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Genome-wide identification of core components of ABA signaling and transcriptome analysis reveals gene circuits involved in castor bean (*Ricinus communis* L.) response to drought

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ABSTRACT

Castor bean (Ricinus communis L.) can withstand long periods of water deficit and high temperatures, and therefore has been recognized as a drought-resistant plant species, allowing the study of gene networks involved in drought response and tolerance. The identification of genes networks related to drought response in this plant may yield important information in the characterization of molecular mechanisms correlating changes in the gene expression with the physiological adaptation processes. In this context, gene families related to abscisic acid (ABA) signaling play a crucial role in developmental and environmental adaptation processes of plants to drought stress. However, the families that function as the core components of ABA signaling, as well as genes networks related to drought response, are not well understood in castor bean. In this study 7 RcPYL, 63 RcPP2C, and 6 RcSnRK2 genes were identified in castor bean genome, which was further supported by chromosomal distribution, gene structure, evolutionary relationships, and conserved motif analyses. The castor bean general expression profile was investigated by RNAseq in root and leaf tissues in response to drought stress. These analyses allowed the identification of genes differentially expressed, including genes from the ABA signaling core, genes related to photosynthesis, cell wall, energy transduction, antioxidant response, and transcription factors. These analyses provide new insights into the core components of ABA signaling in castor bean, allow the identification of several molecular responses associated with the high physiological adaptation of castor bean to drought stress, and contribute to the identification of candidate genes for genetic improvement.

1. Introduction

Abiotic stresses, such as drought, salt, and temperature, are the major environmental factors that affect the plant's geographical distribution, limiting agricultural productivity and threatening food security (Zhu, 2016). Because plants are sessile organisms, they evolved a variety of signaling mechanisms to adapt to adverse environments. Abscisic acid (ABA) is the major phytohormone involved in drought/salt stress in

plants, playing a central role in stomatal closure, regulating water loss under stress conditions. The core components of ABA signaling have been described, involving ABA receptors, metal-dependent protein phosphatases and sucrose nonfermenting 1-related protein kinases (Lim et al., 2022).

The ABA receptors pyrabactin resistance 1 (PYR1), PYR1-like (PYL), and regulatory components of the ABA receptor (RCAR) family bind ABA in response to stress and inducing ABA response (Ma et al., 2009;

Abbreviations: ABA, Abscisic acid; PP2C, protein phosphatase 2C; PYR, pyrabactin resistance 1; PYL, pyrabactin resistance 1 – like; SnRK2, ucrose nonfermenting one-related protein kinase subfamily 2; RCAR, regulatory components of the ABA receptor.

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Park et al., 2009; Santiago et al., 2009; Gonzalez-Guzman et al., 2012; Antoni et al., 2013). When ABA is present, it binds to PYL, and together they interact and inhibit group A protein phosphatase 2C (PP2C) activity, thereby activating the sucrose nonfermenting one-related protein kinase 2 (SnRK2). Subsequently, SnRK2s regulate multiple downstream transcription factors and other proteins to trigger ABA responses, such as ABF/AREB/ABI5, SLAC1, and other ABA-regulated genes (Fujii et al., 2009; Geiger et al., 2009; Lee et al., 2009). The function of PYL-PP2C-SnRK2 genes in developmental processes and in response to abiotic stress has been characterized in plants, mainly in arabidopsis and rice (Allen et al., 1999; Tahtiharju and Palva, 2001; Ohta et al., 2003; Umezawa et al., 2004; Saez et al., 2004; Kuhn et al., 2006; Diédhiou et al., 2008; Ma et al., 2009; Santiago et al., 2009; Park et al., 2009; Rubio et al., 2009; Saavedra et al., 2010; Umezawa et al., 2010; Sun et al., 2011; Kim et al., 2012; Singh et al., 2015a; Tian et al., 2015; Dev et al., 2016).

Castor bean (Ricinus communis L.) belongs to the Euphorbiaceae family. Its high drought tolerance is an important trait (Weiss, 2000). This plant presents a high amount of oil in the seeds, in a proportion of 50% of the total biomass (Shrirame et al., 2011). Castor oil is predominantly composed of ricinoleic acid, a naturally hydroxylated omega 9 fatty acid, has many applications in different industrial sectors, and it is mainly used in cosmetics, pharmaceuticals, lubricants, and biodiesel production (Baldwin and Cossar, 2009). Castor bean is an oilseed of great importance in Brazil, considered the third largest producer in the world, with 35,800 ton in 2020 (Tridge, 2022). Castor bean is easy to grow and resistant to water shortages and can be grown in the extensive semi-arid region of the Brazilian Northeast, where it is one of the few productive alternatives for about millions of people through family farming. With the limitation in the supply of oil, or the fluctuation of its prices, and the growing concern with the issue of global warming, great attention has been given to the redefinition of the model of the world energy matrix, and the use of biodiesel derived from castor bean has been shown to be an option in this scenario. Furthermore, due to the high adaptation of castor bean to limiting water conditions, the characterization of genes involved with the processes of perception and tolerance to water stress can allow the development of new strategies for management, selection, and manipulation of these characteristics not only in this one, but also in other cultures.

As expected, castor bean seed production and quality are adversely affected by abiotic stresses, such as responses to drought and high salinity, which are regulated mainly by ABA signaling. Despite the importance of the ABA signal transduction pathway during the dehydration stress response, castor bean *PYL*, *PP2C*, and *SnRK2* gene families have not been analyzed, and their functions remain unknown. Besides that, the molecular basis castor bean high adaptation to limiting water conditions remains still unknown.

In model plants, such as arabidopsis and rice, genes associated with stress adaptation have been analyzed during drought responses (Shinozaki et al., 2003; Nakashima et al., 2009). However, different plant species have developed distinct mechanisms in response to drought. In general, these mechanisms are complex, associated with different metabolic pathways, and display several layers of response from perception to transduction of signal, which in the end will result in the physiological response. Indeed, the high tolerance to drought of castor bean is an important trait. In this context, the combination of the genome-wide identification of gene families related to the central core of ABA signaling and transcriptomic analysis of the global gene expression response of castor bean plants exposed to drought stress is an important task to allow the identification of molecular responses associated with physiological adaptation to drought stress.

To better understand the castor bean response to drought stress and to evaluate the roles of the *RcPYL*, *RcPP2C*, and *RcSnRK2* genes in this response, a combination of genome-wide analysis of these three gene families and transcriptomic analysis by RNAseq was used. Our analysis identified 7 *RcPYL*, 63 *RcPP2C*, and 6 *RcSnRK2* genes in the castor bean

genome. Comprehensive analyses of the primary structures, chromosomal distribution, phylogeny, and duplication events of the *RcPYL*, *RcPP2C*, and *RcSnRK2* genes were carried out. The general expression profile of castor bean was investigated by RNAseq in root and leaf tissues in response to drought stress. These analyses allowed the identification of differentially expressed genes, including genes involved in central core ABA signaling, genes related to photosynthesis, the cell wall, energy transduction, antioxidant responses, and transcription factors. These analyses contribute to the understanding of the core components of ABA signaling in castor bean and allow the identification of several molecular responses associated with the high physiological adaptation of castor bean to drought stress.

2. Material and methods

2.1. Sequence retrieval and identification of the RcPYL, RcPP2C and RcSnRK2 genes

The PYL, PP2C, and SnRK2 genes from the castor bean genome were identified by BLASTP tool (Altschul et al., 1997) searches using databases from Phytozome (https://www.phytozome.net) using protein sequences previously identified in (Kerk et al., 2002; Schweighofer et al., 2004; Xue et al., 2008) and cassava (Zhao et al., 2019) as baits (Table S1). The putative castor bean PP2C proteins were analyzed by Pfam (https://pfam.xfam.org/) through HMMER software (https://hmmer.org/). Proteins without the PP2C catalytic domain (PF00481) were removed from the analysis. The molecular weight (MW) and isoelectric point of the castor bean PYL, PP2Cs, and SnRK2 proteins were predicted with the online tool ExPaSy (https://web.expasy.org/protparam/), and their protein subcellular localization was predicted with WoLF PSORT (https://wolfpsort.hgc.jp/).

2.2. Phylogenetic and exon/intron analyses

Multiple sequence alignments of PYL, PP2C, and SnRK2 proteins from castor bean, cassava, and arabidopsis were performed with the MUSCLE tool (Edgar, 2004). Phylogenetic analyses were performed using the maximum likelihood method under the best model selection in MEGA 7.1 software (Tamura et al., 2013) with 1000 replicates of bootstrap statistics. The exon/intron structures were examined using the online Gene Structure Display Server (GSDS: https://gsds.cbi.pku.edu.ch) based on their corresponding genomic sequences (Hu et al., 2015).

2.3. Protein domain analysis

To analyze protein domains present in the RcPP2C proteins used the MEME program (meme-suite.org/tools/meme) to determine conserved protein motifs. The maximum number of motifs was set to 10, and the optimum width of motifs was set from 15 to 50.

2.4. Chromosomal gene positions, synteny and collinearity analysis

The location of the genes on the *Ricinus communis* and *Manihot esculenta* chromosomes was shown by Circos (Krzywinski, et al. 2009). Detection of putative gene duplication events was done with MCScanX (E-value 1×10 -10) in each genome and with a comparison between both genomes and visualized using Advanced Circos of TBtools software v1.098769 (Chen et al., 2020). Tandem duplication events were defined as two or more homologous genes located on a chromosomal region within 200 kb (Wang et al., 2012). Collinearity between *R. communis* and *M. esculenta* genomes was done with MCScanX (E-value 1×10 -10) and visualized by TBtools v1.098769 (Chen et al., 2020)

2.5. Calculation of Ka/Ks and divergence time

The nucleotide and amino acid sequences of duplicated gene pairs

were aligned and the number of non-synonymous substitutions per non-synonymous site (Ka), synonymous substitutions per synonymous site (Ks), and Ka/Ks ratio were estimated using KaKs_Calculator 2.0 software (Wang et al., 2010). The divergence time was calculated according to T = Ks/(2 \times 8.1 \times 10⁻⁹) MYA for vascular plants (Lynch and Conery et al., 2000).

2.6. Promoter analysis

For the identification of *cis*-acting elements, the upstream genomic sequence (1000 bp upstream from the translation start codon) of each gene was retrieved from the Dicots Plaza4.5 database, and the presence of *cis*-regulatory elements was identified by the Plant Promoter Analysis Navigator from the PlantPAN 3.0 database (https://plantpan.itps.ncku.edu.tw/promoter.php) (Chow et al., 2019).

2.7. miRNA target prediction

To search whether the *RcPYL*, *RcPP2C*, and *RcSnRK2* genes could be regulated by miRNAs, all 63 *R. communis* mature miRNA sequences deposited in the release 22.1 of the miRBase database (Griffiths-Jones, 2004) were used as input in the psRNAtarget prediction tool (Dai et al., 2018). Default parameters were used to compute predictions.

2.8. Analysis of RcPYL, RcPP2C and RcSnRK2 expression in different organs

Expression analysis in leaves, roots, male flowers and seeds at different developmental stages was conducted using previously published transcriptome data (Brown et al., 2012, ENA sequence read archive (SRA) under accession ERA047687). The gene expression data were visualized in the form of heat maps using a log2-fold change scale with relative values to the gene expression average.

2.9. Plants, growth conditions and drought stress experimental design

The castor bean plants (IAC Guarani cultivar) were sown in 15 L plastic pots with sandy loam soil. Plants were grown under continuous irrigation and a natural photoperiod until the complete expansion of the third pair of leaves (approximately 2 months) when the drought treatment was started. A suspension of irrigation protocol was employed. The plants were divided into two groups: a control group in which irrigation was continued and a group in which irrigation was suspended until a water potential of -1.0 MPa was reached. Water potential was measured daily at predawn with a Scholander-type pressure chamber. The conditions used are considered of a "moderate stress" according to Hsiao (1973). Six plants were used for each condition in the experiment. The control and water-deficit-treated plants were collected at the same time. The tissues were immediately frozen in liquid nitrogen and stored at -80 °C until processing.

2.10. RNA isolation, library construction, and sequencing

Eight cDNA libraries were sequenced, four prepared from leaves and four from roots, including two biological replicates for each condition. A mixture of three different plants was used for each library. Total RNA was isolated from 100 mg of each sample. Frozen samples were ground in liquid nitrogen. The RNeasy Plant Mini Kit (Qiagen) was used, and $>\!10~\mu g$ RNA was used for each sample. Concentration and RNA purity were determined with a NanoDrop 1000 spectrophotometer (Thermo Scientific). RNA integrity was tested on a 1% formaldehyde agarose gel. The RNA samples were pretreated with RNase-free DNase I (Promega). Subsequent RNA processing and sequencing were performed by Fasteris SA (Plan-les-Ouates, Switzerland).

The mRNA was separated with poly-dT beads, fragmented and used to prepare cDNA with random primers to start first-strand synthesis.

cDNA ends were repaired, and 3'A was added before ligation of adapters with indexes. A strand-specific protocol was followed, leading to a library of the type first strand, with an average insert size of 190 bp. After pre-amplification by PCR, the process of cluster generation and sequencing followed. A HiSeq 2500 Illumina instrument was used for sequencing in High-Output mode, and the TruSeq SBS v3 kit (Illumina) and one lane were used to obtain paired-end 100-base reads. Demultiplexing was performed to separate the various sequence libraries.

2.11. Sequence quality, genome, read mapping and post-alignment quality control

The quality of the reads was analyzed using the R functions seeFastq and seeFastqPlot (Backman and Girke, 2016). Sequences containing 20 base calls with a Phred score below 20 were discarded. Subsequently, adapter trimming was performed. A look at the processed data with FastQC v0.11.5 indicated a high quality and results compatible with further analysis. A total of 20 million reads are representative of the size of each library at this stage. The number of such reads (MAPQ>=30) in each sample was between 17.6 and 44.6 million. The reference genome sequence and corresponding annotation files of Ricinus communis were downloaded from Phytozome (v.12.1, Taxonomy ID:3988) cultivar Hale, Rcommunis 119 TIGR.0.1, (https://genome.jgi.doe.gov/pages/ dynamicOrganismDownload.jsf?organism=Phytozome). This genome is approximately 350.6 Mb in total size, arranged in 25,878 scaffolds and with 31,221 genes. The genome was indexed with bowtie2-build (bowtie2 v2.2.9 64bits), and tophat2 (v2.1.1 Linux x86-64 bits) was used for the mapping of the reads to the genome (Langmead et al., 2009; Kim et al., 2013). Principal Component Analysis (PCA) was performed using the "prcomp" function in R version 4.3.1 to evaluate the uniformity of the samples based on gene expression data obtained from RNA-Seq experiments (Supplementary Figure S1).

Position-indexed alignments were visualized with the use of IGV (Integrative Genome Viewer, v 3.3.75) (Robinson et al., 2011) and the Samtools indexed bam files of the accepted hits obtained in the mapping stage (samtools v. 0.1.19) (Li et al., 2009). Post-alignment quality and sequence statistics were accessed using samtools (view and flagstat) and SAMStat 1.5.1 (Lassmann et al., 2011). HTSeq 0.6.1 htseq-count was used to count the reads corresponding to each exon and accumulated for each gene in intersect-strict mode and with the option stranded = reverse and minaqual = 1 (Anders et al., 2015).

2.12. Transcriptomic analysis in response to drought

The relative expression of the *RcPYL*, *RcPP2C* and *RcSnRK2* genes was shown through heatmaps, and the functional protein association network was created using the STRING v11.0 database (Snel et al., 2000) and analyzed using MEDUSA (Hooper and Bork, 2005) and VIACOMPLEX software (Castro et al., 2009). The differential expression analysis of plants submitted to drought was performed with the use of DESeq2 (Love et al., 2014) as part of the R Bioconductor suite. (Java 1.8.0_91, R version 3.2.3, Rstudio 0.99.903, DESeq2 1.10.1). The DESeqDataSetFromHTSeqCount function was used to prepare the DESeqDataSet class object, and functions DESeq and results to make the differential expression analysis and the results table. Minimal prefiltering was performed to exclude rows of genes with 0 or 1 count overall. A table of library-size normalized counts was obtained from the DESeqDataSet object with the DESeq function (counts (object, normalized = TRUE)).

The tables of differentially expressed genes include rows, from the results table above, in which the adjusted p-value for multiple testing is lower than 0.01 and the absolute value of log2FoldChange is >1.8, which corresponds to a fold change of approximately 3.5. Samples in which the original counts in both conditions had two values below 50 were left out of the tables. The MAplot function from the DESeq2 package was used to plot appropriate data.

Another GO group analysis was performed using GSEA (Gene Set Enrichment Analysis) (Subramanian et al, 2005, Mootha et al, 2003). The analysis was performed using javaGSEA software v 2.2.3 downloaded from the BroadInstitute GSEA site (https://software.broadinstitute.org/gsea/downloads.jsp). In this analysis, all the genes were used, in contrast to the enrichment analysis above, in which only the differentially expressed genes were analyzed.

2.13. GO enrichment analysis and GSEA

An enrichment analysis using the differentially expressed genes was performed with the use of the Bioconductor clusterProfiler 2.4.3 package (requires the DOSE package) (Yu et al, 2012; Yu et al, 2015). The enricher function used makes a hypergeometric test of overrepresentation of different biological groups, such as the GO groups used here. The parameters for the enricher function used were a p value cutoff = 0.05, the padjust method = 'BH' (Benjamini and Hochberg, false discovery rate), a minimum group size of 5 and a q value cut-off = 0.2. A visualization tool from the DOSE package, enrichMap, was also used. Venn diagrams were performed using the vennCounts and vennDiagram functions of the limma package in R. A second GO enrichment analysis was performed with the agriGO web-based tool and database (Du et al., 2010), with the default parameters: Fisher statistic method, Yekutieli multitest adjustment method (FDR under dependence), significance level of 0.05, with a minimum number of mapping entries of five and complete GO selection as the gene ontology type, using the differentially expressed genes as input and the castor bean genome as reference.

Another GO group analysis was performed using GSEA (Gene Set Enrichment Analysis) (Subramanian et al, 2005, Mootha et al, 2003). The analysis was performed using javaGSEA software v 2.2.3 downloaded from the BroadInstitute GSEA site (https://software.broadinstitute.org/gsea/downloads.jsp). In this analysis, all the genes were used, in contrast to the enrichment analysis above, in which only the differentially expressed genes were analyzed. A. gmt file was prepared, which lists, for each GO group, the genes that are included in the group. The parameters modified from the default values of the program were: collapse: false, metric: log2 Ratio of Classes, permutation type: gene set, minimum set size: 10, maximum set size: 500, number of permutations: 1000, plot top 100. The GO groups accepted had a nominal p-value <= 0.05 and a false discovery rate q-value <= 0.1.

2.14. Data submission to SRA

All of the sequence reads have been deposited in SRA (Sequence Read Archive) (https://www.ncbi.nlm.nih.gov/sra). The submission SUB3009450 is linked to bioproject PRJNA401329 and biosamples SAMN07602879, SAMN07602890, SAMN07602875, and SAMN07602877.

2.15. Total RNA isolation and quantitative PCR (RT-qPCR) analysis

The analysis of expression using RT-qPCR were fulfilled with the same cDNA synthetized for the transcriptome analysis. After synthesis, cDNAs were diluted 100 times in sterile water. The reactions were repeated four times, and the expression data analyses were performed after comparative quantification of the amplified products using the $2-\Delta\Delta Ct$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). RT-qPCR reactions performed in 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Reaction mixtures contained $2.5\,\mu L$ diluted cDNA, $0.3\,\mu M$ of each primer, and SYBR® Selection Master Mix (Applied Biosystems) in a total volume of $10\,\mu L$. The sequences of each primer pair used in RT-qPCR experiments are indicated in Table S2. Reaction mixtures incubated for 2 min at 50 °C and then 5 min at 95 °C; followed by 40 amplification cycles consisting of 15 sec at 95 °C and 1 min at 60 °C. Analyses of melting curves were performed immediately after the completion of the RT-qPCR. The RT-qPCR

expression data were analyzed by T-test assuming different variances between control and stress samples. The expression data of the RNAseq and RT-qPCR were used for linear regression. The level of significance obtained by ANOVA test. The correlation data analysis was performed on Excel 2019 using extension data analysis.

3. Results

3.1. Genome-wide identification, chromosomal synteny, and duplication analysis of RcPYL, RcPP2C, and RcSnRK2 genes in castor bean

PYL, *PP2C*, and *SnRK2* constitute the core components of ABA signaling. To identify these genes in the castor bean genome, sequences previously identified in arabidopsis (Xue et al., 2008) and cassava (Zhao et al, 2019) were used as queries against the castor bean genome. While arabidopsis is the main eudicot model, cassava is the closest member of the Euphorbiaceae family, with a genome sequence available. A total of 7 *PYL*, 63 *PP2C*, and 6 *SnRK2* genes were identified. Based on their locus IDs, these genes were named *RcPYL1* to *RcPYL7*, *RcPP2C1* to *RcPP2C63*, and *RcSnRK2.1* to *RcSnRK2.6*. All the basic information and the predicted features of the PYL, PP2C and SnRK2 proteins are summarized in Table 1

To analyze the physical locations and duplication events of the RcPYL RcPP2C and RcSnRK2 genes, we located their positions on each chromosome based on the information obtained from the castor bean genome database (NCBI) (Fig. 1A). The linked genes are highlighted with redline. This analysis indicated that the RcPYL2/RcPYL6/RcPYL7 are involved in segmental duplication events. No tandem duplication events were found in the PYL family. Previous works in monocot and eudicot species have demonstrated that the PP2C family was expanded mainly through duplication events (Xue et al., 2008; Cao et al., 2016; Yang et al., 2018; Yu et al., 2019). Here, we found 11 pairs of paralogous RcPP2C genes (RcPP2C10/RcPP2C14, RcPP2C10/RcPP2C35, RcPP2C14/RcPP2C35, RcPP2C56/RcPP2C62, RcPP2C3/RcPP2C26, RcPP2C1/RcPP2C41, RcPP2C17/RcPP2C29, RcPP2C13/RcPP2C50, RcPP2C2/RcPP2C43, RcPP2C24/RcPP2C60, and RcPP2C28/RcPP2C52), indicated to be involved in segmental duplication events, while only one tandem duplication gene pair (RcPP2C33/RcPP2C34) was found. In RcSnRK2 family, two duplication gene pairs (RcSnRK2.2/RcSnRK2.4, RcSnRK2.3/RcSnRK2.6) was observed. These analyses indicate that the RcPYL, RcPP2C, and RcSnRK2 genes are unevenly distributed on different chromosomes and those duplication events also contributed to the expansion castor bean PP2C and SnRK2 families. Additionally, a collinearity analysis with the Arabidopsis thaliana and Manihot esculenta genome was made and the orthologous genes are show in Fig. 1B and Table 1.

To investigate the relationship between the genetic divergence and gene duplication events in paralogous pairs, their Ka/Ks ratios were determined. The values obtained to RcPP2C10/RcPP2C14, RcPP2C10/RcPP2C35, RcPP2C14/RcPP2C35, RcPP2C3/RcPP2C26, RcPP2C1/RcPP2C41, RcPP2C17/RcPP2C29, RcPP2C13/RcPP2C50, RcPP2C24/RcPP2C60, RcSnRK2.2/RcSnRK2.4, and RcSnRK2.3/RcSnRK2.6 pairs were smaller than 1, indicating that these duplication events might have undergone purifying or stabilizing selection (Table 2). Beside that, the Ka/Ks ratios to RcPP2C56/RcPP2C62, RcPP2C2/RcPP2C43, RcPP2C33/RcPP2C34, and RcPP2C28/RcPP2C52 are >1, implying a Darwinian selection, which may have led to advantageous new paralogues.

3.2. Phylogenetic analysis, gene structure and distribution of conserved motifs in the RcPYL, RcPP2C, and RcSnRK2 genes

Evaluation of the evolutionary relationships in the *PYL, PP2C*, and *SnRK2* families in castor bean was conducted through phylogenetic analysis based on full-length protein sequences from arabidopsis, cassava and castor bean (Fig. 2). Consistent with previous studies, the *PYL* family is divided into three groups (1–3), PP2C proteins are classified

Table 1

Physicochemical parameters and subcellular predictions from RcPYL, RcPP2C, and RcSnRK2 genes in castor bean. Gene name: proposed nomenclature; locus ID; size in amino acid, mass - molecular weight; pI - isoelectric point. The MW and pI were predicted by the online tool ExPaSy, the protein subcellular localization was predicted by WoLF PSORT, and the chromosome location was identified in the Phytozome database. (chlo, Chloroplast; pero, peroxisome; cyto, cytosol; nucl, nucleous; mito, mitochondria; cysk, cytoskeleton; vacu, vacuole).

Gene name	Locus ID	Size (aa)	Mass (kDa)	pΙ	Subcellular localization	Group	Orthologues in A. thaliana	Orthologues in M. esculenta	
RcPYL1	29729.m002290	209	22.80	5.298	chlo	3	AtPYL5/6	MePYL2/11/13	
RcPYL2	29742.m001442	186	20.93	6.134	pero	1	_	MePYL6/7/8	
RcPYL3	29794.m003335	189	21.20	5.488	cyto	2	AtPYL2	MePYL9/12	
RcPYL4	29820.m001002	207	22.64	7.345	chlo	3	_	MePYL4/11	
RcPYL5	29827.m002533	215	23.99	5.188	nucl	2	AtPYL1	_	
RcPYL6	30169.m006525	195	21.74	5.834	cyto	1	AtPYL8/10	MePYL5/6/8	
RcPYL7	30190.m010824	196	22.29	5.809	cyto	1	_	MePYL5/6/8/10	
RcPP2C1	28152.m000889	387	43.08	7.321	cyto	D	AtPP2C63	MePP2C44	
RcPP2C2	28211.m000131	282	30.97	5.371	cyto	F	_	MePP2C70/71/74	
RcPP2C3	28329.m000062	702	78.64	5.66	nucl	C	AtPP2C23/66	MePP2C7/8/78	
RcPP2C4	28823.m000015	390	42.76	5.046	chlo	I	AtPP2C57	MePP2C60	
RcPP2C5	28966.m000537	504	55.62	5.209	nucl	E	_	-	
RcPP2C6	29613.m000363	385	43.50	7.347	mito	D	AtPP2C68	MePP2C41	
RcPP2C7	29619.m000250	388	43.02	4.99	nucl	G	_	MePP2C47	
RcPP2C8	29646.m001072	416	44.36	6.155	chlo	K	_	MePP2C15	
RcPP2C9	29648.m001926	571	62.39	4.988	chlo	F	_	_	
RcPP2C10	29706.m001323	536	57.39	4.742	chlo	Α	AtPP2C7/16/77	MePP2C11/16/17/18	
RcPP2C11	29726.m003939	433	47.75	5.201	cyto	L	AtPP2C35	MePP2C32	
RcPP2C12	29726.m004033	495	53.31	4.819	nucl	C	AtPP2C40	MePP2C14	
RcPP2C13	29726.m004098	369	40.97	6.283	cyto	E	_	MePP2C57	
RcPP2C14	29739.m003582	550	59.48	4.647	chlo	A	-	MePP2C11/16/17/18	
RcPP2C15	29751.m001870	393	43.20	4.7	cyto	A	AtPP2C3/24/78	MePP2C24/25/26/31	
RcPP2C16	29757.m000727	525	59.25	5.671	nucl	В		MePP2C19	
RcPP2C17	29784.m000355	395	43.92	7.563	chlo	D	AtPP2C46/59	MePP2C34/37	
RcPP2C18	29792.m000609	718	80.19	6.49	nucl	В	AtPP2C61/72		
RcPP2C19	29794.m003349	345	37.55	5.422	cysk	E	_	MePP2C64	
RcPP2C20	29805.m001514	372	41.53	7.257	chlo	D	_	MePP2C39/58	
RcPP2C21	29814.m000731	374	40.74	6.45	chlo	В		MePP2C52	
RcPP2C22	29820.m001025	463	51.33	5.606	nucl	E	AtPP2C16	MePP2C36	
RcPP2C23	29827.m002557	749	83.08	5.325	nucl	C	-	-	
RcPP2C24	29830.m001460	384	41.62	5.499	nucl	G	AtPP2C47	MePP2C4/21/46	
RcPP2C25	29844.m003353	577	63.54	5.699	chlo	I	AtPP2C50	MePP2C9/10	
RcPP2C26	29846.m000188	697	77.78	5.349	nucl	C	AtPP2C4/23/36/66	MePP2C6/8/78	
RcPP2C27	29848.m004494	296	31.83	4.736	cyto	F	_	MePP2C66	
RcPP2C28	29848.m004528	361	39.68	4.92	nucl	I	-	-	
RcPP2C29	29869.m001174	397	44.10	7.565	chlo	D	AtPP2C64	MePP2C37	
RcPP2C30	29883.m001952	1058	118.12	5.309	vacu	J		MePP2C12	
RcPP2C31	29889.m003313	471	52.57	6.152	nucl	D	AtPP2C42	MePP2C50/51	
RcPP2C32	29889.m003359	375	41.30	4.622	nucl	G	_	MePP2C48	
RcPP2C33	29908.m006034	283	31.16	6.869	chlo	F	_	-	
RcPP2C34	29908.m006036	280	30.61	7.123	nucl	F	-	MePP2C68/69	
RcPP2C35	29912.m005309	537	58.89	5.079	plas	A	AtPP2C77	MePP2C11/16/17/18	
RcPP2C36	29912.m005442	399	43.30	4.819	chlo	A	_	-	
RcPP2C37	29912.m005470	1077	119.42	4.963	chlo	L	-	-	
RcPP2C38	29912.m005562	384	41.82	5.336	cyto	G	AtPP2C22	MePP2C49	
RcPP2C39	29929.m004572	499	54.86	5.534	chlo	E	_	MePP2C34	
RcPP2C40	29940.m000404	657	72.60	5.542	cyto	H	_	- M. DD0044	
RcPP2C41	29970.m001035	309	34.08	6.563	cyto	D	- 4.PP0C44	MePP2C44	
RcPP2C42	29981.m000594	282	31.87	8.868	cyto	F	AtPP2C44	MePP2C77	
RcPP2C43	29983.m003152	283	31.06	6.02	cyto	F	- 4.PP0/21 /50	MePP2C70/74	
RcPP2C44	29983.m003238	473	52.23	5.236	chlo	E	AtPP2C1/52	- MaDD2C2O	
RcPP2C45	30066.m000733	428	45.88	7.069	chlo	H	AtPP2C15	MePP2C30	
RcPP2C46 RcPP2C47	30075.m001168 30076.m004522	328 907	36.37 100.87	4.755 5.30	cysk nucl	F C	_	MePP2C79 MePP2C2/3	
RcPP2C47 RcPP2C48	30076.m004522	907	100.87 32.76	5.39 5.071		G	_	MePP2C2/3 MePP2C35/38	
RcPP2C48 RcPP2C49	30079.m000454	289 376	40.71	6.363	cyto chlo	В	- AtPP2C2/25	MePP2C45 MePP2C45	
RcPP2C49 RcPP2C50	30128.m008870	376 359				E E	AtPP2C34/41/73	MePP2C45 MePP2C57	
RcPP2C50 RcPP2C51			39.84	5.891	cyto	E H	ACF2634/41//3	WEFF2U3/	
RcPP2C51 RcPP2C52	30128.m008876 30131.m006877	436	47.13	6.737 5.084	chlo	H I	=	=	
RcPP2C52 RcPP2C53	30143.m001179	338 415	37.37 45.36	5.084 5.222	cyto nucl	A	_	– МеРР2С24/25/26/31	
RcPP2C53	30147.m014150	398	43.38	5.558	nucl	A	_		
RcPP2C55	30153.m000742	349	38.49	6.288		I	_	_	
RcPP2C56	30169.m006515	352	39.18	5.758	cyto mito	A	_	– MePP2C55	
RcPP2C56 RcPP2C57	30169.m006520	352 262	29.30	5.758	chlo	F	_	MePP2C55 MePP2C61	
RcPP2C57	30170.m013899					r H		MCFF2C01	
RcPP2C58 RcPP2C59	30170.m013899 30170.m014008	425 512	46.17 55.13	5.185	vacu	H K		– MePP2C15	
RcPP2C59 RcPP2C60	30170.m014008 30170.m014230	512 387	55.13 42.22	6.479 5.134	nucl cysk	G G	- AtPP2C27	меРР2С15 МеРР2С21/46	
RcPP2C60 RcPP2C61	30189.m001658			7.938	•	F	AIPP2G2/ _	MCFF2021/70	
RcPP2C61 RcPP2C62		295 350	32.44		chlo		=	— MaDD2C55	
RcPP2C62 RcPP2C63	30190.m010829 37745.m000013	350 271	37.66 29.53	5.307 4.712	cyto	A K	_	MePP2C55	
RcPP2C63 RcSnRK2.1	28725.m000317	362	41.06	4.712	cyto	1	_	– MeSnRK2.6	
AUSIIAAZ. I	20/23.IIIUUU31/	302	71.00	1.061	cyto	1	_		
								(continued on next need)	

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Table 1 (continued)

Gene name	Locus ID	Size (aa)	Mass (kDa)	pI	Subcellular localization	Group	Orthologues in A. thaliana	Orthologues in M. esculenta
RcSnRK2.2	29676.m001638	363	41.09	4.698	cyto	2	SnRK2.2/3	MeSnRK2.1/2
RcSnRK2.3	29772.m000313	336	38.06	5.544	cysk	3	_	MeSnRK8/9/10
RcSnRK2.4	29780.m001320	357	40.87	5.555	cysk	2	_	_
RcSnRK2.5	29822.m003504	338	38.29	5.488	cyto	3	_	_
RcSnRK2.6	29908.m006067	313	35.62	5.28	cyto	3	-	MeSnRK2.8/9/10

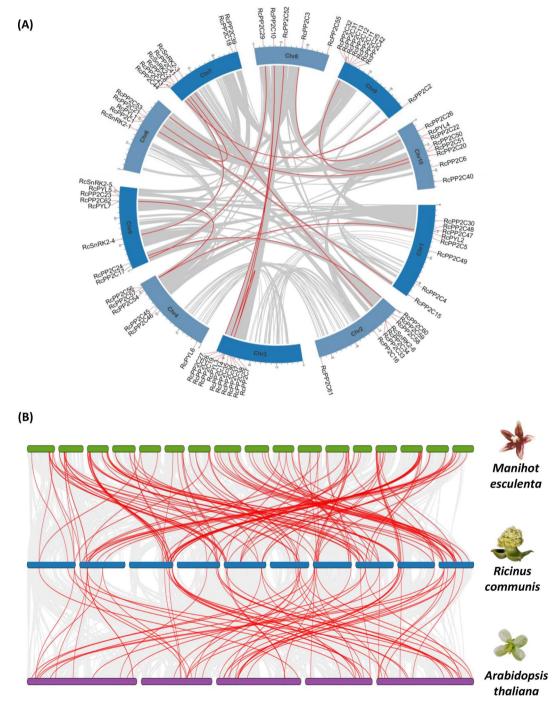


Fig. 1. Chromosomal distribution, synteny, and collinearity. Chromosomal positions and inter-chromosomal groups of duplicated *PP2C*, *PYL*, and *SnRK* gene pairs in *Ricinus* communis (A) and collinearity with Arabidopsis thaliana and Manihot esculenta (B). Gray lines in the background demonstrate all syntenic blocks and the red lines exhibit the segmental or tandem duplication network zones. The approximate location of PP2C, PYL, and SnRK genes is marked with a red line outside chromosome names. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Table 2

Ka/Ks analysis and divergence time between the duplicated RcPP2C and RcPYL gene pairs. Ka. Nonsynonymous substitution rate; Ks. Synonymous substitution rate; MYA. Million years ago.

Gene Family	Group	Gene 1	Gene 2	Туре	Ka	Ks	Ka/Ks	Date (MYA)
PP2C	Α	RcPP2C10	RcPP2C14	segmental	0,955	1,186	0,805	73,2
	A	RcPP2C10	RcPP2C35	segmental	0,818	1,630	0,502	100,6
	A	RcPP2C14	RcPP2C35	segmental	0,967	1,134	0,853	70,0
	A	RcPP2C56	RcPP2C62	segmental	1,081	0,736	1,469	45,4
	C	RcPP2C3	RcPP2C26	segmental	0,986	1,066	0,925	65,8
	D	RcPP2C1	RcPP2C41	segmental	0,958	1,169	0,819	72,2
	D	RcPP2C17	RcPP2C29	segmental	0,08	1,477	0,054	91,2
	E	RcPP2C13	RcPP2C50	segmental	0,189	1,514	0,125	93,5
	F	RcPP2C2	RcPP2C43	segmental	1,013	0,952	1,064	58,8
	F	RcPP2C33	RcPP2C34	tandem	1,017	0,939	1,084	57,9
	G	RcPP2C24	RcPP2C60	segmental	0,958	1,18	0,811	72,9
	I	RcPP2C28	RcPP2C52	segmental	1,023	0,916	1,116	56,6
SnRK2	2	RcSnRK2.2	RcSnRK2.4	segmental	0,976	1,101	0,886	68,0
	3	RcSnRK2.3	RcSnRK2.6	segmental	0,987	1,05	0,940	64,8

into 12 groups (A-L) and SnRK2 into three groups (1-3).

Three PYL proteins were observed in group 1, two in group 2, and two in group 3 (Fig. 2A). Nine RcPP2C proteins (RcPP2C10, RcPP2C14, RcPP2C15, RcPP2C35, RcPP2C36, RcPP2C36, RcPP2C53, RcPP2C54, RcPP2C56, RcPP2C62) are included in group A, the more characterized subfamily involved in the ABA signaling pathway (Tahtiharju and Palva, 2001; Fuchs et al., 2013; Singh et al., 2015b) (Fig. 2B). In addition, four PP2C proteins were found in group B, five in group C, seven in group D, seven in group E, 10 in group F, six in group G, four in group H, five in group I, one in group J, three in group K and two in group L (Fig. 2B). Only one SnRK2 protein was observed in group 1, two in group 2, and three in group 3. (Fig. 2C).

To analyze the structural features of the castor bean *RcPYL*, *RcPP2C*, and *RcSnRK2* genes, exon–intron organization was verified (Fig. 3A). In the *RcPYL* family, the number of introns was reduced, ranging from zero to two. Genes from group 2 (*RcPYL3* and *RcPYL5*) and from group 3 (*RcPYL1* and *RcPYL4*) are intronless. In the *RcPP2C* family, the gene length and intron number are highly divergent. The gene length ranged from 816 bp to 19572 bp, while the number of introns ranged from zero to 16. Nevertheless, the majority of RcPP2C genes have three or four introns. Among *RcPP2C* genes, only *RcPP2C63* is intronless. The exon–intron organization appears to be uniform in RcSnRK2. Except for *SnRK2.3*, which shows six introns, all other genes display eight introns each. As expected, for all families, all paralogous pairs identified here show a similar exon–intron organization.

To compare the motifs shared within PYL, RcPP2C, and SnRK2 proteins, the MEME motif search tool was employed (Fig. 3B). The motif composition is very similar to all PYL and SnRK2 proteins. The motif composition of PP2C appears to be similar for each group with the exception of groups I and K. Groups F and G show the same composition pattern, displaying motifs 4, 10, 8, 3, 6, 2, 1 and 7. Motif 9 was observed only in group D. This distinct motif composition of PP2Cs suggests specific functions for each group and reinforces the evolutionary relationships among the *RcPP2C* genes within the same group.

3.3. Cis-regulatory elements in the RcPYL, RcPP2C, and RcSnRK2 promoters and miRNA target prediction

The analysis of *cis*-regulatory elements in promoter regions is a useful strategy to better understand gene regulation. To identify putative *cis*-regulatory elements in the promoter regions from the *RcPYL*, *RcPP2C*, and *RcSnRK2* genes, 1,000 bp upstream from the translation start site from each gene was retrieved and analyzed. All *cis*-regulatory elements predicted are summarized in Fig. 4 and Table S3.

Several *cis*-acting elements related to hormone responsiveness were observed. As expected, the *cis*-acting elements related to ABA responsiveness (ABRE and MYC) seem to be present in higher numbers, reinforcing the idea that these genes are linked to the core regulatory

network of ABA signaling in castor bean. Environmental stress-responsive elements were also found, including DRE core, MBS, WUNmotif, WRE3, LTR, GC-motif, ARE, STRE, and TC-rich repeats. As expected, *cis*-acting elements related to drought and dehydration responses, such as MBS, ABRE, DRE, and MYC, are abundant.

Regulation of gene expression by microRNAs (miRNAs) has been shown to be important in several aspects of plant physiology and development (Reinhart et al., 2002). To evaluate whether the RcPYL, RcPP2C, and RcSnRK2 genes could be regulated by miRNAs, we used the castor bean miRNA sequences present in the miRbase to obtain conserved miRNAs and search for putative targets in the transcripts. A total of 19 conserved miRNA mature sequences were identified that target RcPYL, RcPP2C and RcSnRK2 transcripts (Table S4). The analysis showed that two RcPYL, 24 PP2C, and three RcSnRK2 genes could be potentially targeted by plant conserved miRNAs. Among them, RcPP2C20 and RcPP2C39 appear to be targeted by three miRNAs. RcPYL1, RcPP2C38, RcPP2C60, and RcSnRK2.1 could be targeted by two miRNAs. RcPYL3, 20 other PP2C genes, RcSnRK2.4 and RcSnRK2.6, appear to be targeted by one miRNA. The experimental validation of the computational predictions will be an important step in evaluating the potential impact of those miRNAs in the regulation of castor bean PYL, PP2C and SnRK2 genes.

Altogether, these analyses can provide additional support to understand the regulation of *RcPYL*, *RcPP2C*, and *RcSnRK2* gene expression in castor bean.

3.4. Expression profiles of the RcPYL, RcPP2C, and RcSnRK2 genes

To investigate the expression profile of the *RcPYL*, *RcPP2C*, and *RcSnRK2* genes under normal growth conditions in leaves, roots, male flowers and in different stages of seed development, we analyzed the RNAseq data published by Brown et al (2012) (Fig. 5).

Although we could not observe any evident expression pattern among the different groups in our analysis, we could see that the *RcPYL*, *RcPP2C*, and *RcSnRK2* genes were generally less expressed in leaves. In roots, the genes with the highest expression levels were *RcPYL6*, *RcPYL5*, *RcPYL1*, *RcPYL4*, *RcPP2C10*, *RcPP2C14*, *RcPP2C53*, *RcPP2C1*, *RcPP2C17*, *RcPP2C29*, *RcPP2C13*, *RcPP2C19*, *RcPP2C39*, *RcPP2C44*, *RcPP2C9*, *RcPP2C27*, *RcPP2C43*, *RcPP2C48*, *RcPP2C51*, *RcPP2C28*, *RcSnRK2.1*, *RcSnRK2.2*, *RcSnRK2.4* and *RcSnRK2.5*.

During seed development, the expression of *RcPYL1*, *RcPYL4*, *RcPP2C56*, *RcPP2C21*, *RcPP2C49*, *RcPP2C61*, and *RcPP2C7* increases, while the expression of *RcPP2C54*, *RcPP2C23*, and *RcPP2C24* is reduced. In male flowers, *RcPP2C53*, *RcPP2C56*, *RcPP2C29*, *RcPPC27*, and *RcPP2C57* were the genes with higher expression levels (Fig. 5).

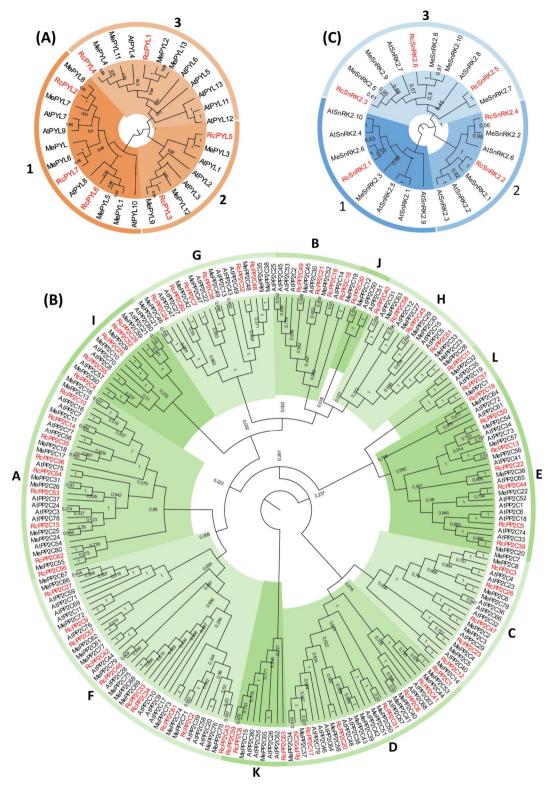


Fig. 2. Maximum-likelihood phylogenetic analysis of the PYL (A) PP2C (B) and SnRK2 (C) families of *Ricinus communis* (*Rc*), *Manihot esculenta* (*Me*) and *Arabidopsis thaliana* (*At*). Sequences were aligned using MUSCLE, and phylogenetic tree reconstructions were made using the maximum likelihood method under the best model selection in MEGA 7.1 software with 1000 replicates of bootstrap statistics (numbers in the branch ramification).

3.5. Differential expression analysis of the castor bean transcriptome in response to drought

To understand castor bean transcriptional modifications leading to physiological adaptation under drought stress, plants with approximately 2 months were submitted to drought treatment through

suspension of irrigation until a water potential of $-1.0\,$ MPa was reached, and global gene expression was analyzed in leaves and roots by RNAseq.

A total of 351,025,404 reads were used in a Tophat2, HTSeq, and DESeq2 analysis for differential gene expression analysis (Table S5). The number of high-quality reads mapped to the reference genome

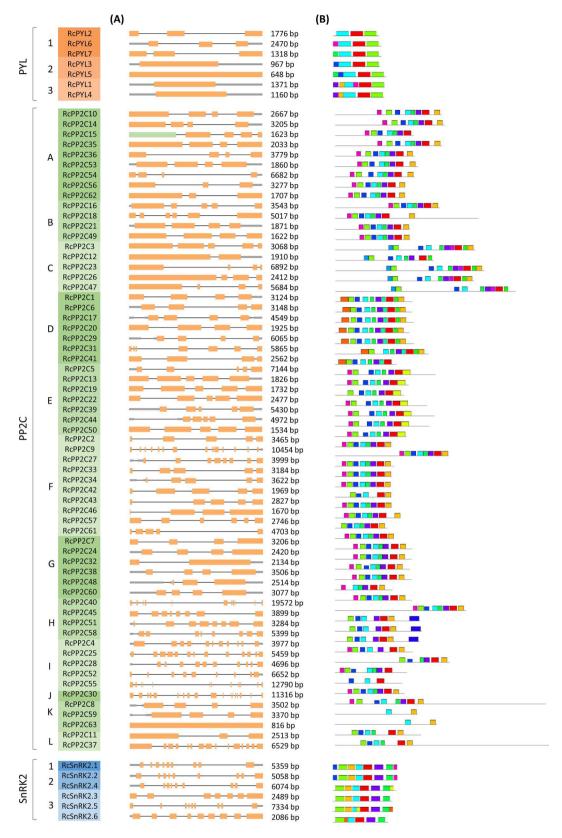


Fig. 3. Exon-intron structure of RcPYL, RcPP2C, and RcSnRK2 genes (A) and conserved motifs of encoded proteins (B). The lengths of exons and introns of each gene are exhibited proportionally. Gene families are grouped and color-coded based on the phylogenetic analysis. For all genes, black lines represent introns, orange boxes represent exons and grey boxes represent UTRs. All conserved motifs in RcPP2C proteins were identified by MEME software and indicated by a colored box. The lines represent the non-conserved sequences, and the length of motifs in each protein is presented proportionally. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

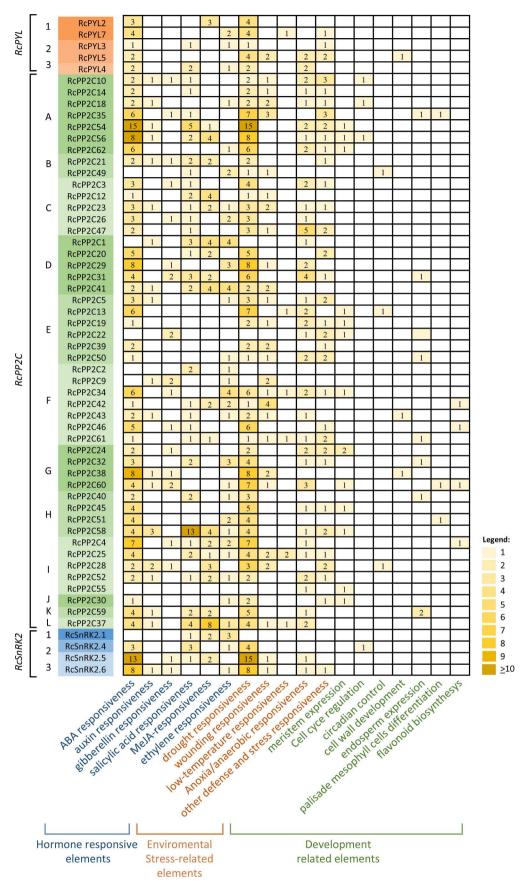


Fig. 4. Number of cis-regulatory elements identified in the promoter regions of the RcPYL, RcPP2C, and RcSnRK2 genes. Cisregulatory elements related to hormone response, environmental stress, and development were identified by Plant Promoter Analysis Navigator 210 from PlantPAN 3.0 database 211 (Chow et al., 2019), using 1000 bp upstream from the translation start site from each gene. In some cases, a 1 kb region was not available, and the analysis was not performed (RcPP2C6, RcPP2C7, RcPP2C8, RcPP2C11, RcPP2C15, RcPP2C16, RcPP2C17, RcPP2C27, RcPP2C44, RcPP2C33. RcPP2C36. RcPP2C48. RcPP2C53. RcPP2C57. RcPP2C63, RcPYL1, RcPYL6, RcSnRK2.2, RcSnRK2.3). The number indicates the sum of various cis-regulatory elements. All cisacting elements summarized here are listed in Table S3. Elements were grouped based on their functional description. ABA responsiveness (ABRE and MYC); auxin responsiveness (TGA-element and AuxRRcore) gibberellin responsiveness (P-box, TATC-box, and GARE-motif); salicylic acid responsiveness (TCA-element and MBS); MeJA responsiveness (TGACG-motif, CGTCA-motif, and JERE); ethylene responsiveness (ERE); drought responsiveness (DRE core and MBS); wounding responsiveness (WUN-motif and WRE3); low-temperature responsiveness (LTR); anoxic/anaerobic responsiveness motif and ARE); other defense and stresses responsiveness (STRE and TC-rich repeats); meristem expression (OCT, CAT-box, and CCGTCC-box); cell cycle regulation (MSAlike); circadian control (circadian); cell wall development (AC-I); endosperm expression (GCN4_motif, AACA_motif); palisade mesophyll cells differentiation (HD-Zip 1); flavonoid biosynthesis (MBSI).

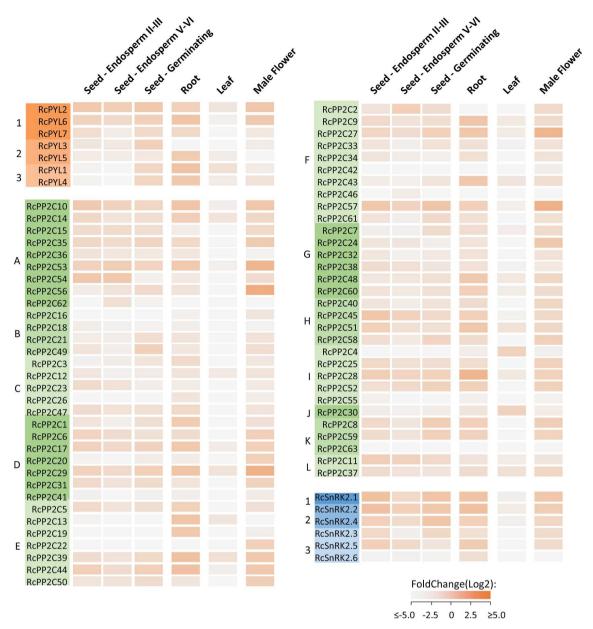


Fig. 5. Heatmap showing the expression patterns of the RcPYL, RcPP2C, and RcSnRK2 genes in different organs from Ricinus communis. The color scale at each dendrogram represents log2 expression values, varying from blue to orange representing the relative expression level scale. The transcriptomic data were obtained from Brown et al. (2012). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(MAPQ>=30) in each sample was between 17.6 and 44.6 million. The counts of reads superimposed on each gene were performed, integrating the values for each exon. The read counts with biological replicates for leaf and root samples are presented in Tables S6 and S7, respectively. The total number of genes included in the tables and the following analysis was 9,153 for leaves and 14,623 for roots. The PCA analysis revealed that the samples (control1/control2 and drought1/drought2) are consistent within each group and distinct between the treatments (Figure S1).

Differential expression analysis was performed with the use of DESeq2 and the results are presented for leaves and roots in Tables S8 and S9, respectively. In addition, Table S10 lists differentially expressed genes common in roots and leaves. The MA-plot of this analysis is shown in Fig. 6A and 6B. The number of genes considered differentially induced with the strict criteria employed was 588 genes induced in leaves and 372 genes induced in roots, of which 112 genes were induced in both organs (Fig. 6C). The total number of genes differentially repressed was 847 for leaves, 975 for roots, and 99 common for the two

organs (Fig. 6D). Heatmap comparisons of differentially expressed genes under drought stress versus control conditions are shown in leaves (Fig. 6E) and roots (Fig. 6F). Very distinct blocks of downregulated and upregulated genes can be seen as a consequence of a strict definition of such groups. The heatmap for genes differentially regulated in both leaves and roots is shown in Fig. 6G.

3.6. Expression profiles of the RcPYL, RcPP2C, and RcSnRK2 genes in plants submitted to drought stress

Considering that PYL, PP2C, and SnRK2 constitute the core of ABA signaling and the central role of this phytohormone in the dehydration stress response, we evaluated the expression of those genes in castor bean plants submitted to drought. Plants with approximately 2 months were submitted to drought treatment through suspension of irrigation until a water potential of -1.0 MPa was reached, and global gene expression was analyzed in leaves and roots by RNAseq.

The expression data show that some of the PYL and PP2C genes are

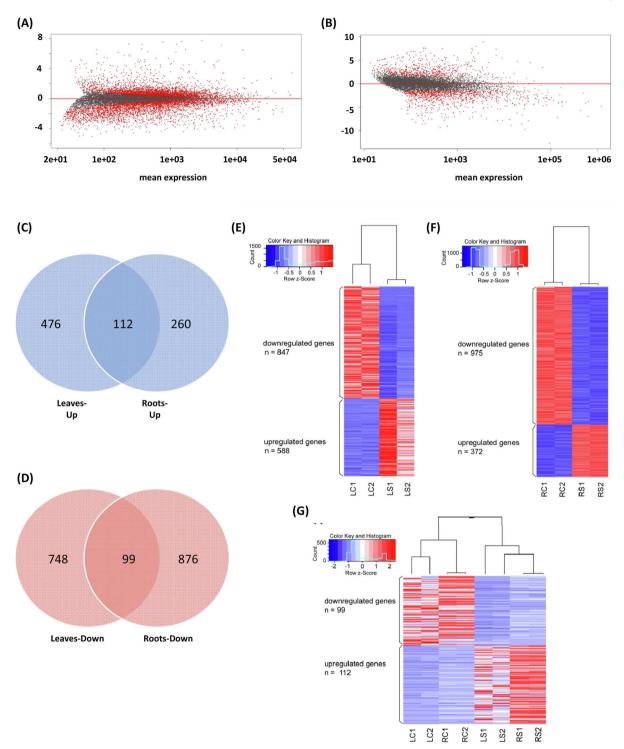


Fig. 6. RNAseq analysis of gene expression in leaves and roots from castor bean plants submitted to drought stress. MA-plot of the results of DESeq2 differential expression analysis in roots (A) and leaves (B). The roots analysis corresponds to values from 14,623 genes, and the leaves analysis corresponds to values from 9153 genes. Genes for which the counts in both conditions had two values below 50 were previously deleted. The graphs present the log fold change (stress condition \times control) versus the mean expression value for each of the genes. Red dots correspond to genes in which the p-adjust value is<0.01. Venn diagrams show the number of common differentially expressed genes in leaves and roots: (C) upregulated genes and (D) downregulated genes. Heatmap comparison of differentially expressed (DE) genes in drought stress conditions compared with control conditions in roots (n = 1347) (E), leaves (n = 1435) (F) and roots and leaves (n = 211) (G). Pairwise sample distances were estimated using the Euclidean distance, and the complete agglomeration method was used for clustering of the samples. Heatmaps were obtained with the heatmap.2 function of the gplot package in R on the normalized transcript counts. Default parameters were used for the distance (Euclidean) and clustering functions (complete agglomeration) with row scaling. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

modulated under drought treatment (Supplementary Fig. S2A). The group A PP2C appears to be the most induced. In addition to these genes, RcPP2C20 (group D) was upregulated in leaves, and RcPP2C2 (group F) was upregulated in leaves and roots. On the other hand, PYL genes are generally downregulated in leaves and roots in response to drought. RcPYL1 and RcPYL4 were significantly repressed in both leaves and roots, while RcPYL7 was significantly repressed only in leaves. In addition to PYL genes, RcPP2C19 and RcPP2C50 are repressed in roots, while RcPP2C4 and RcPP2C8 are repressed in leaves (Supplementary Fig. S2A).

These results were confirmed by analyzing an association network of the *RcPYL*, *RcPP2C*, and *RcSnRK2* genes in castor bean in response to drought stress (Supplementary Fig. S2B). Thus, a PYL/PP2C/SnRK2-mediated interaction network was created, and 34 interactive proteins (with high confidence; score > 0.9), including five RcPYL, 24 RcPP2C, and five RcSnRK2 proteins, were identified (Fig. 6B). In leaves, drought stress induces the coexpression of some *RcPYL RcPP2C* and gene pairs (Supplementary Fig. S2C), while *RcSnRK2* does not appear to be induced. On the other hand, in roots, a higher induction of *RcPYL*, *RcPP2C*, and *RcSnRK2* coexpression was observed (Supplementary Fig. S2D).

3.7. GO enrichment analysis of expressed genes in leaves and roots submitted to drought stress

To identify the general strategy of the transcriptional response of castor bean to drought, the overrepresentation of different GO groups among the genes considered differentially expressed (adjusted p-value < 0.01 and abs (log2FoldChange) > 1.8) was analyzed by GO enrichment analysis performed with the Bioconductor clusterProfiler DOSE packages (R) (Yu et al, 2012, 51 Yu et al, 2015) (Tables S11, S12, S13 and S14) and with the AgriGO tool (Du et al., 2010) (Tables S15, S16, S17 and S18). A third approach used was GSEA (gene set enrichment analysis). In this case, all genes with GO classes were included in the analysis, and the goal, in this case, was to determine whether a set of genes (a specific GO) showed statistically significant, concordant differences between the two conditions (control and stress) (Tables S19, S20, S21 and S22). The strategies gave a similar result, with a higher number of genes in the various GO classes for GSEA, in general. The results below focus on the genes with decreased or increased expression, allowing us to point out several biological processes that seem to be regulated during the drought-stress response. The overrepresentation of differentially expressed genes was used as a basis for the discussion, and GSEA results are presented as needed.

Consistent with the important role of PP2C proteins in the drought response, the GO categories "protein serine/threonine phosphatase activity" and "protein dephosphorylation" were enriched among the genes with increased expression in leaves and roots submitted to drought stress. Eight PP2C genes were identified among the genes with increased expression in leaves: 30169.m006515 (PP2C56), 28211.m000131 (PP2C2), 30147.m014150 (PP2C54), 29805.m001514 (PP2C20), 29751. m001870 (PP2C15), 30143.m001179 (PP2C53), 29912.m005442 (PP2C36) and 29706.m001323 (PP2C10) (Table S11). Five of those genes, 28211.m000131 (PP2C2), 29751.m001870 (PP2C15), 29912. m005442 (PP2C36), 30143.m001179 (PP2C53) and 30169.m006515 (PP2C56), were also observed in the enrichment analysis of upregulated genes in root tissue submitted to drought (Table S13). The GSEA results confirm the increased expression of phosphatase 2C genes in leaves, with the same eight genes induced and with the noticeable result of eleven PP2C increased in roots (Tables S19 and S21).

Regarding all the other genes, in leaves, the differentially induced genes showed GO categories mainly related to general processes, such as "metabolic process", "primary metabolic process", "cellular process", and "cellular metabolic process" (Supplementary Fig. S3A, Tables S8, and S15). Another important GO category detected in differentially expressed genes in castor bean was the "lipid metabolic process". Upon

stress treatment, genes related to lipid metabolism, such as putative triacylglycerol lipase (28470.m000422), GDSL esterase/lipase (30170. m014261 and 28962.m000454), phospholipase A (29929.m004538), lipase member N (30174.m008713), monoglyceride lipase (30167. m000881 and 29113.m000030), mitochondrial carnitine/acylcarnitine carrier (30146.m003605), acyltransferase-like (30060.m000520), 3-hydroxyacyl-CoA dehydrogenase (29726.m004067 and 29912. m005496), acyl-CoA synthetase (29844.m003365) and peroxisomal acyl-CoA oxidase (29713.m000177 and 29646.m001117), are induced in leaves. Indeed, lipid metabolism is an important aspect of castor bean and is recognized as a major source of ricinoleic acid (12-hydroxyoctadec-cis-9-enoic acid; 18:1-OH).

Other enriched GO categories observed in upregulated genes from leaves were related to galactose metabolism (Tables S8 and S11). Castor bean activates at least five galactinol-sucrose galactosyltransferases/stachyose synthases (28543.m000400, 29761.m000419, 29933. m001398, 30147.m013837 and 30076.m004626).

On the other hand, photosynthesis appears to be one of the major processes downregulated in castor bean leaves submitted to drought stress. Several GO categories related to this process, such as "photosynthesis", "light reaction", "light harvesting", and "photosynthetic electron transport chain", had genes with decreased expression in our analysis of stressed leaves (Supplementary Fig. S3B, Tables S8, S12, S16, and S20). These results indicate general repression of photosynthetic machinery under stress conditions. A similar downregulation of photosynthetic genes was observed in a progressive drought stress treatment in other plant species, such as arabidopsis and tomato (Harb et al., 2010; Gururani et al., 2015; Iovieno et al., 2016).

Previous reports have shown that during several types of abiotic stress, the expression of different membrane transporters is induced (Bray, 2004; Ranjan et al., 2012). In contrast, castor bean exposed to water stress seemed to present an opposite trend in leaf tissue. Under our conditions, GO categories related to transport activities, such as "transmembrane transport", "transporter activity", "ammonium transmembrane transporter activity" and "integral component of membrane", were found among the repressed genes in stressed leaves (Tables S8, S12, S16, and S20). The analysis of these categories indicates that different types of putative transporters have been repressed in leaves under drought stress, such as eight putative aquaporins, three nitrate transporters, four ammonium transporters, three nucleotide permeases, six amino acid transporters, seven metal transporters, one cation/H⁺ antiporter, six sugar transporters, two oligopeptide transporters, three dicarboxylate transporters, one auxin transporter-like protein, two ABC transporters, one urea-proton symporter, one vacuolar cation/proton exchanger, four NRT1/PTR proteins, two polyol transporters, one nucleobase-ascorbate transporter and one GABA transporter. Taken together, these data indicate that there is reduced transport of various osmolytes in castor bean leaves during water deficit, and this unusual response may contribute to the differential response of castor bean plants during water deficit.

In roots, a differential induction of genes in GO categories related to energy transduction pathways was shown, such as "generation of precursor metabolites and energy", "oxidative phosphorylation", "electron transport chain", "energy coupled proton transport", "energy derivation by oxidation of organic compounds" and "cellular respiration" (Supplementary Fig. S3C, Tables S9, S13, and S17). Between them, we found one putative phosphofructokinase, one phosphoenolpyruvate carboxylase, two NADH dehydrogenases, three NADH:ubiquinone oxidoreductases, three cytochrome *C* oxidases, one mitochondrial phosphate carrier and eight ATP synthase subunits. Indeed, it has been recognized that the increase in respiration rates in response to severe water stress is possibly a consequence of enhanced metabolism due to osmoregulation and water stress-induced senescence processes (Flexas et al., 2005).

Similar to that observed in leaves, the GO category related to galactose metabolism was also enriched among the root drought-upregulated genes. In this organ, three galactinol synthase genes and

one UDP-glucose 4-epimerase gene were induced upon stress treatment (Tables S9 and S13).

GSEA also detected six genes related to the DNA mismatch repair activity induced in the drought-stressed roots (Table S21). Indeed, DNA repair mechanisms have been considered important biological processes involved in stress adaptation and response (Wojtyla et al., 2016).

On the other hand, the GO analysis indicated that the main downregulated genes in roots were involved mainly in the stress response pathway (Supplementary Fig. S3D, Tables S9, S14, S18, and S22). GO categories such as "response to stimulus", "response to stress", "response to chemical stimulus", "response to oxidative stress" and "response to biotic stimulus" group genes showed decreased expression in response to drought stress. Despite the importance of enzymatic and nonenzymatic antioxidant defense systems to precisely control reactive oxygen species (ROS) levels in response to stress, we verified the downregulation of genes related to ROS metabolism. These genes include 11 genes related to superoxide dismutase (SOD) activity, 10 glutathione-S-transferase (GST) and 25 peroxidases related to the oxidative stress response. A similar repression of eight peroxidases and four SOD genes was also observed in the GSEA of leaves (Table S20). These results corroborate the idea that the repression of the oxidative stress machinery seems to be a consistent strategy of castor bean response to stress treatment. These results indicate that the castor bean response seems to be different from the common strategy of a general upregulation of antioxidant enzymes (You and Chan 2015; 61 Al Hassan et al., 2017).

The analysis of the castor bean transcriptome response to drought stress also revealed the repression of cell wall-related genes in response to drought (Tables S9, S14, S18, and S22). Genes coding putative proteins related to cell wall modification, such as seven endoglucanases, six pectinesterases, 11 polygalacturonases, six xyloglucan endotransglucosylases/hydrolases, 12 expansins, three glucan endo-1,3-betaglucosidases, five pectate lyases, three alpha-galactosidases, six betagalactosidases, nine beta-glucosidases, one beta-xylosidase/alpha-Larabinofuranosidase, two mannan endo-1,4-beta-mannosidases, two endo-1,4-beta-xylanase, one glucomannan 4-beta-mannosyltransferase, five chitinases, 10 cellulose synthases, nine laccase and two ligninforming peroxidases, are downregulated in root tissue. Cell wall metabolism genes, such as four proteins with peptidoglycan-binding lysin domains, one endochitinase and one wall-associated receptor kinase, were induced in roots upon stress. The change in the expression of cell wall-related genes in roots is likely to result in the regulation of cell wall extensibility and cell expansion (Hadfield and Bennett, 1998; Bray, 2004) and confirms the pivotal role of roots in stress perception, signaling, and response.

Additionally, gibberellin biosynthesis and signaling were also repressed in roots under drought stress. Ten putative oxoglutarate-dependent dioxygenase genes, which catalyze the final steps in the GA biosynthesis pathway, one gibberellin 3-beta-dioxygenase 1 and one gibberellin receptor (GID1), were repressed in roots under water stress conditions (Tables S9 and S14). The GSEA results showed a similar decrease in GA biosynthesis (Table S22).

The GSEA of roots exposed to the stress treatment showed that a particular class of transporters was repressed (Table S22). Eleven different WAT1-related genes have been observed to be repressed in roots. In arabidopsis, the WAT1 gene ("walls are thin") encodes a member of the plant drug/metabolite exporter (P-DME) family, with at least 46 different members present in the Arabidopsis genome. The WAT1 protein is a tonoplast auxin transporter that is involved in integrating auxin signaling and secondary cell wall formation in fibers (Ranocha et al., 2010; Ranocha et al., 2013).

3.8. Transcription factors differentially expressed in response to drought

Modulation of transcription factors is an important step in adjusting the plant gene expression program and allows the organism to adapt to new environmental conditions. The identification of many transcription factors operating in drought stress responses in different plant species demonstrates that multiple regulatory pathways are involved in drought stress responses (Yamaguchi-Shinozaki and Shinozaki, 2006; Todaka et al., 2015). The identification of those networks is necessary to characterize the response operating in each plant system.

A search for differentially expressed transcription factors was performed using the lists obtained from the Plant Transcription Factor Database v4.0 (https://planttfdb.cbi.pku.edu.cn/index.php?sp=Rco) and ITAK (https://itak.feilab.net/cgi-bin/itak/index.cgi). These lists were merged with our differentially expressed genes, and a total of 105 differentially expressed transcription factors of different families were obtained (Table S23). A total of 34 families were found, 43% of the 79 families described in castor bean transcription factor databases. Among them, bHLH (basic/helix-loop-helix proteins) and ERF (AP2/ERF superfamily) are the most numerous, with >10 representative genes each.

Some of the identified transcription factors are upregulated in both leaves and roots, such as two homeobox-leucine zippers (27964. m000347 and 30089.m001006), an NAC domain-containing protein (28219.m000090), a MYB (29933.m001439), a zinc finger CCCH domain-containing protein (29912,m005346) and a bZIP (basic leucine zipper protein) (30170.m013868). Indeed, in rice, it has been demonstrated that OsbZIP23 (Os02g52780), which shows the most similarity with 30170.m013868, is a key transcription factor for conferring ABA sensitivity and tolerance to drought and salinity (Xiang et al., 2008). Another 30170.m013868 homologue in rice, OsTRAB1 (Os08g36790), is also related to the ABA transduction pathway in response to drought. Interestingly, OsbZIP23 and OsTRAB1 are activated through phosphorylation mediated by SnRK2 homologous (Hobo et al., 1999; Kagaya et al., 2002; Kobayashi et al., 2005). In addition, the putative homeoboxleucine zipper proteins 27964.m000347 and 30089.m001006 are homologous to arabidopsis ATHB-7 (AT2G46680) and ATHB12 (AT3G61890), implicated in ABA signaling and drought stress responses (Söderman et al., 1996; Valdés et al., 2012; Ré et al., 2014; Pruthvi et al., 2014). Among the downregulated transcription factors in both leaves and roots, we found putative ethylene-responsive transcription factors RAP-type (29986.m001656) and PAR2 (29883.m002042).

Transcription factors regulated in an organ-related manner could also be observed. We observed mainly putative WRKY-, NAC-, C2H2-, bZIP-, and bHLH-type transcription factors among the leaf upregulated genes. Among the transcription factor families downregulated in leaves, we identified mainly ethylene-responsive factors (ERFs) and bHLH-type transcription factors. In roots, we observed transcription factors from the bHLH, heat shock factor (HSF), NAC, WRKY, and bZIP families were upregulated. Among the bZIP-type transcription factors induced only in roots, 29625.m000702 shows more similarity with the arabidopsis ABA insensitive 5 (ABI5) protein (AT2G36270), which participates in the core of the ABA signaling pathway, being phosphorylated and activated by SnRK2 homologous (Finkelstein and Lynch, 2000; Nakamura et al., 2001). On the other hand, bHLH, ERF, LBD (LOB domain-containing protein) and MYB-type proteins are the main transcription factors repressed in roots in response to drought. Among these transcription factors, MYB and WRKY family members have also been described as ABA-dependent transcription factors (Golldack et al., 2011; Chen et al., 2012). Thus, these genes may indicate important transcription factors regulating ABA responsiveness and drought stress response pathways in castor bean.

3.9. Validation of expression profile by RT-qPCR analysis

To confirm the expression data obtained with the RNAseq analysis, the expression profile of some genes considered differentially expressed in response to drought was analyzed by RT-qPCR (Fig. 7). The RT-qPCR analysis of genes from the core regulatory network of ABA signaling pathway confirms that *RcPYL1* and *RcPYL7* were downregulated on roots and leaves under drought stress (Fig. 7A and 7C), while *RcPYL7* was induced only in leaves (Fig. 7B). On the other hand, the PP2C from

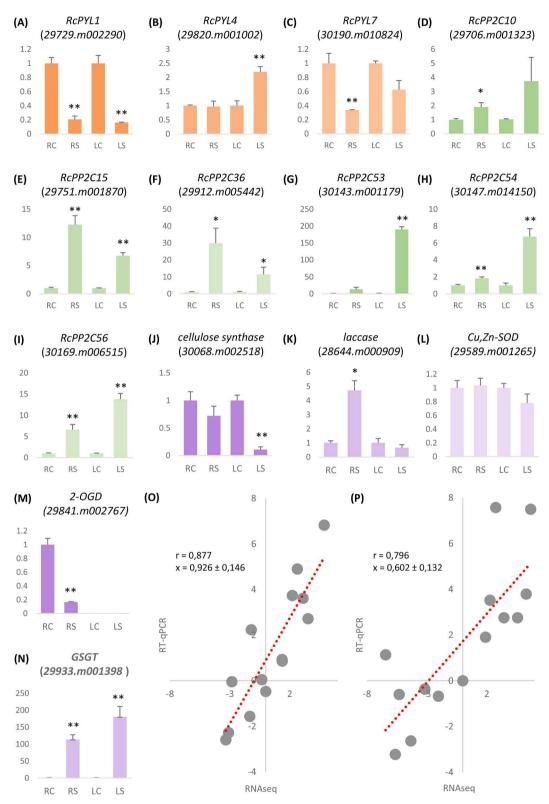


Fig. 7. RT-qPCR analysis of differently expressed genes identified on RNAseq. The expression profile of RcPYL1 (A), RcPYL4 (B), RcPYL7 (C), RcPP210 (D), RcPP215 (E), RcPP236 (F), RcPP236 (G), RcPP2C54 (H), RcPP2C56 (I), cellulose synthase (J), laccase (K), Cu,Zn-Superoxide Dismutase (Cu,Zn-SOD) (L), 2-oxoglutarate-dependent dioxygenase (2-OGD) (M), and galactinol-sucrose galactosyltransferase (GSGT) (N). The RT-qPCR analysis were performed using root and leaf in control (RC/LC) and under drought stress (RS/LS) conditions. Bars represent standard error, (*) and (**) represent statistical difference between stress and control samples with p < 0.05 and p < 0.01, respectively, according to T-test. The correlation between RNAseq and RT-qPCR expression data were performed by linear regression in root (O) and leaf (P). When correlation coefficient (r) is > 0 it is a positive correlation.

group A RcPP2C10, RcPP2C15, RcPP2C36, RcPP2C53, RcPP2C54, and RcPP2C15 were upregulated in both organs under drought stress (Fig. 7D, 7E, 7F, 7G, 7H, and 7I). Additionally, were analyzed representative genes from different GO categories. Differentially expressed genes related to cell wall modification (cellulose synthase/30068. m002518 and laccase/28644.m000909), reactive oxygen species (Cu, Zn-superóxido dismutase/29589.m001265), gibberellin biosynthesis (2oxoglutarate-dependent dioxygenase/29841.m002767) and galactose metabolisms (galactinol-sucrose galactosyltransferase/29933. m001398) were also analyzed. The cellulose synthase/30068.m002518 was downregulated in leaf and present no significant changes in root (Fig. 7J), while the laccase was induced in root, showing no significant changes in leaf (Fig. 7K). The Cu,Zn-superoxide dismutase have not significant changes at organs, 2-oxoglutarate-dependent dioxygenase was repressed in roots and have not expression on leaf, and galactinolsucrose galactosyltransferase was induced in both root and leaf (Fig. 7L, 7M, and 7N). To compare the expression data obtained from RNAseq analysis with the RT-qPCR we perform a correlation analysis with the expression data from the 14 genes tested in the RT-qPCR experiments. The correlation coefficient (r) indicates a positive correlation among the expression data (Fig. 70 and 7P). The linear correlation is stronger in root than leaf (root r = 0.8773, p = 0.000038/ leaf r =0,7963, p = 0,00065). Therefore, the expression profiles of the selected genes were consistent with the transcriptomic results obtained by RNAseq.

4. Discussion

Despite castor bean high adaptation to limiting water conditions, the molecular basis of this response was until uncharacterized. In order to better understand the molecular strategies used by castor bean to respond to drought stress, we perform the analysis of the components of the central ABA signaling as well as a deep transcriptomic analysis of its response upon drought treatments in leaves and roots exposed to drought.

Drought has become one of the most serious problems restricting the worldwide productivity of different crops. This reality is reinforced due to global climate change, freshwater shortages, and increasing populational demand. To improve stress tolerance and reduce productivity loss due to unfavorable environmental conditions, many approaches, such as marker-assisted selection, genome-wide association, and reverse genetics screening, have been applied, and significant progress has been achieved (Taunk et al., 2019).

In plants, ABA plays an important role, regulating developmental processes and responses to abiotic stresses, such as salt and drought stress (Fujii and Zhu, 2009; Yoshida et al., 2014). In plants, ABA plays an important role, regulating developmental processes and responses to abiotic stresses, such as salt and drought stress (Fujii and Zhu, 2009; Yoshida et al., 2014).

In this study, the PYL, PP2C, and SnRK2 genes were identified in the castor bean genome. The PYL family can be divided into three groups (Hauser et al., 2011). Castor bean has three genes in PYL group 1, two genes in group 2 and two genes in group 3. Compared with other gene families, the PP2C gene family is recognized as one of the largest families in the plant kingdom (Yang et al., 2018). Previous genome-wide analyses have identified 80 PP2C genes in Arabidopsis (Xue et al., 2008), 90 in rice (Singh et al., 2010), 88 in hot pepper (Kim et al., 2014), 104 in maize (Wei and Pan, 2014), 91 in tomato (Kim et al., 2014), 86 in Brachypodium distachyon (Cao et al., 2016), 87 in banana (Hu et al., 2017), 94 in alfalfa (Yang et al., 2018), 117 in Populus euphratica (Li et al., 2018a), 257 in wheat (Yu et al., 2019) and 80 in cassava (Zhao et al., 2019). Here, we found 63 PP2C genes in the castor bean genome, divided into 12 groups (A-L). Indeed, evolutionary analysis indicates that PP2C genes can be divided into 11 to 13 groups in different high plants, while in lower plants, such as Chlamydomonas reinhardtii, Physcomitrella patens, and Selaginella tamariscina, the PP2C gene families are

much smaller (Yang et al., 2018). The increase and expansion of the *PP2C* family in higher plants could be correlated with adaptations to complex environmental conditions.

During evolutionary processes, large segmental duplications and small-scale tandem duplications are recognized as two major mechanisms to generate new genes, contributing to plant genome complexity (Cannon et al., 2004). Accordingly, previous works have demonstrated that the PP2C family was expanded through chromosomal duplications in different species (Cannon et al., 2004; Xue et al., 2008, Yang et al., 2018, Cao et al., 2016, Yu et al., 2019; Lu et al., 2019).

In agreement with these results, tandem and segmental duplications can also be responsible for the expansion of castor bean *PP2C and SnRK2* gene families. The mean value of Ka/Ks for *PP2C and SnRK2* paralogous gene pairs was approximately 0.1, which was significantly <1. These data indicate that most mutations that occurred in the genomic sequences of *PP2C and SnRK2* during evolution were detrimental to plant survival. It is likely that the mutated genes were gradually eliminated during long-time selection.

Analysis of upstream regulatory sequences of the *RcPYL*, *RcPP2C*, and *RcSnRK2* genes revealed mainly the presence of ABREs and other elements responsive to ABA and drought stress. We also verified the presence of elements responsive to other phytohormones, such as salicylic acid, ethylene, jasmonates, GA and auxin. These data suggest that, in addition to ABA, these genes may be modulated by other phytohormones. Indeed, previous works have demonstrated that salicylic acid suppresses the degradation of PP2C group A mediated by ABA (Manohar et al., 2017), and brassinosteroid signaling is inhibited by ABA signaling via ABI1 and ABI2 (Wang et al., 2018).

Among the *PYL* genes identified in castor bean, *RcPYL1* and *RcPYL4* were repressed in response to drought in both leaves and roots. These genes are the two members of group 3 PYL and are homologous to *AtPYL4*, *AtPYL5* and *AtPYL6*. Previous works show that AtPYL4, AtPYL5 and AtPYL6 are able to inhibit different group A PP2C phosphatase activities in an ABA-dependent manner: AtPYL4 was demonstrated to inhibit AtHAB2, AtPYL5 inhibits AtHAB1, AtABI1 and AtABI2, and AtPYL6 inhibits AtHAB1, AtABI1 and AHG3 activities (Santiago et al., 2009; Hao et al., 2011). In addition, as verified in arabidopsis in response to ABA treatment (Santiago et al., 2009), castor bean exposed to drought showed a strong downregulation of group 3 PYL. These data suggest the importance of RcPYL1 and RcPYL4 in ABA signaling in castor bean response to drought.

Previous studies have demonstrated that group A PP2C is a second central component of the ABA signal transduction pathway, negatively controlling ABA and stress responses in plants (Fuchs et al., 2013; Singh et al., 2015a). Our phylogenetic analysis identified nine group A RcPP2C genes. In agreement with previous reports in other species, most members of group A from castor bean are induced in response to drought in both leaves and roots. RcPP2C15 (homologue to arabidopsis HAI1, HAI2 and HAI3), RcPP2C53 (homologue to AtAHG3), RcPP2C56 (homologue to AtRDO5), RcPP2C36 (homologue to AtABI1, AtABI2 and AtHAB genes) RcPP2C10 (homologue to AtHAB1 and AtHAB2) and RcPP2C54 (homologue to AtAHG1) are significantly induced in leaves and roots in response to drought (considering GO enrichment and GSEA analysis). In addition to these, group A RcPP2C35 is induced in roots. In arabidopsis, ABI1 and ABI2 have been characterized as the main components of the ABA signaling pathway during development and in response to abiotic stresses (Fuchs et al., 2013; Li et al., 2018b) and HAB genes were demonstrated to have unique drought resistance functions (Bhaskara et al., 2012). These data indicate that, among the PPC2 genes, seven out of nine of the group A genes are induced (PP2Cs RcPP2C15, RcPP2C53, RcPP2C56, RcPP2C36, RcPP2C10, RcPP2C54, and RcPP2C35) and may be relevant components of ABA signaling during drought response. Other RcPP2C induced in leaves and roots in response to drought was RcPP2C2, homologue to arabidopsis PIA1 (AtPP2C20) and member of group F PP2C. In arabidopsis, PIA1 was demonstrated to activate defense-related genes and promote the accumulation of defense

hormones, such as ethylene and salicylic acid (Widjaja et al., 2010).

A global analysis of the transcriptional response to stress was also performed to identify genes associated with castor bean adaptation to drought. Transcriptome analysis showed that the main GO category of differentially expressed genes in response to drought are related to photosynthesis-related processes, which were repressed in leaves. A similar downregulation of photosynthetic genes was observed in a progressive drought stress treatment of arabidopsis and tomato plants (Harb et al., 2010; Gururani et al., 2015; Iovieno et al. 2016). Photosynthesis is one of the key processes affected by water deficit via decreased CO₂ diffusion to the chloroplast and reduction in the contents and activities of photosynthetic enzymes from the carbon reduction cycle, including the key enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) (Reddy et al., 2004; Pinheiro and Chaves, 2011).

Cell wall modification is another process affected during drought stress. Several genes involved in cell wall metabolism or cell wall remodeling were downregulated in castor bean leaves and roots exposed to stress. In plants, the extracellular matrix performs several functions, being involved in plant growth and acting as a barrier to pathogens. Instead of being a static structure, the plant cell wall must remain flexible to respond to developmental and external stimuli, allowing a suitable remodeling response. Several genes encoding proteins involved in the hydrolysis and/or synthesis of cell wall components present modulated expression, suggesting that cell wall modifications may be an important step in stress homeostasis (Bray, 2004; Tenhaken, 2015; Houston et al., 2016). In this context, the huge transcriptional response of castor bean genes involved in cell wall composition, mainly in roots, is an important feature of this analysis. This suggests that cell wall remodeling could be related to a castor bean strategy to rearrange its extracellular matrix to be more adapted to drought stress.

Genes related to oxidative stress are another important GO category of differentially expressed genes in castor bean submitted to drought. Although ROS have been recognized as key players in the complex signaling network of plant stress responses, high ROS accumulation under abiotic stress conditions causes oxidative damage and eventually results in cell death. Indeed, drought stress is reported to increase ROS production, which needs to be precisely controlled by enzymatic and non-enzymatic antioxidant defense systems (Kar, 2011; Noctor et al., 2014; You and Chan 2015). However, surprisingly, we show here that in castor bean roots and leaves, a decrease in the expression of antioxidant genes, such as SOD, GST and peroxidases, is observed in response to drought. Similar to our findings, previous works also show a decrease in SOD and GST isoforms in arabidopsis (Noctor et al., 2014) and thylakoid ascorbate peroxidase (APX) in rice (Jardim-Messeder et al., 2018). These data corroborate the idea that the repression of some oxidative stress machinery components may lead to ROS accumulation, which is necessary to activate different signaling pathways (Waszczak et al., 2018) and indicates that the castor bean response seems to be different from the most common strategy of a general upregulation of antioxidant enzymes (You and Chan 2015; Al Hassan et al, 2017).

The analysis of the castor bean transcriptome response to drought stress reveals a decrease in GA biosynthesis and signaling in roots. Under drought conditions, the major morphological characteristic of plants is growth reduction, considered an adaptive change of plants to avoid high-energy costs under unfavorable conditions. Since GAs stimulate plant growth, it has been proposed that crosstalk between drought stress signals and GA signals results in antagonist interactions to regulate plant growth (Verma et al., 2016). Inhibition of GA biosynthesis is probably involved in the strategy to avoid energetic costs under stress conditions. Previous work demonstrated that the response to severe water stress requires an additional energy apport (Flexas et al., 2005). Thus, this increased energetic requirement justifies mechanisms to avoid energy costs and may also justify the induced expression of genes related to energy transduction pathways.

Our data indicate that the activation of genes involved in the metabolism of raffinose family oligosaccharides (RFOs) seems to be another strategy used by castor bean plants during drought stress responses. The protective role of RFOs, such as galactinol, raffinose, and stachyose, during different abiotic stress responses has been demonstrated in different plants (Taji et al., 2002; Kaplan et al., 2004, Kaplan et al., 2007; Panikulangara et al., 2004; Peters et al., 2007; Kim et al., 2008; Sicher, 2011). These sugars act as osmoprotectants, stabilizing cellular membranes, and ROS scavengers, protecting the cell components against oxidative stress (Nishizawa et al. 2008). The biosynthesis of RFOs involves three types of key enzymes, galactinol synthase, raffinose synthase and stachyose synthase, and different works show that the activity of these enzymes is involved in drought and cold stress tolerance (Taji et al., 2002, Wang et al., 2009, Lü et al., 2017; Vinson et al., 2020; Li et al., 2020), as well as in stress signaling (Kim et al., 2008; Sicher, 2011). In castor bean, drought stress induced the expression of galactinol synthase and stachyose synthetase genes, indicating possible RFO accumulation contributing to conferring high dehydration tolerance.

The search for castor bean transcription factors differentially expressed in response to drought allowed us to identify members from 34 different families. Among them, homologues of genes *OsZIP23*, *OsTRAB1* and *AtABI5*, a subgroup A bZIP transcription factor. Previous works have shown that these transcription factors play a pivotal role in ABA signaling and in the drought stress response in plants, and their activation mechanisms are conserved in different species (Lu et al., 2009). In response to ABA signaling, SnRK2 kinases interact and phosphorylate bZIP transcription factors for transcriptional activation, which is essential to the stress response (Kagaya et al., 2002; Kobayashi et al., 2005; Chae et al., 2007; Fujii et al., 2007; Fujii and Zhu, 2009; Yoshida et al., 2010; Kim et al., 2015). The combination of these different responses appears to be particularly relevant and highlights castor bean drought stress-responsive genes that may be involved in adaptation to limited water conditions.

5. Conclusion

In the present study, identifying the core regulatory network of ABA signaling pathways combined with an evaluation of drought stress-responsive genes highlights the mechanisms involved in castor bean adaptation to limited water conditions. Here, we identified and characterized the *RcPYL*, *RcPP2C* and *RcSnRK2* genes and identified several pathways regulated in response to drought. This analysis indicates interesting pathways regulated during castor bean adaptation to drought stress. Since castor bean plants are highly tolerant to water stress, this analysis allows the identification of gene circuits that may be responsible for this feature and leads to new biotechnology strategies for drought response improvement in this and other cultures.

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CRediT authorship contribution statement

Douglas Jardim-Messeder: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition. **Daniela Cassol:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition. **Ygor Souza-Vieira:** Methodology, Validation, Formal analysis. **Marcelo Ehlers**

Loureiro: Conceptualization, Methodology, Resources. Thomas Girke: Conceptualization, Writing – review & editing, Supervision, Funding acquisition. Mariana Boroni: Methodology, Data curation, Writing – review & editing. Régis Lopes Corrêa: Methodology, Investigation, Writing – review & editing. Ana Coelho: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing. Gilberto Sachetto-Martins: Conceptualization, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Our data are present in supplementary files and have been deposited in SRA (Sequence Read Archive) (https://www.ncbi.nlm.nih.gov/sra).

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2023.147668.

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