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Soybean physiology and gene expression during drought

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ABSTRACT. Soybean genotypes MG/BR46 (Conquista) and BR16, drought-tolerant and -sensitive, respectively, were compared in terms of morphophysiological and gene-expression responses to water stress during two stages of development. Gene-expression analysis showed differential responses in *Gmdreb1a* and *Gmpip1b* mRNA expression within 30 days of water-deficit initiation in MG/BR46 (Conquista) plants. Within 45 days of initiating stress, *Gmp5cs* and *Gmpip1b* had relatively higher expression. Initially, BR16 showed increased expression only for *Gmdreb1a*, and later (45 days) for *Gmp5cs*, *Gmdefensin* and *Gmpip1b*. Only BR16 presented down-regulated expression of genes, such as *Gmp5cs* and *Gmpip1b*, 30 days after the onset of moisture stress, and *Gmgols* after 45 days of stress. The faster perception of water stress in MG/BR46 (Conquista) and the better maintenance of up-regulated gene expression than in the sensitive BR16 genotype imply mechanisms by which the former is better adapted to tolerate moisture deficiency.

Key words: Photosynthesis and real-time quantitative PCR; Drought; *Glycine max*

INTRODUCTION

Drought, high salinity and low temperature are the most common environmental stress factors that adversely affect plant growth and development; they are major limitations to crop productivity worldwide. To increase yields or reduce yield losses under such adverse conditions, it is necessary to improve tolerance to environmental stress (Shinozaki and Yamaguchi-Shinozaki, 2007).

The effects of environmental stress can be quantified as reduction in plant growth and concomitant reduced dry weight. With moisture deficiency, stomatal conductance decreases, thus reducing water loss from leaves and photosynthetic rate is reduced due to decreased intercellular CO_2 concentration (Cornic and Briantais, 1991). As the relationship between photosynthetic rate and stomatal conductance is normally curvilinear, photosynthetic rate is much more dependent on mesophyll resistance than on stomatal conductance; therefore, photosynthetic rate may be unaffected while stomatal conductance decreases to a certain extent.

For millions of years, plants have been developing mechanisms to tolerate or escape water deficits. These mechanisms range from morphological modifications, such as development of more root hairs, deepening of roots, and rolling of leaves, to physiological adaptations, such as alterations in carbon partitioning and isotope discrimination, osmotic adjustment, and alterations in rate and efficiency of photosynthesis (Taji et al., 2004). Each response to water deficit follows molecular events that are initiated by stress perception. This perception seems to be related to changes in the cell volume caused by dehydration and, consequently, alterations in cell-wall pressure and osmotic potential (Panikulangara et al., 2004). These modifications alter cell structure, activating enzyme complexes that trigger molecular events in cascade, leading to expression of many categories of genes involved in the activation of defense responses.

Molecular responses to water deficiency have mainly been investigated in terms of survival of stress. There are changes in expression of regulatory genes that potentially could be manipulated (Iuchi et al., 2000). Abscisic acid (ABA) plays a key role in the molecular signal that is triggered by the onset of drought. This phytohormone apparently works as a second messenger after stress perception, inducing stomatal closure and activating several stress-related genes. On the other hand, some research has shown that there are regulatory systems that are ABA-independent (Yamaguchi-Shinozaki and Shinozaki, 2005).

Various stress-induced genes, such as *rd29a* in *Arabidopsis thaliana*, are induced through an ABA-independent pathway. The protein DREB1 (dehydration-responsive element-binding) is a transcription factor that binds to the promoter of genes such as *rd29a*, thereby inducing expression in response to drought, salinity, or low temperatures. Many genes activated under water-stress conditions by transcription factors such as DREB1 have been identified (Maruyama et al., 2004); their gene products present high similarity with known proteins.

Gene products involved in water-deficit responses can be classified into two groups: the first includes proteins, osmolytes, and other compounds that probably confer direct tolerance to abiotic stresses, including chaperones, heat-shock proteins (HSPs), such as HSP70 (Sung et al., 2001), late embryogenesis abundant (LEA) proteins, such as LEA14 (Singh et al., 2002), mRNA-binding proteins, such as glycine-rich protein (Bocca et al., 2005), key enzymes for osmolyte biosynthesis, such as galactinol synthase and delta-1-pyrroline-5-carboxylate synthetase, involved in synthesis of raffinose-family oligosaccharides (Hannah et al., 2006) and proline (Schafleitner et al., 2007), respectively, and water-channel proteins, such as PIP1b (aquaporin; Aharon et al., 2003), which are multi-

functional proteins involved in the facilitation of transport of solutes and water in leaves and roots.

The second group comprises molecules involved in further regulation of signal-transduction and stress-responsive gene expression. These molecules are regulatory proteins, represented by various transcription factors such as DREB1, ABA-responsive element, nitrogen assimilation control protein, ethylene-response factors, kinases, phosphatases, enzymes involved in phospholipid metabolism, and other signaling molecules, such as calmodulin-binding protein (Shinozaki and Yamaguchi-Shinozaki, 2007).

Tolerance of drought is a complex phenomenon, because it changes according to drought intensity and duration, and the plant's developmental stage during which drought occurs. Also, more than one stress may affect the plant simultaneously, activating many genes in the stress response. Plant acclimation to stresses in general, including drought, induces, at least to some extent, common reactions, such as signaling pathways, target-gene expression, and biochemical/metabolic changes. As a result, differences in water-stress tolerance among cultivars, or within a cultivar at various developmental stages, may result from differences in the expression of genes in signal-perception and transduction mechanisms (Chinnusamy et al., 2004).

Much information about drought-tolerance mechanisms in soybean has been reported in recent decades. However, much effort will still be needed if the molecular mechanisms involved in defense and regulation responses against cell dehydration are to be understood. Physiological and agronomic characterizations have shown that two soybean genotypes, BR16 and MG/BR46 (Conquista), are drought-sensitive and -tolerant, respectively (Oya et al., 2004). Here, we analyzed physiological and gene-expression responses to dehydration in these contrasting genotypes, looking for correlations with drought-tolerance capacity.

MATERIAL AND METHODS

Plant materials

The MG/BR46 (Conquista) genotype presents a deep root system and productivity aspects that make it tolerant of drought. BR16 presents high productivity, but is sensitive to drought conditions. In our previous examination of many genotypes of soybean for drought tolerance, MG/BR46 (Conquista) and BR16 were among the most discrepant (Oya et al., 2004). Seeds of both genotypes were sown in 10-L pots containing washed sand and grown (one plant per pot) under greenhouse conditions (30 ± 2°C during the day and 25 ± 2°C at night, with relative humidity near 50%).

Experimental design

Two hundred and forty plants were divided into two groups: control plants were maintained at 15% gravimetric humidity (GH) (near field capacity) throughout, and stressed plants were exposed to 5% GH (Jones, 2007) from 45 days after planting. Moisture stress was initiated by withholding irrigation, until sand humidity reached 5% GH; the control group was kept at 15% GH until the conclusion of the experiment (90 days later). Pots were weighed twice per day (early in the morning and late afternoon) and water was added to maintain the sand at the desired values of GH. Twice per week, balanced nutrient solution at pH 6.6 was applied instead of water (Hewitt, 1963). Plants from additional pots receiving the same treatments as the experimental pots were harvested and weighed weekly. The plant weight was

subtracted from pot weight to maintain precise control of the gravimetric water content of the soil. The experimental design was a completely randomized factorial (cultivar x water-stress levels), with 10 replicates. Samples for physiological and molecular analysis were collected at 30 and 45 days after the onset of stress, designated "control 30 days" (C30), "stress treatment 30 days" (S30), "control 45 days" (C45), and "stress treatment 45 days" (S45).

Morphophysiological parameters

The parameters evaluated were leaf area, relative growth rate (RGR) - including root (RRGR), shoot (SRGR), leaves (LRGR) and total (TRGR) - net photosynthetic rate, stomatal conductance, internal CO_2 concentration and carbon-isotope discrimination (CID) ($^{13}C/^{12}C$). Photosynthetic rate, stomatal conductance and internal CO_2 concentration were measured in the middle leaflet at the third completely expanded leaf from the top in all replicates of each treatment. Leaf area was measured with an LI-3100 Area Meter (LI-COR, Lincoln, NE, USA). The RGR was calculated using the formula RGR = ln (FDW) - ln (IDW) / (t_2 - t_1), where FDW is the final dry weight, IDW is the initial dry weight and t_1 and t_2 are the times in days at the beginning and at the end of the experiment (Chiariello et al., 1991). Values of photosynthetic rate, stomatal conductance and internal CO_2 concentration were determined using a portable photosynthesis meter, model LI-6400 (LI-COR), at an equipment-programmed light intensity of 1000 μmoL m^{-2} s^{-1}. CID values were determined after analysis using a Delta XP Plus stable spectrophotometer (ThermoFinnigan, Bremen, Germany). ANOVA was performed on the physiological data, using the SAS program, comparing treatment effects within each cultivar. Means were compared using the Tukey test ($\alpha = 0.05$).

Gene-expression analysis

At 30 and 45 days after initiation of water-deficit stress, roots from 10 plants 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. For reverse transcription and synthesis of complementary DNA (cDNA), Moloney murine leukemia virus (Invitrogen) reverse transcriptase was used, as described by Schenk et al. (2003).

Primer Express program 3.0 (Applied Biosystems, Foster, CA, USA) was used to design the primers for real-time polymerase chain reaction (PCR). The primer sequences were determined on the 3' regions of the genes with putative amplicons of 75 to 150 bp (Table 1). Six genes well known to encode proteins related to response to drought (Shinozaki and Yamaguchi-Shinozaki, 2007) were chosen: *Gmdreb1a* (dehydration-responsive binding protein, GenBank accession No. AF514908.1), part of the signal transduction cascade in response 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; *Gmereb* (ethylene-response factor, GenBank accession No. AF537220), and *Gmp5cs* (Δ^1-pyrroline-5-carboxylase synthetase, GenBank accession No. AY492005), a key enzyme in proline synthesis. The *18S rRNA* gene was chosen as the endogenous control (GenBank accession No. X02623.1) for normalization, because its gene expression level is unaffected by abiotic stresses (Stolf, 2007).

Table 1. Primer sequences of genes utilized in real-time quantitative PCR.

Genes	Primer sequences	
	Foward (5'-3')	Reverse (5'-3')
Gm18SrRNA	AAACGGCTACCACATCCAAG	CCTTCAATGGATCCATCGTTA
Gmdreb1a	CGACCAGGAGGGCAGTGAT	GCTTTTCGGCGAATGGAAT
Gmp5cs	TGTCTCTCAGATCAAGAGTTCCAC	CAGCCTGCTGGATAGTCTATTTTT
Gmgols	TGGAAATCAAGTGTGATCCAAG	GAAAAGCCGGGACACATAAA
Gmereb	GAGTCCACAGCCAAGAAACC	ATCCCCTGAAAACGAGGTCT
Gmdefensin	TTTGAGTGACACCAACTGTGG	AACAATGTTTGGTGCAGAAGC
Gmpip1b	TCATGGGTTTCAAAAAGGAGA	GCTTGCAATAAAAGCACAAGC

Gm18SrRNA was used as a reference gene.

Quantitative PCR analysis was performed with a 7300 Real-Time System (Applied Biosystems) thermocycler and a 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 target genes and of the endogenous controls. The results were captured by the Sequence Detection program (Perkin Elmer, Waltham, MA, USA) and analyzed by REST version 2.0.7 (Pfaffl et al., 2002).

RESULTS AND DISCUSSION

At 30 days of water deficit, MG/BR46 (Conquista) RRGR was reduced by 22%, whereas no effect was observed in BR16. Reductions in SRGR of approximately 30% were observed at 45 days of water deficit in both cultivars. LRGR values were reduced by 37 and 60%, in MG/BR46 (Conquista) plants after 30 and 45 days of water deficit, respectively; after 45 days of stress, BR16 had a large reduction in LRGR (82%). TRGR values were reduced only in MG/BR46 (Conquista), by 17 and 27%, after 30 and 45 days of stress, respectively. No significant effects were observed for water-deficit treatment or genotype in terms of root/shoot ratio (Table 2). Leaf areas of MG/BR46 (Conquista) and BR16 plants were reduced by 28 and 38%, respectively, at the first sampling date (S30), and by 34% in both genotypes at the second sampling date (S45; Table 3).

Table 2. Relative growth rate (RGR), including root, shoot, leaf, total, and root/shoot ratio of MG/BR46 (Conquista) (water-stress tolerant) and BR16 (sensitive) cultivars under control conditions (15% gravimetric humidity - GH) and submitted to moderate water deficit (5% GH) for 30 and 45 days of treatment (C30 and C45 corresponding to control and S30 and S45 corresponding to water stress treatment - for the two periods).

Treatments	RGR (mg g^{-1} day^{-1})				Root/shoot ratio
	Root	Shoot	Leaf	Total	
MG/BR46 (Conquista)					
C30	0.0558^{a}	0.0410^{ns}	0.0306^{a}	0.0443^{a}	0.4488^{ns}
S30	0.0433^{b}	0.0369	0.0192^{b}	0.0366^{b}	0.3933
C45	0.0254^{ns}	0.0345^{a}	0.0247^{a}	0.0342^{a}	0.2005^{ns}
S45	0.0195	0.0244^{b}	0.0099^{b}	0.0248^{b}	0.2773
BR16					
C30	0.0397^{ns}	0.0325^{ns}	0.0234^{ns}	0.0382^{ns}	0.4283^{ns}
S30	0.0517	0.0326	0.0187	0.041	0.7178
C45	0.0188^{ns}	0.0226^{a}	0.0123^{a}	0.0290^{ns}	0.3566^{ns}
S45	0.0091	0.0155^{b}	0.0021^{b}	0.0197	0.3207

Different superscript letters denote statistical difference between means by the Tukey test ($P \leq 0.05$, $N = 10$). ns = not significant.

Table 3. Leaf area, photosynthetic rate, stomatal conductance, carbon isotope discrimination of MG/BR46 (Conquista) (water-stress tolerant) and BR16 (sensitive) cultivars under control conditions (15% gravimetric humidity - GH) and submitted to moderate water deficit (5% GH) for 30 and 45 days of treatment (C30 and C45 corresponding to control and S30 and S45 corresponding to water stress treatment - for the two periods).

Treatments	Leaf area (cm^2)	Photosynthesis rate ($\mu mol\ m^{-2}\ s^{-1}$)	Stomatal conductance ($mmol\ m^{-2}\ s^{-1}$)	Carbon isotope discrimination ($^{13}C/^{12}C$)
MG/BR46 (Conquista)				
C30	246.4 ± 16.0^{A}	14.3 ± 0.8^{B}	432.9 ± 37.0^{A}	-28.147 ± 0.2^{B}
S30	177.6 ± 14.0^{C}	8.90 ± 1.2^{C}	208.7 ± 22.5^{C}	-26.643 ± 0.3^{A}
C45	190.5 ± 22.1^{a}	6.40 ± 1.9^{b}	230.0 ± 41.0^{b}	-27.942 ± 0.2^{b}
S45	128.8 ± 11.2^{b}	6.90 ± 0.6^{b}	190.2 ± 24.2^{b}	-26.670 ± 0.2^{a}
BR16				
C30	215.7 ± 15.6^{B}	16.5 ± 1.0^{A}	465.1 ± 43.6^{A}	-28.185 ± 0.2^{B}
S30	133.5 ± 9.3^{D}	12.8 ± 1.8^{B}	296.5 ± 38.6^{B}	-27.482 ± 0.2^{A}
C45	150.6 ± 15.0^{b}	9.30 ± 1.1^{a}	310.2 ± 35.1^{a}	-28.627 ± 0.1^{b}
S45	99.40 ± 11.3^{c}	9.50 ± 0.7^{a}	233.5 ± 19.0^{b}	-27.148 ± 0.2^{a}

Data are reported as means ± SD. Statistical analysis was performed for each treatment and cultivar separately. Superscript capital letters represent results after 30 days of treatment and superscript lower case letters represent the results after 45 days of treatment. Different letters denote statistical difference between means by the Tukey test ($P \leq 0.05$, $N = 10$).

Leaf area was reduced in both genotypes and by both periods of water-deficit treatment. This leaf-area reduction constitutes a strategy to decrease water loss, since less surface area exposed to solar radiation reduces loss of water via evapotranspiration from leaf tissues (Boeger and Wisniewski, 2002). Although leaf-area reduction diminishes water loss, it also decreases useful leaf area for photosynthesis and carbon assimilation, which was also observed in these genotypes. Moreover, decreases in LRGR in both genotypes probably resulted from abscission and reduced production of new leaves.

The reduction of the RRGR in MG/BR46 (Conquista) exposed to water deficit for 30 days could be due to decreased production of new roots or to the death of roots already formed. Relative growth rate expresses plant development as a function of dry weight accumulation over time, and is considered an appropriate physiological index for comparing treatment effects on various agronomic traits, because it is relative rather than absolute. It is known that MG/BR46 (Conquista) develops a larger root system in response to drought; the 22% reduction that we observed may have been due to root constriction in the pots, inducing internal signals to reduce root growth.

Root-system development is important for plant adaptation to water deficits by maintaining moisture uptake. Anatomical, morphological, physiological, and molecular characteristics are directly related to drought tolerance. For example, over-expression of *pip1b* (a water-channel protein, aquaporin, which may increase hydraulic conductivity and facilitate the uptake of water from soil) in BR16 at the second sampling date (Table 4) could be a root-adaptation response to drought stress. Morphological, anatomical, and physiological adaptations of roots to drought stress regulated by molecular mechanisms can provide useful information for molecular-assisted breeding programs, or for development of genetic-engineering strategies to improve drought tolerance (Huang and Fry, 1998).

After 30 days of water stress, both genotypes showed reductions in photosynthetic rate: 24% for MG/BR46 (Conquista) and 40% for BR16. Reductions in stomatal conductance were observed in both cultivars at 30 days, MG/BR46 (Conquista) by 65% and BR16 by 50%. These effects were not detected after 45 days of stress treatment in BR16, whereas for MG/BR46 (Conquista), stomatal conductance was reduced by 79%. Comparing control and stressed plants, CID was higher in MG/BR46 (Conquista) (5.3%) than in BR16 (2.5%) after 30

days of stress. After 45 days, MG/BR46 (Conquista) maintained similar discrimination rates, whereas BR16 increased discrimination to 5.5% higher than control plants (Table 3). Reduction in stomatal conductance during water deficit, as in MG/BR46 (Conquista) after 30 days of stress, and the consequent reduction in photosynthetic rate, is well documented. Lack of moisture is the environmental stress most injurious to photosynthesis and plant growth, since stomatal closure decreases photosynthesis due to CO_2 depletion in the mesophyll (Warren, 2004). Even so, increased internal CO_2 concentration in MG/BR46 (Conquista) plants (data not shown) indicated that the reduction of the stomatal conductance is not uniquely responsible for decreased photosynthetic rate. Water deficit can also limit photosynthesis by affecting the activity of ribulose-1, 5-bisphosphate carboxylase (RuBisCo), damaging the biochemical CO_2-fixation machinery (Machado-Filho et al., 2006). Reduction in the efficiency of RuBisCo may be caused by an increase in mesophyllic resistance due to stomatal closure, constraining CO_2 uptake into chloroplasts and increasing the oxygenase activity of RuBisCo, with a consequent increase in photorespiration. Moreover, ribulose-1, 5-bisphosphate regeneration may also be reduced by drought due to a decrease in ATP synthesis by ATPase (Kron et al., 2008).

Table 4. Gene expression for real-time quantitative PCR of MG/BR46 (Conquista) (water-stress tolerant) and BR16 (sensitive) genotypes under control conditions (15% gravimetric humidity - GH) and submitted to moderate water deficit (5% GH) for 30 and 45 days, for six genes: *Gmdreb1a*, *Gmp5cs*, *Gmgols*, *Gmereb*, *Gmdefensin*, and *Gmpip1b*.

	Gm18SrRNA	*Gmdreb1a*	*Gmp5cs*	*Gmgols*	*Gmereb*	*Gmdefensin*	*Gmpip1b*
	Ref.	TRG	TRG	TRG	TRG	TRG	TRG
	0.83[a]	1.00	0.87	0.83	0.96	1.00	0.89
MG/BR46 (Conquista): 30 days after water stress							
Expression	1.00	2.34	0.23	13.99	0.74	12.82	3.15
95%CI		[2.03-2.75]	[0.13- 0.35]	[4.49-32.89]	[0.57-0.97]	[9.28-18.05]	[2.38-4.10]
P (H1)		0.00	0.05	0.05	0.10	0.07	0.03
Result		Up					Up
MG/BR46 (Conquista): 45 days after water stress							
Expression	1.00	1.70	11.43	1.55	1.18	1.44	4.93
95%CI		[0.25-5.46]	[5.79-27.85]	[0.90-2.47]	[0.60-2.62]	[0.80-2.63]	[2.74-7.77]
P (H1)		0.49	0.00	0.10	0.69	0.21	0.04
Result			Up				Up
BR16: 30 days after water stress							
Expression	1.00	4.74	0.14	1.00	0.95	1.02	0.03
95%CI		[2.81-10.22]	[0.14-0.15]	[0.84-1.12]	[0.60-1.94]	[0.51-1.80]	[0.02-0.04]
P (H1)		0.00	0.03	0.71	0.90	0.90	0.00
Result		Up	Down				Down
BR16: 45 days after water stress							
Expression	1.00	2.15	13.75	0.48	1.77	40.36	38.03
95%CI		[1.75-2.55]	[3.60-37.83]	[0.34-0.76]	[0.94-2.95]	[15.85-85.63]	[12.71-125.81]
P (H1)		0.05	0.00	0.00	0.13	0.03	0.00
Result			Up	Down		Up	Up

Gm18SrRNA was used as a reference (Ref.) gene; TRG = target gene; [a]efficiency 95%CI = confidence interval at 95%; P (H1) = probability of the H1 differential expression hypothesis.

There were no differences in photosynthetic rate of MG/BR46 (Conquista) plants after 45 days of moisture stress, possibly because they were entering senescence. The results for BR16 plants suggest only partial closure of stomata, with sufficient entry of CO_2 to maintain intercellular concentration of CO_2 (data not shown) and photosynthesis. Consistent with this,

the reduction of CID (5.3%) in MG/BR46 (Conquista) plants was twice that observed in BR16 (2.5%). Discrimination of carbon isotopes in leaves during photosynthesis is physiologically linked to water use efficiency (WUE), defined as aerial biomass yield per unit of water used. This trait has been proposed as a criterion for yield improvement under drought. The extent to which C_3 plants discriminate against the isotope ^{13}C during carbon assimilation correlates with low WUE (Monti et al., 2006). It has been suggested that carbon-isotope analysis may be a useful tool in selection for improved WUE in breeding C_3 species.

For gene-expression evaluations, real-time PCR analysis was performed on root samples, since initial perception of drought usually occurs at the roots. The molecular signal transduction that starts in the roots eventually is reflected in physiological responses in the plant as a whole. For example, ABA biosynthesis in roots triggers defense responses in leaves, regulating a series of physiological responses based on altered hormone metabolism and subsequent alteration in expression of transcripts and in activation of proteins (Vasquez-Robinet et al., 2008).

In real-time PCR analysis, the higher expression of *Gmdreb1a* in MG/BR46 (Conquista) and BR16 roots, detected after 30 days of water stress (Table 4), could be related to the reductions in photosynthetic rate and stomatal conductance (Table 3). Li et al. (2005) working with *Gmdrebc*, observed induction by salinity, drought, low-temperature, and ABA treatments in roots of seedlings, suggesting that transcription factors involved in drought responses such as DREB1A and DREBc in roots are more responsive to unfavorable environments than in leaves, consistent with what we found. DREBc may play roles in more general processes in leaves and function, especially in roots during response to stress. This is reasonable, since roots usually sense stress signals first, due to contact with the soil; plants need to express defense-related genes in their roots in time to promote survival under severe conditions (Li et al., 2005). Shen et al. (2003) found that *Ah*DREB1, a DREB-type gene from *Arabidopsis hortensis*, was induced in roots, but not in stems or leaves.

The increase in *Gmp5cs* expression in the last period of treatment for both genotypes and the decrease only in BR16 roots in the first period might be an adaptation to overcome the stress condition, supplying energy for growth and survival, thus helping the plant to survive (Table 4). The drought-tolerant genotype that we studied seemed to increase synthesis of osmotic regulators for protection against water-deficit damage, whereas, in the drought-sensitive genotype, *Gmp5cs* was down-regulated. Furthermore, proline may play other roles, such as an enzyme-stabilizing agent, having the ability to mediate osmotic adjustment, stabilizing sub-cellular structures and scavenging free radicals. Proline has hydrophilic properties, a feature that may facilitate replacement of water molecules in contact with nucleic acids, proteins and membranes during moisture shortage, helping structural maintenance of these molecules (Bayoumi et al., 2008).

Gmgols presented a different profile of expression from that of *Gmdreb* (Table 4). GolS is a key enzyme in the production of raffinose-family oligosaccharides; it represents the galactynol from UDP-galactose and myoinositol, which serves as a precursor of galactosyl to form raffinose, stachyose and verbascose. Studies of *A. thaliana* have shown that the transcription factor DREB up-regulates expression of *gols*; accumulation of raffinose in plants is associated with tolerance of environmental stresses like cold and dehydration (Downie et al., 2003). However, in our study *Gmgols* gene expression was down-regulated in both genotypes. Taji et al. (2004) have shown that two types of *gols* genes, *gols1* and *gols2*, are induced by drought and salinity stress; interestingly, the expression of *gols3*, a third gene in the same family, seems to be insensitive to regulation by the transcriptional activator DREB1. In contrast,

no DREB-recognition sites were detected within the upstream promoter region of *gols1* and *gols2*; in plants engineered with *dreb1a*, an increase in the level of expression of *gols3* was noted, indicating that *gols3* is activated by transcription factor DREB1A (Panikulangara et al., 2004). The *Gmgols* that we analyzed may be similar to *gols1* or *gols2*, since the data do not correspond to the profile of *Gmdreb1*.

Gmereb did not present differences in expression profile in either genotype (Table 4). Multiple ethylene-response factors (ERFs) are induced by disease-related stimuli, such as the phytohormone ethylene, jasmonic acid and salicylic acid, or pathogen infection. Also, many *ereb* genes are regulated by wounding and abiotic stresses, especially salinity and dehydration stresses (Tang et al., 2007).

The differences in expression of *Gmdefensin* for BR16 at 45 days (Table 4), suggest that the tolerant genotype MG/BR46 (Conquista) has a more dynamic and rapid response to moisture stress, since it increased in the latter genotype; therefore, this may be the main factor conferring tolerance, among other morphological and physiological aspects. Plants of the sensitive genotype, BR16, showed increased expression of this gene in the last period of treatment, likely involving the same defense mechanism, albeit with different timing. The higher expression of *Gmdefensin* that we observed in the last period of treatment with the sensitive genotype, contrasts with the data of Maitra and Cushman (1998) that showed a 10-fold up-regulation in tolerant soybean plants under water deficiency. Yamada et al. (2003) also reported an increase in the expression of a *Gmdefensin* homologue in *Nicotiana excelsior* under conditions of salinity stress, indicating involvement of this gene in responses to cell dehydration.

Although, there were no significant differences in RRGR and SRGR values (Table 2), differences in *Gmpip1b* were detected in MG/BR46 (Conquista) and BR16 plants at 30 and 45 days of water stress, but only in BR16 roots was it down-regulated; this contrasts with the more common finding of moisture-stress-induced down-regulation of *pip* expression in roots (Porcel et al., 2006). However, increased expression of *Gmpip1b* in the drought-sensitive genotype in the last period of treatment suggests that up-regulation of the root *pip* gene is a direct effect of the low moisture content of the substrate and concomitant stomatal closure. It is noteworthy that Hill et al. (2004) and Maurel and Chrispeels (2001) have proposed that aquaporins function as osmosensors in plant membranes and are involved in the control of water movement between plant cells. We suggest that the higher expression of *Gmpip1b* helps in recuperation from water deficit and influences the development of tolerance in these plants.

Biochemical mechanisms affecting WUE are complex, considering the distinct physiological processes involved, such as stomatal conductance and photosynthetic rate (Bohnert et al., 1995). Differences in molecular expression levels and in the perception of stress probably exist between tolerant and sensitive cultivars, as found here for MG/BR46 (Conquista) and BR16.

In our study, two genotypes with different strategies to ensure reproduction and survival were examined. Moisture-stress-tolerant MG/BR46 (Conquista) showed some physiological and molecular responses to drought at the first sampling date of stress; it probably perceives water stress more quickly than the sensitive genotype BR16, allowing it to adapt and ensure reproduction. In contrast, BR16 presented later perception of, and defense to, stress, conferring survival ability with low grain yield. In fact, if we considered only physiological aspects, especially photosynthesis and stomatal conductance, we would deduce that both genotypes sense drought similarly. However, our molecular data, especially gene-expression (water channel, raffinose biosynthesis and defensin metabolism) values, suggest that the same molecular mechanisms operate in both genotypes at different stages in response to drought.

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