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Original Article

Genome-wide analysis of maize PR-1 gene family and expression profiles induced by plant hormones and fungal phytopathogens

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Abstract: Objective: In order to find similarity of the protein X in maize with other species we performed a BLASTP search to identify the maize ZmPR-1 family genes. Methods: We used a BLASTP search to identify the maize ZmPR-1 family genes that may show similarities between the protein X in maize and other species. Results: A total of 17 ZmPR-1 genes were identified and these genes were unevenly distributed on 8 chromosomes of maize. All ZmPR-1 gene predicted proteins contained a conserved CAP domain, according to the results of multiple sequence alignment and gene structure analysis. Phylogenetic tree analysis of a total of 85 PR-1 protein sequences from maize, sorghum, rice and Arabidopsis showed that the PR-1 family proteins were divided into four categories, and the maize ZmPR-1 was closely related to sorghum PR-1. In the promoter of maize ZmPR-1 gene, hypothetical cis-elements related to fungal induction, defense stress response, plant hormones, low temperature and drought response were detected. Microarray data analysis showed that ZmPR-1 displayed a tissue-specific expression pattern at different developmental stages, and responded to the infections of five maize pathogens. In addition, we further verified that four ZmPR-1 genes (ZmPR-1-5, 12, 14 and 16) were not only significantly up-regulated after Setosphearia turcica infection, but also affected by exogenous cues such as SA, ABA, MeJA and H_2O_2. Conclusion: The ZmPR-1 family may be important in plant disease resistance. This study's data provide important clues for future research on the function of ZmPR-1 family genes.

Keywords: Maize, whole genome, disease-related proteins-1, gene expression profile, plant disease resistance

Introduction

The induction and accumulation of various disease-related (PR) proteins are an important feature of plants' defense response to pathogen attacks [1]. So far, at least 17 PR protein families have been isolated from dicots and monocots [2, 3]. PR-1 protein is the first PR family protein that was discovered in the 1970s. It was discovered in tobacco infected with tobacco mosaic virus (TMV) [4]. Since then, homologs of tobacco PR-1 protein have been discovered in barley, tomato, corn, rice and other plants [5, 11]. Although the biological activity of the PR-1 protein is still unknown, it is frequently used in many plant species as a marker for systemic acquired resistance (SAR) [1].

PR-1 protein is highly conserved in different plant species, and their homologs also exist in other organisms, including fungi, insects, animals and humans [6]. Plant PR-1 proteins are classified into two types, acidic and alkaline according to their isoelectric point (pI) [1]. All PR-1 proteins have similar structural features. The main translation product of the PR-1 gene contains a hydrophobic signal sequence. According to the tomato PR-1 protein p14a as the structural model, the standard PR-1 protein contains six conserved cysteine residues that form disulfide bonds, contains four α-helices and four β-strands, and between the helices arranged in anti-parallel [7].

Previous studies have found that PR-1 protein in plants accumulates in large quantities after

pathogen infection. For example, the PR-1 protein content in tobacco leaf tissues infected with TMV accounts for 1-2% of the total leaf protein [8]. In vitro, tomato PR-1c inhibited the germination of P. *infestans* spores and the length of the germ tube. In vivo, it inhibited the diseased area of P. infestans-infected leaves [9]. TMV-induced overexpression of a basic PR-1 gene in tobacco pepper increased resistance to *P. parasitica* var. *nicotianae*, *Ralstonia solanacearum* and *P. syringae* pv. T*abaci* [10]. These findings suggest that the PR-1 protein mediates host plant resistance to pathogens [12]. The PR-1 gene family is a multi-gene family in plants. However, studies have revealed that only a few members of this family have inducible expression and pathogen-inhibitory activity. For example, there are 22 and 39 PR-1 genes in Arabidopsis and rice, respectively, but only 1 and 2 genes were found to be induced by pathogens or insect attacks, respectively [3]. The three tested PR-1 genes in apple (*Malus domestica* B.) did not show induced expression after inoculation with pathogenic bacteria or treatment with SAR elicitor [13]. Other studies also found that the PR-1 gene is not only related to host defense, but also responds to abiotic stresses such as light [14], trauma [15], cold damage [16], salt [17] and drought [18]. In addition, PR-1 gene expression is also regulated by plant hormones and chemical inducers, such as salicylic acid (SA), abscisic acid (ABA), jasmonic acid (MeJA), benzothiadiazole (BTH) and isonicotinic acid (INA) [2, 5, 21, 51]. There is evidence that the PR-1 protein plays a role in plant growth or development that is independent of stress response [19, 20, 21].

Corn is an important food and economic crop in the world, and it is also widely cultivated in Northeast China. During the growth of corn, it is often infected by various pathogens, such as *Setosphearia turcica*, *Sesamia nonagrioides*, *Sporisorium reilianum*, *Fusarium moniliforme*, *Phytophthora cinnamomi* and *Colletotrichum graminicola*. Among them, the large leaf spot disease caused by *Setosphearia turcica* infection is the most devastating in the corn-producing areas of Northeast China. Genetic improvement of disease resistance is crucial to the prevention and control of corn diseases. Josep et al. [22] first cloned a PR-1 gene induced by the fungus *Fusarium moniliforme* from corn germinated seeds in 1991. After that, Jeffrey et al. [23] purified a PR-1 protein from corn roots. Up to now, only two PR-1 genes have been reported in maize. However, little is known about the other P1 members. In order to systematically understand the composition of maize PR-1 family members, gene structural characteristics and their role in disease resistance, it is necessary to conduct systematic research on the basis of maize genome. In this study, by searching the maize genome database, we identified 17 maize ZmPR-1 genes and performed bioinformatics analysis, including phylogeny, gene structure, conserved motifs, chromosome location and promoter cis-regulatory elements and many more. Furthermore, the transcription level of ZmPR-1 in different maize tissues and the transcription level of ZmPR-1 in seedling leaves after inoculation with fungal pathogens and plant hormones were determined. The findings of this study will be useful in future research on the function of maize ZmPR-1 family genes.

Materials and methods

Maize PR-1 family gene isolation and sequencing

According to previous reports, 22 Arabidopsis and 32 rice PR-1 protein sequences were downloaded from the Arabidopsis genome TAIR database (http://www.arabidopsis.org) and the rice (Oryza sativa L.) TIGR database (http://rice.plantbiology.msu.edu). Arabidopsis thaliana and rice PR-1 protein sequences were used as queries for BLASTP searches in the Gramene database (http://www.gramene.org/Multi/blastview) to obtain all maize PR-1 genes. All predicted homologous protein sequences of PR-1 family members are downloaded if the E value of BLASTP is greater than E 1010 [24]. The maize PR-1 gene's full-length cDNA sequence was obtained from the Gramene database. Then, to confirm all predicted maize PR-1 genes, Pfam (http://pfam.xfam.org/) and SMART (http://smart.embl-heidelberg.de/) were used [25]. The ExPASy server (http://web.expasy.org/protparam/) was used to calculate the amino acid count, theoretical isoelectric point (pI), and molecular weight (MW) of the PR-1 protein obtained. The subcellular localization of PR-1 protein was predicted using CELLO v.2.5: sub-cellular localization predictor (http://cello.life.nctu.edu.tw/) [26] and pSORT predic-

tion software (http://www.genscript.com/wolf-psort.html) [27].

Analysis of phylogenetic trees and multiple sequence alignment

Clustal X 2.11 software [28] was used to perform multiple sequence alignments on the amino acid sequences of maize PR-1 family genes, and MEGA 6.0 software was used to construct and analyze phylogenetic trees of maize, Arabidopsis, rice, and sorghum PR-1 family members. The Neighbor-Joining (NJ) method of genetic distance was used to build the phylogenetic tree, which was then self-checked and given a bootstrap value of 1000 for the verification parameter [29].

Analysis of the maize chromosome's location, gene organization, conservative motif, and cis-elements gene ZmPR-1

We located the ZmPR-1 family genes' chromosomal locations using the maize sequence database (http://ensembl.gramene.org/Zea mays). We analyzed and drew the gene structure maps of the ZmPR-1 gene family members using the web tool GSDS (http://gsds.cbi.pku.edu.cn/) [30]. The conserved domain structure of the PR-1 protein was examined using the online tool MEME (http://meme-suite.org/) [31]. To investigate the cis-elements in the ZmPR-1 promoter sequence, a DNA sequence 2000 bp upstream of the initiation codon (ATG) was downloaded from the B73 genome. The Promoter 2.0 (http://www.cbs.dtu.dk/services/Promoter/) and PLACE (http://www.dna.affrc.go.jp/PLACE/) software packages were used to predict promoter structure [32].

Microarray data collection and maize ZmPR-1 gene expression profile analysis

PLEXdb (http://www.plexdb.org) maize transcriptome data was used to analyze the temporal and spatial specific expression behavior of the maize ZmPR-1 gene. The microarray data came from a genome-wide gene expression analysis of maize inbred line B73 (GSE27004) and transcriptomics analysis of maize senescence induction [33, 34]. Downloaded from GEO database (www.ncbi.nlm.nih.gov/geo/) the Affymetrix gene chip array data of gene expression during the infection of maize by 5 fungi: the accession numbers are GSE31188 (*Colletotrichum graminicola*) [35], GSE19501 (*Fusarium moniliforme*), GSE27626 (*Phytophthora cinnamomi*) [36], GSE28244 (*Sesamia nonagrioides*) [37] and GSE29747 (*Sporisorium reilianum*) [38]. GeneSpring12.5 software was used to analyze the microarray datasets. The expression data of ZmPR-1 family members were extracted and normalized, and a heat map was created using the MultiExperiment Viewer (MeV, version 4.8.1) software to visualize the data [24].

Plant materials and treatment

The inbred maize line B73 was used the model plant in the study. The seeds were sown in plastic pots (20 cm in diameter, 10 plants per pot) filled with sterile soil, and then grown in an artificial climate incubator (CIMO, China) under 16 hours of light (100 μmol photons $m^{-2}s^{-1}$)/8 h in darkness, the temperature was 25°C/22°C (day/night), and the relative humidity was 70% RH [24]. Corn seedlings cultivated to the 4-leaf stage were subjected to stress tests for induction of all plant hormones and fungal pathogens.

Setosphaeria turcica inoculation treatment: A strain of *S. turcica* isolated from the field was preserved in our laboratory for the infection experiment. The bacterium was cultured on potato dextrose agar (PDA) medium, and it was cultured in an alternating cycle of 12 h light/12 h at 25°C for 7 days. Corn seedlings at the 4-leaf stage were sprayed with a conidia suspension (1×10^6 mL^{-1} in 0.02% of Tween-20), and then placed in a humidified incubator (100% relative humidity) at 25°C [39]. After 0, 6, 12, 24, 48, and 72 hours, 1 gram of seedlings were removed and immediately frozen in liquid nitrogen before being stored at 80°C for expression analysis.

Treatment with phytohormones: The seedlings were treated with 2 mM salicylic acid (SA), 0.1 mM methyl jasmonate (MeJA), and 10 mM H_2O_2 diluted in 0.05 percent (V/V) ethanol. Seedlings in control plants were sprayed with distilled water (containing 0.05 percent (v/v) ethanol) [40]. After 0, 1, 3, 6, 9, 12, and 24 hours of stress treatment, 0.2 grams of each leaf were cut for RNA extraction. In all experiments, samples were immediately frozen in liquid nitrogen and stored at 80°C until analyzed.

Table 1. Primers for qRT-PCR of ZmPR-1 and actin genes

Name	Forward primer	Reverse primer	Products (bp)
ZmPR-1-5	TGCTCTGCCTGCTCCTCTTCTC	TGGAGTAACCGCCGCCTGAC	198
ZmPR-1-12	TGGTGTGTTTAGCTCTGGCG	ACGTTCTCATCCCACGACAC	139
ZmPR-1-14	TACGACCACGACACCAACAG	AGTCTAGTAGGGGCTCTCGC	183
ZmPR-1-16	CACATACACACATCCCGGCT	GCTGTTTTGGGGAGTGAGGT	165
ZmGAPDH	CTTCGGCATTGTTGAGGGTTTG	TCCTTGGCTGAGGGTCCGTC	84

RNA isolation and quantitative real-time PCR (RT-qPCR) analysis

A plant RNA extraction kit was used to extract total RNA from all samples (Invitrogen, USA). The Nanodrop 2000 spectrophotometer was used to determine the quality and quantity of RNA (Thermo Scientific, USA). The RNA sample was then digested with DNase I (Promega, USA). The total RNA was then reverse transcribed into the first strand of cDNA using the cDNA reverse transcription kit (TakaRa, Japan) per the manufacturer's instructions.

RT-qPCR was used to determine the expression level of the maize PR-1 gene after induction by pathogens and plant hormones. The RT-qPCR reaction was carried out on a CFX96 fluorescent quantitative PCR machine (Bio-Rad, USA) with the SYBR Green I Master Mix kit (TransGenBiotech, China). 4 pairs of ZmPR-1 gene specific primers (**Table 1**) were designed for RT-qPCR reaction using Primer6.0 software. The maize ZmGAPDH gene was used as an internal reference gene [41]. The following were used in the RT-qPCR reaction: 2 L cDNA template, 0.5 L upstream and downstream primers (10 μM), 10 L SYBR Green Realtime PCR Master Mix, and 7 L ddH_2O, for a total reaction volume of 20 L. The RT-qPCR cycling program was as follows: 3 minutes of pre-denaturation at 94°C; 40 cycles of denaturation at 94°C for 5 seconds, 1 minute of annealing at 57°C, 30 seconds of prolonged growth at 72°C, and measurement of fluorescence. The $2^{-\Delta\Delta ct}$ method was used to calculate the maize ZmPR-1 transcription level [42]. All RT-qPCR experiments had three biological replicates and three technical replicates.

Data analysis

Samples were randomized for each experiment, with at least three replicates for each independent trial. SPSS 20 software was used for statistical analysis, with $P<0.05$ or $P<0.01$ being considered as significant [43]. Shapiro-Wilk test was used to assess the normal distribution of variables. Student's t and Mann-Whitney U test were used to compare the two groups of variables.

Results

Identification of PR-1 family genes in maize

We identified 17 ZmPR-1 genes from the maize genome. According to their order on chromosomes 1 to 10, they were named as ZmPR-1-1 to ZmPR-1-17, respectively. BLAST analysis of PFAM and SMART databases showed that all genes include a CAP domain (Pfam code PF00188). The DNA sequence length of the 17 ZmPR-1 genes was from 561 to 1174 base pairs (bp). Except for ZmPR-1-4, all other genes showed no introns. The predicted ZmPR-1 protein showed a length from 175 to 256 amino acids (aa), the molecular weight ranged from 7.52 to 133.14 kDa, and the theoretical isoelectric point (pI) ranged from 4.42 to 11.10 (**Table 2**). The results of subcellular localization analysis showed that most of the ZmPR-1 protein is mainly distributed in the periplasm, extracellular and chloroplasts. However, ZmPR-1-11 is also present in the nucleus, cytoplasm and outer membrane.

Multiple sequence alignment, gene structure and conservative motif analysis of ZmPR-1 gene family

We next compared the 17 amino acid sequences of ZmPR-1 proteins and the amino acid sequence of tomato P14a proteins (the crystal structure of the first resolved PR-1 family protein, see Fernandez et al. [7]). The results showed that in addition to ZmPR-1-4, conserved residues (including 6 strictly conserved cysteine residues) and 15 hydrophobic conserved amino acids were identified in the other 16 ZmPR-1 and P14a proteins (**Figure 1**). It was also found that most of the residues were locat-

Table 2. List of identified PR-1 genes in maize along with their detailed information and localization

Gene Name	Gene ID	Chromosome location	Exons	Gene (bp)	Protein (aa)	MW (kDa)	pI	Localization
ZmPR-1-1	Zm00001d029558	chr1:76160276-76161100	1	825	179	19.16	9.5	Pe[a], Ex[b]
ZmPR-1-2	Zm00001d033902	chr1:278098777-278099860	1	1084	203	20.75	9.03	Pe[a], Ex[a], Ch[b]
ZmPR-1-3	Zm00001d004089	chr2:81606608-81607480	1	873	213	23.01	8.73	Ex[a], Ch[b]
ZmPR-1-4	Zm00001d007448	chr2:231980703-231981965	2	1263	177	18.20	9.48	Pe[a], Ex[b]
ZmPR-1-5	Zm00001d041230	chr3:106717846-106718719	1	874	261	26.68	5.25	Ex[a,b]
ZmPR-1-6	Zm00001d052068	chr4:178359682-178360242	1	561	186	20.00	6.92	Pe[a], Ex[b]
ZmPR-1-7	Zm00001d018321	chr5:218439422-218440318	1	897	176	18.41	9.91	Pe[a], Ex[a,b]
ZmPR-1-8	Zm00001d018322	chr5:218451341-218452129	1	789	175	18.72	9.27	Pe[a], Ex[a], Ch[b]
ZmPR-1-9	Zm00001d018323	chr5:218455869-218456651	1	783	206	22.31	8.05	Pe[a], Ch[b]
ZmPR-1-10	Zm00001d018324	chr5:218456946-218457946	1	1001	182	19.68	8.3	Pe[a], Ch[b]
ZmPR-1-11	Zm00001d039212	chr6:172606517-172607690	1	1174	256	28.36	8.98	Pe[a], Ou[a], Cy[a], Nu[b]
ZmPR-1-12	Zm00001d018734	chr7:3398288-3399149	1	862	167	17.74	8.65	Ex[a], Pe[a], Ch[b]
ZmPR-1-13	Zm00001d018737	chr7:3534280-3535055	1	776	169	17.80	4.99	Pe[a], Ex[a,b]
ZmPR-1-14	Zm00001d018738	chr7:3653537-3654275	1	739	163	17.22	4.38	Pe[a], Ex[a,b]
ZmPR-1-15	Zm00001d019364	chr7:29877961-29878744	1	784	171	18.04	8.23	Ex[a,b]
ZmPR-1-16	Zm00001d009296	chr8:52923704-52924587	1	884	167	18.45	8.92	Pe[a], Ex[a,b]
ZmPR-1-17	Zm00001d009772	chr8:80441910-80443053	1	1144	245	27.57	8.61	Pe[a], Ch[b]

Abbreviations: Chr, Chromosome; CDS, coding DNA Sequence; cDNA, complementary DNA; MW, Molecular Weight; pI, Isoelectric point; bp, base pair; aa, amino acid; kDa, kilodalton; Cp, Chloroplast; Ec, Extracellular; Cy, Cytoplasm; Mt, Mitochondria; Nu, Nucleus; Pm, Plasma-membrane. [a]Localization prediction by CELLO v.2.5 (http://cello.life.nctu.edu.tw/). [b]Localization prediction by pSORT (http://www.genscript.com/wolf-psort.html).

ed at important positions in the specific secondary structure of PR-1, including 4 α helices (αI, αII, αIII and αIV) and 4 β sheets (βI, βII, βIII and βIV), as well as small 3_{10}-Spiral pattern. These results indicate that the maize ZmPR-1 protein and tomato P14a have the same three-dimensional structure, further implying that the 17 predicted ZmPR-1 (except ZmPR-1-4) belong to the PR-1 family of genes.

In order to better understand the structural characteristics of ZmPR-1 genes, we used Gene.Display Server 2.0 to analyze the exon/intron structure of ZmPR-1. The results showed that, except for ZmPR-1-4 which contained 1 intron, the other ZmPR-1 members had no introns (**Figure 2B**).

In order to gain insight into the diversity of ZmPR-1 proteins, the conserved motifs were predicted using the MEME program. A total of 8 conserved motifs were identified in ZmPR-1 proteins. The distribution of each motif was shown in **Figure 2C**. The results revealed that ZmPR-1 in Cluster II showed similar motif composition, and each member shared Motif-1, 2, 3, 5, 6, 8. The motif composition of ZmPR-1 was different in the branch I (Cluster I) and the branch III (Cluster III). For example, ZmPR-1-5 and ZmPR-1-8 did not contain Motif-1, and Motif-7 was found to be unique to ZmPR-1-11 and ZmPR-1-17 in Cluster III. ZmPR-1-4 only contained three Motifs (Motif-1, 2, 8).

Analysis of the phylogenetic relationship between corn PR-1 protein and Arabidopsis, rice and sorghum PR-1 protein

In order to analyze the phylogenetic relationship between maize ZmPR-1 proteins, we used MAGA6.0 software to perform multiple sequence alignments of 17 ZmPR-1 full-length amino acid sequences, and constructed a phylogenetic tree. As shown in **Figure 2A**, ZmPR-1 could be divided into three branches (Cluster I, II, and III) according to the distance of kinship, each containing 8, 6, and 3 ZmPR-1 respectively. To further explore the evolutionary relationship between plant PR-1 gene families, we used neighbor-joining analysis to analyze a total of 85 from corn (red dots), sorghum (blue dots), rice (yellow dots) and Arabidopsis (green dots). A phylogenetic analysis of the PR-1 amino acid sequence was performed (**Figure 3**). Finally, the PR-1 family proteins of three monocotyledonous plants and one dicotyledonous plant were divided into four classes: I, II, III and IV, each contained 32, 27, 23 and 3 genes, respectively.

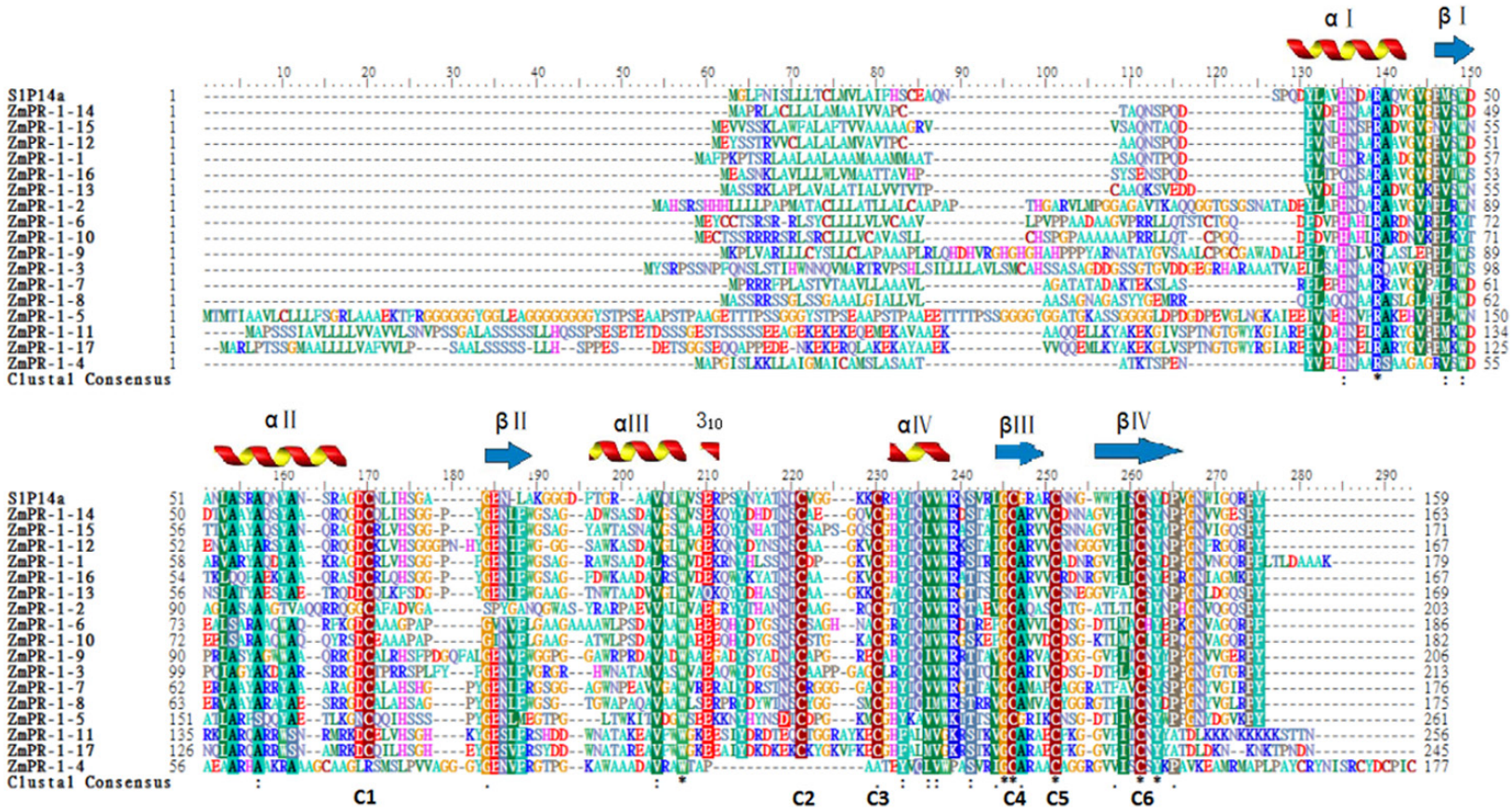

Figure 1. The schematic illustration of amino acid sequence alignment of the PR-1-like domain in the corn ZmPR-1 protein and the tomato P14a protein. The amino acids were colored in the colors defined by the Clustal W program. Identical residues were indicated by asterisks, and conserved hydrophobic residues are indicated by double dots. The secondary structure of six conserved cysteine residues, four α-helices and four β-strands corresponding to the P14a protein were shown at the top of the arrangement.

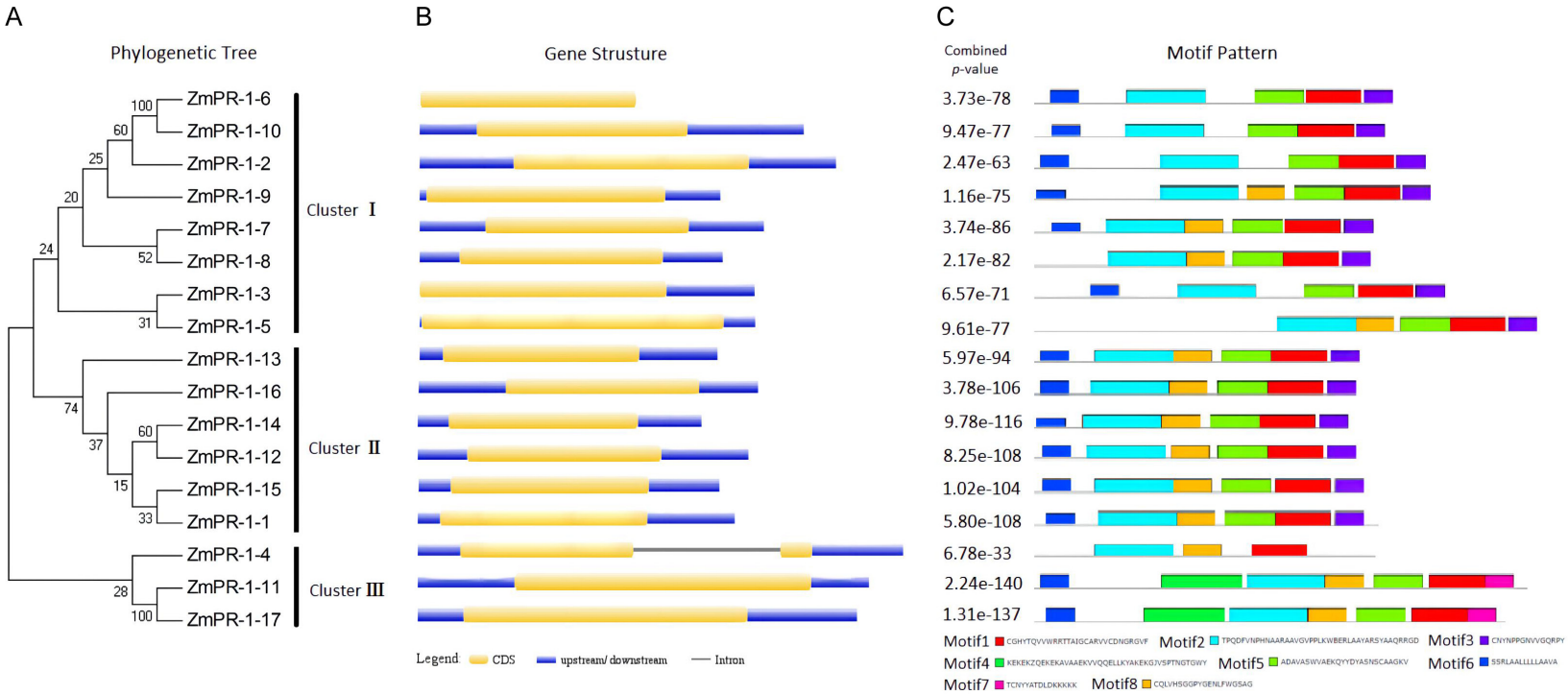

Figure 2. Phylogenetic relationships, gene structure and architecture of conserved protein motifs in PR-1 genes from maize. A. Phylogenetic tree was constructed using MEGA 6.0 by the neighbor-joining (NJ) method with 1000 bootstrap replicates. B. Gene structure of maize PR-1 genes. Exons, introns and upstream/upstream were denoted by yellow boxes, black lines and blue boxes, respectively. C. Seventeen ZmPR-1 proteins with complete ORFs were used for motif prediction. Motif composition of ZmPR-1 proteins using MEME online software, deduced amino acid sequence of each motif.

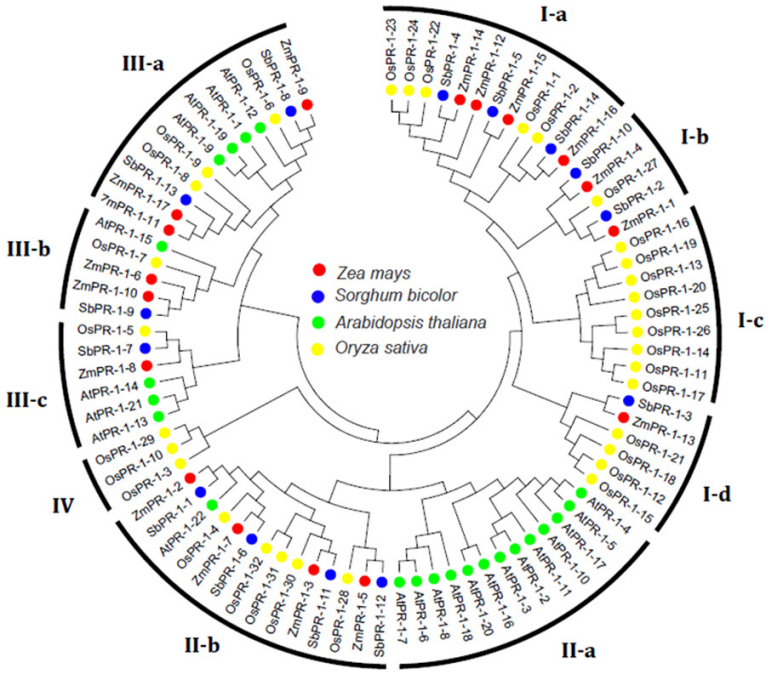

Figure 3. Phylogenetic tree of maize ZmPR-1 gene and PR-1 gene of other plants. A total of 85 spieces including *Zea mays* (17 pieces, marked with a red dot), *Sorghum bicolor* (14 pieces, marked with a blue dot), *Oryza sativa* (32 pieces, marked with a yellow dot) and *Arabidopsis thaliana* (22 pieces, marked with a green dot) were included for PR-1 protein sequence analysis, using MEGA6.0 software. The Neighbor-Joining (NJ) method of genetic distance was used to construct a phylogenetic tree, the phylogenetic tree was self-checked, and the verification parameter bootstrap value was set to 1000.

Among them, class I could be divided into four subcategories I-a, I-b, I-c and I-c3; Class II could be divided into two subtypes: II-a and II-b; Class III could be divided into three subtypes: III-a, III-b and III-c. In the 10 subgroups, the IV and I-c subtypes were unique to rice, and the II-a subtype was unique to Arabidopsis. The PR-1 genes of most maize shared close homology with sorghum PR-1. These results are consistent with the fact that maize and sorghum are both gramineous plants and have close phylogenetic relationships. The degree of PR-1 protein sequence homology is very high, indicating a close evolutionary relationship.

Chromosome localization of ZmPR-1 family genes and analysis of promoter cis-acting regulatory elements

The distribution of all ZmPR-1 gene loci (17 in number) on the maize chromosomes was extremely uneven. These genes were mainly distributed on chromosomes 1 to 8 (**Figure 4**). Chromosomes 5 and 7 contained the largest number of ZmPR-1 genes, and chromosomes 5 and 7 respectively contained 4 tightly clustered ZmPR-1 genes. Chromosomes 1, 2 and 8 each contained two ZmPR-1 genes, while chromosomes 3, 4 and 6 contained only one ZmPR-1 gene.

We used PlantCARE to analyze the 5'-upstream promoter (2 kb) region of 17 ZmPR-1 genes to identify the presence of stress-specific cis-elements. Many kinds of stress response cis-elements were found in this analysis, such as wound and fungal elicitor response element (W-BOX), defense and stress response element (TC-rich), anaerobic induced response element (ARE), salicylic acid response element (TCA), abscisic acid response element (ABRE), methyl jasmonate response element (CGTCA-box and TGACG-box), ethylene response element (ERE), auxin response element (AuxRR-core and TGA-element), gibberellin response element (GARE and TGA), Low temperature response element (LTR) and drought response element (MBS) (**Table 3**). The ZmPR-1-8 gene promoter contained the largest number of cis-elements (14) while ZmPR-1-5 contained only 3 cis-elements. Among the 17 ZmPR-1 genes, 16, 15 and 10 ZmPR-1 genes contained plant hormones response-related elements ABA, MeJA, and SA respectively. The fungal elicitor response elements (W-BOX) was identified in 9 ZmPR-1 genes. The cis-elements play an important role in the regulation of gene expression by controlling the efficiency of the promoter. The study of cis-elements can provide important information for elucidating functional roles and regulation of the ZmPR-1 gene family.

ZmPR-1 family gene expression profiles in various tissues and organs

We used microarray data of gene expression levels from 60 tissues at different developmental stages of maize to identify the spatiotempo-

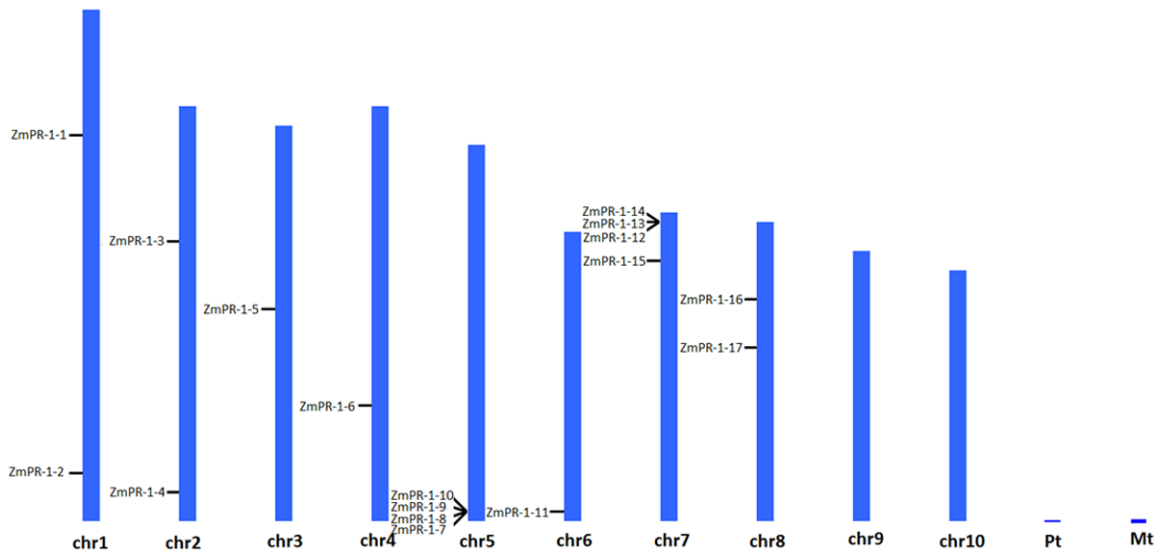

Figure 4. The distribution of ZmPR-1 gene on maize chromosomes. According to the maize (B73) genome, the chromosome position of each ZmPR-1 gene was located. The detailed data were shown in **Table 2**. The chromosome number was shown at the bottom of each chromosome.

ral expression pattern of the ZmPR-1 gene [33]. The heat map revealed that all 17 detected genes were involved in various biological processes and expressed in most tissues, but their levels of expression varied (**Figure 5** and Table S1). ZmPR-1-1 was found in high concentrations in both reproductive organs (seed, endosperm, and coleoptile) and vegetative organs (primary root, internode, leaf, stem, and SAM (Stem and SAM)). Primary roots had significantly higher transcript levels than other tissues. The expression patterns of ZmPR-1-12, 14, 16 were similar, with a high expression level observed in the reproductive organs (endosperm, coleoptile) and vegetative organs (primary root, leaf), stem and SAM (stem and SAM), and the highest expression level was found in leaves (V1-, V3-, V5-, V7-, R2-). The expression patterns of ZmPR-1-7 and 8 are similar, with higher expression levels in both vegetative organs and reproductive organs. ZmPR-1-5, 11, and 17 had the highest expression levels in Anthers and low expression in seeds. ZmPR-1-4 showed low expression in all 60 tissues. In summary, our results indicate that the ZmPR-1 gene plays multiple roles in the growth and development of maize.

Expression profile of ZmPR-1 gene after biological stress

Many studies have shown that the PR-1 gene is involved in plant disease resistance. To investigate whether ZmPR-1 is involved in disease resistance, we used microarray data to analyze the differential expression of ZmPR-1 gene in maize after pest infection. In this study, the transcriptomic profiles of 5 pathogens including *Sesamia nonagrioides*, *Sporisorium reilianum*, *Fusarium moniliforme*, *Phytophthora cinnamomi* and *Colletotrichum graminicola*, were analyzed. As shown in **Figure 6A** and Table S2, after *S. nonagrioides* infection, ZmPR-1-5, -12, -14, -16 were up-regulated, while ZmPR-1-1 and -3 expression was inhibited; after *S. reilianum* infection, ZmPR-1-3, -8, -12, -14, -16 were up-regulated, while ZmPR-1-1 and -5 expression was suppressed; after *F. moniliforme* infection, ZmPR-1-1, 3, 12, 14, 16 showed up-regulation, while the expression of ZmPR-1-5 and -8 was inhibited; after *P. cinnamomi* infection, the expression of ZmPR-1-1, -5, -12, -14, -16 was up-regulated, while the expression of ZmPR-1-3 and -8 was inhibited. After C. graminicola infection, ZmPR-1-1, -8, -12, -14, -16 showed up-regulation, while ZmPR-1-5 expression was inhibited; ZmPR-1-3 was up-regulated at 36 h and down-regulated at 96 h. In addition, we further analyzed the expression profiles of ZmPR-1-5, 12, 14 and 16 after *Setosphearia turcica* infection by qRT-PCR. The results in **Figure 6B** showed that after S. turcica infection, the expression of these 4 ZmPR-1s could be up-regulated in the leaves of maize seedlings, among which ZmPR-14 and 16 reached the highest expression 24 h post-infection, and

Table 3. Numbers of known stress-related cis-regulatory elements present in the upstream region of *ZmPR-1* genes

Motifs Gene name	W box	TC-rich repeats	ARE	TCA-element	CGTCA-motif	TGACG-motif	ABRE	ERE	AuxRR-core	TGA-element	GARE-motif	P-box	LTR	MBS	Total
	TTGACC	GTTTTCTTAC	AAACCA	CCATCTTTTT	CGTCA	TGACG	ACGTG	ATTTCATA	GGTCCAT	AACGAC	TCTGTTG	CCTTTTG	CCGAAA	CAACTG	
ZmPR-1-1		1	1				3	1		1				1	8
ZmPR-1-2		1	1	1	1	1	1								6
ZmPR-1-3	1	1	1	1	1	1	1		1	1	1		1		11
ZmPR-1-4			1	1	1	1	3						1	1	9
ZmPR-1-5			1				1					1			3
ZmPR-1-6	1		1	1	1	1		1						1	7
ZmPR-1-7			1	1	1	1	1	1							6
ZmPR-1-8		1	1	1	1	1	3			1	1	1	1	2	14
ZmPR-1-9			1		1	1	1	1				1	1		7
ZmPR-1-10	1		1		1	1	3	1		1			1	1	11
ZmPR-1-11					1	1	1			1				1	5
ZmPR-1-12	1		1		1	1	3			1	1	1			10
ZmPR-1-13	1		1		1	1	1	1				1		1	8
ZmPR-1-14	1		1	1	1	1	3		1	1			1		11
ZmPR-1-15	1		1	1	1	1	1					1			7
ZmPR-1-16	1	1	1	1	1	1	1						1	1	9
ZmPR-1-17	1		1	1	1	1	3			1		1	1	1	12
Total	9	5	16	10	15	15	30	6	2	8	3	7	8	10	144

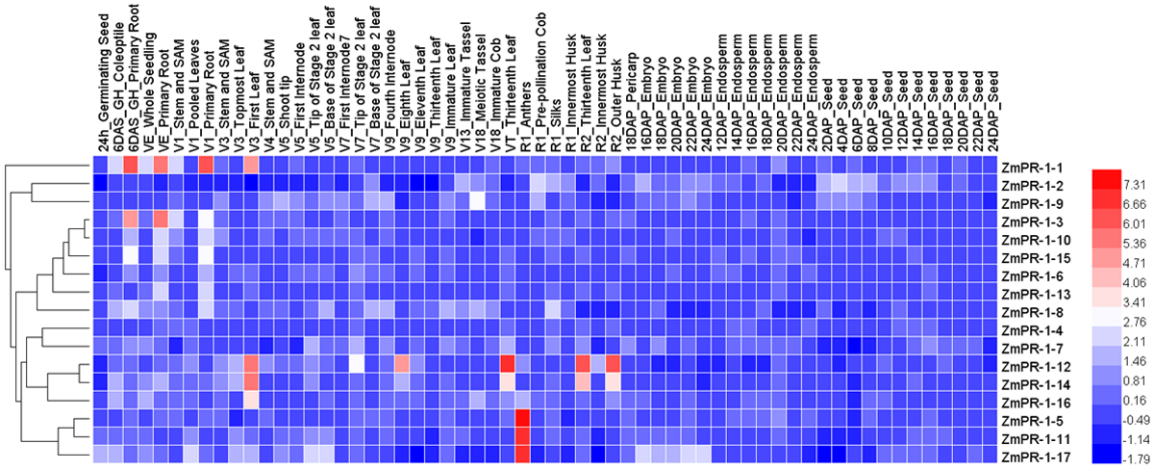

Figure 5. Organ-specific expression patterns of ZmPR-1 genes determined using microarray data. Log2 ratios of expression were used to generate the heat map. Red indicates higher expression, while blue signifies lower expression in 60 tissues.

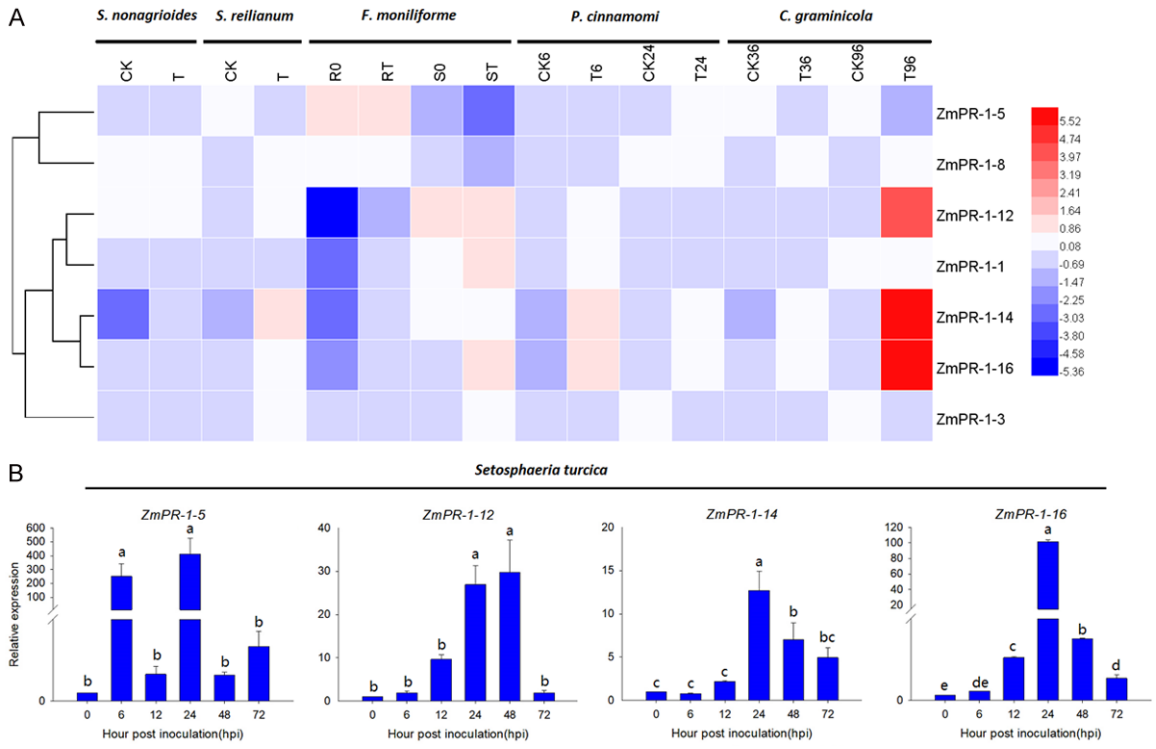

Figure 6. The expression pattern of ZmPR-1 gene under various biological stresses. A. The microarray data was used to determine the expression pattern of the maize ZmPR-1 gene after infection by the pathogens *Sesamia nonagrioides*, *Sporisorium reilianum*, *Fusarium moniliforme*, *Phytophthora cinnamomi* and *Colletotrichum graminicola*. The expression rate of Log2 was used to draw this heat map, and the up-regulation and down-regulation level was represented by red and blue respectively. B. qRT-PCR was performed to quantify the expression profile of 4 selected ZmPR-1 genes after *Setosphearia turcica* infection. All of the relative expression levels were log2 transformed. Bars represent the mean values of three replicates ± standard deviation (SD). Different small letters indicate significant differences at P<0.05.

ZmPR-1-12 reached its highest value at 24 h-48 h, while ZmPR-1-5 showed highest expression at 6 h and 24 h, respectively. In conclusion, our results show that ZmPR-1-5, -12, -14

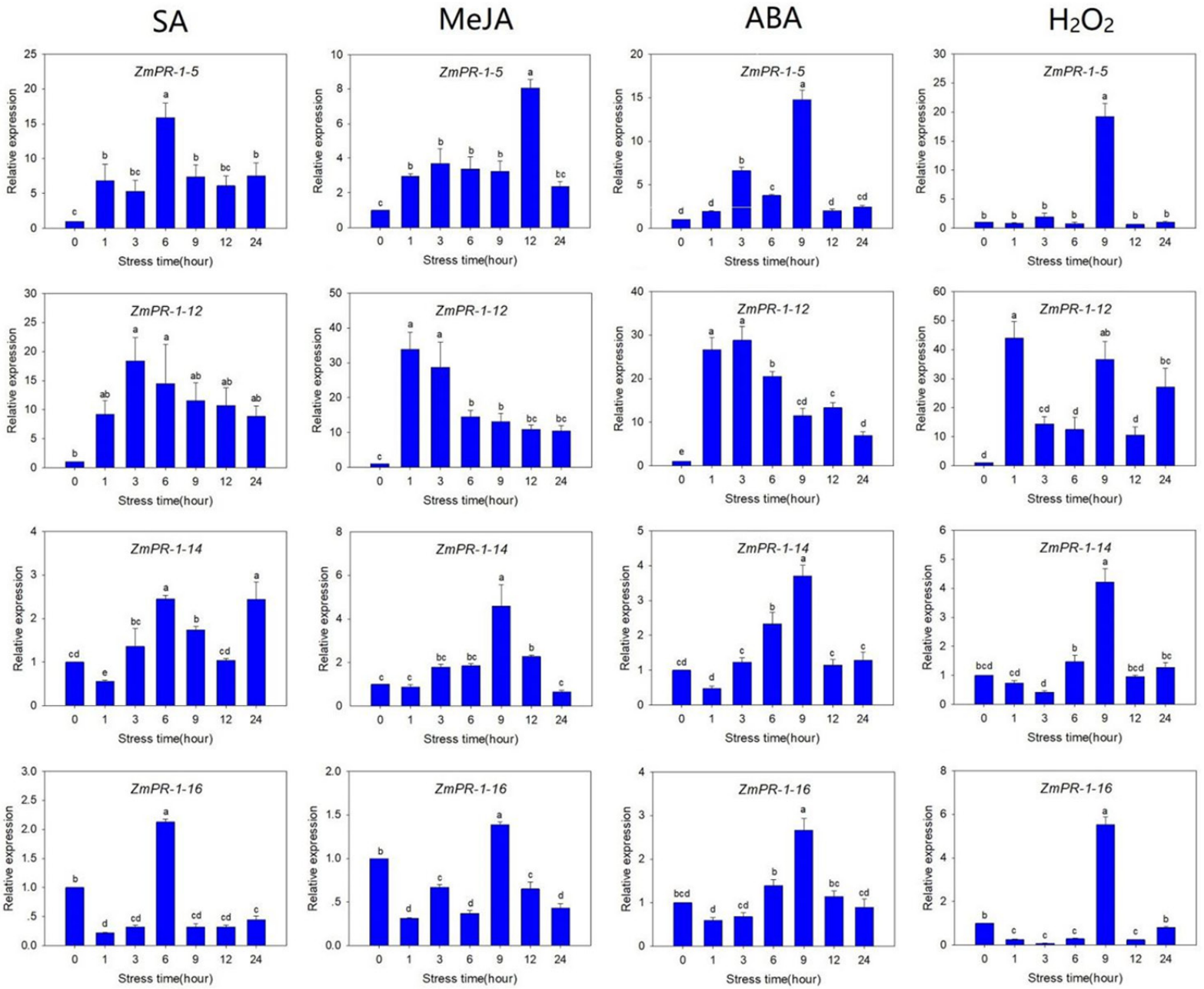
SA
MeJA
ABA
H2O2
ZmPR-1-5
ZmPR-1-12
ZmPR-1-14
ZmPR-1-16
Relative expression
Stress time(hour)
0 1 3 6 9 12 24

Figure 7. RT-qPCR was performed to analyze the expression profiles of 4 selected ZmPR-1 genes after SA, ABA, MeJA and H_2O_2 treatments. All of the relative expression levels were log2 transformed. Bars represent the mean values of three replicates ± standard deviation (SD). Different small letters indicate significant differences at $P<0.05$.

and -16 genes are widely induced by pathogenic bacteria, indicating that these ZmPR-1 genes play an important role in the interaction between maize and these pathogens.

Expression profile of ZmPR-1 gene after SA, ABA, MeJA and H_2O_2 treatment

Previous analysis of cis-acting regulatory elements revealed that the promoter of the ZmPR-1 gene contains several putative cis-regulatory elements related to plant hormone responses. Therefore, we speculate that their expression could be induced by a variety of plant hormones. We used qRT-PCR technology to analyze the transcriptional expression profiles of 4 ZmPR-1 genes after treatment with exogenous plant hormones (SA, ABA, MeJA) and H_2O_2 in 4-leaf stage seedlings. The results in **Figure 7** show that SA, ABA, MeJA and H_2O_2 have significant inducing effects on the four ZmPR-1 (ZmPR-1-5, 12, 14, and 16), but their expression patterns are different after 24 hours of treatment. For example, after SA treatment, the expression patterns of ZmPR-14 and 16 were similar. As the induction time prolonged, their expression level first decreased, then gradually increased. Their expression level reached the highest value at 6 h, which increased by 2.45 and 2.13 times as compared with the control (0 h). After that, it gradually decreased and reached the second peak in 24 hours. However, the expression of ZmPR-5 and 12 gradually increased with the prolonged induction time, reaching the highest value at 6 h and 3 h respectively, and the expression level increased by 19.29 and 16.21 times compared with the control (0 h), and then gradually decreased.

After MeJA treatment, ZmPR-12 expression level increased rapidly, the expression reached the highest value at 1 h, and then gradually decreased; while ZmPR-16 showed the opposite trend, with the expression reaching the lowest value at 1 h; while the expression of ZmPR-5 and 14 gradually increased with the prolonged induction time, reaching the highest value at 12 h and 9 h, respectively. Their expression level increased by 8.05 and 4.09 times compared with the control (0 h), and then gradually decreased.

After ABA treatment, the expression patterns of ZmPR-14 and 16 were similar. As the induction time prolonged, the expression level first decreased, then gradually increased, reaching the highest value at 9 h, and the expression level increased by 3.71 and 2.66 times respectively compared with the control (0 h), and then gradually decreased. The expression level of ZmPR-12 reached the highest value at 1 h, and then gradually decreased; the expression level of ZmPR-5 peaked at 3 h and 9 h, respectively.

After H_2O_2 treatment, the expression patterns of ZmPR-5, 14 and 16 were similar. The expression level reached the highest value at 9 h, while ZmPR-12 increased rapidly, and the expression level peaked at 1 h and 9 h, respectively. The above results indicate that ZmPR-1 genes are involved in the stress response mediated by plant hormones, and differential ZmPR-1 gene expression may play an important role in maize under biotic and abiotic stress.

Discussion

Structural characteristics and evolution of maize PR-1 family genes

Previous reports have shown that there are 39, 22, and 23 PR-1 family genes in Arabidopsis, rice and wheat, respectively, some of which have a regulatory role in biotic and abiotic stress [3]. Although it has been reported that two PR-1 genes have been cloned in maize before, the PR-1 gene family in maize has not been fully studied so far. In this study, we used the latest maize (B73) genome data to identify the 17 ZmPR-1 genes. These analyses provide the first complete list of maize PR-1 gene families. Although the maize genome is larger than that of rice and Arabidopsis, the number of PR-1 family genes in maize is less than that of rice (22 OsPR-1 genes) and Arabidopsis (39 AtPR-1 genes). Gene structure analysis showed that, except for ZmPR-1-4 in maize, which contains 1 intron, the coding regions of other PR-1 genes do not have introns (**Figure 2B**). Our results show the similar findings with rice PR-1 genes [44]. Generally, genes without introns are considered to be putative pseudogenes [45]. Furthermore, when compared to the struc-

tures of other plant gene families, most members of the maize PR-1 genes have only one exon with no clear functional annotation. The PR-1 protein family is part of a distinct and highly conserved protein family [10]. Several studies have shown that different plant PR-1 proteins share homology and structural motifs, indicating that PR-1 genes are conserved [2]. Similarly, the phylogenetic tree analysis revealed that among the four plants, maize and sorghum had the closest phylogenetic relationship, with a high level of homology in their PR-1 protein sequences. These findings support the previous hypothesis that phylogenetically close species share more homology in the same gene members.

Maize PR-1 gene expression in different tissues

The PR-1 protein is not only involved in host defense. A large number of reports show that the PR-1 gene responds to abiotic stimuli, implying that it also plays an important role in the response to abiotic stress [14-18]. There is also evidence that the PR-1 protein plays a role in plant growth or development that is independent of the stress response [19-21]. PR-1 protein, for example, is found in the senescent leaves of adult flowering plants [46]. Lotan et al. found that the PR-1 protein accumulates in the sepals of developing flowers, implying that it may play an important role in flowering plants [47]. Possible role We investigated the expression patterns of 17 maize PR-1 genes in various maize tissues and growth periods and discovered that ZmPR-1 genes are differentially regulated in a variety of biological processes, including maize growth and development. Their expression levels are different (**Figure 4**). Collectively, our data indicate that ZmPR-1 genes play multiple regulatory roles in the growth and development of maize.

The role of maize PR-1 gene in biological stress

A growing body of evidence indicates that PR-1 plays a role as a defensive protein in plant-pathogen interactions [21]. The PR-1 gene is a multi-gene family, and studies have found that only a few members of this family have inducible expression and inhibitory activity against pathogens. For example, there are 22 and 39 PR-1 genes in Arabidopsis and rice, respectively, but only 1 and 2 genes were found to be induced by pathogens or insect attacks, respectively [3]. Three PR-1 genes analyzed in apple (Malus domestica B.) did not show induced expression after inoculation with pathogenic bacteria or treatment with SAR elicitor [13]. In this study, the analysis of microarray data found that after the infection with *S. nonagrioides*, *S. reilianum*, *F. moniliforme*, *P. cinnamomi* and *C. graminicola* in maize, there were 4, 5, 5, 5 and 5 ZmPR-1 genes being up-regulated, and this result is consistent with previous studies of PR-1 genes in other plants. Interestingly, we found that ZmPR-1-5, -12, -14 and -16 genes are widely induced by pathogenic bacteria, indicating that these PR-1 genes have potential roles in broad-spectrum disease resistance.

Expression of corn PR-1 gene after induction of plant hormones

Previous research has shown that the PR1 gene in Arabidopsis is only up-regulated by SA or INA (2, 6-dichloroisonicotinic acid), but not by MeJA or ET [48]. PR1 expression in tobacco can be induced not only by SA, but also by the combination of ET and MeJA; however, neither MeJA nor ET can induce PR1 expression in tobacco [49]. In rice, SA induces the PR1b gene only weakly, but exogenous JA strongly activates it [50]. PR1 family members' expression is constitutive in pear trees and is unaffected by BTH or SA treatment, indicating that they are not regulated in the SAR response [51]. In this study, it is found that in the leaves of maize seedlings, the ZmPR-1 gene was not only strongly induced by SA, but also strongly up-regulated by MeJA and ABA. The results of cis-element analysis also revealed the functional diversity of the maize ZmPR-1 genes, which mainly engages the response sto biotic and abiotic stresses, and the induced responses to plant hormones. Altogether, these results indicate that maize ZmPR-1 may have unique regulatory patterns and functional roles, which is different from previously reported RP-1 genes in Arabidopsis, tobacco and rice.

Conclusions

In summary, we have identified 17 ZmPR-1 genes in maize, which are unevenly distributed on 8 maize chromosomes. We have predicted the conserved motifs and domains of these

genes, chromosomes and subcellular locations, and sequence homology with other plant PR-1, which provides the possibility for the further interrogation of their structures and function. In addition, the expression profiles of ZmPR-1 genes in growth and development, pathogen infection and plant hormone induction were studied, and the results showed that ZmPR-1 gene is not only implicated in the process of growth and development, but also has important regulatory functions in disease resistance of maize. Our data provide important clues for future investigation of ZmPR-1 family genes in different plant responses.

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Disclosure of conflict of interest

None.

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