Research Article

Genome-Wide Identification and Characterization of PRR Gene Family and their Diurnal Rhythmic Expression Profile in Maize

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As essential components of the circadian clock, the pseudo-response regulator (PRR) gene family plays critical roles in plant photoperiod pathway. In this study, we performed a genome-wide identification and a systematic analysis of the PRR gene family in maize. Nine ZmPRRs were identified, and the gene structure, conserved motif, evolution relationship, and expression pattern of ZmPRRs were analyzed comprehensively. Phylogenetic analysis indicated that the nine ZmPRR genes were divided into three groups, except for ZmPRR73, two of which were highly homologous to each of the AtPRR or OsPRR quintet members. Promoter cis-element analysis of ZmPRRs demonstrated that they might be involved in multiple signaling transduction pathways, such as light response, biological or abiotic stress response, and hormone response. qRT-PCR analysis revealed that the levels of ZmPRRs transcripts varied considerably and exhibited a diurnal rhythmic oscillation expression pattern in the given 24-h period under both SD and LD conditions, which indicated that the level of transcription of ZmPRRs expression is subjected to a circadian rhythm and modulated by light and the circadian clock. The present study will provide an insight into further exploring the biological function and regulatory mechanism of ZmPRR genes in circadian rhythm and response to photoperiod in maize.

1. Introduction

The perception of daily changes in photoperiod and temperature enables plants to adapt to different latitudes and achieve successful reproduction. The circadian clock is an autonomous oscillator that produces endogenous biological rhythms of a period of about 24 h and plays crucial roles in coordinating the development and metabolism with daily and seasonal changes through the synchronous expression of genes involved in related biological processes. Many clock-associated genes constitute an interlocked transcription and translation feedback loops to maintain the function of the circadian clock, in which clock-associated genes regulate each other to generate oscillations with expression peaks at specific times during the daily cycle [1–5]. Pseudo-response regulators are important components of the core of circadian clock. The Arabidopsis PRR family has five members: TIMING OF CAB EXPRESSION 1 (TOC1/PRR1), PRR3, PRR5, PRR7, and PRR9.

Numerous studies have indicated the significant roles of PRRs in circadian clock [6]. TOC1 is an essential component of the Arabidopsis circadian oscillator, which exerts its function by directly repressing the transcription of morning-expressed oscillator genes CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) at the core of the clock [7, 8]. PRR9, PRR7, and PRR5 are transcription repressors and repress the transcription of CCA1, LHY, REVEILLE 8 (RVE8), NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED 1 (LNK1), LNK2, LNK3, LNK4, and PRR genes expressed during earlier phases [4]. In addition, PRRs were reported to directly interact with and stabilize CONSTANS (CO) protein, thereby increasing the binding of CO to the promoter of FLOWERING LOCUS T (FT), leading to enhanced FT transcription and early flowering under long-day conditions [9].

The PRR gene family has a highly conserved protein structure. Each member contains a pseudo-receiver (PR)
domain of approximately 120 amino acids at its N-terminus and a CCT motif of approximately 50 amino acids at the C-terminal end [10–12]. The PR domain of PRRs allows oligomerization between the PRRs and bridges interactions of the PRRs and other proteins [5, 7, 11, 13–15]. The transcriptional repression activity of PRRs relies on the presence of a functional CCT domain to recognize the target genes [7, 16–18]. PRR9, PRR7, and PRR5 have a transcriptional repression motif in an intervening region (IR) between the PR and CCT domains; however, this motif is not present in the IR of TOC1 [17].

Rhythmic gene expression and protein accumulation of PRR family members is crucial for proper clock function. PRRs are sequentially expressed from early daytime until around midnight, and each PRR protein works at a specific time. PRR9 functions during early daytime, PRR7 is active from early daytime until midnight, PRR5 works from noon until midnight, and TOC1 is expressed during nighttime [17]. The protein levels of the five PRRs in Arabidopsis thaliana peak sequentially throughout the day, starting with PRR9 3 to 4 h after dawn, followed by PRR7, PRR5, PRR3, and TOC1/PRR1 peaking 1 to 3 h after dusk [6, 7, 12, 15, 17]. PRR9, PRR7, PRR5, and TOC1 proteins are degraded during the night, so they mainly accumulate during the day when they repress the transcription of genes encoding other clock components such as LHY and CCA1 [5, 6, 8, 19–21].

Research on different plants has contributed to our understanding of the function of PRRs. Dicotyledonous and monocotyledonous plants might share the evolutionarily conserved molecular mechanism underlying the circadian rhythm. Rice also has five members of the OsPRR family: OsPRR1, OsPRR37, OsPRR37, OsPRR95, and OsPRR59. Each of OsPRR5 encodes a protein highly homologous to each one of the AtPRR1/TOC1 quintet members: the OsPRR1 gene is the ortholog of AtPRR1; OsPRR95 and OsPRR59 are classified into the same group, to which APRR9/APRR5 belong; OsPRR73/OsPRR37 considerably resemble either APRR7 or APRR3, respectively [12]. There are five copies of the PRR genes in the genomes of Sorghum bicolor, Vitis vinifera, Brassica rapa, and Carica papaya, whereas seven PRR copies have been found in Populus trichocarpa [22–25]. Many PRR genes have been identified in a wide range of crop species. Ppd-H1, Ppd-1, TaPRR73, BvBTC1, PRR37, DTH7, Ghd7.1, OsPRR37, and ShPRR37 encode PRR proteins in barley, wheat, beet, rice, and sorghum, respectively. Allelic variation at these genes confers natural diversity in flowering time [26–32]. Phylogenetic analysis indicates that there are three clades of PRR genes in land plants, including the TOC1, PRR7/3, and PRR5/9 groups based on their similarity to their respective Arabidopsis proteins [33].

Although PRR genes have been investigated in various plants, few studies have explored these genes in maize, and information on the molecular characterization of the PRR family in maize (Zea mays L.) is minimal. Based on the assumption that other plants might share the molecular mechanisms underlying the circadian rhythm, we determined whether maize also has a set of PRRs and whether or not they are associated with the circadian rhythm. Systematical analyses including genome-wide identification, gene classification, chromosomal localization, gene structure analyses, phylogenetic analyses, and expression profiling were conducted in this study. The results might provide a basic understanding of the maize ZmPRR genes and facilitate future researches to further elucidate the potential function of PRR genes in maize development.

2. Materials and Methods

2.1. Plant Material, Photoperiod Treatment, and Tissue Collection. Maize inbred line CML288 plants, which is native to the tropical zone and highly sensitive to photoperiod, were planted in an artificial climate chamber at 28°C with photoperiod conditions of 12 h of light and 12 h of dark and an environmental humidity of 50%. To characterize the diurnal expression pattern of ZmPRRs, when the fifth leaves completely expanded, the plants were transferred to chambers at 28°C under SD conditions (9-h light, 15-h dark) and LD conditions (15-h light, 9-h dark), respectively. Ten days after SD or LD treatment, leaves were harvested in 3-h intervals during a 48-h period, starting at Zeitgeber time 0 (0 hr after the lights were turned on). Three biological replicates were collected at the same time. Then the samples were rapidly frozen in liquid nitrogen and immediately stored at -80°C until RNA extraction.

2.2. Identification of Putative PRR Genes in the Maize Genome. To identify all putative PRR proteins in the maize genome, a hidden Markov model profile and a BLAST search were performed. Each PRR gene contains the response regulator receiver domain of approximately 120 amino acids at its N-terminus and CCT motif of approximately 50 amino acids at the C-terminal end. Based on these criteria, HHMmer search was performed by using the Pfam profile of response regulator receiver domain (PF00072) and CCT motif (PF06203) against the maize proteome sequence from the maize genome sequence project database to identify all putative PRR proteins in the maize genome. Simultaneously, the known sequences of PRR proteins from Arabidopsis and rice were also used as queries to search against the maize protein database with the BLASTP program. The AtPRR and OsPRR gene family information from Arabidopsis and Oryza sativa is listed in Table S1. E-value 1e-10 was used as the cut-off value in HHMmer and BLAST searches. The candidate ZmPRR protein sequences were verified based on the presence of conserved response regulator receiver domain and CCT motif by searching against Pfam and SMART databases. Finally, redundant sequences were removed manually in terms of sequence alignment results. The basic physical and chemical parameters of each ZmPRR were calculated by the online ProtParam tool. Plant-mPLoc was used to predict the putative subcellular localization of maize PRR proteins [34].

2.3. Chromosomal Localization, Exon-Intron Structure Analysis, and Regulatory Cis-Element Analysis. All ZmPRR genes were mapped on chromosomes by identifying their
chromosomal positions in the MaizeGDB database. The DNA and cDNA sequences corresponding to each predicted gene from the maize genome and annotation database were downloaded. The exon-intron structures of all genes were obtained using the online Gene Structure Display Server with coding and genomic sequences [35]. The cis-acting elements were searched from the 1500-bp sequences upstream of the start codon (ATG) of the nine members of the ZmPRR gene family by using the PlantCare software [36].

2.4. Sequence Alignments, Phylogenetic Construction, and Motif Analysis. Multiple sequence alignments were performed on the ZmPRR family protein sequences using ClustalX2 with default parameters [37]. A phylogenetic tree was constructed with the aligned PRR protein sequences using MEGA7.0 by employing the neighbor-joining method with the pairwise deletion option and Poisson correction. Bootstrap analysis was performed using 1000 replicates on each node [38]. The MEME program was used to identify conserved motifs, with the following parameters: number of repetitions: any; the maximum number of motifs: 15; and the optimum motif widths: 6-200 amino acid residues [39]. Visualization of the motifs in proteins was created by TBtools [40].

2.5. Gene Duplications, Synteny Analysis, and Calculation of Ka/Ks Values and the Duplication Event Dating. MCScanX was applied to identify and analyze ZmPRR duplication and collinearity relationship among maize, rice, and sorghum, and the graph of synteny relationship was displayed by TBtools [40, 41]. The ratio of nonsynonymous to synonymous nucleotide substitutions (Ka/Ks) was calculated to estimate the divergence time (T) of each duplicated ZmPRR gene pair, based on a rate of λ substitutions per synonymous site per year. Divergence times (T) were estimated with a formula $T = \frac{Ks}{2\lambda} \times 10^{-9}$ Mya ($\lambda$ was kept as 6.5 × 10−9 as reported for grasses and used in earlier studies) [42].

2.6. Expression Pattern Analysis of the ZmPRR Genes. To elucidate the spatial and temporal expression patterns of ZmPRR genes, comprehensive expression analysis was accomplished based on publicly available genome-wide transcription data of maize different tissues at different developmental stages in the SRA (Sequence Read Archive) database under the accession numbers PRJNA171684 and SRR010680, released by Stelplug et al. [43]. FPKM (fragments per kilobase of exon per million fragments mapped) values from averaged biological replicates were used to measure the expression levels of genes. The average FPKM was then log2 transformed and used to generate a heat map using TBtools [40].

To carry on the diurnal expression profile analysis of ZmPRR genes, the relative expression levels of ZmPRRs during 48 h in inbred line CML288 plants under LD and SD conditions were detected by real-time quantitative PCR (qRT-PCR). Total RNA from leaves was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instruction. Each RNA sample was treated with DNase I to eliminate any residual genomic DNA. Then the RNAs were reverse transcribed into the first-strand cDNA by the RevertAid Premium Reverse Transcriptase (Thermo Scientific™ EP0733). Quantitative real-time PCR was performed on a LightCycler480 Software Setup (Roche) using SG Fast qPCR Master Mix (2X) (BBI) according to the manufacturer’s instructions. Reaction volumes were 20 μl consisting of 10 μl SYBR Green qPCR Master Mix (2X), 0.4 μl 10 μM forward primer, 0.4 μl 10 μM reverse primer, 7.2 μl ddH2O, and 2 μl cDNA template. Each reaction was performed in triplicate using the following conditions: 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 7 s, 57°C annealing for 10 s, and extending at 72°C for 15 s, followed by a melting-curve analysis to check the amplification specificity. The constitutive gene ubiquitin was used as endogenous control to normalize expression in maize tissues. Relative gene expression levels were calculated relative to ubiquitin using the 2−ΔΔCt method. Gene-specific primers for qRT-PCR are presented in Supplementary Table S4.

3. Results

3.1. Genome-Wide Identification and Chromosomal Localization of PRR Genes in Maize. For the identification of all putative PRR proteins in the maize genome, HMMER search using the Pfam profile PF00072 (response regulator receiver domain) and PF06203 (CCT motif) as a query and BLASTP program using the known sequences of PRR proteins from Arabidopsis and rice as a query were performed by against the maize proteome sequence (B73 RefGen_v4) from the maize genome sequence project database. The basic information for PRR genes in Arabidopsis and rice is provided in Table S1. Only candidates that contained a PR domain at the N-terminus and a CCT motif at the C-terminal end were regarded as actual maize PRR proteins (ZmPRRs). As a result, nine candidate proteins were identified as members of the PRR gene family in maize (Table 1). To further verify the reliability of these candidate sequences, we performed the SMART analysis of the nine putative ZmPRR protein sequences, which detected the response regulator receiver domain and CCT motif sequences in all of these candidate sequences. For each of the PRRs identified in maize, a name was given according to the evolutionary relationship with PRRs in Arabidopsis and rice (see the section of phylogenetic analysis). We used the ProtParam tool from ExPASy to analyze the protein size, molecular weight, and theoretical isoelectric point (pI) of these ZmPRR proteins. These ZmPRR genes encode polypeptides ranging from 517 (ZmPRR1-2) to 766 (ZmPRR73) amino acid residues, with a transcript length from 2132 (ZmPRR1-1) to 3538 (ZmPRR73). The molecular weights of the identified ZmPRR proteins ranged from 57.56 kDa (ZmPRR1-2) to 83.76 kDa (ZmPRR73), and the theoretical isoelectric point varied from 6.12 (ZmPRR95-1) to 9.67 (ZmPRR37-1). The prediction of the subcellular localization of maize PRR...
Table 1: The detailed characteristics of ZmPRR genes identified in maize.

| Gene name  | Gene ID         | Location               | Ave. residue weight (g/mol) | Charge | Isoelectric point | Molecular weight (g/mol) | Number of residues | Exon number | Transcript length (bp) | Grand average of hydropathicity | Subcellular location |
|------------|-----------------|------------------------|----------------------------|--------|-------------------|--------------------------|--------------------|-------------|------------------------|-------------------------|---------------------|
| ZmPRR1-1   | Zm00001d051114  | 4:143867613:1438707    | 111.473                    | 2.5    | 6.859             | 60864.39                 | 546                | 7           | 2132                   | -0.593                  | Nucleus              |
| ZmPRR1-2   | Zm00001d017241  | 5:190060998:1900639    | 111.333                    | 2      | 6.7272            | 57559.37                 | 517                | 7           | 2169                   | -0.6                    | Nucleus              |
| ZmPRR37-1  | Zm00001d007240  | 2:225519590:2255243    | 107.252                    | 2.5    | 9.6686            | 63492.96                 | 592                | 7           | 2381                   | -0.933                  | Nucleus              |
| ZmPRR37-2  | Zm00001d022590  | 7:180509884:1805436    | 108.969                    | 9      | 7.4884            | 82162.67                 | 754                | 9           | 2838                   | -0.859                  | Nucleus              |
| ZmPRR73    | Zm00001d047761  | 9:141194049:1412029    | 109.353                    | 1      | 6.5579            | 83764.05                 | 766                | 12          | 3538                   | -0.873                  | Nucleus              |
| ZmPRR59-1  | Zm00001d004875  | 2:145683678:1456888    | 108.792                    | 17.5   | 8.2365            | 75610.19                 | 695                | 8           | 2750                   | -0.734                  | Nucleus              |
| ZmPRR59-2  | Zm00001d052781  | 4:200737914:2007440    | 108.216                    | 18     | 8.2039            | 75101.76                 | 694                | 8           | 2649                   | -0.63                   | Nucleus              |
| ZmPRR95-1  | Zm00001d006212  | 2:201957643:2019639    | 112.081                    | -4     | 6.1166            | 70722.9                  | 631                | 9           | 2348                   | -0.82                   | Nucleus              |
| ZmPRR95-2  | Zm00001d021291  | 7:148065548:1480712    | 110.775                    | -1.5   | 6.3568            | 71006.56                 | 641                | 10          | 2407                   | -0.681                  | Nucleus              |
proteins revealed that all these ZmPRRs were localized in the nucleus.

To gain insights into the evolution of the nine ZmPRR genes, we analyzed their genomic distribution. As shown in Figure 1, the nine putative ZmPRR genes were found on chromosomes 2, 4, 5, 7, and 9. Chromosome 2 contained three ZmPRR members (ZmPRR59−1, ZmPRR95−1, and ZmPRR37−1), chromosomes 4 and 7 contained two ZmPRR members, and chromosomes 5 and 9 only comprised one ZmPRR member.

3.2. Phylogenetic Analysis. To further study the evolutionary relationship between ZmPRR genes and PRR genes from other plants, a phylogenetic tree was constructed using the neighbor-joining algorithm with PRR amino acid sequences from Arabidopsis, rice, and maize by MEGA7. 19 PRR homologs, including 5 Arabidopsis PRRs, five rice PRRs, and 9 ZmPRRs were used to construct the phylogenetic tree (Figure 2). According to the generated unrooted phylogenetic tree, the PRR proteins were clustered into three distinct groups, the same as the classification of OsPRR genes.
Here demonstrated that maize has nine
the Ka/Ks values for each duplicated
ZmPRR

duplicated
PRR
alysis was conducted to elucidate the origin of the

species and interspecies synteny analysis were conducted to
strate the evolutionary relationship of the
PRR
gene family, intra-

PRR
genes pairs among maize, rice, and sor-
gum, respectively. As shown in Figure 3 and Table S2, we
ally identified 10 orthologous gene pairs between maize
rice, whereas 8 orthologous gene pairs between maize
and sorghum. More details about these orthologous gene
pairs were shown in Table S2. As expected, the two

PRR
genes in the same clade matched the same PRR
genes in sorghum or rice, except that ZmPRR59-2 did not
match any orthologous gene in rice and ZmPRR37-2 did
not match any orthologous gene in sorghum.

3.3. Intraspecies and Interspecies Synteny Analysis. To illus-
trate the evolutionary relationship of the
PRR
gene family, intra-

PRR
sequences to conserve the ancestral state. In

PRR
orthologous
gene pairs was estimated to have
occurred between 10.48 Mya (million years ago) and
163.93 Mya.

To further illustrate the evolutionary relationship of the

PRR
gene family, we applied comparative analysis to identify
orthologous

PRR
genes pairs among maize, rice, and sor-
gum, respectively. As shown in Figure 3 and Table S2, we
ally identified 10 orthologous gene pairs between maize
rice, whereas 8 orthologous gene pairs between maize
and sorghum. More details about these orthologous gene
pairs were shown in Table S2. As expected, the two

PRR
genes in the same clade matched the same PRR
genes in sorghum or rice, except that ZmPRR59-2 did not
match any orthologous gene in rice and ZmPRR37-2 did
not match any orthologous gene in sorghum.

3.4. Gene Structure Analysis and Identification of Conserved Motif. To study the structural diversity of ZmPRR genes, the exon/intron organization of individual ZmPRR genes was analyzed by comparing cDNA sequences with the corre-
sponding genomic DNA sequence. The detailed gene structures are schematically shown in Figure 4(a). The number of introns ranges from 6 (ZmPRR1-1, ZmPRR1-2, ZmPRR37-1, and ZmPRR37-2) to 11 (ZmPRR73) in ZmPRR genes. Most ZmPRR genes contained six or seven introns. Gene structure analysis indicated that these ZmPRRs are highly homologous to AtPRRs and OsPRRs not only in their amino
acid sequences, but also in their structural designs. Two
extraordinary long introns of 11562 and 18729 bp were
found in ZmPRR37-2, which was much larger than that in
other ZmPRR genes, which were consistent with the gene
structure of OsPRR37 and OsPRR73 in rice. The amino acid
sequence of ZmPRR1 is notably similar to that of AtPRR1
and OsPRR1. The ZmPRR1-1 and ZmPRR1-2 genes
are unique in the sense that the coding sequence of their CCT

| Paralogous pairs                              | Ka      | Ks      | Ka/Ks   | Purifying selection | Divergence time (Mya) | Duplicate type         |
|-----------------------------------------------|---------|---------|---------|---------------------|-----------------------|------------------------|
| ZmPRR95-1/ZmPRR95-2                          | 0.0582  | 0.1715  | 0.3396  | Yes                 | 13.19                 | WGD or segmental       |
| ZmPRR37-1/ZmPRR37-2                          | 0.0386  | 0.1362  | 0.2835  | Yes                 | 10.48                 | WGD or segmental       |
| ZmPRR37-2/ZmPRR73                            | 0.3274  | 1.2088  | 0.2708  | Yes                 | 92.98                 | WGD or segmental       |
| ZmPRR1-1/ZmPRR1-2                            | 0.0564  | 0.2290  | 0.2463  | Yes                 | 17.61                 | WGD or segmental       |
| ZmPRR59-2/ZmPRR95-2                          | 0.7097  | 2.1311  | 0.3330  | Yes                 | 163.93                | WGD or segmental       |

(Murakami et al. 2003). Zm00001d017241 and Zm00001d05114 were classified into group 1, to which AtPRR1 and OsPRR1 belong, so they were designated as ZmPRR1-1 and ZmPRR1-2, respectively. Zm00001d007240, Zm00001d022590, and Zm00001d047761 were classified into group 2, to which AtPRR3, AtPRR7, OsPRR37, and OsPRR73 belong. Group 2 was further subdivided into three sub-
groups. Zm00001d007240 and Zm00001d022590 were classed in the same subgroup with OsPRR37, so they were designated as ZmPRR37-1 and ZmPRR37-2. Zm00001d047761 was classed into the same subgroup with OsPRR73, so it was designated as ZmPRR73. Similarly, Zm00001d004875, Zm00001d052781, Zm00001d006212, and Zm00001d021291 were classified into group 3, to which AtPRR5, AtPRR9, OsPRR9, and OsPRR95 belong. Zm00001d004875 and Zm00001d052781 were classed into the same subgroup with OsPRR95, so they were designated as ZmPRR95-1 and ZmPRR95-2, respectively. Zm00001d006212 and Zm00001d021291 were classed into the same subgroup with OsPRR95, so they were designated as ZmPRR95-1 and ZmPRR95-2, respectively. In short, we
here demonstrated that maize has nine ZmPRR genes
(ZmPRR1, ZmPRR1-2, ZmPRR37-1, ZmPRR37-2, ZmPRR73, ZmPRR95-1, ZmPRR95-2, ZmPRR95-1, and
ZmPRR95-2). Except for ZmPRR73, two of these genes were
highly homologous to each of the AtPRR or OsPRR quintet
members.

Table 2: Ka/Ks analysis and estimated divergence time for the duplicated ZmPRR paralogs.

| Paralogous pairs                              | Ka      | Ks      | Ka/Ks   | Purifying selection | Divergence time (Mya) | Duplicate type         |
|-----------------------------------------------|---------|---------|---------|---------------------|-----------------------|------------------------|
| ZmPRR95-1/ZmPRR95-2                          | 0.0582  | 0.1715  | 0.3396  | Yes                 | 13.19                 | WGD or segmental       |
| ZmPRR37-1/ZmPRR37-2                          | 0.0386  | 0.1362  | 0.2835  | Yes                 | 10.48                 | WGD or segmental       |
| ZmPRR37-2/ZmPRR73                            | 0.3274  | 1.2088  | 0.2708  | Yes                 | 92.98                 | WGD or segmental       |
| ZmPRR1-1/ZmPRR1-2                            | 0.0564  | 0.2290  | 0.2463  | Yes                 | 17.61                 | WGD or segmental       |
| ZmPRR59-2/ZmPRR95-2                          | 0.7097  | 2.1311  | 0.3330  | Yes                 | 163.93                | WGD or segmental       |

Ka/Ks ratio indicates what selection has been placed on this gene. It is commonly
accepted that Ka/Ks < 1 means purifying selection, and Ka/Ks > 1 means positive selection, which
means evolutionary pressure would eliminate deleterious mutations in the species to conserve the ancestral state. In
our research, all the Ka/Ks values for ZmPRR duplicated
gene pairs were < 1 indicating that the maize
PRR
gene family is highly conserved during evolution and are deduced to
be under purifying selection which would eliminate deleterious
mutations in the species. According to a substitution
rate of 6.5 × 10^-9 substitutions per synonymous site per year,
as previously proposed for maize (Murakami et al. 2003), the divergence time of
5 duplicated ZmPRR gene pairs was estimated to have
occurred between 10.48 Mya (million years ago) and
163.93 Mya.
motif is not separated by an intron, whereas other ZmPRR genes contain an extra intron which was consistent with AtPRR1 and OsPRR1 [12].

Conserved motif and gene structure analysis also verified the classification of the phylogenetic analysis. MEME4.12.0 was used to conduct conserved motif analysis, and 15 motifs were detected in ZmPRR proteins (Figure 4(b) and Table S3). The CCT motif (motif 2), motif 5, and the REC domain, composed of motif 3, motif1, and motif 4, were the most conserved motifs found in almost all nine ZmPRR members. It is worth noting that motif 3 and motif 4 were absent in ZmPRR37-1, whereas motif 5 was absent in ZmPRR1-1 and ZmPRR1-2. The genes in the same group usually share group specific conserved motifs. For instance, motif 6 and motif 9 were only found in ZmPRR1-1 and ZmPRR1-2, whereas motif 7 and motif 10 were only found in ZmPRR73, ZmPRR37-1, and ZmPRR1-2, and motif 8 and motif 15 were only found in ZmPRR59-1/-2 and ZmPRR95-1/-2. In general, the structure of ZmPRR proteins was conserved throughout the ZmPRR gene family.

To identify putative regulatory cis-acting elements enriched in maize PRR genes, the promoter sequences in 3 kb of genomic DNA upstream of the start codon (ATG)
were extracted and searched against the PlantCARE database. Consistent with the fact that PRR is highly regulated by light, 26 putative cis-elements related to light response were detected in ZmPRRs. Sp1 and G-box were the most frequently found cis-elements in all the nine ZmPRR members, followed by circadian found in 5 ZmPRRs, and ACE and AE-box found in 4 ZmPRRs (Table 3). ZmPRR73 had the highest number of light-responsive cis-elements, followed by ZmPRR37-1.

Thirteen putative cis-elements known to be regulated by hormones in some plant genes were identified in ZmPRR members: 3 cis-acting elements involved in abscisic acid responsiveness (ABRE, CE1, and CE3), 3 cis-acting elements participating in gibberellin responsiveness (TATC-box, P-box, and GARE-motif), 3 cis-acting regulatory elements involved in auxin responsiveness (TGA-box, TGA-element, and AuxRR-core), 2 cis-acting regulatory elements associated with MeJA-responsiveness (CGTCA-motif and TGACG-motif), a cis-acting component associated with ethylene-responsive (ERE), and one cis-acting element involved in salicylic acid responsiveness (TCA-element) (Table 3). Among them, ABRE, CGTCA-motif, and TGACG-motif were the most frequently found cis-elements in all the nine ZmPRR members.

In addition, putative cis-elements associated with biotic and/or abiotic stress adaptive elements, such as anaerobic induction (ARE and GC-motif), cold and dehydration-responsiveness (C-repeat/DRE), heat stress responsiveness (HSE), low-temperature responsiveness (LTR), drought-inducibility (MBS), and defense and stress responsiveness (TC-rich repeats), were detected in a series of members.

3.5. The Expression Patterns of ZmPRR Genes in Different Tissue and Developmental Stages. To further investigate the tissue-specific expression patterns of ZmPRR genes, comprehensive expression analysis was accomplished based on publicly available genome-wide transcription data of maize tissues at different developmental stages in the SRA (Sequence Read Archive) database at NCBI (National Center for Biotechnology Information) under the accession numbers PRJNA171684 and SRP010680, released by Stelpflug et al. [43]. The tissue-specific expression patterns revealed the potential roles of ZmPRRs at maize special developmental stages. It can be seen from the heat map that nine ZmPRR genes express in all tissues and have distinct expression levels in different tissues at different developmental stages, as shown in Figure 5. Among the nine ZmPRRs, ZmPRR73 showed the highest expression in almost all tested tissues, followed by ZmPRR95-2. All the ZmPRRs show relatively high expression in reproductive organs such as Immature_Tassel_V13, Meiotic_Tassel_V18, and Immature_Cob_V18. ZmPRR37-1 and ZmPRR37-2 exhibited relatively low expression levels in the root and seed at different developmental stages and relatively high expression levels in the
Table 3: Putative cis-elements enriched in the promoters of ZmPRR family genes.

| Site name     | Sequence        | Gene Function                                                                 |
|---------------|-----------------|-------------------------------------------------------------------------------|
| CE1           | TGCCACCGG       | Cis-acting element associated to ABRE, involved in ABA responsiveness          |
| ABRE          | TACGTGTC        | Cis-acting element involved in the abscisic acid responsiveness                |
| CE3           | GACGCGTGTCA     | Cis-acting element in ABA and VP1 responsiveness                               |
| TGA-element   | AACGAC          | Auxin-responsive element                                                       |
| TGA-box       | TGACGTAA        | Part of an auxin-responsive element                                            |
| AuxRR-core    | GGTCAT          | Cis-acting regulatory element involved in auxin responsiveness                 |
| TCA-element   | GAGAAGAATA      | Cis-acting element involved in salicylic acid responsiveness                  |
| CGTCA-motif   | CGTCA           | Cis-acting regulatory element involved in the MeJA-responsiveness              |
| TGACG-motif   | TGACG           | Cis-acting regulatory element involved in the MeJA-responsiveness              |
| ERE           | ATTTCAAA        | Ethylene-responsive element                                                    |
| GARE-motif    | AAACAGA         | Gibberellin-responsive element                                                 |
| P-box         | CTTTTTG         | Gibberellin-responsive element                                                 |
| TATC-box      | TATCCCA         | Cis-acting element involved in gibberellin-responsiveness                     |
| MSA-like      | CCAACGGT        | Cis-acting regulatory element related to meristem regulation                   |
| CAT-box       | GCCACT          | Cis-acting regulatory element required for endosperm expression               |
| CGGTCC-box    | CGGTCC          | Cis-acting regulatory element related to meristem specific activation         |
| Skn-1_motif   | GTCAT           | Cis-acting regulatory element required for endosperm expression               |
| GCN4_motif    | CAAGCCA         | Cis-regulatory element involved in endosperm expression                       |
| CCAAT-box     | CAACGG          | MYBHv1 binding site                                                           |
| TC-rich repeats | ATTTTCTCCA    | Cis-acting element involved in defense and stress responsiveness             |
| HSE           | AAAAAATTTTC     | Cis-acting element involved in heat stress responsiveness                      |
| ARE           | TGTTTT          | Cis-acting regulatory element essential for the anaerobic induction           |
| GC-motif      | CCCCCG          | Enhancer-like element involved in anoxic specific inducibility                |
| MBS           | CAACTG          | MYB binding site involved in drought-inducibility                             |
| C-repeat/ DRE | TGCCGCGAC       | Regulatory element involved in cold- and dehydration-responsiveness          |
| LTR           | CCGAAA          | Cis-acting element involved in low-temperature responsiveness                |
| ACE           | ACGTGGAG        | Cis-acting element involved in light responsiveness                           |
| Circadian     | CAANNNNATC      | Cis-acting regulatory element involved in circadian control                   |
| C-box         | CTGACGTCA       | Cis-acting regulatory element involved in light responsiveness                |
| G-box         | CACGTT          | Cis-acting regulatory element involved in light responsiveness                |
| as-2-box      | GATAtGATG       | Involved in shoot-specific expression and light responsiveness                |
| 4cl-CMA2b     | TCTCACAACC      | Light responsive element                                                       |
| Box I         | TTTCAA          | Light responsive element                                                       |
leaves. The expressions of the remaining ZmPRRs were far lower. ZmPRR1-2 exhibits a very low expression level in leaves at earlier developmental stages, whereas ZmPRR1-1 exhibits far lower expression level in leaves after V9 developmental stages compared to other tissues. Specifically, ZmPRR1-1 was expressed at high levels in endosperms of 12-16 days after pollination, which was remarkably more than any other ZmPRRs. ZmPRR59-1, ZmPRR59-2, and ZmPRR95-2 show relatively high expression levels in all tissues except for ZmPRR95-2 in seeds. Among all tested tissues, ZmPRR73 exhibits the highest levels in leaves, and ZmPRR95-2 exhibits the highest levels in internode. These results indicated the diverse functions among the members of the ZmPRR gene family in different stages of maize development.

3.6. The Expression Patterns of ZmPRR Genes under Diurnal Changes. A feature shared by circadian genes is that their abundance is subject to diurnal oscillation. To characterize the diurnal expression pattern of ZmPRRs, CML288 plants were grown under LD and SD conditions, and the relative expression levels of ZmPRRs during 48 h were detected by qRT-PCR (Figure 6). qRT-PCR analysis revealed that the levels of ZmPRRs transcripts varied considerably and exhibited a diurnal rhythmic oscillation expression pattern in the given 24-h period under both SD and LD conditions. Each mRNA started accumulating after dawn sequentially at approximately 3-h intervals in the order of ZmPRR73, ZmPRR37, ZmPRR95, ZmPRR59, and ZmPRR1. The transcript levels ZmPRR73 increased immediately from dawn and peaked 9 h after dawn and then gradually decreased under LD condition, while gradually increased at 3 h before dawn and peaked at 6-9 h after dawn before gradually declining in the afternoon SD conditions. The transcript levels ZmPRR37-1 and ZmPRR37-2 gradually increased after dawn in the morning and peaked at 6-9 h after dawn under both LD and SD conditions. The transcript levels ZmPRR59-1, ZmPRR59-2, ZmPRR95-1, and ZmPRR95-2 gradually increased 3 h after dawn in the morning and peaked at 9 h after dawn regardless of the photoperiod conditions. The transcript levels ZmPRR1-1 and ZmPRR1-2 gradually increased at 6 h after dawn in the morning under both LD and SD conditions while reaching the peak three hours earlier under SD condition than LD condition. These results indicated that the level of transcription of ZmPRRs is subjected to a circadian rhythm and modulated by light and the circadian clock.
Figure 5: Expression profiles of ZmPRR genes across different tissues. The color scales for fold-change values are shown at the right. The expression values mapped to a color gradient from low (green) to high expression (red). The expression data were gene-wise normalized and hierarchically clustered.
Figure 6: Expression pattern of ZmPRRs under diurnal changes. Expression levels of ZmPRRs in leaves of CML288 throughout a 48-h period of LD or SD treatment. The relative expression levels are normalized to ZmUBQ. The data are means ± SE of three biological replicates.
4. Discussion

4.1. Copy Numbers of PRR Family Genes in Maize. PRR genes are conserved among angiosperm evolutionary lineages. At least five PRR genes have been identified in angiosperm genomes. In eudicotyledonous plants, five copies of PRR genes have been identified in *A. thaliana*, *V. vinifera*, and *C. papaya*; seven copies have been found in *P. trichocarpa*, and eight copies have been identified in *B. rapa* [12, 22, 24, 44]. In monocotyledonous plants, there are five copies of PRR genes in the genomes of *O. sativa* and *S. bicolor*. In contrast to the five copy numbers in sorghum and rice, nine ZmPRR orthologs of the five PRR genes were identified and mapped to the chromosomes in maize in this study. This finding raises a complex question: why are the copy numbers of ZmPRRs different from *O. sativa* and *S. bicolor*?

Angiosperm genomes have undergone several whole-genome duplication (WGD) events. Maize, rice, and sorghum are members of the grass family (Poaceae), which share an ancient WGD dating to approximately 70 million years ago [45]. Maize and sorghum are members of the tribe Andropogoneae. These two species are estimated to have diverged approximately 12 million years ago, the same point in time as the initial diversification of Andropogoneae [46]. In addition to ancient WGD shared by all grasses, the maize genome has undergone a tetraploidy event approximately 5 to 12 million years ago after its divergence from sorghum, producing two functionally distinct maize subgenomes, maize 1, and maize 2 [47, 48]. The tetraploid history in maize resulted in the maize genome having a significantly higher number of orthologous genes than its close relative, *S. bicolor* and the core of the tribe Andropogoneae [45]. By contrast, sorghum and rice have experienced relatively few interchromosomal rearrangements since the ancient WGD shared by all grasses. Genes whose products participate in transcriptional regulatory networks are more likely to be retained as duplicate gene pairs after polyploidy events and subsequent gene deletion events because the imbalance associated with the loss of one member of transcription factors is likely to influence the regulatory network and downstream signals, which may decrease fitness [45, 48]. The regulatory network of the *Arabidopsis* clock system has maintained a degree of the organization throughout the dynamic changes of copy numbers and functions of clock-related genes [24].

Clock-related transcription factors, such as *CCA1/LHY/RVE* and *PRR* gene families, typically display preferential retention after WGD [4, 49, 50]. PRR genes in *P. trichocarpa* and *B. rapa* have expanded more than those in other plant species, and this expansion apparently resulted from polyploidy events. Some reports have shown that 43% of maize-sorghum syntenic genes are retained as homologous pairs in the maize genome. The frequency of genes encoding transcription factors is 4.3 times greater among the retained genes than the fractionated genes following WGD [51]. If the maize tetraploidy behaved as other known polyploidy in plants, the retained genes should be enriched in those clock-related transcription factors? Consistent with this hypothesis, nine ZmPRR orthologs were identified as members of the PRR gene family in maize in this study. Except for PRR73, two copies of each of the five PRRs were identified, which were significantly different from the copy number of the PRR gene family in other grass family members, such as rice and sorghum [24]. Circadian clocks may have become more intricate networks if additional genes have roles in circadian networks. However, the functions of these additional ZmPRR genes in the maize clock system, how these additional copies of PRR genes evolved in monocots, and how they are incorporated in the regulatory network of the clock system in the evolutionary history of maize remain to be elucidated.

4.2. Divergence of the Three PRR Clades Occurred Independently within Monocot and Eudicot Lineages. Ancient PRR genes have clearly diverged into three clades (PRR1/TOC1 clade, PRR3 and PRR7 clade, and PRR5 and PRR9 clade) in angiosperms before the speciation of monocots and eudicots [24]. The copy numbers of PRR genes independently increased in each lineage as a result of ancient chromosomal duplication events. In most plant species, one copy of the PRR1/TOC1 gene was retained in the PRR1/TOC1 clade, whereas at least two copies were found in the PRR3 and PRR7 clade and the PRR5 and PRR9 clade. Phylogenetic analysis showed that the PRR1/TOC1 clade consisted of two different clusters, each exclusively consisting of dicotyledonous PRR1 or monocotyledonous PRR1 genes, indicating that divergence of PRR1 occurred after the speciation of monocots and dicots [24]. Although the PRR3 and PRR7 clade and the PRR5 and PRR9 clade contained two copies of PRR genes in monocots and dicots, the gene duplication events of the PRR3 and PRR7 clade and the PRR5 and PRR9 clade occurred independently within monocot and eudicot lineages, respectively [24]. In eudicots, the gene duplication events between PRR3 and PRR7 and between PRR5 and PRR9 are derived from the γ polyploidy event that took place before the speciation of *Vitales* (*V. vinifera*) and *eurosid* species. In monocots, the ancestral PRR3/PRR7 was duplicated into PRR37 and PRR73 in the p polyploidy event that occurred before the speciation of *O. sativa* and *S. bicolor*. The monocot PRR59 and PRR95 genes showed an earlier gene duplication that may have occurred in a common ancestor of monocots and eudicots.

In this study, two copies of PRR1/TOC1 genes, ZmPRR1-1 and ZmPRR1-2, in the PRR1/TOC1 clade were identified to be orthologous to PRR1 genes. In the PRR3 and PRR7 clade, three copies of PRR3 or PRR7 genes were identified. ZmPRR37-1 and ZmPRR37-2 were identified to be orthologous to the monocotyledonous PRR37 gene, whereas only ZmPRR73 was identified to be orthologous to the monocotyledonous PRR73 gene. Gene duplication events producing ZmPRR37 and ZmPRR73 might have occurred in a common ancestor of maize and rice. In contrast, the gene duplication events producing ZmPRR37-1 and ZmPRR37-2 occurred independently after the tetraploidy event in maize approximately 5 to 12 million years ago.

In the PRR5 and PRR9 clade, four copies of PRR5 or PRR9 genes were identified in maize. ZmPRR59-1 and ZmPRR59-2 and ZmPRR95-1 and ZmPRR95-2 were identified to be orthologous to the monocotyledonous PRR59...
and PRR95 genes, respectively. The gene duplication events producing PRR95 and PRR59 or PRR5 and PRR9 may have occurred in a common ancestor of monocots and dicots. In the evolution process of maize, the ZmPRR95 gene was duplicated into ZmPRR95-1 and ZmPRR95-2, whereas the ZmPRR59 gene was duplicated into PRR59-1 and PRR59-2 after the tetraploidy event in maize.

5. Conclusions

In this study, nine PRRs were identified and classified into three subgroups in maize based on phylogenetic evolution relationship, conserved motifs, and introns/exons analysis. In addition, ZmPRRs were systematically analyzed, including identification of conserved motif, the gene structure analysis, promoter cis-acting element predication, chromosome localization distribution, estimation of Ka/Ks, duplications, and collinearity analysis. Moreover, whole-genome duplication (WGD) and dispersed duplication (DSD) might be highly contributed to the expansion of ZmPRRs. Finally, the diurnal expression pattern of ZmPRRs under LD and SD conditions results and genome-wide transcription data of maize tissues at different developmental stages showed that the expression of ZmPRRs has a noticeable rhythmic expression trend and tissue-specific expression. It is speculated that ZmPRRs had a significant role in photoperiod response in maize. Our results provide us a strong base for studying the function of PRRs in maize.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Table S1: the basic information for PRR genes in Arabidopsis and rice. Table S2: list of PRR orthologous gene pairs identified in maize, rice, and sorghum. Table S3: motif sequence of PRR genes family in maize. Table S4: list of primers used in qRT-PCR analysis. (Supplementary Materials)

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