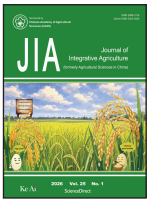
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RESEARCH ARTICLE

Genome-wide characterization and expression analysis of the cultivated peanut *AhPR10* gene family mediating resistance to *Aspergillus flavus*

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Highlights

- Comprehensive identification of 54 *AhPR10* genes in cultivated peanut, phylogenetically classified into eight distinct subgroups with supported gene structure and motif conservation.
- Segmental duplication was identified as the primary driver for the expansion of the *AhPR10* gene family, as revealed by chromosomal distribution and synteny analysis.
- The recombinant AhPR10-33 protein demonstrated significant antifungal activity by inhibiting *Aspergillus flavus* mycelial growth *in vitro*, highlighting its potential role in pathogen resistance.

Abstract

The pathogenesis-related protein PR10 plays a vital role in plant growth, development, and stress responses. This study systematically identified and analyzed *PR10* genes in cultivated peanut (*Arachis hypogaea* L.), examining their phylogenetic relationships, conserved motifs, gene structures, and syntenic relationships. The analysis identified 54 *AhPR10* genes, which were classified into eight groups based on phylogenetic relationships, supported by gene structure and conserved motif characterization. Analysis of chromosomal distribution and synteny demonstrated that segmental duplications played a crucial role in the expansion of the *AhPR10* gene family. The identified *AhPR10* genes exhibited both constitutive and inducible expression patterns. Significantly, *AhPR10-7*, *AhPR10-33*, and *AhPR10-41* demonstrated potential importance in peanut resistance to *Aspergillus flavus*. *In vitro* fungistatic experiments demonstrated that recombinant AhPR10-33 effectively inhibited *A. flavus* mycelial growth. These findings provide valuable insights for future investigations into AhPR10 functions in protecting peanut from *A. flavus* infection.

Keywords: cultivated peanut, PR10, phylogenetic analysis, expression pattern, *Aspergillus flavus*

1. Introduction

Pathogenesis-related (PR) proteins comprise a group of proteins synthesized by plants in response to biotic stress (e.g., bacterial, fungal, or viral infections) or abiotic stress (Xie *et al.* 2008). Based on their primary structure, genetic relationships, and biological activities, PR proteins have been classified into 17 different families (PR1–PR17), among which PR1, PR2, PR3, and PR9 exhibit alkaline protease, β-1,3-glucanase, chitinase, and peroxidase activities, respectively (Loon *et al.* 1994, 2006; Christensen *et al.* 2002; Agarwal and Agarwal 2014). The PR10 family (nuclease activity) represents the largest PR protein subfamily. Following the initial identification of PR10 proteins in parsley cell suspension cultures (Somssich *et al.* 1988), numerous members of this

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family were characterized in monocots, including lily (Huang *et al.* 1997), *Zea mays* (Xie *et al.* 2010; Zandvakili *et al.* 2017), rice (Mcgee *et al.* 2001), and sorghum (Lo *et al.* 1999), as well as in dicots, such as apple (Gao *et al.* 2005), *Juglans sigillata* (He *et al.* 2014), *Vitis vinifera* (Lebel *et al.* 2010), hot pepper (Park *et al.* 2004; Arcos-Ortega *et al.* 2010), soybean (Crowell *et al.* 1992), *Salvia miltiorrhiza* (Zhao *et al.* 2015), alfalfa (Bahramnejad *et al.* 2010; Zhang *et al.* 2011), *Solanum surattense* (Liu *et al.* 2006), and peanut (Chadha and Das 2006). PR10 proteins (15–18 kDa) are predominantly acidic intracellular proteins homologous to nucleases, lacking a signal peptide (Liu and Ekramoddoullah 2006). The genes encoding PR10 proteins typically contain two exons and one intron, with an open reading frame (456–489 bp) encoding a protein (151–162 amino acids) with an isoelectric point (pI) typically ranging from 4.75 to 6.65 (Hoffmann-Sommergruber *et al.* 1997). While PR10 protein sequences demonstrate considerable variation among family members, they share a conserved P-Loop structural domain (GXGGXG), which functions as the nucleic acid-binding site associated with nuclease activity (Liu *et al.* 2006). In plants, PR10 proteins undergo self-activation through phosphorylation, resulting in ribonuclease activity that specifically degrades pathogen genetic material (Van *et al.* 2006). Research in pepper demonstrates that phosphorylated CaPR10 exhibits 12.4-fold higher ribonuclease activity compared to its unphosphorylated form (Park *et al.* 2004). Additionally, PR10 ribonuclease activity correlates with conserved amino acids, including glutamic acid, tyrosine, and histidine (Zhou *et al.* 2002; Wu *et al.* 2003; Cheng *et al.* 2013). Therefore, both phosphorylation and the conserved amino acid sequence in the P-Loop domain play essential roles in determining PR10 protein ribonuclease activity.

PR10 proteins are widely distributed throughout plants and play crucial roles in biotic and abiotic stress resistance. In maize, *ZmPR10.1* and *ZmPR10.2* expression is induced by darkness, mechanical damage, freezing conditions, and bacterial infections (Xie *et al.* 2010). The expression of ZmPR10 in transgenic tobacco leaves inhibits *Aspergillus flavus* growth (Chen *et al.* 2006). In rice, *RSOsPR10* expression is induced by drought, salinity, and rice blast disease (Hashimoto *et al.* 2004). Furthermore, overexpression of grape *VPPR10.1* significantly reduces *Plasmopara viticola* mycelial and sporangial densities, enhancing downy mildew resistance (Su *et al.* 2018). The overexpression of *GmPR10* enhances resistance of transgenic tobacco and soybean plants to *Phytophthora sojae* (Xu *et al.* 2014). Similarly, overexpression of potato *PR-10a* (El-Banna *et al.* 2010), peanut *AhSIPR10* (Jain *et al.* 2012), and cotton *GbPR10* (Liu *et al.* 2022) increases tolerance to various biotic and abiotic stresses. Recent research has focused on understanding the mechanism regulating PR10 antibacterial activities. Agarwal *et al.* (2014) demonstrated that plant PR10 proteins possess ribonuclease activity, which enables protection against phytopathogenic bacteria. This ribonuclease activity has been confirmed in various natural and recombinant PR10 proteins, including Minjiang lily LrPR10-5 (Chen *et al.* 2017), rice JIOsPR10 (Kim *et al.* 2008), pepper CaPR-10 (Park *et al.* 2004), *S. miltiorrhiza* SmPR10 (Zhao *et al.* 2015), pea PR10.4 (Krishnaswamy *et al.* 2011), maize ZmPR-10 (Chen *et al.* 2006), and grape VpPR10.1 (Su *et al.* 2018) and VpPR10.2 (He *et al.* 2012). Notably, certain recombinant plant PR10 proteins demonstrate antimicrobial activities *in vitro*. For instance, recombinant *Jatropha curcas* JcPR10a protein inhibits *Rhizoctonia solani* mycelial growth (Agarwal *et al.* 2013), while peanut AhPR10 fusion protein inhibits *Fusarium oxysporum* and *R. solani* mycelial growth (Chadha and Das 2006). Additionally, recombinant SsPR10 exhibits inhibitory effects on rice blast mycelial growth (Liu *et al.* 2006). Collectively, these findings indicate that PR10 proteins serve as key regulators in plant defense mechanisms against pathogen infections.

Cultivated peanut (*Arachis hypogaea* L.) exhibits vulnerability to various pathogens. Notably, infection by *A. flavus* significantly reduces peanut product quality, while the resulting aflatoxin contamination poses serious food safety concerns (Huang 2010). Therefore, preventing aflatoxin contamination requires identifying candidate genes that mediate resistance to *A. flavus* and elucidating their functions, which has important implications for developing new peanut varieties resistant to *A. flavus* infections (Cui *et al.* 2022). Previous research has demonstrated the significance of AhPR10 in peanut pathogen defense mechanisms. For instance, the peanut AhPR10 protein isolated by Chadha and Das (2006) contains a P-Loop and a Bet v 1 domain. Furthermore, the recombinant AhPR10 demonstrates nuclease activity *in vitro* and inhibits *F. oxysporum* and *R. solani* mycelial growth. The nuclease activity of peanut ARAhPR10 was subsequently verified (Xie *et al.* 2009). This suggests the potential existence of additional PR10 proteins capable of enhancing peanut resistance to *A. flavus*. The availability of genomic information for cultivated peanut (*A. hypogaea* cv. Tifrunner), an allotetraploid (AABB 2n=4×=40), and its wild relatives (diploids: *Arachis duranensis* and *Arachis ipaensis*) (Bertioli *et al.* 2016; Zhuang *et al.* 2019) has enabled genome-wide analyses leading to the identification of numerous cultivated peanut gene families, including *bZIP* (Wang *et al.* 2019), *PLP* (Gao *et al.* 2021), and *AP2/ERF* (Cui *et al.* 2021). However, a genome-wide bioinformatics analysis of the *PR10* gene family in cultivated peanut remains unexplored.

In this study, 54 *AhPR10* gene family members were identified in the *A. hypogaea* L. genome. A comprehensive bioinformatics analysis was performed examining conserved protein domains, phylogenetic relationships, chromosomal locations, *cis*-acting elements, gene duplication events, and syntenic relationships. Furthermore, *AhPR10* expression patterns were analyzed at various time points following kernel inoculation with *A. flavus* spores. The functions of selected *AhPR10* genes related to peanut resistance against *A. flavus* were predicted through bioinformatics analysis and validated experimentally. This study represents the first comprehensive analysis of *AhPR10* genes in cultivated peanut. The findings establish a foundation for future systematic investigations of peanut *AhPR10* gene functions associated with plant resistance to *A. flavus*, while providing valuable insights for genetic improvement of cultivated peanut.

2. Materials and methods

2.1. Identification of *AhPR10* gene family members

To identify *AhPR10* genes in the *A. hypogaea* cv. Tifrunner genome, multiple databases were systematically searched. Genome sequences were obtained from an online peanut genome database (GCA_003086295.3; https://www.ncbi.nlm.nih.gov/assembly) and each AhPR10 protein was identified using the Bet v 1 domain profile (PF00407) from the Pfam database (http://pfam.janelia.org). HMMER 3.1 software (E-value<1e^{-5}) was employed to precisely identify genes (Finn *et al.* 2011; Potter *et al.* 2018). The obtained *Bet v 1* gene sequences were utilized to screen the NCBI (https://www.ncbi.nlm.nih.gov/cdd) and Pfam (http://pfam.xfam.org/) databases for candidate genes encoding conserved AhPR10 structural domains (Mistry *et al.* 2021). The molecular weight and pI of AhPR10 candidate proteins were predicted using ExPASy (https://web.expasy.org/protparam/) (Gasteiger *et al.* 2003), while Plant-Ploc (http://www.csbio.sjtu.edu.cn/bioinf/plant/) was utilized to predict the subcellular localization of AhPR10 proteins (Chou and Shen 2008).

2.2. Sequence alignment and phylogenetic analysis

All cultivated peanut AhPR10 amino acid sequences underwent multiple sequence alignment and comparison using DNAMAN v9 and ClustalW (Thompson *et al.* 1994; Zhu *et al.* 2015). MEGA 7.0 was employed to construct a phylogenetic tree using the maximum likelihood method with 1,000 bootstrap replicates (Kumar *et al.* 2016). The resulting phylogenetic tree was visualized using Evolview (He *et al.* 2016).

2.3. Conserved motif and gene structure analyses

Information regarding *AhPR10* intron–exon structures was obtained using a cultivated peanut genome annotation file and GSDS 2.0 (http://gsds.cbi.pku.edu.cn/) (Hu *et al.* 2015). The MEME online tool (http://meme-suite.org/tools/meme) was utilized to predict the conserved motifs encoded in *AhPR10* genes, employing default parameters with the motif number set to 10 (Bailey *et al.* 2015). Evolutionary relationships, gene structures, and conserved motifs were visualized using TBtools (Chen *et al.* 2020).

2.4. Chromosome localization, duplications, and evolutionary analysis

The physical locations of *AhPR10* genes on chromosomes were determined using a cultivated peanut genome annotation file. MapChart 2.3 software was employed to map the distribution of *AhPR10* genes on chromosomes (Voorrips 2002), with genes designated according to their chromosomal positions. The *AhPR10* replication events within and between species were analyzed and visualized using MCScanX in TBtools.

2.5. Promoter analysis

For each *AhPR10* gene, the 2,000 bp sequence upstream of the start codon was analyzed using PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to predict the type, quantity, and location of promoter *cis*-acting elements (Lescot *et al.* 2002), which were subsequently visualized using TBtools.

2.6. Plant materials, *Escherichia coli* strains, and plasmids

As outlined in our previous study (Cui *et al.* 2022), 'J11' (resistant cultivar) and 'Zhonghua 12' (susceptible cultivar) kernels inoculated with *A. flavus* spores were collected at multiple time points (0 h (T0), 24 h (T1), 48 h (T2), 72 h (T3), 120 h (T5), and 168 h (T7)). Plant tissues were collected at specific developmental stages: roots, stems, and leaves from seedlings at the three-leaf stage, flowers during full bloom, and kernels from fruits 35–85 days after flowering (with controlled pollination). *Escherichia coli* DH5α cells were obtained from Tsingke Biotechnology (Beijing) Co., while Rosetta (DE3) cells and the pET30a vector were sourced from Boyuan Biotechnology (Wuhan) Co., China.

2.7. *In silico* analysis of *AhPR10* expression using RNA-seq data

RNA-seq data from cultivated peanut samples collected at 0 h (T0), 24 h (T1), 48 h (T2), 72 h (T3), 120 h (T5), and 168 h (T7) post-inoculation with *A. flavus* were obtained from the NCBI Sequence Read Archive database (accession number: PRJNA825125) (Appendix A) (Cui *et al.* 2022). The fragments per kilobase million (FPKM) value, representing *AhPR10* transcript abundance across samples, was calculated using the following eq.:

$$\mathrm{FPKM}=\frac{10^6 C}{NL/10^3}$$

where C represents the number of fragments mapped to a specific gene, N denotes the total number of fragments mapped to reference genes, and L indicates the number of bases in a specific gene. The differential expression of *AhPR10* genes was visualized through heat maps generated using OmicShare (https://www.omicshare.com/).

2.8. RNA extraction and reverse transcription quantitative PCR

Total RNA was extracted from control and treated peanut samples using an RNAprep Pure Plant Plus Kit (Polysaccharides & Polyphenolics-rich) (Tiangen Biotech, Co., Beijing, China). Subsequently, cDNA was synthesized using a FastKing RT Kit (With gDNase) (Tiangen Biotech, Co.). Reverse transcription quantitative PCR (RT-qPCR) primers (Appendix B) were designed for 12 genes across different groups using Primer-BLAST (NCBI). A class III alcohol dehydrogenase gene (A class III alcohol dehydrogenase gene (*AhADH3*), *Arahy.VYWU26.2*) functioned as the internal control (Brand and Hovav 2010) for RT-qPCR analysis (20 μL reaction volume), performed using the ABI QS5 RT-qPCR Detection System (ABI, USA) and Talent qPCR PreMix (SYBR Green) (Tiangen Biotech, Co.). The RT-qPCR protocol consisted of: 95°C for 3 min; 40 cycles of 95°C for 5 s and 58°C for 15 s. Three biological replicates, each comprising three technical replicates, were analyzed. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

2.9. Construction of a prokaryotic *AhPR10-33* expression vector

Using the full-length *AhPR10-33* open reading frame as a template, primers were designed for PCR amplification: pET30a-F (5′-GCCATGGCTGATATCGGATCATGGCCGTCTTCACTTTCGAGGATG-3′) and pET30a-R (5′-GTGGTGGTGGTGGTGCTCGAATATTGAGCAGGGTTGGCCAAGACG-3′); the GCCATGGCTGATATCGGATC and GTGGTGGTGGTGGTGCTCGA sequences contain *BamH*I and *Xho*I restriction enzyme sites, respectively. PCR amplification was conducted for 30 cycles with an annealing temperature of 50°C. The PCR products underwent digestion with *BamH*I and *Xho*I before insertion into pET30a digested with identical restriction enzymes to express a fusion protein containing a C-terminal 6× His affinity tag under T7 promoter control.

2.10. Prokaryotic expression and purification of AhPR10-33

The pET30a vector containing *AhPR10-33* was transformed into *E. coli* Rosetta (DE3) cells. The expression of *AhPR10-33* was induced using 0.2 mmol L^{-1} isopropyl β-dthiogalactoside (IPTG) during a 16 h incubation at 18°C. For protein purification, bacterial cells were pelleted and resuspended in lysis buffer. The lysate underwent centrifugation at 12,000×g followed by filtration of the supernatant through a 2 μm pore membrane. The filtrate (20 mL) was applied to a Ni-NTA chromatography column pre-washed five times with elution buffer and equilibrated 10 times with binding buffer. The recombinant protein was eluted using buffer containing imidazole. The eluate was desalted using a buffer comprising 20 mmol L^{-1} Tris-HCl and 150 mmol L^{-1} NaCl (pH 8.0) to obtain the purified protein.

2.11. Confirmation of AhPR10-33 nuclease activity and its inhibitory effects on *Aspergillus flavus in vitro*

The RNase activity of AhPR10-33 was evaluated using PBS buffer (negative control), 8 μg heat-denatured AhPR10-33 recombinant protein in PBS buffer (negative control), and 8 μg native AhPR10-33 recombinant protein in PBS buffer. For each assay, 20 μL of solution was combined with *A. flavus* RNA at a final concentration of 100 ng μL^{-1} and incubated at 37°C for 30 min. The reaction was terminated by adding 20 μL chloroform. After cooling on ice for 10 min, the samples were analyzed by 1% agarose gel electrophoresis.

The antifungal activity of recombinant AhPR10-33 was investigated. DG-18 solid medium was inoculated with *A. flavus* and incubated at 30°C for 3–4 days. From plates containing *A. flavus* colonies, five sterile filter discs (10 mm diameter) were positioned uniformly around the colonies. Aliquots of 40, 60, and 80 μL of purified recombinant protein solution (1 mg mL^{-1}) or ddH_2O and buffer (blank controls) were applied to the filter discs before incubation at 30°C for 36 h. The antifungal efficacy of recombinant AhPR10-33 was determined by measuring its inhibitory effects on *A. flavus* mycelial growth (Liu *et al.* 2018). The mean inhibition of *A. flavus* mycelial growth was calculated from three replicates.

3. Results

3.1. Identification and characterization of peanut *AhPR10* family genes

Previous research established that the Bet v 1 superfamily consists of 11 subfamilies, including major latex protein, cytokinin-specific binding protein, and PR10 (Wang *et al.* 2021). In this study, screening of the peanut protein database using a hidden Markov model file (PF00407) identified 127 Bet v 1 superfamily members. A total of 54 *AhPR10* genes were identified in cultivated peanut and designated AhPR10-1 to AhPPR10-54 based on chromosomal position. Analysis of protein physicochemical properties revealed lengths of 70–169 amino acids, molecular weights of 7.68–19.28 kDa, and pI of 4.7–5.84. All 54 AhPR10 proteins were predicted to localize in the cytoplasm (Appendix C).

3.2. Sequence alignment and phylogenetic analysis of *AhPR10* genes

To examine the evolutionary relationships among cultivated peanut *AhPR10* genes, a phylogenetic tree was constructed using 54 AhPR10 proteins, 25 soybean PR10 proteins, and 20 alfalfa PR10 proteins. Based on clustering information and protein sequence characteristics, *AhPR10* gene family members were classified into eight Groups (Groups 1–8), aligning with the phylogenetic tree-based grouping of *AhPR10* genes (Fig. 1; Appendix D). The close genetic relationships among peanut, soybean, and alfalfa as legumes were evident in the phylogenetic tree analysis. Analysis of the full-length AhPR10 amino acid sequences using DNAMAN revealed that, similar to PR10 proteins in *Zea mays* (Xie *et al.* 2010; Zandvakili *et al.* 2017), rice (Mcgee *et al.* 2001), and soybean (Crowell *et al.* 1992), AhPR10 proteins contained the P-Loop domain. Among the 54 AhPR10 proteins, 44 possessed a complete P-Loop domain (GXGGXG), while 10 exhibited a mutated P-Loop domain due to individual base mutations in the corresponding gene sequence. Notably, the AhPR10 proteins with a complete P-Loop domain belonged to Group 1 (Appendix E). Phylogenetic analysis indicated that while AhPR10 family members were highly conserved, variations in their amino acid sequence lengths and structures suggested functional diversity among groups.

3.3. Analysis of *AhPR10* gene structures and conserved motifs

Analyses of intron–exon structures revealed that most *AhPR10* genes contained two exons (Fig. 2-B). The conserved structural domains encoded by *AhPR10* genes primarily comprised motifs 2 and 3 (Fig. 2-C), which included the conserved P-Loop domain (GXGGXG) and the Bet v 1 domain, respectively (Fig. 2-D). AhPR10 proteins within the same group exhibited similarities in motif number and type. However, inter-group differences were observed, potentially contributing to functional diversity among groups.

3.4. Chromosomal location and duplication of *AhPR10* genes

The *AhPR10* genes demonstrated uneven distribution across

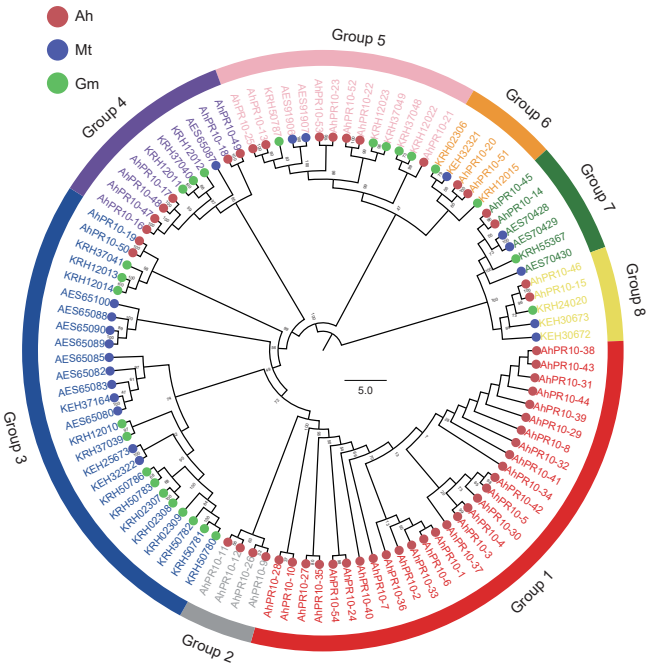


Fig. 1 Phylogenetic analysis of PR10 family proteins from *Arachis hypogaea* L., *Glycine max* L., and *Medicago truncatula* L. Red, purple, and green dots represent PR10 family members in peanut, alfalfa, and soybean, respectively. Groups 1 through 8 are denoted by red, gray, blue, purple, pink, orange, green, and yellow colors, respectively.

eight peanut chromosomes, with most genes positioned relatively close to chromosome ends. Chromosome 11 contained the highest number of *AhPR10* genes (20), while chromosomes 7 and 17 each contained only one *AhPR10* gene (Fig. 3).

Gene duplication events are common in plant genomes. Investigation of the *AhPR10* gene family expansion in cultivated peanut through intraspecific and interspecific collinearity analyses identified 12 pairs of tandemly duplicated *AhPR10* genes and 16 pairs of segmentally duplicated *AhPR10* genes (Appendix F), indicating these duplication events facilitated the expansion of the *AhPR10* gene family (Fig. 4-A). Furthermore, analysis of *PR10* gene homology and chromosomal segments among different species revealed numerous duplicated gene pairs between cultivated peanut, alfalfa, and soybean (Fig. 4-B). Notably, the non-synonymous substitution rate/synonymous substitution rate (K_a/K_s) values for these duplicated genes were less than 1, suggesting high conservation of gene structures and functions during the expansion of *PR10* gene families (Appendix G).

3.5. Analysis of *AhPR10* promoter *cis*-acting elements

Promoters, which are essential for regulated gene expression, contain *cis*-acting elements. To elucidate the mechanism underlying *AhPR10* gene responses to biotic and abiotic stresses, we analyzed the *cis*-acting regulatory elements in the *AhPR10* promoter region using PlantCare. The analysis revealed that nearly all *AhPR10* promoters contained at least one phytohormone response element, including the abscisic acid response element (ABRE), gibberellin response element (GARE-motif), TATC-box, and salicylic acid response element (TCA-element). Significantly, biotic stress response elements, such as a wound response element (WUN-motif) and a defense and stress response element (TC-rich repeat) (Appendix H), along with abiotic stress response elements, including a low-temperature stress response element (LTR), an ARE, and a drought response element (MBS) (Fig. 5-A), were identified in the examined promoter regions. Furthermore, stress response elements constituted 19% of the promoter *cis*-acting elements in Group 1 and 8% in Groups 4 and 6 (Fig. 5-B). These observations suggest that Group 1 *AhPR10* genes may play critical roles in peanut stress resistance.

3.6. Analysis of *AhPR10* expression in response to *A. flavus*

To initially screen for *AhPR10* family members mediating peanut resistance to *A. flavus*, the expression patterns of *AhPR10* at different time points during *A. flavus* infection were analyzed using publicly available RNA-seq transcriptome data (NCBI accession number: PRJNA825125). Significant fluctuations in *AhPR10* transcript levels were observed, indicating the comprehensive role of *AhPR10* family members during the response to *A. flavus*. Notably, most Group 1 *AhPR10* genes exhibited significantly higher expression in resistant material compared to susceptible material at the selected time points following *A. flavus* inoculation (Appendix I).

Representative genes from each group were selected to verify the differential expression of *AhPR10* genes at various time points after inoculating 'J11' and 'Zhonghua 12' with *A. flavus*. The RT-qPCR analysis of *AhPR10* expression in response to *A. flavus* provided essential information for identifying candidate genes mediating plant resistance to *A. flavus*. The expression levels of *AhPR10-12* (Group 2), *AhPR10-14* (Group 7), *AhPR10-18* (Group 4), *AhPR10-25* (Group 5), *AhPR10-33*/*41* (Group 1), and *AhPR10-46* (Group 8) were significantly higher in 'J11' (resistant) than in 'Zhonghua 12' (susceptible) (Fig. 6). These findings, combined with the identified cis-acting elements in *AhPR10* promoter regions, suggest that *AhPR10-7*, *AhPR10-33*, and *AhPR10-41* may enhance cultivated peanut resistance to *A. flavus* (Appendix J). Expression pattern analysis of these three genes in different peanut plant tissues and kernel developmental stages revealed expression across various tissues, with highest levels in kernels, followed by flowers and stems. In developing kernels, *AhPR10-33* expression was approximately 10 times higher than *AhPR10-7* and *AhPR10-41* expression at 85 days after flowering (Appendix K).

3.7. Expression of recombinant AhPR10-33 in *E. coli* and analysis of its inhibitory effects on *A. flavus*

Due to the hormone- and stress-related *cis*-acting elements identified in the *AhPR10* promoter region and the elevated expression of *AhPR10-33* in the *A. flavus*-resistant cultivar, we hypothesized that AhPR10-33 may play a crucial role in cultivated peanut's resistance to *A. flavus* infection. To investigate this, recombinant AhPR10-33 expression was

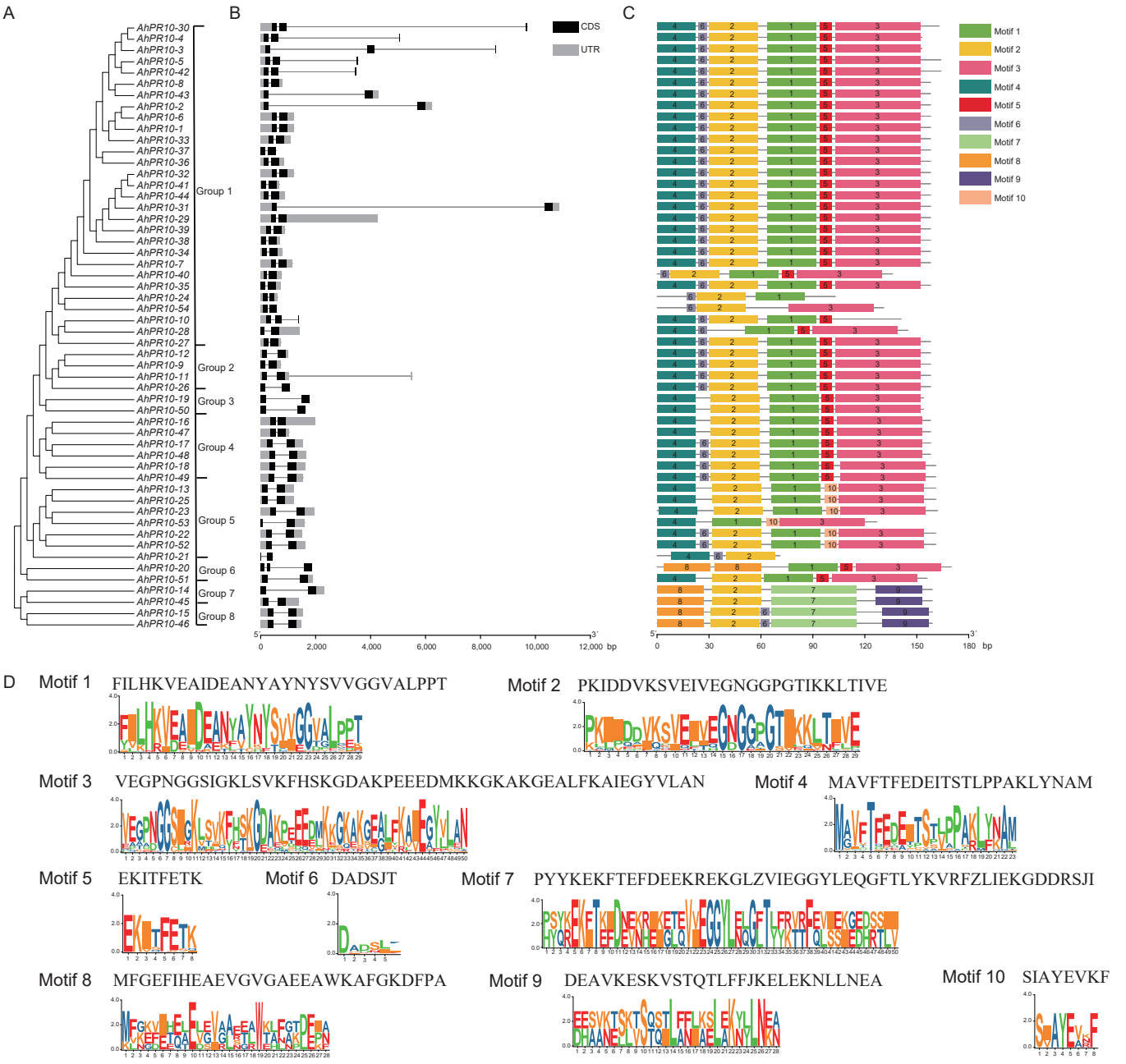


Fig. 2 Evolutionary relationships, gene structures, and conserved motifs in the *AhPR10* gene family. A, phylogenetic tree of the AhPR10 family. B, *AhPR10* gene structures. Black box indicates coding sequence (CDS); gray box represents 3′ or 5′ untranslated region (UTR). C, distribution of conserved domains among peanut *AhPR10* genes. Ten distinct motifs are differentiated by color. The motif lengths can be estimated using the scale provided at the bottom. D, distribution and sequence patterns of conserved motifs in AhPR10 proteins.

induced in *E. coli* cells using 0.2 mmol L^{-1} IPTG (16 h incubation at 18°C) and verified through SDS-PAGE electrophoresis. The SDS-PAGE gel revealed AhPR10-33 as a band at approximately 25 kDa, aligning with the predicted protein molecular weight (Fig. 7-A). The results confirmed that recombinant AhPR10-33 was produced as a soluble protein in *E. coli*.

The nuclease activity of AhPR10-33 was assessed using *A. flavus* total RNA. The introduction of AhPR10-33 to *A. flavus* total RNA (lane R+P) led to substantial RNA degradation, corresponding with the positive control results (RNase and *A. flavus* total RNA; lane R+R). The negative control samples (lanes R, R+BP, and R+H) showed no detectable degradation of *A. flavus* RNA (28S and 18S) (Fig. 7-B). These findings demonstrated that recombinant AhPR10-33 possessed RNase activity capable of degrading *A. flavus* RNA. Further evaluation revealed the fungistatic activity of recombinant AhPR10-33. While filter discs containing buffer or ddH_2O did not affect *A. flavus* mycelial growth, the filter disc with recombinant AhPR10-33 exhibited inhibitory effects. The fungistatic effect intensified with increasing volumes of recombinant AhPR10-33 solution (1 mg mL^{-1})

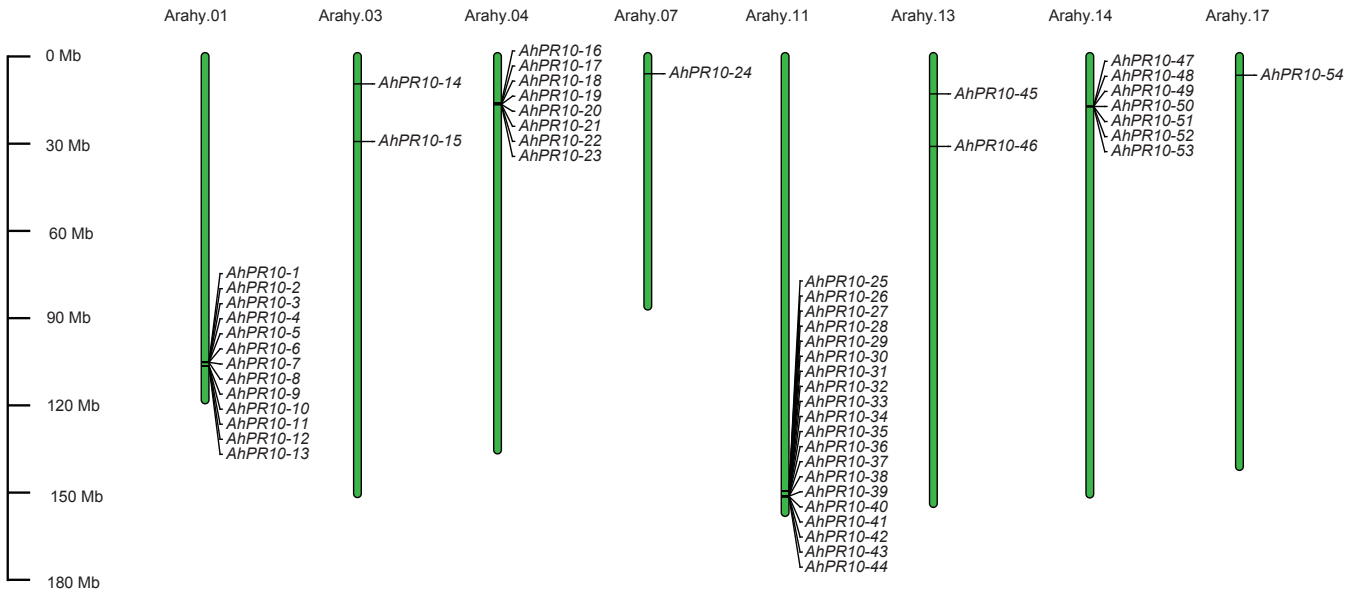


Fig. 3 Chromosomal distribution of *AhPR10* genes. Gene designations appear on the right side of each chromosome. Chromosomal distances are indicated in 30-Mb intervals.

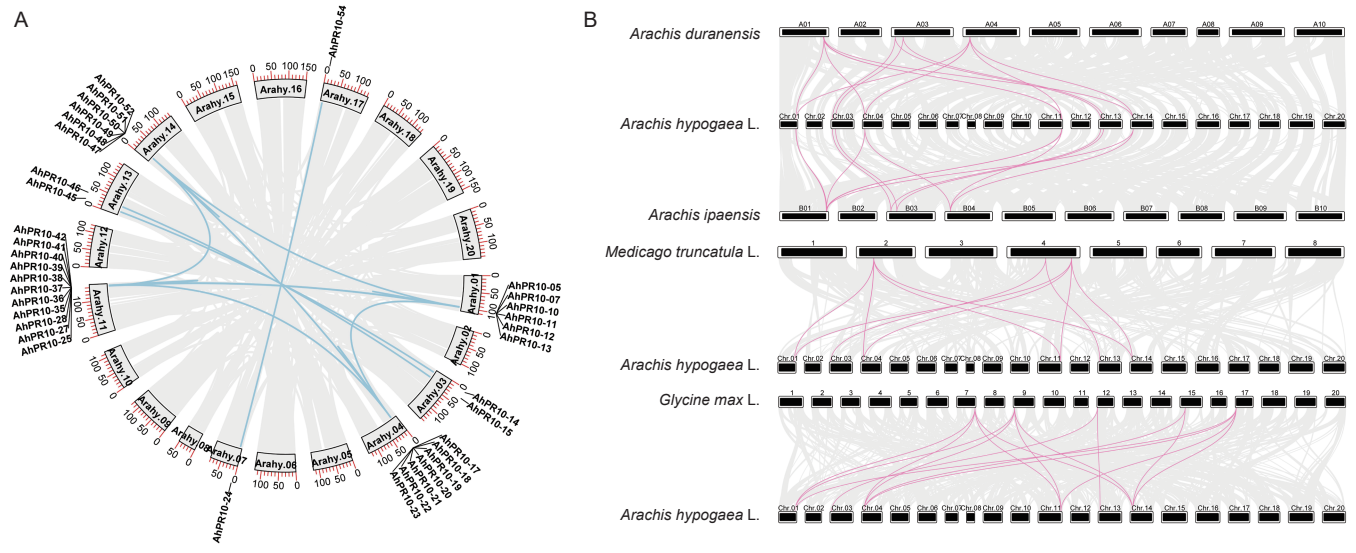
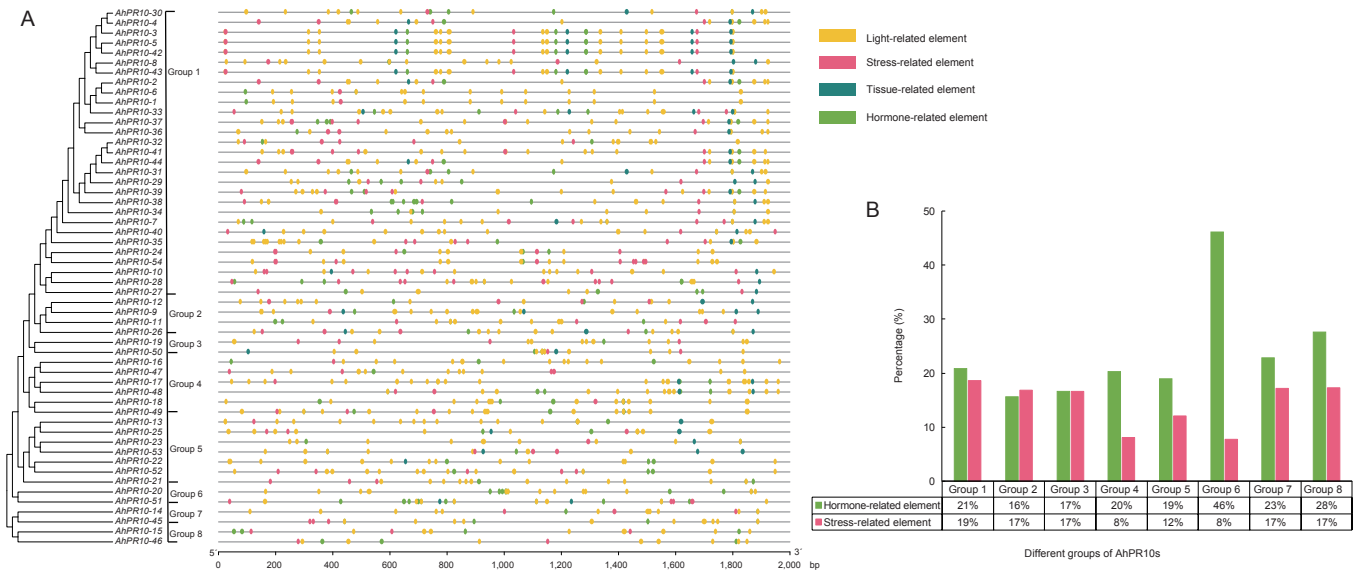


Fig. 4 Syntenic relationships. A, schematic representation of the inter-chromosomal relationships among *AhPR10* genes. Gray lines indicate syntenic blocks in the *Arachis hypogaea* L. genome, while blue lines indicate duplicated *AhPR10* gene pairs. The chromosome number is shown above each chromosome. B, syntenic relationships among *PR10* genes in *A. duranensis*, *A. ipaensis*, *Medicago truncatula* L., *Glycine max* L., and *A. hypogaea* L. genomes.

(Fig. 7-C). The average inhibition zones measured 11.3, 12.5, and 16.7 mm for 40, 60, and 80 μL recombinant AhPR10-33 protein treatments, respectively (Fig. 7-D; e.g., blue line), demonstrating significant inhibitory effects on *A. flavus* growth.

4. Discussion

The *PR10* gene family plays a vital role in plant growth, development, and resistance to biotic stresses, particularly pathogen infections (Liu *et al*. 2006). However, comprehensive research on the *AhPR10* gene family in cultivated peanut remains limited. This investigation aimed to identify *AhPR10* genes contributing to peanut resistance against *A. flavus*. The study utilized the *A. hypogaea* Tifrunner genome to systematically identify *AhPR10* gene family members and elucidate their chromosomal distribution, gene structures, and promoter region *cis*-acting elements. *AhPR10-33* was identified as a candidate resistance gene, and subsequent verification of its encoded protein's fungistatic effect established a foundation for future research on the evolution and potential functions of the peanut *AhPR10* gene family.



	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Hormone-related element	21%	16%	17%	20%	19%	46%	23%	28%
Stress-related element	19%	17%	17%	8%	12%	8%	17%	17%

Fig. 5 Analysis of *cis*-acting elements within the 2-kb *AhPR10* promoter region. A, phylogenetic analysis and distribution of *AhPR10* promoter *cis*-acting elements. Four distinct categories of *cis*-acting elements are distinguished by color coding. The sequence lengths can be determined using the scale provided at the bottom. B, distribution analysis of hormone- and stress-responsive elements across different groups.

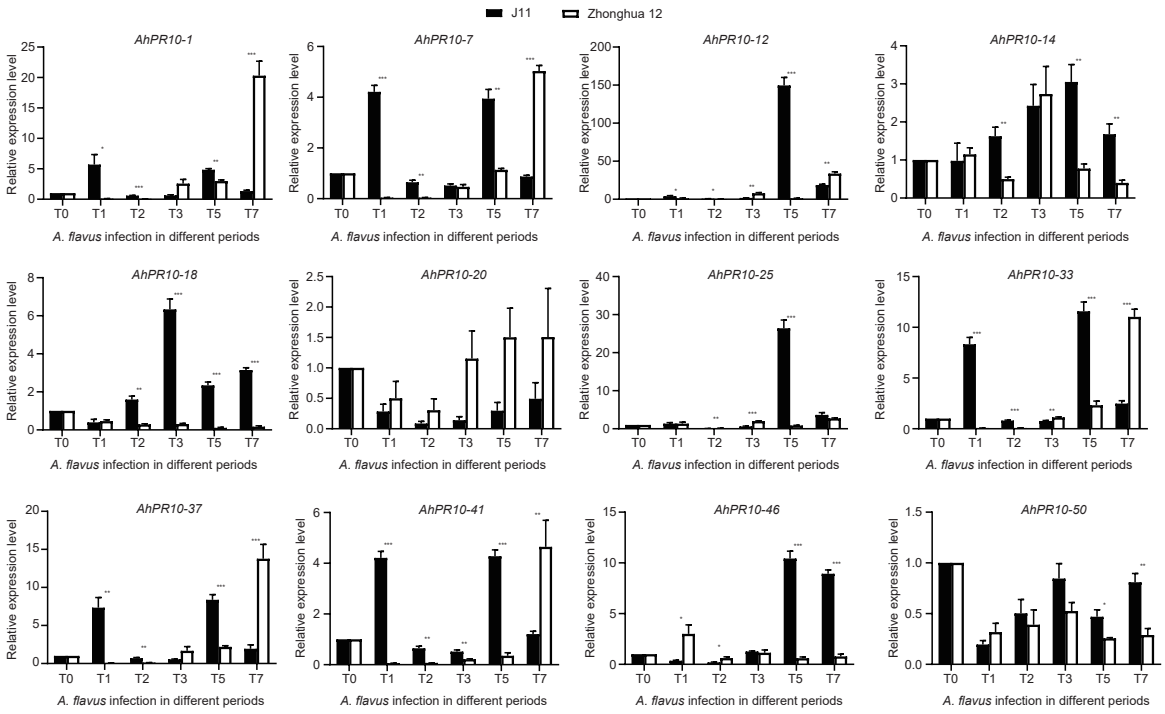


Fig. 6 RT-qPCR analysis of 12 selected *AhPR10* genes in samples infected with *Aspergillus flavus*. Expression levels were normalized against the expression in the untreated (0 d) control group. T0–T7 represent the days 0, 1, 2, 3, 5 and 7 after the peanut kernels are infected with *A. flavus*. Error bars represent three biological replicates. Data are presented as the mean±standard error of three independent biological replicates (n=3). Asterisks denote significant differences between the control and treatment groups at each time point as determined by Student's t-test (*, P<0.05; **, P<0.01; ***, P<0.001).

4.1. Evolutionary relationships among *AhPR10* genes

To elucidate the evolutionary history of the *AhPR10* gene family in cultivated peanut, we constructed a phylogenetic tree incorporating PR10 family members from peanut, soybean, and alfalfa. Based on sequence homology and evolutionary relationships, the analysis classified 54, 25, and 20 PR10 proteins from peanut, soybean, and alfalfa, respectively, into eight distinct groups. While Group 1 consisted exclusively of peanut PR10 family members, some peanut PR10 proteins were also present in Groups 3 and 5.

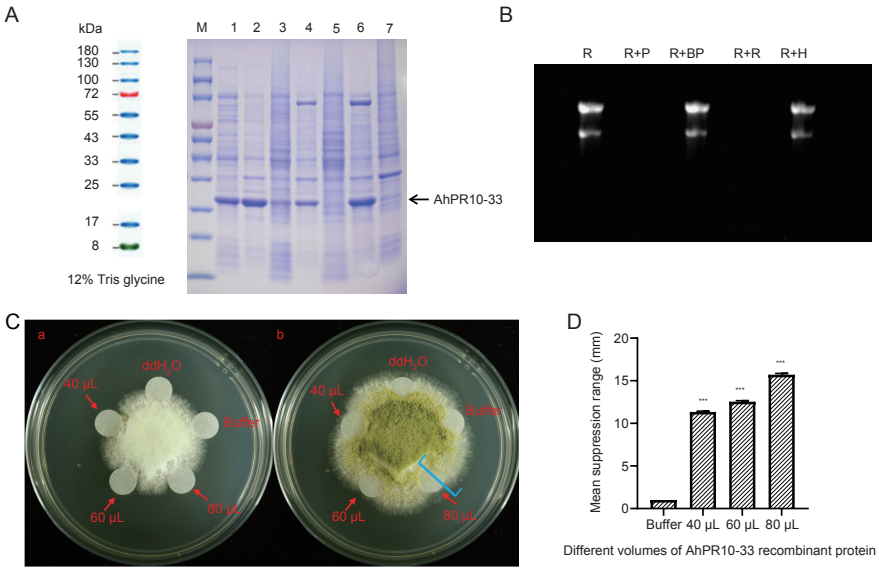


Fig. 7 Expression of recombinant AhPR10-33 in *Escherichia coli* and analysis of its nuclease activity and inhibitory effects on *Aspergillus flavus* growth. A, solubility of recombinant AhPR10-33. Lane M, molecular weight marker; 1, 18°C, cell lysate supernatant; 2, 18°C, cell lysate precipitate; 3, 28°C, cell lysate supernatant; 4, 28°C, cell lysate precipitate; 5, 37°C, cell lysate supernatant; 6, 37°C, cell lysate precipitate; 7, non-induced. B, RNase activity of recombinant AhPR10-33. Lane R, RNA+PBS buffer (negative control); R+P, RNA+AhPR10-33; R+BP, RNA+boiled AhPR10-33 (negative control); R+R, RNA+RNase (positive control); R+H, RNA+nuclease inhibitor (negative control). C, evaluation of the inhibitory effects of varying volumes of recombinant AhPR10-33 solution (1 mg mL^{-1}) on *A. flavus*. a, *A. flavus* growth inhibition at 12 h post-treatment with different volumes of AhPR10-33 solution. b, *A. flavus* growth inhibition at 36 h post-treatment with different volumes of AhPR10-33 solution. Both ddH_2O and buffer served as controls. D, mean inhibitory effect of different volumes of AhPR10-33 protein solution on *A. flavus* mycelial growth; the effect of the buffer alone was established as the baseline (***, $P<0.001$).

The 54 *AhPR10* genes identified in cultivated peanut surpassed the number of *PR10* genes found in soybean and alfalfa (Fig. 1). This expansion likely occurred during peanut evolution when *A. duranensis* (AA) hybridized with *A. ipaensis* (BB), followed by chromosome doubling. Furthermore, the analysis revealed that *AhPR10* gene structure correlates strongly with phylogeny. The two genes associated with the most gene pairs exhibited identical motif composition, suggesting potential functional similarity in their encoded proteins. Notably, motif 2, containing the P-Loop domain, was conserved across all AhPR10 proteins and is encoded by all *AhPR10* genes (Fig. 2).

4.2. Syntenic relationships among *AhPR10* genes

Gene families typically expand through several mechanisms, including genome-wide duplications, tandem duplications, and chromosomal fragment duplications (Vision *et al.* 2000; Cannon *et al.* 2004). The present study identified 54 *AhPR10* genes distributed unevenly across eight chromosomes, with numerous genes resulting from tandem or segmental duplications. Segmental duplications emerged as the primary mechanism driving the expansion of the *AhPR10* gene family. Collinearity analysis of *PR10* genes in cultivated peanut, wild relatives (*A. duranensis* and *A. ipaensis*), alfalfa, and soybean revealed multiple linearly arranged orthologous genes within legume genomes (Fig. 4). The *AhPR10* genes in cultivated peanut demonstrated 23, 23, 18, and 33 orthologous genes in *A. duranensis*, *A. ipaensis*, alfalfa, and soybean, respectively. The K_a/K_s values below 1 indicate that *PR10* genes in leguminous plants underwent strong purifying selection during evolution (Appendix G). These findings align with recent research on other gene family expansion events in peanut (Cui *et al.* 2021). Additional analysis of collinearity among peanut, alfalfa, and soybean would further illuminate the degree of genomic homology among species and the evolution of *PR10* genes.

4.3. Transcriptional regulation of *PR10*

Transcriptional regulation plays a fundamental role in plant stress responses. Transcription factors bind to *cis*-acting elements in promoter regions of target genes to modulate their expression through inhibition or activation. Therefore, identifying *cis*-acting regulatory elements within gene promoter regions provides valuable insights into gene expression patterns and levels. Research has shown that two *PmPR10-1-13* promoter regions (−1,316 to −930 and −309 to −100) contain cis-acting elements linked to pathogen- and wound-inducible gene expression in *Pinus monticola* (Liu *et al.* 2005). Studies in rice demonstrate that the W-box (WLE 1) cis-acting element in the *OsPR-10a* promoter serves an essential function in salicylic acid-mediated *PR10* expression, with WLE 1 mutations abolishing salicylic acid-regulated expression of *OsPR-10a* (Hwang *et al.* 2008). Additional research (Xu *et al.* 2010) revealed that GATA and Dof (TAACAAA) *cis*-acting elements in the *PsPR10* promoter correlate with tissue-specific expression in *Pinus strobus* under biotic and abiotic stress conditions. The presence of stress-related *cis*-acting elements in plant PR10 promoters

suggests their significance in regulating plant stress resistance. In this study, analysis of the 2,000 bp region upstream of *AhPR10* genes revealed multiple hormone- and stress-related *cis*-acting elements (including ABRE, GARE-motif, MBS, and TC-rich repeat), indicating PR10 family members may participate in peanut hormone metabolism, stress-related signaling, and other biological processes (Appendix H). Furthermore, the higher proportion of stress response cis-acting elements in Group 1 (19%) compared to Groups 4 and 6 (8%) suggests Group 1 may have greater significance in peanut stress resistance (Fig. 5).

4.4. *PR10* family genes are widely involved in plant biotic stress responses

Previous studies have demonstrated that most PR10 proteins exhibit nuclease activity and antibacterial properties; the expression of *PR10* genes can be induced by pathogenic microorganisms, indicating their role in plant defense mechanisms (Cheng *et al.* 2013). The enhanced expression of plant *PR10* genes under stress conditions likely reflects plant evolutionary adaptations to various environmental stresses. Research has shown that *PR10* expression in peanut increases following *A. flavus* infection (Luo *et al.* 2005; Guo *et al.* 2008), suggesting PR10's involvement in the peanut's response to *A. flavus*. Among the 54 identified *AhPR10* genes, *AhPR10-7*, *AhPR10-33*, and *AhPR10-41*, which belong to Group 1, demonstrated higher expression in 'J11' (resistant) than in 'Zhonghua 12' (susceptible) from T1 to T7 (Fig. 6). The *AhPR10-33* gene was cloned from 'J11'. Sequence analysis revealed the predicted open read frame (471 bp) encodes a protein with 157 amino acids and a pI of 5.04. This protein contains a P-Loop motif, which remains highly conserved in the PR10 family. Additionally, the AhPR10-33 amino acid sequence shares 97.47% similarity with the amino acid sequence of ARAhPR10 (gb|EU661964.1), which has been linked to peanut resistance to *A. flavus* (Xie *et al.* 2009). The *E. coli*-based prokaryotic expression system, among the most established and reliable protein expression systems, facilitates rapid expression of exogenous genes from various species. In this study, the pET30a vector was utilized to express a recombinant AhPR10-33 fusion protein in *E. coli*. Analysis of the recombinant protein verified the nuclease activity of AhPR10-33. However, not all plant PR10 proteins characterized thus far exhibit nuclease activity. While PR10 proteins isolated from cotton (Zhou *et al.* 2002), hot pepper (Park *et al.* 2004), *Panax ginseng* (Lee *et al.* 2012) demonstrate *in vitro* nuclease activity, PR10 proteins from potato (Matton and Brisson 1989) and *Medicago truncatula* (Samac *et al.* 2011) lack nuclease activity *in vitro*, possibly due to structural and property variations among PR10 family members. The *AhPR10-33* gene encodes the P-Loop conserved domain, which is preserved in the disease response-related protein family. In the fungistatic activity assay, AhPR10-33 significantly inhibited *A. flavus* mycelial growth (Fig. 7-C), aligning with previous reports of antimicrobial effects in grape PR10 protein (He *et al.* 2012). A maize *PR10* gene was isolated and demonstrated inhibitory effects against *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *F. oxysporum*, *Verticillium dahliae*, and *Alternaria solani* (Zandvakili *et al.* 2017). Plant PR10 proteins represent widespread disease response-related proteins exhibiting nuclease activity and antimicrobial properties that provide protection against biotic and abiotic stresses. These characteristics suggest potential applications in enhancing plant stress resistance.

5. Conclusion

This study identified 54 *AhPR10* genes and categorized them into eight distinct groups. These genes exhibited highly similar exon-intron structures and conserved motifs. The expansion of the *AhPR10* gene family occurred through segmental duplications during evolutionary processes under strong purifying selection. Analysis of *cis*-acting elements revealed that hormones and various environmental factors regulate the expression of *AhPR10* genes. Furthermore, RT-qPCR data demonstrated that the expression levels of most *AhPR10* genes in Group 1 were significantly elevated in resistant materials compared to susceptible materials at various time points following *A. flavus* infection. Studies of nuclease activity and *A. flavus* growth inhibition demonstrated that recombinant AhPR10-33 effectively degrades *A. flavus* RNA and inhibits *A. flavus* growth, indicating its potential role in peanut defense against *A. flavus* infections.

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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