Genomic identification of organelle-localized PPR genes conferring resistance to Phytophthora capsici in Capsicum annuum L.

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Research Article

Keywords: PPR, Capsicum annuum L., plant immunity, Phytophthora capsici, chloroplasts, mitochondria

Posted Date: November 13th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-7901650/v1

Additional Declarations: No competing interests reported.

Abstract

Background Pentatricopeptide repeat (PPR) gene family is one of the largest gene families in land plants. PPR genes play an important role in regulating chloroplast and mitochondrial gene expression, and participating in plant development, male sterility restoration, and biotic- and abiotic-stress responses. However, current knowledge about its exact role of *Capsicum annuum* in pathogens resistance remains uncharacterized.

Results In this study, we identified and analyzed PPR genes in two varieties: the resistant of *Zunla-1* and susceptible of *Zhanshugang*, with 497 and 199 PPR genes, respectively. Our finding suggested that there are distinct differences in gene abundance and duplication patterns between the two varieties. Additionally, *Zunla-1*exhibited a greater number of differentially expressed PPR genes than *Zhangshugang*after *P. capsici* invasion. Generally, *Zhanshugang* exhibits limited adaptation to biotic stress compared to *Zunla-1*. This discrepancy may attribute to the PPR genes in *Zunla-1* have more complex duplication history and regulatory of stress-responsive gene expression.

Conclusions These findings highlight that chloroplast- and mitochondrial-localized PPR proteins plays a critical role in resistance to biotic stresses in pepper. Thus, our study provided a novel insight into PPR-mediated biotic resistance mechanisms and identifies candidate genes for developing disease-resistant pepper varieties.

Background

Chloroplasts and mitochondria are essential semi-autonomous organelles in plants, which are deeply involved in primary metabolism, developmental processes, and immune regulation through producing diverse defense-related signaling molecules [1, 2]. Recent studies have revealed the complex regulatory networks involving chloroplast- and mitochondrial-related factors in plant immunity [2, 3]. These organelles act as central hubs for immune signaling, coordinately regulating programmed cell death (PCD) and defense responses via reactive oxygen species (ROS), reactive nitrogen species (RNS), hormone homeostasis, RNA editing, or signaling pathway activation during pathogen invasion [4–6]. Despite the post-transcriptional modifications of chloroplast- and mitochondrial-encoded genes rely on nuclear-encoded proteins are clear [7, 8], the identification and functional mechanisms of related regulatory genes remain to be investigated.

As one of the largest nuclear-encoded protein families in plants [8], pentatricopeptide repeat (PPR) proteins mostly targeted to chloroplasts and mitochondria. They are involved in post-transcriptional processing of organellar genes, including RNA editing, splicing, degradation, stabilization, and translational regulation [9–11]. The PPR proteins are characterized by tandem repeats of 31–36 amino acid residues. Generally, they can be categorized into three types: canonical P-type (35-amino acid residues), L-type (35–36 amino acids), and S-type (31 amino acids) [8, 12, 13]. Based on these motifs, the PPR genes are divided into two subfamilies of P and PLS, which contains only P domains or typically

includes additional C-terminal domains such as E, E+, and DYW domains [8, 14]. These domains appear individually or in combination, then contributing to the functional diversity of PPR proteins [8, 12, 13].

Due to specific functions of PPR proteins were determined by their domains, the P-class PPR proteins primarily function in splicing of group II introns within mitochondrial transcripts. Whereas PLS-class proteins predominantly catalyze cytidine-to-uridine RNA editing, which is the most prevalent form of RNA editing in mitochondria and chloroplasts [8, 15, 16]. Through the regulation of mitochondrial and chloroplast gene expression, PPR proteins take part in diverse essential plant processes, including photosynthesis, growth and development, and responses to both abiotic- and biotic-stress [8, 17]. In addition, the functional diversity of PPR proteins were further enhanced by auxiliary domains such as E, E+, and DYW in PLS-class proteins, contributing to specific roles in RNA editing and gene regulation [12].

Emerging evidence underscores the pivotal role of PPR proteins in regulating plant immune defense pathways. For instance, overexpression of the mitochondrial-encoded OCP3, initially identified as a PPR protein, facilitates RNA editing of the chloroplast *ndh8* transcript, thereby enhancing resistance to the necrotrophic fungus *Plectosphaerella cucumerina* [18]. Furthermore, the nuclear-encoded PPR protein RTP7, governed by the *RTP7* gene, functions as a negative regulator of plant immunity [19]. These discoveries highlight the evolutionary adaptability and functional diversity of PPR proteins for crops disease-resistant.

Capsicum annuum L., a major globally cultivated vegetable crop, has garnered limited research attention on its PPR gene family. During cultivation, peppers exhibit susceptibility to pathogens such as *P. capsies*, which cause significant yield and quality losses and remains challenging to manage [20]. Despite evidence indicating that PPR genes regulate plant immunity across diverse species, their role in enhancing pepper resistance to *P. capsici* remains poorly understood. The evolutionary dynamics and functional mechanisms of PPR genes in peppers remain largely unexplored. Given the rapid evolution of *P. capsici* and declining resistance in susceptible pepper varieties, developing disease-resistant cultivars constitutes the most economically sustainable strategy for managing *P. capsici* infections. Consequently, identifying and characterizing PPR genes involved in pepper immunity is critical for enhancing disease resistance in pepper breeding programs.

Thus, this study utilized the resistant variety *Zunla-1* and the susceptible variety *Zhangshugang* genomes to investigating the PPR gene family. By comparing the evolutionary trajectories, functional divergence, and response patterns of PPR genes in these two varieties under *P. capsici* infection, we sought to elucidate the mechanisms underlying PPR-mediated pepper immunity. Our findings will provide theoretical insights and practical targets for enhancing pepper resistance breeding, while advancing our understanding of PPR gene functions in pepper disease resistance.

Materials and methods

Plant and strain materials

In the current study, pepper of *Zunla-1* was used as a resistant variety, while *Zhangshugang* was used as a susceptible variety. The genome data of *Zunla-1* was available at the National Genomics Data Center (NGDC) under accession number PRJCA025503 [21]. Equally, the genome of *Zhangshugang* was also accessible at the same center under accession number PRJNA800056 [22] (Table 1). Pathogen strain of *Phytophthora capsica* LT1534 is maintained in our laboratory for this study. In order to inoculate peppers, *P. capsica* was cultured at 25°C under dark illumination on V8 medium for vegetative growth, then used for pepper leaf inoculation. The plants were cultivated in plastic flowerpot and inoculated with colonies of 5 mm in diameter at the leaf surface by a dispenser at the 4–6 true leaf stage. Accordingly, the inoculated plants were grown at room temperature in a controlled environment. While the final disease leaves were collected at 24- and 72-hours post-inoculation (hpi) with four replications for transcript sequencing.

Identification of PPR genes in Zunla-1 and Zhangshugang

To identify the PPR gene family in *Zunla-1* and *Zhangshugang*, we downloaded the seed file (PF01535) from the Pfam V31.0 database (http://pfam.xfam.org/; accessed on 9 September 2023) based on the Hidden Markov Model (HMM). We applied the HMMER 3.0 program with E-value threshold of < 10 to filter significant matches, enhancing confidence in identifying true PPR genes. Subsequently, the SMART program (http://smart.embl-heidelberg.de/; accessed on 11 September 2023) was employed to analyze the protein domains of the candidate PPR genes from both genomes. Finally, we utilized the HMMER 3.0 matrix defined by conserved domains of PPR gene subfamilies (P, PLS, E1, E2, E+, and DYW) from *Arabidopsis thaliana* to search, analyze, and classify these protein sequences. Proteins lacking PPR motifs or containing fewer than two such motifs were excluded from further analysis.

Subcellular localization and analysis of PPR genes

The number of amino acids, molecular weight (MW) and isoelectric point (pI) of PPR proteins were calculated using ProtParam (https://web.expasy.org/protparam/). Subcellular localization predictions for PPRs were made via Wolf PSORT (https://psort.hgc.jp/).

Chromosome location and structure analysis

MCScan V1.1.12 [23] was employed for collinearity analysis. The genome positions of PPR genes were visualized using Circos V0.69. Mapchart (https://www.wur.nl/en/show/Mapchart.ht) was utilized to create distribution plots of PPR genes on chromosomes. MEME (https://meme-suite.org/meme/tools/meme) was used to identify conserved protein motifs within sequences, and characteristic diagrams of these conserved domains were generated with TBtools [24].

Cis-acting element analysis of PPR genes

To analyze the *cis*-acting elements within the major promoter regions of PPR genes, a 2000-base pair region upstream of the transcription start site was selected. PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to obtain all *cis*-acting elements in each gene's promoter region, and key responsive elements were selected through screening. Plots were visualized using TBtools [24].

Phylogenetic analysis and gene ontology (GO) enrichment analysis

PPR proteins of the *Zunla-1* and *Zhangshugang* were aligned using the MAFFT method [25], and the alignment was trimmed with TrimAL V1.4 [26] with the minimum threshold and gap threshold set at 20%. Phylogenetic trees were constructed using the maximum likelihood method in IQ-TREE [27] with the optimal evolutionary model "LG + F + R10". ITOL was used to visualize the phylogenetic tree and add subclass information [28]. Gene annotation was performed using the EggNOG mapper v2 website (http://eggnog-mapper.embl.de/) with default parameters. Pathway and GO enrichment analyses were conducted for genes using the ClusterProfile V3.8 package in R V4.4.1, and the GO results were classified into three categories: molecular function (MF), biological process (BP), and cellular component (CC). Visualization of these results was using R V4.4.1.

Collinearity and duplication event analysis of PPR genes

To identify orthologous PPR genes between *Zunla-1* and *Zhangshugang*, we initially performed homology alignment of genes from both genomes using Blastp [29]. The top hits from the alignment were then selected as candidate orthologs and further validated using Orthofinder V2.5.2 [30] and MCScan V1.1.12 [23]. We employed DupGen_finder (https://github.com/qiao-xin/DupGen_finder) to identify various types of duplicated genes and statistically analyzed the quantity and proportion of each duplication type of PPR genes across the entire genome [31].

Expression patterns of PPR genes under P. capsici stress

In this study, Hisat2 was employed to map reads of each sample to the *Zunla-1* and *Zhangshugang* genomes, respectively. FeatureCounts [32] was then utilized to calculate gene expression levels with read counts for each sample. The differentially expressed genes (DEGs) between the two varieties were identified using DESeq2 [33] with criteria of (log_2FoldChange) > 2 and FDR < 0.01. Heatmaps and volcano plots were generated using pheatmap V1.0.12 and EnhancedVolcano V1.20.0 to visualize DEG profiles. Venn diagrams were created using VennDiagrams V1.7.3.

Analysis of gene expression by RT-qPCR assays

To analyze the expression levels of differently expressed genes, pepper leaves were collected at 0, 24, 72 h post-inoculation, frozen in liquid nitrogen, and stored at -80 ℃. Total RNAs were extracted from leaves of pepper using MagZol BD reagent (Magen Biotech, R4803-02), and first-strand cDNA was synthesized

using HiScript III RT SuperMix for qPCR (+ gDNA wiper) (Vazyme, R323-01). The primers used in RT-qPCR assays were shown in Supplementary Table S1. The housekeeping gene Ubi3 was selected as the reference gene. Relative quantification of genes was tested by RT-qPCR assay using the 2 × RealStar Fast SYBR qPCR Mix (High ROX; Genstar, A303) in a 20 μL reaction volume. Thermal cycling conditions were 95℃ for 5 min, followed by 40 cycles of 95℃ for 15 s, 60℃ for 30 s, and 72℃ for 30s. All experiments were performed with three independent biological replicates. The relative expression levels of target genes were estimated by the $2^{-\Delta\Delta CT}$ (cycle threshold) method, and expression differences were analyzed by one-way and two-way ANOVA assays.

Results

Identification and classification of PPR genes in two pepper varieties, Zunla-1 and Zhangshugang

To investigate PPR genes associated with disease resistance in *Capsicum annuum*, we selected the pepper varieties of *Zunla-1* and *Zhangshugang*, which are resistant and susceptible to *P.capsici*, respectively (Fig. 1A). This study systematically identified 479 and 199 PPR genes in the *Zunla-1* and *Zhangshugang* genomes, respectively. Phylogenetic analysis was conducted using the full-length amino acid sequences from both varieties, employing the maximum likelihood (ML) method. The results demonstrated that PPR genes from both varieties were predominantly classified into two subfamilies: P and PLS (Fig. 1B). Specifically, *Zunla-1* contained 216 P-type genes and 263 PLS-type genes, while *Zhangshugang* had 98 P-type and 101 PLS-type genes (Fig. 1C).

Further analysis of conserved motifs revealed that the 263 PLS-type genes in *Zunla-1* were categorized into 73 DYW, 13 E1-type, 128 E2-type, 11 E+-type, and 38 PLS-type, while the 101 PLS-type genes in *Zhangshugang* were categorized into 34 DYW, 3 E1, 57 E2, 3 E+, and 4 PLS (Fig. 1C and Supplementary Table S2). This distribution pattern suggests that the PLS subfamily is more prevalent in both varieties, and *Zunla-1* harbors a significantly larger prepertoirs of PPR genes, which likely enhances resistance to *P. capsici*.

Structural analysis of pepper PPR genes demonstrated that *Zunla-1* contain 332 single-exon genes, 79 with two-exon genes, 22 three-exon genes, 13 four-exon genes, 6 five-exon genes, and 27 genes with six or more exons. In contrast, *Zhangshugang* contains 120 single-exon genes, 37 two-exon genes, 19 three-exon genes, 15 four-exon genes, 5 five-exon genes, and 5 multi-exon genes (Fig. 1D). Statistical comparisons revealed that the proportions of single-exon, double-exon, and multi-exon (≥ 6) genes were significantly higher in *Zunla-1* than *Zhangshugang.* However, there were no significant differences in the distributions of three-exon and four-exon genes showed between the two varieties (Fig. 1D).

Distribution and function analysis of PPR genes in Zunla-1 and Zhangshugang

To determine the distribution of PPR genes within these pepper varieties, we investigated their chromosomal localization and functional genes distribution. Our analysis revealed that *Zunla-1* and *Zhangshuguang* exhibited a gene-wide distribution across all 12 chromosomes, albeit with non-uniform

pattern. Strikingly, P-class and PLS-class genes displayed conserved chromosomal clustering in both varieties, with chromosome 1–3 containing the highest number of PPR genes. In contrast, *Zunla-1* had the lowest PPR gene count on chromosome 10, whereas *Zhangshugang* had the minimum on chromosome 5 (Fig. 2A and Supplementary Fig.S1A-B). Synteny analysis revealed that *Zunla-1* contains 25 syntenic gene clusters, whereas *Zhangshugang* contains only one cluster, indicating substantial evolutionary divergence between the two varieties (Fig. 2B). This suggests that *Zunla-1* has undergone more complex genomic rearrangements during its evolutionary history, including frequent gene duplications and chromosomal rearrangements, leading to significant expansion of the PPR gene family. Such structural complexity likely contributes to enhanced functional diversity in gene expression, enabling *Zunla-1* to better adapt to diverse biotic and abiotic stresses. In contrast, *Zhangshugang* exhibits a relatively simple PPR gene organization with fewer recombination events during evolution, resulting in a smaller and more conserved gene family (Fig. 2B). This structural limitation may restrict *Zhangshugang's* adaptability to environmental challenges, particularly in combating biotic stresses.

Due to PPR proteins mainly function as RNA-binding proteins, playing critical roles in regulating chloroplast and mitochondrial gene expression in plant cells. We predicted the subcellular localization of the PPR proteins in pepper. Subcellular localization analysis revealed that pepper PPR proteins are distributed across various organelles and cellular compartments, including chloroplasts, mitochondria, nucleus, peroxisomes, vacuoles, cytoskeletons, cytoplasm, and plasma membranes (Table 2). Specifically, *Zunla-1* contained 278 PPR genes in chloroplasts, 33 in mitochondria, 31 in nuclei, and 97 in the cytoplasm, while *Zhangshugang* had 130 in chloroplasts, 21 in mitochondria, 13 in nucleus, and 27 in the cytoplasm (Table 2 and Supplementary Table S3). These differences in subcellular distribution underscore distinct functional specializations and environmental adaptation strategies between the two varieties. The greater diversity in *Zunla-1* likely enhances its regulatory flexibility for managing complex physiological processes, whereas the relatively uniform distribution in *Zhangshugang* may restrict its adaptability to environmental changes.

To elucidate the functions of pepper PPR genes, we performed GO and pathway enrichment analyses on PPR genes specifically expressed in *Zunla-1* and *Zhangshugang*. These results indicated significant enrichment in chloroplast- and mitochondrial-related functions in both varieties, including RNA processing, modification, metabolic processes, and gene expression (Fig. 2C). However, *Zunla-1* exhibited dual enrichment in both Biological Processes (BP) and Molecular Functions (MF). *Zunla-1* also showed evolutionary enrichment in specific functions such as adenylate cyclase activity, cytidine to uridine editing, mitochondria-nucleus signaling pathway, cyclase activity, and phosphorus-oxygen lyase activity (Fig. 2C-D). Pathway enrichment analysis demonstrated that *Zunla-1* and *Zhangshugang* achieve functional specialization through differential regulation of metabolic networks. *Zunla*-1 is engaged in amino acid synthesis and diverse metabolic processes, with a higher number of genes enriched in amino acid synthesis pathways and secondary metabolic pathways (Fig. 2D). In contrast, *Zhangshugang* prioritizes energy metabolism and fundamental biosynthetic efficiency, showing significant gene enrichment in energy metabolism and protein synthesis pathways (Fig. 2C-D). These findings highlight

the synergistic mechanisms between organelles and the nuclear genome regulatory networks, contributing to phenotypic differentiation.

To investigate the structural characteristics of PPR genes, especially those involved in mitochondrial and chloroplast function, we conducted comparative analysis of coding sequences between *Zunla-1* and *Zhangshugang*. Variations in exon composition across different genes directly reflect differences in protein-coding potential and structural complexity. PPR genes contain both exons and introns, including 5' and 3' untranslated regions essential for post-transcriptional regulation (Supplementary Fig.S2). These findings indicated that *Zunla-1* not only has a significantly higher number of genes than *Zhangshugang* but also exhibits greater gene structural diversity, suggesting a broader functional repertoire.

Expression patterns of PPR genes in two pepper varieties in response to P. capsici invasion

To investigate the role of PPR genes in pepper response to *P. capsici* invasion, we analyzed high-throughput sequence data from *Zunla-1* and *Zhangshugang* at both 24 and 72 hpi to characterize temporal expression dynamics of PPR genes. The results demonstrated significant differences in transcript abundance between these two varieties after pathogen infection (Fig. 3A). Differential expression analysis revealed that *Zunla-1* exhibited 37 and 58 downregulated genes, while 7 and 19 upregulated genes at 24 hpi and 72 hpi, respectively (Fig. 3B). Conversely, *Zhangshugang* showed 17 and 65 downregulated genes, while 4 and 8 upregulated genes at the same stage (Fig. 3B). Based on the screening criteria of log2 Fold Change > 2 and adjusted *P*-value < 0.01, 56 DEGs were identified in *Zunla-1*, whereas 22 DEGs detected in *Zhangshugang* (Fig. 3C-D). These data suggested that *Zunla-1* likely enhances disease resistance by exhibiting a greater number of differentially expressed PPR genes.

Identification and analysis of variety-specific and shared PPR genes via comparative genomics in Capsicum spp.

To identify variety-specific and shared PPR genes between pepper varieties of *Zunla-1* and *Zhangshugang*, we performed a comparative genomic analysis. These results revealed that *Zunla-1* contained two hundred and eighty-five unique PPR genes, whereas *Zhangshugang* contained only five PPR genes, with one hundred and nighty-four shared PPR genes between the two varieties (Fig. 4A). Among these unique PPR genes in *Zunla-1*, one hundred and forty-four genes exhibited no significant expression differences at either 24 or 72 hpi. Of these, fifty-four genes were upregulated only at 72 hpi, fourteen genes were significantly downregulated at 72 hpi (Fig. 4A). Additionally, two genes were upregulated at 24 hpi but showed no difference at 72 hpi, and one gene was downregulated at 24 hpi but no significant difference at 72 hpi. Furthermore, sixteen genes were continuously upregulated and three were downregulated at both 24 and 72 hpi (Fig. 4A). Among shared genes, one hundred and thirteen genes exhibited no expression differences. Sixty-nine genes were upregulated exclusively at 72 hpi, whereas eight genes were significantly downregulated at that time. Additionally, one gene was upregulated at 24 hpi but showed no difference at 72 hpi (Supplementary Fig.S3A).

Expansion of gene families is crucial for plant adaptive evolution. The emergence of variety-specific genes, likely driven by neo-functionalization, sub-functionalization, and dosage effects through gene duplication, suggests that *Zunla-1* may have more duplication events. Our analysis revealed that among *Zunla-1*-specific differentially expressed genes, the number originating from WGD/segmental duplication increased from six to twenty-seven, while those from singleton duplication increased from eight to twenty, dispersed duplication from seven to twenty-three, and tandem duplication from one to ten. Interestingly, only four proximal duplication genes appeared in *Zunla-1* at 72 hpi (Fig. 4B). In shared DEGs, WGD/segmental and singleton duplication genes increased from eleven to twenty-eight, and six to twenty-four, respectively. Dispersed duplication genes rose from five to nineteen, whereas tandem and proximal duplication each had three DEG PPR genes (Fig. 4B). The results indicated that these divergences reflect distinct adaptive strategies in plant evolution under varying environmental pressures.

PPR proteins are characterized by their role in regulating organellar gene expression via RNA binding activities such as splicing, editing, and stability maintenance. Subcellular localization analysis results indicated that *Zunla-1*-specific and shared PPR proteins are significantly enriched in chloroplast, cytoskeleton and mitochondrial (Fig. 4C). This suggests potential roles in modulating chloroplast and mitochondrial respiration and stress responses.

Further analysis of *Zunla-1*-specific and shared DEGs revealed that among *Zunla-1*-specific genes, *PHT91829* was significantly upregulated following pathogen infection. Nevertheless, *PHT67124*, *PHT91722*, and *PHT6711*5 exhibited no significant changes at 24 hpi but showed significant upregulation as infection progressed. Meanwhile, *PHT89812*, *PHT93075*, *PHT72035* and *PHT82891* were significantly downregulated as infection progressed (Fig. 4D). Notably, all these specific DEGs were localized to chloroplasts. *PHT91829*, *PHT91722*, *PHT67115*, and *PHT93075* originating from WGD or segmental duplications, while *PHT82891* originating from dispersed regions, *PHT67124* from tandem duplication, and *PHT72035* and *PHT89812* from singleton duplication events (Fig. 4B, D).

For the shared genes, *PHT87853/Caz03g13560* and *PHT87794/Caz03g14790* were located in chloroplasts, and originated from WGD/ segmental duplications events, these genes were upregulated upon pathogen infection. Conversely, *PHT75258/Caz07g01890* was localized to the nucleus and showed significantly decreased expression levels as infection progressed (Fig. 4B-D and Supplementary Table S4). RT-qPCR was used to detect the expression levels of these genes, and the results verified the findings presented above (Fig. 5A-K). These suggested that shared chloroplast PPR genes might serve as a foundational defense module for pepper resistance to pathogens, while *Zunla-1* unique genes have independently evolved to enhance the intensity and persistence defense responses.

Furthermore, these analyses demonstrated that the significantly upregulated genes in *Zunla-1* are involved in mRNA modification, potentially optimizing RNA processing efficiency in chloroplasts or mitochondria to enhance stress-responsive gene expression precision and stabilize pathogen-related pathways (Supplementary Fig.S3C). Among the significantly downregulated genes, *PHT77589* regulates the mitochondria/chloroplast-nucleus signaling pathway, *PHT70048* mediates mitochondrial mRNA/RNA

processing and modification, and *PHT90893* is involved in chloroplast RNA processing. These findings suggest that *Zunla-1* may reprogram the "organelle-nuclear" signaling network to reduce non-essential metabolic consumption and prioritize energy allocation to defense pathways. For the shared significantly downregulated genes, *PHT75258*/*Caz07g01890* is associated in mitochondrial RNA metabolism, kinase regulation, translational activation, chloroplast RNA processing, cysteine metabolism, and mRNA modification modules (Supplementary Fig.S3C and Supplementary Table S4).

Function analysis of Zunla-1-specific and shared core differentially expressed PPR genes

To clarify the functional roles of these core differentially expressed PPR genes, we identified *Zunla-1*-specific DEGs, including the upregulated genes included *PHT91829*, *PHT67124*, *PHT91722*, and *PHT67115*, as well as downregulated genes of *PHT72035*, *PHT82891*, *PHT89812*, and *PHT93075*. In addition, shared DEGs between the two varieties were characterized, comprising the upregulated genes of *PHT87853* and *PHT87794*, and the downregulated gene *PHT75258* (Fig. 4D and Fig. 5A-K). Our further analysis focused on the gene structure of these core differentially expressed PPR genes. The results revealed significant variations in motif types and distribution patterns, as well as in the exon-intron structural organization, among the core differently expressed PPR genes. These structural differences may be closely associated with the functional divergence of the genes and their roles in biological processes, thereby providing crucial structural insight for elucidating the functional regulatory mechanisms and evolutionary relationships of these genes (Fig. 6A-B).

We further predicted the *cis*-elements in the promoters of those PPR genes to investigate their regulatory mechanisms. The results demonstrated that these elements wereprimarily associated with light responsiveness, hormonal responses, growth and development regulation, metabolism and environmental responses, defense and stress responses (Fig. 6C-D). Interestingly, hormone-responsive elements including those related to abscisic acid, auxin, gibberellin, methyl-jasmonate, and salicylic acid responsiveness. These elements enable functional specificity through diverse *cis*-element combinations. For example, *Zunla-1* specifically upregulated genes *PHT91829, PHT67115*, and *PHT91722* play distinctive roles in defense and stress respons, and MeJA response. Additionally, two shared upregulated genes of PHT87794 and PHT87853, while only PHT82891 of *Zunla-1* specifically downregulated genes contain MeJA response element. The differential distribution of *cis*-acting elements may be closely related to the functional divergence of genes, which may contribute to their specific functional regulation in response to pathogen infection (Fig. 6C-D).

Correlation analysis of Zunla-1-specific and shared core differently expressed PPR gene

To investigate the functional relationships among core differentially expressed genes in *Capsicum annuum* during pathogen infection, we conducted a comprehensive correlation analysis to elucidate the regulatory networks and functional interactions among these genes. The study focused on *Zunla-1*-specific and -shared genes to identify key regulatory pathways and their roles in stress responses. These results revealed that upregulated genes were positive correlations with other upregulated genes and negative correlations with downregulated genes, whereas downregulated genes were showed negative

correlations with upregulated genes and positive correlations with downregulated genes (Fig. 7A-B). GO enrichment analysis further indicated that the differentially expressed PPR genes *PHT74548* and *PHT70048* were enriched in mitochondrial RNA modification, mitochondrial RNA metabolic, and mitochondrial mRNA processing; *PHT75969* was associated with mitochondrial RNA metabolic processes, mitochondrial RNA processing, mitochondrial mRNA processing, and mitochondrial gene expression; *PHT90893* participated in chloroplast RNA processing processes; and the *PHT95235* gene was related to chloroplast RNA modification (Fig. 2C, Fig. 3B-D). At 24 hpi, *PHT74548* was significantly negatively correlated with *PHT87794* and *PHT91829*; conversely, *PHT75969* and *PHT90893* demonstrated significant positive correlations with *PHT75258* and *PHT72035*, *PHT81891*, *PHT93075*, respectively. Additionally, *PHT95235* showed significant negative correlations with *PHT87853* and *PHT91722*, while displaying significant positive correlations with *PHT75258* and *PHT89812* (Fig. 7A).

Conversely, at 72 hpi, the differentially expressed PPR genes *PHT72035*, *PHT75258*, *PHT82891*, *PHT93075* were positively correlated with *PHT95235*, *PHT90893*, *PHT75969*, *PHT70048*. In contrast, *PHT91722* and *PHT91829* exhibited significant negative correlations with *PHT95235* and *PHT70048*. Furthermore, *PHT89812* demonstrated positive correlatioins with *PHT95253*, *PHT75969*, and *PHT70048*, while *PHT67124* showed negatitive correlations with *PHT95235* (Fig. 7B).

In summary, this study investigated differences in PPR gene families between resistant and susceptible pepper varieties, and explored dynamic expression trends of PPR genes during pathogen infection in *Zunla-1* and *Zhangshugang*. Furthermore, comparative genomic and transcriptomic analyses identified unique and shared differentially expressed genes. In addition, we analysis their subcellular localization, evolutionary characteristics, and functional features. Specifically, eleven core differentially expressed genes were identified in *Zunla-1*, and their structural features, promoter elements, functional enrichment, and inter-gene correlations were analyzed systematically. These findings revealed a complex regulatory network of PPR gene expression during pathogen infection, primarily involving RNA modification, RNA processing, RNA metabolic, gene expression, kinase activity, phosphorylation-related pathways, chloroplast signal transduction, cell cycle regulation and stress response. The results indicated that *Zunla-1* has exhibited evolutionary adaptation in pathogen response, highlighting defense responsiveness of the complex interactions between organelle and nuclear genes in pepper.

Discussion

PPR proteins constitute a large family of proteins widely distributed in plants and certain protists, characterized by their RNA-binding properties. These proteins regulate gene expression through post-transcriptional mechanisms in mitochondria, chloroplasts, and the nucleus through the modulation of RNA modification, processing, and translation [34–36]. In plant organelle RNA editing mechanisms, PPR proteins are essential for maintaining metabolic homeostasis via processes such as intron splicing, RNA editing, and translational regulation [34, 36]. Typically, P-class PPR proteins participate in multiple stages of RNA processing in plant organelles, while PLS-class PPR proteins primarily function in RNA editing [37]. Mutations in PPR gene-coding can lead to mitochondrial and chloroplast dysfunction, resulting slow

plant growth, pollen sterility, abnormal seed development, and impaired responses to biotic and abiotic stresses [8, 38, 39].

Previous studies have identified PPR gene families in multiple plant species, such as *Oryza sativa*, *Arabidopsis thaliana*, *Populus trichocarpa*, *Citrullus lanatus*, and *Camellia sinensis*, involved in regulating chloroplast and mitochondrial gene functions [9, 40–44]. For instance, PGN of *Arabidopsis thaliana* participates in the editing of *cox2* and *nad6* in mitochondria [45], while *SLG1* and *AHG11* are involved in the editing of *nad3* and *nad4* in mitochondria [38, 46]. Moreover, *SLO2* is essential for mitochondrial RNA editing at multiple sites [47]. In rice, *WSL* plays a role in the splicing of *rpl2* in chloroplasts [48], and *POCO1* is involved in mitochondrial RNA editing [49].

Although 552 PPR genes have been identified in pepper previously, research on the disease resistance functions of PPR genes in pepper remains limited [50]. In this study, we performed whole-genome analysis of pepper varieties *Zunla-1* and *Zhangshugang*, identified 479 and 199 PPR genes, respectively. Phylogenetic analysis confirmed the classification of PPR genes into P and PLS subfamilies based on conserved domain analysis, and further classified them into six subgroups (Fig. 1A-C). These genes unevenly distributed across 12 chromosomes, primarily located in mitochondria and chloroplasts, aligning with their functional roles in maintaining organelle metabolic homeostasis (Fig. 2A-D and Supplementary Fig. 1).

Previous studies indicated that over 50% of PPR genes in angiosperms contain a single exon [42]. Similarly, *Zunla-1* and *Zhangshugang* exhibited 69.31% and 60.3% of PPR genes, respectively, contain a single-exon. However, *Zunla-1* exhibited higher diversity in PPR gene structure, and the number of PPR genes with two or more than six exons significantly differ between the two varieties, suggesting a potential link to disease resistance (Fig. 2D). Gene family expansion primarily driven by gene duplication and selective retention, with WGD/segmental duplication events playing a vital role in plant evolution [51–52]. Our study confirmed that *Zunla-1* contains a higher number of PPR genes via WGD/segmental duplication compared to *Zhangshugang* (Fig. 4B). Additionally, transposable elements (TEs) account for over 80% of the pepper genome, contributing to gene family expansion through mechanisms such as transposition and functional divergence [53, 54].

Generally, PPR proteins are participated in various plant processes, including growth, development, photosynthesis, responses to abiotic and biotic stresses. Recent evidence suggests that PPR proteins also play a key role in plant defense responses [1–3]. Emerging studies on chloroplast and mitochondrial pathways in plant immunity revealed that the subcellular localization of PPR proteins correlates with their functional diversity. These proteins regulate post-transcriptional processing of chloroplast and mitochondrial RNA, influencing electron transport chain assembly, stress tolerance, and pathogen defense [19, 55–57]. For instance, *OsPGL1* regulates electron transport chain assembly in rice, enhancing abiotic stress tolerance [58], while *OsNBL3* enhances resistance to pathogens and abiotic stresses [17, 57]. Nuclear-localized *OsNPPR1* regulates mitochondrial function through nuclear splicing

events [59]. *AtRTP7* regulates pathogen resistance in *Arabidopsis* by modulating mitochondrial gene editing [19, 55]. These findings present the importance of PPR proteins in plant immune networks.

Correspondingly, we also identified several chloroplast- and mitochondrial-localized PPR genes in pepper that significantly respond to pathogen invasion. In the resistant pepper variety *Zunla-1*, several chloroplast-localized PPR genes, such as *PHT91829*, *PHT82891*, *PHT93075*, and *PHT75258* were identified. These results suggested that the pathogen response may involve organelle-mediated RNA modification, synthesis, and metabolic regulation pathways (Fig. 2C-D, Fig. 4C and Fig. 5A, E, F, K). By comparative genomic and transcriptomic analysis of pepper-pathogen interaction, we identified both unique and shared differentially expressed PPR genes in the resistant variety of *Zunla-1*. Core promoter functional element analysis revealed that unique-upregulated genes of *PHT91829*, *PHT67124, PHT97122* and *PHT67115*, as well as shared-upregulated genes *PHT87853* and *PHT87794*, contain defense and stress response elements, methyl-jasmonate- and abscisic-acid response elements (Fig. 4A, D and Fig. 6A-B). These differentially expressed genes exhibit a complex correlation pattern, indicating the multiple relationships between upregulated and downregulated genes during stress adaptation. This dynamic interplay may further enhance resistance against *P. capsici* by modulating key processes such as mitochondrial RNA modification, mitochondrial RNA metabolic processes, mitochondrial RNA processing, mitochondrial mRNA processing, mitochondrial gene expression, as well as chloroplast RNA modification and processing. These findings highlight the complexity of the regulatory network governing pepper gene expression during pathogen infection, where specific core differentially expressed PPR genes likely play distinct roles in stress response, adaptive mechanisms, and potential defense pathways (Fig. 7A-B).

These PPR genes in pepper are likely involved in plant immune responses to pathogen invasion. These genes may form more complex interaction networks through gene duplication or transposition events, thereby enhancing plant resistance to biotic and abiotic. Notably, *Zunla-1*-specific and -shared differentially expressed PPR genes were predominantly localized to chloroplasts, mitochondria, and the nucleus. Further analysis revealed that *Zunla-1*-specific PPR genes, especially those in the E + subfamily, significantly enhanced the pathogens resistance compared to shared PPR genes (Fig. 4C, Fig. 6A-B and Supplementary Fig. 3B). These specific PPR genes also exhibited more specialized gene duplication patterns and unique chloroplast regulatory mechanisms. We hypothesize that these differential genes may be involved in pathogen defense via RNA editing and signal transduction mechanisms. Duplication events of WGD/segmental and dispersed may contribute to novel functions in regulating pathogen invasion, leading to functional divergence and sub-functionalization within the PPR gene family. Tandem duplication of PPR genes in *Zunla-1* may confer new functions at specific times (Fig. 4B). The expansion of chloroplast and mitochondrial-localized PPR genes in pepper appears crucial role in pathogen immunity.

Previous studies generally suggest that organelle-localized PPR genes predominantly regulate plant immune responses by modifying organelle-encoded RNA or influencing gene expression [1, 2, 17]. Functional enrichment analysis of core differentially expressed PPR genes responsive to pathogen

invasion revealed that these genes primarily involved in kinase activity regulation and phosphorylation pathways. Several chloroplast-localized PPR genes exhibit a negative correlation with *PHT75258* during pathogen infection (Fig. 7). This gene is homologous to BFA2 of *Arabidopsis thaliana*, which potentially regulates chloroplast ATP synthase, impacting plant growth, development, and stress tolerance. These findings suggest that organelle-localized PPR genes in pepper may modulate pathogen defense responses through direct or indirect interactions with chloroplast or mitochondrial RNA editing. These differential genes likely evolved via accelerated gene duplication or structural rearrangement within PPR gene families, resulting in gene family expansion, functional diversification, and the development of more complex signaling networks to address biotic and abiotic stresses.

Conclusions

Overall, this study systematically elucidates the dynamic response patterns of pepper PPR genes to pathogen invasion, providing the first evidence of specific PPR family members' roles in *P. capsici* resistance. These PPR genes probably utilize unique replication mechanisms to enhance pathogen defense regulation. GO enrichment analysis indicates their involvement in mitochondrial and chloroplast RNA processing, metabolism, and inter-organelle signaling pathways. Our study provides some useful information for comprehending functions of pepper PPR gene family in plant immune regulatory networks. But still need to perform additional experiments to further decision the complicated functions of these PPR genes in future. These results perhaps establish a theoretical framework and actionable molecular targets for optimizing crop breeding to enhance disease resistance.

Abbreviations

PPR Pentatricopeptide repeat

hpi Hours post-inoculation

HMM Hidden Markov Model

GO Gene ontology

MF Molecular function

BP Biological process

CC Cellular component

DEG Differentially expressed gene

ML Maximum likelihood

WGD Whole genome duplication

Declarations

Acknowledgements

Not applicable.

Author contributions

Jiajia Wan and Qifu Liang initiated, coordinated, and supervised the project. Qianqian Ye, Xiaoke Jia, Yi Zhang and Junfeng Wang conducted the experiments and gathered the data. Jiajia Wan and Qifu Liang analyzed the data and drafted the manuscript. Jiajia Wan, Qifu Liang, and Qinghe Chen reviewed and revised the manuscript. All authors have read and approved the final version of the manuscript.

Funding

This work was supported by grants from the National Natural Science Foundation of China (32302311) and the Hainan Province Natural Science Foundation of China (325RC668). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data availability

The data generated or analyzed during this study are included in this published article and its supplementary materials. Further inquiries can be directed to the corresponding author.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Tables

Tables are available in the Supplementary Files section.

Figures

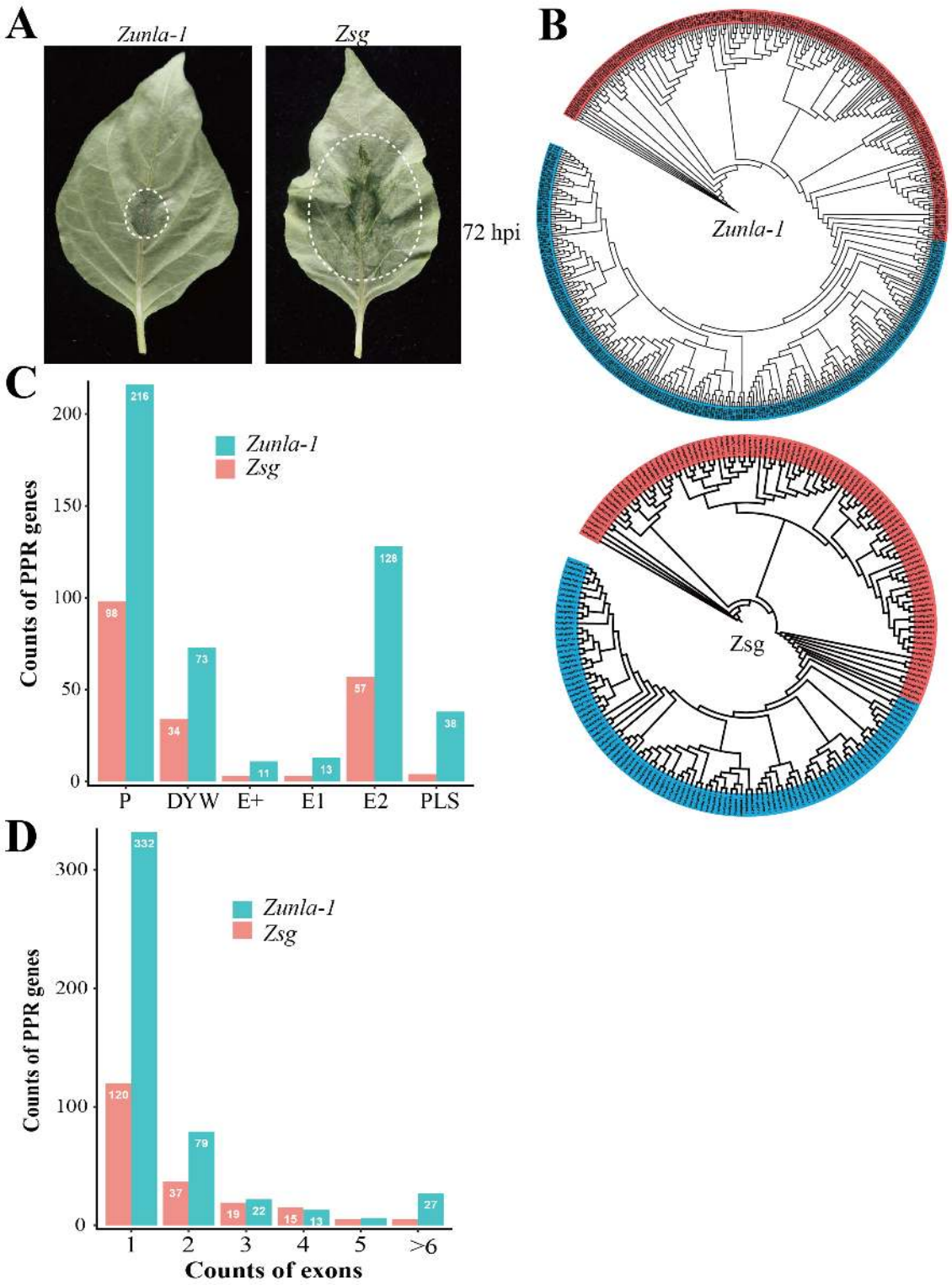

Figure 1

Identification and classification of the PPR gene family in *Zunla-1* and *Zhangshugang*.

(**A**) Phenotypic observation of pepper leaves at 72 hours post-inoculation (hpi). Left: leaf of *Zunla-1*, right: leaf of *Zhangshugang*. White dashed circle mark indicated the areas of diseased spots. Zsg, Zhangshugang. (**B**) Phylogenetic trees of PPR genes in two pepper varieties. Maximum likelihood (ML)

trees were constructed using IQ-TREE V2.2.3 software with 1000 bootstrap replicates. Blue represents the PLS subfamily, while red symbolizes the P subfamily. Bootstrap values are shown for the two varieties. Prior to tree construction, the LG+F+R10 model was identified as the optimal model. (**C**-**D**) Statistics of PPR genes in different subgroups and distribution of exon counts in two pepper varieties.

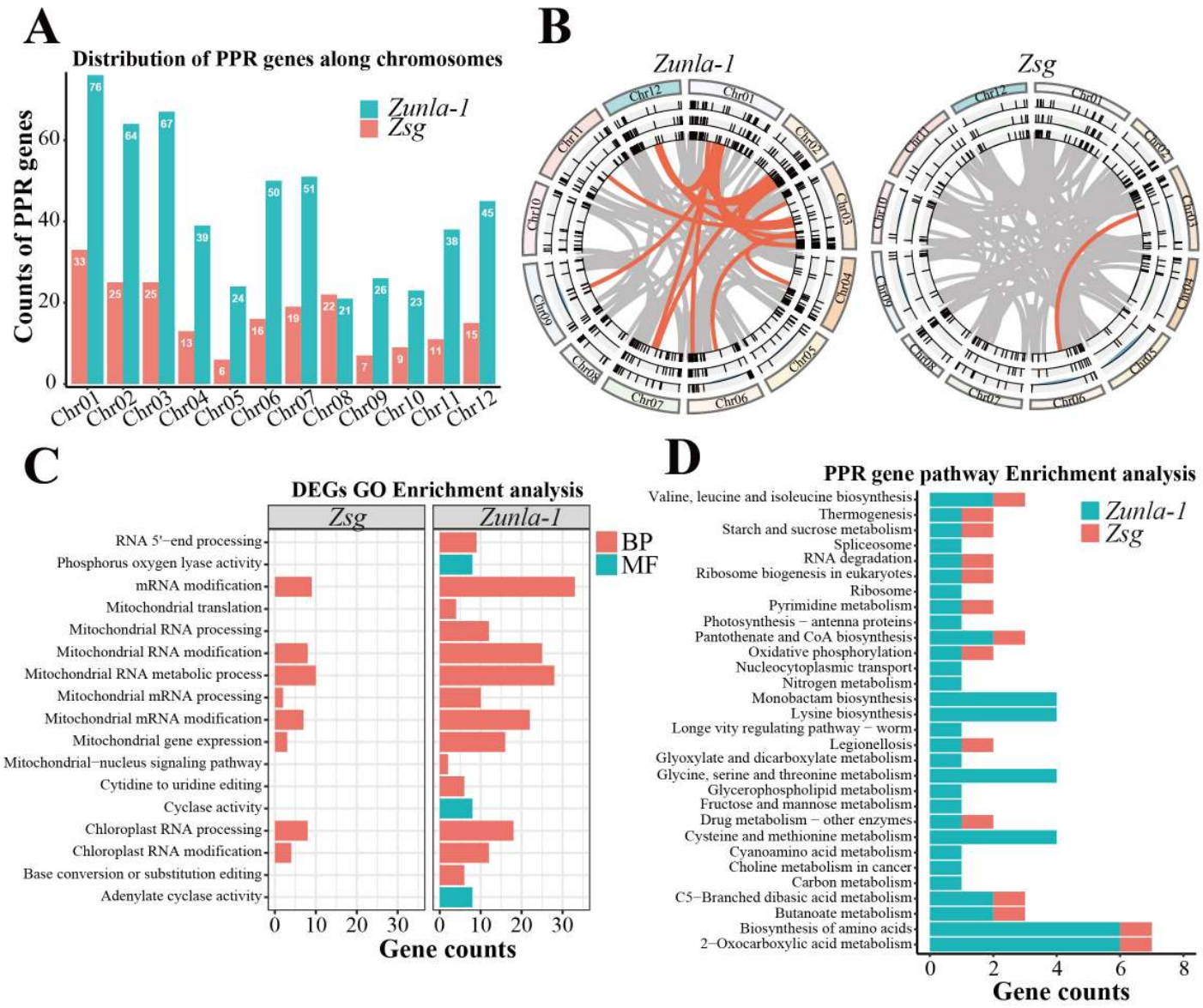

Figure 2

Genome-wide distribution and functional characterization of PPR genes.

(**A**) Distribution and quantity of PPR genes on 12 chromosomes in two pepper varieties. (**B**) Density and collinearity of PPR genes presented in 2 Mb windows along each chromosome. Left: *Zunla-1*, right: *Zsg*. The Circos plot features concentric layers from the outermost to the innermost as follows: density of P-class PPR genes, density of PLS-class PPR genes, and over all PPR gene density. Gray links illustrate genome-wide collinear relationships, while red links indicate specific collinearity among PPR genes. (**C**) GO enrichment analysis of PPR genes, with red and blue colors correspond to biological process (BP)

and molecular function (MF), respectively. (**D**) Pathway enrichment analyses for PPR genes, where blue and red colors correspond to *Zunla-1* and *Zsg*, respectively. Zsg, Zhangshugang.

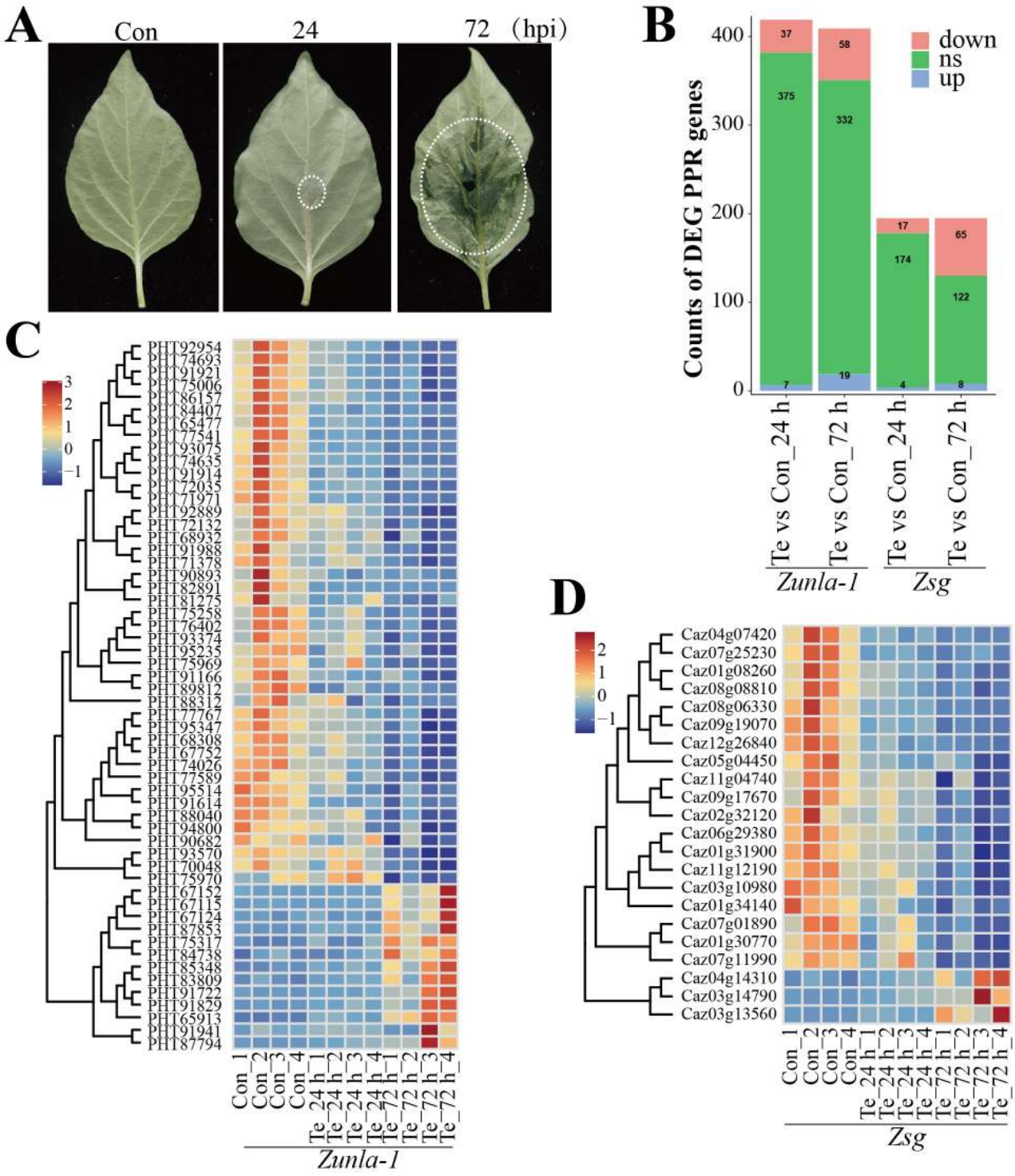

Figure 3

Expression patterns and differentially expressed PPR genes in *Zunla-1* and *Zhangshugang*

(**A**) Phenotype of pepper leaves inoculated with *P. capsici.* Mock, pepper leaf without *P. capsici* inoculation; 24 hpi, pepper leaf at 24 hours post-inoculation with *P. capsici*; 72 hpi, pepper leaf at 72 hours post-inoculation with *P. capsici*. The white dashed circle area indicates the diseased spots. (**B**) Statistics of differentially expressed PPR genes in *Zunla-1* and *Zhangshugang*, based on a log2 fold change > 1 with a *P*-value < 0.01. (**C**-**D**) Heatmaps of significant differentially expressed PPR genes expression in *Zunla-1* and *Zhangshugang*, plotted using normalized TPM values with log2 fold change > 2 with a *P*-value < 0.01. Con, control; Zsg, Zhangshugang.

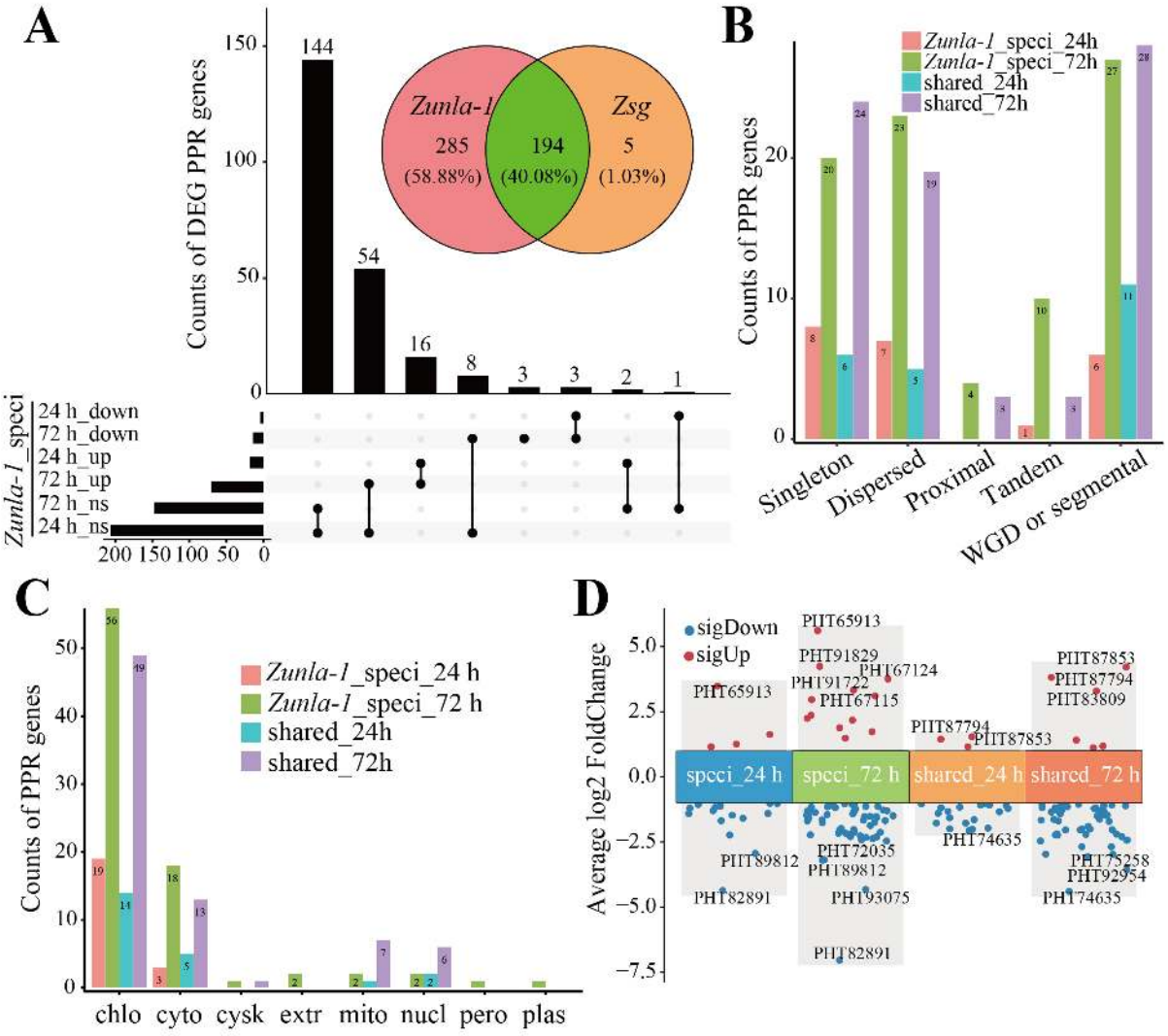

Figure 4

Expression profiles and differentially expressed PPR genes among variety-specific and shared genes between *Zunla-1* and *Zhangshugang*.

(**A**) Analysis of variety-specific and shared PPR genes between *Zunla-1* and *Zsg* along with statistical data on differentially expressed PPR genes within these gene sets. (**B**-**C**) Gene duplication types and locations of differentially expressed PPR genes in variety-specific and shared genes between two pepper varieties. (**D**) Volcano plots illustrating differentially expressed genes under various treatments for both

variety-specific and shared genes, using thresholds of log2 fold change > 1 with *P*-value < 0.01. Red dots indicate up-regulated genes, while blue dots represent down-regulated genes. Zsg, Zhangshugang.

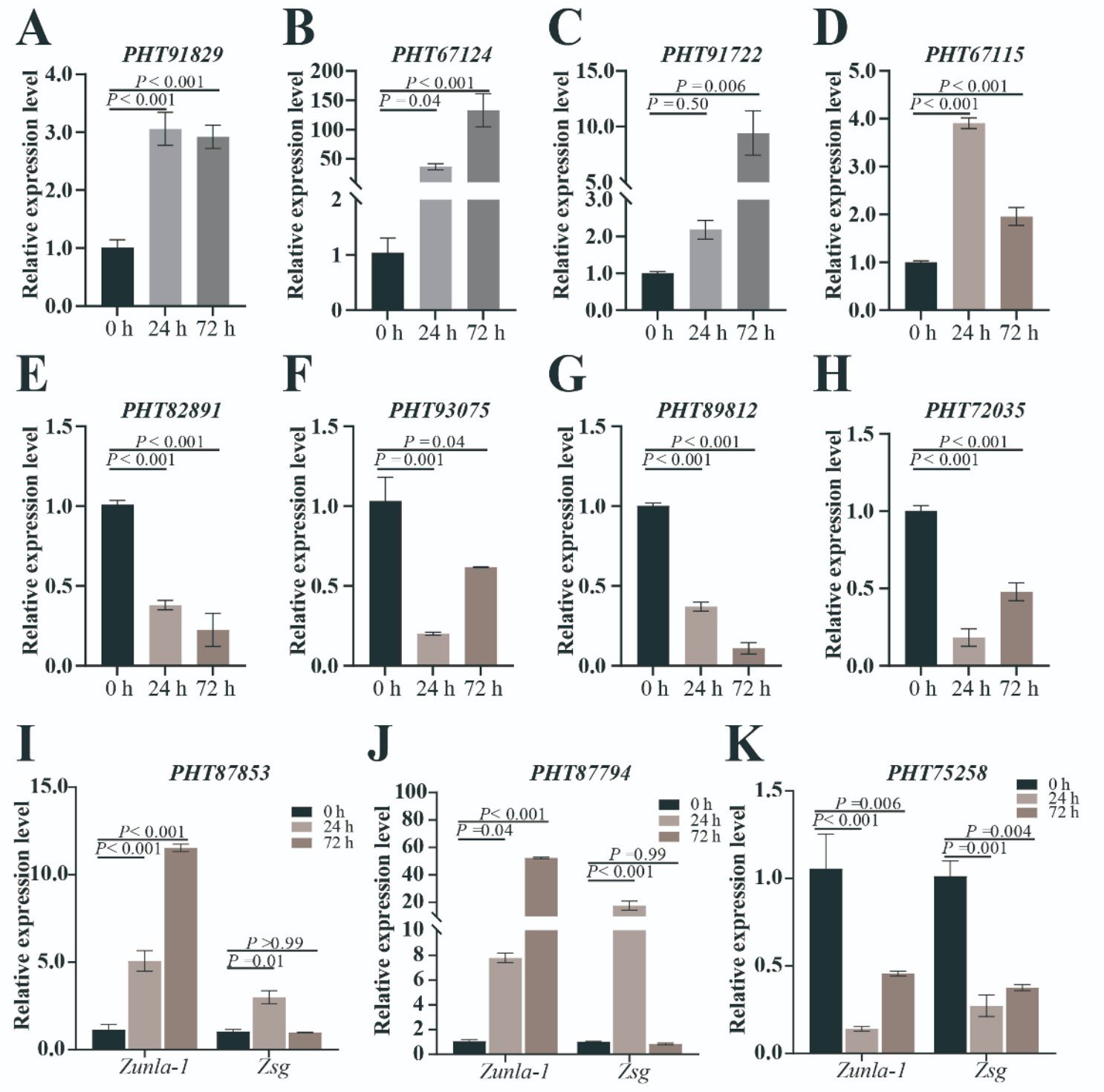

Figure 5

Identification of expression among variety-specific and shared core PPR genes between *Zunla-1* and *Zhangshugang*.

(A-K) RT-qPCR assay for the variety-specific and shared core PPR genesof *PHT91829, PHT67124, PHT91722, PHT67115, PHT82891, PHT93075, PHT89812, PHT72035*, PHT87853, PHT87794 or

PHT75258 expression in *Zunla-1* and *Zsg* at 24- and 72-hours post-inoculation with *P.capsici*, respectively. The non-inoculation plant was used as the control. Bars represent means ± SD from three independent experiments. Zsg, Zhangshuguang.

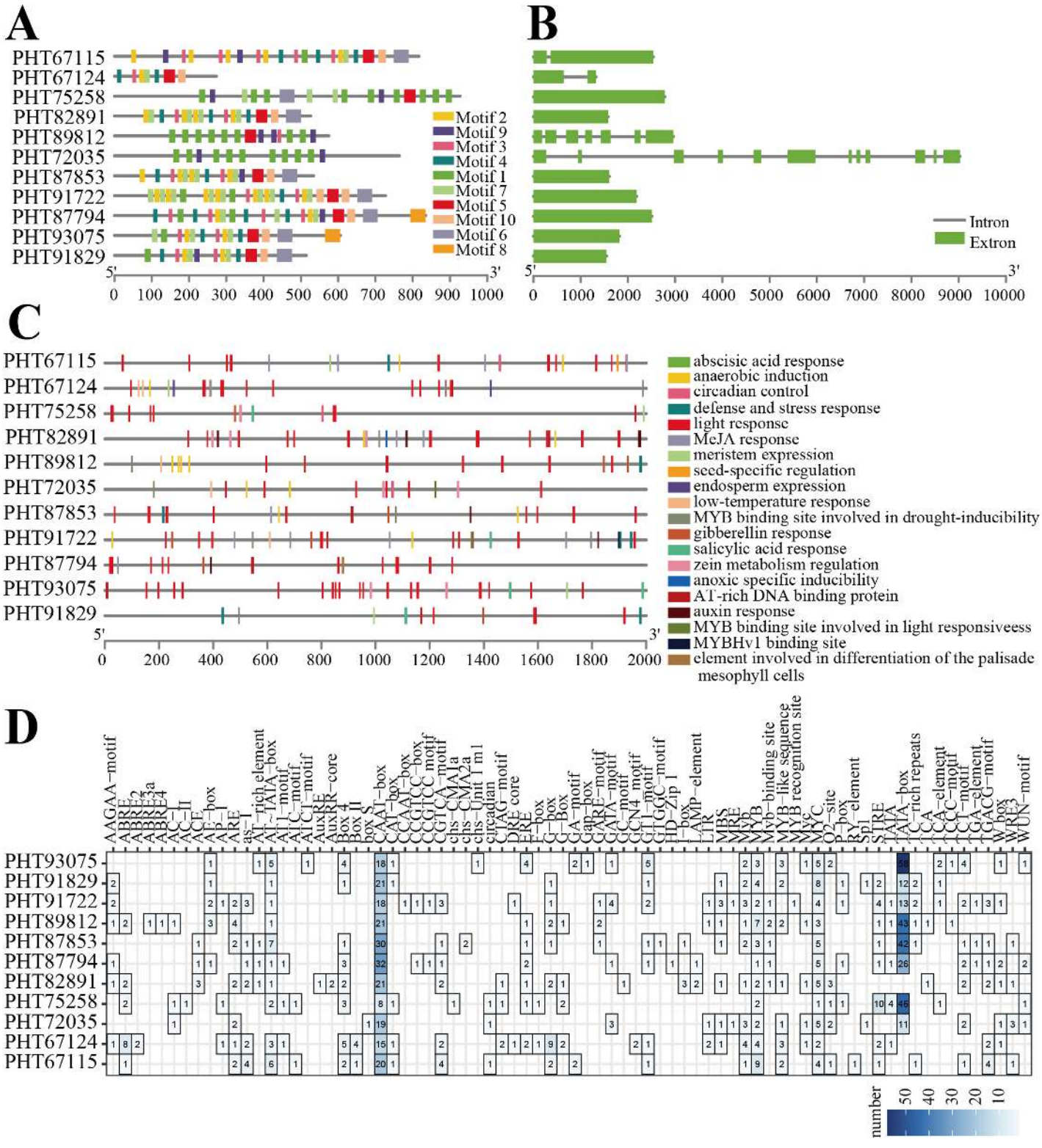

Figure 6

***Cis*-acting element analysis of significantly different expressed PPR genes.**

(**A**-**B**) Distribution of conserved motifs and gene structures of differentially expressed PPR genes. A total of 10 motifs were identified by MEME and are represented by different colors. (**C**-**D**) Distribution and quantities of *cis*-acting elements within the 2,000 bp upstream region of the promoter. Different colors represented distinct functions of *cis*-acting elements, as indicated on the right. Red and black colors denoted *Zunla-1*-specific and -shared differentially expressed PPR genes, respectively.

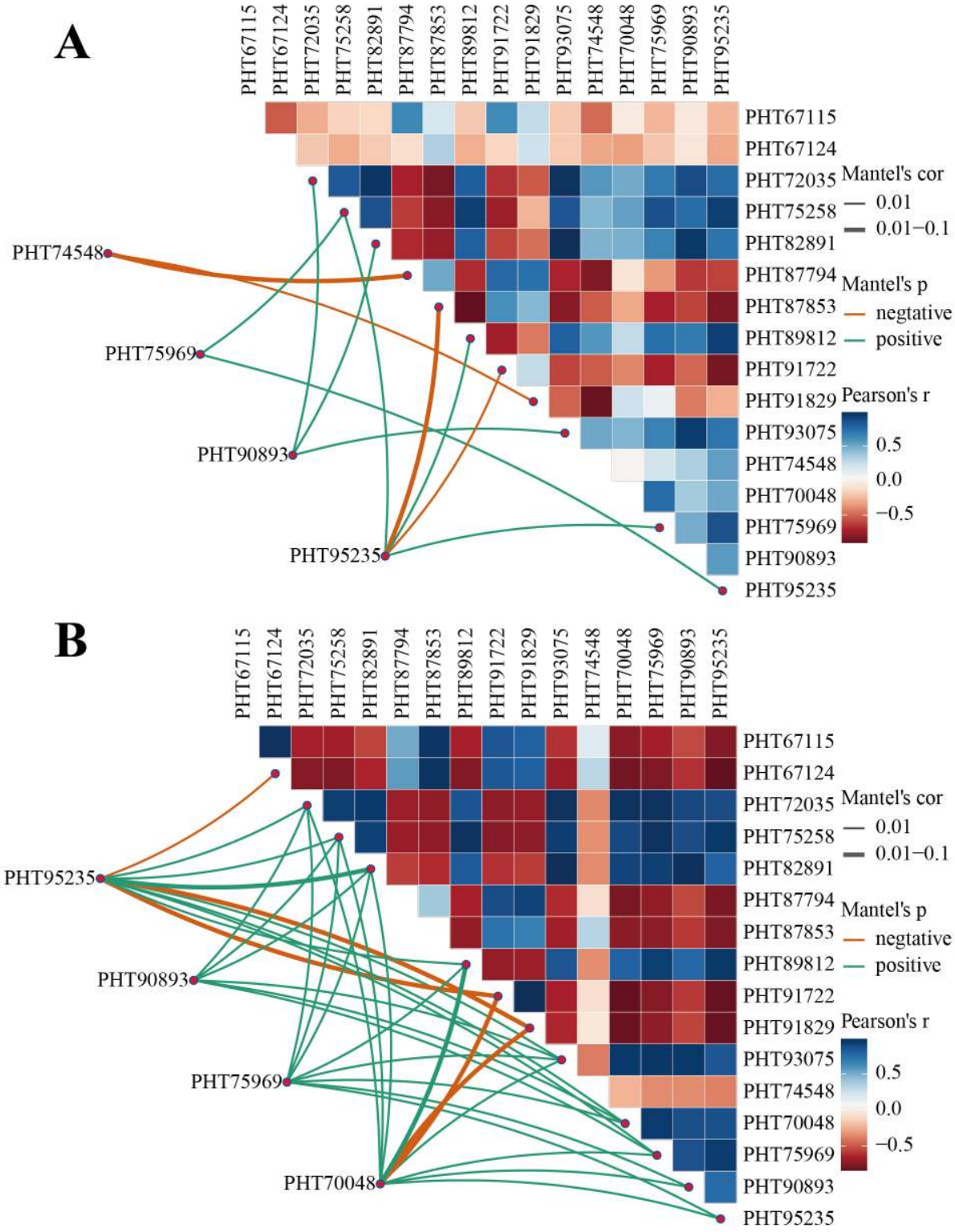

Figure 7

Correlation and co-expression network analysis of significantly expressed PPR genes in *Zunla-1* and *Zhangshugang*.

(**A**-**B**) Pepper leaves were sampled at 24 and 72 hpi with *P. capsici*. Nodes represent PPR genes, with orange and green edges indicating positive and negative correlations between gene pairs, respectively. The heatmap and edges collectively illustrate the association patterns among PPR genes, revealing their co-expression network.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.xlsx
- Table2.xlsx
- SupplementaryFig.S1.jpg
- SupplementaryFig.S2.jpg
- SupplementaryFigS3.jpg
- SupplementaryTableS1.xlsx
- SupplementaryTableS4.xlsx
- SupplementaryTableS3.xlsx
- SupplementaryTableS2.xls