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Source: Canadian Journal of Plant Science, 102(6) : 1151-1163

Published By: Canadian Science Publishing

URL: https://doi.org/10.1139/cjps-2022-0019

Canadian Journal of Plant Science

Article

Genome-wide identification and expression analysis of the polyamine oxidase gene family in soybean

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Abstract

Polyamine oxidases (PAOs) are flavin adenine dinucleotide-dependent enzymes that are involved in polyamine catabolism and play an essential role in growth and developmental processes as well as the response to abiotic stresses. Although the *PAO* gene families have been intensively studied in many plants, the soybean (*Glycine max* (L.) Merr.) *PAO* gene family has not been systematically identified. Here, we identified six *PAO* genes in the soybean genome and named them *GmPAO1–GmPAO6*. The phylogenetic analysis revealed that plant PAO proteins are divided into four classes. GmPAO1 and GmPAO4 belong to class I; GmPAO2, GmPAO5, and GmPAO6 belong to class IV. Similar to most dicotyledonous plants, soybeans do not contain class II. Interestingly, we identified an additional SWIRM-domain *PAO* gene *GmPAO3*, which exists between classes III and IV. *GmPAO3* had a different gene structure and expression. To determine the individual roles of *GmPAOs*, we analyzed their expression levels in various tissues and under abiotic stress. Each *GmPAO* gene can respond in a specific tissue under specific abiotic stress. The data can help to clarify the role of *GmPAOs* in abiotic stress responses in soybean and provide a breeding basis for enhancing soybean tolerance to abiotic stresses.

Key words: soybean, polyamine oxidase, bioinformatics, issue specificity, expression analysis, abiotic stress

Résumé

Les polyamine oxydases (PAO) sont des enzymes qui dépendent du flavine-adénine dinucléotide (FAD). Ces enzymes interviennent dans le catabolisme de la polyamine (PA) et jouent un rôle essentiel dans la croissance et le développement des plantes ainsi que dans leur réaction aux stress abiotiques. Bien qu'on ait étudié de façon intensive la famille des gènes codant les *PAO* chez de nombreux végétaux, ces gènes n'ont pas été systématiquement identifiés chez le soja [*Glycine max* (L.) Merr.]. Les auteurs ont identifié six gènes *PAO* dans le génome du soja, qu'ils ont baptisés *GmPAO1* à *GmPAO6*. L'analyse phylogénétique révèle que les protéines végétales PAO se répartissent en quatre classes. GmPAO1 et GmPAO4 appartiennent à la première, tandis que GmPAO2, GmPAO5 et GmPAO6 appartiennent à la quatrième. Comme c'est le cas pour la plupart des dicotylédones, le soja ne possède pas de protéines de la classe II. Fait intéressant, les auteurs ont identifié un gène *PAO* supplémentaire du domaine SWIRM — *GmPAO3*, situé entre les classes III et IV. La structure et l'expression de *GmPAO3* diffèrent. Pour préciser le rôle de chaque gène *GmPAO*, les auteurs en ont analysé le degré d'expression dans divers tissus et en présence d'un stress abiotique. Chaque gène *GmPAO* réagit à un stress abiotique précis dans un tissu spécifique. Ces données serviront à éclaircir le rôle des gènes *GmPAO* quand le soja subit un stress abiotique et permettront d'améliorer la tolérance de la plante à de tels facteurs. [Traduit par la Rédaction]

Mots-clés : soja, polyamine oxydase, bio-informatique, spécificité des tissus, analyse de l'expression, stress abiotique

Introduction

Soybean (*Glycine max* (L.) Merr.) is an important oilseed crop. However, soybean growth and yield are severely reduced by abiotic stresses, such as drought, salt, and cold (Kidokoro et al. 2015; Valliyodan et al. 2017; Cao et al. 2018). Therefore, it is necessary to understand the environmental adaptability of soybean and enhance its tolerance to abiotic stresses.

Polyamines (PAs) are low-molecular-weight organic cations that are widely present in prokaryotes and eukaryotes (Alcázar et al. 2010; Mattoo et al. 2010). PAs include diamine putrescine (Put), triamine spermidine (Spd), tetramines spermine (Spm), and thermospermine (T-Spm) (Takano et al. 2012; Sobieszczuk et al. 2017). It has been proposed that in plants, these PAs play important roles in various physiological processes, such as growth, development, and the responses to environmental stresses (Rakesh et al. 2014; Heba et al. 2017). Many studies have confirmed that the level of Spm will increase under drought stress in plants. Spm activates antioxi-

dants and promotes reactive oxygen species (ROS) scavenging under drought stress, saving biomolecules and membranes from damage. (Hasan and Jahan 2021). Polyamine oxidase (PAO, EC 1.5.3.11) is an enzyme involved in the PA catabolic process (Tavladoraki et al. 2016; Wang et al. 2019). It is a flavin adenine dinucleotide-dependent enzyme (Wu et al. 2003). Studies on *PAO* genes in a range of plant species have revealed two pathways that catalyze the oxidation of PAs at the secondary amino group: the back conversion (BC) pathway and the terminal catabolism (TC) pathway (Kusano et al. 2008). In the TC pathway, PAOs convert Spm and Spd to N-(3-aminopropyl)-4-aminobutanal and 4-aminobutanal, respectively, together with 1,3-diaminopropane (Moschou et al. 2008; Wang and Liu 2016). In the BC pathway, PAOs can convert Spm (or T-Spm) to Spd and then Put; e.g., all five *Arabidopsis PAO* (*AtPAO1*–*AtPAO5*) genes can catalyze PAs in this way (Tavladoraki et al. 2006; Kamada et al. 2008; Moschou et al. 2008; Fincato et al. 2011). Moreover, both of these pathways can produce H_2O_2 (Fincato et al. 2012).

PAO proteins in plants are usually divided into four subfamilies (Ono et al. 2012; Sagor et al. 2015; Bagni et al. 2001; Yu et al. 2019). Members of subfamily I primarily participate in BC pathway and are located in the cytoplasm; members of subfamilies III and IV also play a role in the BC pathway, and the former is located in the cytoplasm and the latter is located in peroxisomes (Kim et al. 2014). The members of subfamily II are responsible for the final catabolism of PAs and are located in exosomes or vacuoles (Gholizadeh and Mirzagadheri 2020). *PAO* genes are involved in plant responses to abiotic stresses. In maize under high salinity stress, ROS are produced by *PAO* genes activity in the apoplast to sustain leaf growth (Rodríguez et al. 2009).

In soybean, NaCl stress can increase *PAO* genes expression (Fang et al. 2020). Exogenous Spd can reduce the harmful effects of NaCl stress and increase the biomass and GABA content. RNA-seq analysis related to NaCl stress also showed that most of the *PAO* genes were upregulated under NaCl stress (Liu et al. 2019).

The identification of members of the *PAO* gene family has been reported in maize (*Zea mays* L.), *Arabidopsis* (*A. thaliana* (L.) Heynh.), rice (*Oryza sativa* L.), barley (*Hordeum vulgare* L.), tomato (*Solanum lycopersicum* L.), tea (*Camellia sinensis* (L.) Kuntze), and other plants (Yoda et al. 2006; Fincato et al. 2011; Ono et al. 2012; Gholizadeh and Mirzagadheri 2020; Li et al. 2020). However, no systematic identification of the soybean *PAO* gene family has been carried out. In this study, we identified six *PAO* genes (*GmPAO1*–*GmPAO6*) from the soybean genome. We observed *GmPAOs* expression under various abiotic stresses.

Materials and methods

Plant materials and abiotic stress treatment

Glycine max, c.v "Williams 82" was used for all experiments and was grown in the public basic experiment center of Northeast Agricultural University, Harbin, Heilongjiang, China. The soybeans were planted in a mixture of vermiculite and soil (1:1, *v*/*v*), and the growth chamber was set to 24 °C with a photoperiod of 16 h/8 h per day (light/dark). When the unifoliolate leaf was fully developed and the first trifoliolate leaf appeared (at the VC growth stage), the seedlings were transferred to water, a 0.9% (*w*/*w*) (~150 mmol/L) NaCl solution, 20% PEG6000, or 4 °C water for abiotic stress treatment. Primary root, stem, and leaf samples were harvested at 0 h, 2 h, 4 h, 8 h, 12 h, and 24 h after treatment, immediately frozen in liquid nitrogen, and stored at −80 °C. Three independent sets of samples were collected for each time point.

Identification of the *PAO* gene family in soybean and sequence alignment

The protein sequences of five AtPAO*s* in *Arabidopsis* were downloaded from the TAIR database (https://www.arabidopsis.org/), using the *Arabidopsis* protein sequences to identify all candidate *PAO* genes in soybean. Systematic BLASTp (https://phytozome-next.jgi.doe.gov/) searches were performed against the soybean reference genome (https://www.soybase.org/) and Phytozome database (https://phytozome-next.jgi.doe.gov/), based on the published sequences of PAO proteins of *Arabidopsis* and other plants as queries. The screening criteria were $E < e^{-5}$ and protein length > 200 aa (Supplementary Table S1). The candidate genes were confirmed online using Pfam (http://pfam.xfam.org/) and the specific gene name and typical functional domains (Pfam: PF01593) of PAO proteins. Finally, the soybean *PAO* genes were identified according to the position on the chromosome; the setting situation names the *PAO* genes in soybeans in turn. The physical and chemical properties of the GmPAO protein were analyzed. Multiple sequence alignments of soybean GmPAO and corn ZmPAO1 proteins were conducted based on DNAMAN software using default parameter values. The resulting files were visualized through GeneDoc software (Fincato et al. 2012; Hao et al. 2018).

Gene and protein structure analyses

The coding sequence length, number of amino acids, and the chromosomal localization of soybean PAO proteins were obtained from the soybean sequence database. The physical and chemical properties of the putative GmPAO protein sequences, including the molecular formula, the number of amino acids, grand average of hydropathicity (GRAVY) index, instability index, isoelectric point, and molecular weight were obtained from the Expasy website (http://web.expasy.org/cgi-bin/protparam/protparam). The schematic diagram of the exon–intron structure of the *GmPAO* gene was analyzed by GeneStructureDisplayServe2.0 (GSDS). (http://gsds.cbi.pku.edu.cn/index.php). The ExPaSy Online website (https://web.expasy.org/protparam/) was used to predict the secondary structure of the GmPAO protein. Phyre 2.0 tool (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) was used to predict the tertiary structure of the GmPAO protein. The subcellular localization of the GmPAO protein was predicted using WoLF PSORT (http://www.genscript.com/psort/wolf_psort.html). The conserved domains of the GmPAO protein were searched using the Pfam and MEME database, and the resulting files were visualized in TBtools software.

Table 1. Primers used for quantitative real-time PCR.

Gene	Forward primer (5′−3′)	Reverse primer (5′−3′)
GmPAO1	CCGTGTACACCAAAATTTTCCT	TATCAGGTACCTGCCAAAATGT
GmPAO2	TGTGATGCAACAACTCAAGAAG	AGCCTCTCATACACATCATCTG
GmPAO3	GAGGAAGGGTTTAAGTAGTGCT	AATACAGTTAACTGAGCACGGA
GmPAO4	GAATGGCACTACACAATCATCC	GCTTTCGTTTTGGTCTGTAGTT
GmPAO5	GTTGCAAGTTCTGTGATCCTTT	CAGCAATTCCTGATATTCCAGC
GmPAO6	TCAATCTTCACAAAGCAACAGG	CGAAGCTTGTCATACACATCAG
GmActin4	GTTTCAAGCTCTTGCTCGTAATCA	GTGTCAGCCATACTGTCCCCATTT

Table 2. Basic information of the six soybean polyamine oxidase (*GmPAO*) gene.

Gene	Gene ID	Location	Length of CDS (bp)	Size of peptide (aa)	Molecular weight (ku)	Isoelectric point	Instability index	GRAVY
GmPAO1	Glyma.02G018800	Chr02:1 617 837–1 622 858	1158	385	43 293.08	5.6	38.08	−0.181
GmPAO2	Glyma.02G240000	Chr02:42 859 034–42 866 507	1485	494	54 903.13	5.81	35.44	−0.026
GmPAO3	Glyma.07G090100	Chr07:8 434 337–8 436 847	2205	734	80 648.84	6.09	54.54	−0.219
GmPAO4	Glyma.09G227500	Chr09:45 184 915–45 192 522	1398	465	52 175.95	5.18	39.87	−0.274
GmPAO5	Glyma.14G209400	Chr14:47 475 271–47 483 030	1485	494	54 652.72	5.6	37.04	−0.031
GmPAO6	Glyma.18G045100	Chr18:3 897 921–3 904 561	1482	493	54 501.47	5.79	28.32	−0.049

Table 3. Protein secondary structure analysis and subcellular predication of six soybean polyamine oxidase (*GmPAO*) gene.

Gene	Extended strand length (aa)	Extended strand proportion (%)	Beta turn length (aa)	Beta turn proportion (%)	Random coil length (aa)	Random coil proportion (%)	Subcellular location prediction
GmPAO1	139	0.361	79	0.2052	146	0.3792	Vacuole
GmPAO2	202	0.4089	97	0.1964	172	0.3482	Peroxisome
GmPAO3	277	0.3774	125	0.1703	294	0.4005	Peroxisome
GmPAO4	167	0.3591	84	0.1806	192	0.4129	Endoplasmic reticulum
GmPAO5	194	0.3927	90	0.1822	183	0.3704	Endoplasmic reticulum
GmPAO6	215	0.4361	83	0.1684	169	0.3428	Peroxisome

Phylogenetic tree construction

The gene accession number reported in the literature was used to download the PAO proteins' sequence of multiple plants from NCBI (https://www.ncbi.nlm.nih.gov/) and Phytozome (https://phytozome-next.jgi.doe.gov/) (Table S1). The amino acid sequences of these PAOs were determined using MEGA6 software, and a phylogenetic tree was constructed via the MEGA 6.0 software using the neighbor-joining algorithm with a bootstrap support value of 1000 replicates (Tamura et al. 2011). The online site iTOL (http://itol.embl.de/) was used to display the data.

Tissue-specific expression of six *GmPAO*s

The Phytozome database (https://phytozome-next.jgi.doe.gov/) was used to analyze *GmPAO* genes' expression. The *GmPAO* genes' expression data (RPKM values) of various tissues and organs of soybean were downloaded, and the heat map was generated from $\log_{10}$(RPKM) transformed values. HEML1.0 software was used for mapping.

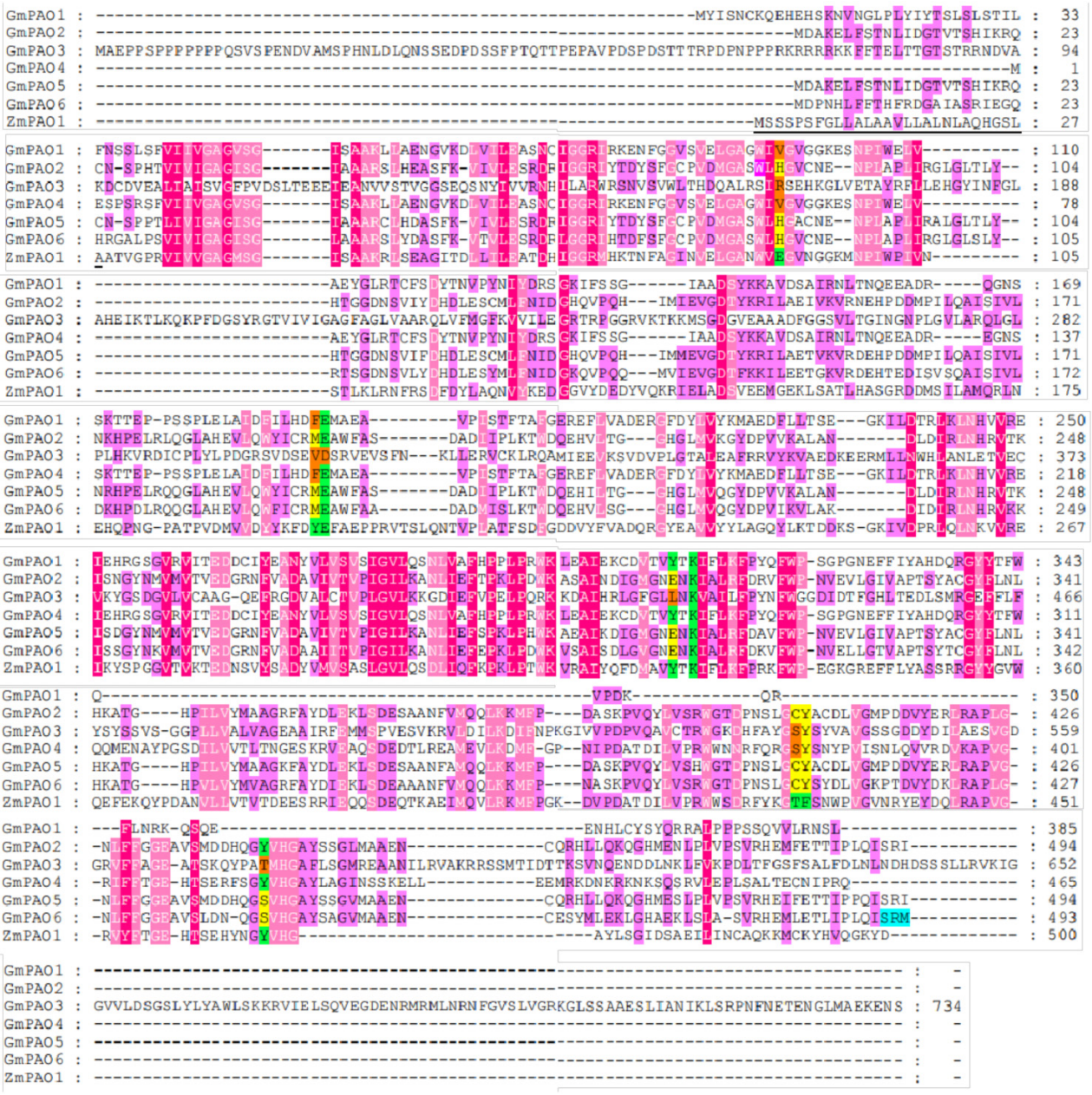
RNA extraction and qrt-PCR analysis

Total RNA was extracted using Trizol reagent, and cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). In this study, all primers were obtained from the qPrimerDB database (shown in Table 1) (Lu et al. 2018). Real-time PCR (rt-PCR) was performed using an ABI7500 instrument and TOYOBO SYBR Green Real-time PCR Master Mix. Quantitative real-time PCR (qrt-PCR) was carried out by initial denaturation at 95 °C for 3 min, 40 cycles at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 5 min. *GmActin4* (*Glyma.12G063400*) served as an internal control (Zhang et al. 2022). The qRT-PCR results were evaluated using the cycle threshold (Ct) values and were calculated using the $2^{-\Delta\Delta Ct}$ method (Czechowski et al. 2005).

Statistical analysis

All of the experiments were performed with at least three biological replicates. To assess the impact of abiotic stress on the expression of *GmPAO* genes, the data were statistically analyzed with a one-way Analysis of variance (ANOVA). Differences were considered statistically significant at $P < 0.05$, and differences were considered highly significant at $P < 0.01$. All analyses were performed using GraphPad Prism 8.0.2.

Fig. 1. Alignment of the amino acid sequences of six soybean polyamine oxidase (GmPAOs) and one maize polyamine oxidase (ZmPAO1) proteins. The signal peptide of ZmPAO1 is underlined. Green boxes indicate the residues of ZmPAO1 protein that are involved in catalytic activity and conserved in the different GmPAO proteins. Conserved residues within GmPAOs are marked with yellow boxes. Non-conserved residues in GmPAOs that are different from those of the PAO catalytic site in ZmPAO1 are marked with orange boxes. In the C-termini of GmPAOs sequences, the putative type-I peroxisomal-targeting signals (PTS1) are indicated with a blue background.

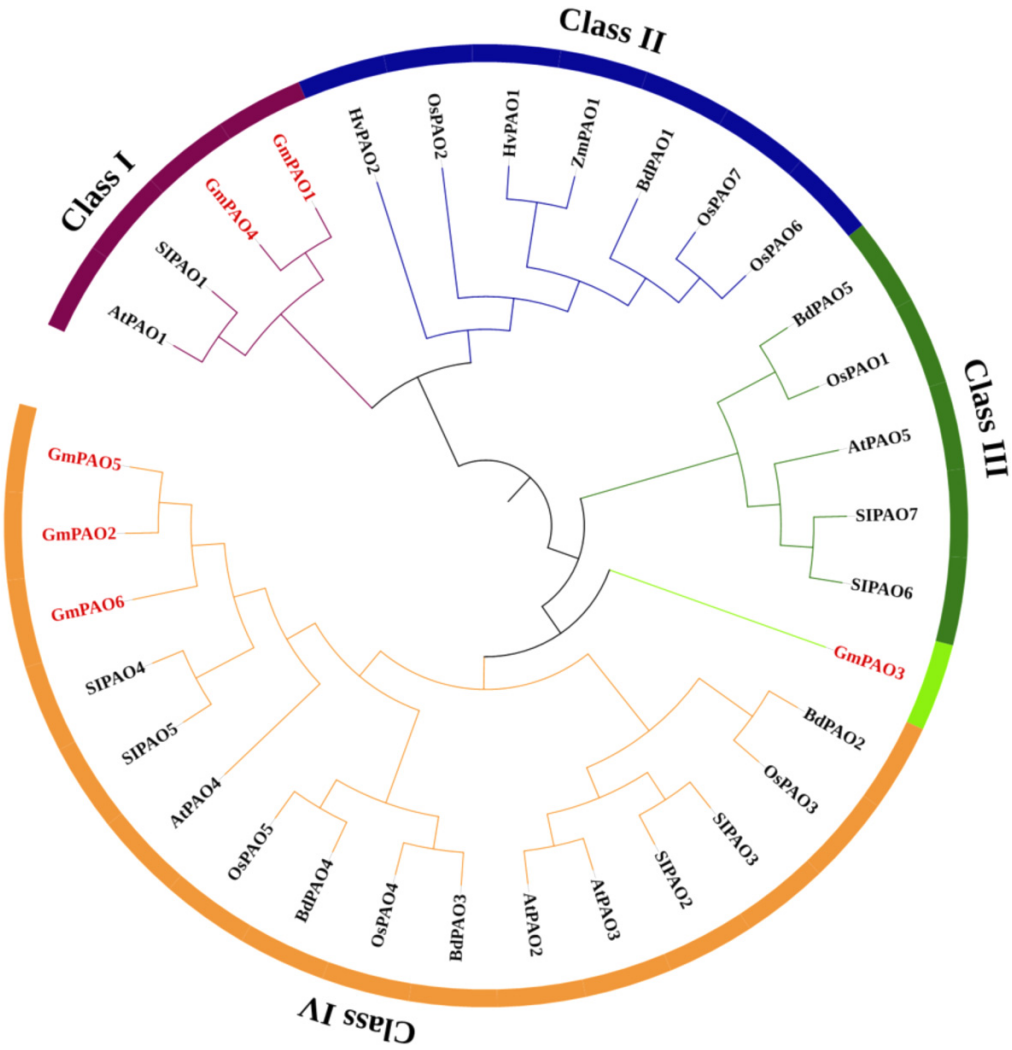


Results

Characterization of polyamine oxidase gene families in soybean

Based on *Arabidopsis* AtPAO proteins, six *PAO* genes were identified in the soybean genome, which were named according to their chromosomal positions, i.e., *GmPAO1–GmPAO6*. Additionally, the physical and chemical properties of the *GmPAO* genes and GmPAO proteins were analyzed using the online website ExPaSy. The encoded proteins of the PAOs ranged from 385 to 734 amino acids, and the predicted molecular weight ranged from 43.3 to 80.6 KDa. The isoelectric point was between 5.18 and 6.09, indicating weak acidity (Table 2). Additional details of these genes are shown in Table 3, including secondary protein structure and subcellular localization predictions. In addition, MEGA6 software was used to multiply the GmPAO and ZmPAO1 alignment of the sequences. The

Fig. 2. Phylogenetic tree of polyamine oxidase (PAO) proteins. The coloured branch indicates the total PAO proteins classified into four groups. GmPAO3 protein exists between class III and class IV, and it was more closely related to class III, but it falls outside of class III and class IV.



results showed that GmPAO members and ZmPAO1 had different sequence homologies, and the GmPAO members also showed differences. The analysis also predicted the presence of peroxisome-targeting sequences in GmPAO6 (Fig. 1).

Phylogenetic analysis of *GmPAO* genes

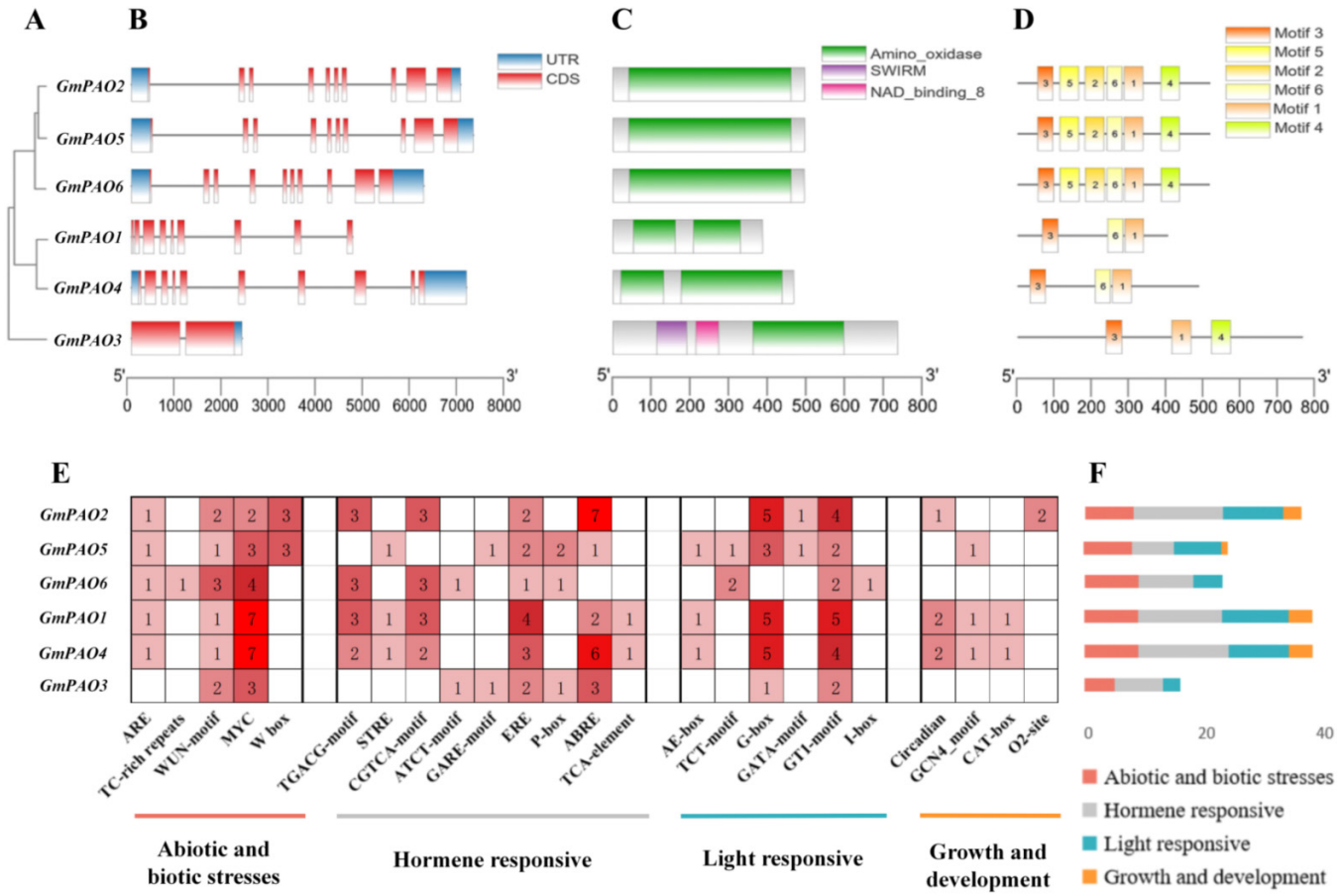
To study the phylogenetic relationship of PAO proteins in different species, a phylogenetic tree was constructed using the neighbor-joining method based on the seven different species (Fig. 2). The full-length amino acid sequences of PAO proteins from maize, *Arabidopsis*, *Brachypodium distachyon* (L.) P.Beauv., rice, barley, and soybean were obtained from the Phytozome. The results showed that all PAO proteins were grouped into four (I–IV) subfamilies in the phylogenetic tree. Among them, class I included two soybean PAO proteins (GmPAO1 and GmPAO4). No GmPAO protein was classified as class II and class III. Class IV included three soybean PAO proteins (GmPAO2, GmPAO5, and GmPAO6). It is worth noting that the phylogenetic analysis placed GmPAO3 protein between class III and IV.

Motif, domain, promoter, and structure analysis of *GmPAO* genes

Phylogenetic analysis of the soybean *PAO* genes alone (Fig. 3A) and corresponding genetic structure analyses using the GSDS website were utilized to reveal the exon and intron structures of *GmPAOs*. The *GmPAO2, GmPAO4, GmPAO5*, and *GmPAO6* genes had 10 exons and 9 introns, and *GmPAO1* had 9 exons and 8 introns, but only two exons (Fig. 3B).

Structural analysis of protein domain for GmPAO1–GmPAO6 demonstrated that all members contained typical amino-oxidase catalytic domain. GmPAO3 protein has an additional SWIRM domain and NAD binding domain. The GmPAO2, GmPAO5, and GmPAO6 proteins had all six motifs, while GmPAO1 and GmPAO4 had motifs 1, 3, and 6. However, the GmPAO3 protein lacked three motifs. Motif 1 and motif 3 were present in all GmPAO proteins (Fig. 3D).

Fig. 3. Bioinformatic analysis of the soybean polyamine oxidase (*GmPAO*) gene family. (A) Phylogenetic tree was generated based on the protein sequences of GmPAOs. (B) Exon–intron structures of *GmPAO* genes, exons, and introns are indicated by red boxes and black lines, and the untranslated regions (UTRs) are indicated by blue boxes. (C) Conserved domains of GmPAOs proteins. (D) Conserved motifs of GmPAOs proteins; each motif is indicated by a colored box. (E) Prediction of *cis*-acting elements within *GmPAO* genes. (F) The number and types of *cis*-acting elements in the promoter regions of *GmPAO* genes.



A large number of light-responsive elements, hormone-responsive elements, and abiotic forced-response elements are distributed in the promoter of *GmPAO* genes (Fig. 3E), suggesting that these genes might play essential functions in light-responsive, hormone-responsive, growth and development, and abiotic and biotic stresses. Furthermore, we also count the number of *cis*-regulatory elements (Fig. 3F). These differences indicate that *GmPAO* was relatively conservative in the evolutionary process, and different classes evolved similar gene structures and structural domain arrangements. *GmPAO3* may have different expression and regulation modes.

These six genes were located on chr 2, chr 7, chr 9, chr 14, and chr 18. A physical map of the location of the *GmPAO* genes on the chromosomes is illustrated in Fig. 4.

Expression profiles of soybean polyamine oxidase genes in diverse tissues

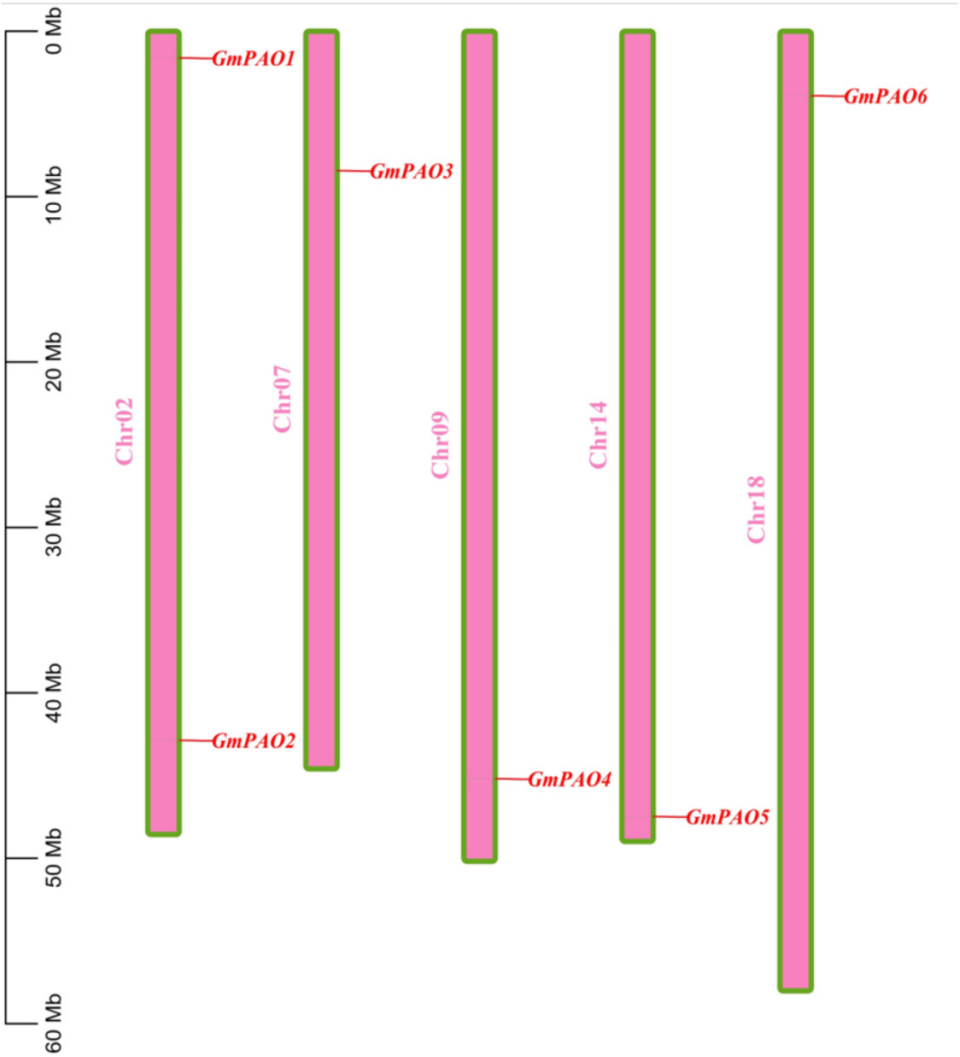
The Phytozome database was used to analyze the expression patterns of *GmPAOs* in different tissues. After transforming the RPKM values using $\log_{10}$, we constructed a heat map for further analysis. As demonstrated in Fig. 5, six *GmPAO* genes were expressed in all tissues, and the expression of *GmPAO* genes varied among the different tissues. However, *GmPAO3* had the most stable expression across the different tissues, while other members were most prominently expressed in flowers relative to other tissues. *GmPAO6* was ubiquitously and highly expressed in all tissues relative to the other *GmPAO* genes, and the expression level of *GmPAO1* in all tissues was relatively low except in the flowers.

The qrt-PCR analysis of the expression of *GmPAO* genes under abiotic stresses

To investigate the expression level of *GmPAO* genes under three abiotic stresses (drought, salt, and low temperature), qRT-PCR was used to measure the expression of the *GmPAO* genes in the roots, stems, and leaves (Figs. 6–8). The qRT-PCR results showed that *GmPAO1, GmPAO4, GmPAO5,* and *GmPAO6* changed significantly under NaCl stress. In roots, *GmPAO4* was upregulated 7-fold under NaCl stress for 8 h. In leaves, *GmPAO4* was also upregulated 12-fold under NaCl stress for 8 h.

Under low-temperature stress (4 °C), almost all *GmPAOs*, including *GmPAO1*, *GmPAO3*, *GmPAO4*, *GmPAO5,* and *GmPAO6*, were upregulated in roots treated for 8 or 12 h, and *GmPAO2*

Fig. 4. Schematic representations of the chromosomal distribution of soybean polyamine oxidase (*GmPAO*) genes on the chromosomes. The names of the *PAO* genes are on the right side; the scale on the left represents the chromosome length.



was upregulated most obviously in leaves. However, *GmPAO3* was upregulated 8-fold in the stems.

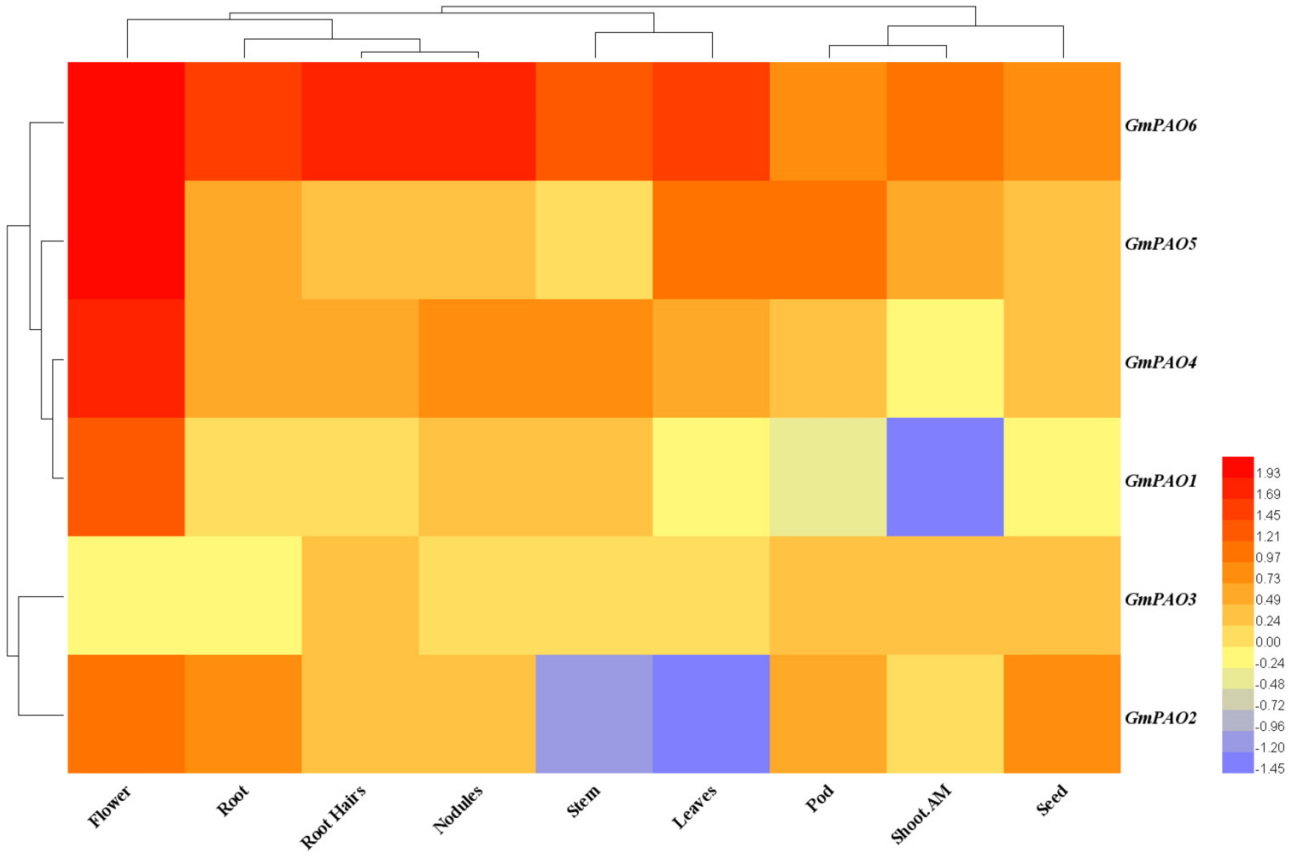
We used 20% PEG6000 to simulate drought stress. Drought stress had a great impact on soybean roots. *GmPAO1* was upregulated 9-fold in roots under drought stress for 4 h, while *GmPAO4* was upregulated 11-fold in roots for 8 h. *GmPAO5* was upregulated 15-fold in roots for 8 h. *GmPAO1* and *GmPAO6* were mainly upregulated in stems, and *GmPAO6* was upregulated up to 5-fold at 8 h under drought stress. In leaves, all the *GmPAOs* changed significantly, and *GmPAO6* was upregulated 34-fold at 12 h under drought stress. The response of *GmPAO* genes to abiotic stress in the stems was not as significant in roots and leaves. *GmPAO3* was upregulated in the stems under low-temperature stress, which was the most obvious at 12 h, i.e., an 8-fold change.

Discussion

Abiotic stresses, including drought, salinity, and cold, can negatively affect soybean growth and yield (Heba et al. 2017; Zhu et al. 2017; Jabeen et al. 2021). Therefore, the identification of tolerant genes to abiotic stress is important for the breeding of abiotic stress-tolerant soybean cultivars. *PAO* genes play a vital role in plant protection against abiotic stresses (Hasan and Jahan 2021). In recent years, the identification of members of the *PAO* family has been reported in multiple species (Yoda et al. 2006; Fincato et al. 2011; Ono et al. 2012; Gholizadeh and Mirzagadheri 2020; Li et al. 2020). However, systematic analysis of the identification, characteristics, and functions of the soybean *PAO* family has not been reported. In this study, six *GmPAO* genes were identified in soybean, and then we used a phylogenetic tree to classify them. Contrary to the previous studies, the six soybean members identified in this study were only included in class I and class IV, while GmPAO3 protein exists between class III and IV, and phylogeny showed that it was more closely related to class III, but it falls outside of class III and IV.

GmPAO1 and *GmPAO4* belong to class I, based on subcellular localization prediction, while the GmPAO1 protein is located in the vacuole, similar to CsPAO6 protein, suggesting that it

Fig. 5. Heat-map of expression levels of soybean polyamine oxidase (*GmPAO*) genes in different tissues. Shoot AM, shoot apical meristem. The red boxes indicate high transcript levels, and blue boxes indicate low transcript levels. The color scale at the right represents the value of $\log_{10}$(RPKM).



may play a role in the regulation of osmotic pressure (Yoda et al. 2006), whereas the GmPAO4 protein is located in the endoplasmic reticulum. These results are consistent with the results of most species for the class *PAO* gene families that catalyze PAs using the BC pathway. Classes II and III PAO proteins have not been identified in soybeans. Three soybean PAO proteins, GmPAO2, GmPAO5, and GmPAO6, belong to class IV. Moreover, based on subcellular localization prediction, GmPAO2 and GmPAO6 proteins are located in the peroxisome, and GmPAO6 has peroxisomal targeting sequences. GmPAO5 is located in the endoplasmic reticulum, and class IV members are involved in the BC pathway (Fincato et al. 2012). Of course, to verify the specific catalytic form of each soybean *PAO* gene family member, biochemical and enzymatic characterizations are still needed.

Tissue expression profile analysis revealed that flower organs were relatively richly expressed, and the expression of class IV was greater than that of class I in soybean, which was similar to that of tomato (Yoda et al. 2006). Except for *GmPAO3*, the other members have similar gene structures. Genes with *GmPAO3*-like structures have also been identified in rice and *Arabidopsis* (Moschou et al. 2012; Ono et al. 2012). The main function of this domain is to catalyze the demethylation of the H3K4 histone lysine, which belongs to the clade of histone lysine-specific demethylases (Moschou et al. 2012).However, due to the apparent lack of class III GmPAO proteins and the unique structure of the *GmPAO3* gene, we hypothesize that the *GmPAO3* gene may act as a coded class III GmPAO protein under undetermined circumstances. Of course, the function of the *GmPAO3* gene needs further study.

In *Arabidopsis*, the AtPAO1 protein belongs to class I; *AtPAO1* gene is mainly expressed during the elongation zone of roots (Fincato et al. 2012). The *PAO1* single mutant and other single mutants were sensitive to salt and drought stress, but the *PAO1 PAO5* double mutant was tolerant to salt and drought stresses, although the exact cause of this phenomenon is unclear (Sagor et al. 2016). GmPAO1 and GmPAO4 proteins belong to class I. Similar to AtPAO1 protein, they are the most sensitive to salt and drought stresses in roots and leaves (Figs. 6 and 8). In tomato, *SiPAO2*, *SiPAO3*, *SiPAO4*, and *SiPAO5* belong to Class IV; all the Class IV members were responsive to cold stress (Hao et al. 2018). GmPAO2, GmPAO5, and GmPAO6 proteins belong to Class IV, and similar to SiPAO2-SiPAO5 proteins, *GmPAO2*, *GmPAO5*, and *GmPAO6* genes also were responsive to cold stress in the roots and leaves.

In conclusion, *GmPAO4* and *GmPAO1* genes had the greatest response in roots and leaves under salt stress. Under low-temperature cold stress, the *GmPAO2* gene was responsive in leaves, and *GmPAO3* was responsive in stems. The most obvious changes in the *GmPAO* genes occurred under drought

Fig. 6. Quantitative real-time PCR analysis of soybean polyamine oxidase (*GmPAO*) genes in response to salt stress. Three independent biological replicates were carried out. Asterisks above bars denote a statistically significant difference by Student's *t* test ($^{*}P < 0.05$ and $^{**}P < 0.01$).

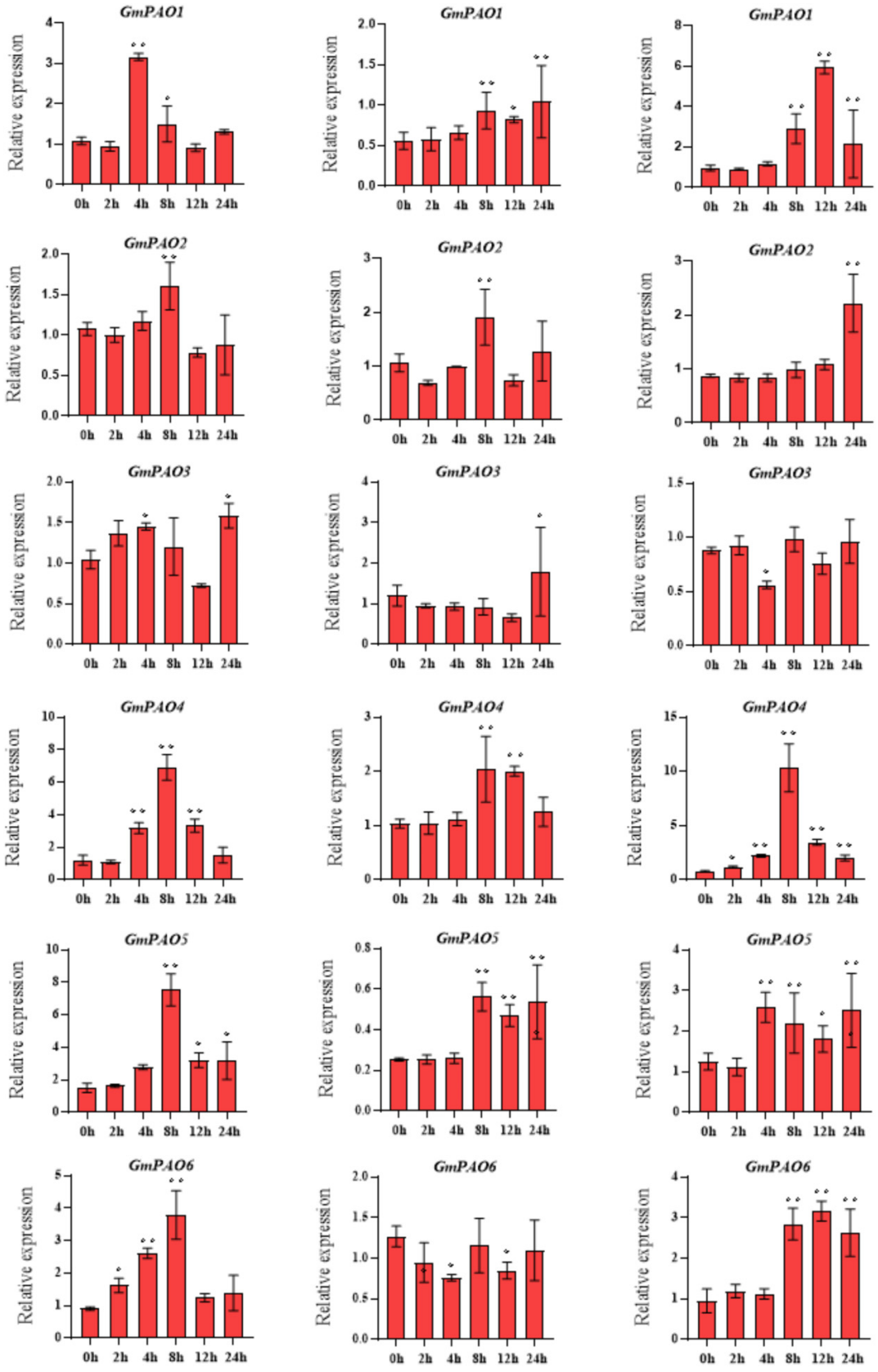


Fig. 7. Quantitative real-time PCR analysis of soybean polyamine oxidase (*GmPAO*) genes in response to low temperature stress. Three independent biological replicates were carried out. Asterisks above bars denote a statistically significant difference by Student's *t* test ($^{*}P < 0.05$ and $^{**}P < 0.01$).

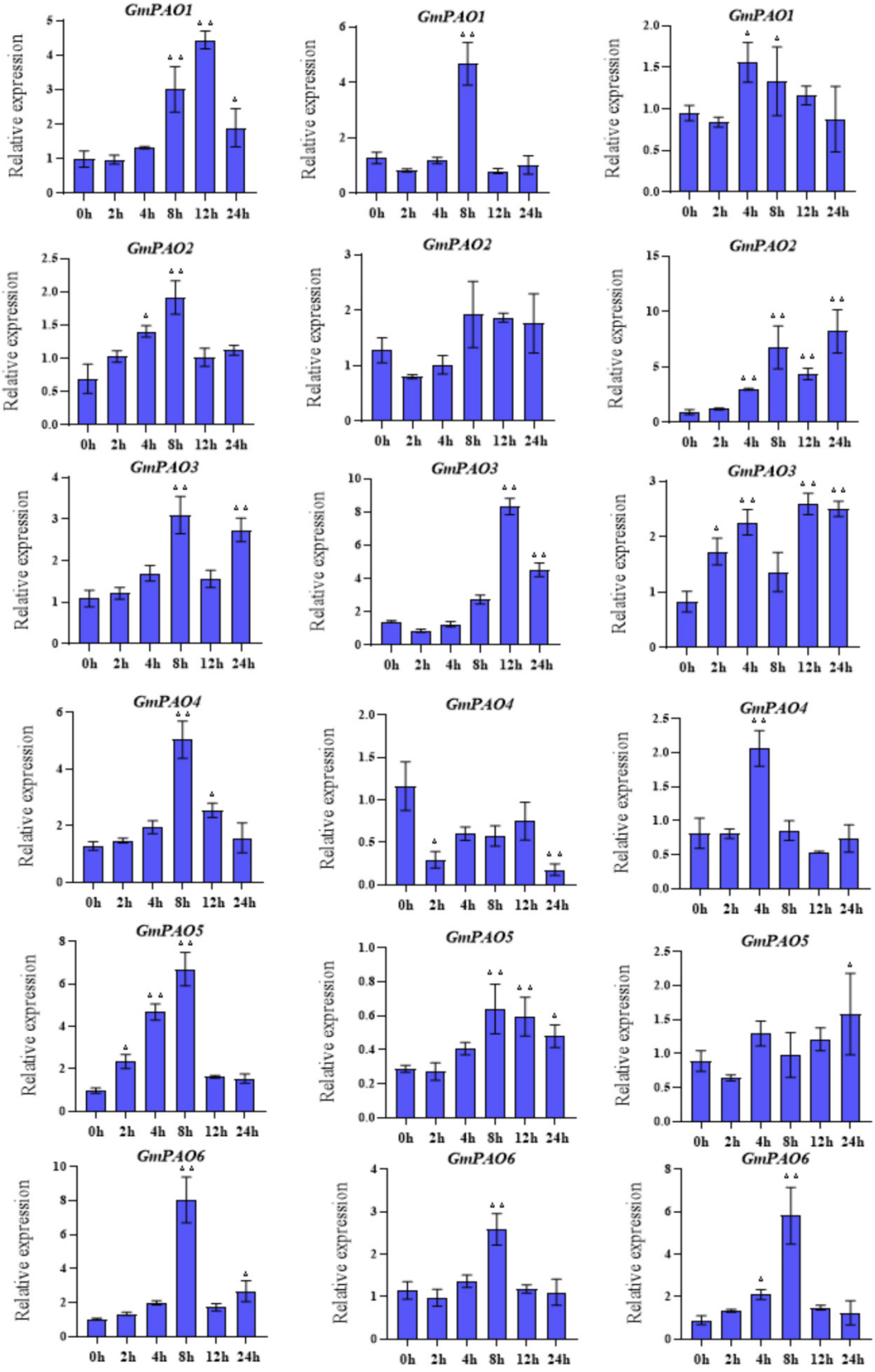
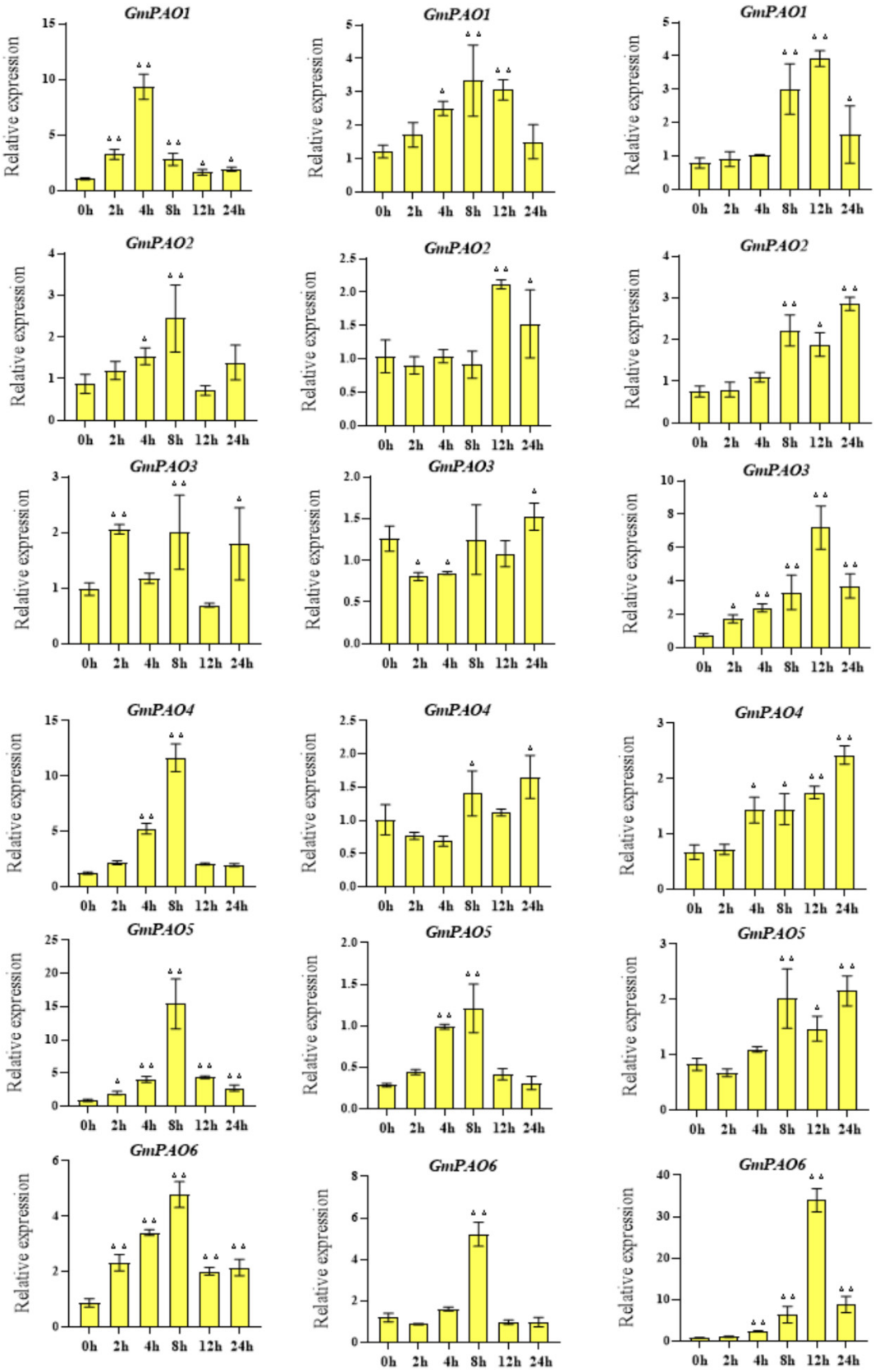


Fig. 8. Quantitative real-time PCR analysis of soybean polyamine oxidase (*GmPAO*) genes in response to low drought stress. Three independent biological replicates were carried out. Asterisks above bars denote a statistically significant difference by Student's *t* test ($^{*}P < 0.05$ and $^{**}P < 0.01$).



stress. Additionally, except for *GmPAO3*, other *GmPAO* genes responded to drought stress in roots, and the response of *GmPAO6* was strongest. To understand the exact mechanism of PAOs, further genetic and biochemical experiments are needed. Our research results provide a reference for further research on the function of *GmPAO* proteins.

Funding

This study was financially supported by the Chinese National Natural Science Foundation (31871650, 31971967, 32001570); the Heilongjiang Provincial Project (GX17B002, C2018016, GJ2018GJ0098, 2019ZX16B01); the National Key R&D Project (2017YFD0101306, 2017YFD0101302); the National Project (2014BAD22B01, 2016ZX08004001-007); the Youth Leading Talent Project of the Ministry of Science and Technology in China (2015RA228); the National Ten-thousand Talents Program; the Postdoctoral Fund in Heilongjiang Province (LBH-Z15017, LBH-Q17015); the national project (CARS-04-PS06); and the "Academic Backbone" Project of Northeast Agricultural University (17XG22). The funding bodies had no role in the study design, data collection, analysis, decision to publish, or preparation of the manuscript.

Article information

History dates

Received: 22 January 2022
Accepted: 29 August 2022
Accepted manuscript online: 10 October 2022
Version of record online: 10 October 2022

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Data availability

All relevant and supporting information files' data are within the paper.

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Author contributions

XZ and YPH conceived and designed the experiments; KWY and CN performed the experiments; KWY and XCZ analyzed the data; KWY, KZZ, YHZ, and NX contributed materials/analysis tools; and KWY prepared the manuscript.

Competing interests

The authors have no conflicts of interest to declare.

Supplementary material

Supplementary data are available with the article at https://doi.org/10.1139/CJPS-2022-0019.

References

Alcázar, R., Altabella, T., Marco, F., and Tiburcio, A.F. 2010. Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. Planta, **231**: 1237–1249. doi:10.1007/s00425-010-1130-0.

Bagni, N., and Tassoni, A. 2001. Biosynthesis, oxidation and conjugation of aliphatic polyamines in higher plants. Amino Acids, **20**: 301–317. doi:10.1007/s007260170046.

Cao, D., Li, Y.Y., Liu, B.H., Kong, F.J., and Lam, S.P.T. 2018. Adaptive mechanisms of soybean grown on salt-affected soils. Land Degrad. Dev. **29**: 4.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., and Scheible, W.R. 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. Plant Physiol. **139**: 5–17. doi:10.1104/pp.105.063743.

Fang, W.W., Qi, F., Yin, Y.Q., and Yang, Z.F. 2020. Exogenous spermidine promotes γ-aminobutyric acid accumulation and alleviates the negative effect of NaCl stress in germinating soybean (*Glycine max* l.). Foods, **9**: 267. doi:10.3390/foods9030267.

Fincato, P., Moschou, P.N., Spedaletti, V., and Tavladoraki, P. 2011. Functional diversity inside the *Arabidopsis* polyamine oxidase gene family. J. Exp. Bot. **62**: 1155–1168. doi:10.1093/jxb/erq341.

Fincato, P., Moschou, P.N., Ahou, A., and Tavladoraki, P. 2012. The members of *Arabidopsis thaliana PAO* gene family exhibit distinct tissue- and organ-specific expression pattern during seedling growth and flower development. Amino Acids, **42**: 831–841. doi:10.1007/s00726-011-0999-7.

Gholizadeh, F., and Mirzaghaderi, G. 2020. Genome-wide analysis of the polyamine oxidase gene family in wheat (*Triticum aestivum* L.) reveals involvement in temperature stress response. PloS ONE, **15**: e0236226. doi:10.1371/journal.pone.0236226.

Hasan, M.M., and Jahan, M.S. 2021. Spermine: its emerging role in regulating drought stress responses in plants. Cells, **10**: 2. doi:10.3390/cells10020261.

Hao, Y.W., Huang, B.B., Jia, D.Y., and Liu, T.B. 2018. Identification of seven polyamine oxidase genes in tomato (*Solanum lycopersicum* L.) and their expression profiles under physiological and various stress conditions. J. Plant Physiol. **228**: 1–11. doi:10.1016/j.jplph.2018.05.004.

Heba, T.E., Nemat, M.H., and Alshafei, M.A. 2017. Exogenous applications of polyamines modulate drought responses in wheat through osmolytes accumulation, increasing free polyamine levels and regulation of polyamine biosynthetic genes. Plant Physiol. Biochem. **118**: 438–448.

Jabeen, Z., Fayyaz, H.A., Irshad, F., Hussain, N., Hassan, M.N., Li, J.Y., and Alsubeie, M.S. 2021. Sodium nitroprusside application improves morphological and physiological attributes of soybean (*Glycine max* L.) under salinity stress. PloS ONE, **16**: e0248207. doi:10.1371/journal.pone.0248207.

Kamada, N.T., Hayashi, M., Fukazawa, M., and Nishimura, M. 2008. A putative peroxisomal polyamine oxidase, AtPAO4, is involved in polyamine catabolism in *Arabidopsis thaliana*. Plant Cell Physiol. **49**: 1272–11282.

Kidokoro, S., Watanabe, K., Ohori, T., and Yamaguchi, S.K. 2015. Soybean DREB1/CBF-type transcription factors function in heat and drought as well as cold stress-responsive gene expression. Plant J. Cell Mol. Biol. **81**: 505–518. doi:10.1111/tpj.12746.

Kim, D.W., Watanabe, K., Murayama, C., and Kusano, T. 2014. Polyamine oxidase5 regulates *Arabidopsis* growth through thermospermine oxidase activity. Plant Physiol. **165**: 1575–1590. doi:10.1104/pp.114.242610.

Kusano, T., Berberich, T., Tateda, C., and Takahashi, Y. 2008. Polyamines: essential factors for growth and survival. Planta, **228**: 367–381. doi:10.1007/s00425-008-0772-7.

Li, M.S., Lu, J., Tao, M.M., Li, M.R., Yang, H., Xia, E.H., and Wan, X.C. 2020. Genome-wide identification of seven polyamine oxidase genes in *Camellia sinensis* (L.) and their expression patterns under various abiotic stresses. Front. Plant Sci. **11**: 544933. doi:10.3389/fpls.2020.544933.

Liu, A., Xiao, Z.X., Li, M.W., and Lam, H.M. 2019. Transcriptomic reprogramming in soybean seedlings under salt stress. Plant Cell Environ. **42**: 98–114.

Lu, K., Li, T., He, J., Chang, W., Zhang, R., and Liu, M. 2018. qPrimerDB: a thermodynamics-based gene-specific qPCR primer database for 147

organisms. Nucleic Acids Res. **46**(D1): D1229–D1236. doi:10.1093/nar/gkx725.

Mattoo, A.K., Minocha, S.C., Minocha, R., and Handa, A.K. 2010. Polyamines and cellular metabolism in plants: transgenic approaches reveal different responses to diamine putrescine versus higher polyamines spermidine and spermine. Amino Acids, **38**: 405–413. doi:10.1007/s00726-009-0399-4.

Moschou, P.N., Sanmartin, M., Andriopoulou, A.H., and Roubelakis, A.K.A. 2008. Bridging the gap between plant and mammalian polyamine catabolism: a novel peroxisomal polyamine oxidase responsible for a full back-conversion pathway in *Arabidopsis*. Plant Physiol. **147**: 1845–1857. doi:10.1104/pp.108.123802.

Moschou, P.N., Wu, J., Cona, A., and Roubelakis-A, K.A. 2012. The polyamines and their catabolic products are significant players in the turnover of nitrogenous molecules in plants. J. Exp. Bot. **63**: 5003–5015. doi:10.1093/jxb/ers202.

Ono, Y., Kim, D.W., Watanabe, K., and Takahashi, Y. 2012. Constitutively and highly expressed *Oryza sativa* polyamine oxidases localize in peroxisomes and catalyze polyamine back conversion. Amino Acids, **42**: 867–876. doi:10.1007/s00726-011-1002-3.

Rakesh, E., Ratilak, E., and Subhash, C.M. 2014. Polyamines and abiotic stress in plants: a complex relationship. Front. Plant Sci. **5**: 175.

Rodríguez, A.A., Maiale, S.J., Menéndez, A.B., and Ruiz, O.A. 2009. Polyamine oxidase activity contributes to sustain maize leaf elongation under saline stress. J. Exp. Bot. **60**: 4249–4262. doi:10.1093/jxb/erp256.

Sagor, G.H.M., Masataka, I., Dong, W.K., and Tomonobu, K. 2015. The polyamine oxidase from lycophyte *Selaginella lepidophylla* (SelPAO5), unlike that of angiosperms, back-converts thermospermine to norspermidine. FEBS Lett. **20**: 3071–3078. doi:10.1016/j.febslet.2015.08.045.

Sagor, G.H.M., Zhang, S.Y., Kojima, S.J., Simm, S.F., and Thomas, B.T. 2016. Reducing cytoplasmic polyamine oxidase activity in *Arabidopsis* increases salt and drought tolerance by reducing reactive oxygen species production and increasing defense gene expression. Front. Plant Sci. **7**: 214. doi:10.3389/fpls.2016.00214.

Sobieszczuk, N.E. 2017. Polyamine catabolism adds fuel to leaf senescence. Amino Acids, **49**: 49–56. doi:10.1007/s00726-016-2377-y.

Takano, A., Kakehi, J.I., and Takahashi, T. 2012. Thermospermine is not a minor polyamine in the plant kingdom. Plant Cell Physiol. **53**: 606–616.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. **28**: 2731–2739. doi:10.1093/molbev/msr121.

Tavladoraki, P., Rossi, M.N., Saccuti, G., and Federico, R. 2006. Heterologous expression and biochemical characterization of a polyamine oxidase from *Arabidopsis* involved in polyamine back conversion. Plant Physiol. **141**: 1519–1532. doi:10.1104/pp.106.080911.

Tavladoraki, P., Cona, A., and Angelini, R. 2016. Copper-containing amine oxidases and FAD-dependent polyamine oxidases are key players in plant tissue differentiation and organ development. Front. Plant Sci. **7**: 824. doi:10.3389/fpls.2016.00824.

Valliyodan, B., Ye, H., and Song, L. 2017. Genetic diversity and genomic strategies for improving drought and waterlogging tolerance in soybeans. J. Exp. Bot. **68**: 8.

Wang, W., and Liu, J.H. 2016. CsPAO4 of *Citrus sinensis* functions in polyamine terminal catabolism and inhibits plant growth under salt stress. Sci. Rep. **6**: 31384. doi:10.1038/srep31384.

Wang, W., Paschalidis, K., Feng, J.C., Song, J., and Liu, J.H. 2019. Polyamine catabolism in plants: a universal process with diverse functions. Front. Plant Sci. **10**: 561. doi:10.3389/fpls.2019.00561.

Wu, T.Y., Yankovskaya, V., and McIntire, W.S. 2003. Cloning, sequencing, and heterologous expression of the murine peroxisomal flavoprotein, N1-acetylated polyamine oxidase. J. Biol. Chem. **278**: 20514–20525. doi:10.1074/jbc.M302149200.

Yoda, H., Hiroi, Y., and Sano, H. 2006. Polyamine oxidase is one of the key elements for oxidative burst to induce programmed cell death in tobacco cultured cells. Plant Physiol. **142**: 193–206. doi:10.1104/pp.106.080515.

Yu, Z., Jia, D.Y., and Liu, T.B. 2019. Polyamine oxidases play various roles in plant development and abiotic stress tolerance. Plants, **8**: 184. doi:10.3390/plants8060184.

Zhang, T.Z., Fang, Q.W., Zheng, J.Q., Li, Z.Y., Li, Y., and Feng, Y. 2022. GmLecRlk, a lectin receptor-like protein kinase, contributes to salt stress tolerance by regulating salt-responsive genes in soybean. Int. J. Mol. Sci. **23**(3): 1030. doi:10.3390/ijms23031030.

Zhu, L.J., Deng, X.J., Zou, L.J., and Lin, H.H. 2017. Enhancement of stress tolerance in cucumber seedlings by proanthocyanidins. Biol. Plant. **61**: 323–332. doi:10.1007/s10535-016-0663-x.