Sucrose Control of Translation Mediated by an Upstream Open Reading Frame-Encoded Peptide[1][W][OA]

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Regulation of gene expression through translational control is common in many organisms. The Arabidopsis (*Arabidopsis thaliana*) transcription factor bZIP11 is translational repressed in response to sucrose (Suc), resulting in Suc-regulated changes in amino acid metabolism. The 5′ leader of the *bZIP11* mRNA harbors several upstream open reading frames (uORFs), of which the second uORF is well conserved among *bZIP11* homologous genes. The uORF2 element encodes a Suc control peptide (SC-peptide) of 28 residues that is sufficient for imposing Suc-induced repression of translation (SIRT) on a heterologous mRNA. Detailed analysis of the SC-peptide suggests that it functions as an attenuator peptide. Results suggest that the SC-peptide inhibits bZIP11 translation in response to high Suc levels by stalling the ribosome on the mRNA. The conserved noncanonical AUG contexts of *bZIP11* uORFs allow inefficient translational initiation of the uORF, resulting in translation initiation of the scanning ribosome at the AUG codon of the *bZIP11* main ORF. The results presented show that Suc-dependent signaling mediates differential translation of mRNAs containing SC-peptides encoding uORFs.

Developmental and physiological processes in organisms depend on regulation of gene expression. Regulation can occur at several levels, from mRNA synthesis to the control of protein activity. The regulation of transcription has been most intensively studied and involves a plethora of transcription factors that operate through several regulatory mechanisms. However, mRNA levels often are not predictive of the levels or activities of the encoded proteins (Conrads et al., 2005; Gibon et al., 2006; Bianchini et al., 2008) and other regulatory mechanisms are of crucial importance as well.

Upstream open reading frames (uORFs) are translational reading frames present in the 5′ leaders of mRNAs. The presence of uORFs usually inhibits translation of the downstream major ORF (main ORF) as eukaryotic ribosomes generally only initiate translation once per mRNA (Luo and Sachs, 1996; Ruan et al., 1996; Morris and Geballe, 2000; Mize and Morris, 2001; Gopfert et al., 2003). Over 3,000 Arabidopsis (*Arabidopsis thaliana*) mRNAs contain uORFs within their 5′ leader sequences, often referred to as 5′-untranslated regions (5′-UTRs; Hayden and Jorgensen, 2007). Similar uORF frequencies are found in the genomes of other eukaryotes, such as yeast (*Saccharomyces cerevisiae*), mammals, and *Drosophila melanogaster* (Morris and Geballe, 2000; Hayden and Bosco, 2008). uORFs are widespread, but their impact on translation has been studied in detail in relatively few cases. From the examples described, several different mechanisms have been uncovered. The uORF of the Arabidopsis *SAC51* gene (encoding a basic helix-loop-helix-type transcription factor) was shown to inhibit translation of the main ORF (Imai et al., 2006). Recently, this translational inhibition was shown to depend on intrinsic proteins of the ribosome (Imai et al., 2008). A uORF in the *AtNMT1* mRNA is feedback-inhibiting translation in response to choline (Tabuchi et al., 2006). In mammals, a highly conserved uORF-encoded hexapeptide controls *S*-adenosyl-Met decarboxylase translation (Mize and Morris, 2001; Hanfrey et al., 2005). In *Xenopus laevis*, the *Connexin41* mRNA is tightly controlled by the three uORFs through a mechanism that depends on the presence of rare codons within the uORF and results in stalled ribosomes (Meijer and Thomas, 2003). The yeast transcription factor GCN4 is translationally regulated through four uORFs within the *GCN4* mRNA and the starvation-dependent phosphorylation status of eIF2 (Hinnebusch, 2005). The Arabidopsis *bZIP11* gene is translationally controlled by Suc (Rook et al., 1998). Suc-induced repression of translation (SIRT) is dependent

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on the second uORF in the *bZIP11* 5′ leader (uORF2). This uORF encodes a conserved Suc control peptide (SC-peptide) present in four other Arabidopsis bZip genes (*bZIP1*, *bZIP2*, *bZIP44*, and *bZIP53*) and in many other bZip genes from other species (Wiese et al., 2004, 2005). The regulatory uORF motif is present in bZip-encoding genes from plants, but is absent from the genomes of other organisms. The molecular details of this plant-specific signaling mechanism are presently unknown.

Sugars are potent signaling molecules in plants. Suc triggers signaling pathways in all plant tissues and thereby alters gene expression (Wiese et al., 2004). Changed Suc levels within the plant affect photosynthesis, metabolism, and developmental processes. High Suc levels inhibit photosynthesis (Koch, 1996) and increase storage through induction of starch synthesis (Hendriks et al., 2003). During starvation, low levels of Suc result in increased photosynthesis and starch mobilization. Suc levels affect secondary metabolism (Teng et al., 2005; Solfanelli et al., 2006), as well as developmental processes, such as flowering and root development (Ohto et al., 2001; Takahashi et al., 2003). How plants perceive Suc is currently unknown, but Suc sensing via Suc transporters has been suggested (Chiou and Bush, 1998; Sivitz et al., 2008). Several genes seem specifically activated by Suc, including *NR1*, encoding nitrate reductase (Cheng et al., 1992), *patatin* (Wenzler et al., 1989; Jefferson et al., 1990), the phloem cell-specific *rolC* gene (Yokoyama et al., 1994), a UDP-Glc pyrophosphorylase gene (Ciereszko et al., 2001), and *MYB75* (Teng et al., 2005). Among the genes repressed by Suc treatment are *PC* (for plastocyanine; Dijkwel et al., 1997) and *ASN1* (Lam et al., 1998). The Suc-controlled bZIP11 transcription factor was shown to regulate genes involved in amino acid metabolism (Hanson et al., 2008), but probably has a much wider role in reprogramming metabolism (J. Hanson, M. Hanssen, and S. Smeekens, unpublished data). The transcription activation potential of bZIP11 and related proteins is enhanced by the Snf1-related kinases (SnRKs) KIN10 and KIN11 that regulate responses to stress resulting from nutrient deprivation (Baena-Gonzalez et al., 2007). Here, it is shown that the SC-peptide is required and sufficient for SIRT, and a model is proposed where Suc in combination with the SC-peptide leads to ribosome stalling and translational inhibition of the main ORF.

RESULTS

Suc-Dependent Translational Regulation of *bZIP11*

SIRT acts within 24 h of Suc addition (Wiese et al., 2004). The stability of the GUS reporter enzyme precluded detection of more rapid changes in response to Suc. A luciferase (LUC)-based transient expression system was developed based on biolistic gene transfer to investigate the kinetics of SIRT in young seedlings in more detail. Seedlings were transformed with a DNA construct expressing the 5′ leader of *bZIP11* followed by the firefly *LUC* gene directed by the 35S promoter (*35S:bZIP11* 5′ leader:*LUC*) and relative LUC activities were determined. Suc-treated seedlings show significantly reduced LUC activities within 3 h of incubation in Suc-containing medium (Fig. 1A). After 24 h of incubation, the LUC activities of seedlings incubated in medium lacking Suc increase 2- to 3-fold, but the levels of the Suc-treated seedlings remain constant (Fig. 1A). Incubation for longer times than 24 h does not result in higher LUC levels (data not shown). Light, as well as sugars, affects the development and physiology of plants. It has been documented that light effects can be mimicked by sugar treatments (Cheng et al., 1992). However, light or darkness has no effect on SIRT (Fig. 1B).

Glc, at twice the molar ratio of Suc, triggers translational repression as well, but to a much lower extent compared to Suc (Fig. 1C). On the other hand, sorbitol treatment is not affecting the SIRT response. SIRT is therefore concluded to be independent of osmotic responses. Several documented examples indicate that hormone signaling and sugar signaling are tightly interconnected in plants (Smeekens, 2000; Rolland et al., 2006). Mutants in hormone signaling were tested to investigate whether hormone signaling is involved in SIRT. Mutants showing defects in ethylene and abscisic acid (ABA) signaling (*ein2aba2*), cytokinin signaling (*ahk2ahk3*), and auxin signaling (*aux1-21*) all show normal Suc-mediated repression of LUC activity (Fig. 1D). Hormone application to wild-type plants (auxin, GA, cytokinin, ABA, 1-aminocyclopropane-1-carboxylic acid) confirmed that SIRT does not depend on hormone signaling (data not shown).

Suc is readily hydrolyzed to Glc and Fru in planta. The major Glc receptor in plants is HEXOKINASE1 (HXK1; Smeekens, 2000; Rolland and Sheen, 2005; Rolland et al., 2006). Whether SIRT depends on Glc signaling through HXK1 was tested. No difference in relative LUC activities is detected between the wild-type and *gin2-1* (HXK1-null mutant) seedlings (Fig. 1E), indicating that HXK1 does not affect SIRT. Suc treatments promote starch accumulation in plants. The *pgm* mutant is deficient in starch synthesis and accumulates excess Suc during the day (Periappuram et al., 2000). Relatively low LUC activities are observed in untreated *pgm* seedlings, possibly due to elevated Suc levels in the *pgm* mutant. However, Suc-treated *pgm* seedlings exhibit SIRT (Fig. 1E) and changed starch levels therefore seem not to be involved in SIRT.

Sequences Downstream of uORF2 Are Dispensable for Suc Repression

The length of the *bZIP11* 5′ leader is 547 nucleotides. The sequence includes four uORFs followed by an intercistronic region of 169 nucleotides before the start

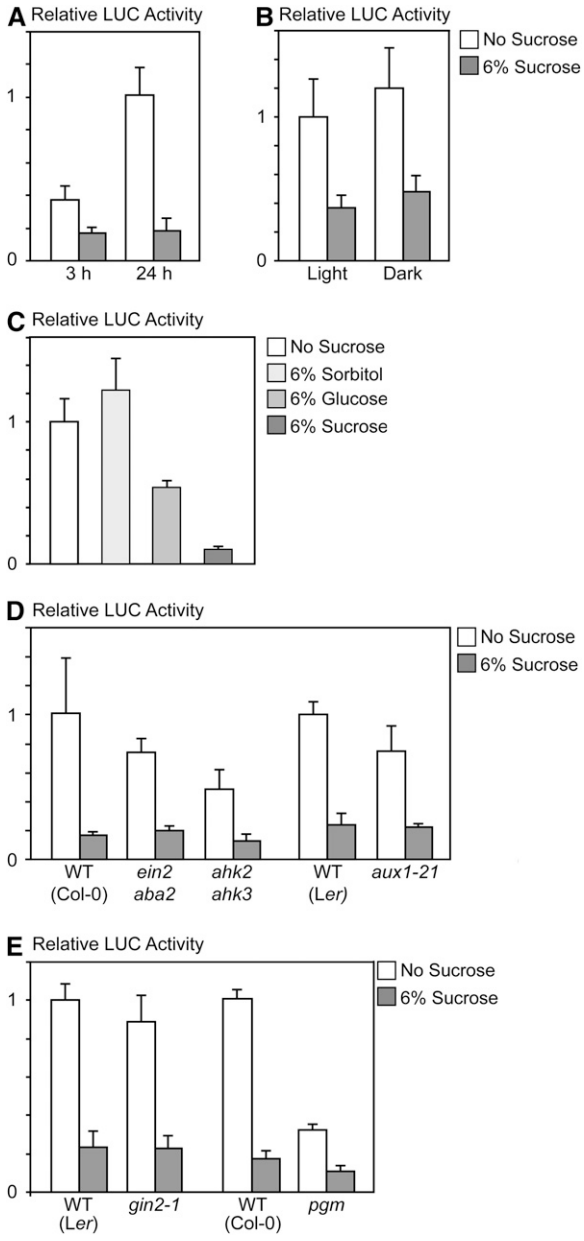

Figure 1. Suc-induced repression of translation is independent of hormone, light, and Glc signaling. Relative normalized LUC activity levels of 10-d-old seedlings transformed with the *35S:bZIP11* 5′ leader:*LUC* construct. Following transformation, seedlings were incubated in medium containing either no Suc (white bars) or Suc (gray bars). Means of at least three biological replicates are presented. Error bars represent SD from the mean. Two or more independent experiments were performed with essentially similar results. A, Relative LUC activity levels of transformed seedlings (accession Col-0) incubated for 3 or 24 h. B, Relative LUC activity levels of transformed seedlings (accession Col-0) incubated for 24 h in either constant light or darkness. C, Relative LUC activity levels of transformed seedlings (accession Col-0) incubated for 24 h in medium supplemented with different sugars. Note that the molar concentration of Glc and sorbitol is close to double that of Suc. D, Relative LUC activity levels of wild-type seedlings compared with that of mutants with altered hormone biosynthesis or signal transduction. Relative LUC levels were compared to that of the corresponding wild-type accessions of the mutants used (*ein2aba2* and *ahk2ahk3* double mutants, Col-0; and *aux1-21*, L*er*). E, Relative LUC activity levels of wild-type seedlings and mutants with altered sugar signal transduction or metabolism. LUC activity levels were compared with that of the corresponding accession of the mutants (*gin2/hxk1*, L*er*; and *pgm*, Col-0). WT, Wild type.

codon of the main *bZIP11* ORF (Fig. 2A). SIRT depends on a AUG start codon within uORF2 (Wiese et al., 2004). To determine whether other regions of the *bZIP11* 5′ leader were important for repression, a series of deletions of the *bZIP11* 5′ leader were tested for repression activity. Suc-dependent repression of LUC levels is detected in seedlings transformed with all constructs containing 330 nucleotides of the leader or more (Δ330, Δ343, Δ392, Δ423, Δ455, and Δ493). In contrast, no repression of LUC levels is detected in seedlings transformed with the shorter construct (Δ313). This indicates that a critical region is located between the Δ313 and Δ330 deletions. This region includes the end of uORF2 and the start codons of uORF3 and uORF4. The possible involvement of uORF3 and uORF4 in SIRT was tested by mutations in which the start codons of these two uORFs were removed, while leaving the amino acid sequence of the uORF2 peptide unaltered. These modified leaders impose SIRT (Fig. 2C), demonstrating that the start codons of uORF3 and uORF4 are dispensable for SIRT. The results further show the importance of the peptide sequence encoded by uORF2, as the mRNA nucleotide sequence in the critical region could be mutated without affecting SIRT. The importance of the peptide sequence is also indicated by the limited conservation of the third codon position compared to the first two positions of the codons of homologous uORFs (Supplemental Fig. S1).

uORF2 Confers SIRT on an Independent mRNA

As previously shown, only the second part of uORF2 starting with the second AUG codon is needed for SIRT (uORF2b; Wiese et al., 2004). This 82-nucleotide-long uORF2 element was transplanted to the unrelated leader of the *At1g20340* (encoding a plastocyanin protein) gene of Arabidopsis, which lacks uORFs. The element encodes the carboxy-terminal 28 amino acids encoded by uORF2. The *At1g20340* leader does not affect the translation of the downstream LUC reporter in response to Suc treatments (Fig. 3). In contrast, Suc-induced repression of LUC activity was detected in the *At1g20340:uORF2* seedlings, indicating that the *bZIP11* element used is sufficient to impose SIRT on the 5′ leader of an unrelated mRNA. Non-Suc-treated seedlings transformed with the *At1g20340:uORF2* construct show approximately 3-fold less LUC activity compared to seedlings transformed with the *At1g20340* construct. This demonstrates the general inhibitory effect of uORF2 on translation of the main ORF.

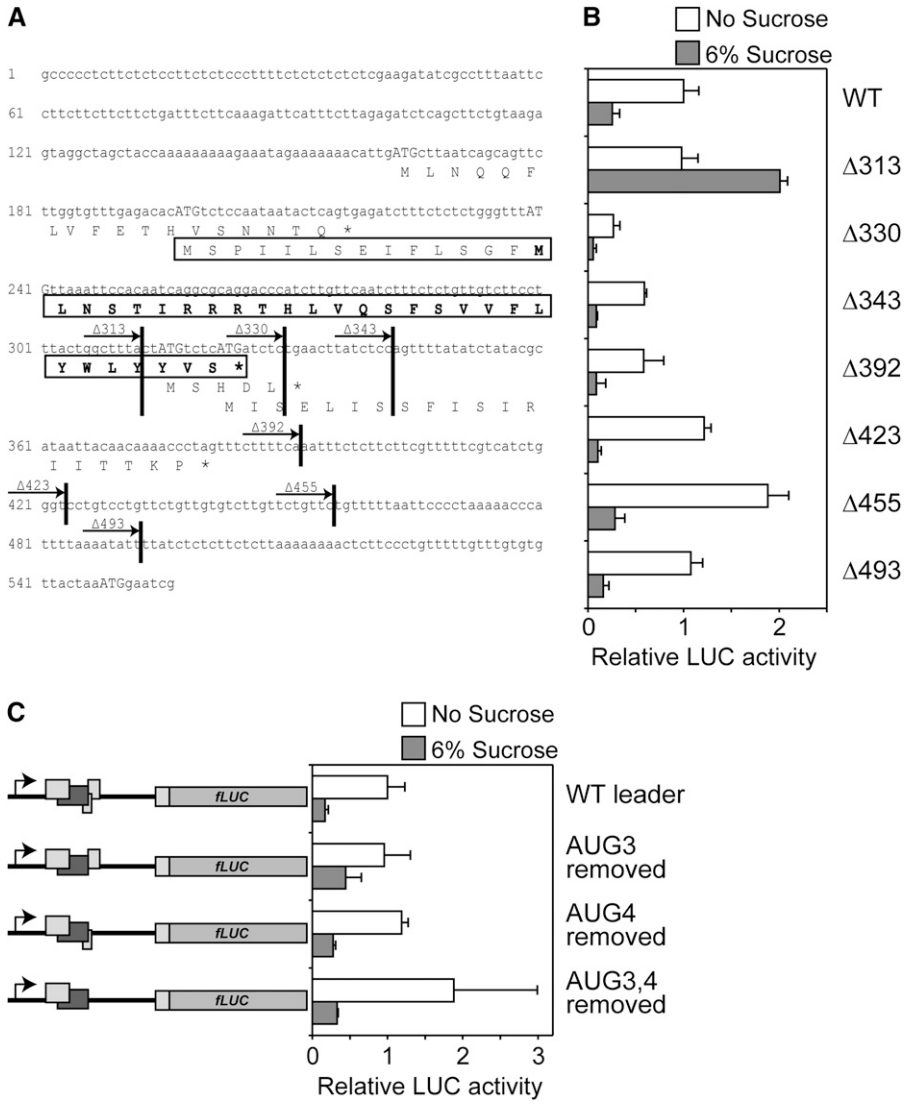

Figure 2. Suc-induced repression of translation is uORF2 dependent. Relative normalized LUC activity levels of 10-d-old seedlings transformed with the *35S:bZIP11* 5′ leader:*LUC* construct or mutant versions thereof. Following transformation, seedlings were incubated in medium containing either no Suc (white bars) or 6% Suc (gray bars) for 24 h. Means of at least three biological replicates are presented. Error bars represent SD from the mean. Two or more independent experiments were performed with essentially similar results. A, Nucleotide sequence of the *bZIP11* 5′ leader. uORFs and the translated peptide sequences are shown below the nucleotide sequence in capital letters. The start codons (ATG) of the respective uORFs are indicated in capital letters. uORF2 is encoding a 42-amino-acid-long peptide (boxed) and the minimal peptide sufficient for Suc-induced repression is indicated in bold. The ATG at position 548 represents the start codon of the *bZIP11* main ORF. The vertical lines illustrate the position of deletions in the 5′ leader. The deletion constructs harbor the 5′ leader from start of transcription to the respective vertical line fused to the *LUC* gene. Names of the deletion constructs are indicated above the arrows. B, Relative normalized LUC activity levels of 10-d-old seedlings transformed with the *35S:bZIP11* 5′ leader:*LUC* construct or indicated deletions. C, Relative normalized LUC activity levels of 10-d-old seedlings transformed with the *35S:bZIP11* 5′ leader:*LUC* construct or constructs lacking start codons of uORFs. Rectangles in schematic drawings (left) represent ORFs. Dark gray rectangles represent uORF2 of the *bZIP11* 5′ leader. WT, Wild type.

Suc Repression Depends on Conserved Amino Acids of the SC-Peptide

The amino acid sequence of the peptide encoded by uORF2b is highly conserved, especially in the carboxy-terminal part of the peptide (Supplemental Fig. S1; Wiese et al., 2005; Hayden and Jorgensen, 2007). This might be a consequence of the conserved nucleotide sequence of the mRNA or reflect an evolutionarily important selection pressure on the peptide sequence. To address the question of whether the mRNA sequence or the peptide sequence is important for SIRT, an extra nucleotide at position 245 in the *bZIP11* 5′

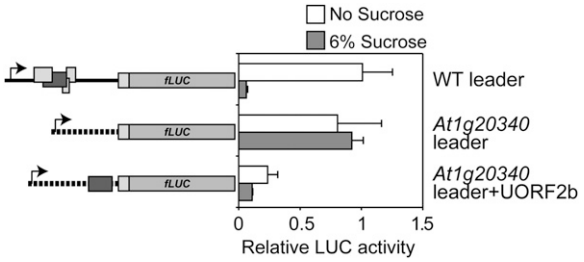

Figure 3. The SC-peptide is sufficient for Suc-induced translational repression. Relative normalized LUC activity levels of 10-d-old seedlings transformed with the *35S*:5′ leader:*LUC* constructs. After transformation, seedlings were incubated for 24 h in medium containing either no Suc (white bars) or 6% Suc (gray bars). Means of at least three biological replicates are presented. Error bars represent SD from the mean. Two or more independent experiments were performed with essentially similar results. Schematic drawings of constructs used for transient expression experiments are indicated to the left. Rectangles represent ORFs. Dark gray rectangles represent uORF2 of the *bZIP11* 5′ leader. The 5′ leader of the *At1g20340* gene is indicated using a dashed line. WT, Wild type.

leader was introduced. The resulting frame shift changes the peptide sequence, whereas the nucleotide sequence of the uORF2 region shown to be sufficient for SIRT remains unaltered. The frame shift mutation totally abolished SIRT as shown by relative LUC activity levels upon transient expression of the modified 5′ leader (Fig. 4). Thus, the peptide encoded by uORF2 mediates SIRT, as previously proposed by Wiese et al. (2004).

To test the relative importance of well-conserved amino acids in uORF2, a series of mutations were introduced in uORF2, all resulting in amino acid substitutions of conserved amino acids in the peptide (Fig. 5). Ser-29, Ser-31, Leu-35, Tyr-39, Ser-42, Arg-21, Arg-22, and Arg-23 were substituted to Ala residues. Leu-35 and Tyr-39 were also changed to their structurally similar amino acids Ile and Phe, respectively. The mutated 5′ leader sequences were tested for SIRT. Mutating the Arg-rich stretch or the Ser-42 residue did not affect SIRT because the resulting normalized expression values do not differ from the values of the wild-type 5′ leader (Fig. 5A). However, mutations affecting either Ser-29 or Ser-31, Leu-35, or Tyr-39 result in loss of SIRT (Fig. 5A). The LUC activity levels of the non-Suc-treated seedlings were not affected significantly by the point mutations. Interestingly, the substitution of Tyr-39 to Ala (Y39A) abolishes Suc repression, whereas replacing it with the structurally related amino acid Phe (Y39F) resulted in a reduced repression (Fig. 5A).

The position of the stop codon within uORF2 is also well conserved (Supplemental Fig. S1). The importance of the stop codon position for SIRT was tested by introducing three mutations in the *bZIP11* 5′ leader that affected the length and sequence of the uORF2. One mutation lengthened the peptide by 13 residues, late stop, and the other shortened the peptide by two residues, early stop. In another mutated 5′ leader, two frame shift mutations in opposite directions were introduced in the beginning of the sequence and in the end, respectively. These mutations give rise to a uORF2 sequence encoding a peptide of wild-type length, but with an unrelated sequence, except for the first two and last two residues (double frame shift). The mutated 5′ leaders are not able to confer SIRT as determined by LUC activity measurements (Fig. 5B). Thus, both the amino acid sequence and stop codon position of the SC-peptide are important for SIRT. Mutations in the mRNA sequence might affect their steady-state levels and therefore mRNA levels were quantified. The results show that the absence of SIRT in the mutants cannot be explained by changes in mRNA levels, in agreement with earlier observations (Supplemental Fig. S2; Rook et al., 1998; Wiese et al., 2004).

Leaky Scanning Is Important for bZIP11 Protein Translation

In eukaryotes, uORFs generally inhibit translation of the downstream main ORF (Vilela and McCarthy, 2003). The SC-peptide encoded by uORF2 of the *bZIP11* 5′ leader efficiently represses bZIP11 translation in response to Suc. Sequences surrounding the AUG codon (the AUG context) affect the efficiency of translational initiation by scanning translational preinitiation complexes (PICs). The AUG contexts of uORF2 differ from the consensus sequence of plants, indicating that initiation of translation of uORF2 is inefficient. In contrast, the AUG context of the main ORF is more similar to the consensus sequence. This

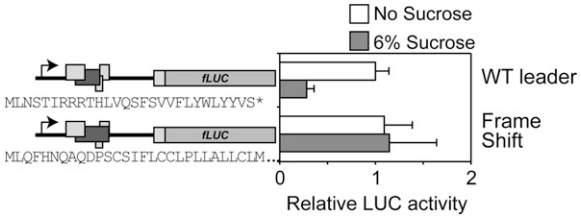

Figure 4. The mRNA sequence of the *bZIP11* 5′ leader is not sufficient to trigger Suc-induced repression of translation. Relative normalized LUC activity levels of 10-d-old seedlings transformed with the *35S*:*bZIP11* 5′ leader:*LUC* construct or a mutated version. Following transformation, seedlings were incubated in medium containing either no Suc (white bars) or 6% Suc (gray bars). Means of at least three biological replicates are presented. Error bars represent SD from the mean. Two or more independent experiments were performed with essentially similar results. Schematic drawings of constructs used for transient expression experiments are indicated on the left. Rectangles represent ORFs. Dark gray rectangles represent uORF2 of the *bZIP11* 5′ leader. The frame shift mutated 5′ leader harbors a single base pair insertion giving rise to a frame shift and therefore a total change of the sequence of the Suc control peptide, as indicated. The nucleotide insertion is situated in the 5′ part of uORF2, which is not required for SIRT (Fig. 3). WT, Wild type.

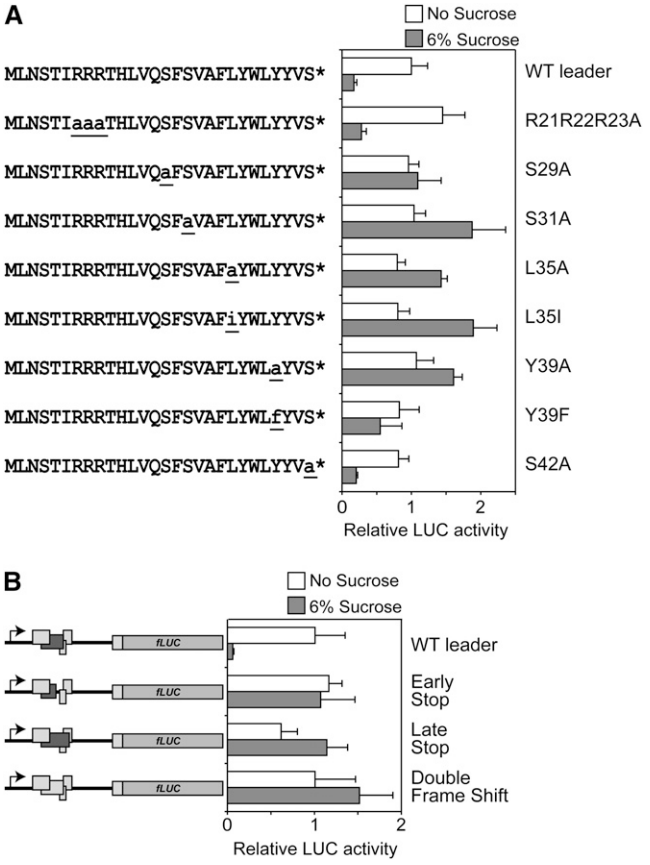

Figure 5. Conserved amino acids and the stop codon position of the Suc control peptide are necessary for Suc-induced repression of translation. Relative normalized LUC activity levels of 10-d-old seedlings transformed with the *35S:bZIP11* 5′ leader:*LUC* construct or versions with mutated 5′ leaders. Following transformation, seedlings were incubated for 24 h in medium containing either no Suc (white bars) or 6% Suc (gray bars). Means of at least three biological replicates are presented. Error bars represent SD from the mean. Two or more independent experiments were performed with essentially similar results. A, The amino acid sequence of the 28 C-terminal amino acids of the SC-peptide or mutated versions used for transformation experiments are indicated on the left. Changed amino acids are indicated in underlined lowercase letters. B, Schematic drawings of constructs used for transient expression experiments are indicated to the left. Rectangles represent ORFs. Dark gray rectangles represent uORF2. The mutated 5′ leaders encode either a shorter uORF2, early stop (the last two residues missing); a longer uORF2, late stop (13 residues added after the intact uORF2 sequence); or a combinatorial mutant, double frame shift, in which a frame shift mutation (Fig. 3) is combined with another frame shift mutation at the end of uORF2. This latter construct encodes a uORF2 peptide in which only the first two and last two residues are the same as in the wild-type counterpart. WT, Wild type.

AUG context pattern also holds for *bZIP11* homologous genes of Arabidopsis, as well as from other species (Fig. 6A). Probably, the scanning PICs are prone to skipping the uORF start codon while efficiently initiating translation of the main ORF. To test this hypothesis, the internal AUG context of uORF2 (uuuAUGuu) was optimized to the aaaAUGgc sequence (Fig. 6B). This consensus sequence has been shown to yield maximal initiation frequency in higher eukaryotes (Joshi et al., 1997) and corresponds to the dicot consensus sequences of initiation context (Rangan et al., 2008). Scanning PICs that pass the weak first start codon of uORF2 should be efficiently captured by an optimized internal AUG context and initiate translation of uORF2. The second start codon was chosen for modification as the first part of the peptide was shown to be dispensable for SIRT (Fig. 3) and due to the fact that only one AUG is needed to cause SIRT (Wiese et al., 2004). Moreover, the second AUG is not fully conserved among SC-peptide homologs; therefore,

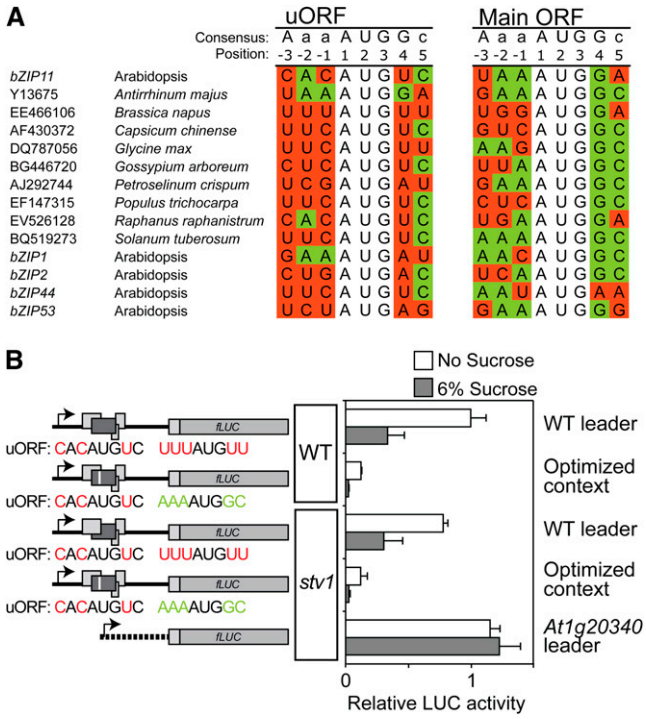

Figure 6. The main ORF is translated through a leaky scanning-dependent mechanism. A, AUG codon contexts of *bZIP11* and homologous genes from other dicotyledonous species and Arabidopsis. The conserved positions are indicated in green if the sequence matches the consensus sequence and in red if the nucleotide does not match the consensus sequence. The indicated consensus sequence is that of dicots (Rangan et al., 2008). B, Relative normalized LUC activity levels of 10-d-old seedlings transformed with the *35S:bZIP11* 5′ leader:*LUC* construct or mutated versions. Following transformation, seedlings were incubated for 24 in medium containing either no Suc (white bars) or 6% Suc (gray bars). The constructs were either transformed into wild-type seedlings (accession Wassilewskija-0) or *stv1* seedlings, as indicated. Means of at least three biological replicates are presented. Error bars represent SD from the mean. Two or more independent experiments were performed with essentially similar results. Schematic drawings of constructs used for transient expression experiments are indicated on the left. Rectangles represent ORFs. Dark gray rectangles represent uORF2 of the *bZIP11* 5′ leader. The AUG contexts are indicated below the schematic drawings and colored in accordance with similarity to the consensus sequence (A). The positions of the mutations are indicated with a white stripe in the boxes. WT, Wild type.

minimal secondary effects of the change are expected (data not shown). The mutated 5′ leader was tested for SIRT using the transient expression assay and compared to the activity of the wild-type 5′ leader. The results of transient expression assays show that improving the AUG context of uORF2 retains SIRT, but dramatically reduces translation of the main ORF (Fig. 6B), providing support for a leaky scanning-dependent mechanism operating at the uORF initiation codons.

Ribosomes probably dissociate after translating uORF2. To test whether translational reinitiation occurs after termination of uORF2 translation, the *short valve1* (*stv1*) mutant was analyzed for SIRT. This mutant was shown to be deficient in translational reinitiation (Nishimura et al., 2005), but was not found to affect SIRT (Fig. 6B). The relative LUC activity of an *At1g20340* construct lacking uORFs was not affected by Suc in the *stv1* mutant, confirming the equal translatability of mRNAs in the mutant treated with or without Suc.

DISCUSSION

Diurnal changes in sugar levels affect the expression levels of approximately one-third of the Arabidopsis genes, highlighting the central role of sugar signaling in plants (Bläsing et al., 2005). The Arabidopsis transcription factor bZIP11 is translationally regulated by Suc (Rook et al., 1998; Wiese et al., 2004) and was shown to regulate a subset of sugar-regulated genes in Arabidopsis (Hanson et al., 2008). bZIP11 activation results in extensive changes in gene expression and appears involved in metabolic reprogramming (Hanson et al., 2008; J. Hanson, M. Hanssen, and S. Smeekens, unpublished data). Likely, bZIP11 is part of a stress-dependent adaptation system that involves the SnRK1 (KIN10, KIN11) protein kinase pathway. In protoplast transfection assays, KIN10 activates the transactivation activity of bZIP11 and other Suc-regulated bZIP proteins (Baena-Gonzalez et al., 2007). It appears that bZIP11 is a node in a signaling network that activates genes in response to stress or starvation. Importantly, bZIP11 translational repression in response to Suc abrogates the downstream bZIP11 response pathway. Here, the mechanism of SIRT has been investigated and a novel mechanistic model is proposed.

Translational Repression of bZIP11 Is Independent of Light and Hormone Signaling

SIRT has previously been shown to act within 24 h of Suc addition. The stability of the GUS reporter protein prohibited more accurate testing of the response (Wiese et al., 2004). A LUC-based transient transformation system showed that SIRT acts within a few hours (Fig. 1A); likely, SIRT acts in a much shorter time frame, but this could not be tested due to initial low LUC activity levels.

Sugar signaling has been shown to be tightly interconnected to light and hormone-signaling pathways (Smeekens, 2000; Rolland and Sheen, 2005; Rolland et al., 2006). For example, the light effect on expression of the *NR* gene is replicated by sugar treatments (Cheng et al., 1992; Ciereszko et al., 2001). Several Glc-insensitive mutants identified were shown to be allelic to ABA-insensitive biosynthetic or signaling mutants and the effects of Glc were suggested to be dependent on increased levels of ABA compared to untreated seedlings (Arenas-Huertero et al., 2000; Huijser et al., 2000; Rook et al., 2001). Sugar signaling is also connected to ethylene signaling (Gibson et al., 2001; Arroyo et al., 2003; Moore et al., 2003; Yanagisawa et al., 2003), cytokinin, and auxin signaling (Ohkama et al., 2002; Ohto et al., 2006). Assaying SIRT in plants subjected to darkness or in various mutant backgrounds impaired in hormonal signaling demonstrated normal SIRT to occur. This indicates the independence of SIRT signaling of these signaling pathways.

HXK1 is an important Glc sensor in plants and was shown to be involved in several sugar responses (Rolland et al., 2006). Suc is readily metabolized to Glc and Fru in plants, but SIRT is not dependent on the HXK1 pathway as determined by the analysis in *gin2* (*HXK1*-null) mutants. Recently, a G protein-dependent Glc-signaling pathway was identified (Grigston et al., 2008). However, the stronger effect of Suc compared to Glc (Fig.1C; Wiese et al., 2004) indicates that SIRT depends on a novel, Suc-dependent signaling pathway. The possibility that starch levels affect SIRT is excluded because the starchless *pgm* mutant shows SIRT. The generally lower LUC levels in *pgm* seedlings compared to wild-type seedlings could be due to the higher sugar levels of the *pgm* mutant during the day (Periappuram et al., 2000).

Suc Repression Is Mediated by a 28-Amino-Acid-Long Peptide

The *bZIP11* 5′ leader contains four partly overlapping uORFs, followed by a 169-nucleotide-long intercistronic region lacking ORFs. Systematic shortening of the *bZIP11* 5′ leader from the 3′ direction and testing for functionality in planta showed that the intercistronic region was dispensable for SIRT. The Δ330 construct displays SIRT, whereas the 17-nucleotide shorter Δ313 construct lacks SIRT activity. The 313 to 330 region of the *bZIP11* mRNA includes the start codons of uORF3 and uORF4, but mutational analysis showed that uORF3 or uORF4 are not involved in the repression mechanism.

Translation of the 28 C-terminal amino acid residues of uORF2 are sufficient to impose SIRT (Wiese et al., 2004). Transplantation of this region to the 5′ leader of the *At1g20340* gene is sufficient to impose SIRT (Fig. 3). This 3′ part of uORF2 sequence shows pronounced conservation at both amino acid and nucleotide levels (Supplemental Fig. S1). The importance of the peptide sequence was demonstrated by insertion of a single base pair in the 5′ nonessential part of the 5′ leader,

which abolishes SIRT (Fig. 4). Mutations in the 5′ leader that did not result in changed amino acid sequence of the SC-peptide did not affect SIRT (Fig. 2). Therefore, nucleotide sequence conservation of uORF2 is most likely due to selection pressure on the peptide sequence, as shown by limited conservation at the third position of the codon of the uORF2 sequence (Supplemental Fig. S1). Together, this indicates the importance for SIRT of the 28-amino acid SC-peptide encoded by the 3′ part of uORF2. This SC-peptide has also been shown to be translated in vitro (Wiese et al., 2004). Mutational analysis has shown that inhibiting translation of uORF2 abolishes SIRT (Wiese et al., 2004). Mutagenesis showed the highly conserved Ser-29, Ser-31, Leu-35, and Tyr-39 to be essential for SIRT. These single amino acid substitutions disrupt functionality of the SC-peptide. Interestingly, changing Tyr-39 to the structurally similar amino acid Phe conferred some repression activity. This highlights the sequence dependence of the repression system and indicates that the SC-peptide interacts with other molecules to repress translation. Such proposed interaction occurs only in Suc-treated plants because SC-peptide amino acid changes specifically affect the translation in plants treated with Suc.

The stop codon position of uORF2 is evolutionarily conserved. Changing the stop position by lengthening the uORF2 abolishes SIRT. uORFs are common within mRNAs of plants and other eukaryotes and most uORFs act in a sequence-independent way (Hayden and Jorgensen, 2007). However, several examples exist in which the protein sequence of the uORF is conserved and therefore is believed to be of regulatory importance. The *AdoMetDC* and the *SAC51* genes in Arabidopsis harbor uORFs with conserved protein sequences (Hanfrey et al., 2005; Hu et al., 2005; Imai et al., 2006). In these cases, the uORFs repress translation like the uORF2 in *bZIP11*.

Translation of the *bZIP11* Main ORF

The eukaryotic translational apparatus first interacts with the 5′ cap structure of the mRNA and then scans the mRNA for the first available AUG codon within an appropriate sequence context to initiate translation. Once the ORF translation is terminated, the ribosomal subunits separate and dissociate from the mRNA. As a consequence, eukaryotic mRNAs generally only encode one ORF. However, several translational mechanisms have been documented that allow translation of ORFs preceded by uORFs (Kozak, 2002). For example, an internal ribosome entry site (IRES) in the mRNA allows the ribosome to directly associate with a sequence within the mRNA. Several plant viral RNAs are translated through IRES sequences (Jaag et al., 2003; Dorokhov et al., 2006; Karetnikov and Lehto, 2007), but so far only one cellular plant mRNA was shown to be translated through an IRES-dependent mechanism (Dinkova et al., 2005). Translation of *bZIP11* does not depend on IRES as all sequences 3′ of the uORF2 can be deleted without affecting translation of the main ORF (Fig. 2A). This excludes the possibility of secondary structures in the mRNA leading to ribosome shunting as documented for several virus RNAs (Xi et al., 2005; Pooggin et al., 2006). Ribosomes are able to reinitiate translation after translating short uORFs (Kozak, 2002), but this seems unlikely for *bZIP11* because, in that case, the AUG context-dependent improvement of uORF2 translational initiation would lead to increased main ORF translation, whereas the opposite is observed. This conclusion is supported by the results from the analysis of the *stv1* mutant, which is impaired in translation reinitiation (Nishimura et al., 2005), but shows normal SIRT. Moreover, reinitiation after translation of a peptide longer than 35 amino acids is impossible in yeast (Vilela and McCarthy, 2003). The complete uORF2 is 42 amino acids long and thus to long for reinitiation. The distance between the uORF and the main ORF can be important for reinitiation (Kozak, 2001). Changing the distance between the stop codon of the SC-peptide and the AUG of the main ORF does not affect SIRT (Fig. 2A). Taken together, we conclude that a mechanism dependent on reinitiation appears unlikely.

Inefficient translation initiation of uORF2 is important for translation of the main ORF (Fig. 6) as the optimization of the AUG2b context strongly represses translation of the main ORF. This observation suggests that ribosomes failing to initiate translation of the weak context of uORF2 continue scanning and translate the main ORF. This notion is also supported by the experiments using the *At1g20340* 5′ leader:uORF2 construct with an improved, but not perfect, context, translation of the main ORF is strongly reduced, further supporting the conclusion that the weak AUG contexts of the uORF are important for efficient main ORF translation (Fig. 3). Most of the known *bZIP11* homologs have noncanonical AUG context sequences of the uORFs, whereas sequences that allow efficient translation initiation surround the start codons of the main ORFs (Fig. 6). This indicates that the main ORF is translated by leaky scanning ribosomes and that this mechanism is evolutionary conserved. bZIP11 translation requires eIF3h (Kim et al., 2004). eIF3h was suggested to increase the processivity of the scanning ribosomes (Kim et al., 2007), which is in agreement with our model of bZIP11 translation. Leaky scanning was also found to be required for the translation of other plant genes, such as *AtLIG1* (Sunderland et al., 2006) and *THI1* (Chabregas et al., 2003). Inserting a strong uORF2b AUG context in uORF2 reduces main ORF translation, but Suc repression is retained (Fig. 6). This important observation excludes a regulatory model in which main ORF translation is regulated by differential initiation of translation of the repressing SC-peptide in response to Suc. Such regulation of translation by uORFs was observed in the translational regulation of *AdoMetDC* (Hanfrey et al., 2005).

We conclude that *bZIP11* is translated by leaky scanning ribosomes and that translation of the SC-peptide is inhibiting translation of bZIP11 in the presence of Suc. Suc concentrations as low as 20 mM already reduce main ORF translation; thus SIRT acts at physiologically relevant Suc concentrations (Wiese et al., 2004, 2005). SIRT is lost in mutants with changed amino acid sequences of the uORF2 encoding SC-peptides, indicating that specific interaction with other biomolecules is important. When translated, nascent peptides move from the peptidyl transfer center through the exit tunnel of the ribosome. The exit tunnel covers approximately 40 amino acids (Matlack and Walter, 1995). Thus, during translation, the SC-peptide interacts with ribosome-associated molecules. The different parts of the translational apparatus are fixed in position relative to the peptidyl transfer center of the ribosome during translation elongation. The stop codon position of the SC-peptide is important for SIRT as shown experimentally (Fig. 5) and by essentially total evolutionary conservation of the stop codon position (Supplemental Fig. S1; Hayden and Jorgensen, 2007). Changes in stop codon position will move the relative position of the SC-peptide in the exit tunnel at termination of translation. Thus, positioning of the SC-peptide is important for SIRT, possibly as the SC-peptide interacts with a component of the translational apparatus, which is located in the exit tunnel. Changing the Tyr-39 residue abolished SIRT, which indicates that the SC-peptide must be translated entirely for biological activity and even the last residue of the SC-peptide is evolutionarily conserved. This implies that SIRT occurs at translational termination of the SC-peptide. In mammalian and cytomegalovirus systems, ribosome stalling depending on the position of the stop codon of the uORF has been documented (Cao and Geballe, 1995; Janzen et al., 2002). Likely the nascent SC-peptide stalls the translating ribosome in the presence of Suc and thereby halts the translation of *bZIP11*. The presence of a stalled ribosome at the mRNA will efficiently block scanning PICs from reaching the *bZIP11* AUG codon and thus inhibit translation of the transcription factor. The nature of the molecule interacting with the SC-peptide remains to be elucidated.

The bZIP11 transcription factor was shown to specifically activate Suc-repressed genes in Arabidopsis (Hanson et al., 2008). Four other closely related bZip transcription factors in Arabidopsis harbor the SC-peptide encoding uORF in the 5′ leader sequence and show SIRT as well (Weltmeier et al., 2009). SIRT thus represents a general molecular mechanism of Suc-controlled gene expression in plants. In this study, the molecular nature of this Suc control was investigated and a ribosomal stalling mechanism suggested for SIRT. This novel translational control mechanism provides further understanding of how Suc regulates gene expression in plants and is important with respect to the proposed biological function of bZip transcription factors in reprogramming metabolism in response to stress and starvation (Hanson et al., 2008).

MATERIALS AND METHODS

Plant Growth Conditions

Wild-type or mutant Arabidopsis (*Arabidopsis thaliana*) seedlings (var Columbia-0 [Col-0], Wassilewkija-0, or Landsberg *erecta* [L*er*]) were grown for 10 d on one-half-strength Murashige and Skoog medium, pH 5.7 (Duchefa), supplemented with 0.2% Suc, 0.5 mg/mL MES (Sigma-Aldrich), and 14 g/L plant agar (Duchefa). The seedlings were grown in growth chambers (Snijders Scientific) at 22°C under constant fluorescent light (100 μmol m^{-2} s^{-1}).

Construction of *bZIP11* 5′ Leader Vectors Used for Transient Expression Experiments

A Gateway-compatible destination vector, p35S-ccdB-LUC, containing the 35S promoter in front of firefly *LUC* (fLUC) was constructed using the *Xba*I/*Sac*I restriction fragment of pGWB35 (provided by Dr. T. Nakagawa, Shimane University, Matsue, Japan) containing the *fLUC* gene with Gateway sites for translational fusion. This was ligated into the 35S promoter-containing vector (pUC19 based). The wild-type *bZIP11* 5′-leader was amplified using primers with Gateway sites (*bZIP11* 5′-UTR FWD, *bZIP11* 5′-UTR REV) and transferred to pDONR-Zeo vector. The resulting plasmids were sequenced and the inserts were transferred to the p35S-ccdB-LUC destination vector by LR reaction, according to the manufacturer's instructions (Invitrogen). The deletion constructs were amplified by using primer *bZIP11* 5′-UTR FWD and *bZIP11*Δ313, Δ*330*, Δ*342*, Δ392, Δ*423*, Δ*455*, and Δ*493* (Supplemental Table S1). For mutated versions of the *bZIP11* 5′ leader, the whole 5′ leader fragment, including Gateway sites, was translocated to the pALTER vector (Promega) using traditional methods (Sambrook et al., 1989). The resulting clone was used as a mutagenesis template using the Altered Sites II in vitro mutagenesis system kit (Promega), according to the manufacturer's instructions and indicated primers listed (Supplemental Table S1). Mutated 5′ leaders were moved to p35S-ccdB-LUC using Gateway technology.

To fuse the 84-nucleotide-long fragment of the uORF2 to the *At1g20340* 5′ leader, this region was amplified using uORF2FWDBamHI and uORF2REV primers (Supplemental Table S1). The PCR fragment was subcloned into pGemT-easy vector (Promega). The *At1g20340* 5′ leader was amplified using the *At1g20340*-leader FWD and *At1g20340*-leader REV *Bam*HI primers and the amplified fragment was subcloned into the pGemT-easy vector (Promega). The uORF2 plasmid was restricted by *Bam*HI/*Nco*I and the resulting fragment was ligated into the *Bam*HI/*Nco*I sites of the *At1g20340* 5′ leader vector. The resulting insert was transferred to the p35S-ccdB-LUC vector using Gateway technology. The *At1g20340* 5′ leader for the control plasmid was amplified using the *At1g20340*-leader FWD and *At1g20340* Gateway REV primers and transferred to the p35S-ccdB-LUC vector using Gateway technology. The integrity of the final constructs was confirmed by sequencing (Macrogen).

Transient Transformation of Arabidopsis Seedlings

Gold particles (1 μm diameter; Bio-Rad) were coated with plasmid DNA according to Giovanna et al. (1998). DNA for coating was premixed in a concentration equivalent of 1.2 mg fLUC vector and 0.4 mg Renilla LUC (rLUC) vector per transient expression experiment. The rLUC vector includes the *rLUC* gene driven by the constitutive 35S promoter. Seedlings were transformed using the Biolistic particle delivery system, model PDS-1000 He (Bio-Rad), according to the manufacturer's instructions using a vacuum adjusted to 28 Pa and 900-psi rupture discs. Prior to particle bombardment, selected seedlings (approximately 50) were transplanted to fresh plates. Following particle bombardment, one-half of the transformed seedlings were transferred into 250-mL flasks containing 50 mL of liquid one-half-strength Murashige and Skoog medium supplemented with 6% Suc and the other half were transferred to flasks containing medium without Suc. The seedlings were incubated in growth chambers, rotary shaking (45 rpm) under constant light for 24 h, and harvested in liquid nitrogen. All experiments were independently replicated and yielded similar results as the ones presented in this article. More than 90% of the seedlings were transformed as determined in

parallel experiments using a plasmid harboring the *GUS* reporter gene and histochemical staining (data not shown).

LUC Activity Assays

Relative LUC levels were determined by the ratio of fLUC activity to rLUC activity. The LUC activities were measured by the Dual-Luciferase reporter assay system kit, according to the manufacturer's instructions (Promega). Approximately 25 mg ground Arabidopsis tissue were lysed using 100 μL passive lysis buffer supplemented with 2 mM DDT and incubated for 15 min at room temperature, followed by 2-min centrifugation (rcf 16,000). Twenty microliters of the supernatant were transferred into a new 2-mL tube and processed according to the Dual-Luciferase reporter assay system kit manual (Promega). LUC activity was measured using the TD-20/20 Glomax luminometer (Promega). All experimental series included transient expression of the wild-type *bZIP11* 5′ leader. The normalized fLUC activity levels were adjusted to the value of the plants transformed with the wild-type 5′ leader treated with medium lacking Suc. All activity levels were measured using three replicates minimum, averaged, and SD from the mean was calculated.

Quantitative PCR Analysis

Total RNA was isolated from 10-d-old seedlings (var Col-0) subjected to transient transformation. Plant tissues were homogenized by grinding in liquid nitrogen and total RNA was isolated using the RNeasy kit (Qiagen). DNA was removed from the preparations using RNase-free DNase I (Fermentas). cDNA was synthesized using M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Due to low RNA yield, RNA preparations from three independent preparations were pooled and used as substrate for cDNA synthesis. Real-time quantitative PCR was performed using an ABI7900HT sequence detector using SYBR Green II master mix (Applied Biosystems). The *ACTIN2* gene (*At3g18780*) was used as an internal reference. Relative RNA levels were calculated by the ΔΔCt method (Pfaffl, 2001). Primer efficiency was determined as described by Rasmussen (2001). Sequences of primers used in quantitative real-time PCR reactions are listed in Supplemental Table S2.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EE466106, AF430372, DQ787056, BG446720, AJ292744, EF147315, EV526128, and BQ519273.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Evolutionary conservation of uORF2b sequences in Arabidopsis and other plant species. Amino acid sequence of uORF2b is more conserved than nucleotide sequence.

Supplemental Figure S2. Different *LUC* mRNA levels in transiently transformed Arabidopsis seedlings do not correlate with LUC activity levels.

Supplemental Table S1. Primers used to construct wild-type and mutated forms of the *bZIP11* 5′ leaders used for transient expression.

Supplemental Table S2. Primers used for quantitative real-time PCR.

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