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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>

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

Kuanwei Yu, Chen Na, Xunchao Zhao, Kezhen Zhao, Yuhang Zhan, Ning Xia, Xue Zhao, and Yingpeng Han 

Key Laboratory of Soybean Biology in Chinese Ministry of Education (Northeastern Key Laboratory of Soybean Biology and Genetics and Breeding in Chinese Ministry of Agriculture), Northeast Agricultural University, Harbin 150030, China

Corresponding authors: **Yingpeng Han** (email: hyp234286@aliyun.com) and **Xue Zhao** (email: xuezhao@neau.edu.cn)

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-aminobutanol and 4-aminobutanol, 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 H₂O₂ (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 *AtPAOs* 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')
<i>GmPAO1</i>	CCGTGTACACCAAAATTTTCCT	TATCAGGTACCTGCCAAAATGT
<i>GmPAO2</i>	TGTGATGCAACAACTCAAGAAG	AGCCTCTACACATCATCTG
<i>GmPAO3</i>	GAGGAAGGGTTTAAGTAGTGCT	AATACAGTTAACTGAGCACGGA
<i>GmPAO4</i>	GAATGGCACTACACAATCATCC	GCTTTCGTTTGGTCTGTAGTT
<i>GmPAO5</i>	GTTGCAAGTTCTGTATCCTTT	CAGCAATTCCTGATATTCAGC
<i>GmPAO6</i>	TCAATCTTCACAAAGCAACAGG	CGAAGCTTGTACATACATCAG
<i>GmActin4</i>	GTTTCAAGCTCTGCTCGTAATCA	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
<i>GmPAO1</i>	Glyma.02G018800	Chr02:1 617 837–1 622 858	1158	385	43 293.08	5.6	38.08	–0.181
<i>GmPAO2</i>	Glyma.02G240000	Chr02:42 859 034–42 866 507	1485	494	54 903.13	5.81	35.44	–0.026
<i>GmPAO3</i>	Glyma.07G090100	Chr07:8 434 337–8 436 847	2205	734	80 648.84	6.09	54.54	–0.219
<i>GmPAO4</i>	Glyma.09G227500	Chr09:45 184 915–45 192 522	1398	465	52 175.95	5.18	39.87	–0.274
<i>GmPAO5</i>	Glyma.14G209400	Chr14:47 475 271–47 483 030	1485	494	54 652.72	5.6	37.04	–0.031
<i>GmPAO6</i>	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
<i>GmPAO1</i>	139	0.361	79	0.2052	146	0.3792	Vacuole
<i>GmPAO2</i>	202	0.4089	97	0.1964	172	0.3482	Peroxisome
<i>GmPAO3</i>	277	0.3774	125	0.1703	294	0.4005	Peroxisome
<i>GmPAO4</i>	167	0.3591	84	0.1806	192	0.4129	Endoplasmic reticulum
<i>GmPAO5</i>	194	0.3927	90	0.1822	183	0.3704	Endoplasmic reticulum
<i>GmPAO6</i>	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 *GmPAOs*

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. HEM1.0 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 $2^{-\Delta\Delta C_t}$ 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.

GmPAO1 :	-----MYISNCKQEHEHSKNVGLPLYYITSLSLSTIL :	33
GmPAO2 :	-----MDAKELFSTNLDGTVTSHIKRQ :	23
GmPAO3 :	MAEPPSPPPPPQSVSPENDVAMSPHNLDLQNSSEDPDSFFPTQTTPPEAVFDSPDSTTRPDNPPPRKRRRRKKFFTELTGTSTRNDVA :	94
GmPAO4 :	-----M :	1
GmPAO5 :	-----MDAKELFSTNLDGTVTSHIKRQ :	23
GmPAO6 :	-----MDPNHLFFTHFRDGAIASRIEQ :	23
ZmPAO1 :	-----MSSSPSFGLLALAAVLALLNLAAHQHSSL :	27
GmPAO1 :	FNSSLSPVIVGAGVSG-----ISAARLAEENGVRDLVLEASNCIGGRIRKENFGVSVELGAGNIVGVGGKESNFIWEIV-----	110
GmPAO2 :	CN-SPTTVIVIGAGISG-----IAAARSHEASFK-VIULESRDFIGGRITYDYDFCPDMGASWTHGVCE--NPLAPIRGLGLTLY--	104
GmPAO3 :	KDCDVEALTAISVCFPVDSLTEEBEIANVSVTVGSGSESNYIVVRNHILARFRSNVSVWLTHDQALRSRSEHKGLVETAYRSLLEHGYINEGL :	188
GmPAO4 :	ESPSRSFVIVGAGVSG-----ISAARLAEENGVRDLVLEASNCIGGRIRKENFGVSVELGAGNIVGVGGKESNFIWEIV-----	78
GmPAO5 :	CN-SPTTVIVIGAGISG-----IAAARCFHDASF-KVIULESRDFIGGRITYDYDFCPDMGASWTHGVCE--NPLAPIRGLGLTLY--	104
GmPAO6 :	HRGALPSVIVIGAGISG-----IAAARSYDASF-KVIULESRDFIGGRITYDYDFCPDMGASWTHGVCE--NPLAPIRGLGLSLV--	105
ZmPAO1 :	AATVGPRVIVGAGMSG-----ISAARRSEAGITDLILALATHIGGRVHKTNFACINELGANVVEGVNGGKMNFIWEPV--	105
GmPAO1 :	-----AEYGLRTCFSTYNVPYNIIDRSKRIFSGH-----IAADSYKKAVDASAIRNLNQEEADR-----QGNS :	169
GmPAO2 :	HTGGDNSVIYHDELSCLMNLIDCHQVPHQ-----IMIEVGDTYKRILAEIVKVRNEHPDDMPILQATISIVL :	171
GmPAO3 :	AHEIKTLKQKPFPGSYRGTVIVIGAGFAGLVAARQLVFMGKGVILEERTREGGVRKTKKMSGDVEAAADFGGSVLTGINGNPLGLVARQLGL :	282
GmPAO4 :	-----AEYGLRTCFSTYNVPYNIIDRSKRIFSGH-----IAADSYKKAVDASAIRNLNQEEADR-----EGMS :	137
GmPAO5 :	-----HTGGDNSVIYHDELSCLMNLIDCHQVPHQ-----IMIEVGDTYKRILAEIVKVRNEHPDDMPILQATISIVL :	171
GmPAO6 :	RTSGDNSVLYHDELSYMLNLIDCHQVPHQ-----MVEVGDTYKRILAEIVKVRNEHPDDMPILQATISIVL :	172
ZmPAO1 :	-----STLKLRLNFRSFYLAQNVKEDGVDYEDYVQKRIELADVEEMGEKLSATLHASGRDIDMSILAMQRNL :	175
GmPAO1 :	SKTTEP-PSSPLELALDILHDEEMAE-----VPSTFFAGREFVADERFDYVYKMAEDFLLTSE--GKILTRKLNHVRE :	250
GmPAO2 :	NKHPELRLQGLAHEVQYICRMHAWFAS-----DADIPLKTQDEHVTG--EHLVQGYDVPVKALAN-----LDIRLNHRVTK :	248
GmPAO3 :	PLHKVRDICPLYPDGRSDVSEVDSRVEVSFN-----KLLERVCKLRQAMIEEKSVDPVPLGTALAEFRVYKVAEDKEERMLNWHANLETVEC :	373
GmPAO4 :	SKTTEP-PSSPLELALDILHDEEMAE-----VPSTFFAGREFVADERFDYVYKMAEDFLLTSE--GKILTRKLNHVRE :	218
GmPAO5 :	NRHPELRLQGLAHEVQYICRMHAWFAS-----DADIPLKTQDEHVTG--EHLVQGYDVPVKALAN-----LDIRLNHRVTK :	248
GmPAO6 :	DKHPDLRLQGLAHEVQYICRMHAWFAA-----DADISLKTQDEHVTG--EHLVQGYDVPVKALAN-----LDIRLNHRVTK :	249
ZmPAO1 :	EHQPNG-PATFVDMVLDYKFDYFAEPPRVTSLQNTVPATFSDGDDVYFVDAQRYEAVYVYLAGQYLTDDKS-GKIVPRLQNKVVRE :	267
GmPAO1 :	IEHRGSGVRVITEDDCIYEANYVLVSISIGVLQSNVAFHEPLERKLEAIEKCDTVTKTELKPEYQEW-SGPGNEFFIYAHDRQGYTFW :	343
GmPAO2 :	ISNGYNMVMVTVDGRNEVDAVIVTPIGILKANLIEFEKLEDMKASAINDIGGNEKIALREDKVFWE-NVEVLGIVAPTSYACGLFLNL :	341
GmPAO3 :	VKYSGDGVVCAAG-QEFGCDVALCTVPLGVKKGDEFEVELELQKDAIHRGLFGLINKVAILSPYNFVGDDIDTFGLHTEDLDMRGFFLE :	466
GmPAO4 :	IEHRGSGVRVITEDDCIYEANYVLVSISIGVLQSNVAFHEPLERKLEAIEKCDTVTKTELKPEYQEW-SGPGNEFFIYAHDRQGYTFW :	311
GmPAO5 :	ISDGYNMVMVTVDGRNEVDAVIVTPIGILKANLIEFEKLEDMKASAINDIGGNEKIALREDKVFWE-NVEVLGIVAPTSYACGLFLNL :	341
GmPAO6 :	ISSGYNKMVMVTVDGRNEVDAVITTPIGILKANLIEFEKLEDMKASAINDIGGNEKIALREDKVFWE-NVELLGTIVAPTSYTCGLFLNL :	342
ZmPAO1 :	IKYSPGGVTYKTDNSVYSADYVMVSASLGVLSQDLQFKEKLETKVRATYQFDVAVTKTELKPEYQEW-EGKGREFFLVASSRRGYGVW :	360
GmPAO1 :	Q-----VPDK-----QR-----	350
GmPAO2 :	HKATG-----HPVLVYMAAGRFAYDLKLSDESAANFVQQKKMF-----DASKPVQYVSRGTPNSLCYACDLYGMPDDVYERAPLQ :	426
GmPAO3 :	YSYSSVS-GGPLLVALVAGEAARFMMSPVESVKRVLDIKRDIENPKGIIVPDPQVACTRGRKFAYSYSYVAIGSSGDDYDIAPESVGD :	559
GmPAO4 :	QQMENAYPGSDILVLTITNGESKRVQAQSDTLREAEVLKDMFGP-NIPDATDILVPRWNNRFQRSSYSNYPISNLQVVRDVKAPVG :	401
GmPAO5 :	HKATG-----HPVLVYMAAGRFAYDLKLSDESAANFVQQKKMF-----DASKPVQYVSRGTPNSLCYACDLYGMPDDVYERAPLQ :	426
GmPAO6 :	HKATG-----HPVLVYMAAGRFAYDLKLSDESAANFVQQKKMF-----DASKPVQYVSRGTPNSLCYSYDLYGKPTDVIDYKRAPLG :	427
ZmPAO1 :	QEFKQYPDANVLVLTITDEESRRISQSDQTKAEIMQVLRKMFPGK-----DVPDATDILVPRWNSDRFYKTESNWPVGNNRYEDVQKRAPVG :	451
GmPAO1 :	---FLNRK-QSQE-----ENHLCYSYQRRALPPSSQVVLRLNSL-----	385
GmPAO2 :	-NFFFGGEAVSMDHOGVHGAYSSGIMAAEN-----CQRHLLQKQGHMENLPLVPSVRHEMFETTIPQISRI-----	494
GmPAO3 :	GRNFFACE-ATSKQYPAMHCAFLSCMREANILRVAKRRSSMTIDTTKSYNQENDDLNLKLVKEDLTFGSFALFDLNLNDHSSSLLRVKIG :	652
GmPAO4 :	-RFFFTGE-HTSERFSGVHGAYLAGINSSKELL-----EEMRKDNKRKNKSQSRVLEPLSALTECNIPRQ-----	465
GmPAO5 :	-NFFFGGEAVSMDHOGVHGAYSSGIMAAEN-----CQRHLLQKQGHMENLPLVPSVRHEMFETTIPQISRI-----	494
GmPAO6 :	-NFFFGGEAVSLDN-QGSVHGAYSAGVMAAEN-----CESVMLEKLGHAEKLSLA-SVRHEMLETLIPQISRI-----	493
ZmPAO1 :	-RYFTGE-HTSEHYNGVHG-----AYLSGIDSABEILINCAQKKMKCYHVQGYD-----	500
GmPAO1 :	-----	-
GmPAO2 :	-----	-
GmPAO3 :	GVVLDSGSLYLYAWLSKKRVIELSQVEGDENRMRMLNRNFGVSLVGRKGLSSAAESLIANIKLSRPNFNETENGLMAEKENS :	734
GmPAO4 :	-----	-
GmPAO5 :	-----	-
GmPAO6 :	-----	-
ZmPAO1 :	-----	-

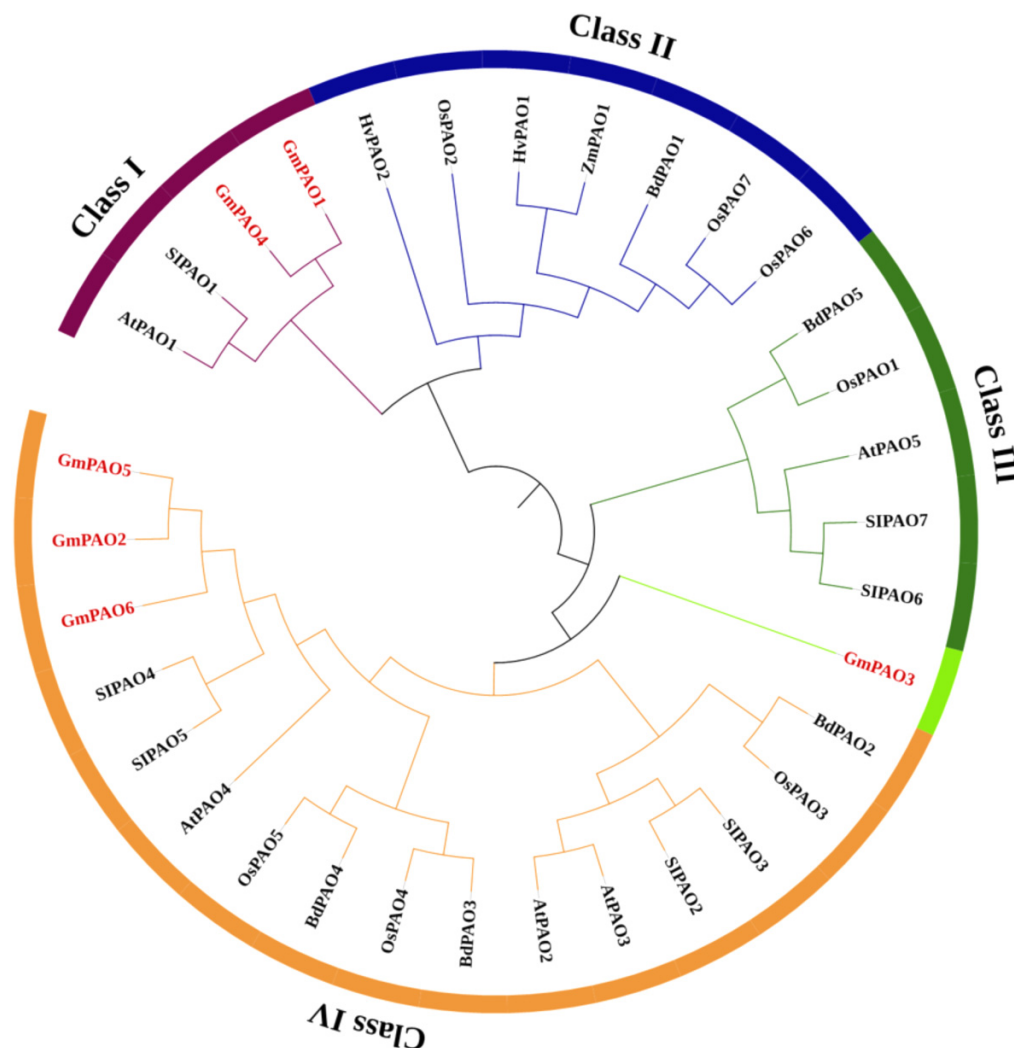
Results

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 Gm-

PAO genes and GmPAO proteins were analyzed using the on-line 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 Phytosome. 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

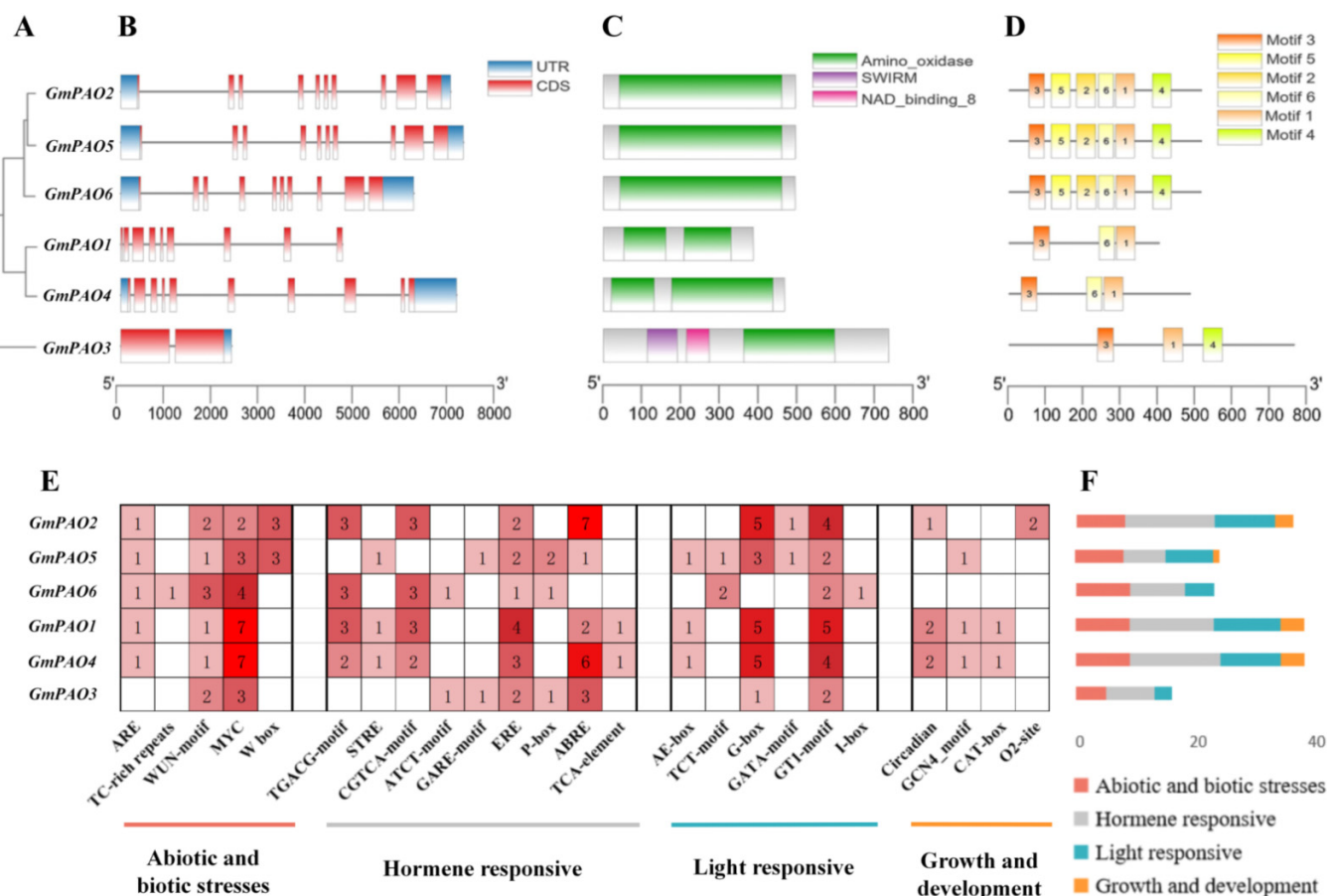
phylogenetic analysis placed GmPAO3 protein between class III and IV.

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*

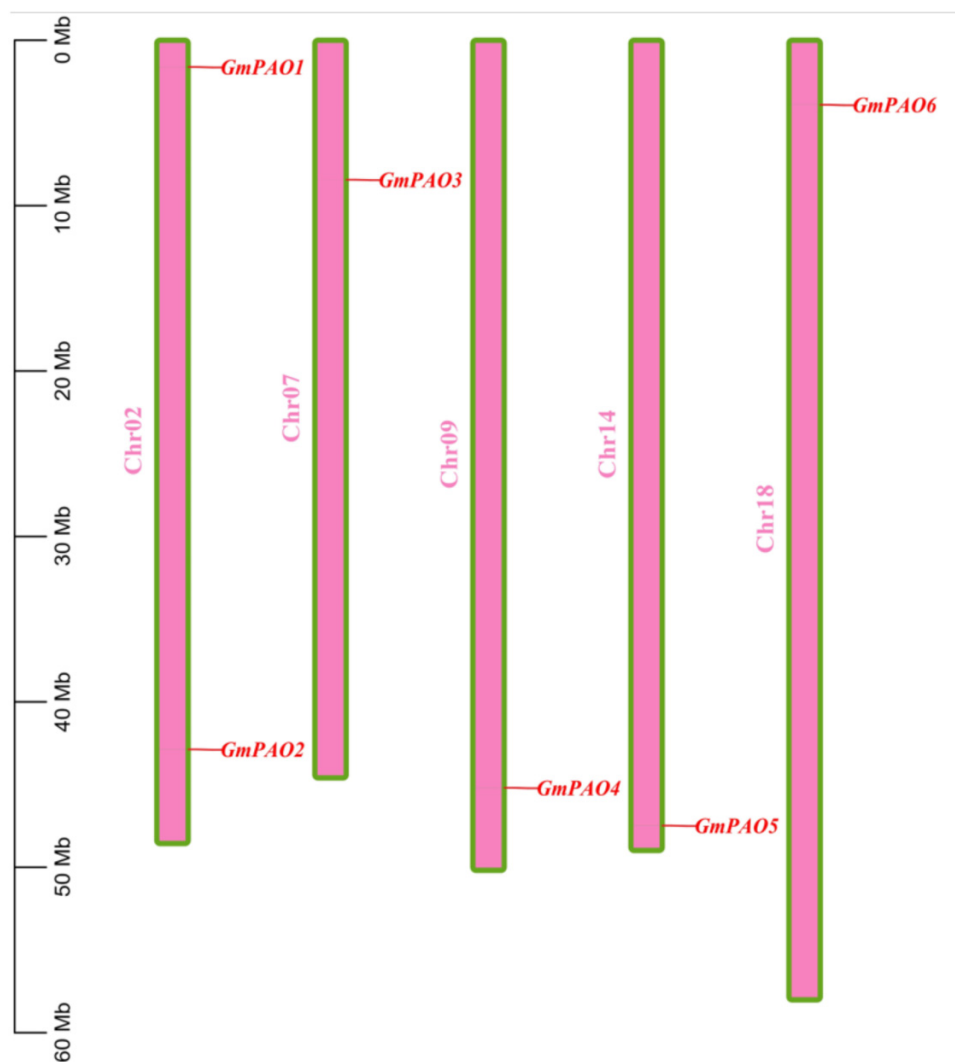
genes were expressed in all tissues, and the expression of *GmPAO* genes varied among the different tissues. However, *GmPAO3* had the most stable expression across the different tissues, while other members were most prominently expressed in flowers relative to other tissues. *GmPAO6* was ubiquitously 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.

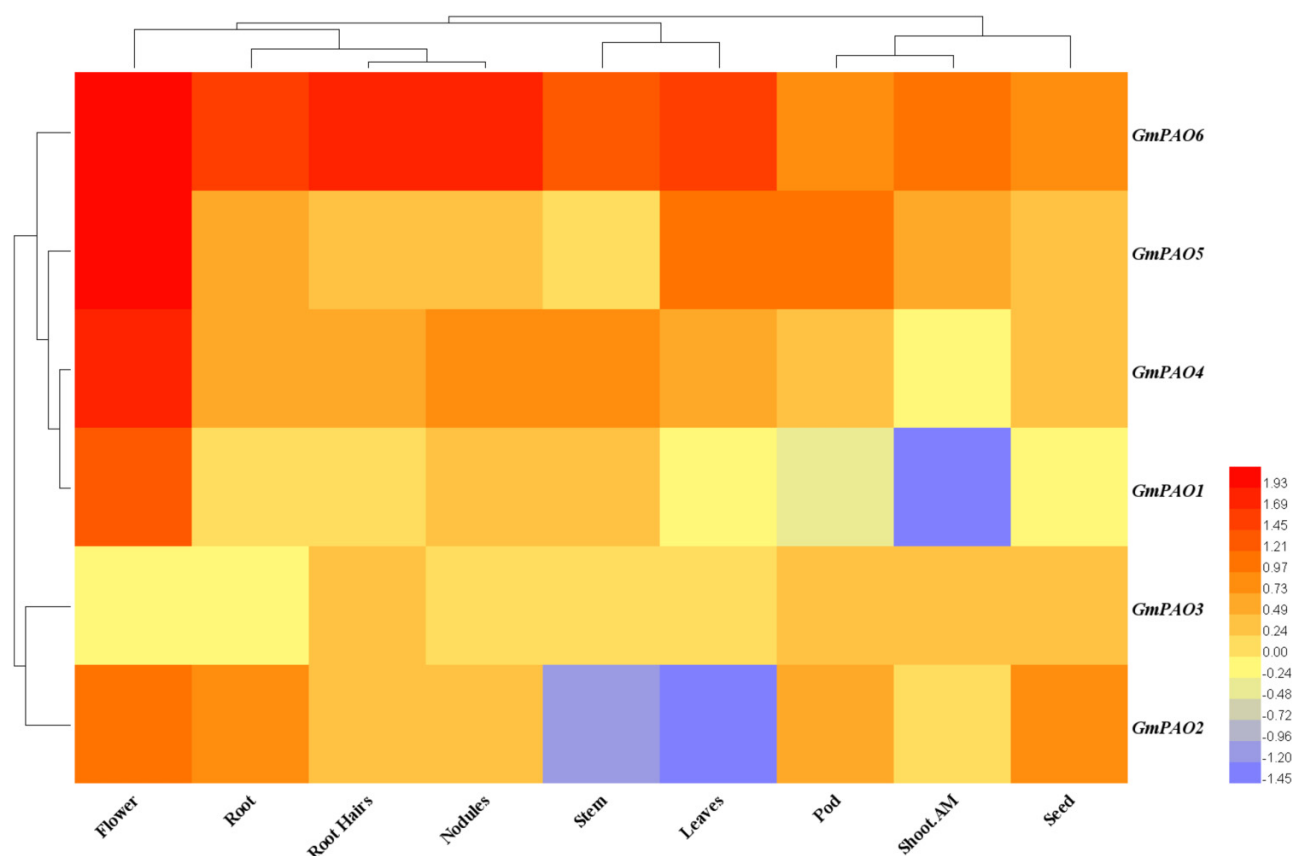
Discussion

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 $\log_{10}(\text{RPKM})$.



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 ** $P < 0.01$).

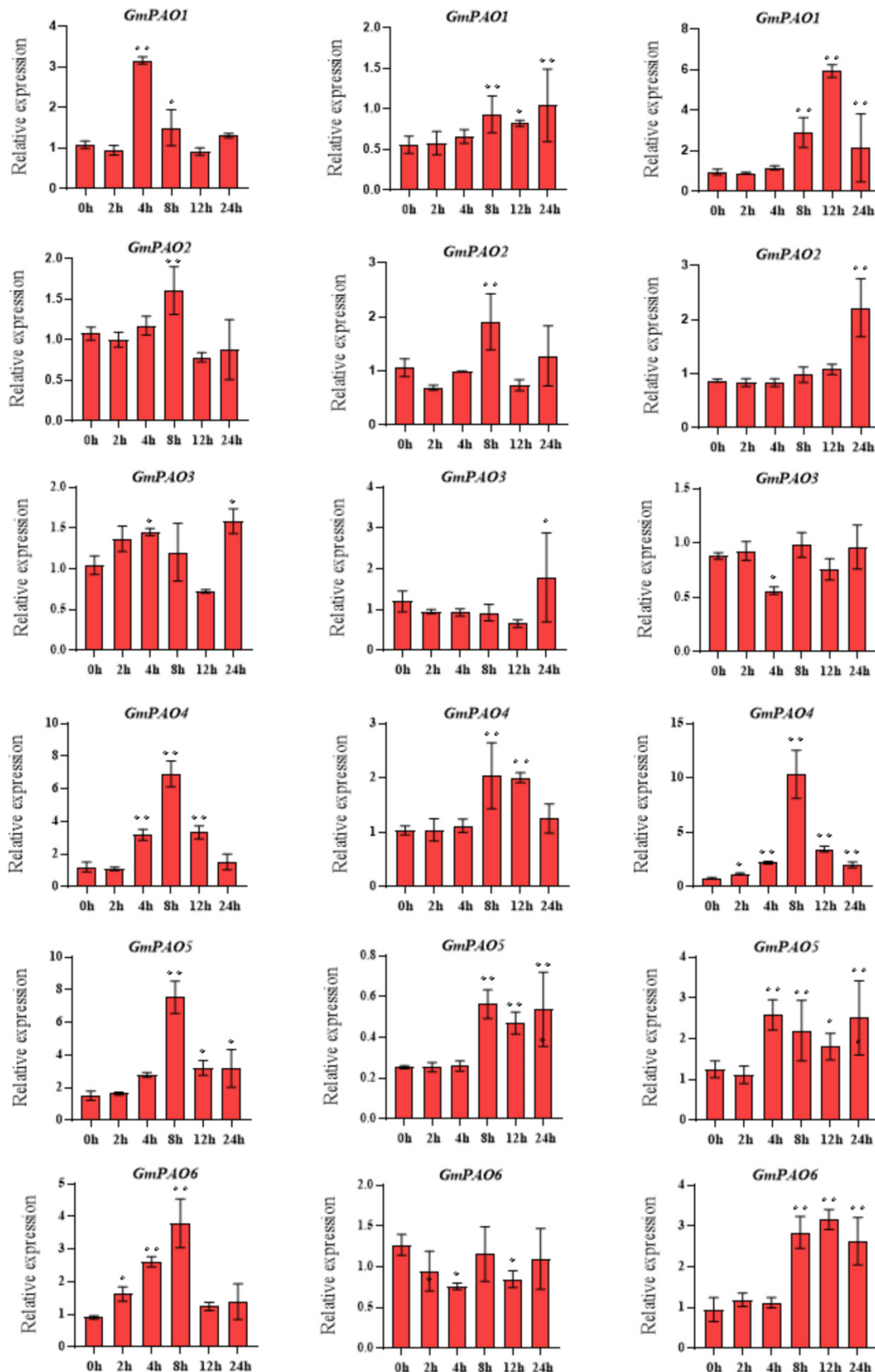


Fig. 7. Quantitative real-time PCR analysis of soybean polyamine oxidase (*GmPAO*) genes in response to low temperature stress. Three independent biological replicates were carried out. Asterisks above bars denote a statistically significant difference by Student's *t* test (**P* < 0.05 and ***P* < 0.01).

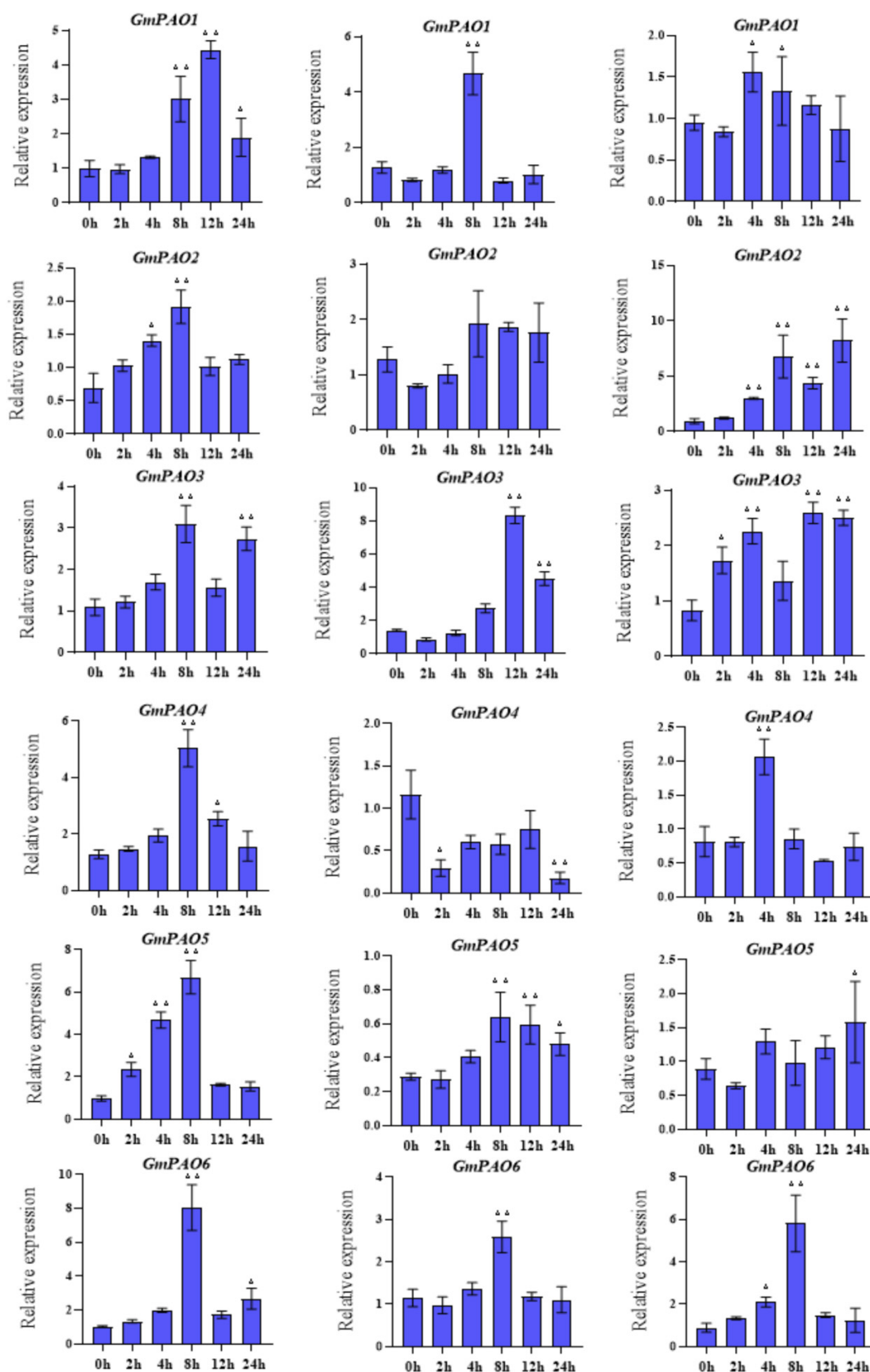
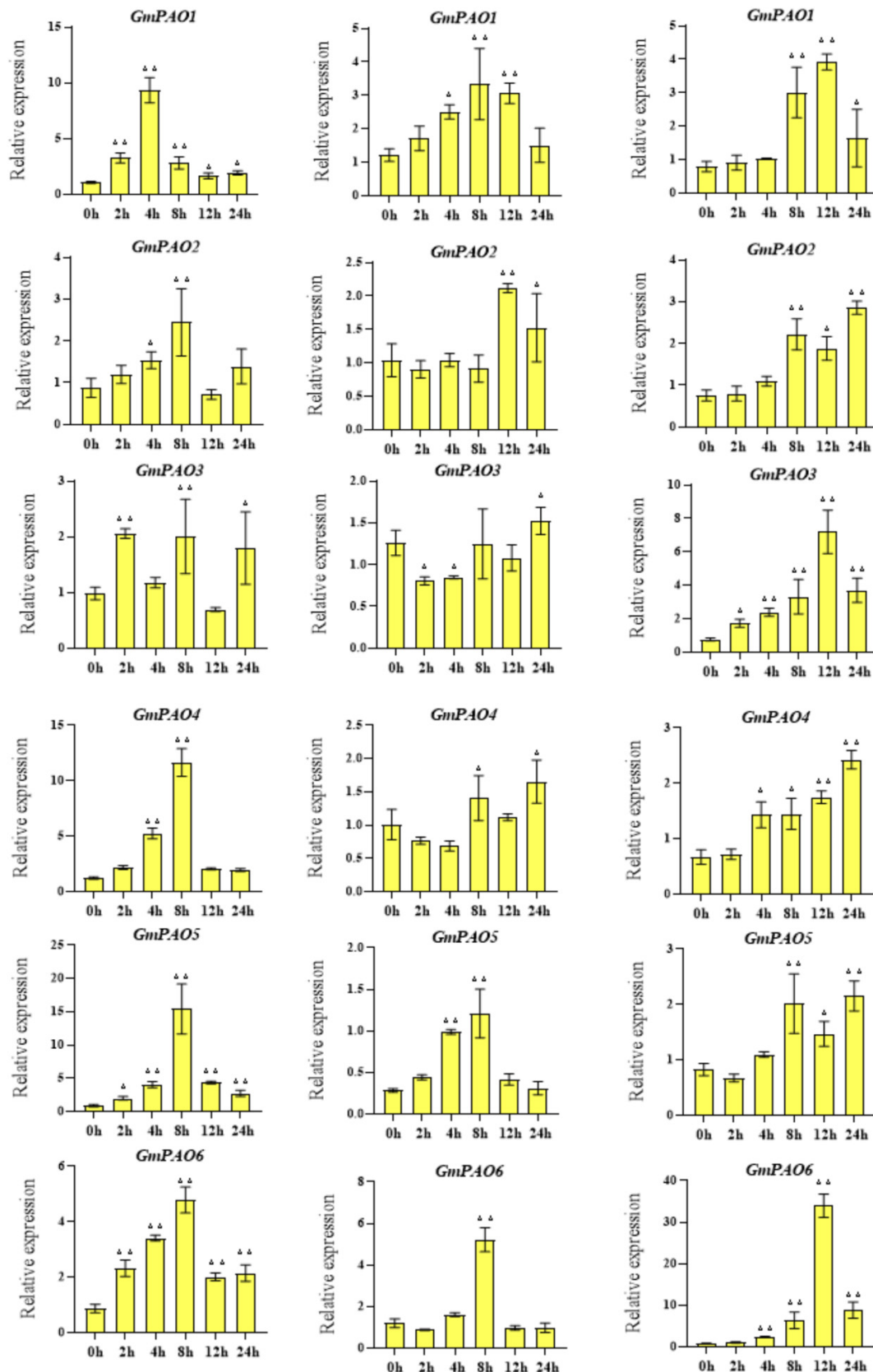


Fig. 8. Quantitative real-time PCR analysis of soybean polyamine oxidase (*GmPAO*) genes in response to low drought stress. Three independent biological replicates were carried out. Asterisks above bars denote a statistically significant difference by Student's *t* test (* $P < 0.05$ and ** $P < 0.01$).



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.

Author information

Author ORCIDs

Yingpeng Han <https://orcid.org/0000-0002-9829-6588>

Author contributions

XZ and YPH conceived and designed the experiments; KKY and CN performed the experiments; KKY and XZ analyzed the data; KKY, KZZ, YHZ, and NX contributed materials/analysis tools; and KKY 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>.

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