Environmental and Experimental Botany 224 (2024) 105821

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Research paper

The differential expression of the *P5CR* and *αTPS6* genes in cowpea plants increases tolerance to water-deficit and high temperatures

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ARTICLE INFO

Keywords:
Vigna unguiculata
Abiotic stress
Osmoprotectants
Proline
Trehalose
Climate change

ABSTRACT

Plants exposed to adverse environmental conditions develop molecular mechanisms of adaptation and/or defense, the osmoprotectors, which function as compatible solutes and contribute to tolerance via prevention systems and protection against cellular damage caused by these abiotic stresses. This study aimed to identify and characterize the osmoprotectors proline and trehalose in cowpea plants cv. Carijó under controlled conditions of water-deficit and heat stress based on the IPCC scenario of 4.8 °C increase in temperature, evaluating their structure and function through computational methods, as well as gene expression by RT-qPCR. The experimental assays were carried out in growth chambers under controlled conditions with different levels of soil water availability, phenological phases and temperature regimes. From the *in silico* analyses, ten *TPS* genes and one *P5CR* gene were identified in *Vigna unguiculata*, and these were named according to their chromosomal location. The *VuP5CR* and *VuTPS* genes play roles in hormone pathway signaling and in the response to light and biotic and abiotic stresses. The genes *P5CR* (proline) and *αTPS6* (trehalose) were induced with increased temperature and lower water availability in the vegetative phase of cowpeas. In addition, *P5CR* also showed induction with 50% water availability at high temperatures. In the pod filling phase, the *P5CR* and *αTPS6* genes were repressed with water availability of 75%, while only the *P5CR* gene was induced when water availability was reduced to 25% under heat stress. *P5CR* and *TPS6* genes were induced in cowpea cv. Carijó in response to associated abiotic stresses (water-deficit and high temperatures), which suggests their participation in the mechanisms of adaptation of the species in adverse environmental conditions.

1. Introduction

Water-deficit and extreme temperatures are among the main environmental restrictions on agricultural productivity in the world, causing morphological, physiological, biochemical and molecular changes, which negatively affect plants (Toscano et al., 2016). For this reason, plants have developed several response mechanisms aimed at their acclimatization and survival in adverse environmental conditions (Carvalho et al., 2017).

When stressed, plant cells are stimulated and signal responses via plant hormones, secondary messengers, signal transducers and transcription regulators, which will coordinate plant growth and development amid stress (Danquah et al., 2014). These stimuli activate genes that encode proteins and enzymes directly involved in stress metabolism, contributing to the acclimatization response to a given stimulus (Casaretto et al., 2016). In conditions of water-deficit and high temperatures, these genes can act in the protection of the cell through the production of important metabolic proteins, thus ensuring the maintenance of turgor in the cell and regulation of target genes (Tokada et al., 2012).

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https://doi.org/10.1016/j.envexpbot.2024.105821
Received 20 February 2024; Received in revised form 12 May 2024; Accepted 16 May 2024
Available online 19 May 2024

These encoded proteins have the function of regulating the concentration of osmoprotective substances, which are small molecules that assist in the plant response to extreme stresses by acting in the adjustment of cellular osmotic pressure, in the detoxification of reactive oxygen species, in the preservation of membrane integrity and in the stabilization of enzymes and proteins (Dar et al., 2016). Among the osmoprotectors are proline and trehalose, which can act as signaling, regulatory, and stabilizing molecules of proteins and biological membranes in plant acclimatization processes in the face of varied conditions of abiotic stresses (Hasanuzzaman et al., 2010).

With changes in climate, abiotic stresses are being intensified due to the frequency of extreme events, such as heat waves and prolonged dry spells (IPCC, 2021), thus there is a need to develop strategies for adaptation of crops of agronomic interest. The cowpea (*Vigna unguiculata* L.) is considered a crop of great socioeconomic importance for the semi-arid regions, especially with regard to food security, because it is a species rich in proteins, minerals, fiber, and essential amino acids (Melo et al., 2018), in addition to presenting low production cost and being more adapted to drought and heat, compared to other agricultural crops (Carvalho et al., 2019).

Several mechanisms of adaptation to drought have been described for cowpeas (Goufo et al., 2017; Ferreira-Neto et al., 2022) and several genes related to water-deficit have been identified, some of which are involved in antioxidant metabolism (Carvalho et al., 2017). Recently, Barros et al. (2021a) evaluated biochemical responses in cowpea, cultivar Carijó, against combined stresses (temperature x water-deficit) in growth chambers and found that there is an increase in the activity of antioxidant enzymes when plants are kept under abiotic stress.

In this context, understanding the nature of signaling cascades, as well as genes differentially expressed in response to abiotic stresses, will be of great importance for the development of plants tolerant to climate change. Thus, the objective of this research was to identify and characterize the osmoprotectors proline and trehalose in cowpea plants cv. Carijó under controlled conditions of water-deficit and high temperatures, evaluating their structure and function through computational methods, as well as their expression by RT-qPCR.

2. Material and methods

2.1. Identification of P5CR and TPS genes in V. unguiculata

Protein sequences of the *P5CR* and *TPS* genes of *Arabidopsis thaliana* were retrieved from the Phytozome database and used as probes in the search for candidate sequences of *P5CR* and *TPS* in the proteome of *Vigna unguiculata* (Vunguiculata_540_v1.2.protein.fa). This was done through a BLASTp using the BioEdit software (Alzohairy, 2011), adopting an *e-value of e-5* as the cutoff point. If multiple results were found for the same gene, only the longest sequence was chosen as a candidate. All protein sequences were manually verified using the Batch CD-search server (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi), using the Pfam database to confirm the presence of the domains P5CR (pfam14748 - P5CR_dimer and pfam03807 - F420_oxidored) and TPS (pfam00982 - Glyco_transf_20 and pfam02358 - Trehalose_PPase).

2.2. Protein characteristics and chromosomal locations

The JvirGel 2.0 software (Hiller et al., 2006) was used to calculate the molecular weight (p.M.) and theoretical isoelectric point (pI) of the VuP5CR and VuTPS proteins. The online server Loctree3 (Goldberg et al., 2014) (https://rostlab.org/services/loctree3/) was used to predict the subcellular location of proteins. Additionally, the prediction of secondary structures was performed through the SOPMA server (Geourjon and Deléage, 1995) (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html). The loci associated with the *P5CR* and *TPS* genes were identified in the GFF file taken from Phytozome (Vunguiculata_540_v1.2.gene_exons.gff3) according to the initial positions on the chromosomes. The TBtools software (Chen et al., 2020) was used to draw the chromosomal distribution images as well as to identify the exon-intron gene structures of the *Vu*P5CR and *Vu*TPS genes.

2.3. Sequence alignment, phenetic analysis and distribution of conserved motifs

The alignments of the sequences were performed using Clustal W (Thompson et al., 1994), and these aligned sequences were used to construct phenetic trees by the *Neighbor-Joining* (NJ) method (Saitou and Nei, 1987) through the MEGA 7 software (Kumar et al., 2016) using the *p-distance* method, with the option of full deletion. The confidence level of the groups was estimated using a *bootstrap* analysis of 2000 resamples. Subsequently, the conserved motifs of *the Vu*P5CR and *Vu*TPS proteins were predicted using MEME (Bailey et al., 2015, https://meme-suite.org/meme/tools/meme).

2.4. Gene ontology and prediction of cis-regulatory elements

The PANNZER2 server (Törönen et al., 2018) (http://ekhidna2.biocenter.helsinki.fi/sanspanz/) was used to perform the analysis of gene ontology in order to obtain information about the biological processes, cellular components and molecular function of *Vu*P5CR and *Vu*TPS genes. The 2 kb region upstream of the CDS of each *Vu*P5CR and *Vu*TPS genes was considered the promoter sequence. The plantCARE software (Lescot et al., 2002, http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to investigate the cis-regulatory elements present in these promoters with the aim of studying the roles of genes in the response to stresses. The image with the distribution of cis-regulatory elements in the promoters of each gene was generated through the TBtools software (Chen et al., 2020).

2.5. Plant material and growing conditions

The experiment was conducted in growth chambers of the Fitotron type, with control of temperature, relative humidity, luminosity, and photoperiod. Seeds of the cultivar Carijó, which is semi-tolerant to increases in temperature (Barros et al., 2021a), were grown in pots with a capacity of 7 L, using the experimental design of a factorial arrangement of 4×3x2, corresponding to soil water availability levels, phenological stages, and temperature regimes, respectively. The levels of water availability in the soil were 25; 50; 75; and 100%, applied in three phenological phases (vegetative, flowering, and filling of the pods). The temperature regimes described in Table 1 were selected from the average temperatures of the last 30 years in the sub-middle region of the São Francisco Valley in northeastern Brazil, and correspond to the average of the minimum values (20 °C), the average (26 °C) and the average of the maximum values (33 °C). The values in the second treatment were adjusted to reflect the anticipated increase of global temperatures (4.8 °C), as projected by the report of the Intergovernmental Panel on Climate Change (IPCC, 2021). The relative humidity, the intensity of the light, and photoperiod were set at 60% ± 5%, 1500 μmol m^{-2} s^{-1}, and 12 hours, respectively.

Irrigation management was performed with the aid of a TDR (Time

Table 1
Temperature regimes used in the cowpea cultivation experiment considering the 4.8 °C increase predicted by the IPCC.

Temperature Regimes	Schedules for each temperature (°C)			
	20:00–6:00	6:00–10:00	10:00–15:00	15:00–20:00
T1 (20–26–33 °C)	20	26	33	26
T2 (24.8–30.8–37.8 °C)	24.8	30.8	37.8	30.8

T1 – control; T2 – IPCC 4.8 °C increase scenario.

Domain Reflectometry) equipment, Campbell's TDR100 model. For this experiment, coaxial cable probes with three rods were used. Initially, the TDR was calibrated for the specific soil type used the experiment, which was an eutrophic red-yellow argisol as described by Batista et al. (2016). Fertilization was carried out three days before planting, based on the results of the chemical analysis of the soil and on the crop-specific recommendations (Cavalcanti, 2008). Irrigation was performed every two days from the data generated by the TDR, with the replacement of the volume of evapotranspired water, maintaining the availability of water in the soil for each treatment.

Before planting, all pots were irrigated to their maximum water retention capacity. Irrigation management involving experimental treatments was initiated fifteen days after planting. The availability of water in the soil was applied in the different phenological phases, divided into three stages. The plants of the first stage were submitted to water-deficit only in the vegetative phase (after the opening of the first compound leaf), ending from the emission of the first flower buds. In the second stage, the plants received different water availability in the flowering phase until the beginning of the filling of the pods. The plants of the third stage were submitted to water-deficit only when the filling of the pods began, ending with the harvest. At the end of each stage, the days that the plants were submitted to different water availability were quantified. Finally, the plant material was collected for total RNA extraction in the phases mentioned above.

2.6. RNA extraction

The total RNA was extracted with the Kit Reliaprep RNA Tissue Miniprep System (Promega), using approximately 200 mg of leaf tissue, and following the manufacturer's recommendations. The protocol adopted includes treatment with DNAse to remove genomic contamination. After extraction, the RNA integrity was verified by electrophoresis in 1% agarose gel stained with ethidium bromide, running at 70 V, 120 A, for 90 minutes. The samples were quantified in Qubit fluorometer (Thermo Fisher Scientific) and immediately used in the synthesis of cDNA.

2.7. Synthesis of cDNA

The cDNA synthesis was performed using 500 ng of total RNA and using the GoScrip Reverse Transcription System Kit (Promega). Initially, RNA samples were added to Oligo *primers* (dT), incubated in a thermoblock at 70 °C for 5 minutes and in ice for 5 minutes. The reaction was added to the reverse transcription reaction mix [4 μL GoScript 5X Reaction Buffer™, 2.6 μL $MgCl_2$ (4 mM), 1 μL PCR Nucleotide Mix (0.5 mM), 1 μL Recombinant RNasin Ribonuclease Inhibitor (25 U/μL), 1 μL GoScrip Reverse Transcriptase (1 u/μL) and 5.4 μL ultrapure water] and incubated in a thermocycler at 25 °C for 5 minutes (annealing), 42 °C for 60 minutes (extension), 70 °C for 15 minutes (RT inactivation) and stored at - 20 °C.

2.8. Design of primers

After the stages of identification and functional characterization of the candidate sequences in *V. unguiculata*, *primers* were designed for the target genes using the software Primer-BLAST (Ye et al., 2012) and Primer3Plus (Untergasser et al., 2007), based on the following criteria: size of the amplicon (70–200 bp), GC content (40–60%), and size of the primer (18–22 bases). The reference genes were obtained in the literature (Amorim et al., 2018) (Table 2). All *primers* were synthesized and purified by desalination at Exxtend Biotecnologia Ltda.

2.9. Analysis of differential expression by RT-qPCR

RT-qPCR reactions were performed in a QuantStudio 5 Real-Time (Thermo Fisher Scientific) equipment using a SYBR Green detection system. In all reactions, biological and technical triplicates were used, as well as negative controls (NTC) for each pair of *primer*. The values of amplification efficiency ($E = 10^{-1}$/slope), correlation coefficient (R), *interception* (y) and *slope* were calculated by the standard curve method using serial dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}). The validations by RT-qPCR followed the recommendations of MIQE (*The Minimum Information for Publication of Quantitative RealTime PCR Experiments*) in order to increase the reliability of the results obtained (Bustin et al., 2009).

The reaction *mix* comprised 1 μL of cDNA, 5 μL of Go-Taq SYBR Green qPCR Master Mix 2x (Promega), 3.4 μL of ultrapure water and 0.3 μL of each primer (5 μM), in a final volume of 10 μL. The reactions were submitted to an initial denaturation step of 95 °C for 2 minutes, followed by 40 cycles at 95 °C by 15 sec and 60 °C by 60 sec, using MicroAmp Optical 96-Well Reaction Plate and MicroAmp Optical Adhesive Film, both from Thermo Fisher Scientific. The dissociation curves were analyzed at 65–95 °C for 20 minutes. The reference genes βTUB, UED21D and UNK were used for the normalization of relative expression results (Table 2). Gene expression levels were evaluated in the REST software (version 2.0.13) (Pfaffl et al., 2002).

3. Results

3.1. Genomic identification and characteristics of VuP5CR and VuTPS proteins

Ten *TPS* genes and one *P5CR* gene were identified in *Vigna unguiculata* through bioinformatics tools and these were named according to their chromosomal location (Table 3). The domain analysis revealed that the *P5CR* sequence had the characteristic domains (pfam14748 - P5CR_dimer and pfam03807 - F420_oxidored) (Figure S1A) and all TPS sequences had the domains TPS (pfam00982 - Glyco_transf_20) in the N-

Table 2
Primers for RT-qPCR validation in leaf tissues of cowpea plants cv. Carijó under abiotic stress.

Gene (access number)	Description	Sequence of *primers*	Amplicon	Reference
Target Gene				
P5CR (XM_028057881.1)	Delta (1)-pyrroline-5carboxylate reductase	(F) GCTTTGGGGCTGATGGAGGAGT (R) ACCATTGATGCTGCTCCCAA	92 bp	This study
αTPS (XM_028058471.1)	Trehalose 6-phosphate synthase	(F) AGACTGCACTTGTATGGTGCT (R) CTTGCTCACACCCTGTGGTT	161 bp	This study
Reference Genes				
βTUB (XM_007147394.1)	Beta-tubulin	(F) CCGTTGTGGAGCCTTACAAT (R) GCTTGAGGGTCCTGAAACAA	117 bp	Amorim et al., (2018)
UED21D	Ubiquitin conjugating enzyme E2 variant 1D	(F) AGAAAAGCCCCCAAGTGTTC (R) CTGCCATCTCCTTTTCTTCAGC	161 bp	Amorim et al., (2018)
UNK (XM_007131494.1)	*Phaseolus vulgaris* unknown gene	(F) ATTCCCATCATGCAGCAAAG (R) AGATCCCTCCAGGTCAATCC	192 bp	Amorim et al., (2018)

Table 3
List of P5CR and TPS genes identified in *V. unguiculata* with their detailed information and chromosomal location.

Gene	Locus ID	Chr	Start	End	CDS length	Exon	Intron	Protein (aa)	MW (kDa)	IP (pH)	Location
VuP5CR1	Vu01g107900.1	1	28035613	28039565	825	7	6	274	28.49	9.47	Cytoplasm (97%)
VuTPS1	Vu02g017600.1	2	6284421	6288960	2586	3	2	861	97.85	5.47	Cytoplasm (84%)
VuTPS2	Vu02g076000.3	2	22795363	22801219	2583	5	4	860	97.14	6.38	Cytoplasm (84%)
VuTPS3	Vu03g093200.2	3	7865203	7875475	2820	19	18	939	106.35	7.19	Cytoplasm (88%)
VuTPS4	Vou03G238000.2	3	39664805	39670069	2577	4	3	858	97.00	6.05	Cytoplasm (84%)
VuTPS5	Vu03g378000.1	3	58138182	58142182	2568	3	2	855	96.70	5.77	Cytoplasm (84%)
VuTPS6	Vu05g178300.10	5	33401588	33424460	2817	18	17	938	105.96	6.70	Cytoplasm (88%)
VuTPS7	Vou07g266000.1	7	38147936	38152423	2559	4	3	852	96.48	5.75	Cytoplasm (84%)
VuTPS8	Vu09g084600.3	9	10703755	10709773	2598	5	4	865	97.95	5.81	Cytoplasm (84%)
VuTPS9	Vu11g044400.1	11	6515844	6533867	2775	18	17	924	104.41	8.23	Cytoplasm (89%)
VuTPS10	Vu11g086200.1	11	25853993	25859286	2601	4	3	866	98.28	5.94	Cytoplasm (84%)

terminal region and TPP (pfam02358 - Trehalose_PPase) in the C-terminal region (Figure S1B). The proteins presented between 274 and 939 aa and were all located in the cytoplasm. The analysis of the physicochemical properties revealed that *Vu*P5CR1 had a pH in the basic scale with about 28.49 kDa while the *Vu*TPS proteins presented isoelectric points varying in the acid and basic scale and weighing between 96.48 and 106.35 kDa (Table 3). Additionally, a varied percentage of α-helices (41.70% - 49.64%), extended strands (13.95% - 18.01%), β-turns (4.66% - 9.49%) and random coil structures (26.28% - 37.42%) were found in the proteins *Vu*P5CR and *Vu*TPS (Table 4).

aa= amino acids, (%) = precision

The multiple alignment of the sequences (Figure S2A) of *Vu*P5CR1 with other plant species revealed that the domains P5CR_dimer and F420_oxidored were well preserved, presenting the characteristic motifs GxxGxG (Rossmann fold), GSxPA (characteristic mark of the dimerization nucleus) and the SP-A/G-GTT motif. In *Vu*TPS (Figure S2B) the results showed that the catalytic centers VAN**R**LP, **GG**L, Y**Y**N, L**W**P, H**D**Y**H**L, FL**HT**, GF**Q**/**H**T, **R**LD, S**K**G, S**R**G and LR**D**G**MNLV**SY**E** of the proteins were highly conserved in *Vu*TPS3, *Vu*TPS6, and *Vu*TPS9, but presented amino acid substitutions in the others, indicating that the *Vu*TPS genes encode active TPS enzymes. The characteristic motifs of the TPP domain Dxxx(T/V), (S/T)Gx and $Kx_{16–30}$(G/S)(D/S)xxx(D/N) were also conserved for most sequences, with the exception of *Vu*TPS3, *Vu*TPS6 and *Vu*TPS9, which suggests that, in these sequences, the TPP domain may be inactive. Additionally, the sequence identity of the 10 *Vu*TPS proteins (Table S1) was calculated by the SIAS server (http://imed.med.ucm.es/Tools/sias.html) and this ranged between 26.19% and 86.80%, with the highest identity between *Vu*TPS2 and *Vu*TPS4 and the lowest between *Vu*TPS3 and *Vu*TPS10. Interestingly, the *Vu*TPS3, *Vu*TPS6 and *Vu*TPS9 sequences presented a higher sequence identity among themselves than in relation to the others, ranging from 69.20% to 80.35%.

3.2. Phenetic analysis and preserved motifs

The ten *Vu*TPS genes were subdivided into two groups: Class I and Class II, as previously reported in other plant species (Lunn, 2014; Mu et al., 2016; Wang et al., 2017; Hu et al., 2020; Dan et al., 2021). Among these, *Vu*TPS3, *Vu*TPS6 and *Vu*TPS9 were classified as Class I and the other seven *Vu*TPS proteins were classified as Class II (Fig. 1A). In addition, the analysis of gene structures revealed that all *Vu*TPS genes were interrupted by introns, with the three members of Class I (*Vu*TPS3, *Vu*TPS6 and *Vu*TPS9) having 18 and 17 introns, while the genes in Class II had a lower frequency, with *Vu*TPS1 and *Vu*TPS5 containing only 2 introns while the other members of this group had 3 or 4 (Fig. 1C). A total of 20 motifs were identified by the MEME server (Fig. 1B) and these were well conserved among the groups. The members of Class I presented 16 motifs, with the exclusion of the motifs 11, 12, 13 and 20 supporting the homology of the sequences in the results described above, which indicates that the members of this class have divergent sequences in relation to the members of Class II.

The ten *Vu*TPS sequences were aligned with 20 protein sequences of *Glycine max*, 11 of *Arabidopsis thaliana* and 11 of *Oryza sativa* (Fig. 2) to investigate the relationships of *TPS* genes in these families. As in *Vu*TPS, the proteins of *V. unguiculata*, *G.max*, *A. thaliana* and *O. sativa* were also grouped into two large groups divided into subgroups. Subgroup I-A presented three sequences of *V. unguiculata*, five of *G.max,* one of *A. thaliana* and one of *O. sativa.* Subgroup I-B presented only sequences of *A. thaliana.* In group II, the subgroups II-A and II-B were the most diverse, presenting, respectively, 3/3 sequences of *V. unguiculata*, 6/7 of *G.max,* 3/3 of *A. thaliana* and 5/2 of *O. sativa.* Subgroup II-C contained only one sequence of *V. unguiculata*, two of *G.max* and one of *A. thaliana* while subgroup II-D grouped only sequences of *O. sativa.* As it is possible to observe, the proteins of *V. unguiculata* and *G.max* were presented in the same subgroups, indicating that they are closely related.

For classification analysis of *P5CR* genes, sequences described and available in the literature of different species were used. The relationship between orthologs was reflected in groupings according to the taxonomy of the groups in monocots and dicots (Figure S3). The main branch of dicotyledons was initiated by the sequence of *Vu*P5CR1 while the monocots branch was initiated by the sequence of *O. sativa.* Additionally, the analysis of motifs identified the presence of 9 conserved motifs where only the sequences of the monocot group, as well as those of *Vitis vinifera* and *Populus trichocarpa,* did not present the motif 8, which indicates a high conservation of P5CR proteins in these plant species.

Table 4
Secondary structures present in *Vu*P5CR and *VuTPS* proteins.

Protein	Alpha helix	Extended strand	Beta turn	Random coil
***Vu*P5CR1**	49.64%	14.60%	9.49%	26.28%
***Vu*TPS1**	41.70%	17.89%	5.92%	34.49%
***Vu*TPS2**	43.37%	17.09%	5.12%	34.42%
***Vu*TPS3**	43.77%	13.95%	5.43%	36.85%
***Vu*TPS4**	42.66%	17.13%	4.66%	35.55%
***Vu*TPS5**	43.63%	18.01%	4.68%	33.68%
***Vu*TPS6**	42.75%	14.82%	5.01%	37.42%
***Vu*TPS7**	45.19%	16.55%	4.93%	33.33%
***Vu*TPS8**	42.43%	16.76%	5.09%	35.72%
***Vu*TPS9**	45.56%	14.18%	5.09%	35.17%
***Vu*TPS10**	42.26%	17.44%	4.85%	35.45%

3.3. Chromosomal location of the VuP5CR and VuTPS genes

The mapping performed for the location of the P5CR and TPS genes in *V. unguiculata* revealed that *Vu*P5CR1 was located on chromosome 01 while the *Vu*TPS genes were distributed on chromosomes 02, 03, 05, 07, 09 and 11 (Fig. 4). Chromosome 03 grouped the largest amount of *VuTPS* genes (*Vu*TPS3, *Vu*TPS4 and *Vu*TPS5) while the other groups had 2 or 1 *Vu*TPS gene. In addition, it was possible to observe that most of the mapped genes were found near the ends of the arms of the chromosomes.

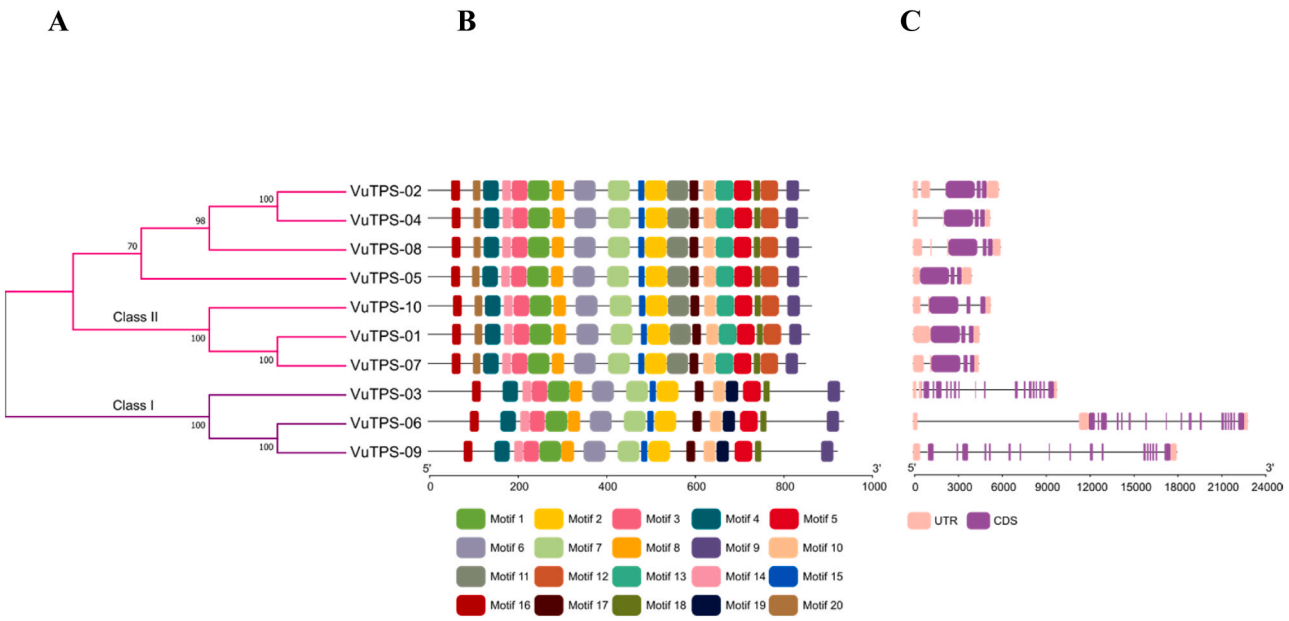

Fig. 1. Phenetic analysis, architecture of conserved protein motifs and gene structure in the *Vu*TPS genes of *Vigna unguiculata*. (A) A phenetic tree was constructed by the NJ method, based on the amino acid sequence of the *Vu*TPS protein domain using the MEGA 7 software. The purple and pink branches represent the sequences of Class I and Class II, respectively. (B) Composition of the motif of the *Vu*TPS proteins. The motifs, numbered 1–20, are displayed in boxes of different colors. (C) Exon-intron structure of *Vu*TPS genes. The numbers near the tree branches represent the values of boostrap based on 2000 resamples.

3.4. Analysis of cis-regulatory elements

We found 67 types of cis-regulatory elements in the *Vu*P5CR and *Vu*TPS promoters, as well as CAAT and TATA box, elements commonly observed in these sequences (Figure S3). These elements were classified in light-responsive elements, such as Box 4, G-box, and TCT-motif; and hormone-responsive elements, such as ABRE (responsive to abscisic acid) and ERE (responsive to ethylene), which were observed in most genes. Additionally, some elements related to plant development, such as O_2-site (involved in the regulation of zein metabolism) and CAT-box (related to meristem expression), were present in *Vu*P5CR1, *Vu*TPS3, *Vu*TPS4, *Vu*TPS5 and *Vu*TPS7. Among the cis-regulatory elements related to biotic and environmental stresses (Fig. 5), elements such as MYB, MYC, TATA, STRE, TC-rich repeats (stress-responsive elements), ARE and WRE3 (essential element for anaerobic induction), as-1 (element responsive to oxidative stress), LTR (responsive to low temperatures), MBS (involved in drought induction), DRE-1 (responsive to drought), W box (element responsive to fungi elicitors and injuries), and WUN-motif (element responsive to injuries) were detected. Our results indicate that the *Vu*P5CR and *Vu*TPS genes play roles in hormonal pathway signaling and in the response to biotic and abiotic stresses.

3.5. Gene ontology of VuP5CR and VuTPS proteins

The analysis of gene ontology resulted in 10 biological processes, four cellular components and three molecular functions annotated in the *Vu*TPS proteins (Figure S4). In terms of molecular function and biological process, it was observed that all *Vu*TPS proteins had alpha-trehalose-phosphate synthase (UDP-forming) activity and participate in the biosynthetic process of trehalose. Most sequences were related to trehalose metabolism in response to stresses. In turn, the analysis of cellular components showed that all proteins were located in the cytosol, with some of them also being integral components of the membrane, in the spliceosomal complex and in the mitochondria (Table S2).

For *Vu*P5CR1, the ontology analysis revealed that this protein had pyrroline-5-carboxylate reductase and nucleotide binding activity. It is involved in various biological processes, such as proline biosynthesis, response to salinity and heat, and is located in the cytosol, cell wall, as well as being an integral component of the membrane (Table S2).

3.6. Analysis of differential expression by RT-qPCR

The flowering phase was discarded from the analysis, because the samples of the control group (water availability of 100%) presented RNA quantification lower than 50 ng/μL, and the two bands corresponding to the ribosomal RNA were not verified after agarose gel electrophoresis. Thus, only the vegetative phase (Phase 1) and the pod filling phase (Phase 3) were used for cDNA synthesis and RT-qPCR reactions.

The *primers* designed for target and reference genes showed efficiency ranging from 90.74% to 106.93% (Figure S5), with correlation coefficients (R^2) ranging from 0.97 to 0.99 and *slope* between −3.16 and −3.56. In addition, the amplification curves were consistent in the dilution points and technical replicas used (Figure S6). In the *melting* curves, single peaks were obtained for all the genes evaluated, confirming the specificity of the *primers* (Figure S7).

In the analyses of relative expression, it was observed that at the lowest temperature (20–26–33 °C) the target genes were constitutive, even under conditions of low water availability (25 and 50%), in both phenological phases (Table 5). In plants exposed to increased temperature (24.8–30.8–37.8 °C) and lower water availability (25%), the *P5CR* and *αTPS6* genes were induced 2.913 and 3.258 times, respectively, when compared to the control group (100%), in the vegetative phase (Phase I). For this same phase, it was observed that at 50% water availability, only the *P5CR* gene was differentially expressed, presenting induction of 1.722 times in relation to the control (Table 5).

In the pod filling phase (Phase 3), the target genes did not present significant expression differences between the groups evaluated at lower temperatures (20–26–33 °C), even under extreme water-deficit (Table 6). On the other hand, with the increase of 4.8 °C in temperature, the *P5CR* gene was induced 1.267 times when compared to its controls in water availability of 25% (Table 6). In addition, the *P5CR* and *TPS6* genes were repressed 0.812 and 0.330 times, respectively, under conditions of moderate water stress (75%), suggesting that physiological and enzymatic activities may have been sufficient to protect the plants against the applied stresses.

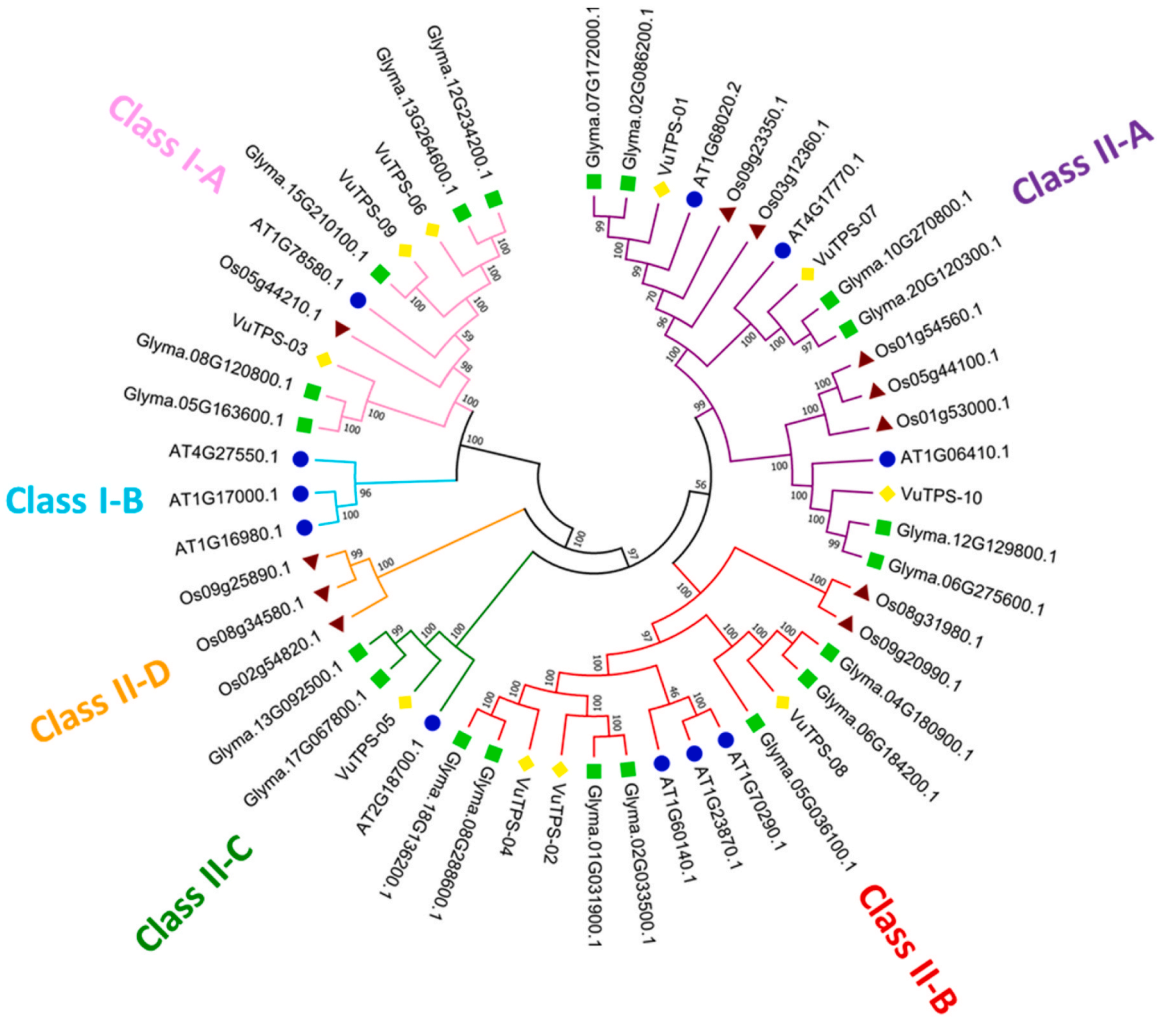

Fig. 2. Phenetic tree constructed by NJ method using 52 TPS protein sequences of *V. unguiculata* and other different species. Each group of species is represented by colors as follows: *V. unguiculata* in yellow; *A. thaliana* in blue; *G.max* in green and *O. sativa* in wine color.

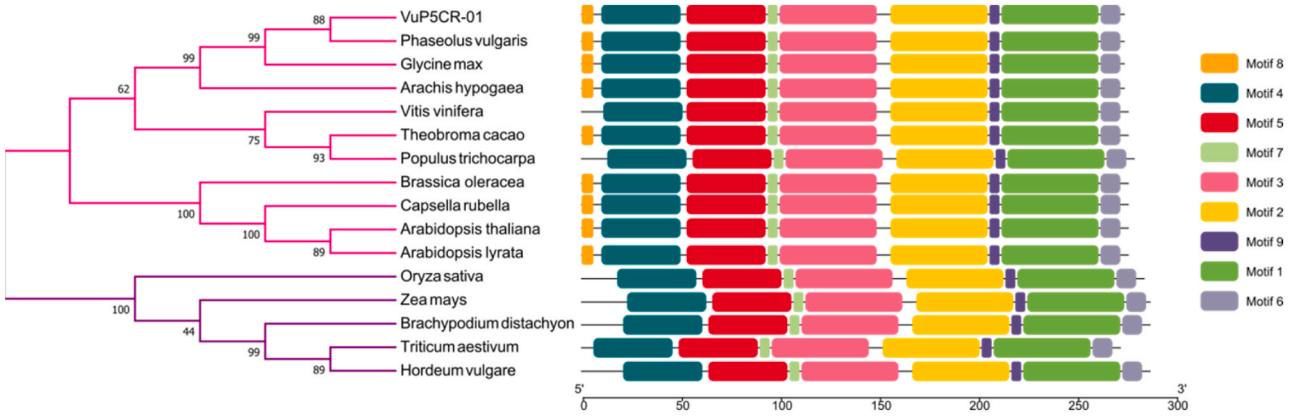

Fig. 3. Phenetic tree of the *Vu*P5CR-01 gene and its orthologs. The bars on the side represent the conserved motifs identified by the MEME servers where each motif is represented by a different color. Numbers next to tree branches represent boostrap values based on 2000 resampling.

4. Discussion

The combination of thermal and water stress affects the growth, development and, consequently, the production of cowpea. Evaluating the impact of water restriction and increased temperature on different phenological phases of cowpea, Barros et al. (2021a) observed greater grain production in plants maintained at water availability of 50, 75 and 100%. Water-deficit (25% availability) in the vegetative phase (Phase 1) reduced grain production by 63.33%, when compared to 100% water availability. In the pod filling phase (Phase 3), water restriction did not cause a decrease in plant production. Furthermore, the increase in temperature and water availability had a positive effect on the dry mass

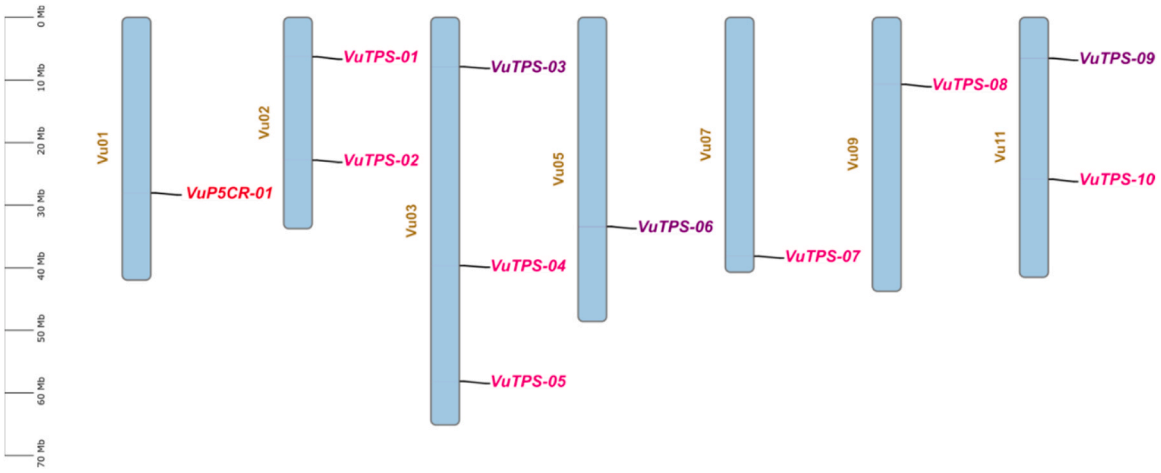

Fig. 4. Chromosomal mapping of *Vu*P5CR and *Vu*TPS *genes*. The names in purple and pink indicate the TPS sequences belonging to Class I and Class II, respectively.

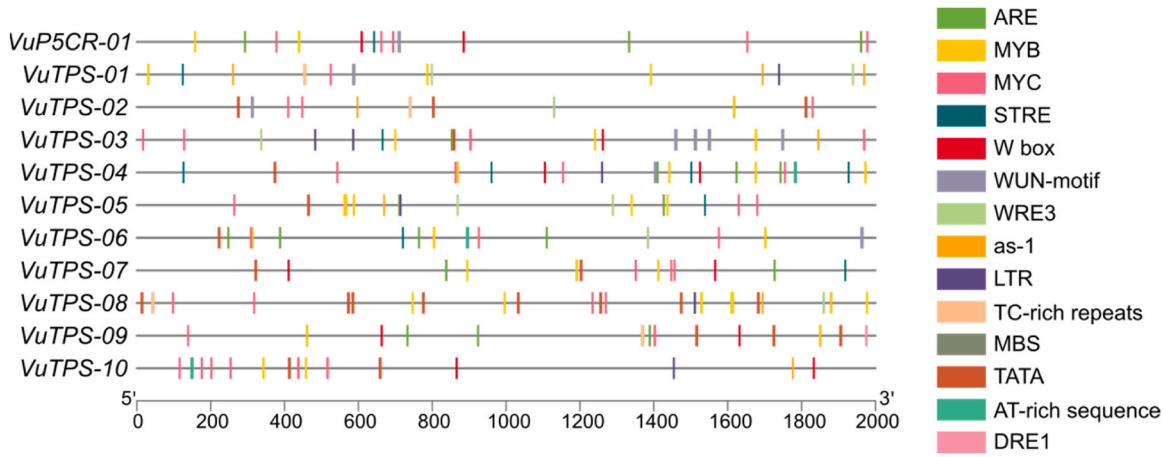

Fig. 5. Distribution of cis-regulatory elements (CREs) found in the *Vu*P5CR and *Vu*TPS promoters of *V. unguiculata*, responsive to biotic and abiotic stresses, identified by the PlantCARE database.

weight of the shoot and root of cowpea plants (Barros et al., 2021a).

Cowpea plants, when cultivated under water stress and/or temperature increase, are observed to rapidly close their stomata, reducing leaf transpiration, resulting in increased leaf temperature, as observed by Barros et al. (2021b). In addition to physiological responses, plants activate enzymatic defense mechanisms, which are correlated with tolerance to water deficit and high temperature (Barros et al., 2021a; Barros et al., 2021b). In the temperature regime (24.8–30.8–37.8 °C) higher enzymatic activities of catalase (CAT), guaiacol peroxidase (GPX) and superoxide dismutase (SOD) were observed (Barros et al., 2021a). Regarding the influence of water availability, there was a reduction in enzymatic activity with an increase in water availability, in the two phenological phases evaluated, regardless of the temperature regime. The SOD enzyme showed a reduction in activity with the increase in water availability only in the vegetative phase (Phase 1), while plants subjected to different water availability in the pod filling phase (Phase 3) showed an increase in the activity of this enzyme with the increase in water supply.

Changes in enzymatic activities under multiple stress conditions, such as water deficit and high temperatures, may be associated with the activation of plants' oxidative defense mechanisms, causing changes at molecular levels, through gene expression. Plant cells stimulate stress through a complex signaling network involving plant hormones, secondary messengers, signal transducers and transcription regulators. These components coordinate plant growth and development under stress, activating genes that encode proteins and enzymes directly related to stress metabolism (Danquah et al., 2014). This response contributes to the plant's adaptation to the specific stimulus (Casaretto et al., 2016).

These proteins trigger a signaling cascade that results in biochemical and physiological responses, including stomatal closure, reduced cell growth and photosynthetic activity, and reduced respiration. When a plant faces stress, it prioritizes the synthesis of proteins related to the stress response (Yamaguchi-Shinozaki; Shinozaki, 2007).

Understanding these plant responses to abiotic stresses is an important step in the development of cultivars with greater tolerance/adaptation to adverse environments. Genes and proteins induced in response to water-deficit may promote a tolerance to dehydration through osmotic adjustment (Nepomuceno et al., 2000). These genes were characterized due to their importance in the synthesis of organic compounds, called osmoprotectors.

During periods of water deficit, specific genes are activated to protect cells against water shortages. They promote the production of important metabolic proteins and adjust osmotic potential, ensuring the maintenance of cellular turgor and regulating target genes (Todaka et al., 2012). These metabolic proteins are involved in the synthesis of glutamine, which plays a crucial role in nitrogen metabolism. It catalyzes the condensation of ammonium and glutamate into glutamine using ATP molecules. Additionally, glutamine regulates proline concentrations, an osmoprotectant that helps adjust cellular osmotic pressure, detoxify

Table 5
Relative expression via RT-qPCR of *P5CR* and *αTPS6* in cDNAs during the vegetative growth phase of cowpea plants cv. Carijó under abiotic stresses.

F1C1 25% x F1C1 100%				
Gene	**Relative expression**	**IF**	**95% CI**	**P(H1)**
P5CR	1200 (NS)	0.628 – 2.344	0.413 – 3.554	0.402
αTPS6	0.847 (NS)	0.348 – 1.883	0.239 – 4.685	0.559
F1C1 50% x F1C1 100%				
Gene	**Relative expression**	**IF**	**95% CI**	**P(H1)**
P5CR	1088 (NS)	0.557 – 2.343	0.258 – 2.942	0.714
αTPS6	1029(NS)	0.423 – 2.317	0.220 – 5.057	0.939
F1C1 75% x F1C1 100%				
Gene	**Relative expression**	**IF**	**95% CI**	**P(H1)**
P5CR	0.793 (NS)	0.427 – 1.635	0.249 – 2.401	0.320
αTPS6	0.668 (NS)	0204 – 2.396	0.092 – 7.249	0.344
F1C2 25% x F1C2 100%				
Gene	**Relative expression**	**IF**	**95% CI**	**P(H1)**
P5CR	2913 (UR)	1.743 – 4.825	1.324 – 6.737	0.000
αTPS6	3258 (UR)	1.302 – 6.661	0.685 – 10.501	0.002
F1C2 50% x F1C2 100%				
Gene	**Relative expression**	**IF**	**95% CI**	**P(H1)**
P5CR	1722(UR)	1.029 – 2.952	0.610 – 4.444	0.012
αTPS6	1382 (NS)	0.558 – 3.813	0.326 – 8.325	0.328
F1C2 75% x F1C2 100%				
Gene	**Relative expression**	**IF**	**95% CI**	**P(H1)**
P5CR	0.865 (NS)	0.467 – 1.580	0.312 – 2.425	0.462
αTPS6	1329 (NS)	0.832 – 2.383	0.345 – 3.229	0.185

F1= Vegetative phase; C1= Chamber 1 (20–26–33 °C); C2= Chamber 2 (24.8–30.8–37.8 °C); NS= not significant; UR= up-regulated; IF= standard error; CI= confidence interval.

Table 6
Relative expression via RT-qPCR of the *P5CR* and *αTPS6* genes in cDNAs of the filling phase of cowpea cv. Carijó under abiotic stresses.

F3C1 25% x F3C1 100%				
Gene	**Relative expression**	**IF**	**95% CI**	**P(H1)**
P5CR	1.301 (NS)	0.836 – 1.938	0.726 – 2.369	0.052
αTPS6	1.227 (NS)	0.654 – 2.390	0.406 – 4.173	0.368
F3C1 50% x F3C1 100%				
Gene	**Relative expression**	**IF**	**95% CI**	**P(H1)**
P5CR	0.827 (NS)	0.593 – 1.071	0.485 – 1.178	0.054
αTPS6	1.529 (NS)	0.917 – 3.116	0.617 – 4.477	0.064
F3C1 75% x F3C1 100%				
Gene	**Relative expression**	**IF**	**95% CI**	**P(H1)**
P5CR	0.837 (NS)	0.569 – 1.140	0.489 – 1.654	0.163
αTPS6	1.019 (NS)	0.519 – 2.083	0.372 – 3.244	0.931
F3C2 25% x F3C2 100%				
Gene	**Relative expression**	**IF**	**95% CI**	**P(H1)**
P5CR	1.267 (UR)	0.952–1672	0.751 – 1.953	0.031
αTPS6	1.110 (NS)	0.632 – 2.417	0.234 – 2.910	0.679
F3C2 50% x F3C2 100%				
Gene	**Relative expression**	**IF**	**95% CI**	**P(H1)**
P5CR	0.939 (NS)	0.608 – 1.376	0.496 – 1.624	0.647
αTPS6	0.606 (NS)	0.245 – 1.638	0.203 – 1.963	0.058
F3C2 75% x F3C2 100%				
Gene	**Relative expression**	**IF**	**95% CI**	**P(H1)**
P5CR	0.812 (DR)	0.660 – 0.977	0.518 – 1.115	0.004
αTPS6	0.330 (DR)	0.169 – 0.681	0.075 – 0.825	0.000

F3= Pod filling phase; C1= Chamber 1 (20–26–33 °C); C2= Chamber 2 (24.8–30.8–37.8 °C); NS= not significant; UR= up-regulated; DR = down-regulated; IF= standard error; CI= confidence interval.

reactive oxygen species, preserve membrane integrity, and stabilize enzymes and proteins (Dar et al., 2016).

It was observed in this study, through bioinformatics analyses, ten *TPS* genes and a *P5CR* gene identified in *Vigna unguiculata*, osmoprotectors that contribute to the tolerance of plants to temperature increase and water-deficit (Hasanuzzaman et al., 2010).

The ten *Vu*TPS genes were subdivided into two groups: Class I and Class II. This difference in the pattern of expression and function between the Class I and Class II *TPS* genes may be related to the wide variation in exon-intron structures. Hu et al. (2020) found that certain introns are required for alternative mRNA *splicing*, and that these could regulate the structure and function of gene-encoded proteins. Some introns may increase mRNA transcription and transport, leading to distinct patterns of specific tissues and varying levels of expression (Reddy et al., 2013; Baek et al., 2008). The results found are in agreement with previous studies that identified 11 *TPS* genes in each of *the Arabidopsis* and rice genomes, which contain four (AtTPS1, AtTPS2, AtTPS3 and *AtTPS4*) and one (*OsTPS1*) Class I, respectively (Zang et al., 2011; Yang et al., 2012).

The *TPS1* gene is directly related to plant growth and tolerance to stress. In *Arabidopsis*, *TPS1* has been shown to be essential for embryogenic and vegetative growth (Dijken et al., 2004). Trehalose-6-phosphate, synthesized primarily by TPS synthase 1, is considered a signaling molecule of carbohydrate status and is linked to the regulation of gene expression related to adverse environmental stresses (O'Hara et al., 2013; Yadav et al., 2014). Trehalose, a non-reducible disaccharide, carries energy and irreplaceable hydrophilic solute that protects cellular proteins and membranes against adverse environmental stresses such as drought and extreme temperatures (Hu et al., 2020).

A striking example is in "resurrection plants", for example, *Selaginella lepidophylla*, *Myrothamnus flabellifolius* and *Sporobolus* spp., which survive extreme desiccation, where up to 99% of their water has been removed. Under water deficiency, resurrection plants accumulate large amounts of trehalose, reaching levels of up to 10–20% of dry weight, which allows them to resist until they are irrigated again (Gibson et al., 2004). This disaccharide is efficient in a condition of water-deficit, because it stabilizes proteins and cellular structures and/or maintains the turgescence of cells through the balance of osmotic pressure, without compromising cellular processes (Ali and Ashraf, 2011). In addition, it was observed that trehalose provided tolerance to water stress through increased cell membrane stability in *Arabidopsis* (Stolker, 2010).

It is important to mention that this disaccharide is present in root nodules of legumes, and that this concentration correlates with the plant's tolerance to drought (Khater et al., 2018). In cowpeas, Khater et al. (2018) reported that trehalose accumulation contributed to the improvement of growth parameters and provided an increase in photosynthetic pigments in fresh leaf tissues of cowpea plants under drought conditions. Ibrahim and Abdellatif (2016) stated that treatment with trehalose (10 mM) induced tolerance to water stress in wheat plants by increasing total soluble sugar, proline and free amino acids.

In addition to *TPS,* the *P5CR* gene *(*pyrroline-5-carboxylate reductase) was also observed in cowpea plants. The enzyme encoded by this gene is induced especially in water-deficit conditions (Sadhukhan et al., 2014), and is involved in the synthesis of proline, an osmoprotector that allows dehydrated plant cells to tolerate dehydration while maintaining turgor. Considering that *P5CR* plays an important role in the synthesis of proline, several efforts have been made in different species to understand the modifications of this gene in order to obtain plants that can grow in the desert or in areas with excess soil salinity (Verbruggen and Hermans, 2008; Hmida-Sayari, 2005).

Dudziak et al. (2019), observed a significant increase in *P5CR* expression in wheat plants, in response to water stress. According to authors Zegaoui et al. (2017) progressive drought triggers the expression of stress response genes, such as proline synthesis, as observed in this study (Tables 5 and 6). Carvalho et al. (2019) also verified the increase in proline accumulation in cowpea plants submitted to water stress, and that this accumulation varied between the cultivars analyzed, which suggests genotype-specific differences, since a greater accumulation of proline was correlated with stress-tolerant plants (Toscano et al., 2016).

In studies carried out with *Glycine max*, *Petunia hybrida*, *Cicer arietinum* and *T. aestivum* it was observed that the overexpression of genes involved in proline synthesis increased the drought tolerance of these transgenics (De Ronde et al., 2004; Yamada et al., 2005; Bhatnagar-Mathur et al., 2009; Vendruscolo et al., 2007). In addition, it is

worth mentioning that proline can act not only in response to stress, but also as a source of energy, since its oxidation produces 30 ATPs (Kishor and Sreenivasulu, 2014). This action may explain the expression of *P5CR* in phase 3 (Table 4), since in this phase the water demand is lower in the species (Murga-Orrillo et al., 2016).

Both P5CR and TPS protect plants against reactive oxygen species (ROS) and maintain homeostasis by adjusting cellular osmotic pressure (Dar et al., 2016). The increase in ROS also induces the synthesis of proline, which can modulate the expression of the P5CR gene to generate a physiological response (Rejeb et al., 2015). Barros et al. (2021a) observed that an increase of 4.8 °C in air temperature and a reduction in water availability causes an increase in enzymatic activities (CAT, APX and SOD) in cowpea, cultivar Carijó. In wheat plants, trehalose neutralized the negative effects of water stress, acting as a direct and indirect ROS scavenger (Stolker, 2010). Fernandez et al., (2010) proposed that trehalose may be a signaling molecule of abiotic stresses that induces plants to accelerate their production rate of ROS, sending a signal to activate enzymatic antioxidants for their elimination, in order to neutralize the stress oxidative.

The response of plants to abiotic stresses is of great interest for research, and the search for genotypes with better performance in adverse environments has been the focus of several studies (Monteiro et al., 2014, Barros et al., 2021b). When grown under stress, plants are affected by metabolic and physiological damage that impairs their growth and development. The results indicate that the *Vu*P5CR and *Vu*TPS genes play roles in the signaling of hormonal pathways and in the response to biotic and abiotic stresses. Thus, studies aimed at a better understanding of the mechanisms of adaptation of plants to unfavorable growing environments are necessary, especially with constant climate change, which is increasingly common (Fariduddin et al., 2013).

5. Conclusion

Our study provided unprecedented information about the identification and characterization of the *P5CR* and *TPS6* genes in the genome of cowpea cv. Carijó. We report for the first time the expression pattern (RT-qPCR) of these genes in response to increased temperature and water stress according to the scenario of a 4.8 °C increase in temperature as predicted in the IPCC report. The induction and/or repression of these genes indicate their fundamental role in the plant's defense response against abiotic stresses.

Funding

This work was financially supported by Embrapa – Empresa Brasileira de Pesquisa Agropecuária (Grant No. SEG-20.19.03.032.00.02), FAPESB (Grant No. BOL0419/2017), FACEPE (Grant No. BFP-0113–5.01/21) and CNPq (Grant No. PQ-306505/2022–3).

CRediT authorship contribution statement

Juliane Rafaele Alves Barros: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Miguel Julio Machado Guimarães:** Writing – original draft, Investigation. **Roberta Lane de Oliveira Silva:** Writing – review & editing, Software, Methodology, Data curation, Conceptualization. **Jessica Barboza da Silva:** Software, Methodology, Formal analysis. **Agnes Angélica Guedes de Barros:** Software, Methodology, Data curation. **Francislene Angelotti:** Investigation, Conceptualization. **Natoniel Franklin de Melo:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2024.105821.

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