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RESEARCH ARTICLE

Remodeling of the cell wall as a drought-tolerance mechanism of a soybean genotype revealed by global gene expression analysis

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Abstract Drought stress is major abiotic stress that affects soybean production. Therefore, it is widely desirable that soybean becomes more tolerant to stress. To provide insights into regulatory mechanisms of the stress response, we compared the global gene expression profiles from leaves of two soybean genotypes that display different responses to water-deficit (BR 16 and Embrapa 48, drought-sensitive and drought-tolerant, respectively). After the RNA-seq analysis, a total of 5335 down-regulated and 3170 up-regulated genes were identified in the BR16. On the other hand, the number of genes differentially expressed was markedly lower in the Embrapa 48, 355 up-regulated and 471 down-regulated genes. However, induction and expression of protein kinases and transcription factors indicated signaling cascades involved in the drought tolerance. Overall, the results suggest that the metabolism of pectin is differently modulated in response to drought stress and may play a role in the soybean defense mechanism against drought. This occurs via an increase of the cell wall plasticity and crosslink, which contributed to a higher hydraulic conductance (K_f) and relative water content (RWC%). The drought-tolerance mechanism of the Embrapa 48 genotype involves remodeling of the cell wall and increase of the hydraulic conductance to the maintenance of cell turgor and metabolic processes, resulting in the highest leaf RWC, photosynthetic rate (A), transpiration (E) and carboxylation (A/C_i). Thus, we concluded that the cell wall adjustment under drought is important for a more efficient water use which promoted a more active photosynthetic metabolism, maintaining higher plant growth under drought stress.

Keywords Gene expression, RNA-seq, Molecular physiology, Water-use efficiency

INTRODUCTION

Drought is the main environmental factor that negatively influences both plant growth and development,

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thus restricting productivity and agricultural expansion. Projections of climate change indicate that drought will become more intense in some areas of the world and therefore the development of tolerant plants is necessary to maintain production (Passioura 2007; Stanke et al. 2013; Spinoni et al. 2017). On the other hand, plants have evolved to create several strategies to cope with drought, including a short life cycle or phenotypical plasticity, enhance in water uptake and reduction water loss, as well as osmotic adjustment, antioxidant capacity, and drought tolerance (Fang and Xiong 2015). The evaluation of tolerance mechanisms and drought-responsive genes from soybean is essential for genetic breeding programs (Rodrigues et al. 2012; Brown 2017). Thus, a transcriptome analysis of contrasting genotypes may contribute to the understanding of the molecular and physiological responses.

The main difficulty in selecting genes as targets for plant breeding that is aimed at drought tolerance is the complexity of the physiological responses to drought stress. Plant survival strategies under drought involve transient responses, such as reduced transpiration, changes in the root system, reduction of leaf area and adjusted osmotic status leading to a minimal water loss and improving water uptake (Hu and Xiong 2014). Transient response and developmental changes require a substantial rebuilding of plant metabolism and changes in the expression of a high number of genes. Global transcriptome analysis has been used to provide a deeper insight into the complexity of plant response to drought stress on the molecular level.

External drought stimuli are perceived by sensors on the membrane, and then the signals are delivered through multiple signaling pathways, resulting in the expression of responsive genes so as to confer drought tolerance in the plants (Zhu 2002; Hirayama and Shinozaki 2010). In general, gene expression studies of various plant species have been performed to classify several groups of genes, which are regulated in response to drought. Among them are those encoding calcium-dependent protein kinases, calmodulin and calmodulin-related calcium sensor proteins and protein phosphatases class 2C (PP2C) (Molina et al. 2008; Guo et al. 2009; Ranjan and Sawant 2015), along with a number of transcription factors (TFs) (Sahoo et al. 2013; Janiak et al. 2018). These signaling proteins are usually classified as ABA-dependent and ABA-independent stress response pathways (Shinozaki and Yamaguchi-Shinozaki 2007). Genes involved in biosynthesis and signaling pathways of other plant hormones, such as auxin, ethylene, jasmonic or salicylic acid, were also identified as differentially expressed under drought (Jakoby et al. 2002; Aimar et al. 2011). Moreover, genes related to anti-oxidation processes, osmo-protectant synthesis and various factors from late embryogenesis abundant (LEA) family were also reported as differentially expressed in response to drought (Shinozaki and Yamaguchi-Shinozaki 2007; Talame et al. 2007).

The main challenge when performing gene expression studies is to identify which genes are not only responsive, but also confer a differential physiological behavior when compared to a sensitive genotype. To achieve this goal, it is necessary to use parental plant genotypes contrasting in drought-tolerance. The physiological response of the genotypes BR 16 (drought-sensitive) and Embrapa 48 (drought-tolerant) under drought conditions was studied by Oya et al. (2004), Carvalho et al. (2015) and our research group (-Mesquita et al. 2020). They found that, in the vegetative stage under drought conditions in the field, the drought-tolerant genotype had the highest number of pods. The studies of the proteome, phosphoproteome and metabolomic profile were also performed by Lima et al. (2019) to detect the metabolic pathways which are affected by drought stress. An integrative overview showed that tolerant plants maintain cell homeostasis and photosynthetic metabolism under stress conditions, as indicated by an abundance of protein and regulation by phosphorylation. Drought-stress marker in roots was also evaluated to understand the mechanism of tolerance in these genotypes. The GmaxRD20A-like and GmaxRD22-like genes, homologs of Arabidopsis genes of the ABA-independent pathway, are highly induced by water-deficit, being these potential drought marker genes in these genotypes (Neves-Borges et al. 2012).

Knowledge about drought-tolerance mechanisms in soybean has been reported in recent decades. However, determinate the main mechanism operating in a given plant genotype is a challenge that requires multiple analytical approaches, involving physiological and gene expression analyzes. The physiological responses revealed that the tolerant genotype Embrapa 48 postpones the leaf dehydration by a mechanism involving a more efficient use and translocation of water from root to the shoot to maintain unchanged the cell homeostasis and the photosynthetic metabolism under the stress (Mesquita et al. 2020). Furthermore, an integrative overview involving proteomic, phosphoproteomic and metabolomic profiles also showed that the Embrapa 48 tolerant plants maintain cell metabolism unchanged under the stress condition in contrast to BR16 sensitive genotype that showed several dysregulated pathways (Lima et al. 2019). Only small deviations of the metabolic pathways were observed for drought-tolerant plants in comparison to the sensitive genotype. These findings indicated that osmoprotection

and/or oxidative protection does not appear to be the major mechanisms for tolerance, as indicated by the accumulation of the metabolite, enzymes assays and the phytohormone profiles from tolerant and sensitive soybean plants (Lima et al. 2019; Mesquita et al. 2020). Here, we analyzed physiological traits essential for drought-tolerance elucidation, such as assimilation rate of CO_2, stomatal conductance (g_s), transpiration (E), water use efficiency (WUEi), carboxylation efficiency (A/C_i), relative water content (RWC), hydraulic conductance (K_f), correlating with data from gene-expression responses to dehydration through RNA-Seq analysis from soybean parental genotypes (Embrapa 48 and BR 16). The metabolic/regulatory pathways and biological processes were explored via Gene Ontology (GO) enrichment and indicated differences in the gene expression reprogramming in the drought-tolerant genotype that correlated with the physiological mechanisms of the tolerance. The results revealed that in response to stress, the tolerant genotype Embrapa 48 has less gene reprogramming, however expressed specifically protein kinases and transcription factors in response to drought tolerance. Expression of the genes relating to pectin remodeling in the leaves may be involved in a mechanism that contributes to the maintenance of leaf turgor and metabolism of the Embrapa 48 genotype under drought stress.

MATERIALS AND METHODS

Plant material, growth and drought-stress treatments

Seeds of soybean genotypes BR 16 and Embrapa 48 were obtained from the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA SOJA, Londrina, Paraná). Seedlings were grown in plastic trays containing Plantmax® commercial substrate, where they remained for 10 days. After germination, seedlings were transplanted to pots containing 10 L of a mixture of soil, sand and manure (2/1/1) each. Plants were grown under natural sunlight in a greenhouse with average daytime temperature 15–35 °C and relative humidity 65–85%.

The plants were grown under normal water conditions until reaching the development stage V3 (fully expanded third trifoliate). The control plants were watered daily with approximately 30 mL water per plant. The plants were exposed to a slow drying soil treatment, which consisted of a reduction in irrigation to 40% of the daily normal until the plant reached the hydraulic potential of $\Psi_w = -1$ MPa (Valente et al. 2009). The hydric regimes were assigned as irrigated (IR) and non-irrigated (NI). The leaf water potential (Ψ_w) was measured in the third emerging trifoliate at dawn using a Scholander pump (Scholander et al. 1965) during the stress period. Samples were collected in liquid nitrogen when the plants had a water potential -1 MPa and then stored at -80 °C until use. For each treatment, 3 pots were used and each pot contained three plants. A trifoliate leaf from each plant from one pot was collected together (3 plants by replicate). Thus, the analyses were performed using 3 distinct pools, resulting in 3 biological replicates. These procedures were performed on both plant cultivars.

RNA extraction, library preparation, and sequencing

Total RNA was extracted from leaves using a Trizol reagent (Invitrogen) according to the manufacturer's instructions. Five micrograms of total RNA was used to prepare paired-end 100 bp libraries using the BIOO NEXT flex Rapid Directional RNA-Seq Kit (Bioo-Scientific, Austin, TX). Library qualities were analyzed with the Bioanalyzer 2100 (Agilent, Santa Clara, CA) and the barcoded libraries were quantified by fluorometry using a Qubit instrument (LifeTechnologies, Carlsbad, CA). The libraries were then pooled in equimolar ratios, quantified by qPCR with a Kapa Library Quant kit (Kapa, Cape Town, South Africa). Three biological replicates of each treatment were sequenced using the Illumina HiSeq 2500 (Illumina, San Diego, CA) from NuBioMol (Center for Biomolecules Analysis—UFV, Brazil).

Raw reads were subsequently subjected to trimming using Trimmometic software (Bolger et al. 2014) with a Phred quality threshold of 20. Reads were aligned to the *Glycine max* genotype Williams 82 primary transcriptome (Wm82.a2.v1) (Schmutz et al. 2010) using the Kallisto aligner (Bray et al. 2016).

Analysis of differentially expressed genes (DEGs)

To identify differentially expressed genes (DEGs), we used the DESeq2 software package, which performs pairwise comparisons (Anders and Huber 2012). DESeq2 analyses were carried out using the Kallisto output. The DEGs were identified using the MA-plot-based method from the package DEGseq version 3.0 (Wang et al. 2009). An absolute fold-change threshold of 2.0 and a false discovery rate (FDR) of ≤ 0.0001 were used to select the DEGs identified by DEGseq.

RT-qPCR

RT-qPCR was performed to validate the differential gene expression data obtained by RNA-Seq analysis. RNA was extracted from leaf tissues of the control and stressed plants using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA quality was analyzed using agarose gel electrophoresis and quantification was performed on a Thermo Scientific NanoDrop 2000c. A total of 4 μg of RNA was used for cDNA synthesis with the SuperScript III kit (Invitrogen) following the manufacturer's instructions.

The gene expression was assessed using an ABI 7500 Fast Thermocycler (Applied Biosystems, Foster City, CA, USA) and Fast SYBR Green Master Mix (Thermo Fisher Scientific). The cycling conditions were as follows: 15 s at 95 °C, 40 cycles of 95 °C for 3 s; 30 s at 60 °C, and final denaturation at 95 °C for 20 s, followed by a melting curve. Specific primers for RT-qPCR were designed using the Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), with a melting temperature (T) between 59–61 °C, a length of 18–23 bp, an amplicon product size of 120–150 bp, and a GC content of 40–60% (Supplementary Information Table S1). Gene expression was normalized using two soybean housekeeping genes, being them UNK2 and Actin. A total of three biological replicates and three technical replicates were performed for each gene. Relative quantification was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Functional classification of the differentially expressed genes

The DEGs were subjected to functional classification using *The Arabidopsis Information Resource* (http://www.arabidopsis.org). The gene ontology (GO) enrichment analysis of each gene set was performed using the ClueGO version 2.0.7 plugin tool (Bindea et al. 2009) in Cytoscape version 3.2.1 (Shannon et al. 2003) using the GO biological process category. Over-represented biological process categories were identified using an (right-sided) enrichment test based on the hypergeometric distribution. To determine significantly the overrepresented GO terms, the terms with a p value lower than 0.05 were considered as a Kappa significant value. Genes classified as significantly over-represented were validated by the Benjamin test.

Measurement of gas exchange and photosynthetic parameters

The assimilation rate of CO_2 (A), stomatal conductance to water vapor (g_s), transpiratory rate (E) and internal and external carbon ratio (C_i/C_a ratio) were determined for the fourth leaf from the apical meristem of each plant using an infrared gas analyzer (IRGA, portable model LI-6400XT, LI-COR Biosciences Inc., Lincon, Nebraska, USA) as described by Mesquita et al. (2020).

Measurement of the relative water content (RWC) of the leaves

The fresh weight (FW) of leaf discs was measured immediately after they were removed from the stem. Then, tissues were incubated in distilled water for at least 4 h in the dark, and the turgid weight (TW) was measured. Finally, dry weight (DW) was measured after incubation at 85 °C until the sample reached a constant weight in the oven. The relative water content (RWC) was calculated using the equation: $RWC\ (\%) = [(FW - DW)/(TW - DW)] \times 100$.

Evaluation of leaf hydraulic conductivity (K_f)

Leaf hydraulic conductivity (K_f) determination was performed using the evaporative flow method (EFM), according to the methodology described by Sack et al. (2002) and Brodribb and Holbrook (2003, 2006), with modifications. The water restriction experiment started when the third trefoil was fully expanded. The leaf water potentials (Ψ_w) were determined before dawn using a Scholander pressure pump and when the leaves reached Ψ of approximately -1.0 MPa, the gas exchange parameters were determined. The measurement of leaf transpiration rate (E) was carried out between eight and ten in the morning of the same day using an infrared gas analyzer (IRGA, portable model LI-6400xt, LI-COR Biosciences Inc., Lincon, Nebraska, USA). K_f was calculated using the equation: $K_f = -E/\Psi$.

RESULTS

Analysis of RNA transcripts

The response of the soybean to moderate drought was investigated at the transcriptional level by an RNA-Seq approach. Previous studies performed by our research group showed greater reduction in the growth of the aerial part, simultaneously with a greater induction of

the growth of the root system under drought, without changing the leaf area, indicating that the tolerant genotype has a differential mechanism in allocating the carbon for the roots (Mesquita et al. 2020). In the present study, gene expression in leaves of two soybean genotypes, BR 16 and Embrapa 48, drought-sensitive and drought-tolerant, were analyzed by Illumina Hi-Seq 2500 (Illumina, San Diego, CA). The initial sample collected after a water-deficit was designated as "NI", and control plants "IR" (Fig. 1a). Approximately 45–50 million reads were generated from each sample. Raw reads were subjected to a pre-processing/trimming step to remove short or low-quality sequences and adaptor/primer sequences. The RNA-Seq analysis workflow is shown in Supplementary Information Figure S1 and was used for data analysis.

To understand further about the similarity of the genotypic responses under water-deficit conditions, we used Principal Component Analysis (PCA) (Fig. 1b). The quality of the data obtained can be observed by the analysis of the sample-by-sample Euclidean distance, which is repopulated in the form of a heat map (Supplementary Information Fig. S2). High-throughput RNA-sequencing analysis was performed using a Kallisto pipeline (Bray et al. 2016) comparing the number of genes differentially regulated in response to drought combinations between controls vs. treatments, for each genotype, using DESEq2 (Anders and Huber 2012). When comparing all genes differentially expressed, we identified for sensitive BR 16 8505 genes, including 3170 genes up-regulated and 5335 genes down-regulated under drought conditions. For the tolerant EMBRAPA 48, 826 genes were differentially expressed, including 355 up-regulated and 471 genes down-regulated (Fig. 1c). The global data show that the transcriptional reprogramming was more pronounced in the sensitive plants, considering the highest number of DEGs in BR 16 genotype. In addition, gene-expression data obtained by RNA-seq strategy correlated with RT-qPCR measurements (Fig. 2), confirming the accuracy of our RNA-seq data.

The gene expression response to drought has been evaluated extensively in several plants including soybeans (Bhargava and Sawant 2013). Thus, to select genes that may be involved not only in general stress response, but also that conferred drought tolerance in soybean, we analyze the list of genes that were differentially expressed only in EMBRAPA 48 genotype and could be correlated with molecular and physiological data previously generated for both genotypes (Lima

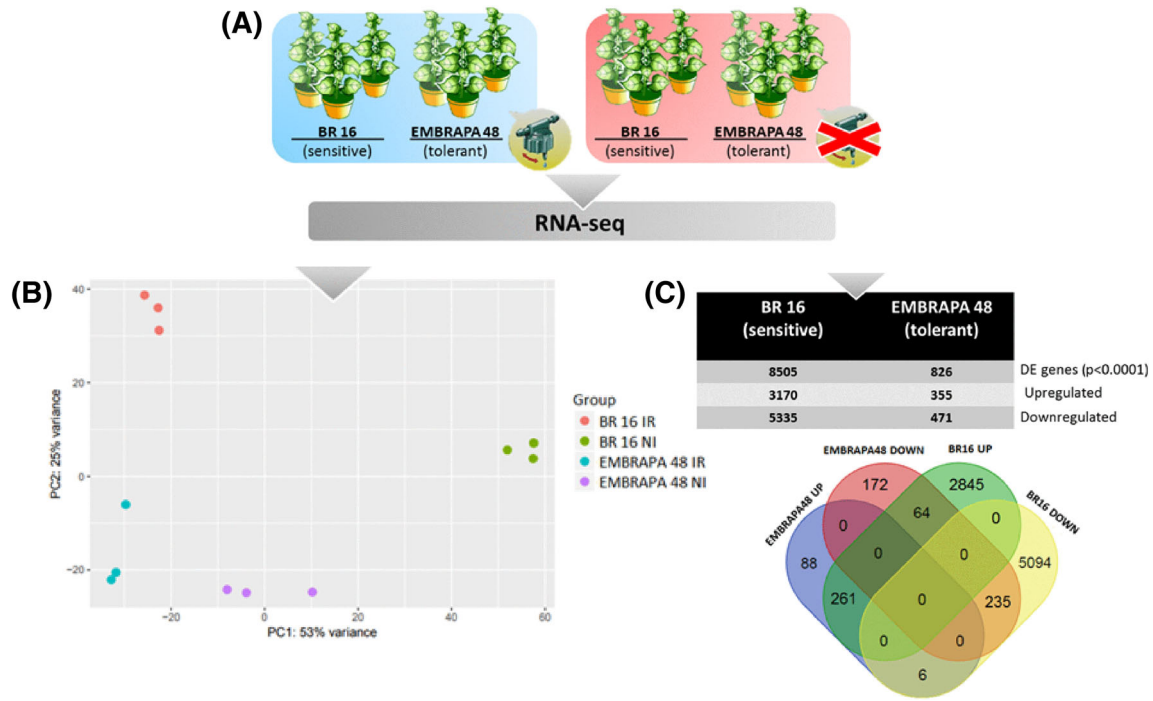

Fig. 1 Overall gene expression in response to drought stress. In **a** soybean plants BR 16 and Embrapa 48 exposed to a gradual drought regime to isolate total RNA for transcriptomic analysis. The water potential was measured by Scholander pump. In the blue box irrigated plants and in the red stressed plants. In **b** transcriptome data used for the principal component analysis, showing distinct clusters of the different soybean genotypes in Irrigated conditions (IR) and not irrigated (NI). In **c** number of differently expressed genes in drought conditions in both genotypes and Venn diagram showing the comparison of the number of genes differentially expressed. Proportion of significant results ($p \leq 0.0001$, log2 fold change ≥ 2 for up-regulated and ≤ -2 for down-regulated genes)

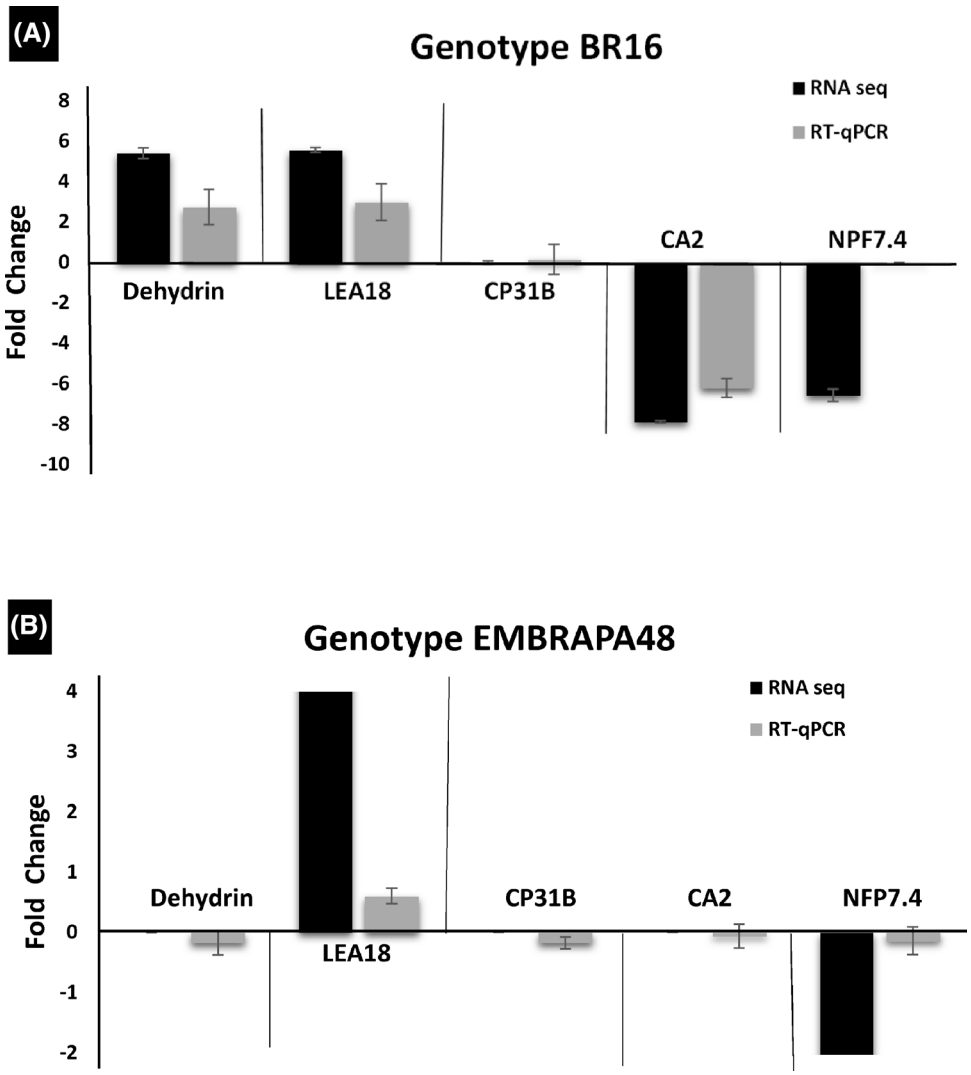

Fig. 2 Validation of (black filled square) RNA-Seq data by real-time (ash filled square) RT-qPCR. The expression variation of the RNAs analyzed in this study in plants submitted to water-deficit compared with controls. The graph in **a** shows the expression variation in the genotype BR16, and the graph in **b** shows the expression variation in the genotype EMBRAPA48. Genes encoding Dehydryn, LEA18, CP31B (chloroplast RNA-binding protein 31B), CA2 (carbonic anhydrase), NPF4.7 (protein NRT1/PTR family 4.7), RGXT2 and RGXT1 were analyzed. The data represent the mean ± SE (n = 3)

et al. 2019; Mesquita et al. 2020). Since the genotype BR16 and EMBRAPA 48 are parental, we believe that the genes differentially expressed only in the genotype EMBRAPA 48 during stress may be responsible for their tolerance. Thus, we first manually inspect the functional categories (ClueGO results) grouped for each genotype to select those enriched only for the tolerant genotype. Followed, the functions of the genes included in these categories were related to molecular mechanisms described for drought tolerance and correlated as physiological traits of both genotypes. This strategy allowed us to select candidates that could play a role in drought adaptation and tolerance, whilst also may explain the lower number of DEGs found in the tolerant genotype. Thus, 260 genes were selected as being differentially expressed exclusively in the tolerant genotype, including 88 up-regulated and 172 down-regulated (Fig. 1c). We identified 20 genes encoding protein kinases (PKs) (Table 1): four genes encoding Serine/threonine-protein kinases (S/T PKs), two were up-regulated (Glyma20G14580.1, Glyma18G18720.1) and two were down-regulated (Glyma09G09800.1, Glyma20G17650.1) under *water-deficit*; three genes encoding Calcium-dependent protein kinases (CDPKs) all were up-regulated (Glyma16G02240.1, Glyma16G14250.1, Glyma15G14340.1); and fourteen other PKs genes, nine of them were down-regulated (Glyma10G19570.1, Glyma07G21590.1, Glyma13G26610.1, Glyma13G24090.1, Glyma18G12450.1, Glyma13G28310.1, Glyma04G19530.1, Glyma07G21660.1) and five were up-regulated genes (Glyma10G22220.1, Glyma13G22430.18, Glyma13G22430.22, Glyma13G22430.11, Glyma17G14990.2).

In addition, 23 genes were identified encoding transcription factors (TFs) (Table 2). These genes were grouped into major groups. The first group contained one auxin response factor (ARF) gene down-regulated (Glyma18G18450.1) in the drought-tolerant genotype.

Table 1 Protein Kinases responsive to dehydration only in the drought-tolerant genotype

Protein kinase		
Gene	Log_2 ratio	Annotation
S/T PKS		
GLYMA09G09800.1	− 0.709405048	CBL-interacting serine/threonine-protein kinase
GLYMA18G18720.1	1.162165	Serine/threonine-protein kinase wnk with no lysine
GLYMA20G14580.1	1.035192	Serine/threonine-protein kinase wnk with no lysine
GLYMA20G17650.1	− 0.624644997	Serine/threonine-protein kinase
CDPKS		
GLYMA16G02240.1	1.123958	Calcium-binding protein
GLYMA16G14250.1	1.102288	Calcium-binding protein cml41-related
GLYMA15G14340.1	0.827979131	WTF9
PKS		
GLYMA10G19570.1	− 1.091893292	Leucine-rich repeat receptor-like protein kinase pepr1-related
GLYMA07G21590.1	− 1.570048333	Leucine-rich repeat N-terminal domain (LRRNT_2)
GLYMA13G26610.1	− 1.640632312	Protein tyrosine kinase (Pkinase_Tyr)
GLYMA13G24090.1	− 0.484742282	AMP-activated protein kinase. gamma regulatory subunit
GLYMA18G12450.1	− 1.188983376	Protein kinase domain (Pkinase)//Leucine Rich Repeat (LRR_1)
GLYMA13G28310.1	− 0.964734668	Cysteine-rich receptor-like protein kinase 27-related
GLYMA04G19530.1	− 1.480822749	SNF1-related protein kinase regulatory subunit gamma-1
GLYMA10G22220.1	1.094759	Cell division protein kinase
GLYMA07G21660.1	− 0.539613982	1-Phosphatidylinositol-3-phosphate 5-kinase fyab1c-related
GLYMA13G22430.18	1.81788	Protein tyrosine kinase
GLYMA13G22430.22	1.648667	Protein tyrosine kinase
GLYMA13G22430.11	1.506839	Protein tyrosine kinase
GLYMA17G14990.2	2.101687	Protein tyrosine kinase

The second group was composed of zinc-finger protein family genes, containing five members (Glyma07G12680.1, Glyma08G03140.1, Glyma05G22440.1, Glyma01G00500.1, Glyma17G15100.1) which were induced by dehydration, and three (Glyma06G19660.2, Glyma12G17890.1, Glyma04G06660.1) were suppressed. The third group was constituted by five MYB family genes down-regulated (Glyma08G02080.1, Glyma19G22220.1, Glyma11G18340.1, Glyma15G14190.1, Glyma12G18470.1). The fourth group consisted of ring-finger family genes; two members (Glyma20G07670.1, Glyma04G03980.4) were induced by dehydration in leaves, and one (Glyma10G12460.4) was suppressed. The fifth group consisted of heat-shock factors (HSFs); two members were induced (Glyma01G21740.1, Glyma09G19060.1) and two suppressed (Glyma08G02590.1, Glyma08G15850.1) by water-deficit. The remaining TF genes encoded members of families AP2/EREBP (Glyma16G01260.4) and NAC domain protein (Glyma11G18200.1).

We found that some genes that code for proteins involved in cell wall dynamics were differentially expressed for both genotypes, which appear to be regulated by drought stress (Table 3). The genes related to the metabolism of Rhamnogalacturonan were only expressed in the tolerant genotype (Glyma08G09360.1, Glyma15G07400.1, Glyma05G22440.1). Two expansion protein genes (Glyma05G06580.1, Glyma20G03390.1) and two responsive xyloglucans transferase genes (Glyma10G15100.1, Glyma05G13870.1) were expressed in both genotypes. Two glycosidases responsive (Glyma10G20000.1, Glyma03G10450.1) were only expressed in the tolerant genotype. A synthase-like D3 Cellulose (Glyma01G23250.1) overexposed in the sensitive genotype and the pectinesterase inhibitor (glyma08G14790.1) down-regulated in the tolerant genotype and up-regulated in the susceptible.

Functional classification of differentially expressed genes

We used the enrichment analysis of DEGs based on up- and down-regulated genes, performed by Cytoscape plug-in ClueGO, which identified significantly over-

Table 2 Transcription factors responsive to dehydration only in the drought-tolerant genotype

Transcription factors (TFS)		
Gene	Log_2 ratio	Annotation
AUXIN-RELATED PROTEIN		
GLYMA18G18450.1	− 0.754799159	Auxin response factor
ZINC FINGER PROTEIN		
GLYMA07G12680.1	1.293328	CCCH Zinc finger protein
GLYMA08G03140.1	1.437808	CCCH Zinc finger protein
GLYMA06G19660.2	− 0.967052969	C3HC4 Zinc finger protein
GLYMA12G17890.1	− 1.279118701	C2H2 Zinc finger protein
GLYMA04G06660.1	− 2.353948183	C2H2 zinc finger protein
GLYMA05G22440.1	1.214217	CCCH Zinc finger protein
GLYMA01G00500.1	1.406536	CCCH Zinc finger protein
GLYMA17G15100.1	1.06469	C2C2 Zinc-finger of the FCS-type
MYB TRANSCRIPTION FACTOR FAMILY		
GLYMA08G02080.1	− 3.100653512	Leucine Rich Repeat (LRR_1)-MYB-LIKE DNA-BINDING PROTEIN MYB // ATMYB103
GLYMA19G22220.1	− 1.554766923	MYB transcription factor
GLYMA11G18340.1	− 0.993510023	MYB transcription fator-MYB 2
GLYMA15G14190.1	− 0.599749666	MYB
GLYMA12G18470.1	− 1.655029264	MYB transcription fator
RING-H2 PROTEIN		
GLYMA20G07670.1	2.672113	Ring finger domain
GLYMA04G03980.4	2.921892	Ring finger domain
GLYMA10G12460.4	− 2.716919566	Ring finger domain
HEAT SHOCK PROTEIN		
GLYMA08G02590.1	− 0.507591383	Heat shock protein 70 kDa
GLYMA08G15850.1	− 0.745310757	Small heat-shock protein 20 KDa
GLYMA01G21740.1	1.480779	Heat stress transcription factor B-2B
GLYMA09G19060.1	1.187051	Heat stress transcription factor C-1
AP2/EREBP FAMILY		
GLYMA16G01260.4	− 0.946836287	AP2 domain
NAC FAMILY		
GLYMA11G18200.1	1.480184	NAC domain protein 61

represented enrichment networks present in both genotypes for the drought treatment (Figs. 3, 4). When the lists of functional categories and networks produced by ClueGO for each genotype were verified, we observed similarities, despite the number of genes grouped in the categories to be very small for tolerant Embrapa 48. This lower number was justified by lower number of dysregulated genes in the tolerant genotype, as describe before. However, some categories and genes were highlighted or present only for Embrapa 48.

The down-regulated genes from the sensitive genotype BR16 showed clusters relating to biological processes, such as regulation of protein catabolic, gibberellin-responsive, acyl-CoA metabolic process, response to glucose, carbohydrate and lipid transport, proteolysis, response to red or far red light, stamen filament development, among others (Fig. 3a). However, for Embrapa 48 (Fig. 3b), the down-regulated genes showed distinct clusters related to lipid transport, membrane fusion, toxin catabolic process, and regulation of secondary cell wall biogenesis.

Analysis applied for up-regulated genes also showed distinct results for the genotypes. Notably, we observed in the sensitive BR16 under drought the predominance of clusters related to the amino acid catabolic process, alcohol biosynthetic process, response to monosaccharide stimulus, hormone signaling pathway, monocarboxylic acid metabolic process, nucleotide salvage

Table 3 Genes coding for proteins involved in cell wall dynamics differentially expressed for both genotypes

Gene	Protein	BR 16-Log^2	EMBRAPA 48-Log^2
GLYMA10G15100.1	Xylosyltransferase MGP4	3.473208	1.975452
GLYMA08G09360.1	Rhamnogalacturonan xylosyltransferase 1 (RGXT1)	NF	2.308536
GLYMA15G07400.1	Rhamnogalacturonan xylosyltransferase 2 (RGXT2)	NF	2.397853
GLYMA05G22440.1	Rhamnogalacturonan specific Xylosyltransferase 1 (RGTX3)	NF	1.214217
GLYMA05G13870.1	Xyloglucan endotransglucosylase 27	3.006961	1.068432
GLYMA10G20000.1	UDP-Glycosyltransferase superfamily protein	NF	3.68817
GLYMA03G10450.1	Hydroquinone glucosyltransferase	NF	1.567052
GLYMA08G14790.1	Pectinesterase inhibitor 51	1.29512	-2.04434
GLYMA05G06580.1	Expansin-like B1	8.871832	3.815932
GLYMA20G03390.1	Expansin -A14	6.47856	NF
GLYMA01G23250.1	Cellulose Synthase-like D3	2.404065	NF

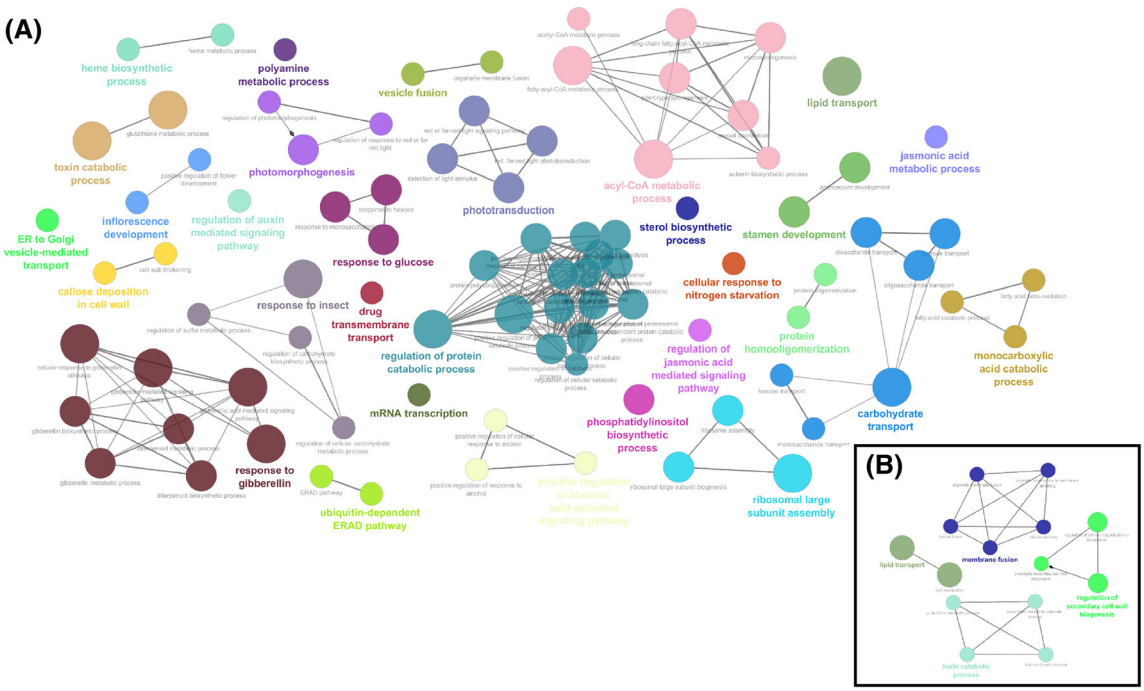

Fig. 3 Over-representation analysis of down-regulated genes using the Gene Ontology biological process database. In **a** clusters containing down-regulated genes in the sensitive genotype BR16. In **b** clusters containing down-regulated genes in the tolerant genotype EMBRAPA 48. The size of the node represents the integration of genes and the thickness of the edge shows a significant Kappa value

(Fig. 4A). For the tolerant genotype, the up-regulation of the DEGs was mainly corresponded to the pathways rhamnogalacturonan II biosynthetic process, endosperm development, serine family amino acid metabolic process as well as photosynthesis and light-harvesting (Fig. 4b).

Drought-stress assays

After the irrigation was interrupted, a reduction on ψ_w was observed. As expected, the decrease on ψ_w was more noticeable in sensitive BR 16 plants (Fig. **5**). The BR 16 plants reached ψ_w values of − 1.0 MPa on the eleventh day, while the Embrapa 48 plants reached the same ψ_w levels on the thirteen day after irrigation suspension. These results were consistent with a more efficient water use by cultivar Embrapa 48 and confirmed this cultivar as drought-tolerant in accordance with Lima et al. (2019) and Mesquita et al. (2020).

Gas exchange and photosynthetic parameters were evaluated (Fig. 6) and also were in accordance with Mesquita et al. (2020). The net photosynthetic rate

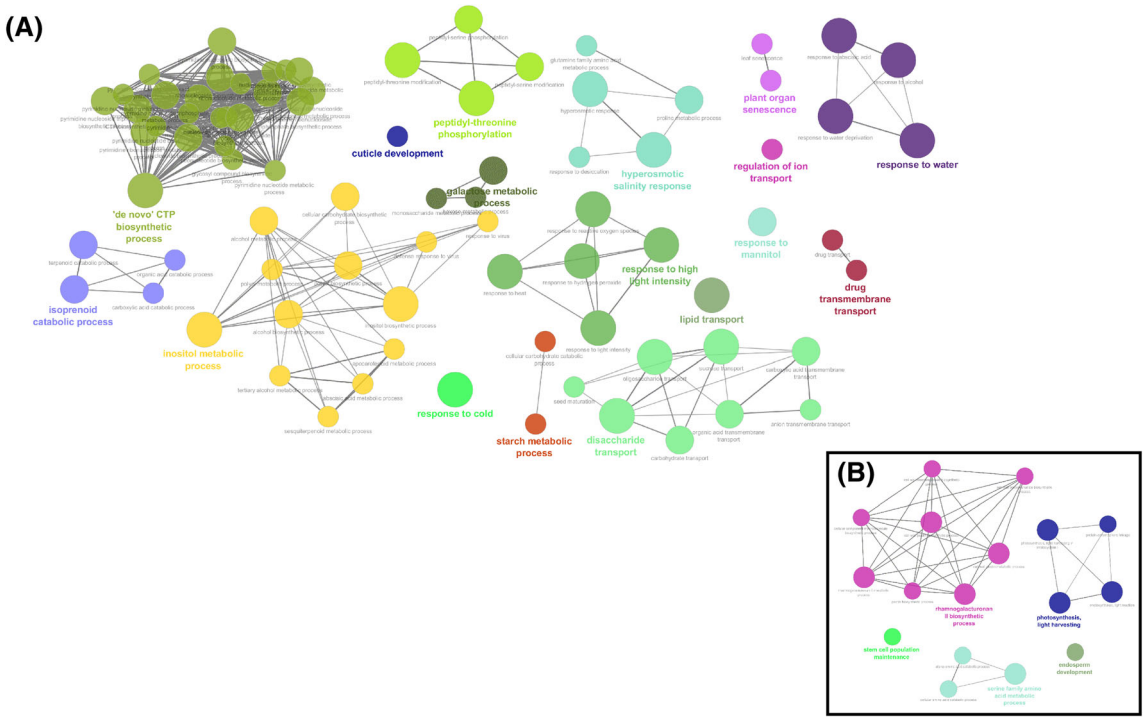

Fig. 4 Over-representation analysis of up-regulated genes using the Gene Ontology biological process database. In **a** clusters containing up-regulated genes in the sensitive genotypeBR16. In **b** clusters containing up-regulated genes in the tolerant genotype EMBRAPA 48. The size of the node represents the integration of genes and the thickness of the edge shows a significant Kappa value

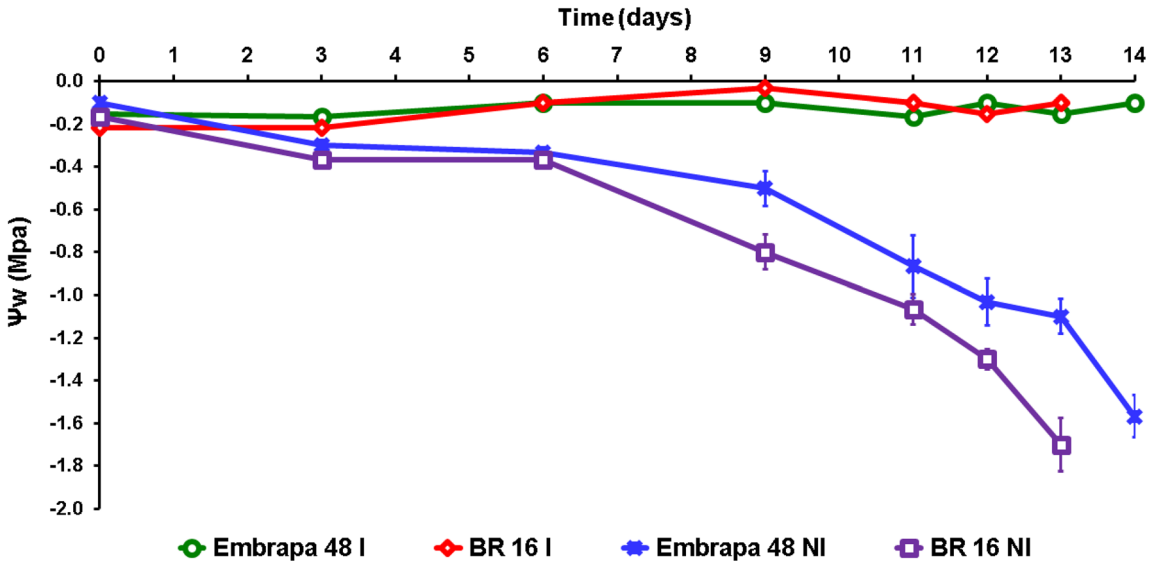

Fig. 5 Temporal profile of leaf pre-dawn water potential (ψ_w) for two soybean cultivars, sensitive (BR 16), and tolerant (Embrapa 48). Each point represents the mean ± standard error (n = 5, where n represents the number of plants), *IR* irrigated, *NI* non-irrigated treatments

(A) decreased for both cultivars under water-deficit; however, the Embrapa 48 maintained higher net photosynthetic rate than BR 16 (Fig. 6a). The stomatal conductance (g_s) showed also higher values in Embrapa 48 (Fig. 6b) while the ratio between internal and external CO_2 (C_i/C_a) concentrations showed slight differences between genotypes (Fig. 6c). The transpiration rate (E) was reduced in both cultivars when water-deficit was imposed, however was significantly higher in the Embrapa 48 cultivar (Fig. 6d). The proportional decrease in E compared to A was greater in Embrapa 48 plants under irrigation and drought conditions,

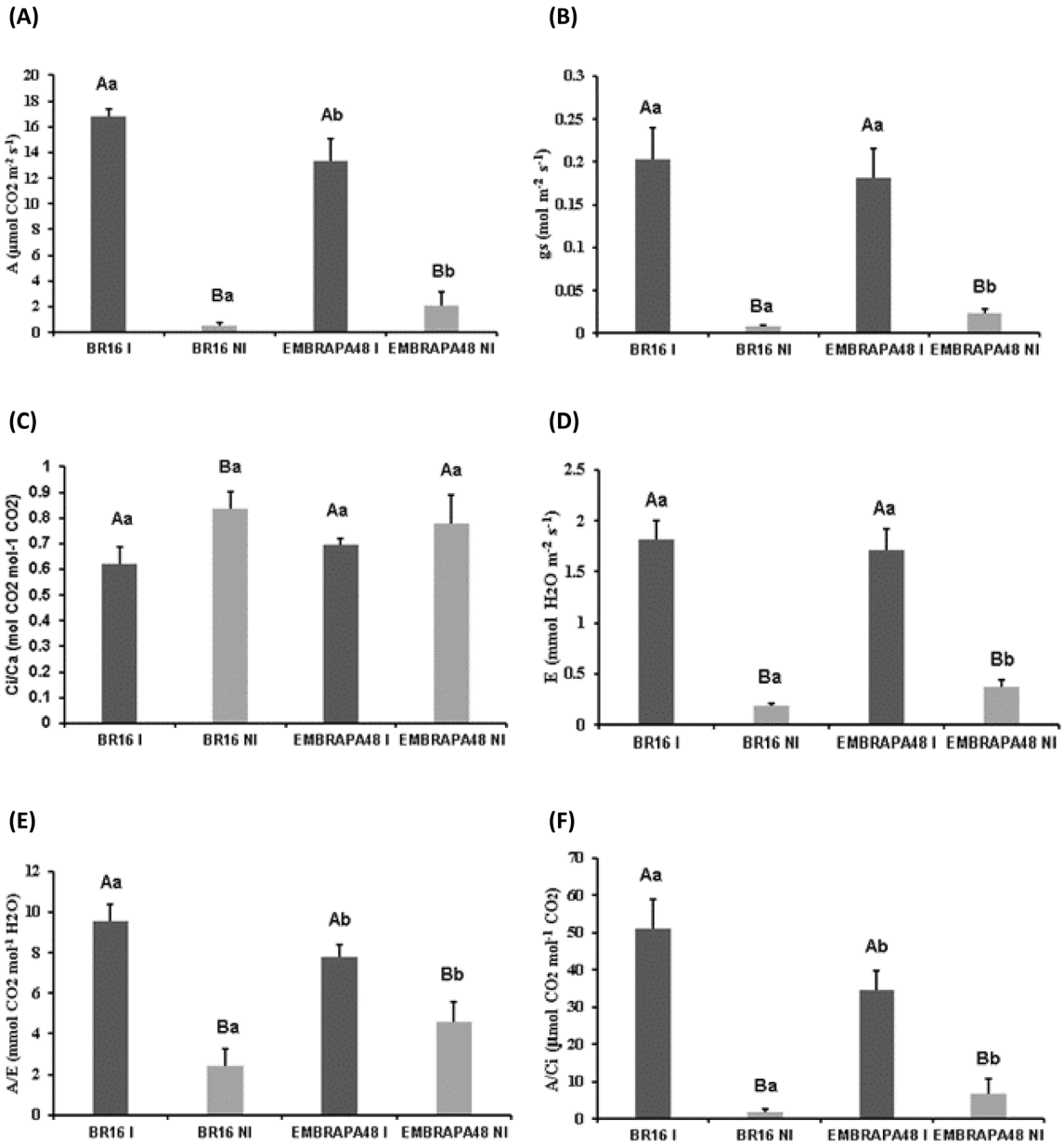

Fig. 6 Effect of water-deficit on the **a** assimilation rate of CO_2 (A), in **b** stomatal conductance (g_s), in **c** ratio C_i/C_a, in **d** transpiratory rate E, in **e** water use efficiency (WUEi) as A/E and in **f** carboxylation efficiency as A/C_i. *IR* irrigated, *NI* non-irrigated treatments. Each bar represents the mean + standard error (n = 5, where n represents the number of plants, t test $p < 0.5$). Different lower case letters indicate significant differences between averages of the same treatment in different cultivars, and capital letters show significant differences between averages within the same cultivar under different treatments

contributing to a greater instantaneous water use efficiency (A/E) in the tolerant genotype (Fig. 6e). The same behavior was also verified for the A/C_i ratio (carboxylation efficiency) being higher in Embrapa 48 (Fig. 6f) under irrigation and drought conditions. In accordance with Mesquita et al. (2020), these results suggest a greater carboxylation efficiency in the tolerant cultivar, associated with its greater photosynthetic capacity.

In conditions of stress due to water-deficit, the decrease in stomatal conductance (g_s) due to stomata closure leads to a drop in water flow, loss of hydraulic load, resulting from cavitation of the xylem and, ultimately, a drop in hydraulic conductivity. The decrease in water flow through the intercellular spaces and the transpiration through the stomates correlated with the drop in water potential. Thus, we studied the correlation between photosynthetic and hydraulic parameters in the soybean genotypes under conditions of normal

irrigation and water-deficit. Leaf hydraulic conductance (K_f) was estimated from the leaf tissues during gas exchanges. As expected, the K_f was abruptly reduced under drought stress. However, the hydraulic conductivities were higher in the Embrapa 48 tolerant genotype under irrigation and drought conditions (Fig. 7a, b). These results are consistent with a better water-absorption efficiency in the Embrapa48 genotype (Lima et al. 2019; Mesquita et al. 2020) and were confirmed by leaf relative water contents (RWC). RWC values decreased with the progression of the stress in both genotypes (Fig. 8). In the leaves under ψ_w = − 1.0 MPa, the RWC declined in BR16 by 49.14% while for Embrapa genotype, the reduction in the RWC was only of 32.67% (Fig. 8).

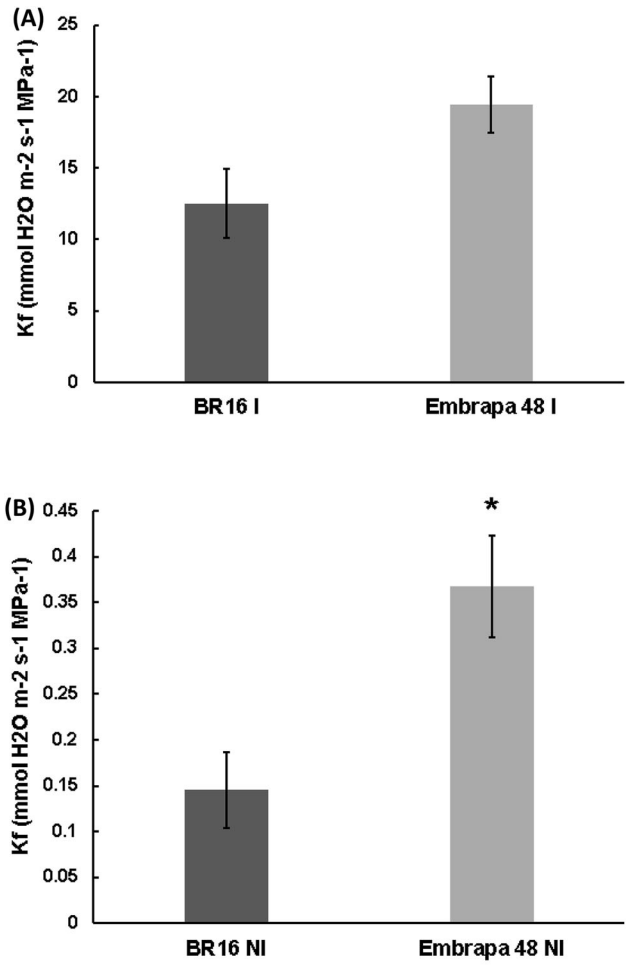

Fig. 7 Leaf hydraulic conductance (K_f) of the soybean genotypes. Each bar represents the mean ± standard error (n = 5, where n represents the number of plants, t test $p < 0.5$). In **a** IR for irrigated and in **b** for NI non-irrigated treatments

DISCUSSION

The early events of plant responses to drought stress are signal perception and subsequent signal transduction, which lead to the activation of various molecular, biochemical and physiological changes (Rejeb et al. 2014; Joshi et al. 2016). With the availability of genomic sequences from various plant species and the recent advances in sequencing technologies, the genes involved in drought/dehydration responses have been identified in a number of plant species, such as Arabidopsis (De Oliveira et al. 2011; Borkotoky et al. 2013; Shariatipour and Heidari 2018), and crops, such as rice and soybean (Prabha et al. 2011; Nakashima et al. 2014; Zhu et al. 2016; Sahebi et al. 2018). Thus, knowledge on gene expression reprogramming in response to drought stress has been obtained thoroughly. However, identifying the genes that contribute the most to the physiological and molecular adaptation mechanisms is a challenge.

In this study, we focused on two soybean genotypes that share a common ancestor (Davis genotype). The general response of Embrapa 48, when compared to the sensitive BR16, showed a very distinct physiological behavior (Oya et al. 2004; Carvalho et al. 2015; Mesquita et al. 2020) and a molecular response (Lima et al. 2019). The overriding feature observed in the gene expression response of BR16 genotype was the transcriptional induction of a relatively large number of genes. As this genotype is drought-sensitive, this large number of differentially expressed genes is in accordance with the other RNAseq studies in other plants (Yates et al. 2014; Fracasso et al. 2016; Yang et al. 2017), showing that sensitive plants dramatically reprogram gene expression under drought stress. This could be explained by the fact that sensitive species undergo greater changes in physiological and biochemical when mitigating the effects of stress conditions, as also observed by Lima et al. (2019) and Mesquita et al. (2020). On the other hand, the drought-tolerant genotype, Embrapa 48, showed a low alteration of gene expression under drought stress, as indicated by the notably lower number of identified DEGs. This general behavior was also observed in the proteomic and metabolomic data (Lima et al. 2019). Thus, the tolerance may reflect a lower level of stress when compared to BR16 and as a consequence result in a reduced reprogramming of the transcriptome (Fig. 1). These data corroborate with those obtained by Rodrigues et al. (2012), who used the Suppressive Subtractive Hybridization (SSH) technique to investigate differentially expressed genes under water-deficit conditions in these genotypes. This "more subtle" response is

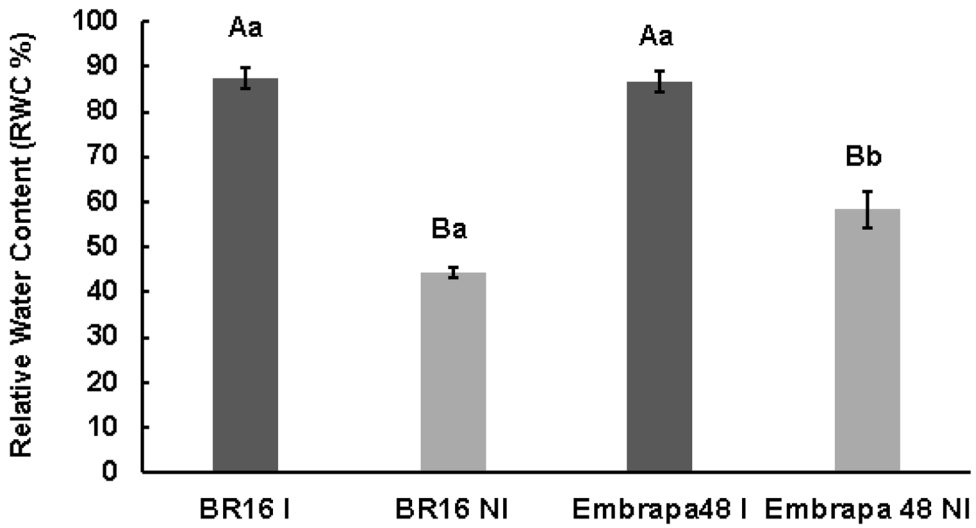

Fig. 8 Relative water content RWC (%) of the leaves of BR16 and Embrapa 48 genotypes under different water potential. Bars represent ± standard error (n = 5, where n represents the number of plants, t test $p < 0.5$). Different lower case letters indicate significant differences between averages of the same treatment in different cultivars, and capital letters show significant differences between averages within the same cultivar under different treatments. *IR* irrigated, *NI* non-irrigated treatments

probably due to differently expressed genes observed in Embrapa 48, when comparing the genetic background among genotypes (Supplementary Information Fig. S3). Gene expression for Transcriptome study performed by Janiak et al. (2018) reveals that drought tolerance in barley may be attributed to stressed-like expression patterns that exist before the occurrence of stress. Our results suggest that drought stress and pathway activation may vary considerably between the two genotypes and involves genes that are expressed even before the onset of drought treatment in the genotype EMBRAPA48, as differentially expressed genes in genetic background by that participate in the pathways of activation of cellular catabolic process, organic cation transport, dicarboxylic acid biosynthetic process, diterpenoid biosynthetic process and responsive to far red light. Yet, these processes may be responsible for the highest photosynthetic rate, stomatal conductance and carboxylation, observed in this genotype even before stress (Mesquita et al. 2020). In fact, gene expression analyses by RNAseq in the present study and by proteomic profiles (Lima et al. 2019) are in accordance with activities for enzymes involved in antioxidant defenses that were higher in the sensitive genotype BR16 (Mesquita et al. 2020). Moreover, levels of oxidative damage (lipid peroxidation) and activity of antioxidant enzymes under water-deficit were always higher in leaves of BR16 and confirmed by a stronger DAB staining in the leaves BR16 compared with Embrapa48 plants (Mesquita et al. 2020).

The ability to tolerate a water-deficit is a complex trait that could be controlled by many genes (Molina et al. 2008; Ergen and Budak 2009). In this context, plant cells detect stress stimulus through sensors or receptors that activate second messengers and initiate the corresponding signaling pathways to transduce the signals (Bhargava and Sawant 2013). In this study, we focused on the gene expression patterns that were distinct between contrasting genotypes aiming to understand the physiological behavior and identify specific candidates that correlate with drought tolerance.

Genes that stimulate the plant to survive better in drought conditions play a role in the regulatory network of gene expression, including several kinase proteins and transcription factors. The higher levels of phytohormone ABA and proline were observed for the sensitive BR 16 in accordance with a more pronounced perturbation in the metabolic pathways under drought for this genotype (Lima et al. 2019). However, when in stress conditions, the genotype Embrapa 48 showed an less changed metabolism, with higher photosynthetic rate, less oxidative damage in leaves and greater root growth compared to the genotype BR 16, indicating different signaling and regulation of the metabolism of the soybean leaves (Mesquita et al. 2020). Three calcium-dependent protein kinases (CDPKs) were up-regulated, and mainly function in the abscisic acid (ABA) signaling pathway and are plant-specific calcium sensors that play important roles in various aspects of plant physiology (Yang et al. 2011; Huang et al. 2012). Several works report that transcript levels of CDPKs are highly induced by drought, suggesting their important roles during abiotic stress responses in soybean

(Hettenhausen et al. 2016), especially in the modulation of ABA signaling to reduce the reactive oxygen species (ROS) (Asano et al. 2012; Neto et al. 2013). Other kinases differentially expressed in the tolerant soybean Embrapa 48 have not been associated to drought response so far.

In recent years, a wide range of TF families holding relevance in drought stress response have been identified, such as AREB, DREB, MYB, WRKY, NAC, ZFP and bZIP (Golldack et al. 2011; Jin et al. 2014; Anbazhagan et al. 2015). Genes that encode C3HC4 and C2H2-type Zinc finger proteins were down-regulated in Embrapa 48 under drought stress (Table 2). Zhang et al. (2016) reported that the family C2H2-type Zinc finger protein negatively regulates the drought response in transgenic *Arabidopsis*, because the plants, overexpressing these genes, might lose large volumes of water by increasing the width/length and number of completely open stoma, leading to drought stress sensitivity. Most of the family CCCH-type zinc finger proteins have shown up-regulation, which has been associated with RNA metabolism by directly binding to RNA targets and have been involved in abiotic and biotic stresses. Studies have indicated that CCCH zinc finger proteins are associated with senescence delaying effect, and they can interact with ABA and drought response regulators (Jan et al. 2013; Bogamuwa and Jang 2016; Chen et al. 2019). In summary, the higher expression of these CCCH-type zinc finger proteins and CDPKs genes in leaves of the tolerant cultivar may be responsible for the lower production of reactive oxygen species and, consequently, less cell damage, as observed in the ROS in leaf assay conducted by Mesquita et al. (2020).

Other TFs, such as the MYB and NAC, were also characterized for their role in stomatal movement controlling pore closure or in inhibiting its opening (Cominelli et al. 2005; Baldoni et al. 2015). Soybean MYB genes may contribute to the coordination of both cellulose and lignin biosynthesis in secondary wall formation. Yang et al. (2017) showed that plants engineered to accumulate less lignin or xylan are more tolerant to drought and activate drought responses faster than control plants. This is an important finding because it demonstrates that modification of cell walls must occur in the primary wall, and the analyses showed a low expression of secondary wall biosynthesis genes in the stress-tolerant genotype. In addition, the soybean orthologue coding Cellulose Synthase-like D3 was up-regulated only in the sensitive genotype and not altered in tolerant Embrapa 48. Evidence suggests that C_2H_2 transcription factors are also involved in the secondary metabolism and cell wall structure (Rao and Dixon 2018).

We found that some genes that code for proteins involved in cell wall dynamics were differentially expressed for both genotypes. Xyloglucan endotransglucosylase/ hydrolase (XTH) and Expansin are cell wall proteins involved in cell wall extension, which appear to be regulated by drought stress as observed for soybean genotypes. Some xyloglucan transferase and glycosidase were also responsive in both genotypes; however, some proteins involved in pectin metabolism were up-regulated in the tolerant genotype Embrapa 48 (Table 3). The major group of polymers in primary dicot cell walls are pectins, a heterogeneous group of homogalacturonic acid, rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Mohnen 2008). Pectins are often modified in plants exposed to drought, facilitating an increase in cell wall plasticity that can contribute to the maintenance of cell turgor or symplast volume (De Diego et al. 2013; Martínez et al. 2007). This plasticity can be correlated with drought tolerance mainly by increasing side chains of the pectic polymers rhamnogalacturonan I and II (RGI and RGII), possibly because the pectin form hydrated gels, which limit the damage to cells (Leucci et al. 2008). Despite its highly complex structure, RG-II is evolutionarily conserved in the plant kingdom as its present in the primary cell wall of all higher plants (O'Neill et al. 1996; Kobayashi et al. 1996). RG-II biosynthesis is a complex process and it is involved in several glycosyltransferases (GTs). One α3-xylosyltransferase (α3-XylT), named RGXT, was able to transfer a xylose residue onto the fucose of the side chain. The Arabidopsis RGXT family has four members linked to RG-II synthesis (Egelund et al. 2006; Liu et al. 2011). Three soybean orthologous genes RGXT1, RGXT2 and RGXT3 were up-regulated only in the genotype Embrapa 48.

An increase in cell wall elasticity can contribute to the maintenance of cell turgor. These results are an indication that the cell wall molecular modifications on the tolerant genotype could contribute to a more efficient water use observed in Embrapa 48. Only in drought-tolerant wheat genotypes, the side chains of rhamnogalacturonan I and II significantly increased in response to water stress (Leucci et al. 2008). The results confirm the role of the pectic side chains during water stress response. In addition, in this study, we also found a gene encoding for pectinesterase inhibitor differentially expressed between genotypes. Pectin is converted by the pectin methylesterase (PME) in pectate and methanol. PME activity is regulated by inhibitor proteins known as the pectin methylesterase inhibitor (PMEI), which plays a key role in wounding, osmotic stress, senescence and seed development. A gene coding for a Pectinesterase inhibitor 51 was down-regulated in

tolerant Embrapa 48 and up-regulated in the sensitive genotype. These results indicate that the metabolism of pectin is differently modulated in response to drought in soybean and may play a role in the plants defense mechanism against water-deficit, through the increase of elasticities and crosslink of the cell wall. Interestingly, the amount of side chains of RGI and/or RGII has been crucial to determine the hydration status of the cell wall matrix (Gall et al. 2015). The comparison with tolerant wheat genotypes indicates an increase in the amount of side chains during water stress, which consequently affects the viscosity status of the cell wall (Piro et al. 2003; Leucci et al. 2008; Gall et al. 2015). In fact, changes in the cell wall in response to abiotic stress, such as drought and cold, have been verified and involve a improve the viscoelastic properties of the primary wall. This is due to increases in the levels of cell wall remodeling and biosynthesis enzymes, as well as by modulating other wall loosening agents, including pectin. Thus contributing to increasing the hydration status of the plant and maintaining turgor pressure for growth (Gall et al. 2015).

The fact that the tolerant genotype leaves were more hydrated under the same water potentials suggests a possible osmotic adjustment; however, the higher levels ABA and proline were observed in the sensitive BR 16 leaves (Lima et al. 2019). Thus, the signal for drought by ABA and proline was more noticeable in the sensitive BR 16. In the same way, amino acids and sugar were more abundant during drought in the sensitive genotype (Lima et al. 2019), which suggests that these compounds were not important for the osmoprotection in the tolerant genotype. Furthermore, we observed also evidences suggesting the participation of a non-stomatal event in the relative drought tolerance of the Embrapa 48 and even though under severe stress, showing lower alteration on the net photosynthesis (Mesquita et al. 2020). Thus, the postponement of water and physiological response suggests that differential hydraulic conductivity may be important to this tolerance. In fact, the relative water content (RWC%) and hydraulic conductance (K_f) were higher in tolerant genotype Embrapa 48. Therefore, the maintenance of higher water content in the leaves in the tolerant cultivar could explain, at least partially, the greater photosynthetic rate of this cultivar.

CONCLUSION

Drought tolerance in plants is performed by different complex mechanisms, and to evaluate which gene is determinant for this phenotype is a challenge, especially because an extensive gene reprogramming is activated. However, we have used two soybean parental genotypes to investigate the molecular responses under drought stress. Although many genes showed similar gene expression patterns in both genotypes, genes involved in the signal transduction cascades and regulation of gene expression, such as kinase of the family CDPKS and TFs of the family C_2H_2 and CCCH, were only differentially expressed in the drought-tolerant genotype. The global transcriptomic study also showed that drought tolerance is operating even before the occurrence of stress and makes the plant ready to respond to adverse environmental conditions. This behavior was confirmed by lower genetic reprogramming in the Embrapa 48 genotype when subjected to drought. Physiological traits combined with proteomic and metabolomic profiles are in accordance with gene expression analysis by RNAseq. The drought-tolerance mechanism of the Embrapa 48 genotype involves in an increase in cell wall elasticity and hydraulic conductance contributes to the maintenance of cell turgor, resulting in the highest leaf RWC, photosynthetic rate (A), transpiration (E) and carboxylation (A/C_i) under conditions of water stress (Mesquita et al. 2020). These genetic traits contribute to maintain higher growth of the Embrapa 48 soybean plants under drought conditions (Mesquita et al. 2020).

Gene expression regulation analysis indicated that cell wall metabolism was changed in this genotype and could be correlated with the more efficient water use. Remodeling of the pectin component of the cell wall may be an important mechanism for the drought tolerance in the Embrapa 48 soybean genotype, promoting a differential hydraulic conductivity and a higher relative water content (RWC%).

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Declaration

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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