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Cloning and quantitative expression analysis of drought-induced genes in soybean

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ABSTRACT. We determined the expression levels of DREB transcription factor (*Gmdreb1*) and of the genes *Gmgols*, *Gmpip1b*, *Gmereb*, and *Gmdefensin* in drought-tolerant (MG/BR46-Conquista) and drought-sensitive (BR16) genotypes of soybean, during drought. The trial was carried out in a controlled-environment chamber, set up to provide drought conditions. Sequences of *Arabidopsis thaliana* DREB-family proteins were used to build a phylogenetic tree through the alignment of the conserved regions near the AP2 domain. We found that *Gmdreb1* is similar to *Atrap2.1*, which is located near the *At*DREB1 and *At*DREB2 families. The amplified fragment was cloned and sequenced; alignment with the sequence available at Genbank showed total similarity. Expression analysis showed that under drought: a) *Gmdreb1* expression increased in leaves and roots of both genotypes and expression level changes occurred that were correlated with the length of the water-deficit period; b) there

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were increased expression levels of *Gmdefensin* in roots of MG/BR46; c) expression of *Gmgols* increased in leaves and roots of the two genotypes; d) *Gmpip1b* expression generally increased, except in roots of BR16, and e) the same was found for *Gmereb*, except in roots of MG/BR46.

Key words: Drought; *Glycine max*; Gene expression; Quantitative PCR; Dreb gene; Water deficit

INTRODUCTION

Abiotic stresses, such as drought, can limit the geographical distribution of plants and limit the growth and yield of economically important species. Substantial efforts have been devoted to determine the nature of the injury caused by these stresses and the plant-protection mechanisms involved in tolerance responses (Bray, 2004). Some genes are up-regulated and others are down-regulated in plants under drought stress. This regulation occurs at distinct levels, from the moment of stress detection to the production of biologically active proteins (Shinozaki and Yamaguchi-Shinozaki, 2007). The scientific community has attempted to determine how plants detect water deficit and process information to alter gene expression.

An important step forward was the identification of a *cis*-acting dehydration responsive element (DRE), the function of which is important for the expression of genes responding to dehydration in *Arabidopsis thaliana* (Yamaguchi-Shinozaki and Shinozaki, 1994). This element has a C-repeat sequence of 5 bp (CCGAC) that is present in one or multiple copies in the promoter region of many plant genes related to dehydration response (Baker et al., 1994).

The transcription factor DREB1 (dehydration-responsive element-binding protein) specifically interacts with the element DRE and induces the expression of genes involved in the stress response of *A. thaliana*. Twelve stress-induced genes were identified as DREB1 target genes by Seki et al. (2001), and Fowler and Tomashow (2002) described 41 genes as DREB target genes. The function of some of these gene products seems to be related to structure and basic cell function maintenance during water deficit, low temperatures and high salinity (Shinozaki and Yamaguchi-Shinozaki, 1996; Kasuga et al., 2004).

Differential expression of genes involved in defense mechanisms against cell dehydration plays a key role in water-deficit tolerance. The understanding of how the expression of individual genes contributes to the final response in cellular, physiological and agronomical terms has enabled the design of strategies for developing plants that are more tolerant to water deficit. The defense mechanisms involved in this process seem to be similar across the plant kingdom. Therefore, research using model plants, such as *A. thaliana* and tobacco (*Nicotiana tabacum*), can help to identify genes with key functions in defense mechanisms in other plant species. Techniques such as real-time quantitative polymerase chain reaction (RT-qPCR), allow precise quantification of the mRNA levels of genes of interest when their expressions are compared under various conditions or treatments (Volkov et al., 2003).

Hence, the objectives of this study were to investigate the expression levels of a soybean DREB transcription factor (*Gmdreb1*), to clone it for further studies and to evaluate the expression of genes that are DREB regulated in *A. thaliana*, such as *Gmgols*, *Gmpip1b*, *Gmereb*, and *Gmdefensin*, in two soybean genotypes, MG/BR46 (Conquista) (referred to as MG/BR46), and BR16, during periods of water deficit, to elucidate their expression profiles.

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MATERIAL AND METHODS

Sequencing

A sequence showing a high degree of similarity with *At*DREB transcription factor was detected in soybean at Genbank, namely accession No. AF514908.1. A phylogenetic analysis was performed in the protein sequences of *A. thaliana* containing the conserved AP2 domain, using the molecular evolutionary genetic analysis (MEGA) version 3.1 software (Kumar et al., 2004). To obtain the coding region for *Gmdreb1*, primers were designed from start and stop codons of the sequence, corresponding to forward: 5' TTT AAT CCG TTA TAT GCC ACC 3', and reverse: 5' TAT CAA TCT TGA AGC TCT TCG 3', producing a fragment of 604 bp.

PCR were performed using a Perkin Elmer 9600 thermocycler (Perkin Elmer, Massachusetts, CA, USA), and samples were purified with a Wizard SV Gel Kit (Promega, Carlsbad, CA, USA), according to manufacturer instructions. Cloning was done using the pGEM-T Easy Vector kit (Promega), and transformation was carried out according to Miller and Nickoloff (1995), using a micro pulser electroporator (Bio-Rad, Foster City, CA, USA). Sequencing of insert-containing plasmids was performed by PCR-cycle sequencing, using the ABI Prism Big Dye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), and the fragments were separated using an ABI Prism 3100 (Applied Biosystems). The chromatograms were passed through a base-calling process using phred (Ewing et al., 1998), which assigned quality values above 20 to the bases.

Gene-expression analysis

Gene-expression analysis was performed using RT-qPCR on tissue samples of leaves and roots of soybean plants of drought tolerant and sensitive genotypes, MG/BR46 and BR16, respectively. Nine days after 10 seeds of each genotype were placed in germitest paper rolls, plantlets were moved to a controlled environment chamber and exposed to the air on paper tissue under the chamber conditions (28 ± 2°C, 60% RH ± 2°C) and water stress treatment for 0, 1, 3, and 5 h.

At the end of each period, roots were collected, placed in liquid nitrogen, and stored at -80°C. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions and subsequently treated with DNAse (Invitrogen). For reverse transcription and synthesis of the complementary DNA (cDNA), Moloney murine leukemia virus (Invitrogen) reverse transcriptase was used, as described by Schenk et al. (2003).

The Primer Express program version 3.0 (Applied Biosystems) was used to design the primers for RT-qPCR. The primer sequences were determined on the 3' regions of the genes with putative amplicons of 75 to 150 bp (Table 1). The genes used in the expression analysis were chosen based on a literature review, where it was found that, in many species, their differential expression is related to drought tolerance. After the identification of candidate genes, a search for similar sequences in soybean was made, such as: *Gmdreb1a* (dehydration-responsive element-binding protein, Genbank accession No. AF514908.1)-signal-transduction cascade in responses to abiotic stress, *Gmdefensin* (drought-induced proteinase inhibitor, Genbank accession No. U12150), *Gmpip1b* (putative channel protein, aquaporin, Genbank accession No. U27347), *Gmgols* (galactinol synthase, Genbank accession No. AY126715) - a key

enzyme in raffinose synthesis. The gene for 18S rRNA was chosen as the endogenous control (Genbank accession No. X02623.1) for normalization, because its level of expression was shown to be constant in the presence of abiotic stresses (Stolf, 2007).

Table 1. Primer sequences of genes utilized in real-time quantitative polymerase chain reation.

Genes	Primer sequences	
	Foward (5'-3')	Reverse (5'-3')
Gmr18SrRNA	AAACGGCTACCACATCCAAG	CCTTCAATGGATCCATCGTTA
Gmdreb1	CGACCAGGAGGGCAGTGAT	GCTTTTCGGCGAATGGAAT
Gmpip1b	TCATGGGTTTCAAAAAGGAGA	GCTTGCAATAAAAGCACAAGC
Gmdefensin	TTTGAGTGACACCAACTGTGG	AACAATGTTTGGTGCAGAAGC
Gmgols	TGGAAATCAAGTGTGATCCAAG	GAAAAGCCGGGACACATAAA

The RT-qPCR analysis was performed with a thermocycler, 7300 Real Time System (Applied Biosystems), and the Platinum®SYBR®Green qPCR SuperMix UDG (Invitrogen). The reaction conditions were 50°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 2 min, 62°C for 30 s and 72°C for 30 s; the data were collected in the last phase (extension phase). The $E = [10^{-1/slope}] - 1$ formula was used to calculate the reaction efficiency both of the target genes and the endogenous control. The results were captured by the Sequence Detection program (Perkin Elmer) and analyzed by the Relative Expression Software Toll (REST) version 2.0.7 (Pfaffl et al., 2002).

RESULTS AND DISCUSSION

A fragment of 604 bp was amplified using primers designed for cloning the coding region of the *Gmdreb1*. After cloning, the fragment was sequenced and its similarity with the coding sequence of the gene *Gmdreb1* was verified. The Blastn aligning of the *Gmdreb1* nucleotide sequence showed 100% similarity to the *dreb* sequence available at Genbank through the accession No. AF514908.1. The conserved regions near the AP2 domain, presented in the phylogenetic tree, were aligned using the ClustalW program (www.ebi.ac.uk/clustalw). The AP2 domain of these transcription factors links to the DRE region located at the promoter region of stress-induced genes. It was found that *Gmdreb1* is similar to the *Atrap2.1* gene, which is located in the *Atdreb1* and *Atdreb2.1* families (Figure 1).

The phylogenetic analysis, performed with all peptide sequences containing the conserved AP2 domain of *A. thaliana* available at Genbank, allowed the detection of three distinct groups, DREB1, DREB2 and RAP2.1, the last of which contains *Gmdreb1*, the object of this study. Studies by Kasuga et al. (1999) showed similarity in the amino acid sequences of proteins *At*DREB1A and *At*DREB2A. Such similarity is in the 58-amino acid conserved region of the DNA linkage domain, present in a large array of plant gene families, including the proteins EREBPs in tobacco and APETALA (AP2) in *A. thaliana*, which are involved in the expression of ethylene response and in floral morphogenesis, respectively.

*At*DREB1A has a 216-amino acid open reading frame encoding a 24.2-kD protein, whereas *At*DREB2A has a 335-amino acid open reading frame encoding a 37.7 kD protein (Kasuga et al., 1999). Each DREB protein has a basic region at the N-terminal end that can function as a nuclear localizing signal and an acid region at the C-terminal end that can func-

tion as an activating domain for transcription. These data suggest that each *dreb* gene encodes a DNA-binding protein that can function as a transcription activator in plants. Also, two homologues of *At*DREB1A (*At*DREB1B and *At*DREB1C) were detected in tandem on chromosome 4 of *A. thaliana* (Gilmour et al., 1998), induced by low temperature, and one homologue of *At*DREB2A (*At*DREB2B), induced by dehydration. Such findings suggest the existence of two independent families of DREB proteins (DREB1 and DREB2) acting as transcription factors in two separate signal-transduction pathways, in response to low temperatures and dehydration.

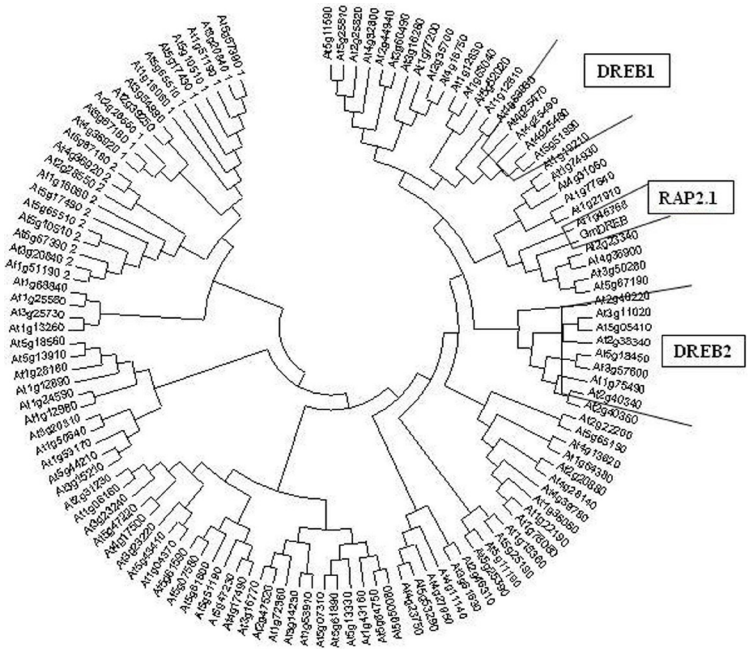

Figure 1. Phylogenetic tree of the DREB family in *Arabdopsis thaliana* containing the AP2 conserved domain.

Mechanisms of adaptation to cold and drought are highly complex, but some of the metabolic pathways are regulated by abscisic acid, which acts as a second messenger, whereas other paths are abscisic acid-independent (Zhang et al., 2004). The expression of these genes (*dreb*) can be altered within minutes of application of cold (Gilmour et al., 1998) or drought (Haake et al., 2002) stress. Two proteins containing AP2, RAP2.6 and RAP2.1 domains were also induced by cold (Fowler and Thomashow, 2002). The RAP2.1 promoter, which contains two copies of the CCGAC sequence of the element CRT/DRE, was highly expressed in transgenic *A. thaliana* plants constitutively expressing DREB, suggesting that it can be a target for DREB activators (Fowler and Thomashow, 2002). The RAP2.1 sequence in *A. thaliana* found at Genbank (accession No. At1g46768) was obtained from plants exposed to cold.

Understanding the molecular-response mechanisms in plants under harsh conditions such as drought is still limited, although the number of genes known to moderate these responses has increased (Kizis et al., 2001). Some of these genes are stress induced and encode

products that confer tolerance to adverse conditions, whereas others encode upstream regulators acting inside the signaling pathway, controlling the stress response.

Increases in the expression of *Gmdreb1* in leaves of MG/BR46, under 1- and 3-h treatments were, respectively, two and three times greater than in the control. In leaves of cultivar BR16 under 1-, 3- and 5-h treatments, the increases in expression were two, two and five times greater than in the control, respectively. The expression in MG/BR46 roots submitted to 1-h treatment increased nine times and, under 3- and 5-h treatments, the increases were 6- and 19-fold, respectively (Table 2). In roots of BR16 under 1- and 5-h treatments, the expression levels of the gene *Gmdreb1* were eight and three times greater than the control, respectively (Table 3).

Table 2. Gene expression for real-time quantitative polymerase chain reaction (qRT-PCR) of MG/BR46 (Conquista) (water-stress tolerant) genotype under water stress treatment (1, 3 and 5 h) in hydroponic system to leaves and roots, for four genes: *Gmdreb1a*, *Gmpip1b*, *Gmdefensin*, *Gmgols*.

Gene		Expression	95%CI	P(H1)	Result	Primer efficiency
Leaf MG/BR46 (Conquista) genotype						
Gmdreb1a	1 h	2.58	[2.08; 3.38]	0.01	Up	1.00
	3 h	3.08	[2.56; 3.93]	0.01	Up	
	5 h	0.06	[0.05; 0.08]	0.07		
Gmpip1b	1 h	10.60	[5.57; 20.38]	0.03	Up	0.89
	3 h	180.81	[107.55; 314.87]	0.00	Up	
	5 h	0.02	[0.01; 0.04]	0.00	Down	
Gmdefensin	1 h	1.55	[1.00; 2.67]	0.10		1.00
	3 h	80.89	[55.57; 144.32]	0.00	Up	
	5 h	0.00	[0.00; 0.00]	0.00	Down	
Gmgols	1 h	157.29	[31.82; 955.99]	0.03	Up	0.83
	3 h	54.74	[0.77; 1270.01]	0.14		
	5 h	0.55	[0.35; 0.77]	0.00	Down	
Root MG/BR46 (Conquista) genotype						
Gmdreb1a	1 h	9.41	[6.53; 16.11]	0.03	Up	1.00
	3 h	6.04	[2.49; 13.11]	0.03	Up	
	5 h	19.31	[13.81; 31.95]	0.03	Up	
Gmpip1b	1 h	15.72	[9.24; 26.89]	0.03	Up	0.89
	3 h	5.42	[0.87; 15.59]	0.15		
	5 h	3.04	[1.76; 5.03]	0.03	Up	
Gmdefensin	1 h	473.83	[244.30; 797.97]	0.01	Up	1.00
	3 h	317.84	[83.21; 700.49]	0.02	Up	
	5 h	55.95	[40.28; 74.45]	0.00	Up	
Gmgols	1 h	2.73	[0.79; 23.61]	0.30		0.83
	3 h	1.65	[0.24; 22.86]	0.71		
	5 h	1.09	[0.19; 11.54]	0.90		

95%CI = confidence interval at 95%; P(H1) = probability of the H1 differential expression hypothesis. The *Gm18SrRNA* was used as a reference.

The increase in *Gmdreb1* expression in plant roots of both genotypes under 1-h treatment implies a more rapid expression response, in comparison to the other periods of exposure. Under 3-h treatment, plant roots of MG/BR46 and BR16 (Tables 2 and 3) showed reductions in the expression levels of the *Gmdreb1*, reaching values lower than those of the control. For the longest treatment period (5 h), MG/BR46 plants maintained expression at the same level as in the 3-h treatment. In the leaves of BR16, there were slower and steadier increases in the expression level during the treatment periods, and MG/BR46 plants barely showed any increases. One possible explanation for BR16 leaves and roots differing in expression of *Gmdreb1* is that the leaves have a defense mechanism to avoid water loss whereas the roots

are more sensitive since they are usually in the soil and not exposed to open air. Since the treatments were applied in an environment-controlled chamber, the exposed roots dried more quickly and probably senesced more rapidly than did the leaves.

Table 3. Gene expression for real-time quantitative polymerase chain reaction (qRT-PCR) of BR16 (water-stress tolerant) genotype under water stress treatment (1, 3 and 5 h) in hydroponic system to leaves and roots, for four genes: *Gmdreb1a*, *Gmpip1b*, *Gmdefensin*, *Gmgols*.

Gene		Expression	95%CI	P(H1)	Result	Primer efficiency
Leaf BR16 genotype						
Gmdreb1a	1 h	2.03	[1.52; 3.18]	0.03	Up	1.00
	3 h	2.48	[2.17; 2.96]	0.00	Up	
	5 h	5.58	[4.87; 6.79]	0.00	Up	
Gmpip1b	1 h	5.97	[3.30; 13.33]	0.00	Up	0.89
	3 h	28.53	[14.03; 52.88]	0.00	Up	
	5 h	20.02	[9.15; 52.63]	0.06		
Gmdefensin	1 h	9.41	[4.15; 29.47]	0.02	Up	1.00
	3 h	2.90	[1.76; 6.66]	0.00	Up	
	5 h	4.57	[1.30; 44.32]	0.00	Up	
Gmgols	1 h	9.58	[1.56; 68.43]	0.00	Up	0.83
	3 h	62.77	[15.63; 210.24]	0.06		
	5 h	102.41	[26.79; 328.80]	0.00	Up	
Root BR16 genotype						
Gmdreb1a	1 h	8.86	[7.57; 10.32]	0.00	Up	1.00
	3 h	0.37	[0.24; 0.66]	0.00	Down	
	5 h	2.67	[1.91; 4.05]	0.00	Up	
Gmpip1b	1 h	0.98	[0.49; 1.65]	0.95		0.89
	3 h	0.00	[0.00; 0.00]	0.00	Down	
	5 h	1.04	[0.56; 2.15]	0.88		
Gmdefensin	1 h	0.56	[0.00; 3020.14]	0.75		1.00
	3 h	0.00	[0.00; 0.05]	0.10		
	5 h	0.15	[0.00; 13.90]	0.59		
Gmgols	1 h	12.68	[1.68; 56.94]	0.00	Up	0.83
	3 h	0.02	[0.01; 0.03]	0.03	Down	
	5 h	9.26	[0.47; 59.76]	0.39		

95%CI = confidence interval at 95%; P(H1) = probability of the H1 differential expression hypothesis. The Gm18SrRNA was used as a reference.

Differential expression of *Gmpip1b* was observed in both genotypes in the various tissues and treatments. In the leaves of MG/BR46 under 1- and 3-h treatments, its expression increased 10 and 180 times, respectively. However, under 5-h treatment, expression was down-regulated in leaves (Table 2), whereas in roots, although not statistically significant, expression increased five times under the 3-h treatment. In BR16, the expression levels of *Gmpip1b* in leaves submitted to 1- and 3-h treatments were five and twenty-eight times higher than in the controls, respectively (Table 3). In roots, the only difference detected was in MG/BR46; under 1- and 5-h treatments, the expression of *Gmpip1b* was fifteen times and three times higher than in the control, respectively. In the first two periods of treatment (1 and 3 h) there was a differential and gradual expression of *Gmpip1b* in leaves of both MG/BR46 and BR16 (Tables 2 and 3). However, under 5-h treatment the expression level in MG/BR46 decreased to values smaller than those of the 0-h control. BR16 plants, although showing lower expression level values of this gene in the 5-h treatment (in relation to the 3-h treatment), maintained the differential expression level in relation to the control. In roots, BR16 did not

show differential expression of the *Gmpip1b* gene; however, roots of MG/BR46 also showed increased expression under 1- and 3-h treatments. The same pattern was seen in MG/BR46 leaves. It is suggested that 5 h under water stress was a critical period for MG/BR46 roots, since they were exposed to open air and may have entered senescence shortly after 3 h.

According to Johansson et al. (2000), the expression of genes encoding aquaporins is regulated in a cell-specific manner, via hormones and by environmental signals such as nematode infection or drought. Over 30 genes encode homologous aquaporins in *A. thaliana*, and the PIP family is located mainly in the plasma membrane. Studies have shown that aquaporins are multifunctional transporters of solutes and water (Maurel and Chrispeels, 2001). The transport of gases, such as CO_2 and NH_3, through aquaporins supports the suggestion that these proteins may be important in leaves and in root-nodule systems (Tyerman et al., 1999).

Transgenic *A. thaliana* (Kaldenhoff et al., 1998) and *Nicotiana tabacum* (Siefritz et al., 2001) plants with down-regulation of gene *pip1*, showed reduced osmotic water permeability in the protoplasts of leaves and roots, respectively, compared to control plants (Martre et al., 2002). Besides, plants with *A. thaliana pip1b* antisense displayed dry root mass five times larger than did control plants (Kaldenhoff et al., 1998). In another study, the *A. thaliana pip2* gene was over-expressed in tobacco, reducing water exchange through the plasma membrane in transformed plants compared to controls (Shukla and Chrispeels, 1998).

In leaves of MG/BR46 submitted to 3 h of water deficit, expression of the *Gmdefensin* gene increased eighty times, whereas, in the same tissue of BR16, increases in expression were nine, two and three times when submitted to 1-, 3- and 5-h treatments, respectively. In the roots of genotype MG/BR46, in the 1-h treatment, *Gmdefensin* expression increased 470 times, and then declined in 3- and 5-h treatments to 317 and 57 times greater than in the control, respectively. No differences in *Gmdefensin* gene expression were detected in the roots of BR16 submitted to these treatments, compared to the control.

In response to water stress, MG/BR46 showed increases in expression levels of *Gmdefensin* that were considerably higher in roots than in leaves. However, the expression in the roots decreased slowly to the last treatment period (5 h). In BR16, *Gmdefensin* expression increased only in leaves with the 1-h treatment.

Defensins are a family of small, cysteine-rich proteins that have antimicrobial activity, protecting cell structures from attack by microorganisms. A recent study pointed out that defensins are also involved in abiotic-stress responses (Maitra and Cushman, 1998). This same study detected a 10-fold increase in the expression of a *defensin*-encoding gene in a drought-tolerant soybean genotype, compared to a sensitive counterpart, when under water deficit. Yamada et al. (1997) also detected increased expression of defensins in tobacco-relative *Nicotiana excelsior* under salt stress, showing that the gene is involved in the response to cell dehydration.

In relation to the control, leaves of MG/BR46 under the 1-h treatment showed increased expression of *Gmgols* of 157 times. For leaves of BR16 under 1- and 5-h treatments, the expression levels were nine and 102 times higher, respectively. In BR16 roots under 1-h treatment, the increase was 12-fold, and after 5 h it increased 9-fold.

Gmgols, which encodes galactinol synthase, showed increased expression in leaves of the MG/BR46 genotype in the early hours of stress, whereas no differences in expression were seen in the roots. In contrast, in roots and leaves of the sensitive genotype, BR16, distinct expression of the gene was observed in all stress periods. Galactinol synthase plays a key role in biosynthesis of raffinose oligosaccharides and soluble carbohydrates, which are

components of sugars transported through the phloem in higher plants (Haritatos et al., 2000). The accumulation of this carbohydrate in plants is associated with tolerance to environmental stresses such as cold, heat, and dehydration (Downie et al., 2003). A number of studies have shown increased expression of *gols* in response to abiotic stresses, as in the vegetative tissues of cold-exposed common bean (*Phaseolus vulgaris*) and in rice (*Oryza sativa*) submitted to cold and osmotic stresses (Liu et al., 1998).

It is noteworthy that leaves of MG/BR46 showed lower expression of all these genes with the 5-h treatment. It is suggested that 5 h was a critical period for MG/BR46, because the plants were already senescent, or their metabolism could be increased for physiologic processes. In roots, only *Gmdreb1*a showed the highest expression in the last period (5-h treatment). In BR16, the *Gmdreb1a* had the same profile as *Gmgols*, suggesting that the gene can regulate *Gmgols* under water-stress conditions, and also the other genes in the cascade.

The two soybean genotypes displayed differences in the expression of the genes analyzed, showing that MG/BR46 has mechanisms to detect water stress more promptly than does BR16. The molecular differences detected between these two genotypes permit the manipulation of important soybean genotypes by genetic engineering to produce plants that are more tolerant to water stress and that can retain viability for longer periods under drought conditions.

Due to the complexity of the processes of adaptation to cold and drought that are under transcriptional control, it is not surprising that transcription factors represent one of the best targets for genetic engineering plants for improved tolerance to cold and drought (Zhang et al., 2004). Yet, not all transcription factors involved in drought-response signal transduction are appropriate targets for biotechnological intervention. Presumably, post-transduction alterations would be necessary for protein activation in transgenic plants (Shinozaki and Yamaguchi-Shinozaki, 2000).

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