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ABSTRACT

In soybean, studies on drought-responses are conducted during vegetative season. Information related to genes triggered in response to water-deficit (WD) in flower and pod is lacking. We performed an RNASeq and an agro-physiological characterization at stages R2 and R4 of soybean cultivar BR16, under WD. Physiological results showed a decrease in gas exchange parameters. Agronomical results showed WD impaired yield. Global Gene Ontology analyses indicated that most of the Differential Expressed Genes (DEGs) were down-regulated in flowers but up-regulated in pods. qRT-PCR revealed that WD triggered hormone biosynthesis changes. Mechanisms such as a decrease in glyceollin levels and cell wall instability were observed. This data showed tissue-specific mechanisms correlated with phenotypic responses such as drought escape, early flowering hormone-induced, and loss in grain weight. We presented here a comprehensive expression profile of flowers and pods soybean genes, which could guide researchers in the development of plants more tolerant to drought.

Introduction

Natural disasters, between 2005 and 2015, cost the agricultural sectors of developing country economies a staggering US$96 billion in damage, crop loss, and livestock production worldwide according to Food and Agriculture Organization of the United Nations (FAO 2018). Drought, which has battered farmers in all corners of the globe, was one of the leading culprits. Eighty-three percent of all drought-caused economic losses documented by FAO's study were absorbed by agriculture, with a price tag of US$29 billion (FAO 2018). In Brazil, where agriculture is a key factor in the national economy, the importance of real and potential prejudices due to drought can be speculated by analyzing some numbers. Especially, in soybean production, an important stanchion on agriculture and Brazilian economy, losses due to WD, added up US$79 billion in financial losses during 1976/1977 and 2013/2014 crop seasons (Ferreira 2016). According to IBGE (Brazilian Institute of Geography and Statistics), in 2015, a year when Brazil went through a serious financial crisis, agriculture was the only economic sector that did not drop its contribution to GDP (Gross Domestic Product) mainly because of soybeans and corn production, 1.8% over the previous year (FAO 2016).

Besides this positive survival in the face of the financial crisis and increasing productivity numbers, upcoming climate predictions are not good for food producers. According to the last report from the Intergovernmental Panel on Climate Change (IPCC 2011), every year from 1961 to 2013, an additional 1 percent of the world's drylands slipped into drought, which will put production, food security and food access in danger. Thus, in the present context and future picture of climatic changes, many alternatives can be used to mitigate and reduce production and financial losses due to drought in soybean. Several studies have been developed to understand soybean plant's response to drought aiming to develop strategies to reduce the harmful effects of WD, to protect vulnerable farming systems and the populations depending on farmers. During the vegetative stage, water shortage can delay seedling emergence and triggers the early transition to the reproductive period. When it occurs at the reproductive stage (flowering and pod formation) can reduce productivity, usually as a result of decreased numbers of flowers, pods, and seeds, induced by different processes. WD also affects key metabolism processes, such as photosynthesis reducing carbon fixation and the availability of photo-assimilates needed for seed formation, which also, impairs grain filling and therefore final productivity (Pinheiro and Chaves 2010).

Although flower, pod, and seed formation are among the main phases affected by WD, most of the available information about drought responses in soybean was assayed in the vegetative and reproductive developmental stage in roots and leaves (Manavalan et al. 2009; Savitri et al. 2013; Shanker et al. 2014; Kidokoro et al. 2015; Xu et al. 2018), leaving a gap about the genes and metabolic pathways involved in the mechanisms of response and acclimatization to WD in soybean reproductive organs, such as flower and pod. Thus, in this context, the present study aimed for the first time to reveal the gene expression profile in flowers (at R2 stage – full bloom) and pods (at R4 stage – pods completely developed) in soybean under WD. This data could help uncover metabolic pathways triggered in reproductive

structures and be a raw material for the development of strategies to increase drought tolerance in plants.

Material and methods

Biological material and greenhouse experiment

Soybean seeds from conventional cultivar BR16, considered drought-sensitive (Oya et al. 2004; Lima et al. 2019) were treated with fungicide Vitavax® Thiram 200 SC (200 g.L^{-1}) (ADAPAR) for health quality purposes and then allowed to germinate on Germitest® paper in the dark in germinating chamber, for 4 days, at 28 ± 1°C and 100% relative humidity (RH). Seedlings were transferred to 5 kg pots filled with substrate mixture 1:1 (fertilized soil and washed sand), one plant per pot. The experiment was carried out in greenhouse conditions under a short-day condition (10 h light/14 h dark) at 28 ± 2°C.

Three experiments were carried out in parallel. The experimental design was in completely randomized blocks, in 2 × 3 factorial arrangement, i.e. two water conditions (water deficit – WD; control – C), three collecting points (R2 to collect flowers, R4 to collect pods and at end of the developmental cycle to assay agronomical parameters), with eight blocks. Pots were maintained at 100% field capacity (FC) through daily irrigation with a fixed water volume sufficient to saturate the substrate until plants reached the phenological stage V7. At this stage, one day before WD induction, all

pots were saturated with water at the end of the afternoon to allow excess water to be drained overnight. In the following morning, pots were wrapped in polyethylene bags, and the central region of each pot was covered with cotton around the stem base to prevent water loss by evaporation (Figure 1). Water deficit was imposed by completely withholding water for 7 days. After water deficit imposition, control plants were keep hydrated by watering them with 180 mL per day.

Physiological and agronomical parameters analysis

Irrigation was kept in C plants, while was withheld in the WD group, which was monitored daily to the stomatal conductance (gs) until plants showed gs values less than 200 mmol H$_2$O m^{-2}.s^{-1} (condition considered as WD stressed) (Flexas et al. 2004; Salinet 2009). When plants were at the R2 developmental stage, i.e. in full flowering which means that there is an open flower at one of the two uppermost nodes, gas exchange parameters were assayed. Photosynthetic rate (A), stomatal conductance (gs), sub-stomatal CO$_2$ concentration (Ci), transpiration rate (E), leaf temperature and vapor-pressure deficit were measured on the central leaflet of the third fully-expanded trifoliate leaf (apex-base direction) using a portable infrared gas analyzer (LCpro-SD, ADC BioScientific). These measurements were performed inside a greenhouse from 9.00 am (Brazilian daylight-saving time) at 1000 μmol
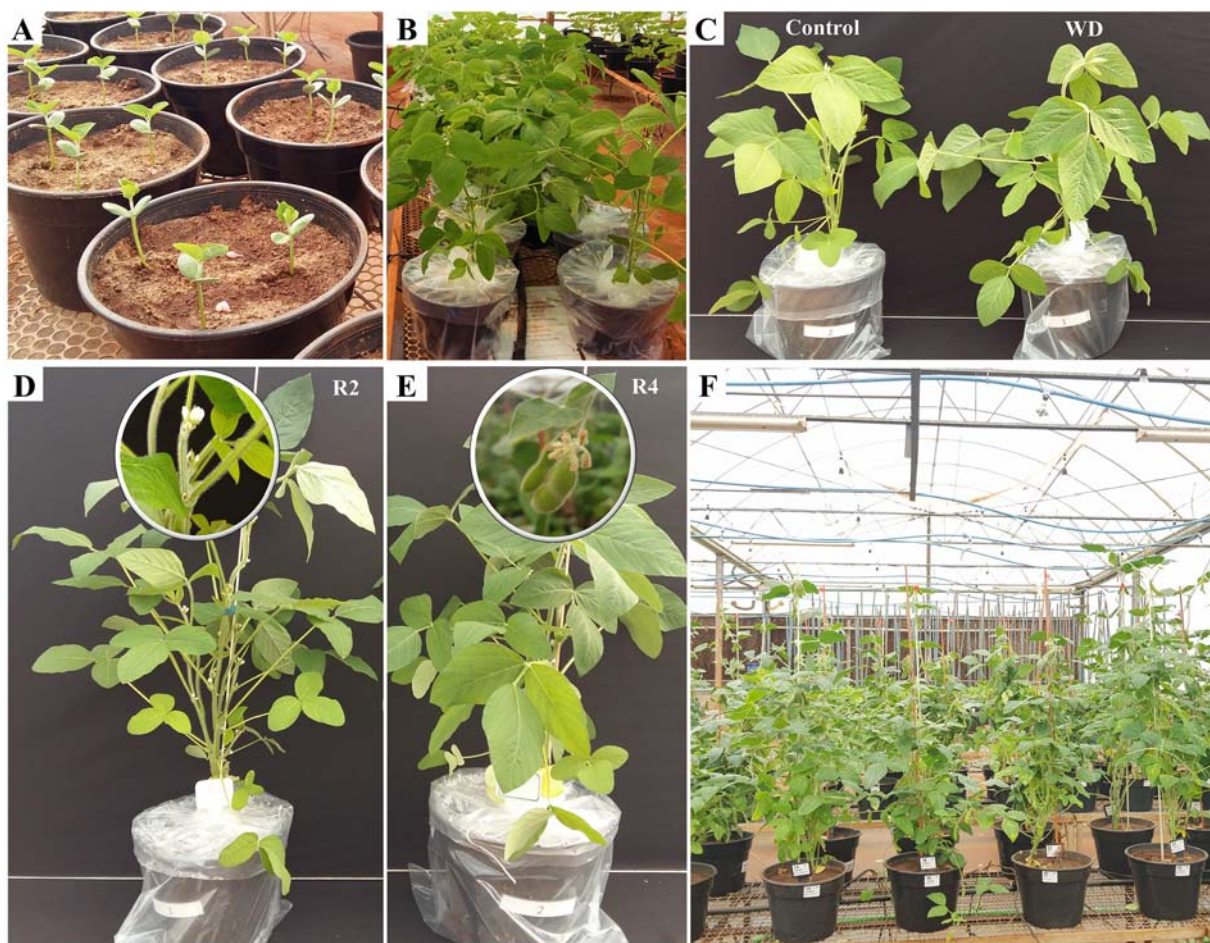
Figure 1. In (A), soybean plants growing in the early stages in greenhouse conditions, showing three plants per pot. Before drought treatment imposition, two plants were removed, keeping more homogenous plants to carry out the experiment, in an attempt to avoid bias due to growth. In (B), plants wrapped in polyethylene bags, and with the central region of each pot covered with cotton around the stem base to prevent water loss by evaporation. In (C), control and treated soybean plants, showing wilting. In (D and E), respectively soybean plants in R2 and R4 developmental stage, showing in detail, flower and pod. In F, general view of soybean plants growing in greenhouse. These plants were assayed for agronomical parameters.

m^{-2}.s^{-1} photosynthetically active radiation (PAR). The intrinsic water use efficiency was obtained through the ratio *A/gs,* as the ratio of carbon assimilation to the correspondent water loss at the leaf level (Medrano et al. 2015). At this point, flowers were collected from WD and C plant groups, individually from each block and the experiment was discarded. After that, other WD parallel experiments were kept until plants reached the R4 developmental stage, i.e. in the full pod, which means pods are 3/4 inch (2 cm) at one of the four uppermost nodes, pods were collected. Physiological parameters were again evaluated at this collecting point and the experiment was discarded. A third experiment with WD and C plant groups was conducted until the end of the reproductive cycle, to evaluate agronomical parameters such as plant height, number of nodes (NN), number of pods with and without seeds, dry mass of pods with and without seeds, number of seeds (NS), dry mass of seeds (DMS), the total number of pods (TNP), dry mass of 100 seeds (DM100S), the mean distance between nodes and average number of seeds per pod. These agronomical results were subjected to statistical normality test (Shapiro and Wilk 1965), variance analysis (ANOVA) and means of 8 biological replicates comparison by Tukey test ($p \leq 0.05$) using Sasm-Agri and R Studio v.3.5.1 software (Canteri et al. 2001; Racine 2012).

mRNA-Seq libraries sequencing

Flowers and pods (without seeds) from soybean plants subjected to WD and C conditions were collected respectively, at the development stages R2 and R4, immediately frozen in liquid N$_2$ and stored at −80°C. Total RNA was extracted using Trizol® reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's specifications.

RNA quantification was performed using the NanoDrop spectrophotometer, according to the following parameters of quality and purity: concentration >600 ng µL, ratio 260/280 ranging between 1.8 and 2, and ratio 260/230 ≥ 2.0. RNA was treated with DNA-free turbo DNAse kit (Life Technologies, Grand Island, NY, EUA). The RNA integrity was evaluated in agarose gel electrophoresis 1% (p/v) with ethidium bromide (1 µg/mL) (Sambrook et al. 1989). Quality samples were again evaluated at Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.) and only samples with RNA Integrity Number (RIN) ≥ 7.00 were used to synthesize the mRNA-Seq libraries. High-quality RNA samples (1000–1800 ng) were sent to Georgia Genomics Facility (GGF), at the University of Georgia (USA), to prepare libraries and sequence the mRNA-Seq libraries. Sixteen libraries, which corresponded to 4 biological repetitions (RNA equimolar libraries) of each treatment and biological material (4 libraries using flowers under WD, 4 libraries using flowers under C, 4 libraries using pods under WD and finally 4 libraries using pods under C) were synthesized using KAPA Stranded mRNA-Seq Illumina® platform kit (Illumina). Libraries were sequenced in Illumina NextSeq 500 1.9 poli-A 75 bp paired-end (Illumina, San Diego, CA, EUA), with about 1X genome coverage.

Bioinformatics analysis

From each library, forward and reverse reads were overlapped to generate longer consensus fragments using COPE v.1.2.5 software (Liu et al. 2012). The quality of these consensus fragments was evaluated using FastQC v.0.11.5 software (Andrews 2010; Patel and Jain 2012). Removal of adaptors and low-quality sequences were carried out through Trimmomatic version 0.36 software (Bolger et al. 2014) standardizing cuts every 4 bases in the extremities of the sequences that presented quality score less than 20 (Phred Quality Score, Q ≥ 20). The quality of resulting fragments was checked again using FastQC v.0.11.5. Soybean reference genome (*Glycine max* Wm82.a2.v1) was downloaded from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax). Genome indexing and alignment of reads were performed using HISAT2 software v.2.1.0., with the final recovery of reads with unique alignments (Kim et al. 2015). Unnatural duplications from the PCR step were removed using the Samtools v.1.5 software (Li et al. 2009). Mapping was carried out using Stringtie v.1.3.3 software (Pertea et al. 2015). The differential expression analysis was performed by EdgeR software v.3.22.3 (Robinson et al. 2010) in RStudio v.3.5.1 (Racine 2012). For each cultivar, DEG was obtained by comparing the C and WD conditions for each tissue (flower and pod). Genes considered differential expressed presented log2 fold-change (Log2FC)≤−2 and ≥+2 values with false discovery rate (FDR) ≤ 0.05 excluding negative logCPM (Molinari 2021). The annotation of the biological function of DEG was performed using the Phytomine tool available at Phytozome (Goodstein et al. 2011). A parametric analysis of gene enrichment (PAGE) was performed using differentially expressed genes sets exclusive from flower and pod and their respective Log2FC (Kim and Volsky 2005).

Candidate genes selection process

Nine differentially expressed genes in flower and pods with biological annotated function were considered to be tested. For flowers, selected genes were: Glyma.06G049900 [(*1-Aminocyclopropane-1-Carboxylate Synthase 8-Related – ACS8,* E.C.4.4.1.14), (primer F 5'ACATATCTCCTGGCTCTTCT3'/primer R 5'GGTAATTGAGTCCGCAAAAG3')], Glyma.11G129300 [(*beta-glucosidase – BG1,* EC.3.2.1.21), (primer F 5'ATAGCCAACATGGTTATGGA3'/primer R 5'AGAAGTTGGTGATGTGAGAC3')], Glyma.10G295300 [(*Glycinol 4- dimethylallyltransferase – G4DT,* EC.2.5.1.36), (primer F 5'TTGTTGTGAAGGCAATCTCT3'/primer R 5'TGCCAATCATTGTGTATGGA3')], Glyma.10G193800 [(*Embryonic Flower 2 – EMF2*), (primer F 5'GTTGATGGGAAGGGGAATAC3'/primer R 5'CAGAAGCAAAGACCAAGAACC3')] and Glyma.09G196500 [(*MYBS2 putative*), (primer F 5'GATCTGACAAACTCTCCTCC3'/primer R 5'TGTCGGCCATTATTGGTAAA3')]. For pods, selected genes were: Glyma.08G044000 [(*pectinesterase – PME,* EC.3.1.1.11), (primer F 5'CTCAAACGCTCAATGAACTC3'/primer R 5'AGTGAAACCATTTTGGCATG3')], Glyma.19G009000 [(*formate dehydrogenase – FDH,* EC.1.17.1.9), (primer F 5'ATTCCTGATGCCAATGTCAT3'/primer R 5'TCAACGTGATCAGAACCAAT3')], Glyma.12G146400 [(*ABA 8'-hydroxylase – CYP707A4,* EC.1.14.14.137), (primer F 5'TGAAGTTGGAATCCTCACAG3'/primer R 5'TTGTCCACAATCCGGTAATT3')] and Glyma.03G042700 [(*WRKY transcription factor 33*), (primer F 5'GAGCCACTAAAGAAACAGGA3'/primer R 5'GGTTTGATTGAGGCTAATGC3')]. Sequences were obtained from Phytozome and specific primers were designed using

Primer3Plus software (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi). Homo and heterodimers were checked using Multiple Primer Analyze software (https://www.thermofisher.com/br/multiple-primer-analyzer.html).

Validation of RNA-Seq gene expression by qRT-PCR

Total RNA (RNA Integrity Number (RIN) ≥ 7.00) was extracted from soybean flower and pod samples using Trizol® reagent, treated with DNAse I kit (Invitrogen, Carlsbad, CA) to remove possible DNA remained. After that, cDNA was synthesized using Super Script® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. qRT-PCR reactions were composed of cDNAs, 0.2 µM F and R primers, and 1x reaction buffer Platinum® SYBR Green® qPCR SuperMix UDG (Invitrogen, Carlsbad, CA). Gene expression calibration was performed using β-actin (Glyma.15G050200 – primer F 5'GAGCTAT-GAATTGCCTGATGG3'/ primer R 5'CGTTTCATGAATTCCAGTAGC3') and $Fyve$ (Glyma.13G114700 – primer F 5'TTCTGTCTTCTGCAAGTGGTG3'/ primer R 5'GATCCCTCATCCATACATTTCAG3') genes, as described by Marcolino-Gomes et al. (2015). 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 (s) followed by 40 cycles of 95°C for 3 s, 60°C for 26 s, and 1 cycle for Melt curve at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. PCR primers efficiencies were estimated using LinRegPCR software v.2012.0 (Ruijter et al. 2009), considering as efficient primers displaying values ≥ 85%. The expression level was determined using the formula $2^{-\Delta\Delta Ct}$ adapted according to the primer's efficiencies (Livak and Schmittgen 2001). Statistical analysis was performed using the t-test ($p \leq 0.05$) from Sasm-Agri software (Canteri et al. 2001). The Pearson correlation between qRT-PCR and RNA-Seq log2FC expression was performed in RStudio v.3.5.1 (Racine 2012).

Results

Physiological and agronomical data analysis

Physiological data obtained revealed that in flowers (R2) and pods (R4), WD treatment decreased gas exchange parameters when compared to C conditions (Figure 2). In the R2 developmental stage, photosynthetic rate (A) decreased from 19.90 to 11.71 µmol CO_2 m^{-2}.s^{-1} in C and treated plants, respectively, differing statistically between treatments. WD also impaired stomatal conductance (gs), declining from 0.31 to 0.07 mol H_2O m^{-2}.s^{-1} in treated compared to C flowers, characterizing the WD condition as moderate (Flexas et al. 2004). Stomal closure severely impacted sub-stomatal CO_2 concentration (Ci), which was reduced by more than 50% in the WD condition, from 260.94 in C to 122.93 µmol CO_2 mol air^{-1} in WD plants. There was also a negative impact on the transpiration rate, which dropped from 3.88 mmol H_2O m^{-2}.s^{-1} (C) to 1.33 mmol H_2O m^{-2}.s^{-1} (WD). WD condition increased vapor pressure (from 1.25 in C to 1.78 kPa in WD) and leaf temperature (from 28.38 to 29.99°C in C and treated plants, respectively), mainly due to altered cellular evapotranspiration. Intrinsic water-use efficiency was increased from 75.38

under C to 165.43 under WD treatment, being statistically different (Tukey test at 5%).

Similar results were obtained in the physiological analyzes performed during the R4 developmental stage. C and treated plants presented average stomatal conductance values of 0.45 and 0.14 mol H_2O m^{-2}.s^{-1}, respectively. Significant reduction in Ci was also identified, decreasing from 274.45 in C to 147.21 µmol CO_2 mol air^{-1} in WD. Transpiration rate was also reduced, from 5.66 (C) to 2.81 mmol H_2O m^{-2}.s^{-1} in WD. Likewise was observed in the R2 developmental stage, in the R4 stage, an increase in vapor pressure (from 1.28 in C to 1.98 kPa in WD plants) and leaf temperature (from 27.21 to 29.88°C in C and treated plants, respectively) was also observed. The change in these parameters resulted in a decrease in the average photosynthetic rate, which dropped from 25.35 in C plants to 17.30 µmol CO_2 m^{-2}.s^{-1} in plants under WD (Figure 2) and a rise in intrinsic water-use efficiency, which increased from 66.88 in C conditions to 141.23 under WD treatment, being statistically different (Tukey test at 5%).

Drought responses in soybean BR16 sensitive cultivar were more pronounced in the R2 stage if compared to the R4 stage. Gas exchange reduction was higher in R2 compared to R4 being 9.44% for photosynthesis, 7.67% for stomatal conductance, 6.53% for sub-stomatal CO_2 concentration, and 15.31% for transpiration, suggesting that flowers were more sensitive to WD than pods. Vapor pressure (12.86%) and leaf temperature (4.11%) reductions were also more prominent in R2 compared to R4.

WD treatment also impaired agronomical parameters (Table 1). R2 and R4 developmental stages were reached respectively, at 40 and 50 days after planting for all treatments (WD and C). WD condition reduced the average number of flowers per plant, from 12 to 8.44 flowers although no statistically significant (Table 1). Likewise, the total number of pods also decreased from 84.78 in C plants to 60 in WD treated plants (Table 1), differing statistically and strongly suggesting that WD might have induced pods abortion.

No differences between soybean plants under C and WD conditions were identified for average height (∼32 cm), number of nodes (∼9 nodes), the mean distance between nodes (∼3 cm), number of pods without seed (∼16) and dry mass of pods without seeds (∼29 g). Statistical differences were observed in the number of pods with seeds (from 66.56 to 47.11), the total number of pods (from 84.78 to 60), dry mass of pods with seeds (from 33.18 to 24.62 g), number of seeds per plant (from 132.89 to 91.67) and dry mass of 100 seeds (from 25.50 to 19.34 g) (Table 1). These results indicated that the application of WD, even in a moderate and controlled manner, resulted in a negative impact on several agronomic parameters in soybean plants.

Expression profile of soybean flower and pod genes under WD

COPE software analyses showed an average of 46% of paired-end reads overlapping (Additional file 1). These overlapped reads represent longer consensus fragments with more accuracy in the nucleotide sequence, increasing quality transcript assembly. Before overlapping, reads showed about 75 bp and after it, reads displayed a length about 130 bp. GC content after cleaning (45–47%) showed that no significant contaminations were present in the samples, thus not impairing reads
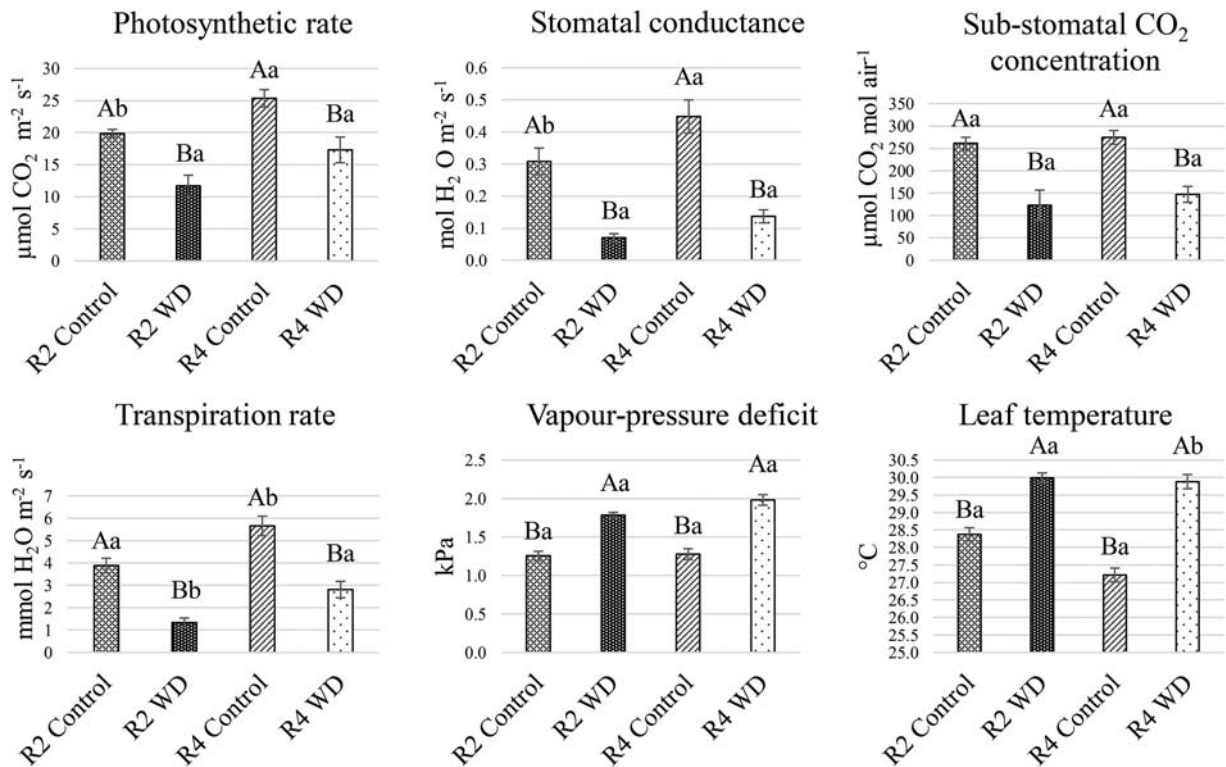
Figure 2. Gas exchange parameters assayed in soybean leaves at R2 and R4 developmental stages under WD and C conditions. Measurements of photosynthetic rate (A) – C.V% = 22.02, stomatal conductance (gs) – C.V% = 38.33, sub-stomatal CO_2 (Ci) – C.V% = 33.27, transpiration rate (E) – C.V% = 26.30, vapor-pressure deficit – C.V% = 9.74 and leaf temperature (°C) – C.V% = 1.64. Capital letters compared C to WD conditions within the developmental stage (R2 and R4) separately. Minor letter compared developmental stages within conditions (C and WD) separately. Means followed by the same letter stand for no significant difference according to means of 8 biological replicates comparison by Tukey test at 5%. Bars mean pattern error. Legend: ANOVA C.V%: Coefficient of Variation.

alignment. About 96.88% of reads showed Phred score ≥ 20, which indicates 1 error for each 100-base call or 99% of real sequence accuracy.

Table 1. Agronomical parameters evaluated at the end of the soybean developmental stage.

	Treatment	Values[a] (±standard error)	Tukey[b] 5%[b]	ANOVA C.V%[c]
Plant height (cm)	C	32.00 ± 1.67	A	13.02
	WD	32.25 ± 1.44	A	
Number of nodes	C	9.22 ± 0.27	A	6.60
	WD	9.67 ± 0.23	A	
Mean distance between nodes (cm)	C	3.48 ± 0.18	A	15.16
	WD	3.00 ± 0.13	A	
Number of pods with seeds	C	66.56 ± 6.29	A	29.34
	WD	47.11 ± 3.93	B	
Number of pods without seeds	C	18.22 ± 2.22	A	31.90
	WD	12.89 ± 1.64	A	
Total number of pods	C	84.78 ± 6.52	A	24.60
	WD	60.00 ± 5.16	B	
The dry mass of pods with seeds (g)	C	33.18 ± 1.96	A	21.81
	WD	24.62 ± 1.72	B	
The dry mass of pods without seeds (g)	C	0.20 ± 0.08	A	125.86
	WD	0.11 ± 0.02	A	
Number of seeds per plant	C	132.89 ± 12.11	A	28.57
	WD	91.67 ± 7.12	B	
The dry mass of 100 seeds (g)	C	25.50 ± 0.50	A	20.23
	WD	19.34 ± 0.86	B	
Number of flowers	C	12.00 ± 1.37	A	41.93
	WD	8.44 ± 1.45	A	

[a]Average values for 8 plants evaluated ($N = 8$).
[b]Capital letters compared C to WD conditions within developmental stages (R2 and R4) separately. Minor letter compared developmental stages within conditions (C and WD) separately. Means followed by the same letter stand for no significant difference according to means of 8 biological replicates comparison by the Tukey test at 5%. Bars mean pattern error.
[c]ANOVA C.V%: Coefficient of Variation.

For libraries alignment, mapping and gene annotation, the genome file in FASTA format (Gmax_275_v2.0.fa.gz), and the annotation file in GFF3 format (Gmax_275_Wm82.a2.v1.gene.gff3.gz) were used. Library alignment was performed, and only single-aligned reads were selected. Multi-aligned and non-aligned reads have been discarded. Obtained results showed that for flower libraries, the percentage of unique read alignment ranged from 85 and 95% (average 94.18%) while for pods libraries, this value oscillated between 77 and 88% (average 88.31%) (Additional file 1). Consequently, the percentage of reads that did not align or showed multiple alignments were lower for flower libraries (between 5 and 15%) and higher for pods libraries (between 12 and 23%). The removal of unnatural PCR duplicates discarded approximately 50% of the total reads, reducing noise, and minimizing false positives. After PCR artifact removal (about 46.90%) the reads alignment against the reference genome was about 94.09%.

For all DEG identified under WD, in flowers, 163 and 62 DEG were down and up-regulated, respectively, while in pods, 23 DEG were down-regulated and 117 were up-regulated (Additional file 1). Considering DEG with biological function annotated, analyses showed 174 genes exclusively in flowers and 104 DEG identified exclusively in pods. Both analyzed tissues showed a remarkable difference when expression profile was assayed: for flowers, 42 genes were up-regulated and 132 down-regulated; while for pods, the opposite pattern was observed, with 84 genes being up-regulated and 20 down-regulated (Additional file 1).

Considering different biological functions, 106 and 98 were found in flower and pod, respectively. Twenty-nine genes corresponding to 14 different biological functions

were found in common for both flower and pod of drought-sensitive cultivar BR16. Among these genes, 8 biological functions presented different profiles (up/down-regulation) in each tissue, and 6 exhibited the same expression profile, being up or down-regulated in both flower and pod. Sequences without annotation were also identified, 33 genes (10 up and 23 down-regulated) being exclusively for flowers, 16 genes (14 up and 2 down-regulated) for pods, and 4 genes identified in common for both tissues, all showing up-regulation (Additional file 2).

For flowers exclusively, among the DEG showing up-regulation, some genes involved in WD responses were identified such as *Late Embryogenesis Abundant proteins* (Glyma.03G144400, Glyma.05G112000, Glyma.10G064400, Glyma.12G001600, and Glyma.13G363300), *Small Heat-Shock Protein Hsp20 Family* (Glyma.19G011400) and *9-cis-epoxycarotenoid dioxygenase (NCED) enzyme* (Glyma.08G176300 and Glyma.15G250100). Down-regulated genes that trigger drought-responses were also identified such as related to hormone (auxin transporter protein – Glyma.03G063900, ABA receptor – Glyma.11G233300 and ethylene – Glyma.18G148000), photosynthesis/stomata closure (protein phosphatase 2c – Glyma.07G164400), sugar biosynthesis and transporter (Glyma.12G032600, Glyma.13G213300 and Glyma.14G159900) (Additional file 2). The statistics of all genes in each Gene ontology category showed in Additional file 2 genes are represented in Figure 3(A).

In pods, a contrasting pattern was identified for some genes involved in abiotic-stress responses when compared to the flower expression profile. Sugar-related genes were up-regulated in pods (Glyma.03G137900 and Glyma.08G009900), as well as *ethylene hormone transcription factor* (Glyma.08G009900) and *ABA hydrolase* (Glyma.12G146400) and photosynthesis/stomata closure (*protein phosphatase 2c* – Glyma.06G290200 and Glyma.18G208100). Other exclusive genes related to WD responses were also up-regulated in pods, such as aquaporin (Glyma.20G170400), *Dehydrin* (Glyma.07G090400), *WRKY transcription factors* (Glyma.03G042700 and Glyma.18G208800) and osmotic-adjustment related (*solute carrier family 35* – Glyma.04G221100) (Additional file 2).

Some genes were shared between both tissues. Among common genes were *salicylate O-methyltransferase*, Protein of unknown function (DUF1298), *EMEA, WRKY, Zinc Finger Fyve domain protein, Sulfate-transporting ATPase, benzoate O-methyltransferase, and extensin related*, which showed contrasting expression profile between tissues and could be represented by different Glyma IDs. On the other hand, *LEA, AWPM-19, ATHB-12, inositol 3-alpha-galactosyltransferase*, and *EMB* genes were up-regulated in both tissues (Additional file 2).

For each GO term identified, genes under each category showed up and down-regulated pathways for pods and only down-regulated pathways for flowers, under WD (Figure 3(A)). Protein binding, membrane, and cellular processes were exclusively up-regulated in pods. Both tissues shared the following GO terms: transition metal ion binding, transferase activity, ion binding, hydrolase activity, and cell part; being up-regulated in pods and down in flowers. Transporter activity, ribonucleotide and purine nucleotide binding, primary metabolic process, nucleotide and nucleotide-binding, nitrogen compound metabolic process, metabolic process, macromolecule metabolic process, gene expression, catalytic activity, carbohydrate metabolic process, binding, ATP binding, adenyl ribonucleotide, and adenyl nucleotide binding were exclusively down-regulated in flowers (Figure 3(A)). In short, GO terms reflected gene expression, as in flowers most GO terms were down-regulated as the DEG (75%) (Figure 3(A)), similarly in pods, up-regulated genes displayed up-regulated GO terms.

Gene expression validation by qRT-PCR

qRT-PCR carried out for DEG in flower and pod showed the same expression profile (down or up-regulation), in different levels, identified in the RNA-Seq, validating the data obtained. These results also suggested that the pipeline analyses applied in this paper were robust enough to reach a strong positive Pearson correlation (0.75) between qRT-PCR and RNA-Seq techniques using log2FC gene expression. Genes from RNA-Seq was selected by higher up/down-regulation with FDR ≤ 0.05.

In flowers, Glyma.06G049900 (*1-Aminocyclopropane-1-Carboxylate synthase 8 – ACS8 –* E.C.4.4.1.14) was up-regulated, showing, respectively 3.12x and 5.76 log2FC in RNA-Seq and qRT-PCR; as well as Glyma.10G193800 (*Embryonic Flower 2 – EMF2)* which presented 5.82x and 1.13 log2FC, and Glyma.09G196500 (*MYBS2 putative*) which displayed 4.52x and 1.02 log2FC in RNA-Seq and qRT-PCR, respectively. Glyma.11G129300 (*beta-glucosidase – BG1 –* EC.3.2.1.21) and Glyma.10G295300 (*glycinol 4-dimethylallyl-transferase – G4DT –* EC.2.5.1.36) were down-regulated in both techniques, presenting respectively,−7.67x and −13.83x and −8.08 and −5.26 log2FC, respectively, at RNA-Seq and qRT-PCR techniques (Figure 3(D)).

For pods, Glyma.12G146400 (*ABA 8'-hydroxylase –* CYP707A4 – EC.1.14.14.137) was up regulated showing, respectively 7.43x and 4.94 log2FC in RNA-Seq and qRT-PCR, as well as Glyma.03G042700 (*WRKY33*) which presented 2.83x and 9.3 log2FC. Down-regulated genes in pods were: Glyma.08G044000 (*pectinesterase- PME –* EC.3.1.1.11) showing −5.79x and −1.14 log2FC in RNA-Seq and qRT-PCR, respectively and Glyma.19G009000 (*formate dehydrogenase – FDH –* EC.1.17.1.9) which presented respectively, −5.67x and −1.88 log2FC, respectively, at RNA-Seq and qRT-PCR techniques (Figure 3(D)).

Discussion

Soybean as an important crop worldwide is affected by WD, and most of the studies currently available on gene expression in response to drought were carried out in leaves and/or roots. Especially in reproductive organs, such as flower and pod, information on genes and the metabolic pathways triggered in response to WD is lacking. This study reported a physiological and agronomical characterization and transcriptome analyses in flower and pod of a drought-sensitive soybean cultivar, subjected to WD treatment.

Physiological data showed that imposed treatment was sufficient to induce primarily responses to cope with WD such as a decrease in gas exchanges. It is known that stomatal conductance reduction, which aims to reduce water loss by transpiration, is one of the first physiological responses in plants to reduce WD damages. Lower stomatal conductance
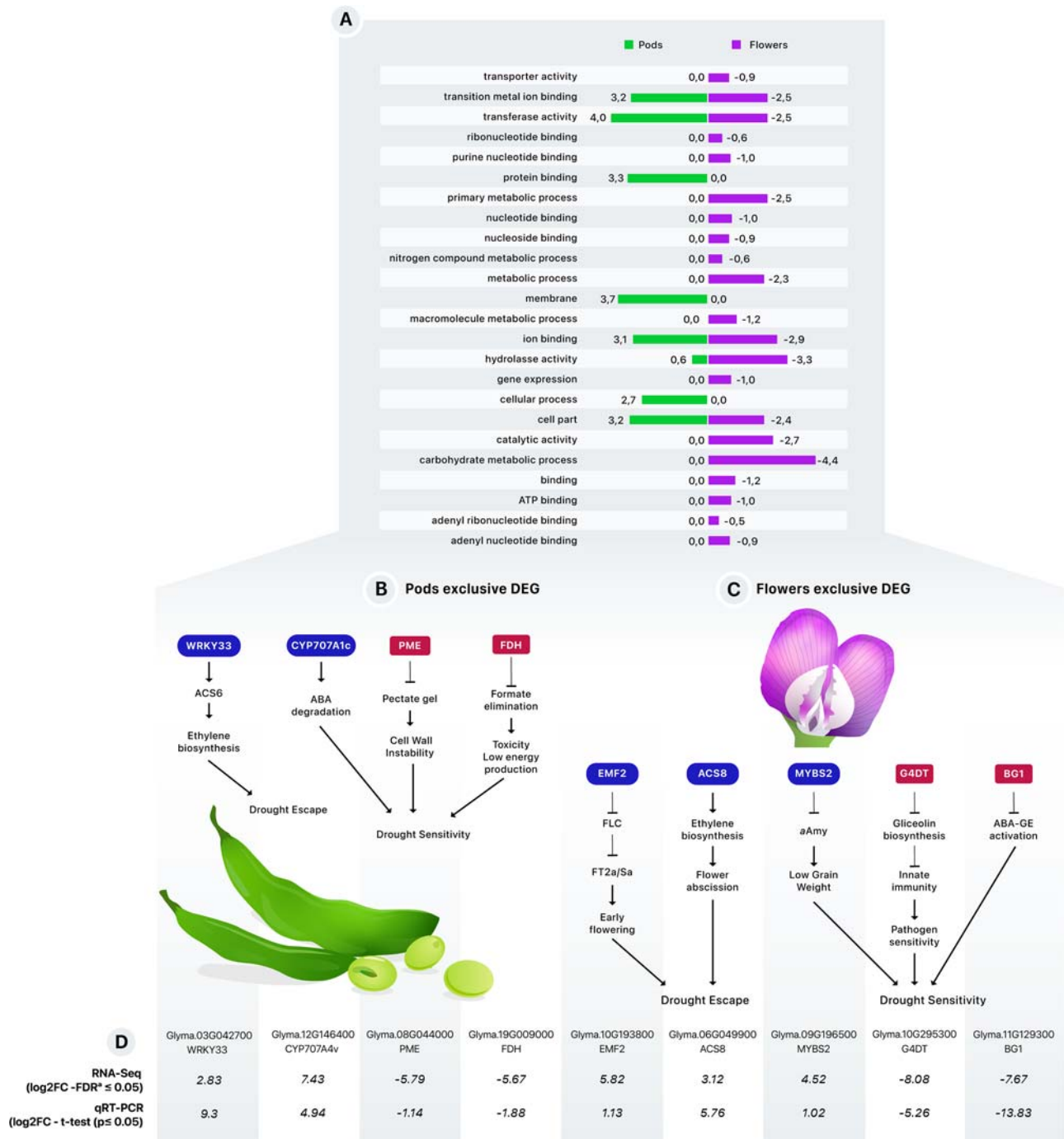
Figure 3. (A). Genetic ontology analysis of DEG identified in flower (R2 developmental stage) and pods (R4 developmental stage) of drought-sensitive soybean cultivar BR 16 subjected to water-deficit conditions. Data include both expression profiles, up and down-regulated genes. Green bars represent genes associated with GO terms identified in pods and purple ones in flowers. Positive and negative numbers associated with the bars, stand for up and down-regulated expression profiles of genes associated with the respective GO term category. (B). Drought-responsive pathways affected by WD condition in soybean cv. BR16. Legend: Blue circles stand for up-regulation and red ones for down-regulation. (C). Comparison of expression analysis performed using RNA-Seq and qRT-PCR techniques for genes differentially expressed in flower and pod of drought-sensitive soybean cultivar BR 16 subjected to WD treatment compared to C condition.

values, however, result in lower CO_2 uptake, which contributes to the reduction of the photosynthetic assimilation rate (Flexas et al. 2004), which may also contribute to enhancing water use efficiency (WUE) in response to WD (Liu et al. 2005). These physiological responses were also reported by Paiva Rolla et al. (2014), which described that photosynthesis and stomatal conductance were reduced in soybean plants under WD conditions. Photosynthetic rate and stomatal conductance also decreased in soybean plants under moderate WD (45% ±2 field water capacity) (Zhang et al. 2016). Besides, corroborating with this study, transpiration rate reduction and an increased in foliar temperature was also

described for soybean subjected to 4 different WD treatments (irrigation with 100%, 50%, 25% and 10% of water) (Inamullah 2005). In field conditions, in a previous study carried out in a dry season, cultivar BR16 showed higher instantaneous WUE and intrinsic WUE (Fuganti-Pagliarini et al. 2017). As observed in our study, plants facing low to moderate WD will frequently enhance WUE (Brock and Galen 2005; Liu et al. 2005; Yin et al. 2005; Medrano et al. 2015), probably due to a protective mechanism against stress, that allows plants to save water, improve water efficient use and to convert available CO_2 into photoassimilates into pods and grain production (Chaves et al. 2009).

Drought occurrence during water-scarcity-sensitive developmental phases, such as flowering and pod filling can also seriously compromise growth and final yield parameters. Here, a decrease in the number of pods with seeds, dry mass of pods with seeds, number of seeds, dry mass of seeds, the total number of pods, and dry mass of 100 seeds was observed in plants under WD compared to C conditions (Table 1). There is a positive correlation between pods, nodes and yield, and nodes and pods and seeds (Egli 2010; Kahlon et al. 2011); thus, a decrease in these parameters will reflect in yield losses. This association is also related to environmental conditions, photosynthesis, crop growth rate, and maturity. Growth parameters, however, appear to have been less affected by WD as plant height and number of nodes did not present reduction under treatment (Table 1). According to the literature, soybean water demand increases during plant development, reaching the maximum necessity at the flowering-pod filling phase, around 7–8 mm of water/day. Besides the developmental stage, water consumption also depends on atmosphere evaporative demand, climatic conditions of the site, sowing season, and rainfall distribution during crop season (Embrapa 2007). In short, results obtained in the greenhouse condition show that drought treatment was applied correctly and reflected in agro-physiological responses (Table 1).

Expression profile and gene ontology analysis of DEG in flower and pod

This is the first study to report a large-scale gene expression profile in soybean flower and pod from a drought-sensitive cultivar. These reproductive organs showed a discrepant expression profile. In flowers, DEG and the average expression (log2FC) of GOs terms significantly expressed were down-regulated (Figure 3(A)), which may negatively affect many pathways. This data also suggested that in flowers, drought conditions tend to inhibit gene expression, being more sensitive to water lack. On the other hand, pods up-regulated genes and pathways indicating that the molecular machinery may have been activated to improve survival and pod formation, as a strategy to preserve and transmit genetic material to the next generation through grain formation, even if it means yield losses in the final production. According to Anjum et al. (2011), this response is disrupted by leaf gas exchanges, which not only impaired the size of the source and sink tissues, but also phloem loading, assimilate translocation and dry matter portioning, resulting in smaller and fewer grains, as described in the following paragraphs.

Among genes identified in our RNA-Seq experiment, *Late Embryogenesis Abundant (LEA)* proteins, *Small-HSPs-Heat Shock (HSPs)* Proteins, and *9-cis-epoxycarotenoid enzyme (NCED)* were up-regulated in flowers, and genes related to photosynthesis/stomata closure, sugar biosynthesis and transporter related genes were down-regulated in this tissue. Under a stressful environmental condition such as WD, *HSPs* act as molecular chaperones assisting protein folding, stabilizing membrane proteins, facilitating protein refolding, reestablishing normal protein conformation, and thus cellular homeostasis (Augustine 2016; Priya et al. 2019). Specifically, small *HSPs* play a distinctive function in the degradation of proteins, keeping membrane integrity under stress conditions (Nakamoto and Vigh 2007; Augustine

2016). Furthermore, *NCED* is an important key rate-limiting enzyme in the ABA biosynthesis pathway. Under WD conditions, an increase in *NCED* transcript levels leads to ABA biosynthesis and accumulation in plants, improving drought tolerance by triggering of WD-defense mechanism via hormone induction (Huang et al. 2018). *NCED* gene has been identified in many plant species and lines overexpressing it have shown improved drought tolerance in Arabidopsis (Tong et al. 2017), cowpea (Iuchi et al. 2001), tomato (Thompson et al. 2000), tobacco (Qin and Zeevaart 2002; Pedrosa et al. 2017), peanut (Wan and Li 2006), rice (Sultana et al. 2014), petunia (Estrada-Melo et al. 2015), and cotton (Souza et al. 2016). Specifically, in soybean plants GM to overexpress *NCED* gene, higher levels of ABA were identified in the WD-treated group (Molinari 2020). In addition, Lima, and co-workers (2019) reported in the conventional cultivar BR16, higher levels of ABA and *ACC* (ethylene precursor) in plants subjected to WD, with increased concentration following the severity of the treatment, corroborating the involvement of these genes under WD.

In flowers, the downregulation of photosynthesis/stomata closure-related genes reflected directly in gas exchange parameters decreases as WD can damage the basic organizational structure of photosynthetic apparatus, inhibiting carbon assimilation, and decreasing photosynthesis rate, usually by stomatal limitation (Wang et al. 2018a). In contrast, the up-regulation of these genes in pods suggested that in this developmental stage all resources were directed to pod filling. In this stage, leaf senescence also allows maximum recovery and remobilization of nutrients to pods formation, increasing in pod CO_2 metabolism, and reduce in energy and ATP use (Bennett et al. 2011). Yet, in soybean, the number of pods can also affect leaf photosynthesis. For instance, in WD condition, the removal of pods reduces CO_2 rates exchange within the plants, probably as a result of stomatal closure, a consequence of increased photoassimilate accumulation within the leaves brought by the availability of fewer sinks to export resources, indicating a dynamic system, in which pods signal their resource necessities to the leaves, initiating remobilization of photoassimilates relative to received signal strength (Bennett et al. 2011).

Aquaporin genes were up-regulated in pods. Under WD conditions, these proteins are responsible to maintain water movement across the plant body, stabilizing homeostasis of the cell membrane (Iwuala et al. 2019). Furthermore, under drought, the alteration in the levels of these proteins could enhance tolerance, as roots water uptake can be enhanced or reduced by the overexpression or loss of one or more PIP genes (Lu et al. 2018). Dehydrins were also up-regulated in pods under WD. These proteins are highly hydrophilic, thermostable and ABA-responsive, being induced under abiotic stress, and participating in membranes, enzymes, and nucleotides stabilization in cells (Hanin et al. 2011; Yu et al. 2018). The up-regulation of osmotic adjustment-related genes suggests that in pods, more than one mechanism was activated to cope with drought effects. Among these osmoprotectants, soluble sugars, which showed up-regulated profile in pods, under WD, regulate cell osmotic status, protecting membrane and contributing to the scavenging of free radical in plant cells (Zivcak et al. 2016). The molecular results, as physiological ones, suggested that pods were less impaired by WD treatment than flowers.

Many genes are involved in the WD stress responses in flowering and pods stages

The qRT-PCR analyses of 9 genes differentially expressed (5 from flowers and 4 from pods) were carried out to validate RNA-Seq expression results. Figure 3(B, C) suggests a schematic chart that compiled all validated genes and the biological processes in response to drought in which these genes are involved.

Results suggested that part of BR16 cultivar sensitivity to drought may be due to the downregulation of important drought-responsive genes. In flowers, Glyma.10G295300 was expressed about 12x less under WD when compared to C conditions (average value between qRT-PCR and RNA-Seq log2FC expression). This gene encodes to G4DT enzyme (*glycinol 4-dimethylallyl transferase*, E.C.2.5.1.36) required to synthesize glyceollin 1 (Akashi et al. 2009; Lygin et al. 2010; Ahuja et al. 2012). In soybean, an important glyceollin is phytoalexin, a specific antimicrobial derivate from the flavonoid pathway, which disrupts or inhibits a wide range of pathogens colonization (Lygin et al. 2010; Sukumaran 2016; Yoneyama et al. 2016). The down-regulation of the phytoalexin pathway under WD may indicate a decrease in the innate immune system during stress (Zernova et al. 2014), suggesting crosstalk signaling between biotic and abiotic responses in plants. Lygin et al. (2010) stated that drought-tolerant plants showed a decrease in pathogen incidences, displaying a positive correlation between glyceollin levels and pathogen resistance. In *Arabidopsis thaliana*, plants infected with nematode showed more susceptibility to drought (Atkinson et al. 2013). Additionally, increased susceptibility to turnip mosaic virus (TuMV) by innate basal defenses, under combined heat and drought conditions was also reported (Prasch and Sonnewald 2013). In this way, BR16 susceptibility to WD may be, among other factors, due to a decrease in soybean innate immunity efficiency, since healthy plants have more probability to survive to hostile environmental conditions such as drought and pathogens infections (Prasch and Sonnewald 2013).

BG1 enzyme (Glyma.11G129300 – *beta-glucosidase*, E.C.3.2.1.21) was also repressed in flowers of BR16, being expressed about 22x less under WD compared to C conditions (average value between qRT-PCR and RNA-Seq log2FC expression). This enzyme hydrolyzes inactive abscisic acid which is conjugated with glucose (ABA-GE) to produce active ABA, in a reversible reaction (Lee et al. 2006; Seiler et al. 2011). Similar to other enzymes involved in ABA biosynthesis, functional deficiency of ABA-glucosidases also alters intracellular ABA level, affecting plant growth, development, and responses to adverse environmental conditions. Interestingly, WD dramatically decreases the enzymatic activity of *AtBG1* by inducing its polymerization, implying that high molecular weight form of *AtBG1* may play an important role in rapid ABA production under stress conditions (Lee et al. 2006). In *A. thaliana,* the loss function of *BG1* caused hypersensitivity to dehydration and defect in stomatal closure during WD (Lee et al. 2006; Xu et al. 2012). On the other hand, the overexpression of *BG1* resulted in higher ABA levels and drought tolerance (Lee et al. 2006; Wang et al. 2011; Han et al. 2012; Saradadevi et al. 2017). This data suggested that at this point, soybean plants may have balanced the down-production of ABA, by overexpressing the *NCED* gene in an attempt to keep ABA levels

enough to cope with WD effects. On the other hand, the downregulation of *BG1* gene in BR16 corroborates with its drought sensitivity and may be also related to early flowering identified for this cultivar, as an escape response strategy to drought, likewise observed in a previous study (Crusiol et al. 2017). Additionally, the downregulation of *BG1* probably led to the down-regulation of *SVP* flowering repressor as well, since they are strictly related genes, and consequently, flowering was induced (Wang et al. 2018b; Negin et al. 2019).

Considering genes down-regulated in pods, Glyma.08G044000, which encodes *PME* enzyme (*pectinesterase*, E.C.3.1.1.11) was expressed almost 7x less under WD when compared to C conditions (average value between qRT-PCR and RNA-Seq log2FC expression). This enzyme is involved in cell wall pectin remodeling (Kashyap et al. 2001), in the control of cell wall porosity and cell wall adhesion (Willats et al. 2001; Jarvis et al. 2003; Wu et al. 2010; Le Gall et al. 2015). Under abiotic stress, pectinesterases contribute to the stiffening of the cell wall by producing blocks of unesterified carboxyl groups that can interact with calcium ions forming a pectate gel, protecting and limiting the damage to cells (Bosch and Hepler 2005; Leucci et al. 2008; Moore et al. 2008; Wolf et al. 2012; Leng et al. 2017; Wu et al. 2018). Wheat, soybean, and tomato showed higher levels of pectin remodeling enzymes in drought-tolerant cultivars (Leucci et al. 2008; An et al. 2014; Iovieno et al. 2016; Landi et al. 2017). In an *Arabidopsis thaliana* mutant to pectinesterase gene, drought tolerance was impaired (Deslattes et al. 2018). Besides, a comparison of cell walls between two wheat cultivars differing in drought tolerance showed that the biosynthesis of pectic polymers under WD was less affected in the tolerant cultivar (Piro et al. 2003; Leucci et al. 2008). Similar results were found in wheat, with a drought-tolerant line displaying more pectin enzymes than the WD-sensitive one (Konno et al. 2008). This result may suggest that, among other factors, the low transcript levels of *PME* in BR16 could be contributing to its sensitivity to drought due to deficiencies in cell wall maintenance in pods.

Another repressed gene in pods, Glyma.19G009000 was expressed about 8x less under WD than C condition (average value between qRT-PCR and RNA-Seq log2FC expression). This gene encodes to FDH enzyme (*formate dehydrogenase,* EC.1.17.1.9), a mitochondrial and NAD-dependent enzyme that catalyzes the oxidation of formate to carbon dioxide in plants while reducing NAD+ to NADH, serving to alleviate any detrimental effects accumulated formate has on the cell (Shiraishi et al. 2000; Alekseeva et al. 2011). Data show that, usually, the expression of FDH is drastically increased when plant tissue is subjected to stressful conditions, such as drought, and pathogen infection (des Francs-Small et al. 1993; Hourton-Cabassa et al. 1998; Suzuki et al. 1998; Li et al. 2001; Li et al. 2002; Choi et al. 2014). Besides that, FDH also acts in formate detoxification (Li et al. 2002), a toxic compound, that can inhibit cellular respiration and root growth, as observed in Arabidopsis (Li et al. 2002; David et al. 2010). Additionally, formate can also restrain water oxidation reaction on the donor side, as well as, in electron transfer on the acceptor side of photosystem II (Feyziev et al. 2000), decreasing photosynthesis, photoassimilates production and resulting in smaller pods and seeds dry mass under WD, in agreement to what was observed for BR16 (Figures 2 and 3(D)).

Glyma.06G049900 that encodes to ACS8 enzyme (*1-Aminocyclopropane-1-Carboxylate synthase 8* – E.C.4.4.1.14) was 6x more expressed in flower under WD than in C conditions (average value between qRT-PCR and RNA-Seq log2FC expression). This enzyme acts in ethylene biosynthesis, a hormone that enables senescence and abscission in flower and pod (Iqbal et al. 2017). Its biosynthesis is affected by several environmental factors including drought (Yoon and Kieber 2013; Song et al. 2016). Studies indicate that ACS synthase is transported from roots (via xylem) to aerial part in response to drought, increasing local ethylene biosynthesis (Tudela and Primo-Millo 1992). In *Arabidopsis*, transcriptional data showed that *ACS8* (AT4G37770.1) is up-regulated in flowers under WD (Zhang et al. 2018), and appears to be circadian clock and photoperiod regulated (Thain et al. 2004). *ACS8* up-regulation in BR16 cultivar may be inducing ethylene biosynthesis and as a consequence causing flower and pods abortion (Figure 3(D)).

Glyma.10G193800 that encodes to transcription factor EMF2 (Embryonic Flower 2) was also upregulated in flower being 7x more expressed under WD when compared to C conditions (average value between qRT-PCR and RNA-Seq log2FC expression). *Arabidopsis thaliana AtEMF2* protein (AT5G51230.1) gene, a Polycomb SUZ2 protein (PcG) (Kim et al. 2010) forms complexes that maintain genes silenced throughout histone modifications (Chen et al. 2009; Costa and Dean 2019). Additionally, *EMF2* interacts with genes related to ABA, such as *Abscisic Acid Insensitive 3* (*ABL3*), *Long Vegetative Phase 1* (*LOV1*), and Flowering Locus C (*FLC*), that control flowering and seed development. *EMF* repress *FLC* (flowering repressor) allowing flower activators, such as *Flowering Locus T* (*FT*) and Suppressor of overexpression of *constans 1* (*SOC1*) to induce flowering (Yoshida et al. 2001; Chanvivattana et al. 2004; Kim et al. 2010). Therefore, the up-regulation of *EMF2* in BR16 cultivar may have induced early flowering, trough *FLC* repression and *FT* and *SOC1* induction. As already discussed, early flowering is a drought escape mechanism adopted by BR16 cultivar (Crusiol et al. 2017) to prioritize grain filling and avoid compromise final productivity.

In flowers, Glyma.09G196500 showed an expression of 6x higher in WD when compared to C conditions (average value between qRT-PCR and RNA-Seq log2FC expression). This gene encodes to a putative *MYBS2* transcription factor, ortholog to MYBS2 (AT5G08520) gene in *Arabidopsis thaliana*, which is responsible for sugar levels maintenance (Chen et al. 2017). In rice, *MYBS2* gene represses amylase (αAmy) production, an enzyme that hydrolyzes starch into sugars, decreasing as a consequence, grain weight in greenhouse and field conditions (Chen et al. 2019). The induction of *MYBS2* in BR16 cultivar may have contributed to low drymass and a low number of seeds under WD compared to C conditions (Figure 3(D)), by repressing αAmy activity and as a result, there was a reduction of available sugar to be mobilized into grains. In addition, Seo et al. (2012) and Gao et al. (2014) reported the involvement of *MYB* genes in drought response in plants, corroborating *MYB* roles in WD-defense mechanisms.

Glyma.12G146400, which encodes a *cytochrome enzyme* (*CYP707A4*), also known as *ABA 8'-hydroxylase enzyme* (EC.1.14.14.137) was expressed about 12x more in pods under WD when compared to C conditions (average value between qRT-PCR and RNA-Seq log2FC expression). In *A. thaliana*, the loss of *CYP707A4* function rescued drought hypersensitivity phenotype by increasing ABA levels (Umezawa et al. 2006). It is important to emphasize here that in both reproductive tissues, ABA metabolism was affected by WD in different ways. Differently from flowers where a balance between *NCED* overexpression to keep ABA levels and a decrease in ABA reactivation by *BG1* gene repression was identified; in pods, ABA catabolism was increased by up-regulation of *CYP707A4*. These mechanisms may have led to lower levels of ABA and as a consequence, drought sensitivity.

The last up-regulated gene validated in pods was Glyma.03G042700 which encodes the *WRKY33* transcription factor and was 12x more expressed on average under WD compared to C conditions (average value between qRT-PCR and RNA-Seq log2FC expression). *WRKY* TFs play important roles in drought response and have been identified in several plants (Fan et al. 2015; He et al. 2016; Xu et al. 2016; Wei et al. 2017). In Arabidopsis, some authors have reported the involvement of WRKY factors in response to drought and osmotic treatment (Chen et al. 2009; Babitha et al. 2012; Zhao et al. 2020). In soybean, the characterization of these transcription factors showed high expression levels under WD and salinity conditions, emphasizing its participation in drought tolerance (Shi et al. 2018). Moreover, predictions from the STRING website, showed that *WRKYs* are involved in four signaling pathways: the jasmonic acid (JA), the salicylic acid (SA), the mitogen-activated protein kinase (MAPK), and the ethylene signaling pathways. According to these authors, *WRKY33* binds directly to the *ACS* gene to promotes ethylene synthesis (Fei et al. 2019). Both reproductive tissues of BR16 showed again a similar hormone response under drought conditions. Ethylene biosynthesis was increased in flower and pods by up-regulation of *WRKY33* and *ACS8* genes, respectively, which contributes to senescence and abortion of these tissues (Figure 3(D)). This increase in ethylene was also reported in BR16 conventional cultivar under drought by Lima and coworkers (2019). It is also important to highlight that early flowering (escape mechanism) is followed by senescence and abortion of less developed flowers, allowing more mature flowers to finish their development. According to the literature, this is a metabolic strategy to deliver water and nutrients for the development of fewer flowers/seeds rather than allocate nutrients to all young organs, under extreme environmental conditions (Richie et al. 1997; Neumaier et al. 2000; Su et al. 2013).

Data obtained here presented a broad expression profile of DEGs in flower and pod of drought-sensitive cultivar BR16, subjected to drought, showing some physiological and molecular mechanisms triggered in response to WD. These results will help researchers to understand how these tissues cope with water scarcity and can provide candidate genes for future projects aiming to develop soybean plants more tolerant to WD.

Disclosure statement

Funding

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