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GENOME-WIDE IDENTIFICATION AND ANALYSIS OF MAIZE *PAL* GENE FAMILY AND ITS EXPRESSION PROFILE IN RESPONSE TO HIGH-TEMPERATURE STRESS

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Abstract

Phenylalanine ammonia-lyase (PAL) plays a crucial role in the process of plant growth and stress response by catalyzing L-phenylalanine deamination. Maize (*Zea mays* L.) B73 genome, mRNA and amino acid databases were used here to assess the functional characteristics and expression patterns of the *PAL* gene family. Through genome-wide bioinformatics analysis, 13 members of the *PAL* family were identified, and predictive analysis were carried out considering the chromosomal locations, differentiation profiles, physicochemical properties and secondary structures of their encoded proteins, as well as their structures, conserved motifs, phylogenesis and expression patterns. The results revealed the 13 maize *PAL* (*ZmPAL*) genes that were unevenly distributed on 10 chromosomes and that their differentiation was achieved through tandem and fragment duplication, occurring approximately 6.16-36.09 million years ago. Most *ZmPAL* proteins are acidic proteins, with secondary structures mainly composed of α-helices. Structure and motif analyses revealed high conservation in the intron number in *ZmPAL* genes and in the overall amino acid sequences. Phylogenetic trees exhibited a clear division into six subfamilies, with the largest number of orthologues in maize, sorghum and rice, and a close genetic relationship among them. In addition, the expression levels of the *ZmPAL* genes were different in various tissues under high-temperature, indicating responsiveness to this stress factor. Furthermore, a synchronous increase in the total phenolics and total flavonoids contents was observed in parallel to the increase in PAL activity under high temperature stress. This outcome indicated that the *PAL* genes can respond to high-temperature stress by promoting the synthesis of total phenolics and flavonoids. These results will facilitate future research on the biological functions of *PAL* genes and its exploitation in the production of high-temperature resistant cultivars.

Key words: Maize (*Zea mays* L.), *PAL*, Gene family, Bioinformatics analysis, Gene expression analysis.

Introduction

Global climate change has greatly restrained crop production, as the rising of temperature causes loses derived from plant damage and development (Fang & Xiong, 2015). According to the Global Climate Report of the National Oceanic and Atmospheric Administration (https://climate.nasa. gov/vital-signs/global-temperature/), the surface temperature of Earth in 2018 was 0.8ºC higher than the average registered in the 20th century. The increase in global temperature has led to a decline in the yield of crops (including rice, wheat, and maize) (Lobell *et al.*, 2011; Bita & Gerats, 2013). It can be predicted that high-temperature stress will continue to occur for a long period; hence the problems derived from the low tolerance of crops to high temperatures needs to be tackled on an urgent basis (Horton *et al*., 2015).

Maize is classified as a *C4* crop (cycling CO_2 into four carbon-sugar compounds) with great production potential. Currently, maize is the second world's largest food crop and is likely to become the largest in the near future (Jones, 2009; Ort & Long, 2014). Being a thermophilic crop, maize generally adapts well to high temperatures. However, when the temperature rises above 35ºC, it has an adversely impact on the growth and development of the plant, such as thinning of the leaves, reduced CO_2 assimilation, photosynthetic rate, grain number per spike and grain weight, and pollen abortion, leading to a decrease in final yield (Ren *et al.*, 2019). High-temperature stress also disturbs the cellular physiological activities of maize, resulting in bursting of reactive oxygen species (ROS) in cells, which induces lipid peroxidation, hence a significant reduction in root vitality (Sun *et al.*, 2017; Yu *et al.*, 2017).

Plants have developed different mechanisms to scavenge reactive oxygen species (ROS) in the course of evolution, including enzymatic antioxidants (such as Catalase, Peroxidase, Superoxide dismutase, *etc*.) and non-enzymatic antioxidants (such as polyphenols, flavonoids, carotenoids, *etc*.) (Mittler, 2002). The phenylpropanoid metabolic pathway is one of the three main secondary pathways in plants. L-phenylalanine or tyrosine are used as substrates for phenylalanine ammonia-lyase (PAL) to produce trans-cinnamic acid, which eventually generates coumarin-CoA. Subsequently, coumarin-CoA is used as a substrate in the production of flavonoids, lignins and coumarins (Fraser & Chapple, 2011). As a other polyphenols, flavonoids possesses one or more phenolic hydroxyl groups, which exhibit strong reducibility and can eliminate ROS produced from environmental stresses (Treutter, 2006). In accordance, environmental stimuli were found to regulate *PAL* transcription levels. Members of the PAL family have been found to perform different functions. In Arabidopsis, nitrogen deficiency can specifically induce the expression of *AtPAL1* and *AtPAL2* genes, with the double mutant *PAL1/PAL2* failing to synthesize flavonoids, demonstrating the involvement of PAL in flavonoid production (Rohde, 2004).

As a key rate-limiting enzyme in phenylpropanoid metabolism, *PAL* is encoded by multiple gene orthologues in a single species. The number of orthologues varies in different species ranging from a few (4 in Arabidopsis, 4 in tobacco, and 8 in bananas) to dozens (26 in tomatoes) (Cochrane *et al.*, 2004; Chang *et al.*, 2008; Reichert *et al*., 2009; Yang *et al*., 2019). Members of the plant *PAL* family respond differently to various stresses, and their expression levels vary amongst tissues and developmental stages. In *Arabidopsis thaliana*, PAL enzymes are encoded by four homologous genes: *AtPAL1-4*. In the stem, *AtPAL1*, *AtPAL2* and *AtPAL4* are highly expressed, whereas *AtPAL3* expression is residual. *AtPAL1* is also expressed in vascular tissue, while *AtPAL2* and *AtPAL4* are expressed in seeds (Fraser & Chapple, 2011). Furthermore, the expression of *AtPAL1* and *AtPAL2* in leaves is induced by low temperature and nitrogen consumption (Olsen *et al.*, 2008). There are 12 *PAL* genes in the watermelon genome, of which 11 are expressed mainly in stems, male and female flowers; and 6 are largely or moderately expressed in fruits under control of ethylene (Dong & Shang, 2013). In contrast, few reports on the systematic analysis of the structure and function of the maize *PAL* (*ZmPAL)* gene family have been published to date.

The completion of the sequencing of the maize genome provides a more convenient and effective strategy for identifying and mining important functional genes at the genomic level using bioinformatics (Lu *et al*., 2015; Jiao *et al*., 2017). This study uses bioinformatics tools to predict the subcellular localization, structure, and physicochemical properties proteins of the *ZmPAL* family, thereby providing a theoretical basis for further in-depth research of the biological functions of this gene family and its implications in the breeding of high- temperature resistant variants.

Materials and Methods

Search and identification of *ZmPAL* gene family: Genomic sequences, mRNA and protein sequences were acquired from the maize B73 genomic database (www.ncbi.nlm.nih.gov /genome?term=Zea+mays+). The Bioedit software was used to establish a local database of amino acid sequences of the whole maize genome (Hall, 2011), and TBlastN (E-value = 0. 001) sequence alignment was conducted using Pfam's HMM (HiddenMarkovModel) sequence (PF00221) as a query to blast against the established local database of maize whole-gene cDNA sequences, so that a preliminary screening of *PAL* candidates was obtained. Next, we searched the corresponding amino acid sequences from the protein database, which were then validated using Pfam (http: // pfam. Wust.l edu / hmmsearch. Shtml) and CDD (www.ncbi.nlm. nih.gov/ Structure / cdd / wrpsb.cgi) to check whether they harbor *PAL* domains. Sequences lacking conserved *PAL* domains were discarded, while the sequences harboring PAL domains were used to elaborate a multiple sequence alignment using DNAMAN. Redundant and overlapping sequences were removed and MapInspect was then used to locate the *ZmPAL* gene. "Chr N" (where N is the chromosome number, 1 - 10) indicating the chromosome number where the sequence was located.

Analysis of *ZmPAL* duplication events and *Ka* / *Ks* value: MCScanX software was employed to assess gene duplications (Wang *et al*., 2012), and the TBtools software was used in the calculation of non-synonymous substitution (*Ka*) and synonymous substitution (*Ks*) rates (Chen *et al*., 2018). *Ka / Ks* <1 is an indicator of purifying selection, *Ka / Ks* = 1 of neutral selection, and *Ka / Ks*> 1 of positive selection (Rozas, 2009; Librado & Rozas, 2009). Differentiation time of homologous genes was determined using the formula $T = Ks / 2\lambda$ ($\lambda = 6.5 \times 10^{-9}$) (Zhang *et al.*, 2011; Peng *et al*., 2013).

Analysis of *ZmPAL* phylogenesis and protein structure :Neighbor-joining (NJ) algorithm was employed in the construction of a phylogenetic tree for the *ZmPAL* gene family using MEGA7.0 software(Imran & Liu, 2016; Kumar *et al.*, 2016); the Protparam online software provided by ExPaSy was used to predict and analyze the *ZmPAL* physicochemical parameters (Imran & Liu, 2016); the online software WOLFPSORT (https: // wolfpsort. hgc.jp/) was used to determine protein subcellular localization; SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/ npsa_automat.pl? page = npsa_sopma.html) was applied for the prediction of secondary structures of ZmPAL proteins; and SignalP4.0 Server (http://www.cbs.dtu.dk/services/SignalP-4.0/) was used for the prediction of signal peptides.

Analysis of *ZmPAL* gene structure and conserved motifs :The genome sequences, coding sequences (CDS) and chromosome localization information of *ZmPAL* were obtained from the NCBI maize database and submitted to the Gene Structure Display Server (GSDS) (http://gsds.cbi.pku.edu.cn) to produce a structural visualization of the exons and introns (Guo *et al.*, 2007); the MEME software (http://meme-suite.org/) was used to predict motifs (Ali *et al.*, 2017; Bailey & Elkan, 1995), setting to 15 the total number of motifs, while other parameters were left as default.

Phylogenetic analysis of ZmPAL proteins in different species: We used ZmPAL1 sequence as a probe to search in the NCBI genbank database for homologous sequences in *Amborella trichopoda*, *Arabidopsis thaliana*, *Glycine max*, *Oryza sativa*, *Physcomitrella patens*, *Ricinus communis*, *Sorghum bicolor*, and *Vitis vinifera* and to investigate the phylogenetic relationship of maize PAL proteins with that of other species. These homologous sequences were submitted to Smart (smart.embl-heidelberg.de/) and CDD (www.ncbi.nlm.nih.gov/ Structure / cdd / wrpsb.cgi) programs to check for the presence of PAL domains. Finally, all homologous sequences were aligned in MEGA7.0 to construct a phylogenetic tree using the neighbor-joining (NJ) algorithm (Bootstrap =1000) (Kumar *et al.*, 2016).

Analysis of *ZmPAL* gene expression profiles: Transcriptome data of *ZmPAL* gene expression profiles in different developmental stages (3-leaf stage, pollination stage), different tissues (bracts, filaments, leaves, male flowers, roots, and stems) and different courses of high temperature stress (38ºC, 0h, 2h, 48h) were obtained from the NCBI SRA database (https://www.ncbi.nlm.nih.gov/sra), using the accession number PRJNA520822 (He *et al.*, 2019). The ggplot2 toolbox in language R was used to plot the gene expression heat map.

Preparation of plant materials and high-temperature treatment: The seeds of maize (AnKe 985) were planted in pots (10*15cm) and placed in an artificial light incubator. The condition was set as following: 28 ± 2ºC, 14h light/10h dark, 80% humidity. After 2 weeks, the seedlings (three-leaf stage) were subjected to high temperature treatment, where the temperature was set to 38ºC, while other conditions remained unchanged. Leaf samples were picked at 0h, 2h, 12h, 24h and 48h during treatment, with three biological replicates per sample. Each sample was immediately treated with liquid nitrogen before storage at -80ºC till further use.

RNA extraction and real-time PCR verification: The Trizol method was used for the extraction of total RNA from the samples. The reverse transcription was performed after DNaseI treatment of the RNA solution. ViiA 7 (ABI company, USA) was used for real-time PCR to analyze *ZmPAL* gene expression. The primers used for *ZmPAL* gene verification were listed in Table 1. The real-time PCR procedure was set as follows: a total 20 ul reaction system, including 10 uL of SYBR Green Master Mix reagent, primers (10 umol·L^{-1}) 0.4 uL each, 1 uL of cDNA, 8.2 uL of ddH_2O. The reaction condition was: an initial 95ºC denaturation for 30 s, followed by 40 cycles of 95ºC for 10 s, and 60 ºCduring 30 s. α-tubulin was used as the internal control, and the relative expression levels of the gene were calculated with 2-ΔΔCt [ΔCt = Ct, Target -Ct,Actin1 ;ΔΔCt = ΔC t,treatment - ΔCt,CK]((Livak & Schmittgen, 2001).

Determination of PAL enzymatic activity, total phenolics and total flavonoids content: PAL enzymatic activity assay: 0.5 g of fresh maize leaves were placed in a pre-cooled mortar, 6 ml of 0.1 mol/L (pH 8.8) sodium borate-boric acid buffer and appropriate amount of quartz sand were added, grinded in an ice bath and transferred into a centrifuge tube. After mixing, the mixture was extracted in a refrigerator at 4ºC for 4h. The extract was centrifuged at 10,000 r/min during 20 min at 4ºC and the supernatant was collected. After, 0.1 mol/L_L-phenylalanine prepared in a sodium borate buffer and 2.8 ml of distilled water were mixed with 0.2 ml of enzyme extract. After shaking the sample was placed to react in a 40 ºC water bath for 30min, before being transferred into an ice bath for reaction termination. The OD290 was determined and PAL activity was expressed as units, representing one unit a change in OD290 of 0.01 per hour (Singh *et al*., 2020).

The Folin-Ciocalteu method was used for colorimetric analyses (Chang *et al.*, 2002). Folin-Ciocalteu chromogenic reagent was added after extracting total phenols in phosphate buffer and measuring the absorbance at 765nm. Gallic acid was used as reference to draw a standard curve for the determination of total phenols.

The content of flavonoids was calculated according to Uarrota's methods (Uarrota *et al*., 2014), with minor changes. Briefly, After extracting flavonoids with phosphoric acid buffer, a reaction system of 2 ml was prepared: 100 uL extract, 0.5 ml 95% ethanol, 0.1 ml 1 mol / L potassium acetate, 0.1 ml 10% $AlCl_3$ solution and 1.2 ml distilled water. After mixing, the reaction was left to take place for 30 minutes prior to measuring the absorbance at 415 nm, and the standard curve was drawn using quercetin as a reference.

Table 1 Primer sequences used in quantitative real-time PCR analysis of *ZmPAL*s

Genes name	Forward Primer sequences	Revers Primer sequences
PAL1	GCTCCTACATGAAGCTTGCC	TCGATCGACTTGGTAGCGAA
PAL2	GGATGGTGGAGGAGTACAGG	ATCATGCTGTCCATGACCCA
PAL3	ATCGCCAACAGGATCAAGGA	CAGGAACACCTTGTTGCACT
PAL4	AAGAAGGTCAACGAGCTGGA	GTTGTCGTTGACGGAGTTGA
PAL5	CACATCTTGGAAGGCAGCTC	AGCGGATGACTTCGATCTGT
PAL6	GCTCATCAGGTCAGACCACT	ACTGCGGAAACTAACAATCCA
PAL7	AGACTCTGAGCACCAACTCC	TGCGAAGCTTCTTCATCAGC
PAL8	TGGGCGTGAGAACTTTGTTG	TTAGCCTCGAAGAGCACAGT
PAL9	GAAGAAAGAGCAACGCCACA	AACGCCAAGGAGAAGAGGAG
PAL10	TCCAGAGGGAGCTCATCAGA	CAGAATCTCGAAGCGGATGC
PAL11	GCGAGATCAACTCCGTCAAC	CTCAGAGAACTGCGCAAACA
PAL12	AAGAAGACCCTGAGCACCAA	TGCGAAGCTTCTTCATCAGC
PAL13	ACTGTGCTCTTCGAGGCTAA	GGTCAGGTGGTCGATGTACT
α-tubulin	CAGTGTGCTGCCCTTATCCC	TTAGAAAAACATCCGCCGAC

Results

Identification and chromosomal localization of *ZmPAL* gene family: We used TBlastN tool to retrieve candidate *PAL* genes by blasting against the whole maize genome the amino acid sequences with *PAL* domains. Then multipule sequence alignment was performed to remove repeat sequences. Unique sequences were then detected using Pfam to obtain genes containing *PAL* domains. A total of 13 maize *PAL* members were identified and named from top to bottom according to their positions on the chromosomes (Table 1) (Wei & Pan, 2014). The distribution characteristics of *ZmPAL* on chromosomes were analyzed by MapInspect tool (www.softsea.com/review/MapInspect.html). The 13 *PAL* sequences were unevenly distributed in ten chromosomes, among which five members are found in chromosome 4, four members in chromosome 5, two members in chromosome 2, one member in chromosomes 1 and 8, and no member in the remaining chromosomes (Fig. 1). Among them, *ZmPAL* members in chromosomes 4 and 5 mainly were found to be part of gene clusters.

Analysis of *ZmPAL* gene duplication events and *Ka/Ks* values: Based on the evolutionary relationship between the family members and the chromosomal localization, five gene pairs were found to be involved in gene duplication events, of which two were found as fragment duplications, accounting for 15.38%, and three were found as a tandem duplication, accounting for 23.07%. These findings suggest that both tandem duplication and fragment duplication are responsible for the diversification of the *PAL* family in *Zea mays*. Tandem duplication occurred in higher proportion, suggesting that gene duplication within the same chromosome was the main mechanism involved in the *PAL* family expansion in maize. Meanwhile, it was found that one tandem duplication and one fragment duplication occurred in *ZmPAL5*, whilst two tandem duplications and 1 fragment duplication occurred in *ZmPAL11*, indicating that *ZmPAL5* and *ZmPAL11* were the most active contributors to gene family expansion. Fragment duplication-induced gene differentiation occurred in 10.50-14.94 million years ago, while tandem duplication-induced gene differentiation occurred in 6.16-36.09 million years ago, suggesting that the differentiation time of tandem duplication started earlier than that of fragment duplication and ended later than that of fragment duplication. It can be seen from Table 2 that the *Ka* values of the five replicate gene pairs were lower than the Ks values, suggesting that synonymous substitution was prior to non-synonymous substitution, and the *Ka / Ks* values of the five gene pairs were all less than 1, indicating that these five gene pairs have undergone strong selection, in spite of their low function diversity (Table 2).

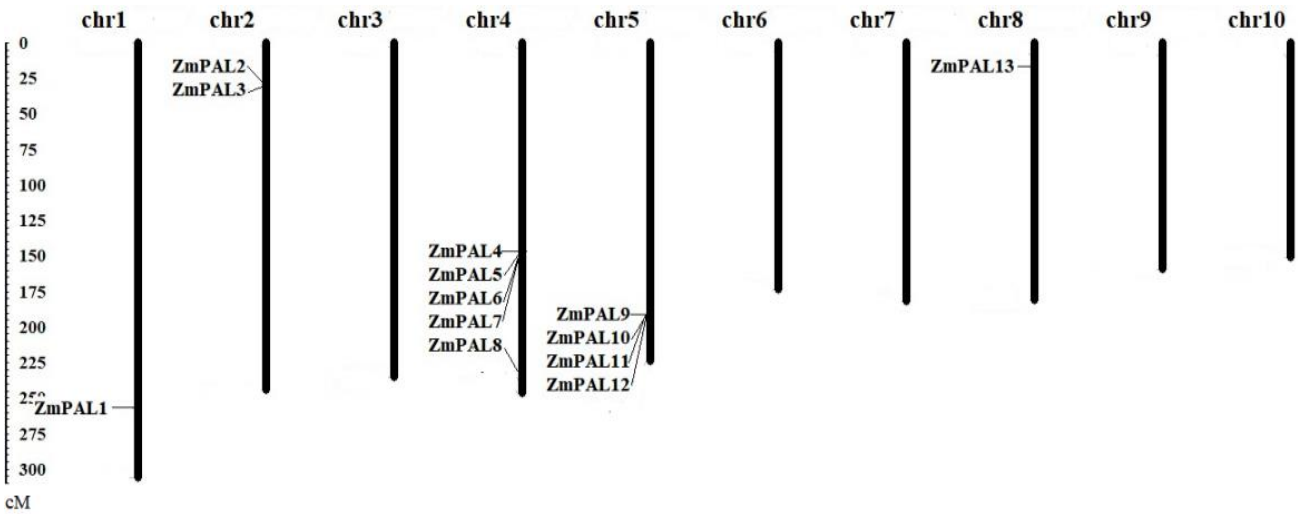

Fig. 1. Chromosomal mapping and gene duplications of *ZmPAL* genes.

Table 2. Analysis of genetic replication events of the *PAL* family in *Zea mays* (L.).

Duplicated genes		*Ka*	*Ks*	*Ka/Ks*	Divergence time (Million years)	Duplicated type
ZmPAL5	*ZmPAL6*	0.0627	0.1264	0.4957	9.72	Tandem duplication
ZmPAL11	*ZmPAL10*	0.0737	0.4692	0.1571	36.09	Tandem duplication
ZmPAL11	*ZmPAL12*	0.0079	0.0801	0.0985	6.16	Tandem duplication
ZmPAL5	*ZmPAL10*	0.0246	0.1366	0.1805	10.50	Segmental duplication
ZmPAL7	*ZmPAL11*	0.0236	0.1942	0.1216	14.94	Segmental duplication

Analysis of ZmPAL protein structures

Analysis of physicochemical properties of ZmPAL proteins: ZmPAL proteins were found to contain 157 to 718 amino acids. Their length differences were large, with molecular weights ranging between 16618.70~77424.43. The isoelectric point of these proteins was between 5.63 - 10.67, where most (12 proteins) falling in the acidic range. Generally, proteins with an instability coefficient greater than 40 are considered unstable proteins. In this study, only ZmPAL13 belongs to this category, while the remaining 12 proteins belong to the stable protein category. Since the hydrophobicity of all the ZmPAL proteins was negative according to total average hydrophobicity analyses, we speculated that these proteins were all hydrophilic (Table 3).

The results of subcellular localization prediction showed that ten proteins were localized in chloroplasts, one in the cytoplasm, one in the plasmodesmata, and one in the peroxisome. The distribution characteristics of *ZmPAL* genes indicated that they might have certain functional diversity, potentially eliminating the excess of ROS in the cells through multiple mechanisms and multi-organelle cooperation, thereby improving stress resistance.

Analysis of protein secondary structures: SOMPA was used to analyze the secondary structures of ZmPAL proteins. From the proportion aspect, it can be seen that α-helix > random coil > extended chain structure > β-turn (except for *ZmPAL13*). The ratio of α-helix accounted about 50 %, while the ratio of β-turn was less than 10 % (Table 4).

Phylogenesis of *ZmPAL* genes and analysis of their structure and conserved motif: As shown in Fig. 2, a rootless phylogenetic tree was constructed based on sequence alignments of all *ZmPAL* genes. According to the phylogenetic tree, the 13 *ZmPAL* genes were divided into three groups, among which group I contained the majority of the members and included six genes *ZmPAL1, ZmPAL3, ZmPAL4, ZmPAL8, ZmPAL9, ZmPAL13*; group II contained *ZmPAL2, ZmPAL5, ZmPAL6, ZmPAL10* ; and Group III had the smallest number of genes, including *ZmPAL7, ZmPAL11, ZmPAL12*.

Gene Structure Display Server online software was used to draw the structure map of *ZmPAL* genes. As shown in Fig. 2, there are minor differences in the numbers and lengths of the 13 *PAL* introns and UTRs. *ZmPAL*9 contained one CDS, two UTRs, and no intron; *ZmPAL13* had only one CDS, while the remaining *ZmPAL* genes had only one CDS and one intron. These findings revealed that the functions of *ZmPAL9* and *ZmPAL13* might have undergone more diverse functions.

Table 3. The physicochemical properties and subcellular localization of PAL in *Zea mays* (L.).

Gene name	Gene ID	No. of amino acids	Molecular weight	Isoelectric point	Coefficient of instability	Grand average hydrophobicity	Subcellular localization
ZmPAL1	109943525	698	75657.70	5.85	32.74	-0.040	plas
ZmPAL2	100281042	715	76777.47	5.63	33.94	-0.079	chlo
ZmPAL3	100384215	703	75655.45	6.10	29.96	-0.074	chlo
ZmPAL4	103653804	704	75300.35	6.83	33.49	-0.104	chlo
ZmPAL5	100285115	718	77424.43	5.97	31.85	-0.116	chlo
ZmPAL6	109946043	157	16618.70	5.93	31.40	-0.204	cyto
ZmPAL7	100281532	716	77200.19	5.75	34.98	-0.087	chlo
ZmPAL8	103655990	415	44893.33	6.72	36.94	-0.081	chlo
ZmPAL9	542258	703	75464.16	5.96	32.36	-0.069	chlo
ZmPAL10	100273579	718	77298.28	6.20	30.49	-0.103	chlo
ZmPAL11	103627433	704	75836.77	5.89	37.13	-0.066	chlo
ZmPAL12	100381820	715	76932.94	5.89	34.43	-0.083	chlo
ZmPAL13	103636788	429	46286.29	10.67	55.56	-0.442	pero

Note: Cyto: Cytoplasm, Chlo: Chloroplast, Plas: Plasmodesmata, Pero: Peroxisome

Table 4. Secondary structure and predicted signal peptide of PAL protein in *Zea mays* (L.).

Gene name	Alpha helix	Beta turn	Random coil	Extended strand
ZmPAL1	380 (54.44%)	39 (5.59%)	220 (31.52%)	59 (8.45%)
ZmPAL2	405 (56.64%)	45 (6.29%)	212 (29.65%)	53 (7.41%)
ZmPAL3	405 (57.61%)	40 (5.69%)	195 (27.74%)	63 (8.96%)
ZmPAL4	405 (57.53%)	46 (6.53%)	189 (26.85%)	64 (9.09%)
ZmPAL5	400 (55.71%)	45 (6.27%)	216 (30.08%)	57 (7.94%)
ZmPAL6	65 (41.40%)	16 (10.19%)	49 (31.21%)	27 (17.20%)
ZmPAL7	410 (57.26%)	51 (7.12%)	193 (26.96%)	62 (8.66%)
ZmPAL8	193 (46.51%)	31 (7.47%)	155 (37.35%)	36 (8.67%)
ZmPAL9	394 (56.05%)	41 (5.83%)	207 (29.45%)	61 (8.68%)
ZmPAL10	429 (59.75%)	45 (6.27%)	197 (27.44%)	47 (6.55%)
ZmPAL11	408 (57.95%)	39 (5.54%)	205 (29.12%)	52 (7.39%)
ZmPAL12	415 (58.04%)	0 (0.00%)	200 (27.97%)	56 (7.83%)
ZmPAL13	97 (22.61%)	28 (6.53%)	247 (57.58%)	57 (13.29%)

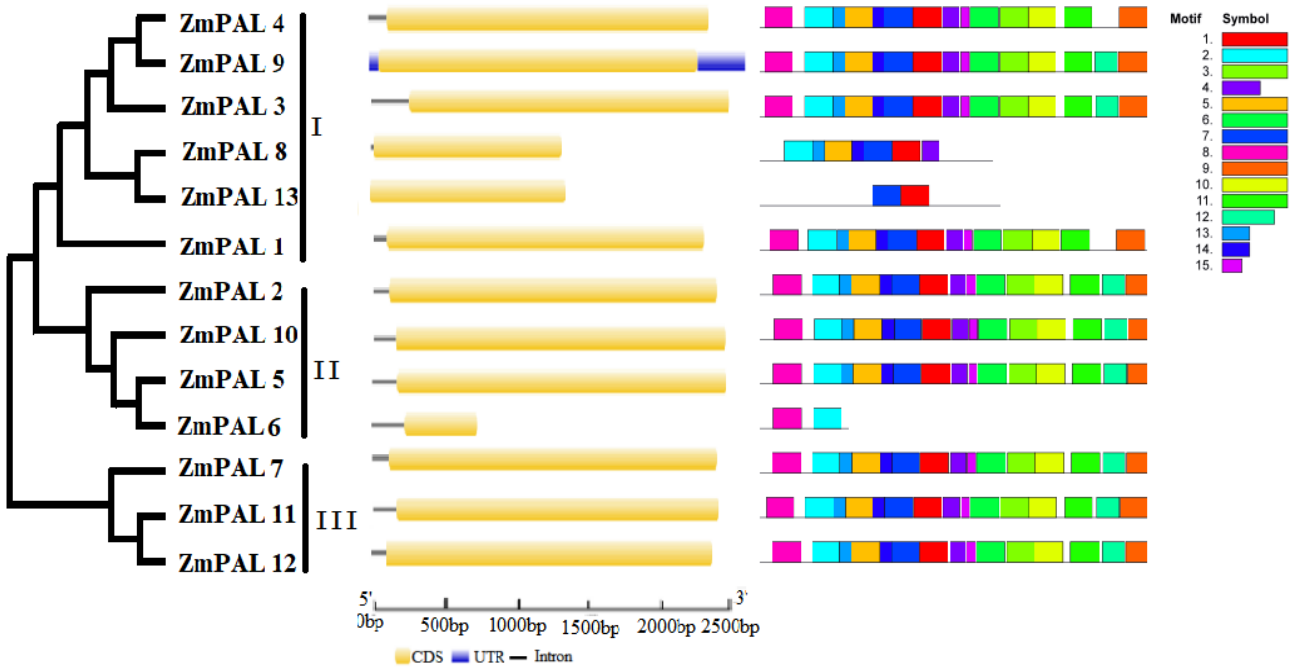

Fig. 2. Gene structure of *PAL* and Conserved motifs in maize.
Note: The yellow boxes represent exons; Line represent introns; The blue boxes represent UTR.

The MEME online tool was used to analyze the conserved motifs of the ZmPAL proteins. The results revealed a total of 15 conserved motifs distributed in ZmPAL protein sequences. Minor changes were observed in the length of the motifs, suggesting that conservation among the ZmPAL family members is relatively high. Further analysis of the distribution of these conserved motifs demonstrated that most of the ZmPAL contained 15 conserved motifs. However, some genes missed a certain number of conserved motifs (Fig. 2). ZmPAL6 and ZmPAL13 had the least conserved motifs (both containing only two), suggesting that the functions of ZmPAL6 and ZmPAL13 may be further differentiated in comparison to other members.

Phylogenetic analysis of ZmPAL proteins in different species: A total of 85 PAL-homologous proteins sequences were identified from eight plant species (including *Sorghum bicolor*, *Oryza sativa*, *Glycine max*, *Vitis vinifera*, *Ricinus communis*, *Arabidopsis thaliana*, *Amborella trichopoda* and *Physcomitrella patens*) through database search. The evolutionary relationship of these PAL proteins sequences was investigated using MEGA7.0 software to built a phylogenetic tree (Fig. 3). According to the map, these proteins sequences can be divided into six groups, with the smallest number of PAL proteins sequences in group A and B, containing nine proteins sequences, the largest number of PAL proteins sequences was found in group F, containing 24 proteins sequences, 12 proteins sequences in group C, 17 proteins sequences in group D, and 14 proteins sequences in group E. The distribution of ZmPAL proteins sequences was: one in group A, three in group C, four in group D and five in group F.We found that a total of 60 PAL proteins sequences were homologues, accounting for about 70.59%. There were 16 pairs of orthologous proteins sequences among species, including three pairs between maize and sorghum, two pairs between rice-sorghum, soybean-grape and maize-rice, and one pair between maize-grape, moss-grape, maize-ricin, maize-soybean, camphor-grape, moss-soybean, soybean-sorghum, soybean-arabidopsis, and moss-rice. Many orthologous protein sequenceswith a close relationship were found between maize, sorghum and rice. No orthologous protein sequences were found between maize and camphor, moss and arabidopsis, and the relationship was relatively distant. There were 14 pairs of paralogous protein sequences in the species, including four pairs from grapes, three pairs from moss, two pairs from camphor; and one pair from sorghum, castor, rice, soybean and maize.

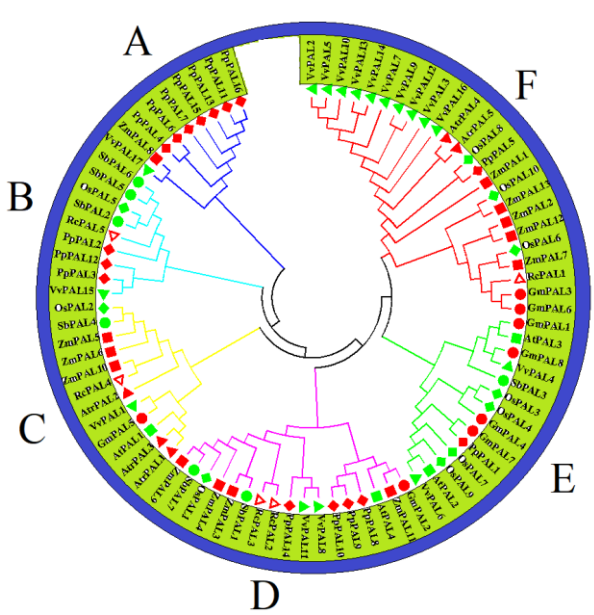

Fig. 3. Phylogenetic tree of PAL protein sequences in maize and 8 species.

Analysis of *ZmPAL* gene expression profiles: In this study, expression data of *ZmPAL* genes in different tissues was obtained and normalized from the SRA database in NCBI. As shown in Fig. 4, *ZmPAL3*, *ZmPAL5*, *ZmPAL9*, and *ZmPAL10* genes were highly expressed, while *ZmPAL8* and *ZmPAL13* genes were less expressed in different tissues (FPKM≤1). *ZmPAL12* was highly expressed in roots, stems, leaves, filaments and tassels, but less expressed in bracts. *ZmPAL4* was highly expressed in roots, stems, leaves and filaments, but less expressed in tassels and bracts. *ZmPAL11* was highly expressed in roots and stems, while *ZmPAL1* was highly expressed only in roots. The number of genes was the highest in roots (11), followed by stems and filaments (8), followed by leaves (7), tassels (6), and bracts (4).

In this study, we found that the expression levels of 13 *ZmPAL* genes were significantly different under high-temperature stress (0 h, 2 h, 48 h) (Fig. 4). The expression of some genes showed a decreasing and then rising trend, such as *ZmPAL4*, *ZmPAL5*, *ZmPAL9* and *ZmPAL10* in bract leaves, *ZmPAL4* in filaments, *ZmPAL7* in headings, *ZmPAL7* and *ZmPAL10* in leaves, *ZmPAL5*, *ZmPAL6*, *ZmPAL7*, *ZmPAL9*, *ZmPAL10*, *ZmPAL11*, and *ZmPAL12* in roots, *ZmPAL2*, *ZmPAL3*, *ZmPAL5*, *ZmPAL6*, *ZmPAL10* and *ZmPAL12* in stems. In contrast, the expression of some genes showed an opposite trend of rising first and then decreasing, such as *ZmPAL3*, *ZmPAL5*, and*ZmPAL6* in filaments, *ZmPAL2* in tassels and leaves and roots, and *ZmPAL7* in roots. Some genes showed a gradual upward trend, such as *ZmPAL6* in filaments, *ZmPAL4* in tassels, *ZmPAL5*, *ZmPAL9*, *ZmPAL11*, and*ZmPAL12* in leaves, and *ZmPAL15* in roots. Some genes showed a decreasing trend, such as *ZmPAL3* in bracts, *ZmPAL2*, *ZmPAL9*, *ZmPAL10*, and *ZmPAL12* in filaments, *ZmPAL4* in leaves, *ZmPAL1*, *ZmPAL3*, and *ZmPAL4* in roots, *ZmPAL9* and *ZmPAL11* in stems.

qPCR analysis of *ZmPAL* genes:The expression patterns of *ZmPAL* genes in leaves in different times were verified in order to dissect the response mechanisms of maize *PAL* genes to high-temperature stress (Fig. 5). The expression of *ZmPAL3*, *ZmPAL4*, *ZmPAL8*, and *ZmPAL13* at 2h, 12h, 24h, 48h treatments was shown to be lower. *ZmPAL2*, *ZmPAL7* had the highest expression at 2h; *ZmPAL1*, *ZmPAL6*, *ZmPAL11*, and *ZmPAL12* had the highest expression at 12h, while *ZmPAL5*, *ZmPAL9*, and *ZmPAL10* had the highest expression at 48h. Furthermore, the expression of *ZmPAL3*, *ZmPAL10*, and *ZmPAL13* showed a downward and then upward trend, *ZmPAL2* (except 12h), *ZmPAL7*, *ZmPAL11*, and *ZmPAL12* showed a upward and downward trend, *ZmPAL5* (except 24h), *ZmPAL9* showed a gradual upward trend, while *ZmPAL4* showed a gradual downward trend.

Effect of high-temperature stress on maize PAL activity, total phenolics and flavonoids contents: As shown in Fig. 6, ZmPAL activity demonstrated a gradual upward trend, reaching a maximum value at 48h with the extension of high-temperature stress time. At 2h, the activity of ZmPAL increased, although not significantly in comparison to the control. At 12h, 24h and 48h, the activity of ZmPAL of the treatment groups was significantly higher than that of the control, and the differences among the treatment groups were pronounced. Total phenol content showed an upward trend after stress and reached the maximum at 48h. At 2h and 12h of stresses, total phenol content in the treatment groups was not significantly different from that in the control group. At 24h and 48h of stresses, total phenol content in the treatment groups was significantly higher than that in the control group, but the difference among the treatment groups was not significant. Total flavonoid content and total phenolic content showed a similar trend, and both showed an upward trend after stress, reaching the maximum at 48h. At 2h and 12h of stresses, total flavonoid content of the treatment groups was not significantly different from that of the control group. At 24h and 48h of stresses, total flavonoid content of the treatment groups was significantly higher than that of the control group, but the differences among the treatment groups was relatively small.

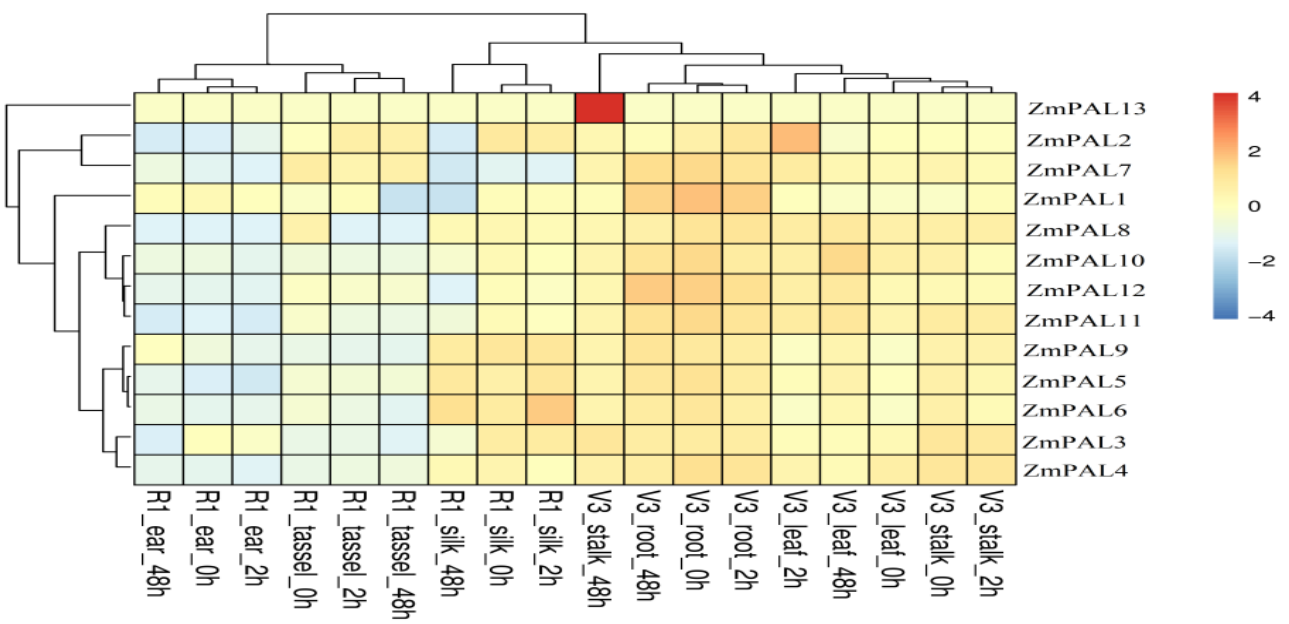

Fig. 4. Expression analysis of *ZmPAL* family gene.
Note: V3: Three leaf stage; R1: Loose powder period; heat stress time: 0h, 2h, 48h.

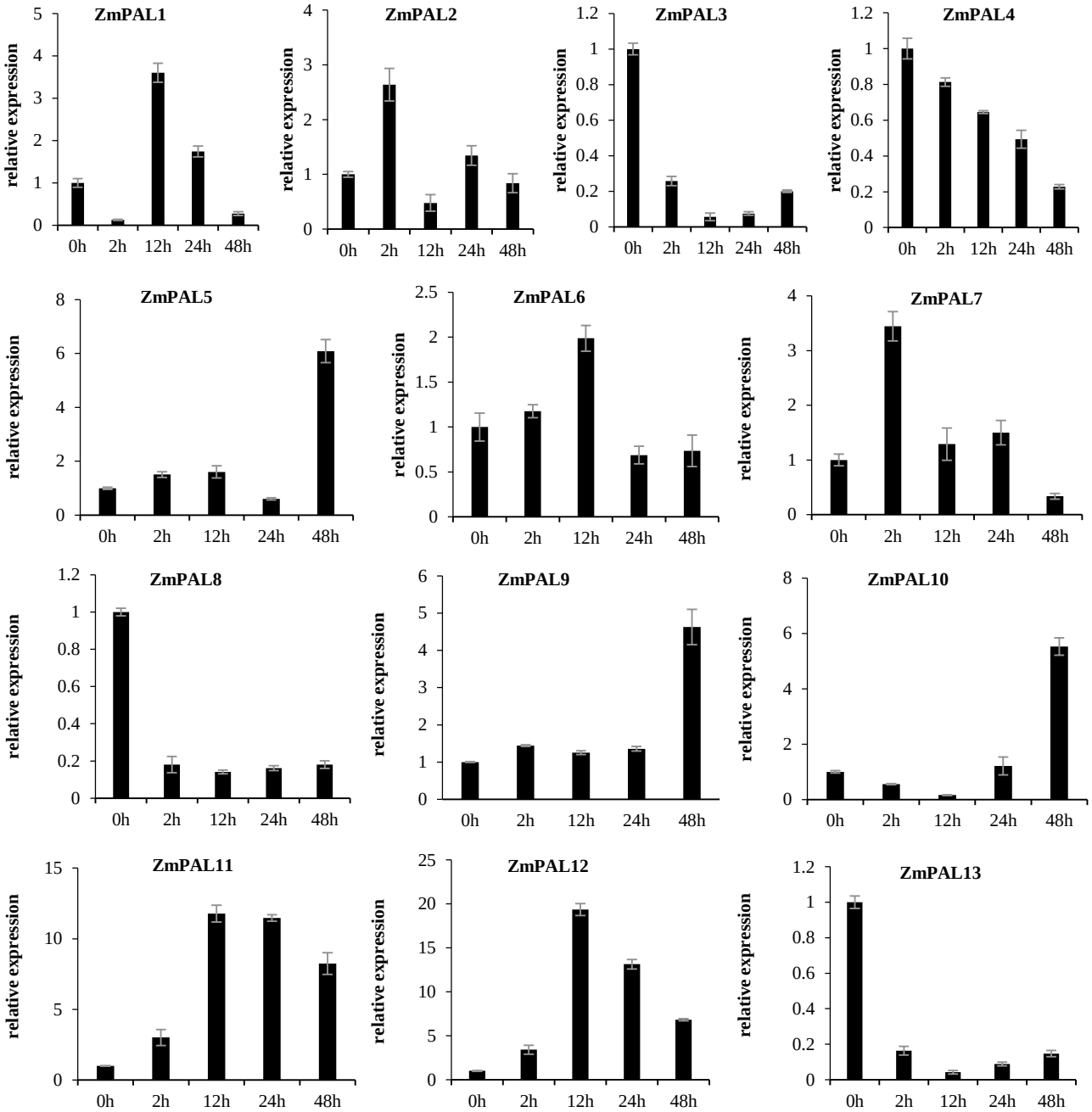

Fig. 5. Expression patterns of *ZmPAL* genes after high temperature treatment.

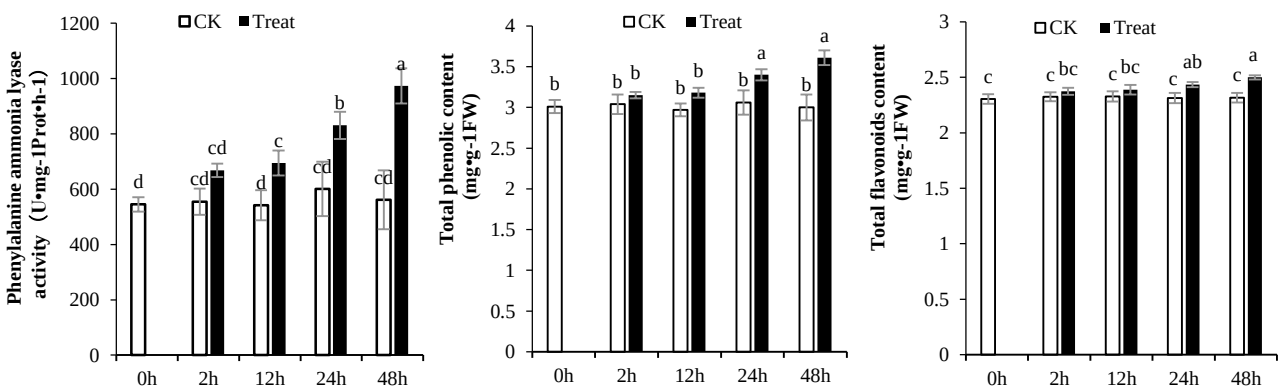

Fig. 6. Changes in PAL activity, total phenolic content, total flavonoid content in Anke No.985 (*Zea mays* L.) after high temperature (38 ℃) treatment.
Note: The X axis indicates treatment time, and the lower case letters indicate significant difference.

Discussion

PAL catalyzes the first-step reaction of the phenylpropanoid metabolic pathway, coupling primary metabolism and phenylpropanoid metabolism. It is a key and rate-limiting enzyme for phenylpropanoid metabolism, where the products play important roles in plant growth and development and in response to stresses (Harakava, 2005). This study identified the *PAL* genes in the maize genome, and demonstrated their expression profiles in response to high-temperature stress. The study explored the physicochemical properties, structures, and evolutionary relationships of the ZmPAL proteins. A total of 13 *ZmPAL* genes were identified in the maize genome. It was reported that the number of *PAL* genes in the genome of arabidopsis (4), tobacco (4), banana (8), rice (9), and tomato (26) (Cochrane *et al*., 2004; Chang *et al*., 2008; Reichert *et al*., 2009; Yu *et al*., 2018; Yang *et al*., 2019), was less than 30, suggesting that *PAL* exists as a small gene family in plants (Achnine *et al*., 2004).

We also demonstrated that gene family expansion could be attributed to gene duplication during the process of species evolution. The maize genome had experienced several rounds of duplication events early in evolution (Paterson *et al*., 2004; Swigonová *et al*., 2004). In this study, we found that five duplication events occurred in 13 *ZmPAL* genes, and six genes were formed due to chromosomal gene duplication. Among them, three were tandem duplications and two were fragment duplications, indicating that the expansion of the *ZmPAL* gene family was achieved by these two mechanisms. An interesting finding was that, although five gene pairs originated from duplications, the number of duplicated genes was not ten but six. That was, *ZmPAL5*, *ZmPAL6*, *ZmPAL7*, *ZmPAL10*, *ZmPAL11*, and *ZmPAL12*. Among them, 1 tandem duplication and 1 fragment duplication occurred in *ZmPAL5*, 2 tandem duplications and 1 fragment duplication occurred in *ZmPAL11*, while only 1 duplication occurred in the other 4 genes, which either occurred with *ZmPAL5* or with *ZmPAL11*, indicating that the contribution of these genes in the process of gene expansion was different, among which *ZmPAL5* and *ZmPAL11* contributed the most. What is the reason behind this interesting phenomenon? What are the common characteristics of genes prone to duplication? What drives some genes to be prone to gene duplication? These questions need to be further studied, and clarifying the above questions is conducive to exploring the dynamics of gene differentiation and the mechanism of species evolution.

According to the phylogenetic relationships, *ZmPAL* genes could be classified into 3 groups: I, II and III, which could be assigned by the analysis of genetic structures and conserved motifs. The structures and conserved motifs of most genes in the same group were similar, indicating that the *ZmPAL* gene family is highly conserved. It was found that the structures of *ZmPAL9* and *ZmPAL13* genes were different from other members, and *ZmPAL6* and *ZmPAL13* genes contained the largest amount of deletions in the conserved motifs, indicating that *ZmPAL6*, *ZmPAL9* and *ZmPAL13* may have different functions. A joint analysis of the phylogeny of the *ZmPAL* gene family and its duplication events revealed that the genes that had duplication events belong to group II and group III. There were seven genes in group II and group III, six of which had duplication events. These genes were clustered due to higher similarity in duplication events (Wei & Pan, 2014).

Analysis of physicochemical properties of *ZmPAL* members showed that the majority of them were acidic proteins, which was consistent with previously reports (Yang *et al*., 2019). Subcellular localization revealed that *ZmPAL* genes were localized in four organelles, most of them in the chloroplast. A previous work proposed that different organelles may cooperate to scavenge ROS through multiple mechanisms, achieve antioxidant function and maintain the dynamic balance of free radicals in vivo, but the chloroplast was the main location to perform their function (Ke *et al*., 2019). In this study, we found that α-helix accounted for the highest proportion in the 12 ZmPAL proteins. Therefore, we speculated that α-helices might play a major role in the secondary structure of ZmPAL proteins.

A highly conserved structure, composed of Ala-Ser-Gly (Yang *et al*., 2019) was found in the amino acid sequence of ZmPAL proteins, which was conserved in PAL proteins of other species. Based on this conserved structure, 85 PAL homologous protein sequences were identified from the NCBI database in this study, among which ten were found in rice, one more than that reported in a previous study (Yu *et al*., 2018;), which may be due to new submission of rice PAL sequences to NCBI. The phylogenetic tree was divided into six subfamilies, and more orthologous genes were found in maize-sorghum and maize-rice, indicating that maize *ZmPAL* genes are closely related to sorghum and rice. These predicted *ZmPAL* genes can be used for variety improvement of these major food crops in the future.

The maize transcriptome data used in this study were derived from the SRA public database (He *et al*., 2019), showing that *ZmPAL* genes were differentially expressed in different tissues (Fig. 4). Some genes were highly expressed in most tissues, such as *ZmPAL12* in roots, stems, leaves, filaments and tassels, and *ZmPAL4* in roots, stems, leaves and filaments. Some genes were highly expressed only in fewer tissues, such as *ZmPAL11* in roots and stems, and *ZmPAL1* in roots only. Some genes, such as *PAL8* and *PAL13*, were less expressed in all tissues. Similarly, *PAL* was differentially expressed in different tissues of *Vitis vinifera*, such as *VviPAL2* and *VviPAL15* in roots, stems, leaves and tendrils, and also in reproductive organs such as flowers, fruits and seeds (Sun *et al*., 2016); *MaPAL4* was highly expressed in *Musa nana* (Yang *et al*., 2019). We speculated that the expression of these genes might occur in specific environments, organs or developmental stages. This study provides an important basis for further studies on the expression and biological function of the maize *PAL* gene family.

Plants are facing many challenges due to global climate change. According to the Global Climate Change Analysis (GCCA), the average temperature will rise by 1 to 3.7°C by the end of the 21st century (Team *et al*., 2014). Different strategies have been adopted to enhance the ability of plants to cope with climate change,

especially in response to high-temperature stress (Ismail *et al*., 2019). Under high-temperature stress, plants could reduce damage through elevated protective enzyme activity (Ren *et al*., 2019). In this study, the expression of *PAL* genes in various tissues of maize increased after high-temperature stress, such as *ZmPAL6* in filaments, *ZmPAL4* in tassel, *ZmPAL5*, *ZmPAL9*, *ZmPAL11*, and *ZmPAL12* in leaves, and *ZmPAL15* in roots, which were further confirmed by quantitative PCR results, indicating that these *PAL* genes may play an important role in maize after high-temperature stress.

Under normal conditions, there is a dynamic balance between the generation and clearance of ROS in plants. However, when plants are subjected to abiotic stresses such as high temperature and drought, this dynamic balance is disrupted, resulting in rapid accumulation of ROS and exacerbated oxidative damage (Mittler, 2002). It was reported that flavonoid metabolites had strong antioxidant activity, scavenging ROS and protecting plants from oxidative damage. Induced flavonoid accumulation and improved antioxidant capacity in *Foeniculum vulgare*, *Arabidopsis*, *Vitis vinifera* and other plants has been reported (Alinian *et al.*, 2016). This study also confirms that PAL enzyme activity, total phenolic content and flavonoid content in maize leaves were significantly increased under high-temperature stress, and the increase of total phenolic content and flavonoid content lagged behind the increase of PAL activity, suggesting that the *ZmPAL* gene may be involved in regulating the synthesis of downstream metabolites to resist abiotic stress (Rohde, 2004).

Conclusion

This study analyzed the structure and function of *ZmPAL* gene family using multiple bioinformatics approaches, which provided a theoretical basis for further studies on the biological function of the *ZmPAL* family and provided evidence on the importance of exploiting high-temperature tolerant maize varieties.

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