BRIEF REPORT



Comparative ABA-Responsive Transcriptome in Soybean Cultivars Submitted to Different Levels of Drought

Mayla Daiane Correa Molinari^{1,2} · Renata Fuganti-Pagliarini³ · Daniel de Amorim Barbosa^{1,4} · Elton Gargioni Grisoste Barbosa¹ · João Matheus Kafer^{1,4} · Daniel Rockenbach Marin^{1,4} · Silvana Regina Rockenbach Marin⁵ · Liliane Marcia Mertz-Henning⁵ · Alexandre Lima Nepomuceno⁵

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Abstract

Background The drought condition is responsible for considerable losses in sovbean production, which in turn may result in billionaire losses. After drought perception, plants activate a cascade of protecting genes against water deficit (WD), many of which are responsive to abscisic acid, the most important phytohormone to plants' adaptation. This work aimed to recover abscisic acid (ABA)-responsive differentially expressed genes (DEG) from an RNA-Seq, carried out from leaves and roots of droughtsensitive (BR16) and tolerant (Embrapa 48) soybean cultivars under mild (MiWD), moderate (MoWD), and severe (SWD) water-deficit treatments. Enriched ABA-responsive pathways important to drought tolerance in soybean were also identified. Results In drought-sensitive and tolerant soybean cultivars, approximately 75% of genes were identified as ABA-responsive by containing more than two ABRE (ABA-responsive elements) in the promoter region. Most of these genes were positively regulated. Roots were the tissue with more ABA-responsive genes and pathways triggered in response to WD in both cultivars, although, on the tolerant cultivar, these pathways were higher expressed. The most important enriched ABA pathways observed in the roots of both cultivars were involved in sugar and sulfur amino acid biosynthesis, osmoregulation, and crosstalk among ABA and ethylene, jasmonate, auxin, and cytokinin. Other pathways enriched were involved in phytoalexin production, ROS homeostasis, and membrane stability by glycerolipid and glycerophospholipid production. ABA-responsive genes were also ordered based on their expression profile in tissue and cultivar, and nine confidence groups could be observed. More than 80% of these clustered genes showed the same regulation profile under MiWd, MoWD, and SWD treatments. Activation of ABA biosynthesis under water deficit was validated by RT-qPCR by increasing the expression level of NCED3, an important enzyme in this pathway, and GOLS, a known ABA-responsive gene.

Conclusions A robust catalog of ABA-responsive genes was made available in this work. Considering ABA's role in droughtresponse mechanisms, the genes in the groups pointed out in this study would be reliable candidates to be used in strategies to develop soybean lines more tolerant to drought. This paper, presented for the first time, ABA-responsive genes and ABAenriched pathways in contrasting soybean cultivars for drought tolerance.

Keywords Glycine max · Abscisic acid · ABRE motif · Water deficit · RNA-Seq

Background

Soybean is one the most important commodity worldwide, showing high socio-economic relevance by participating actively in the economy of a lot of countries such as Brazil,

Mayla Daiane Correa Molinari and Renata Fuganti-Pagliarini equally contributed to this study.

Renata Fuganti-Pagliarini renatafuganti@gmail.com

Extended author information available on the last page of the article

Argentina, and the US. Periods of drought can severely damage the crop, implying billion-dollar losses (Ferreira 2016; Fuganti-Pagliarini et al. 2017; Bijalwan et al. 2022).

Unfortunately, given the current climate change, for the next few years, it is expected that the average temperature of the planet will increase and extreme weather conditions, such as drought, will intensify, jeopardizing agriculture and the global food supply in the future (Intergovernmental Panel on Climate Change (2022). To minimize expected production losses, intensive research efforts around the world are being made to develop more drought-tolerant soybean cultivars

(Fuganti-Pagliarini et al. 2017; Honna et al. 2016a; Ortiz 2019; Molinari et al. 2020, 2021a; Suhartina et al. 2022).

Among the most important pathways of plant adaptability to drought is the ABA-responsive (Shinozaki and Yamaguchi-Shinozaki 2007; Yoshida et al. 2014; Aslam et al. 2022). This phytohormone is responsible for triggering the first defensive mechanisms and controlling a variety of different molecular, physiologic, and metabolic responses under drought conditions (Munemasa et al. 2015; Bulgakov et al. 2019; Pizzio 2022). ABA-responsive genes can be identified by containing in their promoter region at least 2 conserved sequences named ABRE motifs (ACGT core) that are required for gene transcription. Usually, this motif is proximally located within the -1 to -2000 bp upstream transcription start site (TSS) (Hobo et al. 1999; Uno et al. 2000; Maruyama et al. 2012; Duarte et al. 2019; Liu et al. 2020).

The present study allowed the identification of ABAresponsive genes and the most expressed pathways in drought-tolerant and drought-sensitive soybean cultivars. Understanding the mechanism of action of the hub genes involved in these ABA-responsive pathways can provide essential information for the development of breeding strategies using biotechnological tools aiming to cope with the lack of water and, ultimately, reduce production losses.

Methods

Plant Materials and WD Experiment

Two soybean cultivars contrasting for drought response were used to determine the gene expression profile under different WD levels. BR16 cultivar is considered droughtsensitive and Embrapa 48 (E48) drought-tolerant (Oya et al. 2004; Rodrigues et al. 2012). Soybean plants were grown in a greenhouse under a hydroponic system following the protocol established by Martins et al. (Martins et al. 2008). Briefly, seeds were pre-germinated on filter paper for four days in a growth chamber at 25 ± 1 °C and 100% relative humidity. Seedlings were placed in plastic boxes in a way that roots were completely immersed in 50% of Hoagland's solution (Hoagland and Arnon 1950), which was continuously aerated and weekly changed. Boxes were kept in a greenhouse under a natural photoperiod of 12/12 h light/ dark cycle with a temperature of 30 ± 5 °C and $60 \pm 10\%$ relative humidity.

The experimental design was randomized blocks with nine biological replicates. When plants reached the V3 developmental stage (Fehr et al. 1971), roots and leaves of both soybean cultivars were collected following WD treatments levels: 0 min (T0—control), 25 min (T25), 50 min (T50), 75 min (T75), 100 min (T100), 125 min (T125), and 150 min (T150). Treatments were applied by removing plants from the hydroponic solution and placing them in empty boxes for the different water deprivation periods (minutes). Roots and leaf samples from all treatments and soybean cultivars were collected, deep in liquid nitrogen, and stored in a freezer until nucleic acids extraction.

mRNA-Seq Libraries Sequencing

Total RNA was extracted from leaves and root tissues from both soybean cultivars using TRIzol® reagent (Invitrogen, California, USA). Removal of remaining DNA was carried out using DNAse I kit (Invitrogen, California, EUA), and the removal of rRNA was performed with RiboMinusTM plant kit (Invitrogen, California, EUA), following manufacturer's instructions. RNA concentration and purity were measured using BioAnalyzer software (Agilent California, EUA), and the integrity was analyzed on a 1% agarose gel. Quantification of the mRNA was carried out by Qubit (Thermofisher, Massachusetts, EUA). Samples with RNA Integrity Number (RIN) \geq 8.0 were used for sequencing.

RNA from all water deficit treatments were equimolar pooled into four categories as follows: T0—control condition; T25–50 min, considered mild water deficit—MiWD; T75–100 min, considered moderate water deficit—MoWD; T125–150 min, considered severe water deficit—SWD. WD levels were chosen based on physiological analyses performed by Rodrigues et al. (Rodrigues et al. 2012) and Martins et al. (Martins et al. 2008), which showed that within prolonged exposure to the lack of water, photosynthesis of soybean cultivars was maximum at time zero but affected and strongly inhibited after 100 min.

The libraries were prepared using Illumina TruSeq[™] SBS v5 kit with 200 ng of mRNA. Single-end libraries of 100-bp size were synthesized by Fasteris company (Switzerland) and sequenced at Illumina HiSeq 2000 platform. A total of sixteen libraries (4 WD treatment levels×2 soybean cultivars×2 tissues) were synthesized, sequenced, and analyzed.

Bioinformatics Analysis

The quality of reads before and after the trimming process was evaluated using FastQC software version 0.11.5 (Andrews 2010). Removal of adapters, low-quality sequences (Phred quality score \geq 30), and sequences shorter than 40 bp were carried out using Trimmomatic software version 0.36 (Bolger et al. 2014). The soybean reference genome (Gmax_Wm82.a2.v1) used was downloaded from Phytozome v13 (Goodstein et al. 2012). Alignment of reads in the reference genome was performed using HISAT2 software version 2.1.0, retrieving unique alignments with higher quality (Kim et al. 2015). PCR artifacts (unnatural read duplications) were removed by Samtools rmdup software version 1.5 to improve mapping quality (Li et al. 2009). Assembly of transcripts was performed by StringTie software version 1.3.3 (Pertea et al. 2015). The StringTie output was used in GFOLD software version 1.1.4 (Feng et al. 2012) to obtain DEGs. For each soybean cultivar and tissue, DGEs were obtained by comparing control and WD treatment levels. Only genes that presented log2 fold-change (Log2 FC) ≤ -1 (downregulated) and ≥ 1 (up-regulated) were considered differentially expressed. This means that an expression level of at least 2 times between the control and WD levels was considered. The adoption of good practices of RNA-Seq (RNA sequencing) analyses was performed according to experimental design (Conesa et al. 2016; Molinari et al. 2021b).

In Silico Recovery of ABA-Responsive DEGs and Ontology

The promoter region of all DEGs from each WD treatment (MiWD, MoWD, and SWD) and plant tissue were separately recovered using the RSAT plant (Contreras-Moreira et al. 2016). To recover only ABA-responsive genes among all DEGs, a script in shell language was applied to the data. Genes with at least two cis-elements ACGT core between -1 to -2000 bp within the promoter region were considered (Maruyama et al. 2012; Narusaka et al. 2003; Shen et al. 2004; Nakashima et al. 2006, 2014; Yoshida et al. 2010; Wang et al. 2019). The biological annotation of ABA-responsive DEGs was performed using the Phytomine tool available at Phytozome (Goodstein et al. 2012).

To retrieve genes expressed in all levels of WD treatments, a Venn diagram was executed (Bardou et al. 2014). This group of genes represents 100% of expression reproducible along with the WD treatments and was highly accurate to evaluate ABA responses, minimizing sequencing biases. ABA-responsive DEGs identified in all three levels of WD levels (MiWD, MoWD, and SWD) were used to generate 09 genic groups according to their expression profile in tissues and cultivars. The KEGG pathways were evaluated using ShinyGO software version 0.66 (*p*-value \geq 0.05) (Ge et al. 2020).

RT-qPCR

To validate the expression of *NCED3* (9-cis-epoxycarotenoid dioxygenase) and *GOLS* (galactinol synthase), known droughtresponsive genes, identified in the RNA-Seq, an RT-qPCR (reverse transcriptase quantitative polymerase chain reaction) was performed. Total RNA was extracted from soybean leaves and root samples from both soybean cultivars using TRIzol[®] reagent and treated with DNAse I kit (Invitrogen, Carlsbad, CA) to remove possible remaining DNA. After that, cDNA was synthesized using SuperScript[®] III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Sets of primers for *GOLS* (Glyma.10G145300—primer F 5'GCTACCCGATGTTCCCCAAG3'/ primer R 5'GGGGTA CACGGGCTCAATCT3'), *NCED* (Glyma.15G250100 primer F 5'CCAGAAGCCTTACCTCAAAT3'/primer R 5'TCATGGTGGGTTCTTTCAAA3') genes were designed. Sequences were obtained from Phytozome v13, and specific primers were designed using Primer3Plus software (Primer-3Plus software. 2022). Homo and heterodimers were checked using Multiple Primer Analyze software (Multiple Primer Analyze software 2022).

RT-qPCR reactions were composed of cDNAs, 0.2 μ M F and R primers, and 1×reaction buffer Platinum[®] SYBR Green[®] qPCR SuperMix UDG (Invitrogen, Carlsbad, CA). PCR primer efficiencies were estimated using LinRegPCR software v.2012.0 (Ruijter et al. 2019), considering efficient primers displaying values \geq 90%. Gene expression relative quantification was performed using three random biologicals and three technical replicates (*n*=9). Reactions were made through 7900HT thermocycler equipment (Applied Biosystems). Cycling conditions used were denaturation at 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s, 60 °C for 26 s, and 1 cycle for the Melting curve at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s.

Gene expression calibration was performed using β -actin (Glyma.15G050200—primer F 5'GAGCTATGAATTGCC TGATGG3'/primer R 5'CGTTTCATGAATTCCAGTAGC3') and Fyve (Glyma.13G114700—primer F 5'TTCTGTCTT CTGCAAGTGGTG3'/primer R 5'GATCCCTCATCCATA CATTTCAG3') genes, as described by Marcolino-Gomes et al. (Marcolino-Gomes et al. 2015). The expression level was determined using the formula $2^{-\Delta\Delta Ct}$ adapted according to the primer's efficiencies (Livak and Schmittgen 2001).

Results

ABA-Responsive DEG in Three Levels of WD Treatment

Results showed that the total number of differentially expressed genes identified in leaves were 5256 and 5279, respectively, in BR16 (sensitive cultivar) and Embrapa 48 (tolerant cultivar). In roots, for BR16 and Embrapa 48, the total number of DEG was 4957 and 5665, respectively (Fig. 1). In Fig. 1, it can also be observed the number of ABA-responsive genes identified in each dataset (3875; 3753; 3875; 4256). Those numbers indicated that an average of 75% of the DEG were identified as ABA-responsive, once at least two cis-elements ACGT core were found between -1 to -2000 bp within their

promoter region. Among the ABA-responsive genes, approximately 40% were differentially expressed in all three levels of water deficit treatments (MiWD; MoWD; SWD). This subset of genes is important for both soybean cultivars as the drought intensifies and has 100% reproducibility within the independent libraries of the present study (756; 1892; 865; 2535 genes identified in all treatments, in leaves and roots of BR16 and Embrapa 48), representing an accurate gene set to study the ABA-enriched pathways among soybean cultivars (Fig. 1). The individual expression of these data sets' genes is available in Additional file 1 and Additional file 2.

Groups of ABA-Responsive Genes

The intersection of ABA-responsive genes (756; 1892; 865; 2535) was clustered into 09 ABA-responsive groups (Fig. 2). In group 01, 105 genes were observed in leaves,

mostly being upregulated (61). Forty-one genes showed downregulation in all WD treatments and soybean cultivars, and 3 genes presented different expression profiles, varying accordingly with WD treatments (MiWD, MoWD, and SWD) (Additional file 3, sheet: group 01). Group 02 comprised 1071 genes expressed in the roots, with 888 and 182 genes showing, respectively, upregulation and downregulation. Only one gene showed expression profile variation among WD treatments (Additional file 3, sheet: group 02).

Groups 03 and 04 clustered ABA-responsive genes classified as cultivar-specific. In group 03, 16 genes were identified as being expressed in the leaves and roots of drought-sensitive cultivar BR16. Among these genes, 12 were upregulated and 1 downregulated, with 3 other genes showing different expression profiles, varying accordingly with treatments (additional file 3, sheet: group 03). Group 04 comprised genes expressed in leaves and roots of



Fig. 1 The total set of differentially expressed genes (DEGs) and the subset of ABA-responsive genes in BR16 (drought-sensitive) and Embrapa 48 (drought-tolerant) soybean cultivars during mild (MiWD—T25–50 min), moderate (MoWD—T75–100 min), and severe water deficit (SWD—T125–150 min) treatments compared

to the control (no WD). The error bar represents the standard error among the number of the average genes in the three water-deficit levels. Venn diagrams represent the intersection of ABA-responsive genes expressed in the three water-deficit levels (756; 1892; 865; 2535)

drought-tolerant cultivar E48. In this group, 40 genes were observed, 13 being upregulated, 19 downregulated, and 8 genes presented expression profiles discrepant among WD treatments (Additional file 3, sheet: group 04).

Groups 05 and 06 included genes expressed, respectively, in leaves and roots of sensitive cultivar BR16. Thus, in group 05, a total of 187 genes were identified, with 129 and 43 showing upregulation and downregulation, respectively, in all three WD treatments. Fifteen genes presented expression profiles differing among MiWD, MoWD, and SWD treatments (Additional file 3, sheet: group 05). In group 06, 381 genes expressed only in the roots of BR16 were identified. Among them, 179 were upregulated, 201 were downregulated, and one gene showed an expression profile varying accordingly with WD treatments (Additional file 3, sheet: group 06). In these groups, most of the genes were downregulated, opposing previous groups.

Groups 07 and 08 included genes expressed, respectively, in leaves and roots of drought-tolerant cultivar E48. Again, in these groups, most of the genes were downregulated. In group 07, from a total of 488 genes, 152 and 301 genes showed, respectively, upregulation and downregulation. Thirty-five genes presented expression profiles varying accordingly with WD treatments (additional file 3, sheet: group 07). In group 08, genes expressed only in roots of E48 were gathered. From a total of 933 genes, 533 were upregulated and 400 were downregulated. No genes showing expression profile variation among WD treatments were identified.

Finally, in group 09, ABA-responsive DEGs were identified in both soybean cultivars (E48 and BR16), and tissues (leaves and roots) in all levels of WD treatments (MiWD, MoWD, and SWD) were collected (Additional file 3, sheet: group 09). In this group, 134 genes were observed, with 125 being upregulated and 9 downregulated. No genes showing expression profile variation among WD treatments were identified.

ABA-Enriched Responsive Pathways in Three Levels of Drought (MiWD; MoWD; SWD)

KEGG analyses indicated 06 ABA-enriched pathways in leaves of BR16. These were involved in sugar, camalexin, glycerophospholipid, ethylene, jasmonate biosynthesis, and reactive oxygen species (ROS) homeostasis. In BR16 roots, 10 enriched pathways were observed, being 06 of them the same as observed in leaves. The remaining four were identified as involved in glycolipid, amines, auxin, and cytokinin biosynthesis (Fig. 3).

In leaves of drought-tolerant Embrapa 48, 07 ABAenriched pathways were observed. They were glycerolipid, sulfur amino acid, sugar, auxin, camalexin, ethylene, and jasmonate biosynthesis. In Embrapa 48 roots, 11 enriched pathways were found, 7 of them as identified in leaves, plus pathways related to amines, glycerophospholipid, auxin, and cytokinin biosynthesis.

Despite the similarities among the ABA responses between soybean cultivars, the number of compounds in each pathway was higher in the tolerant cultivar than in the sensitive one. This was seen mainly for membrane components like glycerolipid and glycerophospholipid in the roots, suggesting that ABA responses may be more intense in Embrapa 48 (tolerant cultivar) (Fig. 3).

RT-qPCR of Known ABA-Responsive Genes

RT-qPCR and RNA-Seq showed the same expression profile of *GmNCED3* and *GmGOLS* genes. Both genes were upregulated in both techniques (Fig. 4). These genes presented higher expression on the roots of both soybean cultivars. Also, more than two ABRE motifs were observed in the promoters of the *GmNCED3* (5 ABRE motifs identified) and *GmGOLS* (6 ABRE motifs identified) genes, confirming the ABA biosynthesis activation under water deficit conditions (Fig. 4).

Discussion

ABA-Responsive DEGs and Enriched Pathways Under WD Treatments

Most of the drought and ABA-related genes identified here are greatly known in literature, increasing the reliability of this approach to identify genes with a real profile under drought in silico. Besides that, the same expression profile (mostly upregulation) of genes, showing slight modulation differences, reinforces the involvement of these genes in WD defense mechanisms and the accuracy of the pipeline employed. This is possible to infer due to all libraries' being completely independent but presenting similar responses to the conditions applied.

In general, a higher number of differential expressed genes were identified in both leaves and roots for both soybean cultivars, with a high percentage being ABA-responsive as well. Most DEGs and ABA-responsive genes identified in root-specific groups (Additional file 2; Additional final 3/group 02/group 06 and group 08) were droughtresponse genes such as heat shock proteins and heat shock TFs (transcription factors), genes related to osmotic adjustment (proline, oligosaccharides, trehalose, and glucose), hormone-related (ethylene, auxin, and gibberellin), LEA (late embryogenesis abundant) and chaperones, water channels (aquaporins) and other WD-response related TFs such

Venn diagrams showing 9 clusters of ABA-responsive DEGs identified in all levels of water deficit treatments, in both soybean cultivars and plant tissues



◄Fig. 2 Venn diagram of 09 clusters of ABA-responsive DEGs identified in all three levels of water-deficit (MiWD—25–50 min, MoWD—75–100 min, and SWD—125–150 min) treatments, in drought-sensitive (BR16) and drought-tolerant (E48) soybean cultivars and tissues (roots and leaves). Legend: L, leaves; R, roots; orange, BR16L, leaves; green, BR16R, roots; blue, E48L, leaves; pink, E48R, roots. Graphics show the total of genes identified in each soybean cultivar, tissue, and WD treatment level

as DREB (dehydration-responsive element-binding protein), NAC (NAM—no apical meristem, <u>A</u>TAF1-2-Arabidopsis transcription activation factor, and CUC2—cup-shaped cotyledon), MYB and WRKY. As reported here, these genes were also described to be associated with drought responses in roots of different plant species (Janiak et al. 2016) and recently identified in RNA-Seq analyses carried out in roots of rice (Zhang et al. 2017), sorghum (Zhang et al. 2019), chickpea (Mashaki et al. 2018), and wheat (Chaichi et al. 2019; Iquebal et al. 2019).

As an organ in direct contact with the soil, roots are capable of precepting and signalizing soil status toward the shoot, which triggers changes in the regulation of gene expression that afterward controls molecular mechanisms of drought responses (Janiak et al. 2016). In this role, ABA plays an important part in regulating drought sensing by roots. The increase in ABA of roots in response to drought was correlated with an increase in foliar-ABA concentrations, suggesting that drought-induced ABA plays a significant role in controlling leaf water potential (Zegada-Lizarazu and Monti 2019). ABA accumulated in roots is transported to trigger stomatal closure, limiting transpirational water loss while up-regulating genes to promote osmotic adjustment in leaves (Haworth et al. 2018; Bharath et al. 2021). This data corroborates and reinforces the expression of the ABA-responsive genes and their role in leaves' response to water deficit.

Among the 134 genes clustered as commonly expressed in both soybean cultivars, tissues, and in all WD levels (Additional final 2/group 09), drought response genes showing upregulation profile were also identified, such as NAC and WRKY TFs, heat shock TFs and heat shock proteins, and NCED. The NAC TF family is involved in various stress responses. High-throughput transcriptome studies of plants submitted to drought showed that NAC-encoding genes were differentially expressed in roots of rice (Moumeni et al. 2011), pine (Lorenz et al. 2011), cotton (Ranjan and Sawant 2014), and common bean (Recchia et al. 2013). In poplar and soybean, differential expression of NAC genes was identified specifically or preferentially in roots (Cohen et al. 2010; Le et al. 2011), corroborating results found here. Many reports have shown the involvement of WRKYs and ABA, mainly in the stomata closure response (Finatto et al. 2018). In Arabidopsis, AtWRKY genes are involved in increasing salt and osmotic stress sensibility (Chen et al. 2010). In tobacco, data showed that ABA participated in WRKY-induced stomatal closure (Chu et al. 2015). In addition, overexpression of *WRKY* genes increased drought tolerance in *Vitis vinifera* (Wang et al. 2014), cotton (*Gossypium hirsutum*) (Yan et al. 2015), rice (Wu et al. 2009), and tobacco (Ding et al. 2015).

Heat shock factors (HSFs) are important transcription factors that are frequently induced by heat along with other abiotic stresses like salt, drought, and cold (Manna et al. 2020). The involvement of HSFs in drought responses has been reported in many crop species, such as chickpea (Ma et al. 2016), maize (Li et al. 2015), wheat (Huang et al. 2016), sorghum (Tashi et al. 2018), and rice (Yoshida et al. 2011).

ABA-enriched pathways were identified in both tissues and soybean cultivars under all three levels of WD treatments. Sugar-related, camalexin, glycerophospholipid, and pathways for ethylene and jasmonate hormones biosynthesis were identified in both leaves and tissues. In addition to these pathways, amines, auxin, and cytokinin pathways were root specific (Fig. 3). Cellular sugar status is essentially maintained during normal growth conditions but is adversely affected during various environmental perturbations. Drought presents such an unfavorable environmental condition that it hinders the photosynthetic fixation of carbon in sugars and affects their transport, decreasing the cellular osmotic potential (Kaur et al. 2021). However, the tolerant cultivar was able to produce greater amounts of these compounds under drought and guarantee their osmoprotection and thus, adaptation to lack of water. Besides, drought reprograms the distribution of sugar in cellular and subcellular compartments. Usually, plants more tolerant to drought present higher levels of sugars, guaranteeing osmotic potential maintenance, protection of photosynthetic apparatus, maintenance of ROS, positive feedback in ABA biosynthesis, biosynthesis of heat shock proteins, increase in numbers of polyamines, proline accumulation, and prevention of water loss by lignin deposition (Kaur et al. 2021) (Fig. 3).

In addition to the sugar content, drought induces changes in glycerolipid metabolism and glycerophospholipid metabolism (Liu et al. 2021a). In general, lipids play important roles in cellular functions, including serving as structural molecules in membranes, affecting membrane proteins' function, and enhancing their protection. As drought induces an increase in fatty acid saturation of plasma membrane lipids and ROS accumulation, this leads to membrane stiffness (Liu et al. 2021a). However, the accumulation of lipids leads to the protection of these membranes in dry conditions. The presence of these pathways in the Embrapa 48 cultivar suggested that, besides many other defense mechanisms triggered by water deficit, this could be an additional one to cope with drought in tolerant cultivars (Fig. 3).

Furthermore, the presence of enriched camalexin pathways in both soybean cultivars shows the well-known



Enriched ABA-responsive pathways

Fig. 3 ABA-enriched responsive pathways under all levels of water deficit treatments (mild, MiWD; moderate, MoWD; severe, SWD) in drought-sensitive (BR16) and tolerant E48 (Embrapa 48) soybean cultivars. Green arrows represent activation; red arrow represents modulation

crosstalk between biotic and abiotic stress responses. The phytoalexin camalexin plays a crucial role in defense against fungal and bacterial pathogens (Mewis et al. 2012), and high levels of camalexin in plants subjected to drought can potentially reduce infection by pathogens (Mewis et al. 2012; Zhang et al. 2014).

The ROS homeostasis pathway was only enriched in leaves of BR16, a drought-sensitive cultivar. Some of the secondary messengers triggered by ABA can participate in plants' adaptation to abiotic and biotic stress. Examples are ROS (enriched pathway identified in leaves of BR16), nitric oxide (NO), and cytosolic-free Ca²⁺ (León et al. 2014; Huang et al. 2019). The generation of ROS in plants under water deficit is due to ABA binding to its receptor, which inactivates protein phosphatase 2C, resulting in the activation of OST1 (open stomata 1) kinase, which stimulates NADPH oxidase (due to phosphorylation) enzyme to generate ROS and then the production of NO. Both ROS and NO can elevate levels of cytosolic Ca²⁺. The high levels of ROS, NO, and Ca²⁺ act either directly or together to activate anion/cation efflux channels while inhibiting the influx channels. The result is the loss of cations/anions from guard cells, resulting in turgor loss and stomatal closure (Agurla et al. 2018), a well-known response triggered by water deficit conditions.

Besides ABA, several other hormones increase when plants are exposed to stress, which closes stomata and helps plant defense responses. As pathways enriched here, auxins, cytokinins, jasmonate, and ethylene usually complement ABA's role in drought conditions responses (Iqbal et al. 2022). Auxins are involved in many plants' developmental processes as well as in a dynamic role in mediating and improving plant tolerance to noninfectious stresses, such as deficiency conditions (Kazan 2013). When plants are exposed to drought, different modulations in the synthesis, metabolism, transport, and activity of auxins take place (Ljung 2013). In tobacco seedlings, auxins promote root branching and have a potential role in drought tolerance mechanisms (Verma et al. 2016; Wang et al. 2018). Molecularly, according to Jung and co-workers (Jung et al. 2015), among auxin-coding genes recognized in rice plants, some genes were activated by drought stress. In addition, the overexpression of auxinrelated genes also resulted in enhanced drought tolerance



Validation of known ABA-responsive-genes

Fig. 4 Results of the expression level of known ABA-responsive genes *GmNCED3* and *GmGOLs* in the RNA-Seq and RT-qPCR. Green bars represent the gene expression in Log2FC for RNA-Seq and $2^{-\Delta\Delta CT}$ for RT-qPCR. The asterisk represents significant expression by *t*-test

 $(p \ge 0.05)$. The blue Web logo represents the core of the ABRE motif (ACGT) in the promoter of both genes. The sequences represent the promoter region between -1 and -2000 bp of TSS (transcription start site) and ABRE motifs are highlighted in light blue

in poplar and potato (Ke et al. 2015). Moreover, in tomatoes, auxin response factors (ARFs) bind directly to the promoters of auxin-responsive genes, allowing them to be activated or repressed transcriptionally and enhancing stress tolerance (Bouzroud et al. 2018). These ARFs also regulate genes involved in drought response and enhanced tolerance in clovers (Zhang et al. 2020a). Auxin also enhanced drought resistance by interacting with other phytohormones. By regulating members of the ACS (1-aminocyclopropane-1-carboxylate synthase) gene family, which is a rate-limiting enzyme in ethylene biosynthesis, drought resistance in plants was enhanced (Colebrook et al. 2014). Overall, auxin has the potential to stimulate drought tolerance by regulating antioxidant enzyme activities, secondary metabolites, ABA content, expression of stress-responsive genes, and oxidative damage to plants (Singh et al. 2022).

Cytokinins are important for plant regulation, growth, and acclimation to drought conditions (Li et al. 2016). The enhancement or reduction of the cytokinin level depends on the period and severity of the drought stress (Zwack and Rashotte 2015). In Arabidopsis, the independent overexpression of CKX1, CKX2, CKX3, and CKX4 genes resulted in transgenic lines with reduced CK levels and subsequently greater drought tolerance (Nishiyama et al. 2011). Ethylene is strongly involved in the response to dehydration. In addition to acting on osmoregulation, it involves the activation of transcription factors of the ERF (responsive factors of ethylene) family, involved in drought tolerance (Kaur et al. 2021). In Arabidopsis, the overexpression of an ERF gene resulted in lines showing drought tolerance by lessening transpiration, squeezing the aperture of the stomata, and thinning the cuticle (Scarpeci et al. 2017). In cotton, ethylene-responsive genes or binding protein elements such as AP2 (apetala2), EREBPs (ethylene-responsive element binding proteins), and Apetala2 were identified in response to heat and drought conditions (Liu and Zhang 2017). Furthermore, in soybean, GmERF3 was induced by drought, ABA, salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), and its overexpression in tobacco plants lead to lines exhibiting more tolerance to drought due to high contents of proline and solubilized sugar (Zhang et al. 2009). It is worth emphasizing that in the present study, the ERF1 gene was upregulated in the roots of both soybean cultivars, corroborating ethylene's role in activating drought responses in plants.

Jasmonate also plays a substantial role in water stress tolerance. Its role is well established in crops such as peanuts, rice, soybeans, and broccoli (Kaur et al. 2021). When this phytohormone is applied exogenously, it leads to the accumulation of soluble sugars among other metabolites, in addition to improving the activity of genes that eliminate ROS, thus improving drought tolerance (Abdelgawad et al. 2014; Shan et al. 2015). This data corroborates our data on sugar and ROS homeostasis-enriched pathways in both BR 16 and Embrapa 48 soybean cultivars. Under normal conditions, the proteins JAI3/JAZ bind to various transcription factors, including MYC2, and limit their activity. However, during drought, the degradation of JAZ proteins occurs, resulting in active transcription factors that upregulate genes of JA, enhancing tolerance (Ollas and Dodd 2016; Ullah et al. 2018).

It is important to highlight that all these hormones normally do not work in exclusive pathways but depend on each other at different stages to control responses to environmental factors (Ullah et al. 2018), such as drought. Thus, via jasmonate, the MYC2 transcription factor was enriched, mainly in the roots of both soybean cultivars assayed here. As a result of ABA and jasmonate activation, soybean exhibit pivoting roots with a great ability to penetrate deeper soils in search of water (Kaur et al. 2021). This strong hormonal relationship in the roots could have led the tolerant cultivar to better adapt to the condition of lack of water, as it is observed that these pathways were more expressive in the tolerant cultivar in a number of genes (Fig. 4). Other examples of these hormones' crosstalk are described below. Exogenous application of auxin enhanced drought tolerance and levels of ABA and JA in *Trifolium repens* (Zhang et al. 2020b). Moreover, JA signaling pathways interact with ABA signaling pathways, suggesting their role in response to drought stress. It has been recently revealed that JA enhanced the hydraulic conductivity of plant roots under drought stress by interacting with calcium and ABAdependent and independent signaling pathways (De Ollas and Dodd 2016).

Drought-Responsive Genes Expressed Under WD Treatments

ABA increased synthesis is a well-known mechanism triggered by plants to cope with water deficit conditions. The NCED enzyme is a key point control in ABA biosynthesis (Bhaskara et al. 2012; Behnam et al. 2013) and the overexpression of NCED genes have improved drought tolerance in many plants such as Arabidopsis (Iuchi et al. 2001; Tong et al. 2017), avocado (Persea americana) (Chernys and Zeevaart 2000), citrus (Citrus sinensis) (Neves et al. 2013; Pedrosa et al. 2015; Rodrigo et al. 2006), common bean (Phaseolus vulgaris) (Oin and Zeevaart 1999), tobacco (Oin and Zeevaart 2002; Pedrosa et al. 2017), rice (Oriza sativa) (Sultana et al. 2014; Huang et al. 2018), petunia (Estrada-Melo et al. 2015), cotton (Gossypium hirsutum) (Souza et al. 2016), cowpea (Vigna unguiculata) (Iuchi et al. 2000), grape (Vitis vinifera) (Soar et al. 2004), peanuts (Arachis hypogaea) (Wan and Li 2006), tomato (Solanum lycopersicum) (Burbidge et al. 1999; Thompson et al. 2000), and turmeric (Curcuma longa) (Ahrazem et al. 2012). In soybean lines overexpressing the NCED3 gene, plants showed higher concentrations of ABA under WD (Molinari et al. 2020), reinforcing the role of this phytohormone in droughtresponse mechanisms as well as corroborating data found here for soybean cultivars BR16 and Embrapa 48.

Galactinol synthase (GOLs) is a key enzyme in raffinose family oligosaccharide (RFO) biosynthesis. In different environmental stresses such as cold, heat, and dehydration drought, RFOs regulate the osmotic potential and protect enzymes and membranes (Crowe et al. 1984; Wang et al. 2004). The expression of some *GOLS* genes was induced by drought, heat shock, salinity, and osmotic shock (Taji et al. 2002; dos Santos et al. 2015). In addition, the involvement of *GOLS* genes in drought responses, as well as the overexpression of these in successfully increasing drought tolerance, has been reported for many plants, such as Arabidopsis (Taji et al. 2002), *Brachypodium distachyon* (Himuro et al. 2014), poplar (Liu et al. 2021b), rice (Takahashi et al. 1994; Shimosaka and Ozawa 2015), maize (Gu et al. 2016), coffee (Santos et al. 2015, 2011), tomato (*Lycopersicon* *esculentum* Mill. cv Moneymaker) (Downie et al. 2003), grape (*Vitis vinifera*) (Pillet et al. 2012), and *Salvia miltior-rhiza* (Wang et al. 2012).

Corroborating our findings here, that the *GmGOLs* gene participates closely in drought defense responses in soybean, Marcolino and co-workers (Marcolino-Gomes et al. 2014) described a diurnal oscillation and induced expression for *GmGOLS*-like gene in lines submitted to water deficit. Gene expression quantification for soybean *GOLS* (Glyma19g40680) confirmed the expression induction in plants submitted to water deficit (Rodrigues et al. 2015). In addition, soybean lines overexpressing *AtGOLS2* showed increased galactinol transcripts, which may have acted as osmoprotectants, leading to higher drought tolerance and survival rate. In field conditions, these plants presented higher yields, probably due to the increased synthesis of RFOs under well-watered conditions (Honna et al. 2016b).

Conclusions

Gene group 09, a hub of drought and ABA-responsive genes, are reliable candidates to be used in projects to develop more drought-tolerant lines, which, in the long-term, can positively impact the soybean crop, minimizing losses due to water scarcity.

Enriched pathways confirmed the complexity of responses triggered in both tolerant and sensitive soybean cultivars by water deficit conditions as well as the large-scale hormonal crosstalk responsible and involved in these defense mechanisms.

Abbreviations ABA: Abscisic acid; ABRE: Abscisic acid-responsive element; ACS: 1-Aminocyclopropane-1-carboxylate synthase; AP2: Apetala2; ARFs: Auxin response factors; At: Arabidopsis thaliana; ATAF1-2: Arabidopsis transcription activation factor 1-2; cDNA: Complementary deoxyribonucleic acid; CKK: Cytokinin dehydrogenase; CUC2: Cup-shaped cotyledon; DEGs: Differentially expressed genes; DNA: Deoxyribonucleic acid; DNAse: Deoxyribonuclease; DREB: Dehydration-responsive element-binding protein; E48: Embrapa 48; EREBPs: Ethylene-responsive element binding proteins; ERF: Responsive factors of ethylene; ET: Ethylene; Gm: Glycine max; GOLS: Galactinol synthase; HSFs: Heat shock factors; JA: Jasmonic acid; JAI3/JAZ: Jasmonate-insensitive/jasmonate-zim; KEGG: Kyoto Encyclopedia of Genes and Genomes; LEA: Late embryogenesis abundant; Log2 FC: Log2 fold-change; MiWD: Mild water-deficit treatment; MoWD: Moderate water-deficit treatment; mRNA: Messenger ribonucleic acid; NAC: No apical meristem; NADPH: Nicotinamide adenine dinucleotide phosphate; NCED3: 9-Cis-epoxycarotenoid dioxygenase; NO: Nitric oxide; OST1: Open stomata 1; RFO: Raffinose family oligosaccharide; RIN: RNA integrity number; RNA: Ribonucleic acid; RNA-Seq: RNA sequencing; ROS: Reactive oxygen species; rRNA: Ribosomal ribonucleic acid; RT-qPCR: Reverse transcriptase quantitative polymerase chain reaction; SA: Salicylic acid; SWD: Severe water-deficit treatment; TFs: Transcription factors; TSS: Transcription start site; WD: Water deficit.

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Author Contribution MDCM conceived and designed the study, performed data analysis and interpretation, and wrote the manuscript. RFP also conceived and designed the study, performed data analysis and results compilation, reviewed the manuscript, and provided scientific editing and language proofreading. EGB reviewed the manuscript and provided scientific editing and language proofreading. DAB, DRM, and SRRM were involved in the experiments and collecting of biological material in a greenhouse. LMMH and ALN are the principal researchers in the project and reviewed the final version of the manuscript. All authors have read and approved the final version of the article.

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Declarations

Ethical Approval and Consent to Participate Not applicable.

Consent for Publication All authors gave their consent for the publication of the research results.

Conflict of Interest The authors declare no competing interests.

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Authors and Affiliations

Mayla Daiane Correa Molinari^{1,2} · Renata Fuganti-Pagliarini³ · Daniel de Amorim Barbosa^{1,4} · Elton Gargioni Grisoste Barbosa¹ · João Matheus Kafer^{1,4} · Daniel Rockenbach Marin^{1,4} · Silvana Regina Rockenbach Marin⁵ · Liliane Marcia Mertz-Henning⁵ · Alexandre Lima Nepomuceno⁵

- ¹ Londrina State University, Londrina, PR, Brazil
- ⁴ Coordination for the Improvement of Higher Education Personnel, Embrapa Soja, Londrina, Brazil
- ² Arthur Bernardes Foundation, Embrapa Soja, Londrina, Brazil
- ³ Piccola Scientific Consulting, Saskatoon, Canada
- ⁵ Embrapa Soja, Londrina, Brazil