GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Overexpression of the activated form of the *AtAREB1* gene (*AtAREB1ΔQT*) improves soybean responses to water deficit

J.P. Leite[1], E.G.G. Barbosa[2], S.R.R. Marin[2], J.P. Marinho[3], J.F.C. Carvalho[5], R.F. Pagliarini[2], A.S. Cruz[2], M.C.N. Oliveira[4], J.R.B. Farias[5], N. Neumaier[5], F.C.M. Guimarães[2], T. Yoshida[6,7], N. Kanamori[6], Y. Fujita[6], K. Nakashima[6], K.Y. Shinozaki[6,7], J.A. Desidério[1] and A.L. Nepomuceno[2]

[1]Departamento de Biologia Aplicada à Agricultura, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal, SP, Brasil
[2]Laboratório de Biotecnologia Vegetal, Embrapa Soja, Londrina, PR, Brasil
[3]Departmento de Biologia Geral, Universidade Estadual de Londrina, Londrina, PR, Brasil
[4]Departmento de Biometria, Embrapa Soja, Londrina, PR, Brasil
[5]Laboratório de Ecofisiologia Vegetal, Embrapa Soja, Londrina, Brasil
[6]Biological Resources and Post-Harvest Division, Japan International Research Center for Agricultural Sciences, Tsukuba, Ibaraki, Japan
[7]Laboratory of Plant Molecular Physiology, University of Tokyo, Bunkyo-Ku, Tokyo, Japan

Corresponding author: A.L. Nepomuceno
E-mail: alexandre.nepomuceno@embrapa.br

Genet. Mol. Res. 13 (3): 6272-6286 (2014)
Received November 11, 2013
Accepted February 3, 2014
Published August 15, 2014
DOI http://dx.doi.org/10.4238/2014.August.15.10

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 (AREB1ΔQT) was created. Several reports claim that plants overexpressing AREB1 or AREB1ΔQT show improved drought tolerance. In our studies, soybean plants overexpressing AREB1ΔQT 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 AREB1ΔQT 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-AREB1ΔQT 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*ΔQT; 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*Δ*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*Δ*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*Δ*QT* vector contained the 4486-bp *35S:AtAREB1*Δ*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 T_0 transformed plantlets were PCR-screened with specific primers (**Table S1**) to identify those containing the *AtAREB1*Δ*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*Δ*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'-TCTCT GCTGCCACGGGACTCG-3'; and R: 5'-CATGCGATTCCCCAGGTATG-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 $2^{-\Delta Ct}$, which was derived from Livak and Schmittgen (2001). Here, -ΔCt 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 *Hin*dIII 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*Δ*QT* was labeled with radioactive α-[^{32}P]-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 χ^2 analysis, rejecting the hypothesis at the 0.05% level.

Relative *AtAREB1*Δ*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*Δ*QT* primers (F: 5'-GGAGGTG GAGGGTTGACTAGA-3'; R: 5'-CACTGCTCTGAAACTCATCAAACG-3'), and the *Gmβ-actin* (accession No. GMU60500) primers for an endogenous control (F: 5'-GAGCTATGAATT GCCTGATGG-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° ± 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° ± 5°C and 60 ± 10% relative humidity. The experimental design was completely randomized, with 10 replicates.

At the V_2 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 V_3 stage. Plant height, leaf number, number of nodes, mean internode length, and leaf area (length x width) were analyzed under both conditions. Stomatal conductance (g_s), photosynthetic rate (*A*), transpiration rate (E), and intercellular CO_2 concentration (C_i) 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 $\mu mol \cdot m^{-2} \cdot s^{-1}$. 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 $\alpha = 0.05$ as the level of significance.

RESULTS

Copy number and segregation ratio of the *AtAREB1*Δ*QT* events

The amplification efficiency of the primers for the *AtAREB1*Δ*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 T_0 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 T_1 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 T_0 generation of line A2651 could not be determined because

inconsistencies occurred between the quantitative and Southern blot methods. However, the T_1 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::AtAREB1ΔQT* in soybean plants of the T_0 and T_1 generations of lines A2639, A2651, and A2654.

Line	Quantity of transgenes (qPCR)	Number of insertions (Southern blot)	Copy number
A2639 T_0	5	4	4
01 T_1	5	4	5
11 T_1	5	5	5
14 T_1	5	ND	2-3
15 T_1	5	4	4
22 T_1	6	4	4
24 T_1	5	ND	2-3
28 T_1	4	ND	2
31 T_1	4	ND	2
34 T_1	1	ND	1
A2651 T_0	1	2	ND
01 T_1	5	ND	ND
02 T_1	3	3	3
04 T_1	2	2	2
07 T_1	2	2	2
16 T_1	3	ND	ND
17 T_1	2	ND	ND
18 T_1	2	ND	ND
19 T_1	4	2	2
27 T_1	3	2	2
28 T_1	3	2	2
31 T_1	5	3	3
32 T_1	3	ND	ND
33 T_1	2	2	2
35 T_1	3	2	2
A2654 T_0	12	4	6
03 T_1	34	4	17
12 T_1	32	4	16
14 T_1	23	4	11-12
16 T_1	27	4	13-14
17 T_1	31	4	15-16

ND = not determined.

In the T_0 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. T_1 plants of this line showed the same banding pattern as the T_0 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.

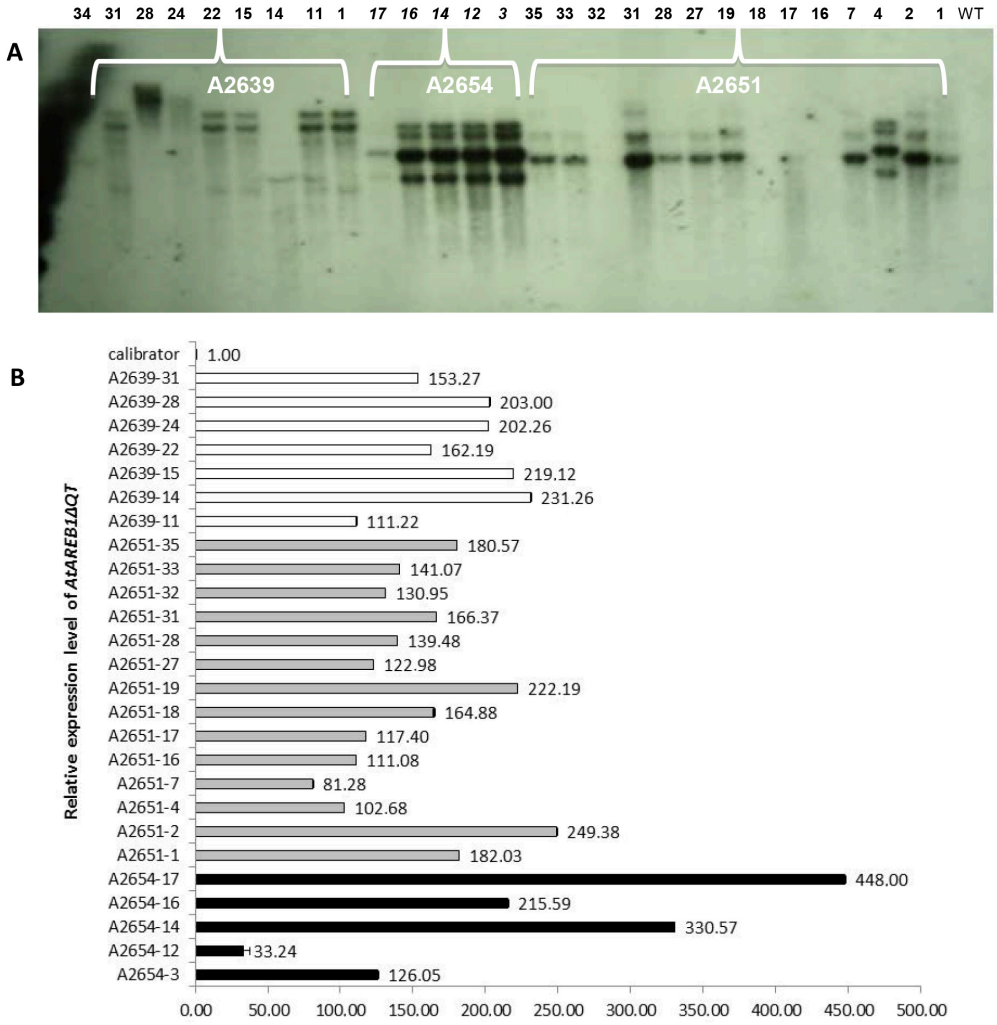

Figure 1. A. Southern blot of T1 plants and negative control (WT = wild-type plants). **B.** Relative *AtAREB1ΔQT* expression by quantitative reverse transcription polymerase chain reaction of the A2639, A2651, and A2654 lines and their respective descendants. The expression level of the calibrator was set to 1.0, which is 164.49 times higher than that of *Gmβ-actin*. Error bars indicate the standard error; N = 3.

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::AtAREB1ΔQT* lines

The results of the plant phenotypic and physiological analyses of the *35S::AtAREB1ΔQT* 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 *AtAREB1ΔQT* 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 *AtAREB1ΔQT* 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 C_i 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 C_i) 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 $H_2O \cdot m^{-2} \cdot s^{-1}$ (Table 2; DS).

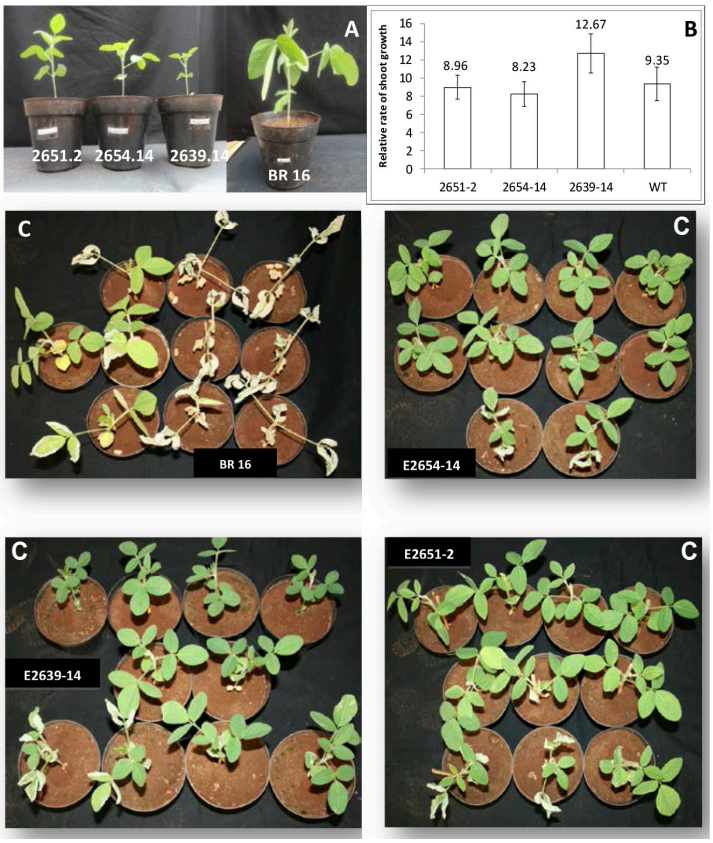

Figure 2. A. Transgenic and BR 16 plants under well-watered conditions at the V4 stage. **B.** Relative rate of shoot growth of the A2651.2, A2654.14, A2639.14, and wild-type (WT) plants after water stress. Values are reported as means (N = 10 plants); the error bars indicate the standard error. **C.** 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 AREB1ΔQT lines under well-watered (C) and water-stressed conditions (DS) (N = 10) at the V2 (C) and V3 stages (DS).

	A	g_s	C_i	LN	NOS	H	IML	WUE_{inst}	LA
C									
WT	14.1^{cA}	0.22^{cA}	263^{aB}	8.9^{aB}	3.0^{aB}	16.2^{aA}	5.1^{aA}	1.86^{aA}	39.8^{aA}
A2651.2	19.6^{aA}	0.40^{aA}	264^{aA}	8.0^{bB}	3.0^{aB}	12.6^{bB}	2.9^{cA}	2.25^{aA}	21.9^{bA}
A2654.14	18.0^{abA}	0.28^{bA}	248^{aA}	7.9^{bB}	2.8^{aB}	11.8^{bcB}	3.7^{bA}	2.28^{aA}	15.6^{cA}
A2639.14	16.9^{bA}	0.27^{bA}	241^{aA}	8.0^{bB}	2.7^{aB}	11.4^{cA}	3.1^{cA}	2.26^{aA}	14.5^{cA}
DS									
WT	0.3^{bB}	0.016^{bB}	400^{aA}	9.6^{bA}	3.6^{aA}	17.0^{aA}	5.1^{aA}	-0.91^{bB}	37.6^{aA}
A2651.2	2.2^{abB}	0.028^{abB}	240^{bA}	11.0^{aA}	4.0^{aA}	13.7^{bA}	3.2^{bA}	2.43^{aA}	21.3^{bA}
A2654.14	2.2^{abB}	0.033^{aB}	257^{bA}	10.0^{aA}	4.0^{aA}	12.7^{cA}	3.7^{bA}	2.53^{aA}	15.3^{cA}
A2639.14	2.6^{aB}	0.030^{abB}	259^{bA}	11.0^{aA}	3.7^{aA}	12.3^{cA}	3.2^{bA}	2.65^{aA}	13.9^{cA}

A = photosynthesis (μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$); g_s = stomatal conductance (mol $H_2O \cdot m^{-2} \cdot s^{-1}$); 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 (mmol 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.

Table 3. Phenotype and gas exchange characteristics of the wild-type (WT) cultivar and AREB1ΔQT lines under well-watered conditions followed by water stress conditions (N = 20) at the V2/V3 stages.

	A	g_s	C_i	LN	NOS	H	IML	WUE_{inst}	LA
WT	7.2[b]	0.09[c]	328.0[a]	9.3[a]	3.4[ab]	16.6[a]	5.1[a]	0.55[b]	38.7[a]
A2651.2	10.9[a]	0.20[a]	252.8[b]	9.5[a]	3.5[a]	13.1[b]	3.0[c]	2.33[a]	21.6[b]
A2654.14	10.5[a]	0.15[b]	252.7[b]	9.3[a]	3.3[ab]	12.2[c]	3.6[b]	2.40[a]	15.4[c]
A2639.14	10.6[a]	0.15[b]	249.7[b]	9.5[a]	3.2[b]	11.9[c]	3.1[c]	2.45[a]	14.2[c]

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.

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), AREB1ΔQT 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, AREB1ΔQT 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 *AtAREB1*Δ*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 *AtAREB1*Δ*QT* expression (Figure 1B), three lines containing a low [A2639.14 (2-3 copies) and A2651.2 (3 copies)] and high [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 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 *35S::AtAREB1FL* construct. Therefore, although the *AREB1* gene plays a key role in the ABA-dependent drought response pathway in plants, different gene constructs (*35S::AtAREB1FL* and *35S:AtAREB1*Δ*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. AREB1ΔQT 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 *Arabidopsis* plants that were transformed with the *35S::AtAREB1*Δ*QT* construct, showed that the transformed plants were slightly smaller than the WT plants throughout their life. In contrast, *35S::AtAREB1* transgenic plants of *Arabidopsis* (Fujita et al., 2005) and soybean (Barbosa et al., 2013) showed a

phenotype that was similar to that of the WT plants. These results may be caused by differences between full-length AREB1 and AREB1ΔQT. 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_s 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_s declines below 0.05 mol $H_2O \cdot m^{-2} \cdot s^{-1}$, 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_s is >50-100 $mmol \cdot m^{-2} \cdot s^{-1}$, regardless of the species. In contrast, when the g_s 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_s that varied between 10 and 30 $mmol \cdot m^{-2} \cdot s^{-1}$; 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 AREB1ΔQT 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-AREB1ΔQT* 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 *AtAREB1ΔQT* 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 g_s 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 *AtAREB1ΔQT*-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.

ACKNOWLEDGMENTS

Research supported by the Science and Technology Research Partnership for Sustainable Development (SATREPS) of the Japan Science and Technology Agency/Japan International Cooperation Agency (JIRCAS) and Empresa Brasileira de Pesquisa Agropecuária, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Faculdade de Ciências Agrárias e Veterinária, Universidade Estadual Paulista (FCAV-UNESP), Jaboticabal, Brazil.

Supplementary material

REFERENCES

Agarwal PK and Jha B (2010). Transcription factors in plants and ABA dependent and independent abiotic stress signalling. *Biol. Plant.* 54: 201-212.

Aragão FJL, Sarokin L, Vianna GR and Rech EL (2000). Selection of transgenic meristematic cells utilizing a herbicidal molecule results in the recovery of fertile transgenic soybean [*Glycine max* (L.) Merril] plants at a high frequency. *Theor. Appl. Genet.* 101: 1-6.

Barbosa EGG, Leite JP, Marin SRR, Marinho JP, et al. (2013). Overexpression of the ABA-dependent AREB1 transcription factor from *Arabidopsis thaliana* improves soybean tolerance to water deficit. *Plant Mol. Biol. Rep.* 31: 719-730.

Bota J, Medrano H and Flexas J (2004). Is photosynthesis limited by decreased Rubisco activity and RuBP content under progressive water stress? *New Phytol.* 162: 671-681.

Bubner B and Baldwin IT (2004). Use of real-time PCR for determining copy number and zygosity in transgenic plants. *Plant Cell Rep.* 23: 263-271.

Cheong YH, Kim KN, Pandey GK, Gupta R, et al. (2003). CBL1, a calcium sensor that differentially regulates salt, drought, and cold responses in *Arabidopsis*. *Plant Cell* 15: 1833-1845.

Corrêa LG, Riaño-Pachón DM, Schrago CG, dos Santos RV, et al. (2008). The role of bZIP transcription factors in green plant evolution: adaptive features emerging from four founder genes. *PLoS One* 3: e2944.

Doyle JJ and Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19: 11-15.

Ennahli S and Earl HJ (2005). Physiological limitations to photosynthetic carbon assimilation in cotton under water stress. *Crop Sci.* 45: 2374-2382.

Fehr WR, Caviness CE, Burmood DT and Pennington JS (1971). Stage of development descriptions for soybeans, *Glycine max* (L.) Merrill. *Crop Sci.* 11: 929-931.

Flexas J and Medrano H (2002). Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. *Ann. Bot.* 89: 183-189.

Flexas J, Bota J, Loreto F, Cornic G, et al. (2004a). Diffusive and metabolic limitations to photosynthesis under drought and salinity in C(3) plants. *Plant Biol*. 6: 269-279.

Flexas J, Bota J, Cifre J, Escalona JM, et al. (2004b). Understanding down-regulation of photosynthesis under water stress: future prospects and searching for physiological tools for irrigation management. *Ann. Appl. Biol.* 144: 273-283.

Flexas J, Bota J, Galmés J, Medrano H, et al. (2006). Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. *Physiol. Plant.* 127: 343-352.

Fujita Y, Fujita M, Satoh R, Maruyama K, et al. (2005). AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in *Arabidopsis*. *Plant Cell* 17: 3470-3488.

Fujita Y, Fujita M, Shinozaki K and Yamaguchi-Shinozaki K (2011). ABA-mediated transcriptional regulation in response to osmotic stress in plants. *J. Plant Res.* 124: 509-525.

Furihata T, Maruyama K, Fujita Y, Umezawa T, et al. (2006). Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. *Proc. Natl. Acad. Sci. U. S. A.* 103: 1988-1993.

Gao SQ, Chen M, Xu ZS, Zhao CP, et al. (2011). The soybean GmbZIP1 transcription factor enhances multiple abiotic stress tolerances in transgenic plants. *Plant Mol. Biol.* 75: 537-553.

Jakoby M, Weisshaar B, Dröge-Laser W, Vicente-Carbajosa J, et al. (2002). bZIP transcription factors in *Arabidopsis*. *Trends Plant Sci.* 7: 106-111.

Jiang CZ, Rodermel SR and Shibles RM (1993). Photosynthesis, rubisco activity and amount, and their regulation by transcription in senescing soybean leaves. *Plant Physiol.* 101: 105-112.

Kim JS, Mizoi J, Yoshida T, Fujita Y, et al. (2011). An ABRE promoter sequence is involved in osmotic stress-responsive expression of the DREB2A gene, which encodes a transcription factor regulating drought-inducible genes in *Arabidopsis*. *Plant Cell Physiol.* 52: 2136-2146.

Kim S, Kang JY, Cho DI, Park JH, et al. (2004). ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance. *Plant J.* 40: 75-87.

Kobayashi F, Maeta E, Terashima A and Takumi S (2008). Positive role of a wheat HvABI5 ortholog in abiotic stress response of seedlings. *Physiol. Plant* 134: 74-86.

Kohzuma K, Cruz JA, Akashi K, Hoshiyasu S, et al. (2009). The long-term responses of the photosynthetic proton circuit to drought. *Plant Cell Environ.* 32: 209-219.

Lawlor DW (2013). Genetic engineering to improve plant performance under drought: physiological evaluation of achievements, limitations, and possibilities. *J. Exp. Bot.* 64: 83-108.

Lawlor DW and Cornic G (2002). Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. *Plant Cell Environ.* 25: 275-294.

Lawlor DW and Tezara W (2009). Causes of decreased photosynthetic rate and metabolic capacity in water-deficient leaf cells: a critical evaluation of mechanisms and integration of processes. *Ann. Bot.* 103: 561-579.

Lee SJ, Kang JY, Park HJ, Kim MD, et al. (2010). DREB2C interacts with ABF2, a bZIP protein regulating abscisic acid-responsive gene expression, and its overexpression affects abscisic acid sensitivity. *Plant Physiol.* 153: 716-727.

Liao Y, Zhang JS, Chen SY and Zhang WK (2008a). Role of soybean GmbZIP132 under abscisic acid and salt stresses. *J. Integr. Plant Biol.* 50: 221-230.

Liao Y, Zou HF, Wei W, Hao YJ, et al. (2008b). Soybean GmbZIP44, GmbZIP62 and GmbZIP78 genes function as negative regulator of ABA signaling and confer salt and freezing tolerance in transgenic *Arabidopsis*. *Planta* 228: 225-240.

Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25: 402-408.

Meyer R, Candrian U and Lüthy J (1994). Detection of pork in heated meat products by the polymerase chain reaction. *J. AOAC Int.* 77: 617-622.

Morran S, Eini O, Pyvovarenko T, Parent B, et al. (2011). Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. *Plant Biotechnol. J.* 9: 230-249.

Nakashima K, Ito Y and Yamaguchi-Shinozaki K (2009). Transcriptional regulatory networks in response to abiotic stresses in *Arabidopsis* and grasses. *Plant Physiol.* 149: 88-95.

Olsson M (1995). Alterations in lipid composition, lipid peroxidation and anti-oxidative protection during senescence in drought stressed plants and non-drought stressed plants of *Pisum sativum*. *Plant Physiol. Biochem.* 33: 547-553.

Oya T, Nepomuceno AL, Neumaier N, Farias JRC, et al. (2004). Drought tolerance characteristics of Brazilian soybean cultivars: Evaluation and characterization of dought tolerance of various Brazilian soybean cultivars in the field. *Plant Prod. Sci.* 7: 129-137.

Quick WP, Chaves MM, Wendler R, David M, et al. (1992). The effect of water stress on photosynthetic carbon metabolism in four species grown under field conditions. *Plant Cell Environ.* 15: 25-35.

Rech EL, Vianna GR and Aragão FJ (2008). High-efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants. *Nat. Protoc.* 3: 410-418.

Reeves WM, Lynch TJ, Mobin R and Finkelstein RR (2011). Direct targets of the transcription factors ABA-Insensitive(ABI)4 and ABI5 reveal synergistic action by ABI4 and several bZIP ABA response factors. *Plant Mol. Biol.* 75: 347-363.

Sambrook J, Fritsch EF and Maniatis Y (1989). Molecular Cloning: A Laboratory Manual. 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, 6-12.

Stolf-Moreira R, Lemos EGM, Abdelnoor RV, Beneventi MA, et al. (2011). Identification of reference genes for expression analysis by real-time quantitative PCR in drought-stressed soybean. *Pesq. Agropec. Bras.* 46: 58-65.

Tezara W, Mitchell VJ, Driscoll SP and Lawlor DW (2002). Effects of water deficit and its interaction with CO_2 supply on the biochemistry and physiology of photosynthesis in sunflower. *J. Exp. Bot.* 53: 1781-1791.

Yoshida T, Fujita Y, Sayama H, Kidokoro S, et al. (2010). AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. *Plant J.* 61: 672-685.