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Research Article

Introduction of the *rd29A:AtDREB2A* CA gene into soybean (*Glycine max* L. Merril) and its molecular characterization in leaves and roots during dehydration

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Abstract

The loss of soybean yield to Brazilian producers because of a water deficit in the 2011-2012 season was 12.9%. To reduce such losses, molecular biology techniques, including plant transformation, can be used to insert genes of interest into conventional soybean cultivars to produce lines that are more tolerant to drought. The abscisic acid (ABA)-independent Dehydration Responsive Element Binding (DREB) gene family has been used to obtain plants with increased tolerance to abiotic stresses. In the present study, the *rd29A:AtDREB2A* CA gene from *Arabidopsis thaliana* was inserted into soybean using biolistics. Seventy-eight genetically modified (GM) soybean lines containing 2-17 copies of the *AtDREB2A* CA gene were produced. Two GM soybean lines (P1397 and P2193) were analyzed to assess the differential expression of the *AtDREB2A* CA transgene in leaves and roots submitted to various dehydration treatments. Both GM lines exhibited high expression of the transgene, with the roots of P2193 showing the highest expression levels during water deficit. Physiological parameters examined during water deficit confirmed the induction of stress. This analysis of *AtDREB2A* CA expression in GM soybean indicated that line P2193 had the greatest stability and highest expression in roots during water deficit-induced stress.

Keywords: Arabidopsis, differential expression, genetically modified organism, water deficit.

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Introduction

Brazil is the world's second largest soybean producer (Empresa Brasileira de Pesquisa Agropecuária - EMBRAPA, 2012), and its main consumer market is China, which represents 67% of Brazilian soybean exports (United States Department of Agriculture - USDA, 2011). According to some estimates, exports involving the soybean complex will represent US$30 billion in 2013 (Associação Brasileira das Indústrias de Óleos Vegetais - ABIOVE, 2013).

However, the 2011-2012 harvest yield was reduced by 12.9% compared to the previous year because of severe droughts in southern Brazil, one of the main soybean-producing regions (Companhia Nacional de Abastecimento - CONAB, 2012). The alternatives available to minimize/reduce problems arising from water deficit include the use of biotechnology to develop cultivars more tolerant to drought. To address this problem, a group of researchers from the *Japan International Research Center for Agricultural Sciences* (JIRCAS) identified, characterized and isolated a family of transcription factors (*Dehydration Responsive Element Binding* - DREB) from *Arabidopsis thaliana* that are associated with increased tolerance to abiotic stressors (Shinozaki and Yamaguchi-Shinozaki, 2000).

Transcription factor (TF) *At*DREB1A, which is responsive to cold, was inserted into soybean and subjected to molecular, anatomical and physiological analysis by Polizel *et al.* (2011) using experimental water deficit conditions. Transcription factor DREB2A, which belongs to the same TF family, was also characterized in *A. thaliana* and responded to drought, high salinity and heat. Overexpression of constitutively active (CA) DREB2A resulted in significant tolerance to drought and heat stress in transgenic *Arabidopsis* plants (Sakuma *et al.*, 2006a,b). *AtDREB2A*-

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homologous genes were studied in maize (Qin *et al.*, 2007), rice (Dubouzet *et al.*, 2003), sunflower (Almogueva *et al.*, 2009), wheat (Terashima and Takumi, 2009) and chrysanthemum (Liu *et al.*, 2008). Recently, Mizoi *et al.* (2013) identified a soybean DREB2 gene, GmDREB2A;2, and showed that its heterologous expression in *Arabidopsis* improved the plants tolerance to stress. These findings indicate that plants overexpressing *AtDREB2A* and *DREB2A-like* proteins have increased tolerance to abiotic stress, drought and heat, which often occur together under field conditions.

There is a pressing need for soybean cultivars that are more tolerant to drought and heat, particularly since data provided by the IPCC (Intergovernmental Panel on Climate Change, 2007) indicate that the Earth's average temperature will increase in forthcoming years and lead to a reduction in land area suitable for growing soybean and other crops. The aim of this study was to insert the TF *DREB2A* isolated from *A. thaliana* into soybean using biolistics and then examine this genes effect on resistance to drought. The number of transgene copies inserted into the soybean genome was quantified using quantitative polymerase chain reaction (qPCR) and Southern blotting. The transgene expression under drought conditions was analyzed by qPCR in leaves and roots. The results described here may be useful in developing soybean cultivars that are more tolerant to drought and heat.

Materials and Methods

Phylogenetic analysis

The similarity between the sequence of *AtDREB2A* (the constitutively active form of CA; JIRCAS) and the soybean *DREB* genes, and the homology of *AtDREB2A* CA with other genes belonging to the same subfamily in other plant species, were assessed by *in silico* analyses done using sequences downloaded from the National Center for Biotechnology Information (NCBI) expressed sequence tags (EST) database. The sequences were aligned using Clustal X software and two phylogenetic trees were created using MEGA 4.0 (Molecular Evolutionary Genetics Analysis) software.

Production of genetically modified soybean plants

Embryos from the conventional soybean cultivar BR 16, which is sensitive to drought (Oya *et al.*, 2004), were co-transformed with the genetic constructs *rd29A:AtDREB2A* CA (Patent no. WO 2006/006236 PCT/JP2004/01003) and *ahas*:*AtAHAS* using biolistics, based on the methods of Aragão *et al.* (2000) and Rech *et al.* (2008). The genetic construct *rd29A:AtDREB2A* CA contains the stress-inducible *rd29A* promoter (Yamaguchi-Shinozaki and Shinozaki, 1994) and the coding region of *AtDREB2A* is in the CA form, which has a deletion in the negative regulation domain between amino acid residues 136 and 165. The *ahas*:*AtAHAS* construct acts as a selection gene that confers tolerance to imazapyr, an imidazolinone herbicide (Aragão *et al.*, 2000).

Characterization of the transgenic plants

Identification of plants containing the AtDREB2A CA gene by conventional PCR

Conventional PCR was used to confirm the positive GM events, chimerization and segregation of the transgene in leaf genomic DNA extracted according to Doyle and Doyle (1987). The primer set used was: forward (F) 5'-GTTTGAACGATCGGGGAAT-3' and reverse (R) 5'-GGGAAGCTTGCCATAGATGCAATTCAATCAAA CT-3', which amplified a 1975 bp fragment between the promoter and terminator regions. The reaction mixture consisted of 5 μM of each F and R primer, 2.5 mM dNTPs (Invitrogen, Grand Island, NY, USA), 50 mM magnesium chloride ($MgCl_2$) (Sigma), 2.5 μL of 10X buffer, one unit (U) of *Taq* DNA polymerase (Invitrogen) and 60 ng of genomic DNA. Amplification was done using an initial denaturation step at 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. Once identified, the transgenic plants were transferred to pots in a greenhouse where they were grown and used as described below.

Quantification of copy number by qPCR

Quantitative PCR (qPCR) was used to determine the number of transgenic cassette copies of *rd29A*:*AtDREB2A* CA inserted into the soybean genome. Sixteen PCR-positive plants from the T_0 generation (P1397, P7186, P7413, P2193, P7195, P7417, P7212, P7418, P7231, P7430, P7256, P7374, P7431, P7174, P7393 and P7531) were analyzed. The endogenous lectin gene *GmLec* (accession no. K00821) was used as the reference gene for normalization since it is a species-specific gene and there is only a single copy in the soybean haploid genome (Meyer *et al.*, 1994). The primer set used for the target gene was: F5'-CCGAGAGTCAACAAAGTGGTTTT-3' and R 5'-CGAGCTGAACGGAGGTATTCC-3', and the probe sequence was 5'-FAMCCGCTACAAAGC-3'. The primer set used for the reference gene was F 5'-TCCCGAGTG GGTGAGGATAG-3' and R 5'- CATGCGATTCCCCAG GTATG-3', and the probe sequence was 5'- TETTCTC TGCTGCCACGGGACTCG-3'. The cycling conditions consisted of 50 °C for 2 min, denaturation at 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

To calculate the number of copies inserted into T_0 generation plants, the formula of Livak and Schmittgen (2001) was modified for $2^{-\Delta Ct}$ since the plants were hemizygous in this generation and the resulting value was multiplied by two to correct for the 2:1 proportion of the endogenous gene corresponding to the transgene. One T_0 generation plant containing a large copy number of *AtDREB2A* CA (P2193) and one containing a low copy number (P1397) were selected for gene expression analysis.

Southern blotting

The number of transgenic cassette insertions was assessed by Southern blotting using soybean genomic DNA obtained from T_0, T_1 and T_2 plants of P2193, which had high transgene expression levels. After extraction, the DNA was digested using the restriction enzymes *Hin*dIII and *Eco*RV and then hybridized using a ^{32}P-labeled radioactive probe that targeted *AtDREB2A* CA (Figure 1). Hybridized bands were detected by autoradiography (Southern, 1974).

Leaf dehydration test using a biological oxygen demand (BOD) incubator

GM plants P2193 and P1397 were grown in Typic Haplorthox (Embrapa, 2006) for 60 days in a greenhouse with drip irrigation (twice per day for 5 min each) at 28 ± 3 °C. The experiment was based on the protocol by Kariola *et al.* (2006), with the following modifications: (a) one leaflet was collected from each plant to be analyzed and divided into three parts, (b) each leaflet part was considered to be one biological replicate, and (c) the initial and final dehydration time points were sampled based on the water loss of the detached leaves. Thirty and 90 min cell dehydration treatments were done by subjecting the detached leaves to a temperature of 30 °C and 60% humidity in a BOD incubator. Leaflets of the non-transformed, conventional soybean cultivar BR 16 were used as negative controls.

Leaf and root dehydration under hydroponic conditions

To simulate a water deficit in the roots of P2193 plants, a hydroponic experiment was done according to the protocol of Martins *et al.* (2008). At the V_3 vegetative growth stage, dehydration treatments lasting zero (control - without stress), 30, 60 and 90 min under stress conditions were applied in triplicate, with each plant being considered as a biological replicate. Triplicate measurements of the photosynthetic rate, stomatal conductance, transpiration and leaf-air temperature difference were obtained during the treatments using an LI-6400 portable photosynthesis system. The data were analyzed statistically using Duncan's test at 5% significance ($p < 0.05$) (SAS software).

Extraction of total RNA and cDNA synthesis

GM plants P1397 and P2193 and the conventional cultivar BR 16 (non-transgenic control plant) were subjected to dehydration experiments after which total RNA was extracted using TRIzol® reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. For cDNA synthesis, 5 µg of total RNA treated with DNase was transcribed using M-MLV reverse transcriptase according to the manufacturer's recommendations. The cDNA was quantified spectrophotometrically (NanoDrop 1000, Thermo Scientific, Wilmington, USA) and its intactness was analyzed electrophoretically in a 1% agarose gel with sodium borate (SB) buffer.

Quantification of the relative gene expression by qPCR

The gene expression levels in transgenic plants P1397 and P2193 and the negative control BR 16 were assessed by quantitative PCR (qPCR). *GmrRNA18S* (accession no. X02623.1) and *Gmβ_actin* (Stolf-Moreira *et al.*, 2011) were used as reference genes for normalization. The primers and probes used were: (a) F 5-GGTTGACAGACTG AGAGCTCTTTC-3 and R 5-CAAATCGCTCCAACTA

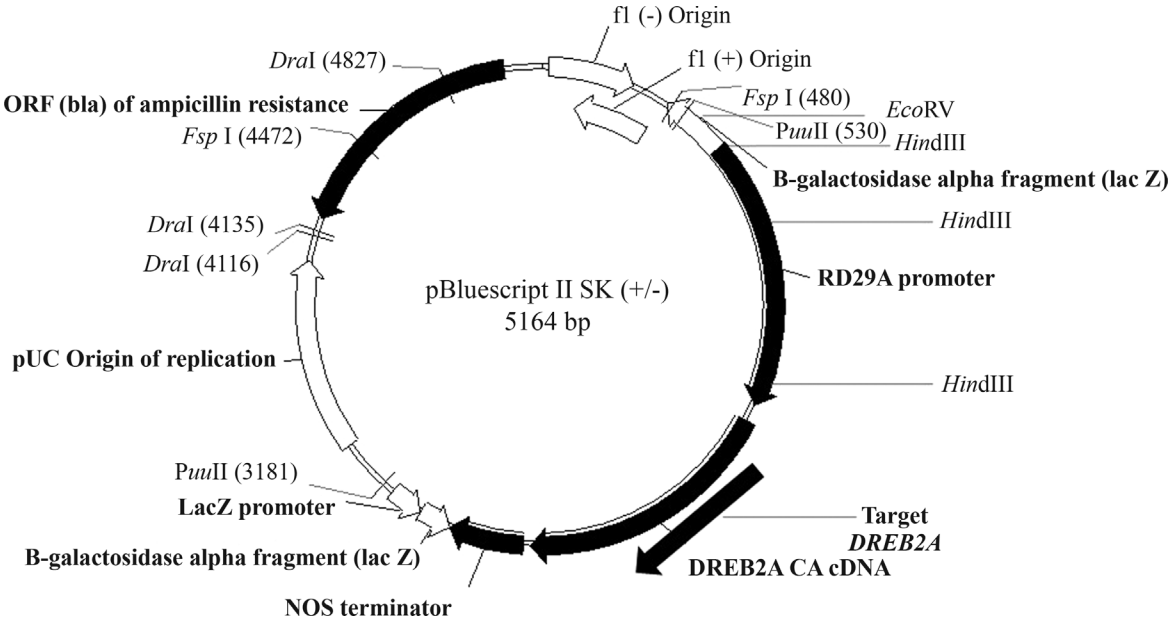

Figure 1 - Plasmid containing the transgene *rd29A:AtDREB2A* CA and the map of restriction enzymes used in this work. The enzymes used in Southern blotting were *Hin*dIII and *Eco*RV.

AGAAC-3 and probe 5-VICCACCACCCATAGAAT CAA-3 for *GmrRNA18S*, (b) F 5-TTGCCTGATGGACA GGTCATT-3 and R 5- CAATCAGAGAAGGCTGGA AC AG-3, and probe 5- AGGTTCTGAGAGATTCCGTTGC CCAGA-3 for *Gm*β*_actin*, (c) F 5-CCGAGAGTCAACA AAGTGGTTTT-3 and R 5-GGGAAGCTTGCCATAGA TGCAATTCAATCAAACT-3, and probe 5-FAMCCGCT ACAAAGCCTCAACTACGGAATACCT-3 for *Primer AtDREB2A*, and (d) F 5-CAGGCCCGAGAGTCAACA AA-3 and R 5-GAGGTATTCCGTAGTTGAGGCTTT-3, and probe 5-FAMTAGCGGATCAAAACCAC-3 for *Assay AtDREB2A*. *Gm*β*_actin* was not used in the cell dehydration experiment with the T_0 generation.

The reactions were done in biological and technical triplicates using a TaqMan® PreAmp Master mix kit (Applied Biosystems) and a 7300 Real Time System thermocycler (Applied Biosystems) under the following cycling conditions: 50º C for 2 min, denaturation at 95º C for 10 min, followed by 40-45 cycles of 95 °C for 15 s and 60 °C for 1 min. The Relative Expression Software tool (RESTmsc software; Pfaffl *et al.*, 2002) was used to analyze the gene expression data.

Results

Sequence alignment

The analysis of various sequences of *DREB*-homologous genes yielded a single phylogenetic tree (Figure 2). Sequence alignment indicated conservation of the *DREB* genes in the species investigated, which suggested that the

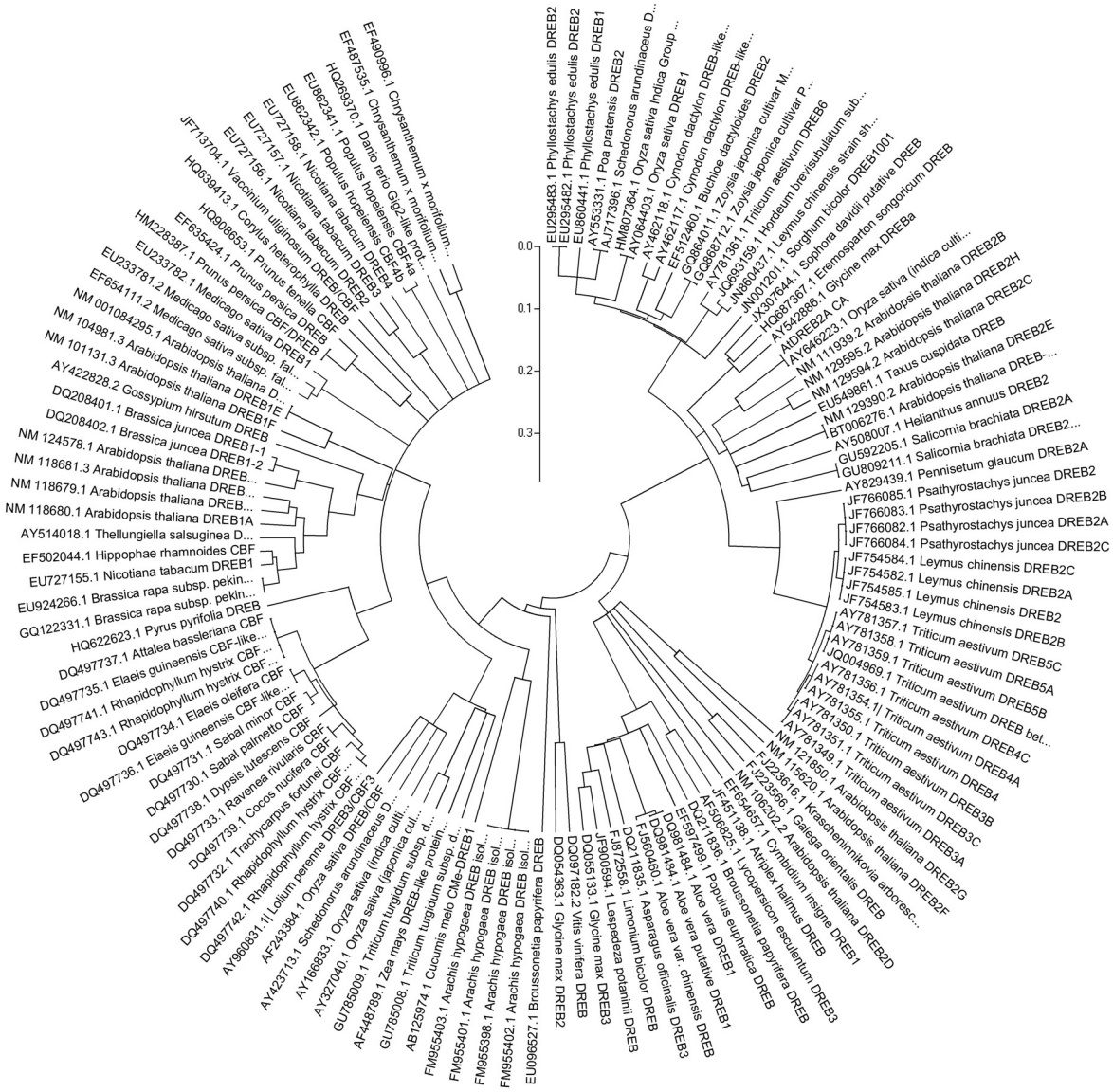

Figure 2 - Phylogenetic tree generated from the DREB-coding DNA sequence (CDS) from various plant species. The sequences were obtained from the NCBI website and were aligned and clustered using Clustal X and MEGA 4.0, respectively.

A. thaliana DREB2A CA gene inserted into soybean could be expressed to activate the endogenous genes responsive to water deficit stress. This conclusion was supported using the phylogenetic tree constructed with soybean *DREB* genes (Figure 3).

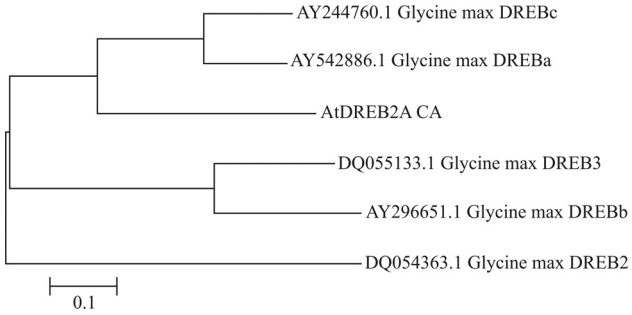

Figure 3 - Phylogenetic tree generated from CDS sequences of the soybean DREB gene. The sequences were obtained from the NCBI website and were aligned and clustered using Clustal X and MEGA 4.0, respectively.

Production of GM soybean plants

A total of 6,537 embryos from cultivar BR 16 were bombarded with the genetic construct of interest. Nine hundred and twenty seedlings from 4,185 multiple shoots were screened for the presence of *AtDREB2A* CA by conventional PCR using a specific primer set, and 78 of these were PCR-positive and produced seeds.

Number of transgene copies in GM soybean plants

The number of copies of the *AtDREB2A* CA transgene was assessed in the T_0 generation of GM soybean plants and ranged from 2-17 (Table 1). No chimeras of P2193 and P1397 were detected, and the number of copies in T_1 generation plants indicated segregation in the transition from T_0 to T_1: plants 1 and 2 of P1397 lost all copies and eight copies, respectively, and plants 1 and 2 of P2193 lost all copies and 48 transgene copies, respectively (Table 1).

Analysis in T_0, T_1, and T_2 generations of P1397 and P2193

Leaf dehydration experiment

Gene expression in GM plants was compared to that in the conventional cultivar BR 16. P2193 showed high

Table 1 - Estimation of AtDREB2A copy number in generations T_0 and T_1 using qPCR and the $2^{-\Delta Ct/2}$ method. The first column displays the selected events, the second and third columns display the results from the independent experiments, the fourth column lists the average of the two experiments, and the fifth, sixth and ninth columns show the standard deviation (SD), coefficient of variation (CV%) and estimated copy number, respectively.

Sample	First experiment	Second experiment	Average $2^{-\Delta Ct}$	SD $2^{-\Delta Ct}$	CV% $2^{-\Delta Ct}$	SD+	SD-	Nº of copies
P 1397	15.95	10.35	13.15	3.96	30.15	17.11	9.18	9 to 17
P 7186	8.39	5.73	7.06	1.88	26.60	8.94	5.18	5 to 9
P 7413	4.95	3.98	4.47	0.69	15.45	5.16	3.78	4 to 5
P 2193	966.38	225.89	596.14	523.61	87.83	1119.74	72.53	High**
P 7195	9.36	6.74	8.05	1.85	23.00	9.91	6.20	6 to 10
P 7417	4.05	2.92	3.49	0.80	22.95	4.29	2.69	3 to 4
P 7212	6.84	5.09	5.97	1.24	20.75	7.21	4.73	5 to 7
P 7418	7.9	5.66	6.78	1.58	23.44	8.37	5.19	5 to 8
P 7231	6.23	4.25	5.24	1.40	26.72	6.64	3.84	4 to 7
P 7430	4.33	3.2	3.77	0.80	21.13	4.56	2.97	3 to 5
P 7256	9.44	4.88	7.16	3.22	45.12	10.39	3.93	4 to 10
P 7374	3 12	2.32	2.72	0.57	20.86	3.29	2.15	2 to 4
P 7431	14	8.56	11.28	3.84	34.07	15.12	7.44	7 to 15
P 7174	6.17	3.88	5.03	1.62	32.26	6.65	3.41	3 to 7
P 7393	4.61	3.15	3.88	1.03	26.62	4.91	2.85	3 to 5
P 7531	9.23	5.91	7.57	2.34	30.94	9.91	5.23	5 to 10
P 1397.1 (T_1)*	0.07	0.05	0.06	0.01	20.41	0.07	0.05	Negative***
P 1397.2 (T_1)	2.94	1.7	2.32	0.88	37.85	3.20	1.44	1 to 3
P 2193.1 (T_1)	0.76	0.53	0.65	0.16	25.29	0.81	0.48	Negative
P 2193.2 (T_1)	4.14	2.12	3.13	1.43	45.68	4.56	1.70	2 to 5

*Samples identified with T_1 descended from the T_0 generation (*i.e.*, generation T_1). The copy number in generation T_1 was divided by two because these plants were hemizygous. The number followed by a dot in T_1 plants indicates the two descendants analyzed of each planta (P 1397 and P 2193), here denominated dot 1 and dot 2. **High fluorescence values caused signal saturation that led to imprecise results; in this case, the plants were considered to have > 50 copies. ***Samples with values < 1 were considered to be negative.

transcript levels of *AtDREB2A* CA in the T_0 generation, with 725-, 535- and 146-fold greater expression after zero (control), 30 and 90 min of leaf dehydration in a BOD incubator, although only the 30 and 90 min treatments were significantly different from the levels in BR 16 ($p < 0.004$ and $p < 0.003$, respectively). In P1397, the highest levels of *AtDREB2A* CA transcripts were observed after 30 min of exposure to stress (89-fold, $p < 0.11$), but none of the values was statistically significant at any dehydration time point. When T_1 generation GM plants were assessed, high levels of *AtDREB2A* CA transcripts were detected at time zero, which was 11-fold higher in P1397 ($p < 0.072$) and 111-fold greater in P2193 ($p < 0.081$). In P1397, the transgene expression levels were reduced, which suggests possible repression of gene expression at the 90 min treatment interval (0.001-fold, $p < 0.022$).

When the results for the T_0 and T_1 generations were compared, P2193 plants showed the greatest gene expression in both cases (Table 2). This finding most likely indicates greater stability of the transgene *rd29A:AtDREB2A* CA expression in P2193 compared to P1397, which only exhibited significant expression in control plants from T_1. Transgene instability in P1397 was further demonstrated by the possible repression of gene expression after a 90 min exposure to stress (Table 2).

Segregation test and copy number analysis by Southern blotting in P2193

The results of the transgene segregation test in P2193 complied with the proportion expected for the T_1 generation according to Mendel's first law; however, the transgene segregation did not comply with Mendel's first law in plants P2193.2, P2193.6 and P2193.7 from the T_2 generation. Since most P2193 descendants segregated independently, Southern blot analysis was used to determine the number of inserts. In T_0, the number of inserts detected for the *AtDREB2A* CA gene was lower than the copy number detected by qPCR. In T_1, however, the results of both methods were similar and indicated three homozygous, two heterozygous and three negative plants. T_2 generation plants had the same insert number as T_2 plants of P2193.3 and P2193.6, indicating that the transgene behaves in a stable manner (Figure 4).

Hydroponic experiment with the T_2 generation of P2193

Compared to gene expression in the non-transformed, conventional cultivar BR 16, the results of the hydroponic experiment with the T_2 generation of P2193 showed that the *AtDREB2A* CA transgene was overexpressed in leaf tissue by 122-, 907-, 1,042- and 509-fold ($p < 0.001$) at zero (control), 30, 60 and 90 min after treatment, respectively

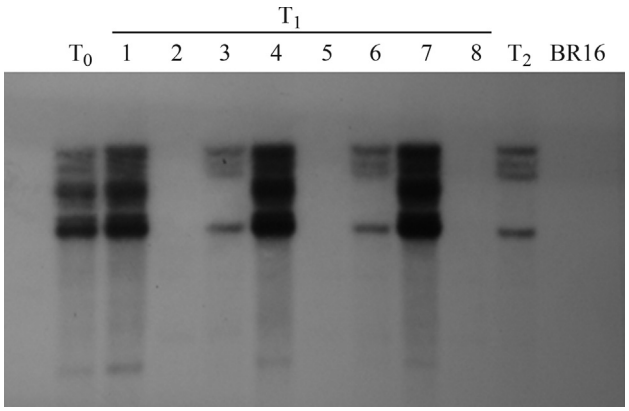

Figure 4 - Southern blot for generations T_0, T_1 and T_2 of genetically modified P2193 containing the transcription factor *At*DREB2A. Lanes 1-8 represent T_1 generation plants. BR16 - control (normal) soybean cultivar.

Table 2 - Relative expression of the *AtDREB2A* transgene in GM plants P1397 and P2193 in response to dehydration.

Dehydration experiment	GM plant	Treatment (min)	Expression	Expression profile*	SE	p
T_0 Generation	P1397	0	1.99	-	± 2.3	0.559
		30	89.2	-	± 138.1	0.110
		90	5.1	-	± 9.8	0.241
	P 2193	0	725	-	± 1744.8	0.144
		30	535.2	Up	± 1308.7	0.004
		90	146.1	Up	± 203.0	0.003
T_1 Generation	P 1397	0	11.3	Up	± 12.9	0.072
		30	0.9	-	± 1.1	0.965
		90	0.001	Down	± 0.002	0.022
	P 2193	0	111.1	Up	± 247.1	0.081
		30	0.28	-	± 0.31	0.545
		90	8.3	-	± 10.5	0.141

The standard error (SE) was calculated for median values, with $p < 0.1$. *The values for up-regulated (UP) profiles were significantly different from the control group when the expression profile during water stress diverged from the control at 10% probability ($p < 0.1$) upon randomization testing (n = 2,000 iterations). All statistical analyses were done using RESTmsc software.

(Table 3). Higher levels of gene expression were detected in roots, with the transgene showing 901-, 858- and 1,363-fold higher expression at zero, 30 and 90 min after treatment ($p < 0.001$), respectively, and a peak increase of 27,750-fold after 60 min (Table 3, Figure 5). Thus, in leaves and roots, the highest levels of transgene expression were detected after 60 min, as indicated above.

During water stress in the hydroponic system the photosynthetic rate (CER), stomatal conductance, transpiration, and the leaf-air temperature difference decreased in BR 16 and P2193 plants during all treatments. GM plants showed a decline in these parameters after 30 min of dehydration, whereas the greatest decrease in the non-transformed control BR 16 occurred after 60 min of dehydration (Figure 6). During dehydration, the CER and stomatal conductance differed significantly between cultivar BR 16 and GM P2193 at time zero and 30 min, with the value at zero min being higher for P2193 compared to BR 16. Intra-group analysis of BR 16 and P2193 showed that the values for both groups of plants differed from each other in the first 60 min, but were similar after 60-90 min of treatment (Duncan's test, $p \leq 0.05$) (Figure 6).

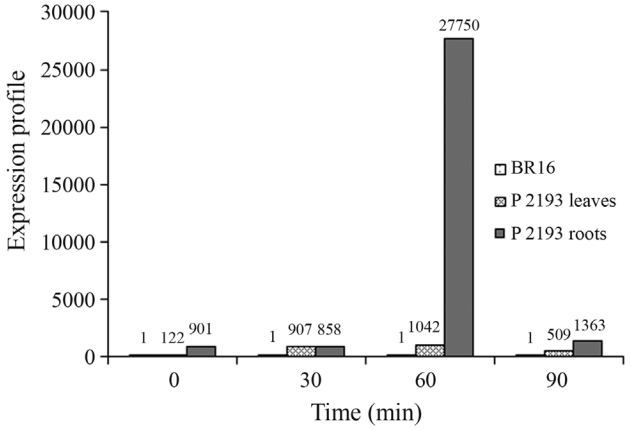

Figure 5 - Relative gene expression of the *AtDREB2A* CA transgene in leaves and roots of the T_2 generation of P2193 during stress induced by a water deficit. Transgene expression was normalized to the reference genes *GmrRNA18S* and *Gm*β*_actin* and then calibrated with the corresponding time points and tissue from control plants (cultivar BR 16) ($p < 0.001$). Statistical analyses were done using RESTmsc software and all treatments of leaves and roots were significantly higher than the control.

Discussion

The phylogenetic tree generated by aligning homologous *DREB2A* sequences from various plant species indicated minor differences in non-conserved regions (Sakuma *et al.*, 2002) of DREB family genes and grouped species that were phylogenetically close to each other. *DREB2A* has highly conserved regions, including a DNA-binding domain known as AP2, which classifies *A. thaliana DREB2A* in the DREB subfamily that contains AP2 and a subgroup referred to as A-2 (Sakuma *et al.*, 2002). As also observed by Li *et al.* (2005), *AtDREB2A* CA clustered with the *GmDREBa* and *GmDREBc DREB* genes in soybean, thereby corroborating the similarity among *DREB* genes (Figure 3).

As with TF *At*DREB1A (Polizel *at al.,* 2011), TF *At*DREB2A CA from *A. thaliana* was successfully inserted into soybean by biolistics. Chimeric plants are a common occurrence in biolistic-based transformations but were not observed in GM plants P1397 or P2193. According to Rech *et al.* (2008), use of the herbicide imazapyr to select transformed plants reduces chimerization because of translocation events and decreases the concentration of herbicide in the apical meristematic region of the embryonic axes. Thus, imazapyr hindered the development of cells that did not receive the insert.

The number of transgene copies (2-17) of *AtDREB2A* CA inserted into the T_0 generation of GM plants is characteristic of genetic transformations by biolistics and corroborated the independence of the events and the chance insertions into the receptor genome (Kohli *et al.*, 2010). For P2193, the *AtDREB2A* CA transgene insertions occurred at

Table 3 - Relative gene expression of the *AtDREB2A* transgene in the leaves and roots of T_2 generation, GM plants P2193, grown under hydroponic conditions.

Organ	Treatment (min)	Expression	Expression profile*	SE	p
Leaves	0	121.7	Up	± 114.5	0.001
	30	907.5	Up	± 146.8	0.001
	60	1042.0	Up	± 145.2	0.001
	90	508.8	Up	± 497.9	0.001
Roots	0	901.3	Up	± 111.4	0.001
	30	857.8	Up	± 120.0	0.001
	60	27749.7	Up	± 137.8	0.001
	90	1363.2	Up	± 182.8	0.001

The data were analyzed using *RESTmsc* software, which performs random comparisons of each treatment with its corresponding control. The standard error (SE) was calculated for median values, with $p < 0.1$. *The values for up-regulated (Up) profiles were significantly different from the control group when the expression profile during water stress diverged from the control at 10% probability ($p < 0.1$) upon randomization testing ($n = 2,000$ iterations).

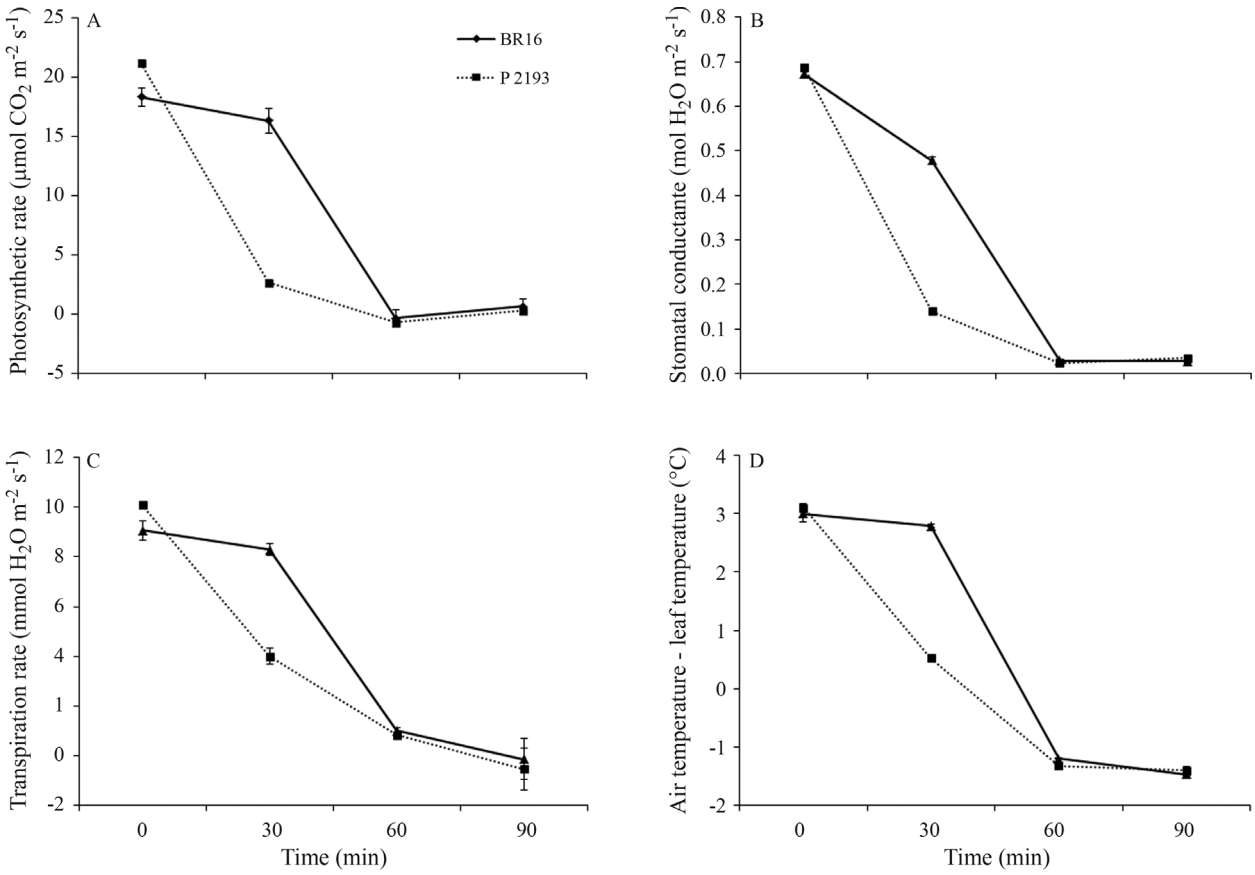

Figure 6 - Physiological responses in control plants (cultivar BR 16) and transgenic P2193 plants during stress for 30, 60 and 90 min. (A) Photosynthetic rate, (B) Stomatal conductance, (C) Transpiration rate and (D) Air-leaf temperature. The points are the mean ± SE of three determinations. The non-overlapping points at 30 min were significantly different from each other ($p \leq 0.05$, Duncan's test).

a single locus since the data from generations T_1 and T_2 showed a pattern of segregation that followed Mendel's first law. This conclusion agrees with the findings of Rech *et al.* (2008), who showed that transgenic plants transformed using biolistics follow the Mendelian model.

Southern blot data for P2193 indicated a large number of inserts in the T_0 generation, a loss of transgene inserts in the following generation and subsequent stability of the transgene, as demonstrated by the finding that generations T_1 and T_2 had the same number of inserts. P2193 had a high number of copies in the T_0 generation, whereas 0-5 copies were detected in samples from T_1 (segregation of non-linked inserts). For P1397, 9-17 transgene copies were detected in T_0 plants and 0-3 copies were observed in T_1 plants.

Loss of the transgene of interest is a common phenomenon during gamete segregation and may become more evident when a construct that includes modifications/deletions is inserted (Kohli *et al.*, 2010). Possible explanations of these losses include mechanisms such as intrachromosomal recombination, gene instability arising from manipulation during tissue culture, patterns of DNA methylation and co-elimination of transgenes activated by a plant genome defensive process (Romano *et al.*, 2005). Nevertheless, as Kohli *et al.* (2006) observed, multiple transgene copies may result in greater transcript expression compared to events with only a single copy. This situation occurred in P2193, which had a high copy number and high levels of gene expression in T_0, T_1 and especially T_2. This finding suggests that some transgene copies may have been inserted in a completely stable manner.

The levels of transgene expression in P2193 and P1397 during cell dehydration in a BOD incubator indicated that *A. thaliana* TF *DREB2A* was activated in soybean. In addition, the high transgene levels detected in T_0 plants, particularly in P2193 after 30 and 90 min of treatment, may be indicative of a defensive response by soybean to the imposed environmental stress, as previously observed by Yamaguchi-Shinozaki and Shinozaki (2006).

Gene activation and expression triggered by the stress-inducible *rd29* promoter was observed in *A. thaliana* (Quist *et al.*, 2009), *Brassica napus* (Dalal *et al.*, 2009), *Arachis hypogaea* L. (Bhatnagar-Mathur *et al.*, 2007) and *Glycine max* L. Merril (Polizel *et al.*, 2011). In the latter study, the investigators inserted and characterized the *rd29A:AtDREB1A* cassette in soybean and described the activation of the *rd29* promoter during leaf water stress. The *AtDREB1A* transgene levels were approximately 2-, 7-

and 3.4-fold after 20, 34 and 41 days of water deficit, respectively. Additionally, genes in the DREB1A-activated cascade, such as *GmPI-PLC*, *GmSTP*, *GmGR-RBP* and *GmLEA14*, exhibited high expression levels during severe stress (Polizel *et al.*, 2011).

High transgene expression was also detected during dehydration in the hydroponic system. Transgene expression levels in leaves and roots peaked after 60 min of water stress, with increases of 1,042- and 27,750-fold, respectively, compared to the controls. This finding suggested differential *AtDREB2A* CA expression in organs of soybean plants. Similar findings have been reported in studies of roots containing DREB2A homologous genes and the constitutive 35S promoter in maize (*ZmDREB2A*) (Qin *et al.*, 2007), *A. thaliana* (Liu *et al.*, 1998) and *Glycine max* (*GmDREBc*) (Li *et al.*, 2005). In soybean, transcription factors may have an indirect action in maintaining leaf and root cells during water stress by triggering the expression of genes associated with the protection of cell structures against dehydration. Zhong *et al.* (2008) demonstrated that transcription factors may be induced by specific conditions, including the duration of experimental stress.

The rate of photosynthesis, stomatal conductance, transpiration and leaf-air temperature difference in *AtDREB2A* CA transgenic plants differed from those of the control cultivar BR 16 for each treatment. These findings confirmed the occurrence of stress in the hydroponic system, with a tendency to reduce the rates of stomatal conductance, photosynthesis and transpiration (Jaleel *et al.*, 2009).

The levels of gene expression and copy numbers of *AtDREB2A* CA in GM plants indicated that this transgene was recognized by the cell enzymatic machinery and resulted in its insertion and expression, most likely in euchromatic regions where transgene methylation and/or transgene silencing did not occur (Kohli *et al.*, 2010). For P2193, the transgene was differentially expressed in three generations (T_0, T_1 and T_2) and in leaves and roots during a water deficit. Future investigations should address the mechanisms involved in this response in P2193 more thoroughly, particularly with regard to the genes associated with the cascade regulated by *AtDREB2A* CA. In addition, the introduction of *AtDREB2A* CA into breeding programs aimed at developing soybean cultivars that are more tolerant to drought and heat should also be considered.

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References

Almogueva C, Prieto-Dapena P, Díaz-Martín J, Espinosa JM, Carranco R and Jordano R (2009) The *Ha*DREB2 transcription factor enhances basal thermotolerance and longevity of seeds through functional interaction with HaHSFA9. BMC Plant Biol 9:1-12.

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.

Bhatnagar-Mathur P, Devi MJ, Reddy DS, Lavanya M, Vadez V, Serraj R, Yamaguchi-Shinozaki K and Sharma KK (2007) Stress-inducible expression of *At* DREB1A in transgenic peanut (*Arachis hypogaea* L.) increases transpiration efficiency under water-limiting conditions. Plant Cell Rep 26:2071-2082.

Dalal M, Tayal D, Chinnusamy V and Bansal KC (2009) Abiotic stress and ABA-inducible Group 4 *LEA* from *Brassica napus* plays a key role in salt and drought tolerance. J Biotechnol 17:137-145.

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

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K and Yamaguchi-Shinozaki K (2003) *OsDREB* genes in rice, *Oryza sativa* L., encoded transcription activators that function in drought, high-salt and cold-responsive gene expression. Plant J 33:751-763.

Embrapa (2006) Sistema Brasileiro de Classificação de Solos. 2nd edition. Embrapa, Rio de Janeiro, 306 pp.

Intergovernmental Panel on Climate Change - IPCC (2007) Contribution of working Group I to the fourth assessment report of the intergovernmental panel on climate change. In: Solomon S, Qin D, Manning M, Marquis M, Averyt K, Tignor MMB, Miller Jr HL and Chen Z (eds) Climate Change 2007: The Physical Science Basis. Cambridge University Press, Cambridge, 996 pp.

Jaleel CA, Manivannan P, Wahid A, Farooq M, Al-Juburi HJ, Somasundaram R and Panneerselvam R (2009) Drought stress in plants: A review on morphological characteristics and pigments composition. Int J Agric Biol 11:100-105.

Kariola T, Brader G, Helenius E, Li J, Heino P and Palva ET (2006) *Early responsive to dehydration 15*, a negative regulator of abscisic acid responses in *Arabidopsis*. Plant Physiol 142:1559-1573.

Kohli A, Melendi PG, Abranches R, Capell T, Stoger E and Christou Paul (2006) The quest to understand the basis and mechanisms that control expression of introduced transgenes in crop plants. Plant Signal Behav 1:185-195.

Kohli A, Miro B and Twyman RM (2010) Transgenic integration, expression and stability in plants: Strategies for improvements. In: Kole C, Michler CH, Abbott AG and Hall TC (eds) Transgenic Crop Plants: Principles and Development. Springer-Verlag, Berlin, pp 201-237.

Li XP, Tian AG, Luo GZ, Gong ZZ, Zhang JS and Chen SY (2005) Soybean DRE-binding transcription factors that are responsive to abiotic stresses. Theor Appl Genet 110:1355-1362.

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K and Shinozaki K (1998) Two transcription fac-

tors, DREB1 and DREB2, with EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought and low-temperature responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10:1391-1406.

Liu L, Zhu K, Yang Y, Wu J, Chen F and Yu D (2008) Molecular cloning, expression profiling and trans-activation property studies of a *DREB2*-like gene from chrysanthemum (*Dendranthema vestitum*). J Plant Res 121:215-226.

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.

Martins PK, Jordão BQ, Yamanaka N, Farias JRB, Beneventi MA, Binneck E, Fuganti R, Stolf R and Nepomuceno AL (2008) Differential gene expression and mitotic cell analysis of the drought tolerant soybean (*Glycine max* L. Merrill Fabales, Fabaceae) cultivar MG/BR46 (Conquista) under two water deficit induction systems. Genet Mol Biol 31:512-521.

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

Mizoi J, Ohori T, Moriwaki T, Kidokoro S, Todaka D, Maruyama K, Kusakabe K, Osakabe Y, Shinozaki K and Yamaguchi-Shinozaki K (2013) GmDREB2A;2, a canonical dehydration- responsive element binding protein2-type transcription factor in soybean, is posttranslationally regulated and mediates dehydration-responsive element-dependent gene expression. Plant Physiol 161:346-361.

Oya T, Nepomuceno AL, Neumaier N, Farias JRB, Tobita S and Ito O (2004) Drought tolerance characteristics of Brazilian soybean cultivars - Evaluation and characterization of drought tolerance of various of Brazilian soybean cultivars in the field. Plant Prod Sci 7:129-137.

Pfaffl MM, Horgan GW and Dempfle L (2002) Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:1-10.

Polizel AM, Medri ME, Nakashima K, Yamanaka N, Farias JRB, Oliveira MCN, Marin SRR, Abdelnoor RV, Marcelino-Guimarães FC, Fuganti R, *et al.* (2011) Molecular, anatomical and physiological properties of a genetically modified soybean line transformed with *rd29A:AtDREB1A* for the improvement of drought tolerance. Genet Mol Res 10:3641-3656.

Qin F, Kakimoto M, Sakuma Y, Maruyama K, Osakabe Y, Tran LSP, Shinozaki K and Yamaguchi-Shinozaki (2007) Regulation and functional analysis of *ZmDREB2A* in response to drought and heat stresses in *Zea mays* L. Plant J 50:54-69.

Quist TM, Sokolchik I, Shi H, Joly RJ, Bressan RA, Maggio A, Narsimhan M and Li X (2009) *HOS3*, an ELO-Like gene, inhibits effects of ABA and implicates an S-1-P/ceramide control system for abiotic stress responses in *Arabidopsis thaliana*. Mol Plant 2:138-151.

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

Romano A, Van Der Plas LHW, Witholt B, Eggink G and Mooibroek H (2005) Expression of poly-3-(R)-hydroxyalkanoate (PHA) polymerase and acyl-CoA-transacylase in plastids of transgenic potato leads to the synthesis of a hydrophobic polymer, presumably medium-chain-length PHAs. Planta 220:455-464.

Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K and Yamaguchi-Shinozaki (2002) DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. Biochem Biophys Res Commun 290:998-1009.

Sakuma Y, Maruyama K, Osakabe Y, Qin F, Seki M, Shinozaki K and Yamaguchi Shinozaki K. (2006a) Functional analysis of an *Arabidopsis* transcription factor, DREB2A, involved in drought-responsive gene expression. Plant Cell 18:1292-1309.

Sakuma Y, Maruyama K, Qin F, Osakabe Y, Shinozaki K and Yamaguchi-Shinozaki K. (2006b) Dual function of an *Arabidopsis* transcription factor DREB2A in water-stress responsive and heat-stress-responsive gene expression. Proc Natl Acad Sci USA 103:18822-18827.

Shinozaki K and Yamaguchi-Shinozaki K (2000) Molecular responses to dehydration and low temperature: Differences and cross-talk between two stress signaling pathways. Curr Opin Plant Biol 3:217-223.

Southern EM (1974) An improved method for transferring nucleotides from electrophoresis strips to thin layers of ion-exchange cellulose. Anal Biochem 34:317-318.

Stolf-Moreira R, Lemos EGM, Abdelnoor RV, Beneventi MA, Rolla AAP, Pereira SS, Oliveira MCN, Nepomuceno AL and Marcelino-Guimarães FC (2011) Identification of reference genes for expression analysis by real-time quantitative PCR in drought-stressed soybean. Pesqui Agropecu Bras 46:58-65.

Terashima A and Takumi S (2009) Allopolyploidization reduces alternative splicing efficiency for transcripts of the wheat *DREB2* homolog, *WDREB2*. Genome 52:100-105.

Yamaguchi-Shinozaki K and Shinozaki K (1994) A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell 6:251-264.

Yamaguchi-Shinozaki K and Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol 57:781-803.

Zhong R, Lee C, Zhou J, McCarthy RL and Ye ZH (2008) A battery of transcription factors involved in the regulation of secondary cell wall biosynthesis in *Arabidopsis*. Plant Cell 20:2763-2782.

Internet Resources

Associação Brasileira das Indústrias de Óleos Vegetais (ABIOVE), http://www.abiove.com.br (May 20, 2013).

Companhia Nacional de Abastecimento (CONAB), http://www.conab.gov.br (May 8, 2012).

Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), http://www.cnpso.embrapa.br (May 10, 2012).

United States Department of Agriculture (USDA), http://www.usda.gov/wps/portal/usda/usdahome (July 6, 2011).

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