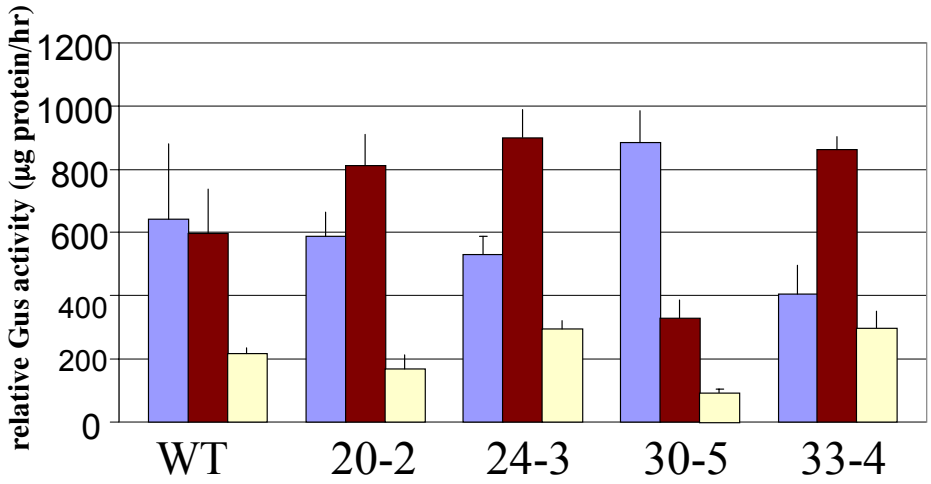


Figure 3: Relative GUS activity levels from seedlings grown with different sucrose concentrations. Propagated seeds from EMS-lines were analyzed for repression using GUS as a reporter. Seeds were grown for 5 days in different concentrations of sucrose in constant light (left bar, middle bar, right bar: 0,20, 100 mM sucrose). WT is represented by line 5-4.

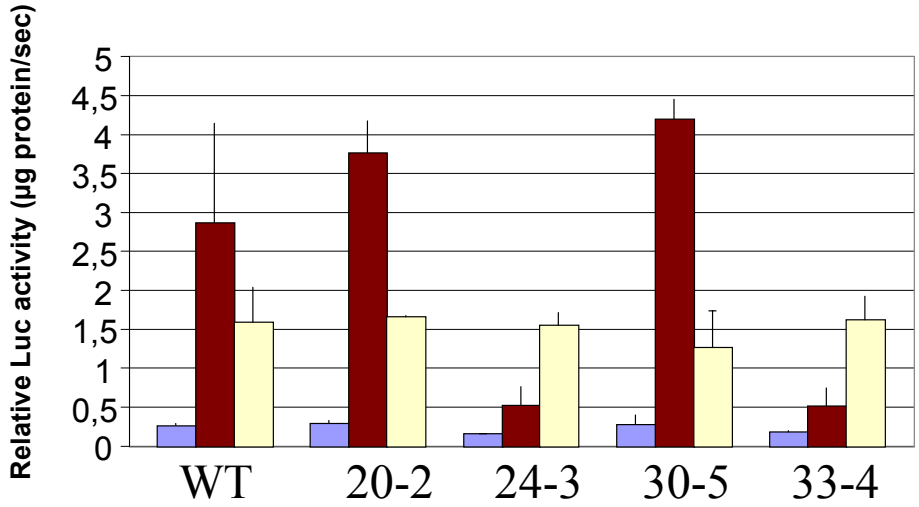


Figure 4: Relative LUC activity levels from seedlings grown on different concentration of sucrose. Seeds from EMS-mutants were tested for their response to different concentrations of sucrose, using the *LUC* gene (left bar, middle bar, right bar: 0,20 100 mM sucrose). Line 5-4 represents WT.

Promoter-up mutants contain mutations in the promoter sequence that causes aberrant RNA levels and thus aberrant expression levels. The possibility of the isolated mutants being promoter-up mutants was excluded by determining RNA levels. The RNA levels of the *ATB2* gene and reporter genes were investigated by quantative PCR. Investigation of the RNA levels for the *ATB2* and *LUC* genes revealed no deviations from wt levels (figure 5). This excludes the possibility that the promoter of the *LUC* transgene has been affected by the mutagenesis. However, the *GUS* mRNA levels are elevated (line 30-5) or not elevated (line 24-3) to increasing sucrose concentrations in the mutant lines.

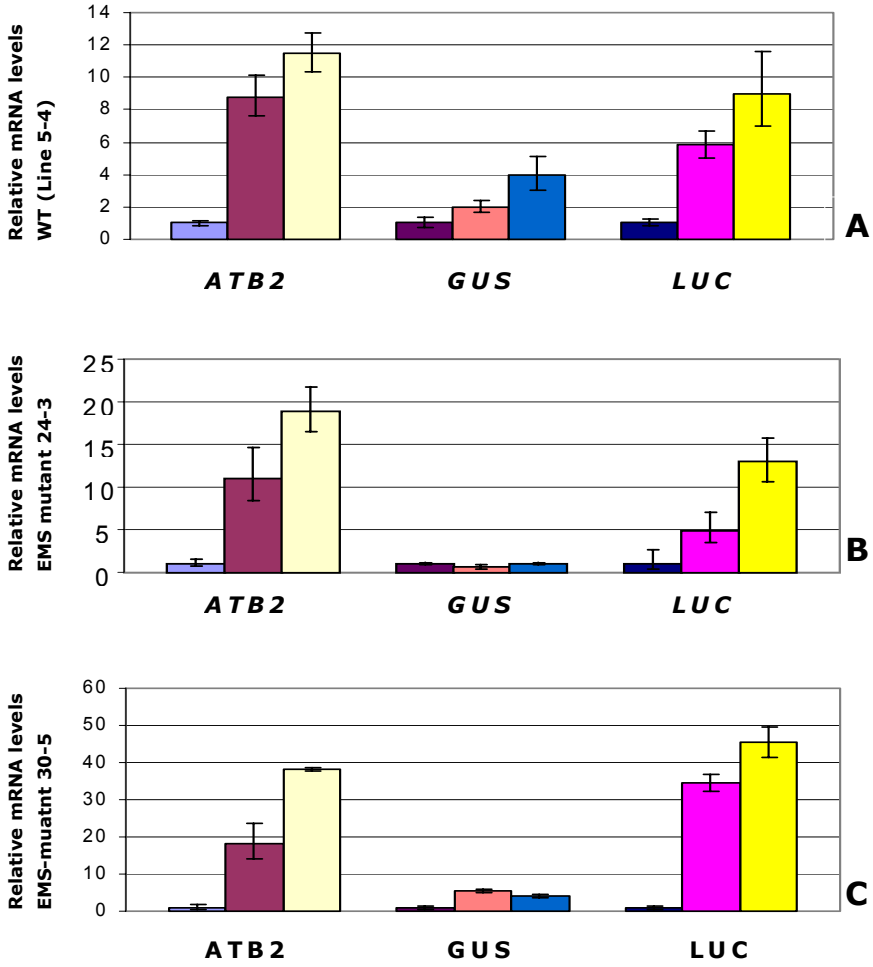


Figure 5: Relative expression levels of the ATB2, GUS and LUC genes from EMS-mutants and wt lines. Seeds were grown in liquid medium containing 0,5 MS and 0, 20 or 100mM sucrose. After 5 days of growth under constant light, the seedlings were harvested. RNA was isolated and used for quantitive analysis by qPCR (left bar, middle bar, right bar: 0,20 100 mM sucrose). Panel A depicts RNA levels from wt (line 5-4), panel B depict RNA levels from EMS mutant 24-3 and panel C depicts RNA levels from EMS line 30-5.

Discussion

Our search for mutants in the sucrose regulatory pathway has, interestingly, only resulted in the isolation of mutants that respond differently to non-repressing sucrose concentrations. Two different reporter genes were used in the EMS screen that was performed to isolate mutants. RNA analysis was used to eliminate promoter-up mutations. The combined results clearly show that the isolated mutants are able to repress translation in a sucrose-dependent manner. We suggest that mutants that lack a functional sucrose repression mechanism are most likely lethal and will thus not survive the EMS-screen. Sugar regulatory pathways have been shown often to be intertwined with other regulatory pathways like development, photosynthesis and hormonal regulation (Laby, R. J. et al. 2000, Lejay, L. et al. 2003, Rylott, E. L. et al. 2003). The *ATB2* gene is involved in the sucrose regulatory pathway and correct expression is required for viability. The combination of incorrect expression of the *ATB2* gene together with other deregulated pathways may cause lethality. An observation that supports this finding is that during the mutant screen, several white seedlings with very high luminescence levels were isolated. These white seedlings did not survive. One white plant flowered when grown on sucrose but no seeds were set. The wt *ATB2* gene is highly expressed in the funiculi of developing seeds (Rook, F. et al. 1998). The high expression levels imply a very important function during the seed filling stage. Aberrant expression of the *ATB2* gene during this stage might explain the inability of the putative mutants to set seed. This assumption is confirmed by the observation that constitutive overexpression of *ATB2* by the *CaMV35S*-promoter, without the translational control of the 5'UTR, causes severe growth phenotypes including lethality and sterility (Wobbes et al, submitted).

The 5'UTR is a target of the sucrose sensing pathway and presumably, the peptide encoded by the conserved uORF, is involved in the regulation. uORF's are known to affect translation and several examples exist in which the regulation is sequence dependent (Degnin, C. R. et al. 1993, Mize, G. J. et al. 1998, Wang, Z. and Sachs, M. S. 1997). The uORF encoded peptide could inhibit or allow translation directly or indirectly by sucrose as has been shown for the conserved "arginine attenuator peptide" (AAP) and the conserved uORF located in the leader of the S-adenosylmethionine decarboxylase gene (Delbecq, P. et al. 2000, Raney, A. et al. 2002). Sucrose may directly be sensed by the uORF-encoded peptide. In this case, the conserved uORF could have easily been missed by a saturated EMS-mutagenesis, since it is encoded by only 129 nucleotides. A small regulatory chain decreases the chance of finding mutants.

The combination of a short regulatory chain and high conservation has influenced the possibility of isolating mutants impaired in sucrose sensing negatively. The high conservation point towards a conserved

regulatory system and mutating this system possibly renders it inactive, therefore mutants in this system are lethal. The inability to isolate mutants in the sucrose regulatory pathway illustrates the fact that the sucrose regulatory chain is vital for the viability of the plant. A strategy for isolating mutants in this pathway should possibly not rely on the isolation of homozygous lines. Mutants that are not lethal might be isolated if activation tagging or 2-hybrid screen is used. These screens do not rely on mutations and therefore do not impair the regulatory system by mutating conserved sequences. Research presented here provides a promising start to further investigate this regulatory system. Analyzing mutated versions of the conserved uORF should provide greater insight into the sucrose regulatory system.

Material and methods

EMS-mutagenesis Approximately 50,000 seeds of double reporter containing line GL5-4 were allowed to imbibe for 5 days at 4ºC. The seeds were dried and subsequently transferred to a 10 ml MilliQ solution supplemented with 12,5 μl ethylmethane sulfonate (EMS, Sigma, Germany). The seeds were stored for 24 hrs at 22ºC in the dark. The EMS treated seeds (M1, for Mutegenized) were carefully washed with MiliQ water and the seeds were divided into 47 batches and sown on soil. The seeds were transferred to white light (16 h light/8 h dark) for EMS M2 seeds production. EMS M2 seed from each individual batch was harvested separately.

Repression Assay Seeds from transgenic Arabidopsis plants were sterilized by the chlorine gas method for 4 hrs. The seeds were transferred to a downflow cabinet and allowed to air for 30 min. For easy dispersal, a 0.1% of agar solution was added and the seeds were allowed to hydrate. The seeds were added to pre-autoclaved 250 ml flasks containing 0,5 MS medium. An 80% filter sterilized sucrose solution was added to a final concentration of 20 or 100 mM. The flasks were covered with aluminum foil and imbibed for 2 days at 4ºC. After imbibition, the seeds were allowed to germinate and grow under continuous fluorescent light at 22ºC for 5 days with agitation (150 rpm). Seedlings were harvested by draining excess medium on a tissue and subsequently freezing in liquid nitrogen. For long-term storage, the harvested material was stored at -80ºC.

Mug assay Ten seedlings per sample (three samples per concentration) were homogenized with extraction buffer (50 mM Na_2PO_4 pH 7.0, 10 mM Dithiothreitol, 1 mM EDTA pH 8.0, 0.1% Sodium Lauryl Sarcosine, 0.1% Trition X-100). The suspension was centrifuged 1 min 14000 rpm and 27 ml pre-warmed assay buffer of 37ºC (22 mg 4-methylumbelliferyl-b-D-glucuronide (MUG) in 50 ml extraction buffer) was added to 3 ml extract. The reaction was incubated at 37ºC for 1 hour and the reaction was stopped by adding 270 ml 0.2M Na_2CO_3. Fluorescence was measured with a Fluorstar at l355/l460.

Luminescence assay Seeds (10 mg per plate for the EMS-screen) from transgenic *Arabidopsis thaliana*, ecotype C24, were sterilized by the chlorine gas method for 4 hrs. The seeds were transferred to a downflow cabinet and

left for 30 min. For easy dispersal, 700 ml 0.1% agar was added and the seeds were allowed to hydrate. The seeds were dispersed on 0,5 MS medium containing 0.7% plant agar and 100 mM sucrose. Following drying, the plates were sealed with parafilm and aluminum foil and incubated at 4ºC for 2 days. Next, the plates were transferred to the growth chamber where the aluminum foil and parafilm were removed. The plates were incubated 4 days with constant light at 22 ºC after which the plates were transferred to the downflow cabinet and sprayed with a 5mM sterile luciferine solution (5 mM luciferine, 10 mM Tris-HCl pH 8.0, 0.01% Trition X-100). After spraying the plates were placed back in the growth chamber, under constant light, for 1 day. Next, the plates were sprayed with a sterile 1 mM luciferine solution and incubated 1 min at room temperature after which luminescence was measured for 10 minutes. Luminescence was measured with a Hamamatsu Argus 20 image processor and c2400-47 VIM camera. Seedlings displaying elevated luminescence compared to the wt were transferred to soil for propagation.

Quantative Luciferase activity assay Transgenic seeds harboring the luciferase gene were grown as described earlier on solid medium. After 4 days incubation, the seeds were sprayed with a 5 mM sterile luciferine solution and were incubated 1 day further in the growth chamber. Seedlings were harvested one day later and luciferase activity was determined with the Luciferase Reporter Gene Assay, high sensitivity kit (Roche, Mannhein, Germany) as described by the manufacturer.

Quantative PCR RNA was isolated using the Purescript, RNA islation kit (Gentra Systems, Minneapolis, USA) according the manufactures instructions. RNA was made DNA-free with the DNA-free^{TM} kit (Ambion Ltd, Cambridgeshire, England).). The DNA free RNA was checked for DNA by PCR with a primerpair for *ATB2* (forward AGACGATCTAACG GCTCAGGTT, reverse TGCGTTGTGATGCTGACACTT) and *TUB4*,(forward TGGACAATGAGGCTC TCTACG, reverse CAGGG AAACGAAGACAGCAAG) cDNA synthesis was performed with M-MLV Reverse Transcriptase from Promega (Madison, USA), according to the manufactures instructions. The qPCR reactions were performed with a FAM/Tamra labeled probe (Isogen, Maarssen, Holland). Taqman Universal Master Mix, No AmpErase UNG, (Applied Biosystems, Roche Molecular Systems) was used in the qPCR reaction. All primers and probes were designed using the Primer Express^{TM} v1.0 software (Applied Biosystems). Q-PCR results were analysed with SDS v1.7 software Applied Biosystems. Results for *AtbZIP2* were calculated using equation $R=(E_{target})^{\Delta Ct_{target}\ (control-sample)}/\ (E_{ref})^{\Delta Ct_{ref}\ (control-sample)}$ as described by (Pfaffl, M. W. 2001a). Relative quantification for all other primer-probe combinations was sufficiently similar to *Actin2* to use the $\Delta\Delta$Ct method (User bulletin #2, ABI Prism 7700 Sequence Detection System, ABI, 2001). Primer-probe efficiencies for all primer-probe sets were determined according to equation $E=10^{(-1/slope)}$ as described by (Rasmussen, R. 2001). See table 1 for sequences of primers and probes which were used for the qPCR reaction.

Table 1: Sequences of primers and probes used in the quantative RT-PCR reaction to measure the relative RNA content of seedlings grown with different sucrose concentrations. Probes and primers for the *GUS*, *LUC*, *ATB2* and *ACT* genes are depicted.

primers	probes
GUS forward 5'AACCCCAACCCGTGAAATC 3'	*GUS* 5'ACTCGACGGCCTGTGGGCATTC 3'
GUS reverse 5'CACAGTTTTCGCGATCCAGAC 3'	
LUC forward 5'TCCATGGTCACCGACGC 3'	*LUC* 5'AAGAAAGGCCCGGCGCCATTCT 3'
LUC reverse 5'GGTTCCATCTTCCAGCGGA 3'	
ACT forward 5'GCTGAGAGATTCAGACTGCCCA3'	*ACT* 5'AAGTCTTGTTCCAGCCCTCGTTTGTGG3'
ACT Reverse 5' CACAGTTTTCGCGATCCAGAC 3'	
*ATB*2 forward 5'TCGTCAGGATCGGAGGAGAGT3'	*ATB2* 5'AACGTAAACAGGAGCTCTCAAACCGTGAA3'
ATB2 Reverse 5'GATCGTCTAGGAGCTTTTGTTTCTTC3'	

Chapter 4

A conserved uORF mediates sucrose-induced repression of translation

with Anika Wiese, Barry Wobbes, Sjef Smeekens

(submitted to the Plant Cell)

Abstract

Sugars have been shown to regulate transcription of numerous genes in plants. Sucrose controls translation of the group S bZIP-type transcription factor *ATB2/AtbZIP11* (Rook, F. et al. 1998). This control requires the unusually long 5'UTR of the gene. Point mutations and deletions of the 5'UTR have uncovered the sequences involved. A highly conserved uORF coding for 42 amino acids is essential for the repression mechanism. It is conserved in 5'UTRs of bZIP-transcription factors from other Arabidopsis genes and many other plants. *ATB2/AtbZIP11* is normally expressed in association with vascular tissues. Ectopic expression of a 5'UTR construct shows that the sucrose repression system is functional in all tissues of the shoot. Another Arabidopsis bZIP-transcription factor gene, *AtbZIP2* harboring the conserved uORF is regulated similarly via sucrose-induced repression of translation. This suggests a general function of the conserved uORF in sucrose-controlled regulation of expression.Our findings imply the operation of a sucrose-sensing pathway that controls translation of several plant bZIP transcription factor genes harbouring the conserved uORF in their 5'UTRs. Target genes of such transcription factors will then be regulated in sucrose-dependent way.

Introduction

Plants are autotrophic organisms and synthesize sugars for growth and storage de novo. Sugars can also function as hormone-like signaling molecules that adjust metabolism, growth and development of plants. Sugar signaling operates at essentially all phases of the plant life cycle and dominates plant metabolism. Most sugar signaling effects appear to be mediated through transcriptional control; changes in the sugar concentration cause induction or repression of gene-transcription (Koch, K. E. 1996). However sugar sensing also affects gene expression post-transcriptional, by changing mRNA stability, translation or protein stability (Chan, M. T. and Yu, S. M. 1998, Cheng, W. H. et al. 1999, Rook, F. et al. 1998, Yanagisawa, S. et al. 2003). The dominant sugars, sucrose and hexoses activate different cellular processes in plants. Investigations of developing seeds suggest that hexoses control metabolism and growth, whereas sucrose regulates differentiation and storage (Wobus, U. and Weber, H. 1999). Sucrose and hexoses are sensed via different, not yet fully understood signaling pathways (Rolland, F. et al. 2002, Smeekens, S. 2000). Hexoses are thought to be sensed either in a hexokinase-dependent or independent manner the latter possibly involving hexose transporters (Rolland, F. et al. 2002, Smeekens, S. 2000). Recently the catalytic function of hexokinase was separated from the sensory function of the enzyme, showing that downstream metabolism is not involved in hexokinase signalling (Moore, B. et al. 2003). It is now well established that molecular sucrose is sensed via independent pathways. Such pathways have not yet been unraveled, but sucrose transporters with unusual long cytosolic loops may act as receptors (Lalonde, S. et al. 1999). Downstream signaling possibly involves SNF-like kinases (SnRKs) (Purcell, P. C. et al. 1998, Tiessen, A. et al. 2003). Molecular sucrose specifically regulates transcription of genes. Such genes are not activated to the same extend by the sucrose breakdown products glucose and fructose. They encode e.g. patatin, rolC and UDP-glucose pyrophosphorylase (Ciereszko, I. et al. 2001, Jefferson, R. et al. 1990, Wenzler, H. et al. 1989, Yokoyama, R. et al. 1994). Sucrose represses a proton-sucrose symporter in sugar beet (Chiou, T. J. and Bush, D. R. 1998). Moreover, the phenotypic deviations caused by constitutive expression of the HDZip transcription factor gene *ATHB13* are induced specifically by sucrose (Hanson, J. et al. 2001). Disaccharide-sensing that is independent from metabolism was observed in experiments with the non-metabolizable sucrose analogues palatinose and turanose. These analogues affect e.g. the expression of the vine hexose transporter *VvHT1*, alpha-amylase in barley embryos and

ADP-glucose pyrophosphorylase in potato. (Atanassova, R. et al. 2003, Loreti, E. et al. 2000, Tiessen, A. et al. 2003). However such sucrose analogues were shown to also activate signaling pathways different from sucrose (Roitsch, T. et al. 2003, Sinha, A. K. et al. 2002).

A post-transcriptional regulation of expression mediated by sucrose was observed for the group S basic region leucine zipper (bZIP) transcription factor *ATB2/AtbZIP11*. Sucrose-regulation of *ATB2/AtbZIP11* expression takes place at the level of translation; elevated sucrose concentrations repress translation of the transcription factor (Rook, F. et al. 1998). Thus, *ATB2/AtbZIP11* is the first known gene being translationally repressed by sucrose. Interestingly, *ATB2/AtbZIP11*-tran scription is stimulated by sugars and light. Transcription is repressed in darkness in a COP1 and DET1-dependent manner (Rook, F. et al. 1998). *ATB2/AtbZIP11* is prominently expressed in the vascular tissues of seedlings and young vegetative tissues. It is highly induced in funiculi of fertilized ovules (Rook, F. et al. 1998). This expression pattern and the translational regulation by sucrose suggests that *ATB2/AtbZIP11* might coordinate metabolism-associated processes in newly established sinks (Rook, F. et al. 1998).

Translational control has been observed for plant genes stimulated by light, hormones and programmed cell death (Bailey-Serres, J. 1999). Next to *ATB2/AtbZIP11*, only one other metabolite-induced translational control system in plants has been described. Polyamines trigger translational repression of a S-adenosyl-L-methionine decarboxylase gene (Hanfrey, C. et al. 2002).

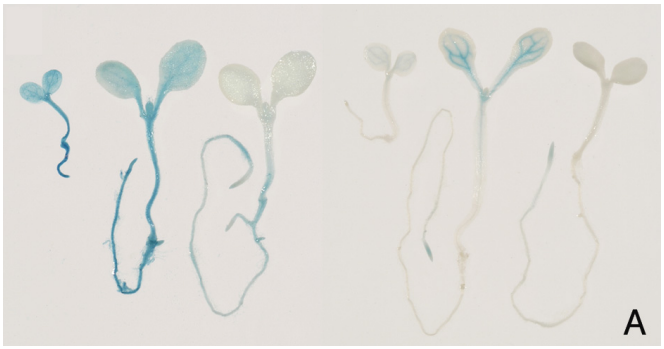
Elements involved in translational control are mostly found in untranslated regions (UTRs). The 5'caps as well as the 3'poly A determine translational efficiency in a more general way. The efficiency of translation is further controlled by various features of the 5' UTR like length, secondary structures upstream start codons (uAUGs) or open reading frame (uORFs), internal ribosome entry sites (IRES) and binding sites for regulatory proteins (Wilkie, G. S. et al. 2003). The 5'-untranslated region (5'UTR) of the *ATB2/AtbZIP11* mRNA was shown to be necessary for sucrose-induced repression of translation (SIRT) (Rook, F. et al. 1998). The unusually long *ATB2/AtbZIP11*-5'UTR (Rook, F. et al. 1998) contains four upstream open reading frames (uORF 1-4). The upstream ORF2 encodes a peptide of 42 amino acids. Interestingly this peptide is highly conserved in uORFs in some other bZIP 5'-untranslated regions in Arabidopsis as well as in other dicotyledonous and monocotyledonous plants. Moreover the overlapping uORF1 of *ATB2/AtbZIP11* is conserved in some 5'UTRs encoding the conserved uORF2. Deletions and point mutations in the 5'UTR allowed us to investigate the involvement

of the uORFs in sucrose induced translational repression. Ectopic expression of a marker gene construct shows that the sucrose repression system functions in all shoot tissues of the seedling beyond the vascular expression pattern of *ATB2/AtbZIP11.* The sucrose induced repression system seems to be absent or weaker in roots.
We propose bZIP-transcription factors harboring the conserved uORF in their 5'UTR to be translationally controlled by sucrose and confirm this suggestion for other Arabidopsis bZIP- transcription factors encoding the conserved uORF in a different 5'UTR context.

Results

The sucrose repression system controls translation in all shoot tissues

Sucrose induced repression of translation (SIRT) of *ATB2/AtbZIP11* was observed only in association with veins due to the tissue-specificity of the *ATB2/AtbZIP11* promoter (Rook, F. et al. 1998). To test the tissue specificity of the repression system, we expressed an *ATB2/AtbZIP11*-5'UTR controlled GUS/GFP chimera terminated by the *ATB2/AtbZIP11*-3'-UTR ectopically by the Arabidopsis *POLYUBIQUITIN10* promoter (*UBQ10*) (Sun, C. W. and Callis, J. 1997). The resulting construct *UBQ10*:5*'UTR-GUS/GFP* was transformed to Arabidopsis thaliana (ecotype Col-0). Histochemical staining of 5 days old seedlings of the selected lines *UBQ10:5'UTR-GUS*-I and II show that the *UBQ10*-promoter is ubiquitously active. Seedlings grown without sucrose or with 20 mM show GUS-expression in root and shoot (figure 1A). A weak repression of GUS activity in the cotyledons is observed in seedlings grown in 20 mM sucrose, whereas seedlings grown in 100 mM sucrose do not show GUS activity in the shoot. Some GUS expression is still observed in roots of seedlings grown in 100 mM sucrose (figure 1A). For comparison, histological staining of Arabidopsis Col-0 seedlings harboring *bZIP11:5'UTR-GUS* (Rook, F. et al. 1998) is presented, which shows the normal expression of the *ATB2/AtbZIP11* gene (figure 1A). Intense GUS activity in the veins is observed in seedlings grown in 20 mM sucrose, whereas repression of GUS activity occurs in seedlings grown in 100 mM sucrose (figure 1A) as presented for the C24 ecotype (Rook, F. et al. 1998).



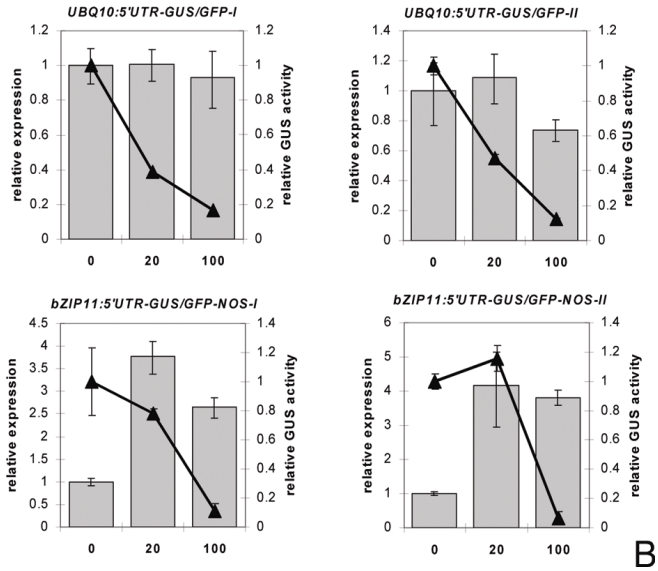


Figure 1 SIRT in transgenic seedlings expressing the ATB2/AtbZIP11-5'UTR controlled marker gene. Seedlings were grown in ½MS medium, containing 0 mM, 20 mM or 100 mM sucrose in liquid culture for 5 days in constant light. (A) Histochemical staining of seedlings. Left three seedlings, UBQ10:5'UTR-GUS/GFP-I; right three seedlings, bZIP11:5'UTR-GUS. Seedling most left in row were grown in ½MS medium without sucrose; middle, 20 mM sucrose; right, 100 mM sucrose. Quantification of GUS activity and mRNA accumulation in lines UBQ10:5'UTR-GUS/GFP-I and bZIP11-5'UTR-GUS/GFP-NOS. Measured GUS activities of seedling extracts was adjusted to total protein content. GUS activities (line and filled triangles) are shown relative to GUS activity of seedlings grown without sucrose. Expression of the marker gene mRNA was quantified by Q-PCR and normalized to the expression of the ACTIN2 gene. Transcript accumulation of the marker gene mRNA (grey bars) is shown as relative expression compared to the marker gene mRNA level of seedlings grown without sucrose. Error bars represent standard deviation of three measurements.

Quantification of GUS activity in UBQ10:5'UTR-GUS seedlings and comparison to the level of GUS-mRNA shows that the repression is not due to changes in the mRNA levels, since only GUS activity decreases with increasing sucrose (figure 1B). This shows that the GUS activity is controlled by SIRT. The ectopic expression with UBQ10:5'UTR-GUS shows a higher sensitivity against the sucrose repression than observed with the ATB2/AtbZIP11 promoter. Here repression takes place already at lower sucrose concentrations (figure 1B). Such differences in sensitivity might be due to differences in the local sucrose concentration or sensitivity of the sensing system in different tissues.
SIRT occurs in all green parts of the seedling but less efficient in the root and might therefore be a general sucrose control mechanism for all shoot tissues. Similar 5'UTR constructs to those tested for *UBQ10*-promoter were also constructed with the *35SCaMV*-promoter. Such *35SCaMV:5'UTR-GUS/GFP*-constructs allow SIRT in transgenic Arabidopsis seedlings. Thus relatively high mRNA levels as they are normally obtained with the *35SCaMV*-promoter also show SIRT.

The *ATB2/AtbZIP11*-5' leader is necessary and sufficient for SIRT

In previous work it was shown that deletion of the 5'UTR results in a loss of SIRT (Rook, F. et al. 1998). The 5'UTR was necessary for sucrose repression, but a possible function of the *ATB2/AtbZIP11*-3'UTR dependent on the 5'UTR was not excluded. Thus it was tested whether the 5'UTR by itself is sufficient for SIRT. For this, a GFP/GUS chimera-coding region terminated by the *NOS* (nopaline synthase) 3'UTR extracted from pCAMBIA1304 was fused to the *ATB2/AtbZIP11*-promoter and -5'UTR. The construct *bZIP11:5'UTR-GUS/GFP-NOS* was transformed to Arabidopsis thaliana, ecotype Col-0 and independent homozygous lines were selected for analysis. GUS activity of the transgenic lines is repressed in seedlings grown in 100 mM sucrose, whereas RNA levels are comparable to the seedlings grown on lower sucrose concentration, indicating a repression of translation (figure 1B). This shows that the exchange of the *ATB2/AtbZIP11*-3'UTR does not affect the SIRT mechanism. Hence the 5'UTR is necessary and sufficient for repression.

Translation of the conserved uORF2 is required for SIRT

The *ATB2/AtbZIP11* 5'UTR harbors 4 upstream open reading frames (uORFs). Starting from the 5'-end of the mRNA, these overlapping uORFs are designated uORF1 (18 amino acids), uORF2 (42 amino acids), uORF3 (5 amino acids) and uORF 4 (19 amino acids) (figure 2A). An additional internal start codon (AUG) is present in uORF2. This uORF can be translated as the long uORF2a, starting at the first AUG codon or as uORF2b, starting at the internal AUG (28 amino acids).

Upstream open reading frames are known to affect translation (Morris, D. R. and Geballe, A. P. 2000), therefore their involvement in SIRT was investigated. Point mutations disrupting the start codons of the uORFs in the 5' leader of *ATB2/AtbZIP11* were introduced to investigate the importance of translation of the uORFs for the SIRT mechanism. Start codons were exchanged to either stop codons or alternative amino acid codons (table 1). These mutated 5'UTRs were inserted into the *bZIP11:5'UTR-GUS* construct (Rook, F. et al. 1998) and transformed into Arabidopsis thaliana. Independent homozygous lines for each construct were selected, and 5 days old seedlings grown in liquid culture were analyzed for SIRT. Results for two representative lines are shown in figure 2B.

First, a construct was created, in which translation of uORF1, 2 and 4 was disrupted (*bZIP11:5-1.2.4-GUS* I and II) by mutation of the respective start codons (table 1, figure 2A). In transgenic seedlings harboring this construct GUS activities remain high when grown in 100 mM sucrose whereas the marker gene mRNA accumulates as expected (figure 2B). In these lines, SIRT is absent, suggesting that one or more of these uORFs is involved in the sucrose repression of translation. Moreover, the small uORF3 alone does not mediate SIRT.

Next we introduced point mutations in single uORFs to identify the ones involved in SIRT. The *ATB2/AtbZIP11* uORF1 and uORF2 show conservation in their sequence with uORFs in other plant bZIP-5'UTRs (see below) and our investigations were concentrated on those. The exchange of the start codon (AUG) of uORF1 to AAG (Lys) in construct *bZIP11:5-1-GUS* (table 1, figure 2A) still allows repression of GUS activity in seedlings grown in 100 mM sucrose. This is due to SIRT since the mRNA of the marker gene does not reflect the repression of GUS activity (lines *bZIP11:5-1-GUS*, figure 2B).

The first AUG of the highly conserved uORF2 (AUG2a) was converted to a GUG (Val) in 5'UTR 5-2a (table 1, figure 2A).

Transgenic seedling of lines *bZIP11:5-2a-GUS* I and II showed repression of GUS activity when grown in 100 mM sucrose (figure 2B). Marker gene mRNA levels did not reflect this repressive effect (figure 2B), thus SIRT occurs normally. Remarkably, the exchange of the internal putative start codon AUG2b (5'UTR 5-2b) to a stop codon (table 1, figure 2A) destroys SIRT. Seedlings harboring *bZIP11:5-2b-GUS* and grown in 100 mM sucrose show an unrepressed GUS activity (figure 2B). Thus, SIRT activity is dependent on translation of the conserved uORF2. Only translation of the highly conserved C-terminal part is essential for SIRT, since usage of the first start codon is not required. In the following the uORF2 of *ATB2/AtbZIP11* and any orthologous uORF from other plants or other Arabidopsis genes will be named the Sucrose-Control-uORF (SC-uORF).

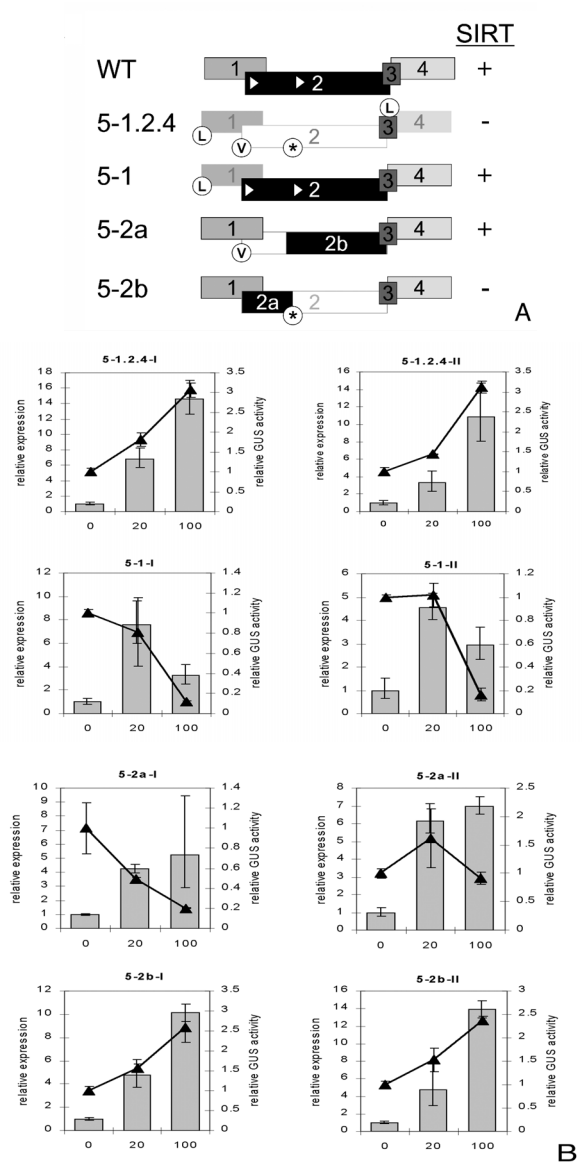


Figure 2. uORF involvement in SIRT.

Schematic illustration of the *ATB2/AtbZIP11*-5'-UTR. Shown are uORF arrangement and effects of the introduced point mutations. *, stop codon; L, lysine; V, valine. White triangles represent start codons in uORF2. Analysis of SIRT mediated by 5'UTRs with different point mutations. Seedlings were grown for 5 days in constant light in 0 mM, 20 mM or 100 mM sucrose. Results presented as described in legend of figure 1.

Table 1. Start codon point mutations introduced into the *ATB2/AtbZIP11*-5'UTR.

Name	Mutated uORF(s)	AUG exchange
5-1	1	AAG (L)
5-2ab	2a/b	5-2a: GTG (V) / 5-2b: TAG (*)
5-2a	2a	GTG (V)
5-2b	2b	TAG (*)
5-1.2.4	1,2,4	5-1, 5-2ab and uORF4: AAG (L)

The effect of the exchange on the codon is shown in parentheses. *stop codon; L, lysine; V, valine.

The SC-uORF is translated *in vitro*

Following the identification of the Sucrose-Control-uORF we tested whether this uORF is translated to the SC-peptide *in vitro*. For this purpose, the *ATB2/AtbZIP11*-5'UTR was subcloned *Eco*RV/*Nco*I into pBluescriptII KS(+), resulting in construct pb5 (figure 3A). The first 43 bp of the 5'UTR are absent in this construct, which does not affect the uORFs. The 5'UTR sequence was transcribed with T3 polymerase and translated with ^{35}S-labeled methionine in wheat germ lysate. Translation of pb5 results in a peptide with a molecular weight of approximately 4 kDa consistent with the calculated molecular weight of the translated uORF2 (figure 3B, lane 2). Translation of the pBluescriptII KS(+) vector alone showed that the additional larger peptides are not resulting from translation of the 5'UTR (figure 3B, lane 4). To verify the identity of the translated peptide as derived from uORF2, mutations were introduced in the 5'UTR. Deletion of the first 172 bp of the 5'UTR including uORF1 and modification of the uAUG2a into a better context (ACAC<u>ATG</u>TCT) (Lutcke, H. A. et al. 1987) in construct pb5-AUGc (figure 3A) results in high level translation of a peptide of the same size as with the wild type 5'UTR (figure 3B, lane 1). This indicates that the *in vitro* translation starts at the first start codon of the conserved uORF. The mutation of the 5'UTR 5-2a with a point mutation in AUG2a (table 1, figure 2A) in construct pb5-2a results in a loss of the 4 kDa SC-peptide (figure 3B, lane 3).

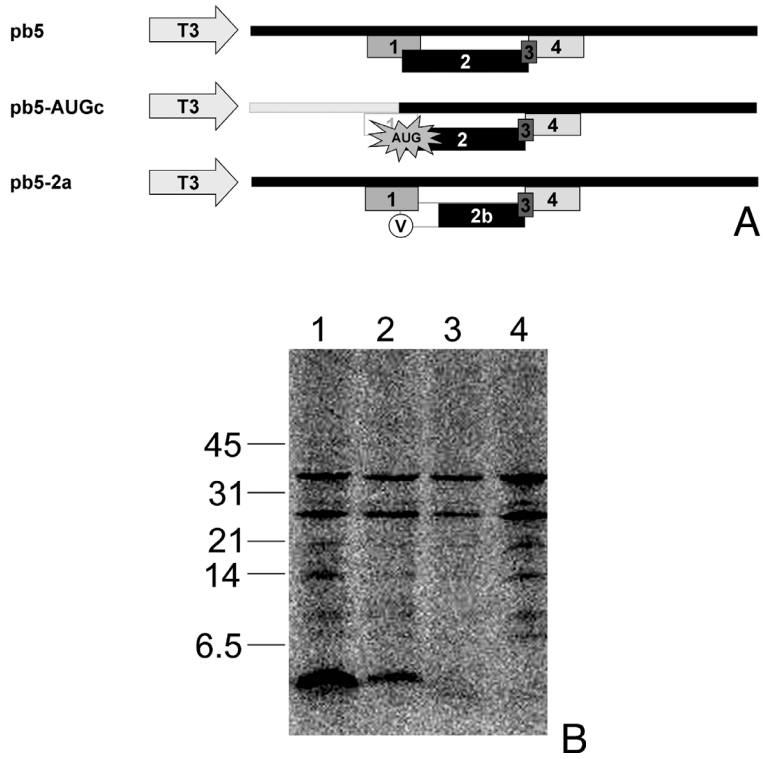


Figure 3: In vitro translation of the *ATB2/AtbZIP11*-5'UTR.(a) In vitro translation-constructs of the *ATB2/AtbZIP11*-5'UTR in pBluescriptII KS(+). The overlapping uORFs are indicated. pb5, wild type *ATB2/AtbZIP11* 5'UTR; pb5-AUGc, 5'UTR with exchange of AUG2a to an improved context; pb5-2a, point mutation in AUG2a, removing the first start codon of uORF2. (b) Gel electrophoresis of coupled *in vitro* transcription/ translation products. Extracts were separated in a 16,5 % tris-tricine SDS gel, ^{35}S-methionine radiolabeled products were analyzed by PhosphoImager following 2 weeks exposure. Molecular weights of a protein standard are indicated at the left in kDa. Lane 1, pb5-AUGc; lane 2, pb5; lane 3, pb5-2a; lane 4, pBluescript)II KS(+) vector.

The SC-uORF is conserved among plants

Protein BLAST searches with the uORF2 amino acid sequence against translated databases (Altschul, S. F. et al. 1990) revealed that uORF2 of *ATB2/AtbZIP11* (SC-uORF) shows high amino acid sequence conservation to other plant uORFs (figure 4A). SC-uORFs were found upstream of four other bZIP transcription factor genes in Arabidopsis and also in bZIP genes of other dicotyledonous and monocotyledonous plant species (figure 4A,B). Remarkably, these bZIP transcription factors all belong to

the group S of bZIP transcription factors (Jakoby, M. et al. 2002). Table 2 shows 21 identified EST, cDNAs or genomic sequences coding for an SC-uORF followed by the complete group S bZIP transcription factor coding sequence. SC-uORFs were not observed in any other genes in the databases. In Arabidopsis thaliana only 5 of the 17 identified S-group bZIP transcription factors (Jakoby, M. et al. 2002) harbor the SC-uORF.

SC-uORFs are present in long and short versions; they vary in length between 41-53 and 22-32 amino acids (figure 4A, table 2), respectively. SC-uORFs show amino acid identities between 28% and 97%. The coding sequence shows variability in codon usage in all third "wobble"-positions of the codons. An effect of a conserved RNA sequence structure is therefore unlikely. All SC-uORFs are highly conserved in their C-terminus; the shorter uORFs lack the N-terminus present in the longer uORFs. Both types of uORFs are found within and between plant species. The distances of the SC-uORF stop codon to the main bZIP reading frame start codon are in the range from 71 (BQ987415, figure 4A) to 255 nucleotides (*ATB2/AtbZIP11*,table2).

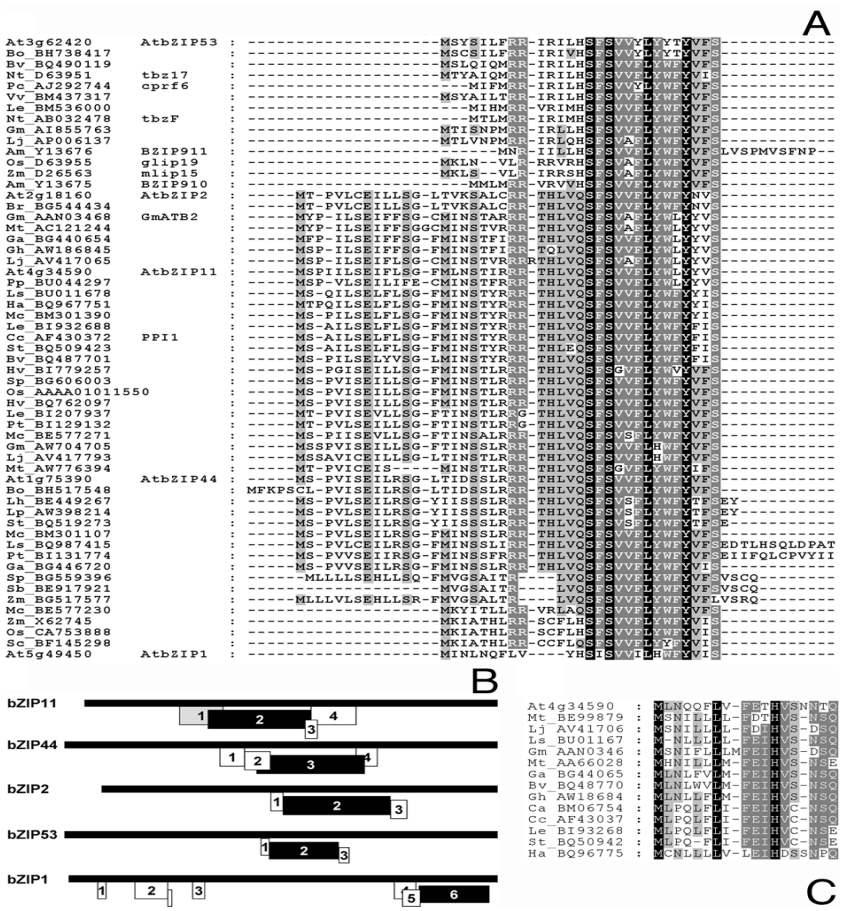


Figure 4: Conservation of uORFs in group S bZIP-5'UTRs.
(A)Alignment of the conserved SC-uORF amino acid sequences, identified in BLAST searches. Black box, white letter, 100% identity; dark grey box, white letter 80% identity; grey box, black letter, 60 % identity. ESTs carry their accession number and a shortcut of the species. Am, Antirrhinum majus (Snapdragon); At, Arabidopsis thaliana (Thale cress); Bo, Brassica oleracea (Cabbage); Br, Brassica rapa (Birdsrape mustard); Bv, Beta vulgaris (Sugar beet); Cc, Capsicum chinense (Pepper); Ga, Gossypium arboreum (Cotton Tree); Gh, Gossypium hirsutum (Cotton); Gm, Glycine max (Soybean); Ha, Helianthus annuus (Sunflower); Hv, Hordeum vulgare (Barley); Le, Lycopersicon esculentum (Tomato); Lh, Lycopersicon hirsutum (wild tomato); Lj, Lotus japonicus; Lp, Lycopersicon pennellii (wild tomato); Ls, Latuca sativa (Lettuce serriola); Mc, Mesembryanthemum crystallinum (Ice plant); Mt, Medicago trunculata (Barrel Medic); Nt, Nicotiana tabacum (Tobacco); Os, Oryza sativa (Rice); Pc, Petroselinum crispum (Parsley); Pp, Prunus persica (Peach); Pt, Populus tremula x Populus tremuloides (Poplar); Sb, Sorghum bicolor (Broomcorn); Sc, Secale cereale (Rye); Sp, Sorghum propinquum (Sorghum); St, Solanum tuberosum (Potato); Vv, Vitis vinifera (Vine); Zm, Zea mays (Maize). (B) Schematic illustration of uORF organisation in 5'UTRs of the five Arabidopsis group S bZIP-genes, encoding the SC-uORF (indicated as black box). The conserved uORF1 of *ATB2/AtbZIP11* is presented in grey. uORFs are numbered starting from 5'end of UTR. An upstream AUG in *AtbZIP1* is indicated as a small unnumbered box. (C) Amino acid sequence alignment of conserved uORFs, overlapping the SC-uORF. ESTs carry their accession number and a shortcut of the species. Black box, white letter, 100% identity; dark grey box, white letter 80% identity; grey box, black letter, 60% identity.

Table 2: Data of 5'UTRs harboring the SC-uORF.

Plant	Gene-name	Name/EST	Intercistronic spacer length (nt)	SCu-ORF length (aa)	SC-uORF pI
Arabidopsis thaliana (Thale Cress)	ATB2/AtbZIP11	At4g34590	255	42	9.69
Arabidopsis thaliana (Thale Cress)	AtbZIP44	At1g75390	164	41	9.97
Arabidopsis thaliana (Thale Cress)	AtbZIP2	At2g18160	116	41	8.84
Arabidopsis thaliana (Thale Cress)	AtbZIP53	At3g62420	182	28	9.82
Arabidopsis thaliana (Thale Cress)	AtbZIP1	At5g49450	10	25	6.69
Nicotiana tabaccum (Tobacco)	tbz17	D63951	240	28	10,27
Nicotiana tabaccum (Tobacco)	tbzF	AB032478	162	25	10,90
Zea mays (Maize)	mLIP15	D26563	170	26	11,00
Zea mays (Maize)	OCSBF-1	X6245.1	188	28	9,78
Antirrhinum majus (Snapdragon)	BZIP910	Y13675.1	166	25	10,90
Antirrhinum majus (Snapdragon)	BZIP911	Y13676.1	130	32	8,36
Oryza sativa (Rice)	glip19	D63955	169	26	11,57
Oryza sativa (Rice)	--	AAAA01011550	254	41	9,98
Glycine max (Soybean)	GmATB2	AAN03468	228	41	9,10
Petroselinum crispum (Parsley)	cprf6	AJ292744	138	25	10,27
Lycopersicon esculentum (Tomato)	--	BI207937	114	41	8,36
Medicago trunculata (Barrel Medic)	--	AC121244	198	42	9,10
Gossypium arboreum (Tree cotton)	--	BG446720	89	41	8,27
Lotus japonicus	--	AP006137	115	28	10,90
Capsicum chinense (Pepper)	PPI1	AF430372	188	41	9,69
Solanum tuberosum (Potato)	--	BM110898	99	42	9,69

Only EST/DNA-sequences were chosen, that code for the complete group S bZIPcoding sequence. SC-uORF distance: nucleotides (nt) following SC-uORF stop codon until main bZIP AUG start codon; SC-uORF length: number of amino acids (aa) encoded by the uORF; SC-uORF-pI, pI calculated with Compute pI/Mw tool, http://www.expasy.org/tools/pi_tool.html.

The peptide sequences, encoded by SC-uORFs have a high, calculated isoelectric point (table 2). These basic peptides mainly contain 2–3 consecutive arginines (figure 4A), a feature also found in the translation inhibiting uORF "BUP" which inhibits the

translation of the b_2-adrenergic receptor (Parola, A. L. and Kobilka, B. K. 1994). Interestingly, the coding sequence of the SC-uORF includes one rare arginine codon cgc or cgg which occurs with a frequency of 3.8 or 4.8 per 1000 (Nakamura, Y. et al. 2000). Such rare codons might be involved in the repression mechanism as was shown for a rare leucin codon in frog (Xenopus laevis) *Connexin41* mRNA (Meijer, H. A. and Thomas, A. A. 2003). So far, we identified a maximum of three long uORFs and one or two short uORFs in rice, Lotus japonicus and ice plant. Arabidopsis encodes five S-group bZIP genes harboring the SC-uORF, *ATB2/AtbZIP11, AtbZIP2, AtbZIP44, AtbZIP53 and AtbZIP1*. The *AtbZIP1* 5'UTR shows features not observed in any of the other genes. Its short SC-uORF sequence lacks consecutive arginines, is not basic (pI 6,69, table 2) and its distance to the start codon of the bZIP coding sequence is only 10 nucleotides long (table 2).

Figure 4B shows the uORF organization of all 5 *AtbZIP* 5'UTRs coding for the conserved uORF. The number of encoded uORFs varies between three and six. Other uORFs overlap the highly conserved SC-uORF in all plant species. In some SC-uORF encoding 5'UTRs, further inspection revealed conservation of an overlapping uORF upstream of the SC-uORF. In *ATB2/AtbZIP11* it is represented by the uORF1 coding sequence (figure 4B, C). These 16-18 amino acid long uORFs show 33-100 % amino acid identity to each other. They overlap the highly conserved SC-uORF sequence in a different reading frame over ca. 20 nucleotides (figure 4B).

The intercistronic region is important for SIRT

The distance between the stop codon of the SC-uORF and the bZIP start generally ranges between 71 and 255 bp (table 1). Such sequences may be important for the SIRT mechanism as well. This was investigated by partial deletions of the *ATB2/AtbZIP11* 5'UTRs (figure 5A). The four overlapping uORFs are located within the middle part of the *ATB2/AtbZIP11* 5'UTR (figure 5A). The 5'UTR starting from *Nhe*I (-401 from bZIP11-AUG) sequence was divided into two parts; the uORF containing 5'-end part and the downstream 3'-end (figure 5A). In deletion 4-11, 166 bp of the 3'-end part of the leader was removed, starting immediately after the stop codon of uORF4 (figure 5A). Thus, all uORFs are present in this construct. Deletion 4-23 represents the 5' end deletion of the uORF containing part. Only the uORF-free 3' terminal 169 base pairs are present, starting with the uORF4 stop codon (figure 5A).
The truncated 5'UTRs 4-11 and 4-23 were inserted into the *bZIP11:5'UTR-GUS* (Rook, F. et al. 1998), resulting in *bZIP11:4-23UTR-GUS* and *bZIP11:4-11UTR-GUS*.
Those were transformed into Arabidopsis (ecotype Col-0). The construct lacking the spacer (*bZIP11:4-11-GUS*) results in high GUS activity in seedlings grown in 100 mM sucrose and this is mirrored by the GUS-mRNA level. Here, SIRT is completely abolished. However, the intercistronic region by itself does not mediate SIRT. Transgenic seedlings harboring *bZIP11:4-23-GUS* do not show SIRT when grown in 100 mM sucrose. Under the experimental conditions, control line *bZIP11:5'UTR-GUS* showed SIRT as expected (not shown).

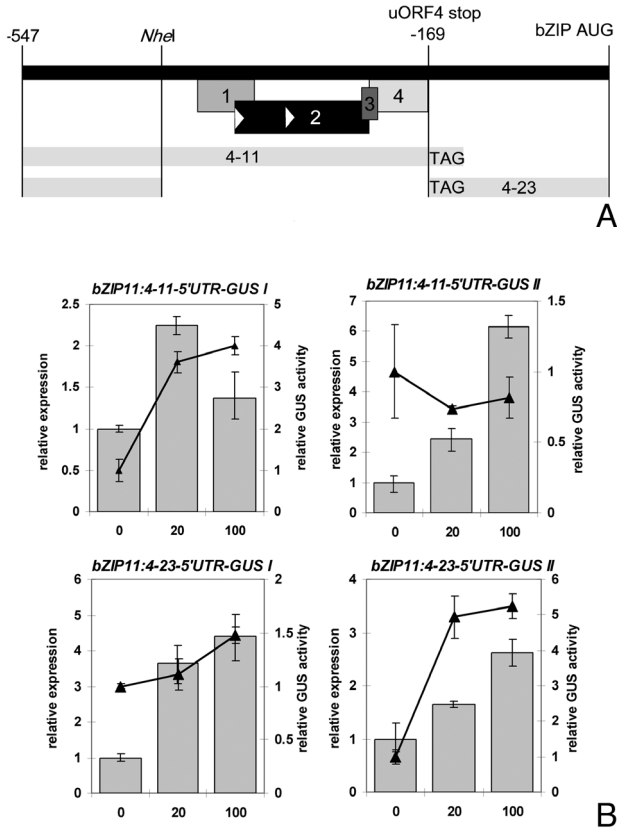


Figure 5: Effects of deletions in the ATB2/AtbZIP11 5'UTR on SIRT. (A)Schematic illustration of *ATB2/AtbZIP11*-5'-UTR, the uORF arrangement and deletions in lines *bZIP11:4-11-5'UTR-GUS* and *bZIP11:4-23-5'UTR-GUS*. (B) Analysis of SIRT mediated by 5'UTRs with deletions. Seedlings were grown for 5 days in constant light in 0 mM, 20 mM or 100 mM sucrose. Results presented as described in legend of figure 1.

SC-uORF containing 5'UTRs mediate SIRT

SIRT in *ATB2/AtbZIP11* requires translation of its conserved SC-uORF. We tested whether other bZIP genes, coding for such a SC-uORF in their 5'UTR show SIRT. The Arabidopsis group S bZIP transcription factor *AtbZIP2* (GBF5) encodes uORFs in its 5'untranslated region similar to *ATB2/AtbZIP11,* including the conserved SC-uORF (figure 4B). Arabidopsis (ecotype Col-0) was transformed with the *AtbZIP2* gene in which 81 basepairs of the

AtbZIP2-coding sequence were translationally fused to the GUS-marker gene, replacing the major part of AtbZIP2-coding sequence (*bZIP2:5'UTR-GUS*; figure 6 A). Further a similar construct with a deletion of the 5'UTR was constructed and tested. Construct *bZIP2:*D*5'UTR-GUS* resembles *bZIP2:5'UTR-GUS*, but carries a deletion of the 5'UTR from the 5'end up to position -86 bp relative to the *AtbZIP2*-start codon.

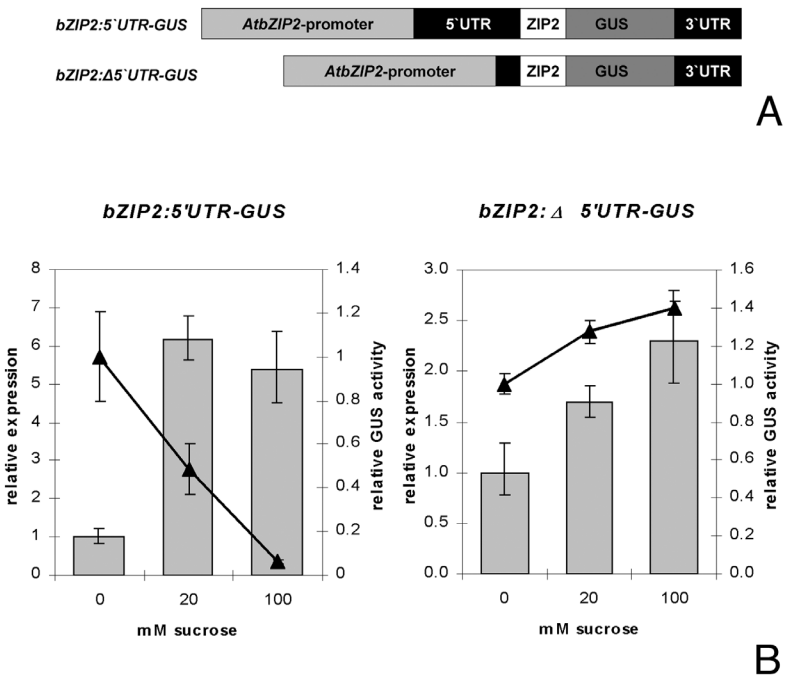


Figure 6. SIRT in *AtbZIP2.*

(A)Schematic illustration of GUS-marker gene-constructs of *AtbZIP2.bZIP2:5'UTR-GUS*, AtbZIP2 5'UTR and 17 amino acids of the *AtbZIP2* coding sequence translational fused to GUS coding sequence. *bZIP2:D5'UTR-GUS* carries a deletion of the 5'UTR from the 5'end up to position -86 from relative to the main start codon. (B) Analysis of SIRT on AtbZIP2. Seedlings were grown for 5 days in constant light in 0 mM, 20 mM or 100 mM sucrose. Results presented as described in legend of figure 1.

In transgenic homozygous seedlings harboring *bZIP2:5'UTR-GUS*, GUS-mRNA increases with the sucrose concentration, whereas the GUS activity is sucrose repressed. SIRT is lost in transgenic seedlings expressing the 5'UTR-marker gene construct with deletion of the major part of the 5'UTR including all uORFs (line *bZIP2:5'UTR-GUS*). GUS activity increases with sucrose concentration and the GUS-mRNA level. 5'UTR-dependent SIRT was also observed for the SC-uORF containing *AtbZIP44* gene, which is expressed in floral tissues (results not shown).

Discussion

The conserved SC-uORF mediates SIRT

Sucrose induced repression of *ATB2/AtbZIP11* translation was shown to depend on the 5'UTR of the gene (Rook, F. et al. 1998). This region is necessary and sufficient for SIRT and was investigated in more detail.

Point mutations in uORF2 start codons in the 5'UTR of this gene revealed that SIRT requires the translation of this uORF, named the Sucrose Control uORF (SC-uORF). Remarkably, BLAST searches revealed a strong conservation of the *ATB2/AtbZIP11*-SC-uORF encoded peptide in 5'UTRs of group S bZIP transcription factors (Jakoby, M. et al. 2002) of all plant species. In these 5'UTRs, the SC-uORF is arranged together with at least two other uORFs. Usually the SC-uORF overlaps with other uORFs.

Five Arabidopsis bZIP-transcription factors harbor the SC-uORF and SIRT was observed in five day old seedlings for *ATB2/AtbZIP11* and *AtbZIP2*. Also for *AtbZIP44* SIRT occurs in sucrose fed floral organs (results not shown).

These Arabidopsis bZIP transcription factors code for long conserved uORFs (41-42 amino acids). Many of the SC-uORF sequences in plants are shorter, (22-32 amino acids), including those in the 5'UTRs of the Arabidopsis *AtbZIP53* and *AtbZIP1* (figure 4A). These represent mainly the more conserved C-terminal part of the *ATB2/AtbZIP11* uORF2. Such a shorter conserved uORF was created by mutation of the first AUG in uORF2 of *ATB2/AtbZIP11* (AUG2a, *bZIP11:5-2a-GUS*). This mutation still allows SIRT suggesting that the N-terminal 14 amino acids of this uORF are not essential for SIRT. This first part of the SC-peptide shows high amino acid sequence conservation and its importance is currently unclear. In lines *bZIP11:5-2b-GUS*, a stop codon replaces the second AUG codon of uORF2 (AUG2b) and this mutation destroys SIRT. Thus,

initiation at AUG2b is sufficient for SIRT to occur. Most likely, *in planta* the larger SC-peptide is synthesized, as was observed in *in vitro* translation studies (Figure 3B). Importantly, *AtbZIP2* and *AtbZIP44* SC-uORF sequences do not contain the internal AUG (Figure 4A), precluding internal initiation of translation in these uORFs. Only long SC-peptides can be translated from these SC-uORFs. Most likely, uORF2 translation is initiated at the first AUG2a. In case of abrogation of AUG2a (*bZIP11:5-2a-GUS*) the shorter SC-peptide initiating at AUG2b, still mediates SIRT. Therefore, we propose that both long and short conserved translated SC-uORFs mediate SIRT. The existence of the conserved SC-uORFs in bZIP-transcription factors was noted before (Martinez-Garcia, J. F. et al. 1998, Strathmann, A. et al. 2001, Yang, S. H. et al. 2001). Here we propose a function of the conserved SC-uORF in sucrose induced translational control.
The conserved uORF1 in the 5'UTR of *ATB2/AtbZIP11* is not involved in SIRT. Repression occurs normally when translation of uORF1 is prevented by mutation of the start-codon (line *bZIP11-5-1-GUS*). Moreover such an additional conserved uORF is also absent in *AtbZIP2* and *AtbZIP44*, supporting the notion that it is not required for SIRT. This conserved uORF is only present in some of the identified plant bZIP 5'UTRs (figure 4C) and its function remains unclear.

The repression mechanism

Different mechanisms of uORF control on translation of the downstream main ORF have been described. Usually the nascent uORF-peptide acts in cis on translation (Damiani, R. D., Jr. and Wessler, S. R. 1993, Degnin, C. R. et al. 1993, Lohmer, S. et al. 1993). Upstream ORFs can hinder translation of the downstream ORF by forcing the ribosomes to reinitiate following termination of the uORF. Such uORFs can also cause stalling of the translational machinery during peptide elongation or at termination. Ribosome stalling seems to depend on the amino acid-sequence of the uORF. Amino acid sequence-dependent uORFs are thought to inhibit translation via ribosome stalling by interaction with RNA or protein components of the translational machinery (Vilela, C. and McCarthy, J. E. 2003).
Translation of the conserved "arginine attenuator peptide" (AAP) in the 5'UTRs of the fungal carbamoyl phosphate synthase causes ribosome stalling in response to an arginine surplus (Wang, Z. and Sachs, M. S. 1997). Such uORF-sequence dependent translational control is also found for the conserved uORF in the cytomegalovirus UL4 gene (Degnin, C. R. et al. 1993). Moreover, the non-conserved MAGDIS uORF of the mammalian S-adenosylmethionine decarboxylase, show a translational control, which depends on the uORF sequence. (Mize, G. J. et al.

1998, Wang, Z. and Sachs, M. S. 1997). The remarkable high conservation of the SC-uORF amino acid sequence implies such a sequence dependence of the encoded SC-peptides as well. Comparison of all known sequence-dependent uORFs did not allow the identification of conserved domains which might be involved in interaction with the translational apparatus or RNA (Tenson, T. and Ehrenberg, M. 2002). Interestingly, the SC-uORF and the conserved BUP-uORFs (b_2-adrenergic receptor upstream peptide) in mammals both contain 2-3 consecutive arginines. A "cis-like"-action of the BUP was suggested, by binding directly to the mRNA of origin following its translation and hindering further scanning of ribosomes. Mutating the consecutive arginines in the BUP-peptide reduced the repressive effect of the peptide, probably due to abrogation of the inhibitory BUP interaction with the mRNA (Parola, A. L. and Kobilka, B. K. 1994).

The coding sequence of the conserved SC-uORFs harbors at least one rare arginine codon inside the double or triple arginine codons (Nakamura, Y. et al. 2000). Such rare codons can be part of a stalling/decelerating mechanism, as was shown for the *Connexin41* gene, which encodes a rare leucine codon in its uORF (Meijer, H. A. and Thomas, A. A. 2003).

The length of the intercistronic region between the SC-uORF and the main start codon varies between 71 bp to 255 bp (table 1) with the exception of *AtbZIP1,* which harbors a spacer of only 10 bp. The length of the intercistronic region is important for reinitiation efficiency, which was observed to increase with the length of the intercistronic spacer (Child, S. J. et al. 1999). Deletion of the intercistronic spacer of *ATB2/AtbZIP11* shows a clear requirement of this region for the sucrose-induced repression of translation. The intercistronic region alone has no repressive effect, thus interaction of the intercistronic region with the uORF-region is required to mediate repression. A similar interdependence of uORF and intercistronic spacer was described for the arginine/lysine transporter gene *cat-1*, where induced translation of an uORF opens up an internal ribosome entry site (IRES), which promotes translation of the major ORF (Yaman, I. et al. 2003). Similarly, the translation of the SC-uORF might induce an inhibitory RNA structure or expose a binding site for an inhibitory RNA binding protein.

A sucrose activated signal transduction process somehow affects the initiation of the SC-uORF translation or the SC-peptide activity such that translation is repressed. The molecular details of this process remain to be uncovered.

The physiological importance of metabolite-induced translational regulation was recently shown for plant S-adenosylmethionine decarboxylases (SAMs) genes (Hanfrey, C. et al. 2002). These genes encode a highly conserved uORF of 50 to 54 amino acids ("small uORF") overlapped by a short uORF of 2 to 4 amino acids ("tiny uORF") (Franceschetti, M. et al. 2001). Polyamines repress translation of this enzyme under physiological conditions in the plant. Deletion or disruption of the conserved "small" uORF in the gene causes a loss of translational control and results in severe growth perturbations in transgenic tobacco due to unrepressed translation of the enzyme (Hanfrey, C. et al. 2002). *ATB2/AtbZIP11* is the only other known metabolite dependent translational control system involving a conserved uORF in plants. Constitutive overexpression of *ATB2/AtbZIP11* in Arabidopsis by *CaMV35S*-promoter construct lacking the translational control of the 5'UTR causes severe growth phenotypes including lethality and sterility (Wobbes et al, submitted). Thus loss of control of *ATB2/AtbZIP11* translation may cause such severe effects as well. Other plant transcription factors also show reduced translational activity due to uORFs (Damiani, R. D., Jr. and Wessler, S. R. 1993, Locatelli, F. et al. 2002, Lohmer, S. et al. 1993), but the inducing stimuli have not been identified.

The bZIP transcription factor *ATB2/AtbZIP11* is expressed in vascular tissues of seedlings. In these tissues sucrose induced translational repression was shown (Rook, F. et al. 1998). Expression of the *ATB2/AtbZIP11* 5'UTR-GUS-construct under control of the *UBQ10*-promoter showed that SIRT is active in all other tissues of the shoot, whereas it seems to be absent in roots. Thus, the sensing system is active in the whole shoot allowing SIRT on other bZIP transcription factors with different tissue specific expression patterns.

Plants undergo 5-10 fold changes in sucrose concentration over the diurnal period (Farrar, J. et al. 2000) and SIRT represents one flexible way to respond to such changes. Sucrose concentrations change substantially during development as well. Hexose and sucrose gradients have been observed during cotyledon differentiation of faba bean embryos or during oilseed rape seed development (Borisjuk, L. et al. 2003, Hill, L. M. et al. 2003). SIRT probably is responsive to such sucrose gradients over tissues and organs.

ATB2/AtbZIP11 expression is light dependent. Photosynthesis results in increased sucrose levels (Farrar, S. C. and Farrar, J. 1987) and activation of sucrose transport processes. The association of the *ATB2/AtbZIP11* expression with vascular

tissues suggests a function in assimilate partitioning such as phloem loading/unloading. Cell-wall invertases are likely targets of *ATB2/AtbZIP11* (Wobbes et al., submitted). These enzymes are thought to be involved in sucrose partitioning between source and sink organs (Sturm, A. and Tang, G. Q. 1999). Thus at low or intermediate sucrose concentrations, *ATB2/AtbZIP11* is translated, resulting in increased cell wall invertase expression and activity. Higher sucrose levels switch off ATB2 synthesis and as a result invertase expression. In developing sink organs such as fertilized ovules, this mechanism would balance the sucrose supply and demand and allow efficient distribution of resources over competing sinks. The physiological importance of SIRT on the expression of *ATB2/AtbZIP11* is currently under investigation.

Many previously characterized S-group transcription factors harboring the SC-uORF are induced by low temperature, salt stress, ABA, ethylene, IAA, JA, pathogen attack or senescence (Aguan, K. et al. 1993, Kusano, T. et al. 1995, Lee, S. J. et al. 2002, Yang, S. H. et al. 2001). Such conditions often promote changes of sucrose concentrations, which in turn could affect the translation of bZIP-proteins and activation of downstream target genes. SC-uORF-containing genes can be regulated by different stimuli, but gene activity is overridden and/or fine-tuned by SIRT, depending on the prevailing sucrose concentration.

Plant bZIP transcription factor have been shown to homodimerize or heterodimerize. The group S bZIP factor CPRF6 heterodimerizes with the group C bZIP factor *CPRF2* (Onate, L. et al. 1999, Rugner, A. et al. 2001, Strathmann, A. et al. 2001). The snapdragon bZIP910 and bZIP911 were found to bind to hybrid c-box/g-box ACGT elements as homodimers, whereas heterodimers of these transcription factors show low affinity for these boxes (Martinez-Garcia, J. F. et al. 1998). Thus a whole range of mechanisms are involved in controlling activity of bZIP transcription factors, including transcription, translation, post-translational modifications and homo- or dimerization with other transcription factors. Such a system allows a flexible, multi-responsive regulation of bZIP target genes.

In conclusion, we propose SIRT to act on a set of plant bZIP transcription factors encoding the SC-uORF. Interaction of transcriptional activation and translational control allows these regulatory genes to respond in a flexible way to rapidly changing stimuli which affect sucrose levels in cells or tissues.

Conclusions

The *ATB2* gene is a bZIP transcription factor and is controlled by the sucrose (Rook, F. et al. 1998). Deletion of the 5'UTR leader sequence abolishes the post-transcriptional regulation by sucrose. The leader sequence by itself can impose sucrose regulation other ORF's like the *GUS* and *LUC* coding sequences. Thus, the leader sequence is necessary and sufficient for sucrose dependent post-transcriptional gene regulation. Expression of the reporter gene is observed throughout the seedling if the ubiqituine or 35S promoters are used. Interestingly, repression by sucrose is only observed in the shoot. Possibly additional factors, expressed in tissues that are exposed to light, are necessary for operation of the sucrose repression system (Carlberg, I. et al. 2003). In order to identify additional factors involved in the sucrose repression system a non-destructive mutant screen was developed. A dual reporter system was used for the mutant screen since it enables identification of false positive mutants. Independent transgenic lines were tested and a line was selected that displayed the expected response to high concentrations of sucrose by the reporter genes. This line was used in a mutagenesis and mutant selection procedure.
EMS-mutants were selected by identifying seedlings that were disturbed for sucrose mediated LUC repression. However, further analysis of these mutants revealed a wild type sucrose repression phenotype for the *GUS* gene. Interestingly all mutants isolated displayed this differential regulation of the reporter genes. A mutation in one of the reporter genes, or regulatory sequences thereof, could be responsible for the differential regulation, but this is unlikely. Possibly, mutants in the sucrose regulatory pathway may be lethal. Mutants with subtle mutations, which cause a mild effect, introduced by EMS, were not identified. The 5'UTR, which is necessary and sufficient, contains a conserved uORF of 129 nucleotides that encodes a peptide of 42 amino acids. Currently we cannot exclude the possibility that the peptide is a direct target for sucrose control. For such a small target, it may be difficult to retrieve mutants. Sucrose may associate directly with the uORF-encoded peptide as was suggested for the UTR encoded peptide of the CPA1 gene (Delbecq, P. et al. 1994).

The leader sequence, and in particular the conserved uORF, was analyzed in greater detail. The length of the intercistronic region between the SC-uORF and the main start codon varies greatly. Deletion of the spacer sequence of the *ATB2* gene showed that this region is required for sucrose induced regulation of translation (SIRT). The length of the intercistronic region is important for reinitiaton efficiency and is related to the length of the spacer sequence (Child, S. J. et al. 1999). Blast searches have revealed additional S bZIP transcription factors which all contain

the conserved uORF (Sucrose Control uORF, SC-uORF) which are present in all plant species (Jakoby, M. et al. 2002). The S-type bZIP transcription factors *ATB2*, *ATBZIP2* and *ATBZIP44* are all controlled by sucrose (data not shown).
In contrast to the other bZIP genes with the SC-uORF, the ATB2 SC-uORF contains an internal AUG. Deleting the first AUG of the SC-uORF still conferred sucrose mediated regulation. However, replacing the internal AUG with a stop codon abolished the sucrose-mediated repression. Thus, the conserved uORF is necessary for SIRT but the N-terminal part of the SC-uORF is dispensable. The coding sequence of the C-terminal part of the SC-uORF harbors at least one rare arginine codon inside the double or triple arginine codons. Rare codons like these can be part of a stalling/decelerating mechanism which was shown for the Connexin41 gene that contains a rare leucine codon in its uORF (Meijer, H. A. and Thomas, A. A. 2003).

In conclusion, the ATB2 S type transcription factor is regulated at the post-transcriptional level by SIRT. Although the precise mechanism is still unkown, it has been shown that a conserved uORF is necessary for SIRT. The identification of additional genes with the SC-uORF suggests a general sucrose regulatory mechanism. Future experiments should provide a greater understanding of the mechanism involved in SIRT.

Material and methods

*Plant transformation and growth conditions, w*ild-type Arabidopsis (ecotype Col-0) were grown in a growth chamber at 22ºC under a 16h-light 8h-dark cycle with a relative humidity of 80%. Floral dip transformations (Clough, S. J. and Bent, A. F. 1998) were performed by using *Agrobacterium tumefaciens* strains GVG 2260 and EHA105. For *in vitro* cultures, seeds were surface sterilized by liquid or vapor-phase (Clough, S. J. and Bent, A. F. 1998) methods. For surface sterilization, seeds were soaked in 96% ethanol for 5 minutes, 10 minutes in 20% commercial bleach and washed 4 times in sterile MilliQ water. After surface sterilization, seeds were stratified for 2 days at 45ºC. Transformed plants were selected by the bar resistance of the T-DNA on solid half-strength MS medium with vitamins (Murashige T. and Skoog F. 1962) containing 15 mg/l phosphinotricine. Homozygous lines were selected and analyzed for GUS- or GFP-mRNA and for GUS-activity. Results of two representative independent lines are presented, named line I and line II of each construct. Transgenic seedlings of selected lines were grown for 5 days in liquid half-strength MS medium with vitamins (Murashige T. and Skoog F. 1962). Filter sterilized sucrose was added to autoclaved medium at indicated concentrations. After imbibition, seedlings were grown under continuous light at 22ºC for 5 days with agitation (150 rpm).

Marker gene constructs, molecular cloning techniques were performed as described (Sambrook, J. et al. 1998). For constructing UBQ10:5'UTR-GUS/GFP, the GUS coding sequence in the ATB2-GUS construct in pBluescript[SK(-) (pbPGA) (Rook, F. et al. 1998) was exchanged for a *GFP-GUS* chimera coding region by restriction of pbPGA with BamHI and of pCambia1304 (www.cambia.org) with BstEII. The overlapping ends were filled in with T4-DNA-polymerase. Both vectors were digested with NcoI and the isolated GFP-GUS coding-sequence was inserted into pbPGA resulting in pbPGGA. The Arabidopsis *POLYUBIQUITIN10* promoter (*PUBQ10*, upstream sequence of At4g05320 −389 to −1307 from ATG) was isolated PstI/BamHI from the vector p3325 (Norris, S. R. et al. 1993) and ligated into PstI/BamHI opened binary vector pGreenII0229 (Hellens, R. P. et al. 2000) resulting in pGreenII0229-PUBQ10. pbPGGA was digested XbaI, for isolation of the *ATB2/AtbZIP11* marker gene construct, which was introduced into the XbaI digested vector pGreenII0229-PUBQ10. In this construct 196 bp of the *ATB2/AtbZIP11* promoter are included.
The exchange of the *ATB2/AtbZIP11*-3'UTR was performed by *SpH*I restriction of pCambia1304 (www.cambia.org) and following fill in. The *GFP-GUS* chimera-coding sequence fused to the *nos* (nopaline synthase) 3'UTR was isolated by a second restriction with *Nco*I. Construct *pbPGA* (Rook, F. et al. 1998) was opened with *Not*I, sticky ends were filled in by Klenow treatment. *Nco*I restriction released the GUS-coding sequence and the *ATB2/AtbZIP11* 3'UTR from pbPGA and the *GFP-GUS* chimera coding sequence with *nos*-poly A was inserted. The whole construct with *ATB2/AtbZIP11* promoter and 5'UTR was inserted via *Xho*I and *Sac*I restriction sites into the binary vector pGreenII0229 (Hellens, R. P. et al. 2000).
For construction of *AtbZIP2*-GUS constructs, the 5' and 3'UTRs were amplified by PCR; subcloned in pGEM[-T easy (Promega) and sequenced. The 3'UTR was amplified with primer bZIP2 3'UTR-F: 5'-GCTAGCTGATTAATAAAATTAATTAAAATAATTAGATG-3', containing an *Nhe*I site (underlined) and 3'UTR-R: 5'-TCAAATCTACCAA GTCAAATTTCACCGCTAGCGGGCCC-3' with *Nhe*I and *Apa*I sites (underlined). This fragment was isolated with *Nhe*I from pGEM-T easy and inserted in pCambia1301. The 5'UTR was amplified with primer bZIP2+5'UTR-F: 5'-AAGCTTGTTAACCTCTTCCTTATCTCCTTAAAA-3' containing *Hind*III and *Hpa*I (underlined) and bZIP2 5'UTR-R with *Nco*I (underlined): 5'-CCATGGTGACGACGGAGTCCGACG-3' a fragment of 501bp 5'UTR and the first 81bp of the coding sequence. The 5'UTR was isolated with *Hin*dIII/*Nco*I from a pGEM-T easy subclone and inserted in the *Hin*dIII/*Nco*I digested pCambia1301 carrying the GUS coding sequence, resulting in *5'UTR-bZIP2 CDS-GUS-3'UTR* in translational fusion. The *AtbZIP2* promoter was isolated with *Bal*I from BAC F8D23 (4.4 kb) and subcloned in the *Sma*I site of pluescriptII[SK(+), subsequently the 2213 bp *AtbZIP2* promoter fragment was isolated with *Eco*72I and *Ear*I. *pCambia1301 5'UTR-bZIP2 CDS-GUS-3'UTR* was digested with *Hpa*I. After a partial digestion with *Ear*I and this construct was ligated to the 2.2 kb *Eco*72I/*Ear*I promoter fragment. Finally, the entire promoter-5'UTR-GUS-3'UTR was isolated from pCambia1301 with *Apa*I/*Bam*HI and ligated in the *Apa*I/*Bam*HI digested pGreenII0229 (Hellens, R. P. et al. 2000) resulting in vector *bZIP2:5'UTR-GUS*.

For construction of the *AtbZIP2*-5‘UTR deletion construct *bZIP2:*D*5’UTR-GUS* primer bZIP2 5’UTR-R (described above) and bZIP2D 5’UTR-F 5’-AAGCTTCAGACAGATCATAAAAAAAAACCAAAC-3’ with a *Hind*III site (underlined) were used to amplify a fragment consisting of 86 bp 5’UTR and 81 bp of the coding sequence. The 5’UTR-D was digested with *Hin*dIII/*Nco*I from a sequenced pGEM-T easy subclone and exchanged for the *Hin*dIII/*Nco*I fragment of pCambia1301. *pCambia1301 5’UTR*-D-*bZIP2 CDS-GUS-3’UTR* was digested with *Hin*dIII, polished with T4 and ligated to the 2.2 kb *Eco*72I/*Ear*I, T4-polished *AtbZIP2* promoter fragment. Finally, the entire *AtbZIP2*-promoter-5’UTR-D-GUS-3’UTR was digested from pCambia1301 with *Apa*I/*Bam*HI and ligated in the *Apa*I/*Bam*HI digested pGreenII0229 (Hellens, R. P. et al. 2000) resulting in vector *bZIP2:*D*5’UTR-GUS.*

Deletion constructs, deletions of the 5’UTR were generated by PCR. The PCR products were *Nco*I/*Nhe*I cloned into the pNE03. The partially deleted leader segments were *Nco*I/*Nhe*I cloned from pNE03 into the PGA vector. The promoter-leader-GUS-trailer was *Kpn*I/*Not*I cloned into pGreenII0229 (Hellens, R. P. et al. 2000). Primers used for the 3’-end deletion 4-11 were NE03 DraIII-SmaI 5’ GGAACAAGAGTCCACTATTA 3’ and NE4-11: 5’-TATCCATGGCTAG GGTTTTGTTGTAATTATGCG-3’. Primers used for the 5’-deletion were GUSA-leader ATB2 5’-GTCCACCAGGTGTTCGGCGTGGTG-3’ and 4-23: 5’- TATAGCTAGCTA GTTTCTTTTCAAATTTCTCTTCTTCG-3’.

*Point mutation constructs, a*n *EcoR*V fragment from *pbPGA* was cloned into pBluescript II KS (+) (Stratagene, La Jolla, USA) creating *pNE03*. Point mutations were created in the *pNE03* vector with the QuickChange[Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) as described by the manufacturer. After checking the desired mutations by sequencing, the *Nco*I/*Nhe*I restriction fragment was cloned into the *pbPGA* vector. Subsequently the *Kpn*I/*Not*I fragment, containing the *ATB2/AtbZIP11*-promoter-5’UTR-GUS-3‘UTR, was fused into pGreenII0229 (Hellens, R. P. et al. 2000). Primers used for mutagenesis were NE05mutorf1 5’-AAACATTGA AGCTTAATCAGC-3’, NE05mutorf2 5’-TTGAGACACGTGTCTCCAATA-3’, NE05mutorf2A 5’-TTTCTCT CTGGGTTTTAGTTAAATTCCA-3’, NE05mutorf4 5’-TATGTCT CAAGATCTCTGAAC-3’.

Constructs for *in vitro* translation, for *in vitro* translation, the *ATB2/AtbZIP11* 5’UTR from +4 to −496 (relative to start codon) was isolated from vector PGA (Rook, F. et al. 1998) in an *EcoR*V and *Nco*I restriction. After fill in of the *Nco*I overlapping ends the fragment was subcloned into *EcoR*V opened pBluescript[II KS(+) (Stratagene) resulting in pb5. In the same manner for pb5-2a, the 5’UTR of the construct *bZIP11:52a-GUS* was subcloned into pBluescript[II KS(+) (Stratagene). The AUG context in the construct pb5-AUGc was modified using PCR on pbPGA (Rook, F. et al. 1998) with the following primers: Primer 1: 5’-CACAATGGCTCCAATAATACTCAGTGAG-3’ (exchanged start codon context underlined) and primer 2: 5’-TCCATGGAGTAACACA CAAACAAAAACAG-3’, for amplification up to the included *Nco*I restriction site (underlined) at the end of the 5’UTR. The PCR product was subcloned in pGEM[-T easy (Promega). After sequencing, the fragment was isolated by *EcoR*I restriction and ligated into pBluescript[II

KS(+) (Stratagene), resulting in pb5-AUGc. Sequencing confirmed the correct orientation of the *ATB2/AtbZIP11* 5′ UTR and its modifications to the T3 promoter.

GUS chemistry and quantification, plant material was stained using GUS-buffer consisting of 0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, 10 mM EDTA, 0.1% (v/v) triton X-100 and 1 mg/ml X-gluc. For GUS quantification, seedlings were ground using a drill with a metal pistil in an eppendorf tube or in a mortar with liquid nitrogen. The glucuronidase activity was quantified using the fluorometric MUG assay (Jefferson, R. A. et al. 1987). Seedlings were ground in extraction buffer (50 mM Na_2PO_4 pH 7.0, 10 mM dithiothreitol, 1 mM EDTA pH 8.0, 0.1% sodium lauryl sarcosine, 0.1% triton X-100). The suspension was centrifuged 1 min 14000 rpm and 27 ml pre-warmed assay buffer of 37ºC (22 mg 4-methylum-belliferyl-b-D-glucuronide (MUG) in 50 ml extraction buffer) was added to 3 ml extract. The reaction was incubated at 37ºC for 1 hour and stopped by adding 270 ml 0.2M Na_2CO_3. Fluorescence was measured with a Fluostar Galaxy (BMG, Offenburg, Germany) at l355/l460 (Gallagher, S. R. 1992). Protein content was determined using the Bradford assay (Bradford, M. M. 1976) to compare activity to protein units.

In vitro translation-analysis, coupled in vitro transcription/translation reactions were performed with the coupled transcription/translation (TNT) Wheat Germ Lysate System (Promega, Madison, USA) according to the manufacturer with T3 polymerase, 1mg plasmid and 10mCi [^{35}S]methionine (Amersham Pharamcia, Buckinghamshire, UK) per 50ml translation mixture. Incubation was performed for 90 min at 30ºC. Translation reaction was directly mixed with (2x) loading buffer consisting of 100 mM Tris-HCl, pH 7.5, 4% SDS, 0.2% bromophenol blue, 15% glycerol, and 200 mM ?-mercaptoethanol. The mixture was heated at 955C for 4 min. Sample mixes were then microfuged (15,000*g* for 1 min). Qualitative analysis of the translated products was performed by separation on a 16,5% tris/tricine polyacrylamide precast gel (BioRAD, Herkules, USA) (Schagger, H. and von Jagow, G. 1987). Radiolabeled translation products were visualized on a PhosphorImager (Molecular Dynamics) after ten days of exposure. Protein size standard was prestained "broad range" (BioRAD).

*RT-PCR measurements, r*elative quantification of the marker gene messenger levels was performed using the Taqman Relative Quantitative Real-Time PCR technology on the ABI 7700 lightcycler (Applied Biosystems, Foster City, USA) with gene-specific primers and probes labeled 5′ FAM and 3′ TAMRA, using the 2x Taqman Q-PCR mix (Applied Biosystems). Primers were used at concentrations of 900 mM and probes at 250 mM. The 5′UTR-GUS-fusion was amplified with 5′-TTGTGTG TTACTCCATGGTCCG-3′ and 5′-CCCAGGCCG TCGAGTTTT-3′ and detected with the probe 5′-CCTGTAGAAAC CCCAACCCGT GAAATCA-3′ alternatively, GUS was detected in PCR with primer GUS fwd 5′-AACCCCAACCCGTGAAATC-3′, GUS rev 5′-CACAGTTTTCGCGATCCAG AC-3′ and the GUS probe 5′-ACTCGACG GCCTGTGGGCATTC-3′. Expression of the GUS/GFP-chimera coding sequence was performed with GFP fwd: 5′-ACGGCATCAAAGCC AACTTC-3′, 5′-TCAGCGAGTTGC ACGCC-3′ and GFP-probe 5′-AGACCC

GCCACAACATCGAAGAC-3'. *Actin2* (At3g18780) mRNA expression was detected as reference with Act fwd 5'-GCTGAGAGATTCAGACTGCCCA-3' Act rev 5'-CACAGTTT TCGCGATCCAGAC-3' and the Act probe 5'-AAGTCTTGTTCCAG CCCTCGTTTGTGG-3'. All primers and probes were designed using the Primer Express™ v1.0 software of Applied Biosystems. Q-PCR results were analysed with SDS v1.7 software from Applied Biosystems. Results for *AtbZIP2* were calculated using equation $R=(E_{target})^{\Delta Ct_{target}\,(control\text{-}sample)}/\,(E_{ref})^{\Delta Ct_{ref}\,(control\text{-}sample)}$ as described by (Pfaffl, M. W. 2001). Relative quantification for all other primer-probe combinations was sufficiently similar to *Actin2* to use the $\Delta\Delta$Ct method (User bulletin #2, ABI Prism 7700 Sequence Detection System, ABI, 2001). Primer-probe efficiencies for all primer-probe sets were determined according to equation $E=10^{(-1/slope)}$ as described by (Rasmussen, R. 2001).

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact Anika Wiese, A.Wiese@bio.uu.nl.

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Conclusions

The *ATB2* gene is a bZIP transcription factor and is controlled by the sucrose (Rook, F. et al. 1998). Deletion of the 5'UTR leader sequence abolishes the post-transcriptional regulation by sucrose. The leader sequence by itself can impose sucrose regulation other ORF's like the *GUS* and *LUC* coding sequences. Thus, the leader sequence is necessary and sufficient for sucrose dependent post-transcriptional gene regulation. Expression of the reporter gene is observed throughout the seedling if the ubiqituine or 35S promoters are used. Interestingly, repression by sucrose is only observed in the shoot. Possibly additional factors, expressed in tissues that are exposed to light, are necessary for operation of the sucrose repression system (Carlberg, I. et al. 2003a). In order to identify additional factors involved in the sucrose repression system a non-destructive mutant screen was developed. A dual reporter system was used for the mutant screen since it enables identification of false positive mutants. Independent transgenic lines were tested and a line was selected that displayed the expected response to high concentrations of sucrose by the reporter genes. This line was used in a mutagenesis and mutant selection procedure.
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contain the conserved uORF (Sucrose Control uORF, SC-uORF) which are present in all plant species (Jakoby, M. et al. 2002a). The S-type bZIP transcription factors *ATB2*, *ATBZIP2* and *ATBZIP44* are all controlled by sucrose (data not shown).
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In conclusion, the ATB2 S type transcription factor is regulated at the post-transcriptional level by SIRT. Although the precise mechanism is still unkown, it has been shown that a conserved uORF is necessary for SIRT. The identification of additional genes with the SC-uORF suggests a general sucrose regulatory mechanism. Future experiments should provide a greater understanding of the mechanism involved in SIRT.

Reference List

(2000) Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature **408**:796-815.

Aguan,K., Sugawara,K., Suzuki,N., and Kusano,T. (1993) Low-temperature-dependent expression of a rice gene encoding a protein with a leucine-zipper motif. Mol.Gen.Genet. **240**:1-8.

Altschul,S.F., Gish,W., Miller,W., Myers,E.W., and Lipman,D.J. (1990) Basic local alignment search tool. J Mol.Biol. **215**:403-410.

Andre,B. (1995) An overview of membrane transport proteins in Saccharomyces cerevisiae. Yeast **11**:1575-1611.

Arenas-Huertero,F., Arroyo,A., Zhou,L., Sheen,J., and Leon,P. (2000) Analysis of Arabidopsis glucose insensitive mutants, gin5 and gin6, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. Genes Dev. **14**:2085-2096.

Atanassova,R., Leterrier,M., Gaillard,C., Agasse,A., Sagot,E., Coutos-Thevenot,P., and Delrot,S. (2003) Sugar-regulated expression of a putative hexose transport gene in grape. Plant Physiol **131**:326-334.

Bailey-Serres,J. (1999) Selective translation of cytoplasmic mRNAs in plants. Trends Plant Sci. **4**:142-148.

Barker,L., Kuhn,C., Weise,A., Schulz,A., Gebhardt,C., Hirner,B., Hellmann,H., Schulze,W., Ward,J.M., and Frommer,W.B. (2000) SUT2, a putative sucrose sensor in sieve elements. Plant Cell **12**:1153-1164.

Beaudoin,N., Serizet,C., Gosti,F., and Giraudat,J. (2000) Interactions between abscisic acid and ethylene signaling cascades. Plant Cell **12**:1103-1115.

Bhat,P.J. and Hopper,J.E. (1992) Overproduction of the GAL1 or GAL3 protein causes galactose-independent activation of the GAL4 protein: evidence for a new model of induction for the yeast GAL/MEL regulon. Mol.Cell Biol. **12**:2701-2707.

Bhat,P.J. and Murthy,T.V. (2001) Transcriptional control of the GAL/MEL regulon of yeast Saccharomyces cerevisiae: mechanism of galactose-mediated signal transduction. Mol.Microbiol. **40**:1059-1066.

Bisson,L.F., Neigeborn,L., Carlson,M., and Fraenkel,D.G. (1987) The SNF3 gene is required for high-affinity glucose transport in Saccharomyces cerevisiae
7. J.Bacteriol. **169**:1656-1662.

Boles,E. and Hollenberg,C.P. (1997) The molecular genetics of hexose transport in yeasts. FEMS Microbiol.Rev. **21**:85-111.

Borisjuk,L., Rolletschek,H., Wobus,U., and Weber,H. (2003) Differentiation of legume cotyledons as related to metabolic gradients and assimilate transport into seeds. J Exp.Bot. **54**:503-512.

BradFord,B.B. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal.Biochem. 72:248-254.

Brandon Moore, Li Zhou, Filip Rolland, Qi Hall, Wan-Hsing Cheng, Yan-Xia Liu, Ildoo Wang, Tamara Jones, and Jen Sheen (2003) Role of the Arabidospis Glucose sensor HXK1 in nutrient, Light, and Hormonal Signaling. Science **300**:332-336.

Carlberg,I., Hansson,M., Kieselbach,T., Schroder,W.P., Andersson,B., and Vener,A.V. (2003) A novel plant protein undergoing light-induced phosphorylation and release from the photosynthetic thylakoid membranes. Proc.Natl.Acad.Sci.U.S.A **100**:757-762.

Carlson,M. (1999) Glucose repression in yeast. Curr.Opin.Microbiol. **2**:202-207.

Chan,M.T. and Yu,S.M. (1998a) The 3' untranslated region of a rice alpha-amylase gene functions as a sugar-dependent mRNA stability determinant. Proc.Natl.Acad.Sci.U.S.A **95**:6543-6547.

Chan,M.T. and Yu,S.M. (1998b) The 3' untranslated region of a rice alpha-amylase gene mediates sugar-dependent abundance of mRNA. Plant J. **15**:685-695.

Chappell,S.A. and Mauro,V.P. (2003) The internal ribosome entry site (IRES) contained within the RNA binding motif protein 3 (Rbm3) mRNA is composed of functionally distinct elements. J.Biol.Chem.

Cheng,W.H., Taliercio,E.W., and Chourey,P.S. (1999) Sugars modulate an unusual mode of control of the cell-wall invertase gene (Incw1) through its 3' untranslated region in a cell suspension culture of maize. Proc.Natl.Acad.Sci.U.S.A **96**:10512-10517.

Child,S.J., Miller,M.K., and Geballe,A.P. (1999) Translational control by an upstream open reading frame in the HER-2/neu transcript. J.Biol.Chem. **274**:24335-24341.

Chinnusamy,V., Stevenson,B., Lee,B.H., and Zhu,J.K. (2002) Screening for gene regulation mutants by bioluminescence imaging. Sci.STKE. **2002**:L10.

Ciereszko,I., Johansson,H., and Kleczkowski,L.A. (2001) Sucrose and light regulation of a cold-inducible UDP-glucose pyrophosphorylase gene via a hexokinase-independent and abscisic acid-insensitive pathway in Arabidopsis. Biochem.J. **354**:67-72.

Clough,S.J. and Bent,A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. **16**:735-743.

Dai,N., Schaffer,A., Petreikov,M., Shahak,Y., Giller,Y., Ratner,K., Levine,A., and Granot,D. (1999) Overexpression of Arabidopsis hexokinase in tomato plants inhibits growth, reduces photosynthesis, and induces rapid senescence
7. Plant Cell **11**:1253-1266.

Damiani,R.D., Jr. and Wessler,S.R. (1993) An upstream open reading frame represses expression of Lc, a member of the R/B family of maize transcriptional activators. Proc.Natl.Acad.Sci.U.S.A **90**:8244-8248.

David R.Morris and Adam P.Geballe (2000) Upstream Open Reading Frames as Regulators of mRNA Translation. Mol.Cell Biol. **20**:8635-8642.

Dean,K.A., Aggarwal,A.K., and Wharton,R.P. (2002) Translational repressors in Drosophila. Trends Genet. **18**:572-577.

Degnin,C.R., Schleiss,M.R., Cao,J., and Geballe,A.P. (1993) Translational inhibition mediated by a short upstream open reading frame in the human cytomegalovirus gpUL4 (gp48) transcript. J Virol. **67**:5514-5521.

Delbecq,P., Calvo,O., Filipkowski,R.K., Pierard,A., and Messenguy,F. (2000) Functional analysis of the leader peptide of the yeast gene CPA1 and heterologous regulation by other fungal peptides. Curr.Genet. **38**:105-112.

Delbecq,P., Werner,M., Feller,A., Filipkowski,R.K., Messenguy,F., and Pierard,A. (1994) A segment of mRNA encoding the leader peptide of the CPA1 gene confers repression by arginine on a heterologous yeast gene transcript. Mol.Cell Biol. **14**:2378-2390.

Dellaporte,S.L., Wood,J., and Hicks,J.B. (1983) A plant DNA minipreparation: version 2. Plant Mol.Biol.Rep. **1**:19-22.

Diba,F., Watson,C.S., and Gametchu,B. (2001) 5'UTR sequences of the glucocorticoid receptor 1A transcript encode a peptide associated with translational regulation of the glucocorticoid receptor. J.Cell Biochem. **81**:149-161.

Eckardt,N.A. (2001) Luc genetic screen illuminates stress-responsive gene regulation. Plant Cell **13**:1969-1972.

Fang,P., Wang,Z., and Sachs,M.S. (2000) Evolutionarily conserved features of the arginine attenuator peptide provide the necessary requirements for its function in translational regulation. J.Biol.Chem. **275**:26710-26719.

Farrar,J., Pollock,C., and Gallagher,J. (2000) Sucrose and the integration of metabolism in vascular plants. PLANT SCIENCE **154**:1-11.

Farrar,S.C. and Farrar,J. (1987) Effects of photon fluence rate on carbon partitioning in barley source leaves. Plant Physiol Biochem:541-548.

Farras,R., Ferrando,A., Jasik,J., Kleinow,T., Okresz,L., Tiburcio,A., Salchert,K., del Pozo,C., Schell,J., and Koncz,C. (2001) SKP1-SnRK protein kinase interactions mediate proteasomal binding of a plant SCF ubiquitin ligase. EMBO J. **20**:2742-2756.

Forsberg,H. and Ljungdahl,P.O. (2001) Sensors of extracellular nutrients in Saccharomyces cerevisiae. Curr.Genet. **40**:91-109.

Franceschetti,M., Hanfrey,C., Scaramagli,S., Torrigiani,P., Bagni,N., Burtin,D., and Michael,A.J. (2001) Characterization of monocot and dicot plant S-adenosyl-l-methionine decarboxylase gene families including identification in the mRNA of a highly conserved pair of upstream overlapping open reading frames. Biochem.J. **353**:403-409.

Gaba,A., Wang,Z., Krishnamoorthy,T., Hinnebusch,A.G., and Sachs,M.S. (2001) Physical evidence for distinct mechanisms of translational control by upstream open reading frames. EMBO J. **20**:6453-6463.

Gallagher,S.R. (1992) *GUS Protocols. Using the GUS Gene as a Reporter of Gene Expression*. Academic Press.

Ganster,R.W., McCartney,R.R., and Schmidt,M.C. (1998) Identification of a calcineurin-independent pathway required for sodium ion stress response in Saccharomyces cerevisiae. Genetics **150**:31-42.

Ghassemian,M., Nambara,E., Cutler,S., Kawaide,H., Kamiya,Y., and McCourt,P. (2000) Regulation of abscisic acid signaling by the ethylene response pathway in Arabidopsis. Plant Cell **12**:1117-1126.

Gopfert,U., Kullmann,M., and Hengst,L. (2003) Cell cycle-dependent translation of p27 involves a responsive element in its 5'-UTR that overlaps with a uORF. Hum.Mol.Genet. **12**:1767-1779.

Gray,W.M., del Pozo,J.C., Walker,L., Hobbie,L., Risseeuw,E., Banks,T., Crosby,W.L., Yang,M., Ma,H., and Estelle,M. (1999) Identification of an SCF ubiquitin-ligase complex required for auxin response in Arabidopsis thaliana. Genes Dev. **13**:1678-1691.

Greer,L.F., III and Szalay,A.A. (2002) Imaging of light emission from the expression of luciferases in living cells and organisms: a review. Luminescence. **17**:43-74.

Hanfrey,C., Franceschetti,M., Mayer,M.J., Illingworth,C., and Michael,A.J. (2002) Abrogation of upstream open reading frame-mediated translational control of a plant S-adenosylmethionine decarboxylase results in polyamine disruption and growth perturbations. J.Biol.Chem. **277**:44131-44139.

Hanson,J., Johannesson,H., and Engstrom,P. (2001) Sugar-dependent alterations in cotyledon and leaf development in transgenic plants expressing the HDZhdip gene ATHB13. Plant Mol.Biol. **45**:247-262.

Hardie,D.G. and CARLING,D. (1997) The AMP-activated protein kinase-fuel gauge of the mammalian cell? Eur.Journal Biochem. **246**:259-273.

Hardie,D.G., CARLING,D., and Carlson,M. (1998) The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu.Rev Biochem. **67:821-55**:821-855.

Harrod,R. and Lovett,P.S. (1995) Peptide inhibitors of peptidyltransferase alter the conformation of domains IV and V of large subunit rRNA: a model for nascent peptide control of translation. Proc.Natl.Acad.Sci.U.S.A **92**:8650-8654.

Hellens,R.P., Edwards,E.A., Leyland,N.R., Bean,S., and Mullineaux,P.M. (2000) pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Mol.Biol. **42**:819-832.

Hemmings-Mieszczak,M. and Hohn,T. (1999) A stable hairpin preceded by a short open reading frame promotes nonlinear ribosome migration on a synthetic mRNA leader. RNA. **5**:1149-1157.

Hilgarth,C., Sauer,N., and Tanner,W. (1991) Glucose increases the expression of the ATP/ADP translocator and the glyceraldehyde-3-phosphate dehydrogenase genes in Chlorella. J.Biol.Chem. **266**:24044-24047.

Hill,J.R. and Morris,D.R. (1992) Cell-specific translation of S-adenosylmethionine decarboxylase mRNA. Regulation by the 5' transcript leader. J.Biol.Chem. **267**:21886-21893.

Hill,L.M., Morley-Smith,E.R., and Rawsthorne,S. (2003) Metabolism of sugars in the endosperm of developing seeds of oilseed rape. Plant Physiol **131**:228-236.

Hinnebusch,A.G. (1993) Gene-specific translational control of the yeast GCN4 gene by phosphorylation of eukaryotic initiation factor 2. Mol.Microbiol. **10**:215-223.

Huijser,C., Kortstee,A., Pego,J., Weisbeek,P., Wisman,E., and Smeekens,S. (2000) The Arabidopsis SUCROSE UNCOUPLED-6 gene is identical to ABSCISIC ACID INSENSITIVE-4: involvement of abscisic acid in sugar responses. The Plant Journal **23**:577-585.

Jakoby,M., Weisshaar,B., Droge-Laser,W., Vicente-Carbajosa,J., Tiedemann,J., Kroj,T., and Parcy,F. (2002) bZIP transcription factors in Arabidopsis. Trends Plant Sci. **7**:106-111.

Jang,J.C., Leon,P., Zhou,L., and Sheen,J. (1997) Hexokinase as a sugar sensor in higher plants 2. Plant Cell 9:5-19.

Janzen,D.M., Frolova,L., and Geballe,A.P. (2002) Inhibition of translation termination mediated by an interaction of eukaryotic release factor 1 with a nascent peptidyl-tRNA. Mol.Cell Biol. **22**:8562-8570.

Jefferson,R., Goldsbrough,A., and Bevan,M. (1990) Transcriptional regulation of a patatin-1 gene in potato. Plant Mol.Biol. **14**:995-1006.

Jefferson,R.A., Kavanagh,T.A., and Bevan,M.W. (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6**:3901-3907.

Jiang,R. and Carlson,M. (1996) Glucose regulates protein interactions within the yeast SNF1 protein kinase complex. Genes Dev. **10**:3105-3115.

Jousse,C., Bruhat,A., Carraro,V., Urano,F., Ferrara,M., Ron,D., and Fafournoux,P. (2001) Inhibition of CHOP translation by a peptide encoded by an open reading frame localized in the chop 5'UTR. Nucleic Acids Res. **29**:4341-4351.

Klaff,P., Riesner,D., and Steger,G. (1996) RNA structure and the regulation of gene expression. Plant Mol.Biol. **32**:89-106.

Koch,K.E. (1996) Carbohydrate-modulated gene expression in plants. Annu.Rev.Plant Physiol.Plant Mol.Biol. **47**:509-540.

Kozak,M. (1987) An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. **15**:8125-8148.

Kozak,M. (1989) The scanning model for translation: an update. J.Cell Biol. **108**:229-241.

Kusano,T., Berberich,T., Harada,M., Suzuki,N., and Sugawara,K. (1995) A maize DNA-binding factor with a bZIP motif is induced by low temperature. Mol.Gen.Genet. **248**:507-517.

Laby,R.J., Kincaid,M.S., Kim,D., and Gibson,S.I. (2000) The Arabidopsis sugar-insensitive mutants sis4 and sis5 are defective in abscisic acid synthesis and response. Plant J. **23**:587-596.

Lalonde,S., Boles,E., Hellmann,H., Barker,L., Patrick,J.W., Frommer,W.B., and Ward,J.M. (1999) The dual function of sugar carriers. Transport and sugar sensing. Plant Cell **11**:707-726.

Law,G.L., Raney,A., Heusner,C., and Morris,D.R. (2001) Polyamine regulation of ribosome pausing at the upstream open reading frame of S-adenosylmethionine decarboxylase. J.Biol.Chem. **276**:38036-38043.

Le,S.Y. and Maizel,J.V., Jr. (1997) A common RNA structural motif involved in the internal initiation of translation of cellular mRNAs. Nucleic Acids Res. **25**:362-369.

Lee,S.J., Lee,M.Y., Yi,S.Y., Oh,S.K., Choi,S.H., Her,N.H., Choi,D., Min,B.W., Yang,S.G., and Harn,C.H. (2002) PPI1: a novel pathogen-induced basic region-leucine zipper (bZIP) transcription factor from pepper. Mol.Plant Microbe Interact. **15**:540-548.

Lejay,L., Gansel,X., Cerezo,M., Tillard,P., Muller,C., Krapp,A., von Wiren,N., Daniel-Vedele,F., and Gojon,A. (2003) Regulation of root ion transporters by photosynthesis: functional importance and relation with hexokinase. Plant Cell **15**:2218-2232.

Lincoln,A.J., Monczak,Y., Williams,S.C., and Johnson,P.F. (1998) Inhibition of CCAAT/enhancer-binding protein alpha and beta translation by upstream open reading frames. J.Biol.Chem. **273**:9552-9560.

Locatelli,F., Magnani,E., Vighi,C., Lanzanova,C., and Coraggio,I. (2002) Inhibitory effect of myb7 uORF on downstream gene expression in homologous (rice) and heterologous (tobacco) systems. Plant Mol.Biol. **48**:309-318.

Lohmer,S., Maddaloni,M., Motto,M., Salamini,F., and Thompson,R.D. (1993) Translation of the mRNA of the maize transcriptional activator Opaque-2 is inhibited by upstream open reading frames present in the leader sequence. Plant Cell **5**:65-73.

Loreti,E., Alpi,A., and Perata,P. (2000) Glucose and disaccharide-sensing mechanisms modulate the expression of alpha-amylase in barley embryos. Plant Physiol **123**:939-948.

Lovas,A., Bimbo,A., Szabo,L., and Banfalvi,Z. (2003) Antisense repression of StubGal83 affects root and tuber development in potato. The Plant Journal:139-147.

Luehrsen KR, de Wet JR., and Walbot V. (1992) Transient expression analysis in plants using firefly luciferase reporter gene. Methods Enzymol:397-414.

Lutcke,H.A., Chow,K.C., Mickel,F.S., Moss,K.A., Kern,H.F., and Scheele,G.A. (1987) Selection of AUG initiation codons differs in plants and animals. EMBO J. **6**:43-48.

Martin,T., Hellmann,H., Schmidt,R., Willmitzer,L., and Frommer,W.B. (1997) Identification of mutants in metabolically regulated gene expression. The Plant Journal **11**:53-62.

Martinez-Garcia,J.F., Moyano,E., Alcocer,M.J., and Martin,C. (1998) Two bZIP proteins from Antirrhinum flowers preferentially bind a hybrid C-box/G-box motif and help to define a new sub-family of bZIP transcription factors. Plant J. **13**:489-505.

Mayordomo,I. and Sanz,P. (2001) Hexokinase PII: structural analysis and glucose signalling in the yeast Saccharomyces cerevisiae. Yeast **18**:923-930.

McCartney,R.R. and Schmidt,M.C. (2001) Regulation of Snf1 kinase. Activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. J.Biol.Chem. **276**:36460-36466.

Meier,C., Bouquin,T., Nielsen,M.E., Raventos,D., Mattsson,O., Rocher,A., Schomburg,F., Amasino,R.M., and Mundy,J. (2001) Gibberellin response mutants identified by luciferase imaging. Plant J. **25**:509-519.

Meijer,H.A., Dictus,W.J., and Thomas,A.A. (2000) Cloning and analysis of the untranslated regions of the Xenopus laevis Connexin30 mRNA. Gene **258**:71-76.

Meijer,H.A. and Thomas,A.A. (2003) Ribosomes stalling on uORF1 in the Xenopus Cx41 5′ UTR inhibit downstream translation initiation. Nucleic Acids Res. **31**:3174-3184.

Messenguy,F., Vierendeels,F., Pierard,A., and Delbecq,P. (2002) Role of RNA surveillance proteins Upf1/CpaR, Upf2 and Upf3 in the translational regulation of yeast CPA1 gene. Curr.Genet. **41**:224-231.

Meyer,S., Melzer,M., Truernit,E., Hummer,C., Besenbeck,R., Stadler,R., and Sauer,N. (2000) AtSUC3, a gene encoding a new Arabidopsis sucrose transporter, is expressed in cells adjacent to the vascular tissue and in a carpel cell layer. The Plant Journal **24**:869-882.

Mikulits,W., Schranzhofer,M., Beug,H., and Mullner,E.W. (1999) Post-transcriptional control via iron-responsive elements: the impact of aberrations in hereditary disease. Mutat.Res. **437**:219-230.

Miller,P.F. and Hinnebusch,A.G. (1990) cis-acting sequences involved in the translational control of GCN4 expression. Biochim.Biophys.Acta **1050**:151-154.

Mize,G.J., Ruan,H., Low,J.J., and Morris,D.R. (1998) The inhibitory upstream open reading frame from mammalian S-adenosylmethionine decarboxylase mRNA has a strict sequence specificity in critical positions. J.Biol.Chem. **273**:32500-32505.

Moore,B., Zhou,L., Rolland,F., Hall,Q., Cheng,W.H., Liu,Y.X., Hwang,I., Jones,T., and Sheen,J. (2003) Role of the Arabidopsis glucose sensor HXK1 in nutrient, light, and hormonal signaling. Science **300**:332-336.

Moreno,F. and Herrero,P. (2002) The hexokinase 2-dependent glucose signal transduction pathway of Saccharomyces cerevisiae
7. FEMS Microbiol.Rev. **26**:83-90.

Morris,D.R. and Geballe,A.P. (2000) Upstream open reading frames as regulators of mRNA translation. Mol.Cell Biol. **20**:8635-8642.

Murashige T. and Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol.Plant **15**:473-497.

Nakamura,Y., Gojobori,T., and Ikemura,T. (2000a) Codon usage tabulated from international DNA sequence databases: status for the year 2000. Nucleic Acids Res. **28**:292.

Nelissen,B., De Wachter,R., and Goffeau,A. (1997) Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of Saccharomyces cerevisiae
5. FEMS Microbiol.Rev. **21**:113-134.

Nemeth,K., Salchert,K., Putnoky,P., Bhalerao,R., Koncz-Kalman,Z., Stankovic-Stangeland,B., Bako,L., Mathur,J., Okresz,L., Stabel,S., Geigenberger,P., Stitt,M., Redei,G.P., Schell,J., and Koncz,C. (1998) Pleiotropic control of glucose and hormone responses by PRL1, a nuclear WD protein, in Arabidopsis. Genes Dev. **12**:3059-3073.

Niu,X., Helentjaris,T., and Bate,N.J. (2002) Maize ABI4 binds coupling element1 in abscisic acid and sugar response genes. Plant Cell **14**:2565-2575.

Norris,S.R., Meyer,S.E., and Callis,J. (1993) The intron of Arabidopsis thaliana polyubiquitin genes is conserved in location and is a quantitative determinant of chimeric gene expression. Plant Mol.Biol. **21**:895-906.

Onate,L., Vicente-Carbajosa,J., Lara,P., Diaz,I., and Carbonero,P. (1999) Barley BLZ2, a seed-specific bZIP protein that interacts with BLZ1 in vivo and activates transcription from the GCN4-like motif of B-hordein promoters in barley endosperm. J Biol.Chem. **274**:9175-9182.

Ozcan,S., Dover,J., and Johnston,M. (1998) Glucose sensing and signaling by two glucose receptors in the yeast Saccharomyces cerevisiae. EMBO J. **17**:2566-2573.

Ozcan,S., Dover,J., Rosenwald,A.G., Wolfl,S., and Johnston,M. (1996) Two glucose transporters in Saccharomyces cerevisiae are glucose sensors that generate a signal for induction of gene expression. Proc.Natl.Acad.Sci.U.S.A **93**:12428-12432.

Parola,A.L. and Kobilka,B.K. (1994) The peptide product of a 5' leader cistron in the beta 2 adrenergic receptor mRNA inhibits receptor synthesis. J.Biol.Chem. **269**:4497-4505.

Pego,J.V., Weisbeek,P.J., and Smeekens,S.C. (1999) Mannose inhibits Arabidopsis germination via a hexokinase-mediated step. Plant Physiol. **119**:1017-1023.

Perata,P., Matsukura,C., Vernieri,P., and Yamaguchi,J. (1997) Sugar Repression of a Gibberellin-Dependent Signaling Pathway in Barley Embryos. Plant Cell **9**:2197-2208.

Pfaffl,M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29**:e45.

Pilipenko,E.V., Pestova,T.V., Kolupaeva,V.G., Khitrina,E.V., Poperechnaya,A.N., Agol,V.I., and Hellen,C.U. (2000) A cell cycle-dependent protein serves as a template-specific translation initiation factor. Genes Dev. **14**:2028-2045.

Pooggin,M.M., Futterer,J., Skryabin,K.G., and Hohn,T. (1999) A short open reading frame terminating in front of a stable hairpin is the conserved feature in pregenomic RNA leaders of plant pararetroviruses. J.Gen.Virol. **80 (Pt 8)**:2217-2228.

Proud,C.G. (2001) Regulation of mRNA translation. Essays Biochem. **37**:97-108.

Purcell,P.C., Smith,A.M., and Halford,N.G. (1998) Antisense expression of a sucrose nonfermenting1related protein kinase sequence in potato results in decreased expression of sucrose synthase in tubers and loss of sucroseinducibility of sucrose synthase transcripts in leaves. Plant J. **14** :195-202.

Randez-Gil,F., Sanz,P., Entian,K.D., and Prieto,J.A. (1998) Carbon source-dependent phosphorylation of hexokinase PII and its role in the glucose-signaling response in yeast
5. Mol.Cell Biol. **18**:2940-2948.

Raney,A., Law,G.L., Mize,G.J., and Morris,D.R. (2002) Regulated translation termination at the upstream open reading frame in s-adenosylmethionine decarboxylase mRNA. J.Biol.Chem. **277**:5988-5994.

Rasmussen,R. (2001) Quantification on the LightCycler. In *Rapid cycle Real-time PCR, methods and applications*, S.Meuer, C.Wittwer, and K.Nakagawara, eds (Heidelberg: Springer Press), pp. 21-34.

Redei,G.P. and Koncz,C. (1992) Classical mutagenesis. In Methods in Arabidopsis research, World Scientific, London), pp. 17-45.

Reifenberger,E., Freidel,K., and Ciriacy,M. (1995) Identification of novel HXT genes in Saccharomyces cerevisiae reveals the impact of individual hexose transporters on glycolytic flux 5. Mol.Microbiol. 16:157-167.

Reinders,A., Schulze,W., Kuhn,C., Barker,L., Schulz,A., Ward,J.M., and Frommer,W.B. (2002) Protein-protein interactions between sucrose transporters of different affinities colocalized in the same enucleate sieve element. Plant Cell **14**:1567-1577.

Reynolds,K., Zimmer,A.M., and Zimmer,A. (1996) Regulation of RAR beta 2 mRNA expression: evidence for an inhibitory peptide encoded in the 5'-untranslated region. J.Cell Biol. **134**:827-835.

Roblin,G., Sakr,S., Bonmort,J., and Delrot,S. (1998) Regulation of a plant plasma membrane sucrose transporter by phosphorylation
4. FEBS Lett. **424**:165-168.

Rodriguez,A., de la,C.T., Herrero,P., and Moreno,F. (2001) The hexokinase 2 protein regulates the expression of the GLK1, HXK1 and HXK2 genes of Saccharomyces cerevisiae
2. Biochem.J. **355**:625-631.

Roitsch,T., Balibrea,M.E., Hofmann,M., Proels,R., and Sinha,A.K. (2003) Extracellular invertase: key metabolic enzyme and PR protein. J.Exp.Bot. **54**:513-524.

Roitsch,T., Bittner,M., and Godt,D.E. (1995) Induction of apoplastic invertase of Chenopodium rubrum by D-glucose and a glucose analog and tissue-specific expression suggest a role in sink-source regulation. Plant Physiol **108**:285-294.

Rolland,F., De Winde,J.H., Lemaire,K., Boles,E., Thevelein,J.M., and Winderickx,J. (2000) Glucose-induced cAMP signalling in yeast requires both a G-protein coupled receptor system for extracellular glucose detection and a separable hexose kinase-dependent sensing process. Mol.Microbiol. **38**:348-358.

Rolland,F., Moore,B., and Sheen,J. (2002) Sugar sensing and signaling in plants. Plant Cell **14 Suppl**:S185-S205.

Rook,F., Corke,F., Card,R., Munz,G., Smith,C., and Bevan,M.W. (2001) Impaired sucrose-induction mutants reveal the modulation of sugar-induced starch biosynthetic gene expression by abscisic acid signalling. The Plant Journal **26**:421-433.

Rook,F., Gerrits,N., Kortstee,A., van Kampen,M., Borrias,M., Weisbeek,P., and Smeekens,S. (1998) Sucrose-specific signalling represses translation of the Arabidopsis ATB2 bZIP transcription factor gene. Plant J. **15**:253-263.

Rook,F., Weisbeek,P., and Smeekens,S. (1998) The light-regulated Arabidopsis bZIP transcription factor gene ATB2 encodes a protein with an unusually long leucine zipper domain. Plant Mol.Biol. **37**:171-178.

Rugner,A., Frohnmeyer,H., Nake,C., Wellmer,F., Kircher,S., Schafer,E., and Harter,K. (2001) Isolation and characterization of four novel parsley proteins that interact with the transcriptional regulators CPRF1 and CPRF2. Mol.Genet.Genomics **265**:964-976.

Rylott,E.L., Gilday,A.D., and Graham,I.A. (2003) The gluconeogenic enzyme phosphoenolpyruvate carboxykinase in Arabidopsis is essential for seedling establishment. Plant Physiol **131**:1834-1842.

Sambrook,J., Fritsch,E.F., and Maniatis,T. (1998) Molecular Cloning - A Laboratory Manual., 2nd ed. Cold Spring Harbor Laboratory Press (N.Y.).

Schagger,H. and von Jagow,G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal.Biochem **166**:368-379.

Schmidt,M.C., McCartney,R.R., Zhang,X., Tillman,T.S., Solimeo,H., Wolfl,S., Almonte,C., and Watkins,S.C. (1999) Std1 and Mth1 proteins interact with the glucose sensors to control glucose-regulated gene expression in Saccharomyces cerevisiae. Mol.Cell Biol. **19**:4561-4571.

Sheen,J., Zhou,L., and Jang,J.C. (1999) Sugars as signaling molecules. Curr.Opin.Plant Biol. **2**:410-418.

Sheu,J.J., Yu,T.S., Tong,W.F., and Yu,S.M. (1996) Carbohydrate starvation stimulates differential expression of rice alpha-amylase genes that is modulated through complicated transcriptional and posttranscriptional processes. J.Biol.Chem. **271**:26998-27004.

Sinha,A.K., Hofmann,M.G., Romer,U., Kockenberger,W., Elling,L., and Roitsch,T. (2002) Metabolizable and non-metabolizable sugars activate different signal transduction pathways in tomato. Plant Physiol **128**:1480-1489.

Slocombe,S.P., Laurie,S., Bertini,L., Beaudoin,F., Dickinson,J.R., and Halford,N.G. (2002) Identification of SnIP1, a novel protein that interacts with SNF1-related protein kinase (SnRK1). Plant Mol.Biol. **49**:31-44.

Smeekens,S. (2000) Sugar-Induced Signal transduction in Plants. Annu.Rev.Plant Physiol.Plant Mol.Biol. **51**:49-81.

Strathmann,A., Kuhlmann,M., Heinekamp,T., and Droge-Laser,W. (2001a) BZI-1 specifically heterodimerises with the tobacco bZIP transcription factors BZI-2, BZI-3/TBZF and BZI-4, and is functionally involved in flower development. Plant J **28**:397-408.

Sturm,A. and Tang,G.Q. (1999) The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. Trends Plant Sci. **4**:401-407.

Sugden,C., Donaghy,P.G., Halford,N.G., and Hardie,D.G. (1999) Two SNF1-related protein kinases from spinach leaf phosphorylate and inactivate 3-hydroxy-3-methylglutaryl-coenzyme A reductase, nitrate reductase, and sucrose phosphate synthase in vitro. Plant Physiol **120**:257-274.

Sun,C.W. and Callis,J. (1997) Independent modulation of Arabidopsis thaliana polyubiquitin mRNAs in different organs and in response to environmental changes. Plant J. **11**:1017-1027.

Tenson,T. and Ehrenberg,M. (2002) Regulatory nascent peptides in the ribosomal tunnel. Cell **108**:591-594.

Tiessen,A., Prescha,K., Branscheid,A., Palacios,N., McKibbin,R., Halford,N.G., and Geigenberger,P. (2003) Evidence that SNF1-related kinase and hexokinase are involved in separate sugar-signalling pathways modulating post-translational redox activation of ADP-glucose pyrophosphorylase in potato tubers. Plant J. **35**:490-500.

Valvekens,D., Van Montagu,M., and Van Lijsebettens,M. (1988) Agrobacterium tumefaciens mediated transformation of A. thaliana root explants by using kanamycin seletion. Proc.Natl.Acad.Sci.U.S.A **85**:5536-5540.

Vilela,C. and McCarthy,J.E. (2003) Regulation of fungal gene expression via short open reading frames in the mRNA 5'untranslated region. Mol.Microbiol. **49**:859-867.

Vincent,O., Townley,R., Kuchin,S., and Carlson,M. (2001) Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. Genes Dev. **15**:1104-1114.

Wang,L. and Wessler,S.R. (2001) Role of mRNA secondary structure in translational repression of the maize transcriptional activator Lc(1,2). Plant Physiol **125**:1380-1387.

Wang,Z. and Sachs,M.S. (1997) Arginine-specific regulation mediated by the Neurospora crassa arg-2 upstream open reading frame in a homologous, cell-free in vitro translation system. J.Biol.Chem. **272**:255-261.

Wang,Z. and Sachs,M.S. (1997) Ribosome stalling is responsible for arginine-specific translational attenuation in Neurospora crassa. Mol.Cell Biol. **17**:4904-4913.

Weise,A., Barker,L., Kuhn,C., Lalonde,S., Buschmann,H., Frommer,W.B., and Ward,J.M. (2000) A new subfamily of sucrose transporters, SUT4, with low affinity/high capacity localized in enucleate sieve elements of plants. Plant Cell **12**:1345-1355.

Wenzler,H., Mignery,G., Fisher,L., and Park,W. (1989) Sucrose-regulated expression of a chimeric potato tuber gene in leaves of transgenic tobacco plants. Plant Mol.Biol. **13**:347-354.

Wilkie,G.S., Dickson,K.S., and Gray,N.K. (2003) Regulation of mRNA translation by 5'- and 3'-UTR-binding factors. Trends Biochem.Sci. **28**:182-188.

Wobus,U. and Weber,H. (1999) Sugars as signal molecules in plant seed development. Biol.Chem. **380**:937-944.

Xiao,W., Sheen,J., and Jang,J.C. (2000) The role of hexokinase in plant sugar signal transduction and growth and development 2. Plant Mol.Biol. **44**:451-461.

Yaman,I., Fernandez,J., Liu,H., Caprara,M., Komar,A.A., Koromilas,A.E., Zhou,L., Snider,M.D., Scheuner,D., Kaufman,R.J., and Hatzoglou,M. (2003) The zipper model of translational control: a small upstream ORF is the switch that controls structural remodeling of an mRNA leader. Cell **113**:519-531.

Yanagisawa,S., Yoo,S.D., and Sheen,J. (2003) Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. Nature **425**:521-525.

Yang,S.H., Berberich,T., Sano,H., and Kusano,T. (2001) Specific association of transcripts of tbzF and tbz17, tobacco genes encoding basic region leucine zipper-type transcriptional activators, with guard cells of senescing leaves and/or flowers. Plant Physiol **127**:23-32.

Yokoyama,R., Hirose,T., Fujii,N., Aspuria,E.T., Kato,A., and Uchimiya,H. (1994) The rolC promoter of Agrobacterium rhizogenes Ri plasmid is activated by sucrose in transgenic tobacco plants. Mol.Gen.Genet. **244**:15-22.

Yu,S.M., Kuo,Y.H., Sheu,G., Sheu,Y.J., and Liu,L.F. (1991) Metabolic derepression of alpha-amylase gene expression in suspension-cultured cells of rice. J.Biol.Chem. **266**:21131-21137.

Zhou,L., Jang,J.C., Jones,T.L., and Sheen,J. (1998) Glucose and ethylene signal transduction crosstalk revealed by an Arabidopsis glucose-insensitive mutant. Proc.Natl.Acad.Sci.U.S.A **95**:10294-10299.

Zourelidou,M., Torres-Zabala,M., Smith,C., and Bevan,M.W. (2002) Storekeeper defines a new class of plant-specific DNA-binding proteins and is a putative regulator of patatin expression. The Plant Journal **30**:489-497.

Samenvatting voor iedereen

Planten kunnen suikers produceren uit licht, water en koolstofdioxide. Dit proces staat bekend als fotosynthese. Het geproduceerde suiker wordt in de vorm van sucrose in grote hoeveelheden gedistribueerd door de plant. De afstemming van de suikerproductie en distributie is van cruciaal belang voor de plant. Een uitgebreid regelmechanisme zorgt ervoor dat deze processen vlekkenloos verlopen. Voor verschillende suikers zijn verschillende regelmechanismen geïdentificeerd. Nog niet alle componenten van het regelmechanisme voor het suiker sucrose zijn ontdekt. Eén component is in het plantje *Arabidopsis thaliana* (zandraket) ontdekt. Tijdens een zoektocht naar licht-gereguleerde eiwitten is de bZIP transcriptie factor ATB2 geïdentificeerd. Nu blijkt dat de transcriptiefactor ATB2 niet alleen door licht, maar ook door sucrose wordt gereguleerd. Tijdens dit promotie onderzoek is het regelmechanisme voor sucrose onderzocht.

Een transcriptiefactor is een eiwit dat genen aan of uit kan zetten. Voordat een eiwit gemaakt wordt, hebben zich al verschillende processen afgespeeld. Een gen is een stukje DNA dat de blauwdruk van een eiwit bevat. Eerst worden er vele kopieën van de blauwdruk gemaakt, het RNA. Van het RNA wordt uiteindelijk de informatie voor het eiwit gelezen. Ribosomen vertalen de informatie van het RNA molecuul tot een eiwit. Er zijn verschillende manieren bekend om de eiwitproductie te reguleren. De meest voorkomende manier om eiwitproductie te reguleren is de regulering van de hoeveelheid RNA. Wanneer er geen RNA gesynthetiseerd wordt, vindt ook geen synthese van eiwitten plaats. Soms komt het echter voor dat er wel synthese van het RNA plaatsvindt, maar geen synthese van eiwitten. Regulatie van de synthese van eiwitten onafhankelijk van de synthese van RNA is de tweede manier van regulatie. Het RNA wordt dan in de normale hoeveelheid gemaakt, maar wordt niet afgelezen door de ribosomen. Wanneer eiwitproductie op de tweede manier gereguleerd wordt, blijkt dat het RNA een stukje extra informatie bevat. Het *ATB2* gen bevat ook deze extra informatie en wordt dus door 2 verschillende systemen gereguleerd. RNA wordt gemaakt onder invloed van licht, maar wordt niet afgelezen als er veel sucrose aanwezig is.

Om meer inzicht te krijgen in het regelmechanisme door sucrose is een techniek ontwikkeld om planten te isoleren die een niet normale regulatie laten zien (deze planten zijn mutant). Mutant isolatie is een veel gebruikte en geaccepteerde manier om meer informatie te verkrijgen over een proces. Mutanten worden

gemaakt door het DNA van kleine fouten te voorzien (muteren). Een mutantverzameling wordt gemaakt door zeer veel planten willekeurig te muteren. In deze mutantverzameling wordt gezocht naar een plant die mutant is voor de regulatie door sucrose. Omdat er veel planten gemuteerd worden, is het noodzakelijk om snel en met zo min mogelijk werk veel planten te bekijken. Normaal gesproken wordt het geproduceerde eiwit zichtbaar gemaakt met behulp van een kleuring. Een groot nadeel is dat planten voor de kleuring opgeofferd moeten worden. Met behulp van een gen uit het vuurvliegje kan het eiwit zichtbaar gemaakt worden, zonder dat het plantje opgeofferd hoeft te worden. Het plantje gaat licht geven op plaatsen waar het eiwit aanwezig is en zo kan er snel bekeken worden of het eiwit wel of niet aanwezig is.

In hoofdstuk één van dit proefschrift wordt de ontwikkeling van deze methode beschreven. Normaal gesproken wordt het effect van sucrose op de eiwitproductie in vloeibaar medium getest en worden de zaailingen na het groeien in vloeibaar medium gekleurd. Deze vloeibare methode is omgezet naar een methode met vast medium. Ook is er naar de meest optimale omstandigheden gezocht, zodat duidelijk onderscheid kan worden gemaakt tussen zaailingen die wel en geen licht uitzenden. Op deze manier zijn planten geïdentificeerd die met veel sucrose weinig licht laten zien. Deze planten zijn gebruikt voor het identificeren van mutanten. In het tweede hoofdstuk wordt omschreven hoe de mutant collectie gemaakt is en gescreend is voor mutanten. Mutanten die geïdentificeerd zijn als gestoord in de sucrose regulatie zijn nader bestudeerd. Het bleek dat de geïdentificeerde mutanten niet de mutanten waren die wij zochten. Waarschijnlijk zijn dergelijke mutanten niet-levensvatbaar en is het onmogelijk om deze mutanten met deze werkwijze te isoleren.

In hoofdstuk drie is de RNA structuur van het *ATB2* gen nader bekeken. Door iedere keer stukjes van het RNA molecuul af te halen, is het gebied dat verantwoordelijk is voor de sucrose regulatie geïdentificeerd. In dit gebied is een structuur geïdentificeerd dat wij het sucrose control-uORF hebben genoemd (SC-uORF). Om te laten zien dat het SC-uORF daadwerkelijk verantwoordelijk is voor deze regulatie, is er een specifieke mutatie aangebracht. Deze mutatie zorgt ervoor dat de extra informatie niet meer beschikbaar is, terwijl het RNA molecuul in tact wordt gelaten. Uit deze experimenten is gebleken dat het SC-uORF inderdaad verantwoordelijk is voor de sucrose regulatie. Om zeker te weten dat andere sequenties van het RNA geen rol spelen, is het SC-uORF in een ander gen geplaatst en is naar de sucrose regulatie gekeken. Het blijkt dat het SC-uORF alleen voldoende is voor sucrose regulatie.

Al is het op dit moment niet duidelijk of andere componenten nodig zijn, het SC-uORF speelt in ieder geval een belangrijke rol in de sucrose regulatie. Met de identificatie van het SC-uORF is een start gemaakt met de ontrafeling van het sucrose specifieke regelmechanisme.

Dankwoord

Zo, klaar! Erg fijn om aan deze pagina te kunnen beginnen. Misschien is dit wel de belangrijkste pagina van mijn boekje aangezien deze het meest gelezen zal worden. Al is het traject anders verlopen dan ik verwacht had, ik ben blij dat het nu ten einde is gekomen. Het is naast een leerzame periode gelukkig ook een leuke periode geweest. Graag wil ik iedereen die direct en indirect een bijdrage heeft geleverd bedanken. Ik geloof dat het de traditie is om een aantal mensen specifiek te noemen en dat zal ik dus ook doen. Ten eerste: Mirjam, jij zal zeker ook blij zijn dat het achter de rug is. Geen chagrijnige gezichten meer omdat het schrijven niet zo best ging. Dank je voor je knuffels die je me gegeven hebt toen ik ze nodig had. Sjef, bedankt voor de vrijheid die je mij gegeven hebt. De mensen van het lab, waarvan ik een aantal mensen specifiek wil noemen: Adillah, Anja, Anne, Auke, Barry, Bas^2, Evelien, Fatima, Jeanine, Jolanda, Lisette, Marco, Marcel, Marjolein, Marten, Shanna, Sjeng, Tita en Wietske. Zonder jullie was het niet zo gezellig geworden. Also I would like to especially thank Johannes Hanson for showing me the Russian way of life. De wortels, voor hun gezelligheid en interesse. Mijn ouders, voor hun interesse en steun. Sebastiaan, mijn broertje, voor de ontspannende borrels die we samen gehouden hebben. En iedereen die ik vergeten ben.

Curriculum Vitae

Nico Elzinga werd op 4 augustus 1974 geboren in Amsterdam. Na afronding van de Mavo in 1990 heeft hij in 1992 het Havo diploma gehaald. In hetzelfde jaar is hij met de studie biotechnologie aan de HLO begonnen. De stage werd gelopen bij voormalig S&G seeds (Novartis) onder begeleiding van Peter de Haan en Tony de Rover. Na het behalen van het HLO-diploma, is hij in hetzelfde jaar begonnen met de studie biologie aan de UvA. Bij Zeneca-Mogen heeft hij stage gelopen onder begeleiding van Maarten Stuiver en Jerome Custers. Na het succesvol afronden van deze studie, is hij in 1998 begonnen met het in dit proefschrift beschreven onderzoek bij de vakgroep Moleculaire Planten Fysiologie aan de Universiteit van Utrecht, onder begeleiding van Sjef Smeekens.

Colour supplement

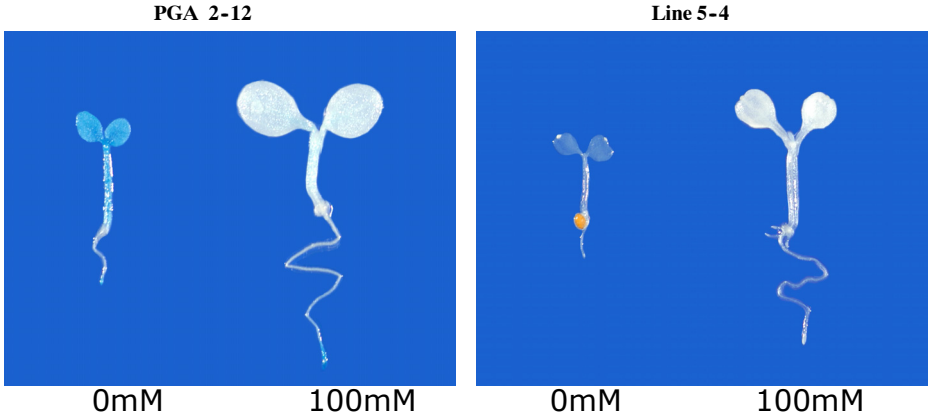

Figure 2: Gus stained seedlings grown in different sucrose concentrations. The *ATB2* transgenic plants harboring different constructs were tested for the response of the reporter gene to sucrose: line PGA 2-12 (4A) (Rook, F. et al. 1998) and line GL 5-4 (4B). Line GL 5-4 contains a double reporter construct. Seedlings were grown for 5 days in constant light with or without sucrose before being harvested and stained.

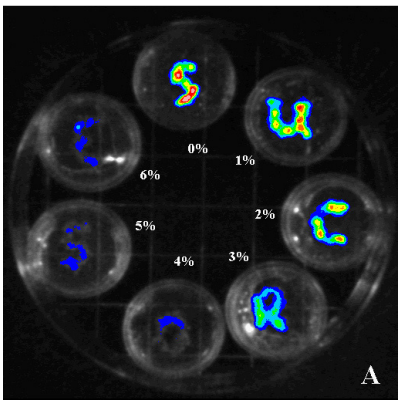

Figure 3: ATB2 repression by sucrose on solid medium. Line GL5-4 (*Arabidopsis thaliana* ecotype C24), containing the dual reporter system, was grown on plates containing 0,5 MS medium with increasing concentrations of sucrose (0-6%). Luciferase (figure 3A) and Glucuronidase activity (figure 3B) were determined by luminescence and enzymatic assays, respectively.

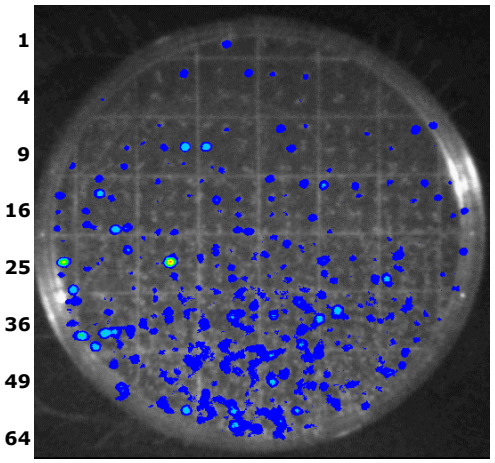

Figure 4: Luminescence levels of seedlings sown in a low to high density order. The top of the plate contains few seedling and the number of seedlings increases towards the bottom of the plate. The numbers indicated left of the plate indicate the number of seeds present in a square. Seeds were grown on solid medium with 4% sucrose for 5 days under constant light before luminescence levels were determined

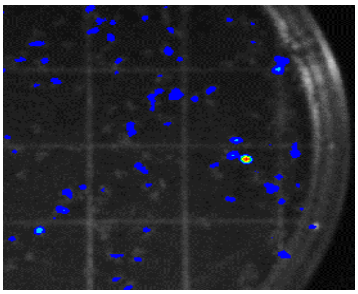

Figure 1: CCD image of an EMS-mutant displaying enhanced luminescence levels. EMS treated seeds were sterilized and sown on solid MS-medium containing 4% sucrose. After incubation of 5 days in the growth chamber the seeds were assayed for luciferase expression by measuring luminescence.

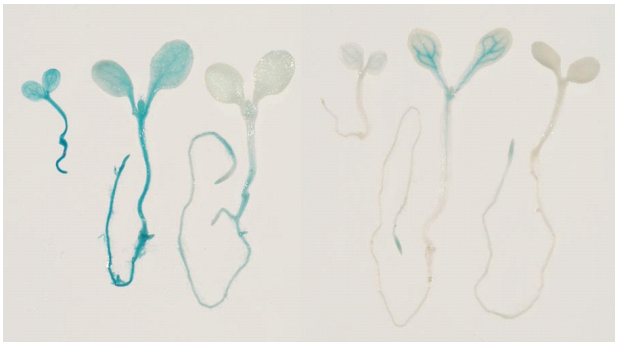

Figure 7: Histochemical staining of seedlings transformed with a construct were the *ATB2* leader sequence was separated from endogenous *ATB2* sequences. The *ATB2* leader sequence, driven driven by the ubiquitine10 promoter (Sun, C. W. and Callis, J. 1997) (7A), is used to confer repressability of the *GUS/GFP* reporter gene, line ubi 6-3 was used. 7B: controle line 7-1 which contains PGA construct (Rook, F. et al. 1998). Seedlings were grown 5 days in liquid medium with different concentrations of sucrose, the seedlings were stained for activity. From left to right 0, 20 and 100 mM sucrose.

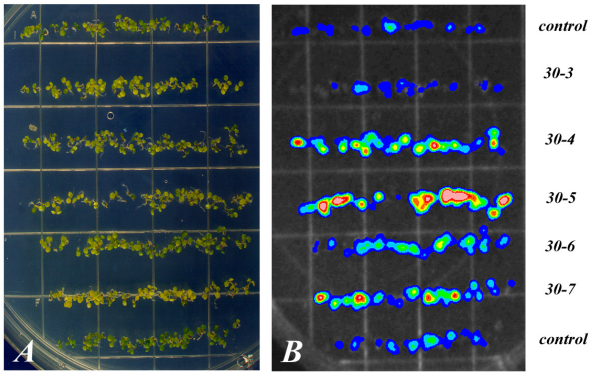

Figure 2A: Picture displaying offspring seeds of primary EMS-mutants that were sown on solid medium containing 0,5 MS-salts and 4% sucrose. Approximately 50 seeds per line were incubated 5 days in the growth chamber under constant light. Figure 2B: CCD image of propagated primary EMS-mutants. Luminescence levels were measured, mutant lines display high luminescence levels.