Chapter 2

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

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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.
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 millimolar 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 ß-glucuronidase coding sequence and the white box represents the Luciferase coding sequence.](image)

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.](image)
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 inch (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 overall 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.
Relative mRNA levels
WT (Line 5-4)

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 quantitative 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 ubiquitine 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 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 than 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 mutagenized 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 promoter 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 Xba1 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 4°C 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 22°C 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% Triton 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 Dellaporote protocol (Dellaporote, 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.

Quantitative PCR RNA was isolated using the Purescript, RNA isolation kit from Gentra Systems (Minneapolis) according to the manufactures instructions. RNA was made DNA-free with the DNA-free™ kit from Ambion Ltd (Cambridgeshire). The DNA free RNA was checked for DNA by PCR with a primerspair for ATB2 (forward AGACGATCTAACGGCTCAGGTT reverse TGCGTGTACGCTGACACTT) and TUB4, (forward TGGACAATGAGGCTCTCTACG, reverseCAGGGAAACGAAGACGAGC )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™ 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})^{\frac{\Delta Ct_{target}}{\Delta Ct_{ref}}} 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 quantitative 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.

<table>
<thead>
<tr>
<th>primers</th>
<th>probes</th>
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<tbody>
<tr>
<td><strong>GUS</strong> forward</td>
<td>5’AACCCCAACCGTGAAATC 3’</td>
</tr>
<tr>
<td><strong>GUS</strong> reverse</td>
<td>5’CACAGTTTTTCGGATCCAGAC 3’</td>
</tr>
<tr>
<td><strong>LUC</strong> forward</td>
<td>5’TCCATGGTCACCGACGC 3’</td>
</tr>
<tr>
<td><strong>LUC</strong> reverse</td>
<td>5’GGTCCATCTTTCAGCGGA 3’</td>
</tr>
<tr>
<td><strong>ACT</strong> forward</td>
<td>5’GCTGAGAGATTCAGACTGCCCA 3’</td>
</tr>
<tr>
<td><strong>ACT</strong> reverse</td>
<td>5’GCTGAGAGATTCAGACTGCCCA 3’</td>
</tr>
<tr>
<td><strong>ATB2</strong> forward</td>
<td>5’TCGTAGATCCGGAGGAGGT3’</td>
</tr>
<tr>
<td><strong>ATB2</strong> reverse</td>
<td>5’GATCGTCTAGAGGCTTTTGTTC3’</td>
</tr>
<tr>
<td><strong>GUS</strong> probe</td>
<td>5’ACTCGACGGCTGTGGCATTC 3’</td>
</tr>
<tr>
<td><strong>LUC</strong> probe</td>
<td>5’AAGAAAGGCCCCGCGCCATCT 3’</td>
</tr>
<tr>
<td><strong>ACT</strong> probe</td>
<td>5’AAGTCTTTCCAGCCCTCGTGG3’</td>
</tr>
<tr>
<td><strong>ATB2</strong> probe</td>
<td>5’AACGTAAACAGGAGCTCTCAAACCGTGAA3’</td>
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