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RESEARCHARTICLE

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Unrevealing Genetic Structure and Evolutionary Relationship of the *Pathogenesis-related* Gene Family in *Vicia Faba* and *Medicago Truncatula*

Tahmina Latif*[1], Muhammad Usman Ghani[1], Aliya Batool[1] and Idrees[1]

[1]Centre of Agricultural Biochemistry and Biotechnology University of Agriculture Faisalabad, Pakistan

Correspondence
latiftahmina7@gmail.com

Abstract

Faba beans (*Vicia faba*) have been grown for years and are a good crop for sustainable and diverse farming systems. With various cultivated forms such as broad, horse, tick, and central Asian varieties. Faba beans have a high nutritional value, often outperforming peas and certain grains. Their ability to thrive in diverse climates and soil types gives them a competitive edge over other legume crops. The limited use of these crops in modern agriculture is attributed to yield instability resulting from biotic and abiotic stresses, especially drought, which impacts cultivation in the Mediterranean region. Faba bean cultivation in Pakistan is limited due to farmers' awareness, seed accessibility issues, and insufficient research and development efforts for the crop successfully sequenced the vast faba bean genome, paving the way for breakthroughs in developing more nutritious, climate, biotic, and abiotic stress resilient legume. Despite its importance, the faba bean genome remains unannotated, and many gene families are yet to be characterized. This study compared the *PR* genes from *Vicia faba* and *Medicago truncatula*, using chromosomal mapping, motif, gene structure, phylogenetic, and synteny analyses to better understand evolutionary patterns and functional characteristics. The outcomes from this study provided insights into plant defense mechanisms and evolutionary processes that would be a valuable resource for crop improvement and stress resilience research.

KEYWORDS
Evolutionary studies, Gene expression, Amino acids, Axon, Intron

1 | INTRODUCTION

Vicia faba is a vital source of nutrition (Singh, 2017). These are part of the legume family, leguminoseae, encompassing species consumed by both human and domestic animals, often in the form of dried grains. *V. faba* possesses high nutritional value and is regarded as superior to peas and various grain legumes in certain regions. They are cultivated not only for consumption but also as green manure, capable of significantly improving the yields of cereals and other crops (Bond *et al.*, 1994). Classification excludes groundnuts (*Arachis hypogaea*) and soybeans (*Glycine max*) primarily cultivated for edible oil. *V. faba* has been grown for thousands of years and now includes a variety of cultivars, complicating its classification. (Mínguez and Rubiales, 2021) Abiotic and biotic factors are major constraints on Faba Bean yield. The expansion of the plant-based protein mandate is incorporating *V. faba* as a strong contender to meet this growing demand (Gutiérrez *et al.*, 2023).

Vicia faba, also known as broad bean, has been cultivated for thousands of years. Today, it exists in various cultivated forms that are challenging to classify. Currently, it is classified into four main categories: 1) *V. faba* var. equine (horse bean) with medium-sized seeds 2) *V. faba* var. major (broad bean) with large broad and flat seeds 3) *V. faba* var. minor (tic bean) with small and round seeds 4) *V. faba* var. pauncijuga (Primitive) with small seeds. Out of these, the last one is grown in Central Asia (Redden *et al.*, 2014).

The *V. faba* plant has its origins in the Near East and the Mediterranean basin, where it has been deliberately grown for a period of about 8,000 to 10,000 years. It is used as a staple in human diets and as

animal feed for pigs, horses, chickens, and pigeons in industrialized nations. *V. faba* possesses high nutritional value and is regarded as superior to peas and various grain legumes in certain regions. The most promising advancements have been observed in the legume species *M. truncatula* (Michno *et al.*, 2020).

The genetic information in faba bean is carried on only 6 pairs of chromosomes. Chromosome 1 (the longest) alone is the size of the 23 chromosomes in the human genome combined. Faba bean is a diploid with 2n = 2x = 12 chromosomes partially cross-pollinated ranging from 4 to 84% and possesses one of the largest genomes among crop legumes (~13000 Mb). For the first time, an international group led by INRAE researchers successfully assembled and annotated the sequence of the enormous faba bean genome that 4 times larger than the human genome. This grown legume, with its protein-rich seeds and minimal requirement for nitrogen fertilizer, provides several benefits to ecologically friendly agricultural methods. *V. faba* removes a barrier to varietal selection in this species and will, in particular, allow for improved variety performance in terms of climate change and pest pressure, as well as yield consistency and nutritional value of the seeds.

In *Vicia faba*, *Pathogenesis-Related (PR)* plays critical roles in the plant's defense mechanisms against pathogens. *PR* genes are a subset of plant genes that are activated in response to pathogen attacks. They encode a wide range of proteins involved in plant defense, including enzymes, signaling proteins, and proteins that directly inhibit pathogen growth. *Chitinase* genes are enzymes that degrade chitin, which is a key component of fungal cell walls. In plants, *Chitinase* genes are frequently classified as *PR genes* (*PR*-3, *PR*-4, *PR*-8, and *PR*-11). These enzymes help to break down invading fungi's cell walls, preventing them from growing and spreading. *PR and Chitinase* genes are typically up regulated in response to pathogen infection. This up regulation is frequently facilitated by signaling molecules such as salicylic acid (SA), jasmonic acid (JA), and ethylene.

PR genes can be activated by abiotic stresses, contributing to an increased level of resistance against pathogens. These genes, responsive to both biotic and abiotic stimuli, hold significant potential for incorporation into breeding programs aimed at enhancing pathogen resistance in plants (Wanderley-Nogueira *et al.*, 2012). The *PR* proteins are currently divided into 17 classes (van Loon *et al.*, 2006). Moreover, their expression is heightened in response to pathogen infection. Initially characterized as proteins exclusively expressed upon pathogen infection, extensive research into various gene classes has revealed that certain family members exhibit expression in one or more plant parts during the normal development and growth of healthy plants (Sudisha *et al.*, 2012). *Chitinase* genes in faba beans are extremely sensitive to fungal infections caused by *Botrytis fabae* and *Fusarium spp*. When a pathogen attacks, the expression levels of *Chitinase* and other *PR genes* rise, adding to the overall defense strategy (Bol *et al.*, 1990). When plant genes exhibit some role during pathogen infections, they are categorized as *Pathogen Related (PR)* proteins because of their role in induce defense. *PR* protein that have enzymatic activity against chitin, which is a fundamental component of the cell walls of fungi, insects, and other organisms (Zribi *et al.*, 2021). The *PR* proteins are currently divided into 17 classes (van Loon *et al.*, 2006). Initially characterized as proteins exclusively expressed upon pathogen infection, extensive research into various gene classes has revealed that certain family members exhibit expression in one or more plant parts during healthy plants' normal development and growth. Moreover, their expression is heightened in response to pathogen infection. (Sels *et al.*, 2008). Multiple classes of *Pathogenesis-Related (PR)* protein genes demonstrate expression in the majority of plant-pathogen interactions, although some display more specific patterns (Sudisha *et al.*, 2012). In specific interactions with Arabidopsis, the expression of diverse *PR* protein gene classes is intricately linked to an elaborate and complex defense response network (Almeida-Silva and Venancio, 2022). *Pathogenesis-related (PR)* proteins serve as crucial defense-related signaling molecules triggered by phytopathogens, playing a pivotal role in thwarting the infiltration of invading pathogens. These *PR* proteins have undergone categorization into numerous families, with distinctions based on their function, molecular weight, amino acid sequence, and other intrinsic properties (Kattupalli *et al.*, 2021). The most promising advancements have been observed in the legume species *Medicago* truncatula. Genome-wide studies have emerged as a valuable approach for identifying genetic intervals linked to phenotypic variations in *M.* truncatula (Michno *et al.*, 2020).

Objectives

1. To explore the genetic and evolutionary history of *V. faba.*
2. Find the syntenic regions to figure out the evolutionary pattern.

Examine the structure of gene and protein sequences for species comparison.

2 MATERIALS AND METHODS

Data Retrieval and Search

The genomes of *V. faba* was downloaded from (https://projects.au.dk/fabagenome) and *Medicago Truncatula* downloaded from (https://www.legumeinfo.org/) along with their gff3 files. Nucleotide and protein sequences of *PR* gene

families of *Medicago truncatula* and *V. faba* were searched and retrieved. These sequences were then used as input in TBtools Biosequence structure Illustrator and visualize domain pattern found was reanalyzed again using NCBI Batch Conserved Domain search (https://www.ncbi.nlm.nih.gov) and selected the option Pfam.

Chromosomal Mapping of *PR* Gene Family of *V. faba* and *M. truncatula*

Gene positions and chromosomal lengths were input into MG2C(Chao *et al.*, 2021), resulting in the construction of a comprehensive chromosomal map for *V.faba*. This map provides a detailed visual representation of gene distribution and chromosomal characteristics within the species. Data that require for this analysis gene ID of *PR* gene family of *V. faba,* start position and end position and chromosomes number and length of chromosomes collect from downloaded gff3 files.

Motif Analysis of *PR* Gene Family of *V. faba* and *M. truncatula*

The MEME Suite Server 4.11.2 (Bailey *et al.*, 2015) was employed to investigate conserved motifs using the keyword *Chitinase* and *PR* gene families. The MEME analysis was configured with 5 motifs. Initiated the search to obtain the desired results server identify conserved motifs in the *PR* gene family by providing DNA sequences from 33 genes of *V. faba* 50 genes of *PR* gene family as input and specifying the analysis parameters. Conserved motifs of *PR genes* nucleotide sequences were with motif length set to 1–50, and motif sites to 2–120. The maximum number of motifs to find was set to 5, the distribution of one single motif was any number of repetitions and the other parameter was searched by Xstream enriched motifs (Bailey, 2021).

Gene Structure Analysis of *PR* gene Family of *V. faba* and *M. truncatula*

The exon structure of genes was determined by performing a comparative analysis between the predicted cDNA sequences and the genomic DNA sequences using specialized bioinformatics tools designed for tuberculosis research TB tools (Chen *et al.*, 2020). The tree layout, presented in the form of a cladogram, was specifically designed with a width of 600 and a height of 800, effectively illustrating the presence and arrangement of exons within the genetic sequences.

Phylogenetic Analysis of *PR* Gene Family of *V. faba* and *M. truncatula*

Phylogenetic tree was constructed using the neighbor-joining method (Hawtin, 1982) with the Poisson correction, and pairwise deletion option parameters enabled. The reliability of the trees obtained was tested using a bootstrap test with 1000 replicates. Images of the phylogenetic trees were drawn by using ITOL https://itol.embl.de/. A multiple alignment analysis was performed with multiple sequence alignment MEGA11 tool (Tamura *et al.*, 2021) and a phylogenetic tree was created using the Genome workbench program (https://www.ncbi.nlm.nih.gov/tools/gbench) (Kuznetsov and Bollin, 2021).

Synteny Analysis of *PR* Gene Family of *V. faba* and *M. truncatula*

TB Tools (Chen *et al.*, 2020) facilitated the synteny analysis, Navigate the genomic relationships between 50 genes in *V. faba* and 63 genes in *Medicago* of *PR* gene family . Through the use of Tb Tools for synteny analysis, significant conserved genomic regions between *V. faba* and *M. truncatula* were unveiled. Synteny relationships among genes were established based on their classification within the same gene families in *V. faba* and *M. truncatula*, utilizing a Pfam search with a threshold of 1e−10 and representing legume gene families (accessible at https://legumeinfo.org/data/public/Gene_families/legume.genefam.fam1.M65K).Additionally, these genes occur in regions with similar genic content, requiring a minimum of 10 homologous genes with a conserved order within a neighborhood of 20 genes surrounding the candidate gene.

3 RESULTS

Chromosomal Mapping of *PR* Genes in *V. faba and Medicago*

The distribution of *PR* genes across the six chromosomes underscores intrinsic variations in gene counts among these chromosomal entities. This observation significantly contributes to the diverse gene distribution pattern observed across the studied chromosomal entities. The distribution of *PR* genes across the 6 chromosomes in *V. faba* demonstrates considerable variability. Specifically, Chr4 and Chr6 display the highest number of genes, containing 18 and 11 genes, respectively. Following these, Chr1L accommodates 7 genes, while Chr1S holds 4 genes. Chr2 and Chr3 contain 3 and 5 genes, respectively. Chr5 exhibits the lowest gene count with only one gene (Fig. 1).

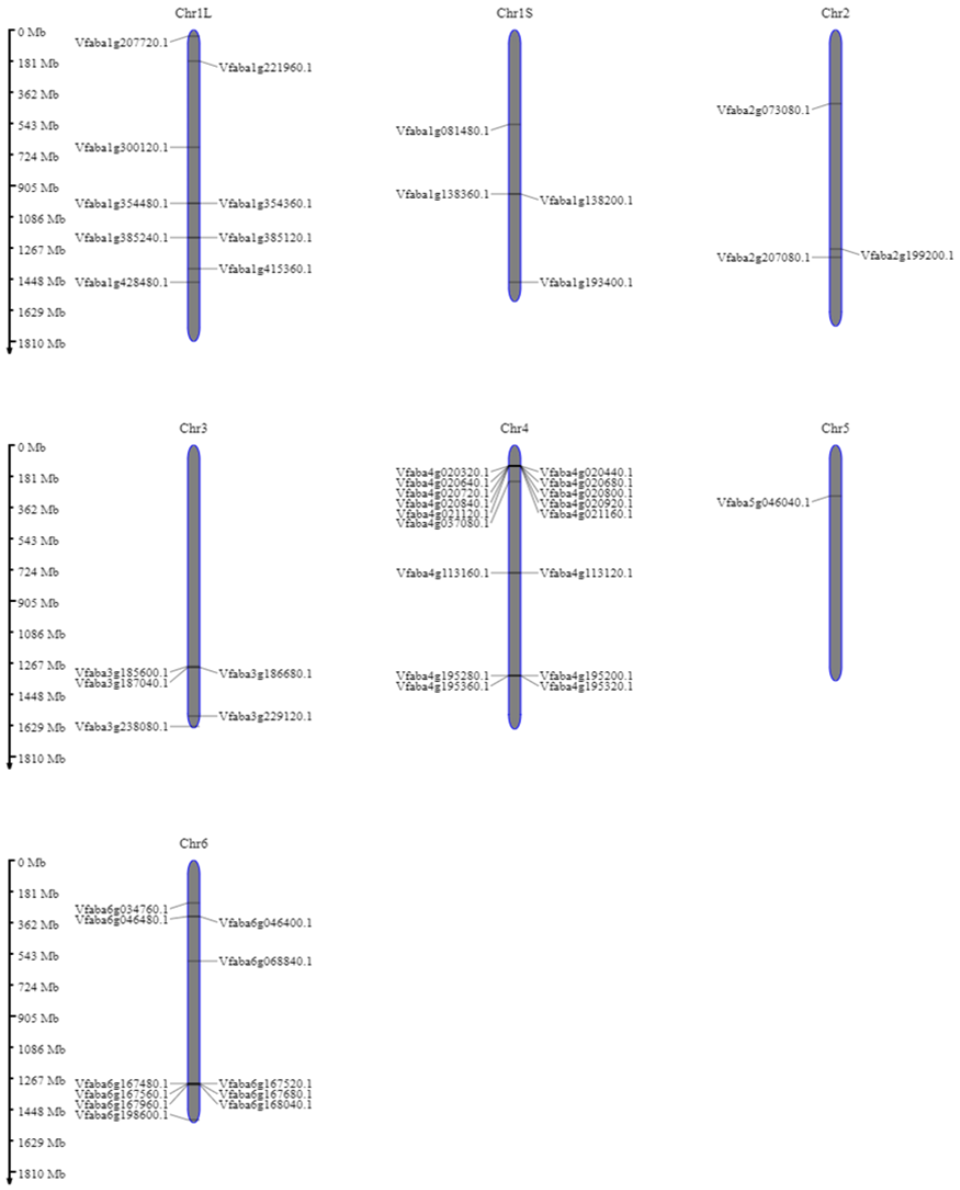

Fig. 1: This figure shows the number of PR genes across the six chromosomes of V. faba. Chromosome 4 has the highest count with 18 PR genes, followed by Chromosome 6 with 11 genes. Chromosome 1L has 9 genes, Chromosome 1S has 4, Chromosome 3 has 5, Chromosome 2 has 3, and Chromosome 5 has the fewest with 1 PR gene. These variations highlight the differing roles of each chromosome in the plant's defense mechanisms.

Motif Analysis of *PR* Gene Family of *V. faba and M. truncatula*

The MEME analysis revealed the presence of distinct motifs within the *PR* gene family of faba beans, including 5 conserved motifs and location of motifs displaying similarity to known functional motifs (Fig. 2). Location of motifs displaying similarity to known functional motifs or domains documented in motif databases, indicating potential functional relevance. Analyzed and annotated the putative functions or conserved domains represented by these identified motifs. Visualized the distribution of identified motifs across the 50 *PR* gene of *V. faba* sequences to observe their positioning and recurrence. The total height of the letter piles at each position indicates the conservation of the sequence at that position. The height of a single letter in the letter piles represents the relative frequency of the corresponding amino acid at that position. These motifs exhibited variability in length and diverse distribution in 2 bits across the *PR* gene sequences (Fig. 3).

Gene Structure Analysis of *PR* Gene Family of *V. faba and M. truncatula*

The analysis revealed a notable range in the number of exons present within the *PR genes* of *V. faba*, varying from one to eight exons. Most of the *PR* genes exhibited a distinctive characteristic, with a significant portion comprising a single exon. Functional Significance is that such diversity hints at potential variations in gene functions, processing, or regulatory mechanisms within *V. faba*. Exon lengths starting from 0 at the 5' end and extending up to 14000 at the 3' end (Fig. 4). The diverse

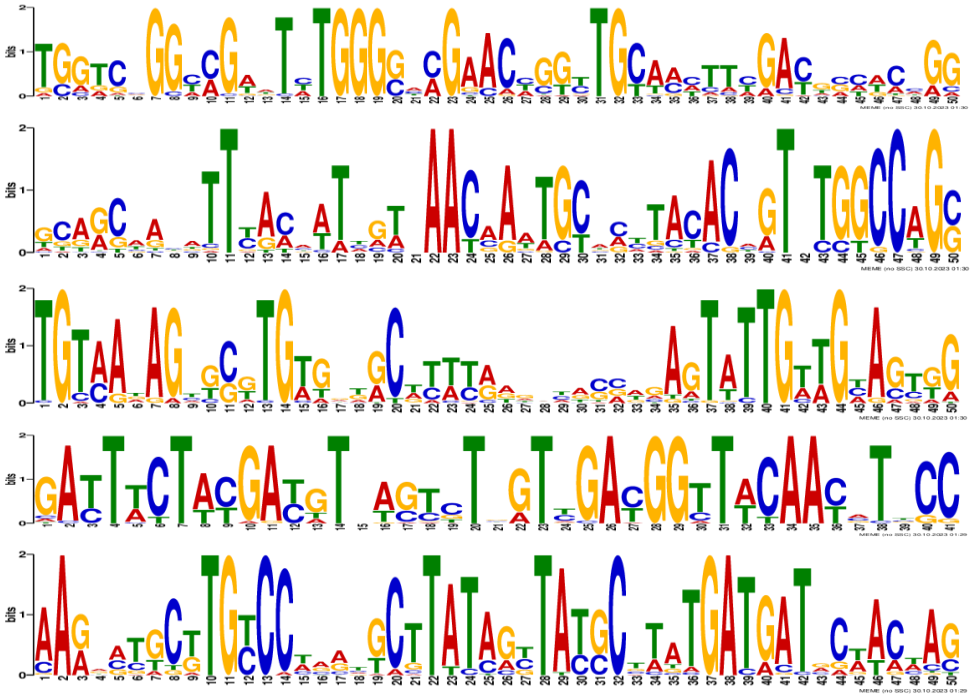

Fig. 2: The graphic depicts a sequence logos representing nucleotide frequencies at each place in a given DNA sequence alignment. The height of each letter represents the relative frequency of the matching nucleotide at that place, while the overall height of the stack shows sequence conservation at that site. The colors represent distinct nucleotides: red for adenine (A), blue for cytosine (C), yellow for guanine (G), and green for thymine.

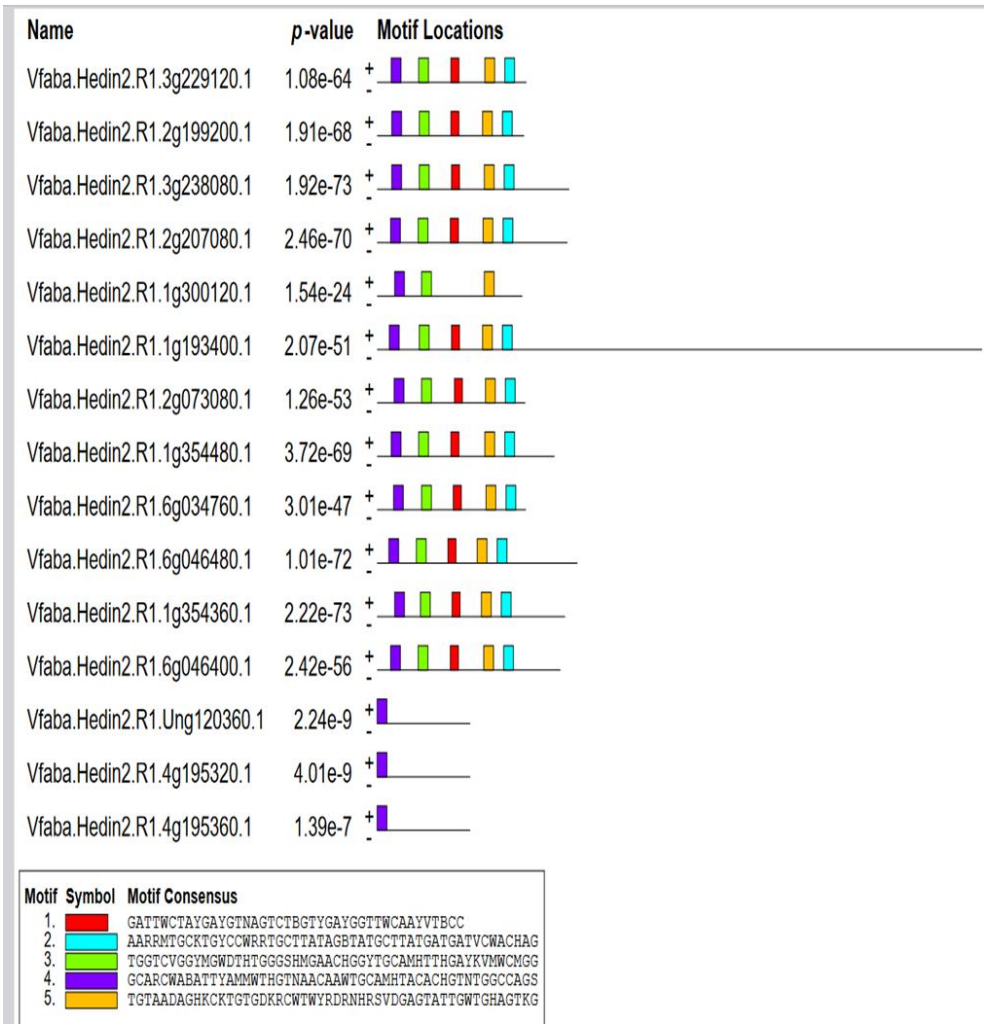

Fig. 3: This figure shows motif analysis for various genes. Each row lists a gene name, its associated ppp-value, and the locations of motifs within the gene. Colored boxes in the "Motif Locations" column indicate where each motif is found. The legend below identifies motifs by color and provides their consensus sequences. These figures are sequence logos that depict the nucleotide composition of two distinct motifs. In each column, the frequency of nucleotides (A, C, G, T) at each position within the motif is shown. The height of each letter in the column represents its relative frequency at that position. The y-axis measures the information content in bits, where taller letters indicate more conserved (less variable) positions in the motif.

Fig. 4: This figure displays the diversity in exon structures of PR genes in V. faba. Panel illustrates the range in the number of exons within PR genes of V. faba, highlighting significant structural diversity from one to eight exons, with a notable prevalence of single-exon genes.

exon length spectrum suggests potential functional and structural diversity within these genes. The analysis of *PR* genes in *V. faba* revealed a wide range in the number of exons, ranging from one to eight. This diversity in exon lengths suggests potential functional and structural variations within these genes. Interestingly, most of the *PR* genes in *V. faba* consist of a single exon, which is a distinctive characteristic. The prevalence of single-exon of *PR* genes in this analysis indicates a common structural pattern within this gene family.

Phylogenetic Analysis of *PR* Gene Family of *V. faba* and *M. truncatula*

The Neighbour Joining method was employed to establish the evolutionary relationship and phylogenetic relationships among the 113 *PR* gene, 50 *V. faba* and 63 *M. truncatula*. An unrooted tree was constructed, emphasizing evolutionary relatedness between the two species, facilitating comparative analysis. Clustering of similar sequences and their presentation in a circular phylogenetic tree enhanced the visual interpretation and facilitated a comprehensive understanding of the evolutionary connections between the *Chitinase* genes of *V. faba* and *M. truncatula*. The circular phylogenetic tree visualization improved the accessibility and interpretability of the analysis results, aiding in the comprehension of the evolutionary relationships and clustering of *PR genes* in the two species. The analysis likely demonstrated distinct clades or groupings representing gene lineages and relationships between *Chitinase* genes of *V. faba* and *M. truncatula*. The visualization and clustering provided insights into the evolutionary patterns and relatedness of *PR* genes that offering a clearer understanding of their evolutionary divergence and potential functional similarities or differences. The phylogenetic tree created with 3 clades is indicating the evolutionary relationships between *V. faba* and *M. truncatula* (Fig. 5).

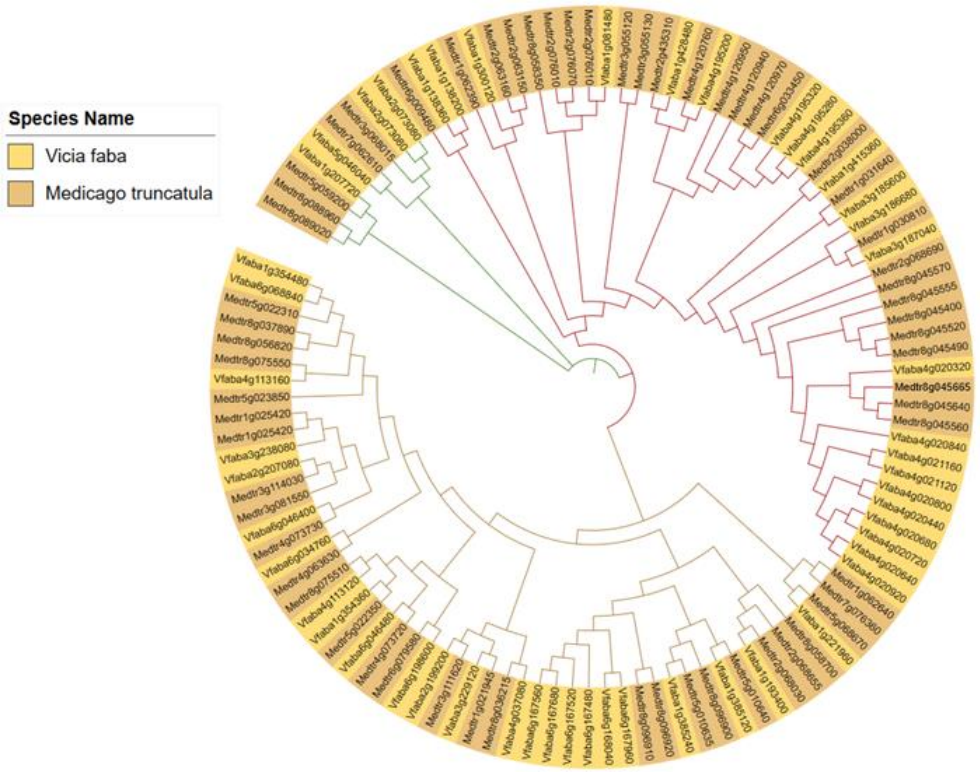

Fig. 5: Phylogenetic tree illustrating the evolutionary relationships among Pathogenesis-Related (PR) genes in V. faba and M. truncatula. The tree is divided into three main clades (Clade 1, Clade 2, and Clade 3). Clades predominantly consist of genes from both species, highlighting shared evolutionary origins and species-specific adaptations. Gene duplication events within clades indicate functional diversification, while the presence of mixed clades suggests conserved defense mechanisms across species.

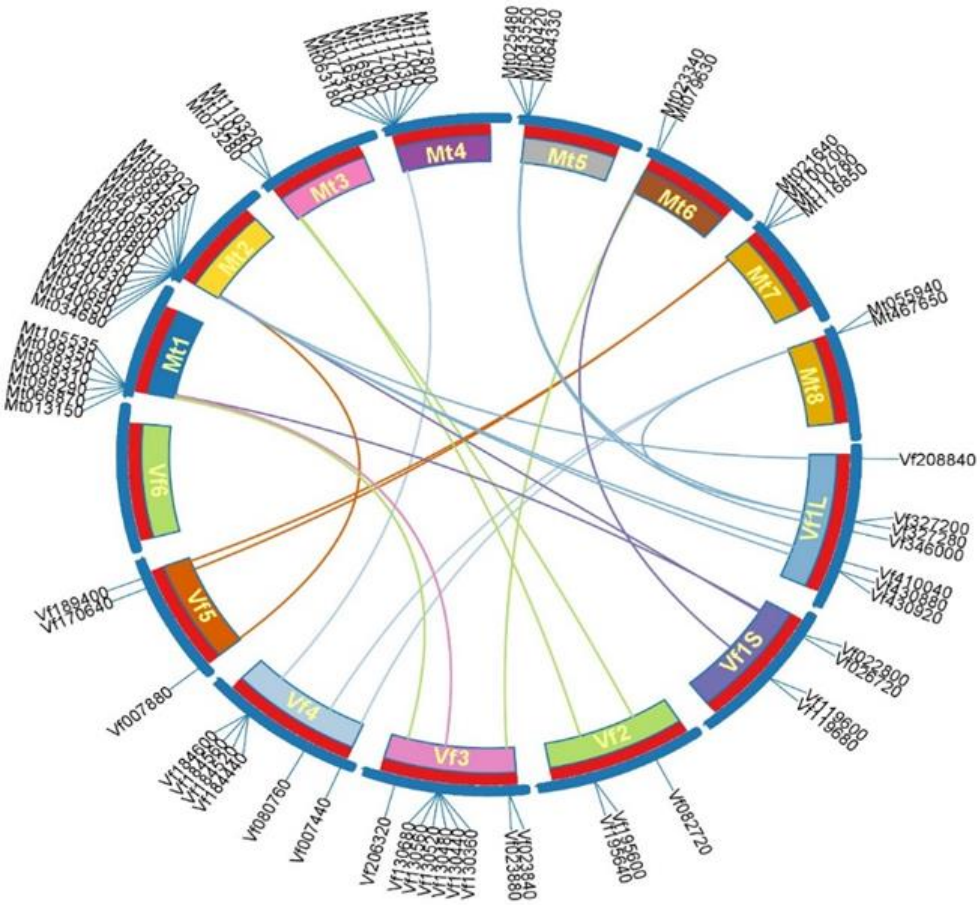

Fig. 6: The circular diagram shows synteny between the chromosomes of Medicago truncatula (Mt1 to Mt8) and V. faba (Vf1 to Vf8). Each colored segment represents a chromosome, labeled with specific gene loci. Colored lines connect syntenic regions between the two species, indicating conserved genomic areas.

Synteny Analysis of *PR* Gene Family of *V. faba* and *M. truncatula*

The synteny analysis uncovered significant conserved genomic regions between *V. faba* 50 genes and *M. truncatula* 63 genes of *PR* gene family. The analysis revealed conserved syntenic blocks, suggesting preserved genetic elements and shared genomic architecture between the two legume species. The results of synteny analysis indicate that in *M. truncatula*, chromosomes Mt7 and Mt8 contain the highest number of genes that exhibit significant synteny with all chromosomes in *V. faba*. This suggests a strong conservation of gene order between these two legume species. This result suggests that specific regions of *M. truncatula* genome (*Mt7 and Mt8*) have a remarkable degree of similarity with multiple chromosomes in *V. faba.* Within the context of phylogenetic analysis, clade 1 presents four genes one gene of *V. faba* (*vfaba*1g207720) while other three genes originate from *Medicago* (Medtr8g089020, Medtr8g088960, and Medtr5g059200). The noteworthy observation is that two genes from chromosomes Mt8 (Medtr8g089020, Medtr8g088960) have conserved gene order with a single gene situated on chromosome *Vf*1L that synteny is highlighted by blue line (Fig. 6).

4 | DISCUSSION

The chromosomal mapping, motif analysis, and phylogenetic and synteny studies of Pathogenesis-Related (*PR*) genes in *Vicia faba* and *Medicago truncatula* provide significant insights into the genetic and evolutionary dynamics of these essential legume species. The distribution of PR genes across *V. faba* chromosomes exhibits considerable variability, with chromosomes 4 and 6 containing the highest number of genes (18 and 11, respectively), while chromosome 5 harbors only one gene. This non-uniform distribution suggests that specific chromosomes play more dominant roles in encoding plant defense mechanisms, possibly reflecting evolutionary pressures and functional specialization. Motif analysis identified five conserved motifs within the PR gene family, highlighting regulatory regions that may influence gene expression. The sequence conservation at specific positions underscores their potential functional significance in regulatory processes. The variability in motif length and distribution across PR genes reflects functional and structural diversity, critical for the adaptation of these genes to various biotic and abiotic stresses.

Gene structure analysis revealed a notable diversity in exon number and length among PR genes in *V. faba*. The predominance of single-exon genes suggests a streamlined structural organization that could facilitate rapid gene expression, potentially advantageous in plant defense responses. This structural characteristic might also hint at evolutionary strategies to optimize genetic resources for enhanced resilience against pathogens. The phylogenetic analysis unveiled three main clades of *PR* genes, indicating shared evolutionary origins between *V. faba* and *M. truncatula*, along with species-specific adaptations. The clustering of similar sequences highlights conserved defense mechanisms, while gene duplication events within clades suggest functional diversification. Mixed clades with genes from both species reflect conserved evolutionary strategies to address common challenges in legume plants.

Synteny analysis uncovered significant conserved genomic regions between *V. faba* and *M. truncatula*, with chromosomes Mt7 and Mt8 in *M. truncatula* showing strong synteny with multiple *V. faba* chromosomes. The conserved gene order emphasizes evolutionary conservation and functional similarities between these species. A notable observation in clade 1 of the phylogenetic tree is the conserved gene order of two genes on *M. truncatula* chromosome Mt8 (Medtr8g089020 and Medtr8g088960) with a single gene on *V. faba* chromosome 1L (Vfaba1g207720). This finding underscores the preserved genomic architecture and the potential functional importance of these regions.

These insights are invaluable for advancing breeding strategies to enhance disease resistance and environmental resilience in legumes. The conserved regions and genes identified in this study offer promising targets for future research and genetic improvement initiatives in legume crops.

Conclusion

This study highlights the diverse distribution, conserved motifs, structural variability, and evolutionary relationships of *PR* genes in *Vicia faba* and *Medicago truncatula*. Chromosomal mapping and synteny analysis reveal significant conservation of gene order, emphasizing shared genomic architecture and functional relevance. The predominance of single-exon genes and conserved motifs underscores streamlined defense mechanisms in legumes. Phylogenetic analysis further elucidates evolutionary relationships and species-specific adaptations. These findings provide valuable insights for improving disease resistance and environmental resilience in legumes, offering a foundation for future genetic and breeding advancements.

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