Overexpression of the activated form of the 
\textit{AtAREB1} gene (\textit{AtAREB1}\textit{\Delta QT}) improves soybean responses to water deficit

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\textbf{ABSTRACT.} Abscisic acid-responsive element binding protein (AREB1) is a basic domain/leucine zipper transcription factor that binds to the abscisic acid (ABA)-responsive element motif in the promoter
region of ABA-inducible genes. Because AREB1 is not sufficient to direct the expression of downstream genes under non-stress conditions, an activated form of AREB1 (AREB1\(\Delta QT\)) was created. Several reports claim that plants overexpressing AREB1 or AREB1\(\Delta QT\) show improved drought tolerance. In our studies, soybean plants overexpressing AREB1\(\Delta QT\) were characterized molecularly, and the phenotype and drought response of three lines were accessed under greenhouse conditions. Under conditions of water deficit, the transformed plants presented a higher survival rate (100%) than those of their isoline, cultivar BR 16 (40%). Moreover, the transformed plants displayed better water use efficiency and had a higher number of leaves than their isoline. Because the transgenic plants had higher stomatal conductance than its isoline under well-watered conditions, it was suggested that the enhanced drought response of AREB1\(\Delta QT\) soybean plants might not be associated with altered transpiration rates mediated by ABA-dependent stomatal closure. However, it is possible that the smaller leaf area of the transgenic plants reduced their transpiration and water use, causing delayed stress onset. The difference in the degree of wilting and percentage of survival between the 35S-AREB1\(\Delta QT\) and wildtype plants may also be related to the regulation of genes that protect against dehydration because metabolic impairment of photosynthesis, deduced by an increasing internal CO\(_2\) concentration, was not observed in the transgenic plants.

**Key words:** AtAREB1\(\Delta QT\); Biolistics; Drought tolerance; Soybean; Transcription factor; Water use efficiency

**INTRODUCTION**

Although plants have gradually evolved a remarkable ability to tolerate periods of water deficit, drought still represents the primary cause of crop loss worldwide. Actually, the use of genetic engineering techniques to modify the expression patterns of drought-responsive genes appears to be a useful tool to modify or regulate metabolic pathways that aim to cope with abiotic stresses and diminish yield losses of economically important crops. The development of drought-tolerant crop plants exclusively through genetic engineering still needs to be proven in real field conditions. However, the available data for numerous engineering strategies to develop drought tolerance suggests that future commercial varieties will be developed using both genetic engineering and traditional molecular marker-assisted breeding selection.

During an abiotic stress event, plants show an array of biochemical and physiological changes through the expression of a large number of stress-responsive genes. These activated genes can be classified as genes involved in cellular metabolism, stress tolerance, and regulatory genes including various kinases, phosphatases, and transcription factors (TFs) (Cheong et al., 2003; Agarwal and Jha, 2010). TFs, individually or combined with others factors, play a key role in the activation or repression of signal transduction pathways once they recognize specific DNA sequences in the regulatory regions of target genes regulating their expression (Barbosa et al., 2013).

Among the nine members of the abscisic acid (ABA)-responsive element-binding
protein (AREB)/ABA-responsive element (ABRE)-binding factor (ABF) TF family identified in *Arabidopsis thaliana*, AREB1 has been reported to regulate environmental stress responses and ABA signaling during the vegetative stage (Jakoby et al., 2002; Fujita et al., 2005; Corrêa et al., 2008; Yoshida et al., 2010). The AREB1/ABF2 TFs are basic leucine zipper (bZIP) proteins that regulate ABA-dependent stress-responsive gene expression, and they act as a major TF under abiotic stress conditions in *A. thaliana* (Kobayashi et al., 2008; Lee et al., 2010).

Several studies have demonstrated the importance and potential of AREB1 and bZIP proteins in abiotic stress tolerance (Fujita et al., 2011; Gao et al., 2011; Kim et al., 2011; Reeves et al., 2011; Barbosa et al., 2013). However, the overexpression of AREB1 under the control of the cauliflower mosaic virus (*CaMV*) 35S promoter (35S-AREB1) on its own is insufficient to induce the expression of downstream genes such as *RD29B* in normal growth conditions because AREB1 requires ABA for full activation. Additionally, its activity is regulated by ABA-dependent multisite phosphorylation within conserved domains (Furihata et al., 2006). The SnRK2 family of kinases has been reported to be involved in the phosphorylation and modification of AREB/ABF TFs that are induced by ABA (Fujita et al., 2005; Nakashima et al., 2009; Yoshida et al., 2010).

To confirm that the N-terminal P region of AREB1 functions as a transcriptional activation domain of the *AREB1* gene, Fujita et al. (2005) constructed a series of effector plasmids bearing N-terminal deletion mutations of AREB1 under the control of the constitutive *CaMV* 35S promoter. A small deletion of 60 amino acids (P region) from the N terminus of AREB1 resulted in a significant decrease in the transactivation of the reporter gene in protoplasts treated either with or without ABA.

Later, Fujita et al. (2005) showed that the AREB1ΔQT mutant protein containing the P region and the bZIP DNA-binding domain, when co-transfected together with *RD29B-GUS*, resulted in a significant activation of the *GUS* reporter gene even in the absence of ABA, confirming that the N-terminal P region of the *AREB1* gene contained a transcriptional activation domain and that AREB1ΔQT was the constitutive active form of AREB1 in plants and protoplasts.

The overexpression of AREB1ΔQT under the control of the *CaMV* 35S promoter in *A. thaliana* activated the expression of the downstream *RD29B* gene in the absence of exogenous ABA. This overexpression also led to the expression of late embryogenesis abundant (LEA) proteins and the activation of ABRE-dependent signal transduction pathways. Transgenic plants of *Arabidopsis* were hypersensitive to ABA, and nearly all survived a lack of water for 12 days in the drought-tolerance test. Overall, the overexpression of AREB1ΔQT resulted in the expression of downstream genes responsible for protecting plants from water deficit stress and enhanced their tolerance to drought (Fujita et al., 2005).

In soybean, the first report of transformation using the full-length *AREB1* gene (35S::*AtAREB1* construct) was presented by Barbosa et al. (2013). These authors showed that the introduction of the foreign *AREB1* gene helped the transgenic plants to cope with five days of water deficit more efficiently than the wild-type (WT) plant. The genetically modified (GM) plants exhibited no growth retardation, high survival rate, and no leaf damage after water stress; additionally, they showed better physiological performance under water deficit (higher relative rate of shoot length, stomatal conductance, and photosynthesis) than the WT plants.

Thus, this study aimed to determine if the insertion of the activated form of the
AtAREB1 gene (AREB1∆QT) in the soybean cultivar BR 16, which is considered to be sensitive to drought (Oya et al., 2004), would improve the drought responses of the transformed plants in glasshouse conditions without other deleterious effects.

MATERIAL AND METHODS

Soybean transformation and polymerase chain reaction (PCR) analysis

Embryos of cultivar BR 16 were co-transformed with two expression vectors, 35S::AtAREB1∆QT (Fujita et al., 2005) and 35S::ahas, by particle bombardment according to the method described by Aragão et al. (2000) and Rech et al. (2008). The transformation method, selection, and regeneration procedures were described in Barbosa et al. (2013).

The pBI35S-AREB1∆QT vector contained the 4486-bp 35S::AtAREB1∆QT construct (Fujita et al., 2005), and the pAC321 vector (Rech et al., 2008) (Figure S1A and S1B) included a 8673-bp fragment containing the mutated Atahas gene (GenBank accession No. AL133315, position 35,863-41,580). The ahas gene was under the control of the Arabidopsis ahas promoter and terminator. The Atahas gene contains a mutation at position 653 of the coding sequence (GenBank accession No. X51514), which results in a Ser-to-Asp substitution that confers imidazolinone herbicide-specific resistance (Aragão et al., 2000).

The T0 transformed plantlets were PCR-screened with specific primers (Table S1) to identify those containing the AtAREB1∆QT and ahas gene constructs. Amplification was performed as described in Barbosa et al. (2013).

Transgene copy number quantification using real-time PCR

For the quantitative PCR (qPCR) analysis, total DNA was extracted as described by Doyle and Doyle (1987). Gene models from the soybean genome (www.phytozome.net) that were similar to the transgene coding sequences (CDS) were retrieved (Glyma02g14880, Glyma07g33600, Glyma13g22060, and Glyma10g08370), as were other previously described Glyma genes (Liao et al., 2008a,b; Gao et al., 2011). Multiple alignments of the transgene CDS with the orthologous soybean sequences were performed using Vector NTI 11 and MEGA 4 in order to design primers that would amplify only the introduced AtAREB1∆QT CDS (AtAREB1: F: 5'-GGAGGTGGAGGGTTGACTAGA-3'; P: 5'-TCAACGAGTATATCGAACCTT-3'; and R: 5'-CACTGCTCTGAAACTCATCAAACG-3'). TaqMan qPCR was performed in a 25-µL mixture containing 1X PCR Master Mix buffer, 1 µL DNA sample, and optimal concentrations of the transgene-specific primers and probe (300 nM AtAREB1 primers and 250 nM probe). The amplifications were conducted in triplicate using a 7300 Real Time System thermocycler (Applied Biosystems, Foster City, CA, USA), and the cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. To verify the quantity of the transgene (Bubner and Baldwin, 2004), the endogenous lectin gene, GmLec (Glyma02g01590.1; accession No. K00821), was used in a multiplex reaction as the reference and as the calibrator gene (GmLec: F: 5'-TCCCGAGTGGGTGAGGATAG-3'; P: 5'-TCAACGAGTATATCGAACCTT-3'; and R: 5'-CACTGCTCTGAAACTCATCAAACG-3'). This gene is species-specific and is present as a single copy per haploid genome (Meyer et al., 1994) or as two allelic copies (homozygous) in the soybean. The transgene copy number was calculated
using the formula $2^{-\Delta C_{t}}$, which was derived from Livak and Schmittgen (2001). Here, $-\Delta C_{t}$ is the value of the sample tested in triplicate, and the number 2 corresponds to the sum of the amplification efficiencies of the target gene (100% = 1) and endogenous control (100% = 1).

Copy number estimates using Southern blots and segregation ratios

Soybean genomic DNA samples were extracted and purified from fresh leaves using the cetyltrimethylammonium bromide method (Sambrook et al., 1989). DNA was quantified by ultraviolet (UV) absorption at 260 nm, and the purity was evaluated based on the UV absorption ratio at 260/280 nm and analyzed using 1% agarose gel electrophoresis (0.5X Tris, borate, ethylenediaminetetraacetic acid) and staining with ethidium bromide. The DNA was digested with the HindIII restriction enzyme (Figure 1A). To verify the number of insertions of the transgene, a PCR-amplified probe obtained from amplifying a 447-bp CDS region (F: 5'-TGATGAGTTTCAGAGCAGTG-3'; R: 5'-TCTCCTGATTCTTTTGCATT-3') was synthesized. PCR products were purified on an agarose gel using the DECA Prime Kit, and the fragment containing AtAREB1∆QT was labeled with radioactive α-[32P]-dCTP overnight according to manufacturer instructions. The number of copies was inferred based on the correlation between data obtained by qPCR and Southern blot analysis. The segregation ratio was determined with a $\chi^2$ analysis, rejecting the hypothesis at the 0.05% level.

Relative AtAREB1∆QT gene expression level

Total RNA was isolated from the T$_1$ plants using TRIzol® reagent. RNA was used for cDNA synthesis using Superscript III™ reverse transcriptase (Invitrogen™). The Platinum® SYBR Green® qPCR Super Mix-UDG with ROX kit was used according to manufacturer instructions to perform qPCR. The cycling conditions were the same as those described above, with an additional extension period at the end of 7 min at 72°C for the dissociation curve. The plates were assembled with cDNAs of the samples, AtAREB1∆QT primers (F: 5'-GGAGGTGGAGGGTTGACTAGA-3'; R: 5'-CACTGCTCTGAAACTCATCAAACG-3'), and the Gmß-actin (accession No. GMU60500) primers for an endogenous control (F: 5'-GAGCTATGAATTGCCTGATGG-3'; R: 5'-CGTTTCATGAATTCCAGTAGC-3') according to Stolf-Moreira et al. (2011). Reactions were calibrated with the GM sample A2639.34, which showed the lowest gene expression among all GM lines obtained. Data were analyzed using the Student t-test with a significance level of 5%.

Phenotypic and physiological analysis under water-deficient conditions

After the molecular characterization, three independent lines were retained for further studies based on their copy number (low-copy lines A2651.2 and A2639.14 and high-copy line A2654.14) (Table 1).

Soybean seeds from the T$_2$ generation of all lines were germinated on filter paper for four days in a growth chamber with the temperature and relative humidity set to 25°C ± 1°C and 100%, respectively. The seedlings were cultivated in pots containing a mixture of soil: sand:manure (1:3:1, 26% water-holding capacity) under well-watered conditions until reaching the V$_2$ developmental stage (Fehr et al., 1971). When necessary, water was added...
until the weight of the pot reached 80% of the weight of the pot at saturation. The conditions in the greenhouse included a natural photoperiod with an approximate 12 h light and 12 h dark cycle at 30°C ± 5°C and 60 ± 10% relative humidity. The experimental design was completely randomized, with 10 replicates.

At the V₃ stage, plant growth was measured (day 0 = control condition, C), the pot was saturated with water, and watering was suspended thereafter. Another measurement was performed 10 days after irrigation was withheld (day 10 = drought stress, DS), at the V₅ stage. Plant height, leaf number, number of nodes, mean internode length, and leaf area (length x width) were analyzed under both conditions. Stomatal conductance (gₛ), photosynthetic rate (A), transpiration rate (E), and intercellular CO₂ concentration (Cᵢ) were also evaluated at C and DS using an LI-6400 Portable Photosynthesis System (LI-COR, Lincoln, NE, USA). The measurements were obtained using the middle leaflet of the youngest soybean leaf, which was fully expanded, under a photon flux density of 1.000 µmol·m⁻²·s⁻¹. The instantaneous water use efficiency (WUE_inst) was determined as the ratio between A and E. Plants were then watered, and the recovery, as well as the percentage of foliar damage, were evaluated three days later. The relative rate of shoot growth (RRSG) was calculated according to the following formula: RRSG = (shoot length at day 10 - shoot length at day 0) / shoot length at day 0 x 100%.

Response variables were statistically analyzed by an exploratory diagnostic check for assumptions of normality and the independence of the residue, the non-additivity of the model, and the homogeneity of treatment variances, followed by analysis of variance. After these analyses and when the F test indicated statistical significance, the Duncan test for multiple comparisons among treatment means was applied with α = 0.05 as the level of significance.

RESULTS

Copy number and segregation ratio of the AtAREB1∆QT events

The amplification efficiency of the primers for the AtAREB1∆QT and GmLec genes were 98 and 95%, respectively. The Ct outputs (data not shown) indicated that only a few lines had a standard deviation above 0.3. The transgene copy number that was introduced into the soybean genome is provided in Table 1.

The T₀ generation of line A2639 showed five copies of the transgene by the quantitative method; that is, two and a half times the quantity of the endogenous lectin gene (Table 1). Taken together with the Southern blot result, these data indicate that line A2639 contains four insertions and four copies of the transgene per haploid genome (n). The T₁ generation copy numbers ranged from four to six copies, or two and a half times to three times the amount of the endogenous lectin gene that was detected by the quantitative method. The Southern analysis results presented by some lines indicated the insertion of tandem copies (Table 1, Figure 1A). However, it was not possible to estimate the number of insertions in other lines by the Southern technique; therefore, the copy number of the transgene was only determined by the quantitative method. Our data showed that the number of copies ranged from one to three (Table 1, Figure 1A).

The copy number of the T₀ generation of line A2651 could not be determined because
inconsistencies occurred between the quantitative and Southern blot methods. However, the T1 generation of plants presented five copies, or one or two and a half times the amount of the endogenous lectin gene that was detected by the quantitative method. Considering these results together with the Southern blot data, it can be inferred that these plants have two to three copies of the transgene in their genome (Table 1, Figure 1A).

**Table 1.** Quantity of transgenes by quantitative polymerase chain reaction (qPCR), number of insertions by Southern blot analysis, and copy number of 35S::AtAREB1∆QT in soybean plants of the T0 and T1 generations of lines A2639, A2651, and A2654.

<table>
<thead>
<tr>
<th>Line</th>
<th>Quantity of transgenes (qPCR)</th>
<th>Number of insertions (Southern blot)</th>
<th>Copy number</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2639 T0</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
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<td>4</td>
<td>5</td>
</tr>
<tr>
<td>11 T1</td>
<td>5</td>
<td>ND</td>
<td>5</td>
</tr>
<tr>
<td>14 T1</td>
<td>5</td>
<td>ND</td>
<td>2-3</td>
</tr>
<tr>
<td>15 T1</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
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<td>6</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>24 T1</td>
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<td>2-3</td>
</tr>
<tr>
<td>28 T1</td>
<td>4</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td>31 T1</td>
<td>4</td>
<td>ND</td>
<td>2</td>
</tr>
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<td>1</td>
</tr>
<tr>
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<td>ND</td>
</tr>
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<td>ND</td>
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<td>2</td>
</tr>
<tr>
<td>A2654 T0</td>
<td>12</td>
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<td>6</td>
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<td>17 T1</td>
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<td>15-16</td>
</tr>
</tbody>
</table>

ND = not determined.

In the T0 generation of line A2654, 12 copies were detected by the quantitative assay, which was six times higher than the endogenous gene. Together with Southern blot data, where four insertions were detected, and taking into account the band intensity, it can be inferred that all copies were inserted in tandem. T1 plants of this line showed the same banding pattern as the T0 plant, and more than 10 copies of the transgene were detected in all plants (Table 1, Figure 1A).

The segregation ratio of the lines (A2639, A2651, and A2654) did not present the Mendelian 3:1 pattern of segregation. Instead, a rather complex segregation pattern (1:6, 2:3, and 1:3, respectively) was observed (Table S2). This data may suggest insertions at different loci and the occurrence of duplication or rearrangement.
Relative expression levels of 35S::AtAREB1ΔQT in transgenic soybean lines

The amplification efficiencies of AtAREB1 and Gmβ-actin genes were 80 and 88%, respectively, and neither primer dimerization nor nonspecific product amplification was observed. As expected, the reverse transcription (RT)-qPCR data revealed that the transgene was overexpressed in all lines that were analyzed (Figure 1B), including the
lines that were selected for phenotypic and physiological studies (A2639.14, A2651.2, and A2654.14).

The expression of the transgene in the low-copy lines A2651.2 and A2639.14 was 249.38 and 231.26 times higher than that of the calibrator, respectively. The expression in the A2654.14 high-copy line was 330.57 times greater than that of the calibrator (Figure 1B).

**Phenotypic and physiological analyses of the 35S::AtAREB1∆QT lines**

The results of the plant phenotypic and physiological analyses of the 35S::AtAREB1∆QT lines and the cultivar BR 16 are shown in Tables 2 and 3. Some traits (leaf number, A, g_s, and Ci) had a significant interaction in the genotype (WT, A2651.2, A2654.14, and A2639.14) x water availability (C and DS) comparisons (N = 10) (Table 2). Other traits (number of nodes, height, internode length, WUE, and leaf area) did not have a significant interaction in the genotype x water availability comparisons and were averaged across C and DS (N = 20) (Table 3).

We observed that soybean transformation with the AtAREB1∆QT gene under the control of the 35S promoter affected the growth characteristics of the transformed plants: the GM plants were shorter than WT plants because of smaller internodes, and they had a smaller leaf area (Figure 2A, Tables 2 and 3). Under well-irrigated conditions, the BR 16 cultivar had more leaves than the transformed plants; however, under water stress, the opposite was true. This behavior shows that the transgenic plants were able to continue growing and produced more leaves than the WT plants under water stress (Table 2).

Despite the alterations in growth patterns, all AtAREB1∆QT soybean lines presented higher rates of recovery (100%) when compared to the WT plants (40%) after 10 days of water deprivation followed by three days of re-irrigation (Figure 2B and C). The percentage of plants without any leaf damage was also higher (80%) in the transgenic plants than in the WT plants (10%); additionally, the leaf damage that was observed in the transgenic plants was less severe (Figure 2C). Another interesting feature of the GM line was that it remained viable for growth even under water stress, which was verified by their similar or even higher RRSG (A2639.14) (Figure 2B) and higher number of leaves (Table 2) than in the WT plants.

Gas exchange measurements showed that the transgenic plants exhibited increased g_s values in well-watered conditions, which resulted in increased A but similar WUE (Table 2) in relation to the BR 16 plants. Under water stress, a difference in A and g_s was observed only for the A2639.14 and A2654.14 lines, respectively. WUE was low and negative for BR 16 plants under water stress, showing that respiratory metabolism was predominant in these plants. The effects of the impaired processes related to photosynthesis under water-stress conditions were more evident in BR 16 plants than in transgenic plants because Ci was greater in BR 16 plants than in transgenic plants (Table 2).

After a period of good water availability followed by water stress, the transgenic plants had higher A due to their higher g_s and improved WUE and CO_2 assimilation (lower Ci) when compared to BR 16 plants (Table 3).

Following the criteria of Flexas et al. (2004a), the soybean plants in our study were subjected to severe water stress after suspending irrigation for 10 days because their g_s was lower than 0.1 mol H_2O·m^{-2}·s^{-1} (Table 2; DS).
Water deficit response in GM soybeans with AtAREB1∆QT

A. Transgenic and BR 16 plants under well-watered conditions at the V4 stage. B. Relative rate of shoot growth of the A2651.2, A2654.14, A2639.14, and wild-type (WT) plants after water stress. Values are reported as means (N = 10 plants); the error bars indicate the standard error. C. Transgenic and WT plants after 10 days of water stress followed by 3 days of re-irrigation. Recovery rates were 100% (all transgenic plants) and 40% (BR 16).

Table 2. Phenotype and gas exchange characteristics of the wild-type (WT) cultivar and AREB1∆QT lines under well-watered (C) and water-stressed conditions (DS) (N = 10) at the V2 (C) and V3 stages (DS).

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>g_s</th>
<th>C_i</th>
<th>LN</th>
<th>NOS</th>
<th>H</th>
<th>IML</th>
<th>WUE_inst</th>
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<td>241</td>
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<td>400</td>
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<td>12.3</td>
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A = photosynthesis (µmol CO_2·m^{-2}·s^{-1}); g_s = stomatal conductance (mol H_2O·m^{-2}·s^{-1}); C_i = CO_2 internal concentration (µmol CO_2/mol); LN = leaf number; NOS = number of nodes; H = height; IML = internode mean length; WUE_inst = instantaneous water use efficiency (nmol C/mol H_2O); LA = leaf area. Means followed by the same lowercase letter in the columns of C or DS and the same capital letter for each genotype in C and DS do not differ by the Duncan test at the 5% probability level.
DISCUSSION

Although the AREB/ABF TFs are induced by abiotic stress, being dependent on ABA to regulate stress-responsive genes (Kobayashi et al., 2008; Lee et al., 2010), AREB1∆QT is a constitutively active form of AREB1 in plants and protoplasts (Fujita et al., 2005), and it is ABA-independent. This means that in both well-watered and stress conditions, AREB1∆QT may regulate the activation or repression of downstream genes related to the protection of cellular functions and homeostasis in water deprivation.

In our studies, soybean plants overexpressing \textit{AtAREB1∆QT}, the activated form of AREB1, were evaluated to determine the insertion copy number, transgene expression level, growth abnormalities, and drought responses in greenhouse conditions. The lines obtained had variable quantities of transgene with respect to both the number of insertions and copy number (Table 1) and a high transgene expression level (Figure 1B). Because most of the GM lines behaved similarly and had high \textit{AtAREB1∆QT} expression (Figure 1B), three lines containing a low [\textit{A2639.14} (2-3 copies) and \textit{A2651.2} (3 copies)] and high [\textit{A2654.14} (11-12)] transgene copy number with good seed production were chosen for phenotypic and physiological analyses and assessment of drought responses under greenhouse conditions.

The high level of transgene expression in the line \textit{A2654.14} supported the idea a high copy number does not always result in a low level of transgene expression due to gene silencing mechanisms. This finding differed from that of Barbosa et al. (2013) where a high copy number caused a low level of expression of the \textit{35S::AtAREB1FL} construct. Therefore, although the \textit{AREB1} gene plays a key role in the ABA-dependent drought response pathway in plants, different gene constructs (\textit{35S::AtAREB1FL} and \textit{35S::AtAREB1∆QT}) might be differently activated in response to water deprivation, but both constructs are useful tools to improve drought responses in soybean.

Data from phenotypic and physiological analyses are shown in Figure 2 and Tables 2 and 3. AREB1∆QT plants presented higher survival under water stress and higher recovery rates after re-irrigation than WT plants (Figure 2B and C). Additionally, leaves of transgenic plants were less damaged than those of WT plants, and the plants sustained growth with water deprivation, producing more leaves (Table 2; DS). However, the transformed plants were shorter due to smaller internodes, and had a smaller leaf area than the WT plants (Figure 2A and Tables 2 and 3). Fujita et al. (2005), working with \textit{Arabidopsis} plants that were transformed with the \textit{35S::AtAREB1∆QT} construct, showed that the transformed plants were slightly smaller than the WT plants throughout their life. In contrast, \textit{35S::AtAREB1} transgenic plants of \textit{Arabidopsis} (Fujita et al., 2005) and soybean (Barbosa et al., 2013) showed a

Table 3. Phenotype and gas exchange characteristics of the wild-type (WT) cultivar and AREB1∆QT lines under well-watered conditions followed by water stress conditions (N = 20) at the V2/V3 stages.

<table>
<thead>
<tr>
<th></th>
<th>$A$</th>
<th>$g$</th>
<th>$C_i$</th>
<th>LN</th>
<th>NOS</th>
<th>H</th>
<th>DML</th>
<th>WUE ($\mu$mol/$s$)</th>
<th>LA</th>
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</table>

Means followed by the same lowercase letter in the columns do not differ by the Duncan test at the 5% probability level. For abbreviations, see legend to Table 2.
phenotype that was similar to that of the WT plants. These results may be caused by differences between full-length AREB1 and AREB1∆QT. According to Kim et al. (2004) and Moran et al. (2011), plant development can be affected by the positional effects of the insertions, and genes related to primary metabolism can be compromised by the constitutive activity of the 35S promoter or by other possible chromosomal rearrangements or disruptions, resulting in growth disturbances.

Gas exchange data revealed that the transgenic plants had higher A than WT plants as a result of higher g_ in the well-irrigated conditions (Table 2; C) and lower C_i with a water deficit (Table 3). A higher WUE under water stress (Table 2) and after well-irrigated conditions followed by a water deficit was also observed (Table 3). It is possible that C_i in BR 16 plants under water stress occurred as a result of a limited supply of ATP (Kohzuma et al., 2009; Lawlor and Tezara, 2009) and/or metabolic impairment. According to Flexas et al. (2004b), when g_ declines below 0.05 mol H_2O·m^2·s^1, C_i commonly increases sharply and may reflect impaired photosynthetic metabolism. It is usually assumed that the presence of non-stomatal limitations or metabolic impairment imposes restrictions to photosynthetic recovery by leaves after rainfall or irrigation (Quick et al., 1992).

Although there is consensus that the main causes of decreased photosynthesis under conditions of water stress are both stomatal closure and reduced mesophyll conductance, metabolic impairment, and decreased activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) (Flexas and Medrano, 2002; Lawlor and Cornic, 2002; Tezara et al., 2002; Bota et al., 2004; Flexas et al., 2004a, 2006; Ennahli and Earl, 2005).

For C_3 plants, rubisco activity remains essentially unaffected by water stress whenever g_ is >50-100 mmol·m^2·s^1, regardless of the species. In contrast, when the g_ value falls below this threshold, Rubisco activity may eventually decline, perhaps depending on the species and/or environmental conditions. The plants that were submitted to water stress in our study presented g_ that varied between 10 and 30 mmol·m^2·s^1; therefore, rubisco activity must have been reduced. Additionally, as shown in soybean by Olsson (1995), ABA and water stress may have induced early senescence in the BR 16 line compared to the transgenic plants, and this could also cause decreased rubisco levels and activity (Jiang et al., 1993). Therefore, it is very likely that the improved drought response of the AREB1∆QT soybean plants was not associated with altered transpiration rates that were mediated by ABA-dependent stomatal closure. It is possible, although, that the smaller leaf area of the transgenic plants reduced their transpiration and water use, causing delayed stress onset (Lawlor, 2013).

We cannot discard, however, that the differences in the degree of wilting and survival between the 35S-AREB1∆QT and WT plants may have been caused by the regulation of genes related to protection against dehydration. Differences in physiological performance were also observed under well-irrigated conditions where the transgenic plants, as well as the BR 16 cultivar, were in water-saturating conditions. In A. thaliana, the overexpression of AtAREB1∆QT activated downstream genes that are thought to protect plants from water deficit stress and enhance their tolerance to drought (Fujita et al., 2005). These authors showed that AREB1 directed expression of ABA- and dehydration-inducible regulatory genes such as the linker histone H1-3, AAA ATPase, and LEA class genes, which are thought to be involved in alleviating water stress. In soybean, Barbosa et al. (2013) also showed that overexpressing the full-length AREB1 gene helped transgenic plants cope with water deficit more efficiently than the WT plants.
The implication of higher gs in the transgenic plants is that it may increase the net rate of carbon gain and favor a strategy to avoid stress during a short growing season through rapid growth and reproduction. On the other hand, it is necessary to determine how the reduction in leaf area affects grain production. Therefore, new insights into the ability of AtAREB1∆QT-transformed plants to remain viable with extremely low water content, the possibility that the protective and regulatory mechanisms have been altered in the transformed plants, and the yield with different water regimes under field conditions are needed.

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Supplementary material

REFERENCES


