Albalawi T (2024)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca
Volume 52, Issue 3, Article number 13854
DOI:10.15835/nbha52313854
Research Article

Unraveling barley's PAL gene family: a genome-wide study on defense mechanisms against *Puccinia graminis* f. sp. *tritici*

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Abstract

Phenylalanine ammonia lyase (PAL) is a pivotal enzyme bridging primary and secondary phenylpropanoid metabolism, influencing plant growth, development, and stress responses. Despite extensive studies on PAL genes across various plant species, their investigation in barley, a critical staple food globally, has been relatively scarce. In this study, we have successfully identified 10 *HvPAL* genes, designated as *HvPAL* genes, in *Hordeum vulgare* (barley). These *HvPAL* genes were categorized based on their conserved sequences, which revealed patterns through MEME analysis and multiple sequence alignment. Interestingly, we found cis elements related to stress in the promoter regions of *HvPAL* genes, indicating their involvement in the response to pathogens. Furthermore, these gene promoters contained components associated with light, development, and hormone responsiveness. This suggests that they may play a role in hormonal developmental processes. MicroRNAs were also identified as regulators of the *HvPAL* genes we identified highlighting their significance in barley. To further investigate these gene expression patterns, we analyzed the RNA-seq data revealed the upregulating of *HvPAL 2*, *HvPAL3,* and *HvPAL8*, and downregulating *HvPAL 5*, *HvPAL 6*, and *HvPAL9* genes in this study. This study focused on the regulation of PAL genes in response to 23 different races of *Puccinia graminis* f. sp. *tritici* in barley. These results suggest ways to improve traits and develop barley varieties that are resistant to pathogens by selectively increasing the expression of certain *HvPAL* genes that were not previously regulated. This thorough investigation aims to expand our knowledge of the versatility of the PAL gene family, providing insights for advancements in host -pathogen genetics.

Keywords: barley; gene regulation; *H. vulgare*; *Puccinia graminis* f. sp. *tritici*; phenylalanine ammonia-lyase (*PAL*); transcriptomic

Introduction

The non-oxidative deamination of L-phenylalanine to trans-cinnamic acid is the first step in these biosynthetic pathways, which are nearly universal among eukaryotes (Jones, 1984). The enzyme phenylalanine ammonia-lyase (PAL; EC 4.3.15) plays an extremely important role in this notable reaction (Weisshaar and Jenkins, 1998), and along with tyrosine and a special compound known as dihydroxyphenylalidone it is responsible for the synthesis of various secondary metabolites including flavonoids, lignin (Whetten and

Received: 15 Apr 2024. Received in revised form: 26 May 2024. Accepted: 02 Sep 2024. Published online: 12 Sep 2024.
From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

Sederoff, 1995; Guo and Wang, 2009). These include jasmonic acid (JA) and abscisic acid at the transcriptional level, and when a plant experiences different types of stresses occurring concurrently, PAL can be triggered by these together more easily than by other enzyme systems (Dixon *et al*., 2002). As a result, there is an important role for PAL in how plants resist both biotic factors, and an increase in expression of the PAL gene to such a height might allow plants to defend against various kinds of stress (Xu *et al*., 2012; Sami *et al*., 2024).

PAL (phenylalanine ammonia-lyase) is the first step in the phenylpropanoid pathway (Guo and Wang, 2009). Capable of catalyzing the deamination reaction to convert L-phenylalanine into trans-cinnamic acid is especially important (Dixon *et al*., 2002). This vital pathway leads to the synthesis of critical secondary metabolites involved in plant growth and development (Rui-Fang *et al*., 2016). Moreover, PAL functions as a crucial component of the plant stress response system and is affected by various factors. Drought-induced water scarcity and attacks by pathogens such as fungi contribute to alterations in their expression (Chen *et al*., 2017). Additionally, wounds and prolonged exposure, along with stressors specific to cut flowers or those exposed to unfavorable temperatures, further influence PAL expression. The swift response of PAL underscores its pivotal role in enabling plants to combat a diverse array of biotic and abiotic stressors (Cass *et al*., 2015). If PAL can be induced to express itself more quickly, this would lead to a tremendous breakthrough in creating plants capable of withstanding various kinds of stress (Dehghan *et al*., 2014). PAL is a key enzyme in the biosynthesis of important secondary metabolites, and plays an essential role in plant growth and development. Importance goes further and is involved in a major part of the plant's response to stress (Bartwal *et al*., 2013). PAL exhibits significant variation in activity based on developmental stages and various cell and tissue types within a plant. These changes in activity are particularly pronounced during heightened stress conditions in a plant life cycle (Liang *et al*., 1989). Consider *Arabidopsis thaliana*, where four genes, denoted as *AtPAL1-4*, encode active PAL isoforms (Dong and Shang, 2013). These genes are differentially expressed in various plant parts. *AtPAL1* is particularly concentrated in vascular tissue, whereas both seeds and serve as major sources of expression for *AtPAL2* and *AtPAL4*; the extent to which they find themselves expressed here depends largely on the sensitivity criteria used (Yu *et al*., 2018). By contrast, agricultural practices involving open fields can have severe negative consequences. Interestingly, among the discovered stressors, only *AtPAL1* and *AtPAL2* exhibit increased activity in response to low temperature and low nitrogen levels, respectively (Hahlbrock and Scheel, 1989). The gene expression of PAL shows potential variations across different plant species, specific plant parts, or even disparate stress environments (Ngumbi and Calla, 2022).

Barley (*Hordeum vulgare* L.) is the fifth-largest cereal grain crop in the world. An early crop domesticated by man approximately 1000 years ago in the Fertile Crescent region is now of historical significance (Zhang and Li, 2010). However, when humans first began consuming barley as a grain for animal feed, it later took the role of an important latecomer after wheat and rice (Smil, 2001). Although harvested mainly for animal feed and malt production, barley remains an important food source in certain societies in Asia and Northern Africa. Barley, the most resilient cereal crop, adapts to all environments from high latitudes and altitude countries down through parched desert land (Ullrich, 2014). In the Himalayas and Ethiopia, it remains a major food source (Ullrich, 2014). However, many abiotic stress factors affect barley physiology and yield and quality. Barley susceptibility to abiotic stress is a major problem in areas that lack stable weather conditions and ideal soil (Forster *et al*., 2000). Barley confronts recurrent fungal attacks from rust, powdery mildew, and *Fusarium* spp., resulting in substantial yield losses and quality concerns (Rana *et al*., 2022). The PAL gene family plays a role in the defense strategies of barley. These enzymes, known as PAL enzymes, help convert phenylalanine into cinnamic acid, which then kickstarts the production of secondary metabolites, such as phenolic compounds, flavonoids, and lignin. This process reinforces the defense mechanisms of plants against fungal pathogens (Chen *et al*., 2017). Elevated PAL gene expression leads to increase PAL activity, which contributes to the production of defence-related compounds. Barley's defense mechanism encompasses induced responses and generation of antimicrobial phytoalexins (Kaur *et al*., 2022). The present study focused on a comprehensive genome-wide analysis and expression profiling of the phenylalanine ammonia lyase gene

family in barley (Li *et al*., 2019). This study aimed to unravel the intricacies of PAL gene regulation with the goal of incorporating genes associated with enhanced PAL activity to bolster fungal resistance in barley varieties.

Materials and Methods

Retrieve amino acid sequences

Amino acid sequences of *H. vulgare* were obtained from the Phytozome v13 and the PAL genes of *H. vulgare* were identified using the Basic Local Alignment Search Tool for Protein Sequences (BLAST-P) program (Zhang *et al*., 2016). The protein sequence containing the PF00221 domain serving as the query in Phytozome v13 (*https://phytozome-next.jgi.doe.gov*; Haider *et al*., 2023).

Physicochemical properties, subcellular localization and of cis- elements prediction

Data on ten HvPAL proteins was gathered from ProtParam and Phytozome. The Phytozome gave the number and position and orientation of chromosomes of the gene within that specific region. Moreover, it includes information on mRNA length (CDS) and peptide size. The pI, molecular weight, Grand Average of Hydropathy (GRAVY), and stability index for these proteins were screened by ProtParam. WoLF PSORT database (<https://wolfpsort.hgc.jp/>) was used to determine the localization of these proteins with the range of 1000-base pair upstream promoter regions. The web tool PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) was used to predict the localization of these proteins based on 5-20 base pairs of upstream sequences from the first nucleotide in each case. These outputs were displayed as a heat map using TBtools for visualization (Bülow and Helh, 2016).

In silico examination of conserved motif domain and exon-intron positioning

An online tool has been implicated for Conserved Motif domain and Exon-Intron positioning i.e. MEME (http://meme.sdsc.edu/meme/website/intro.html) followed by the Conserved Domain Database (CDD; https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The genomic and CDS sequences of the HvPAL gene family were processed using the Gene Structure Display Server (GSDS) web tool at (http://gsds.cbi.pku.edu.cn/) to analyze exon and intron distributions (Barre *et al*., 2009).

Phylogenetic analysis

A phylogenetic tree was established by aligning the amino acid sequences of the HvPAL proteins with those from *H. vulgare, O. sativa, A. thaliana, and S. bicolor*. The software MEGA 11 was employed utilizing the neighbor-joining (NJ) method for tree construction, and bootstrapping was carried out with 1000 replications. The resulting trees were inspected visually. The Interactive Tree of Life (iTOL) program (<https://itol.embl.de/>) was then used to modify the tree, providing a user interface for exploring and annotating phylogenetic relationships as described in (Rehman *et al*., 2022).

Prediction of miRNA

The website PmiREN (<https://www.pmiren.com>) was utilized to determine the target sites of all the HvPAL gene families. By employing the PsRNA online server tool (<https://www.zhaolab.org/psRNATarget/>) with default settings, comparisons were made between the CDS sequences of the genes and mature miRNA sequences. Lastly, the interactions between the target genes and predicted miRNAs were visualized using Cytoscape (Mazhar *et al*., 2023).

Evolutionary, gene structure and gene ontology (GO) term analyses

The research on the evolution of PAL genes in barley involves the examination of duplication and synteny. To determine the rate at which each pair of genes evolved, we calculated the Ka/Ks substitution rates by aligning protein sequences using MUSCLE program and TBtools 1.108 with default settings. The Ks value was applied to the equation T = Ks/2, where $\lambda = 6.5 \times 10\text{-}9$, to estimate the time of divergence between these genes. Additionally, we used MCScanX v1.0 with default settings to identify gene duplication events (Haider, *et al.,* 2023; Luo *et al*., 2022). Furthermore, TBtools aided in creating maps that visually depict the synteny between paralogous genes in barley (Mazhar *et al*., 2023).

The gene structure display server (GSDS v2.0) (*http://gsds.cbi.pku.edu.cn/*) has been carried to predict the gene structure analysis to discern intron-exon positioning. Similarly, PlantCare database (*http://bioinformatics.psb.ugent.be/webtools/plantcare/html*) has also been carried out for prediction of cis-regulatory elements following the method of Abdulla *et al*. (2023).

The objective of conducting the GO enrichment analysis on barley was to explore the functions of these genes. The UniProt online database (<https://www.uniprot.org/>) (Langenbacher *et al*., 2020) was utilized to obtain information about their activities and involvement in various biological processes. Next, the PAL gene sequences were submitted to the ShinyGo v0.741 web tool (<http://bioinformatics.sdstate.edu/go/>) (Mazhar *et al*., 2023) for GO word enrichment analysis.

Protein-protein Interaction and expression analyses

The study utilized the STRING database v0.741 (<https://string-db.org/>) to further explore the protein interactions among PAL genes in barley. This online tool effectively showcases the interplay among proteins within the PAL genes of the barley plant.

Gene expression profiling of 23 races of Puccinia graminis f. sp. tritici

To analyze the RNA-seq data of HvPAL genes and investigate the expression profiles in response to various races of *Puccinia graminis* f. sp. *tritici*, data were retrieved from the NCBI GEO database ([https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE130423] (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE130423)) under accession GSE130423 (Sharma *et al*., 2019). This study aimed to explore the intricate interactions between barley and different pathogen races. Specifically, the expression patterns of 10 PAL genes (phenylalanine ammonia lyase), denoted as HvPAL 1 through HvPAL 10, were examined in response to pathogen races, including WM-1, R29M, R29JB, R29JA, R11c, QCCJ, P84-16, AC-12, A-5, A-48, A-21, A-15, A-14, A-12, 81AC46, 81AC34, 81AC28, 79_20, 79.2, 79-1, 72_00, 640 °C, and 370 °C. To gain an understanding of the differences in gene expression, we utilized Statistix 8.1, a pairwise comparison tool. This tool helped us identify variations between conditions and provided insights into potential targets for further studies.

Results

Identifying PAL genes in Hordeum vulgare L. and their localization

A comprehensive analysis of 10 *HvPAL* genes was conducted to elucidate their molecular characteristics. Notably, *HvPAL 6* displayed the smallest molecular weight at 13,647.51, whereas *HvPAL4* exhibited the highest molecular weight at 78,012.73. The isoelectric point (pI) values ranged from a minimum of 36.31 in *HvPAL9* to a maximum of 61.39 in *HvPAL6*. Consistently, negative Gravy scores for all genes indicated their hydrophilic nature. Regarding coding sequence (CDS) length, *HvPAL* 5 had the shortest length at 1,410 bp,

while *HvPAL2* boasted the longest at 2,688 bp. Furthermore, the peptide length varied from 127 in *HvPAL6* to 723 in *HvPAL* 4.

In our exploration of *HvPAL* genes at the genomic level, we delineated their positions and orientations on the chromosomes. Among the 10 *HvPAL* genes examined, *HvPAL8* was located on chromosome 1, *HvPAL 6* on chromosome 2, and *HvPAL2*, *HvPAL5*, *HvPAL3*, and *HvPAL4* were clustered on chromosome 2. *HvPAL7* is located on chromosome 3, whereas *HvPAL10*, *HvPAL9*, and *HvPAL1* are located on chromosome 6. Further scrutiny of the gene orientations revealed that *HvPAL6*, *HvPAL5*, *HvPAL3*, *HvPAL4*, and *HvPAL9* were oriented in the forward (F) direction, whereas *HvPAL8*, *HvPAL2*, *HvPAL7*, *HvPAL10*, and *HvPAL1* were oriented in the reverse (R) direction (Table 1).

Table 1. PAL gene family information for 10 non-redundant genes discovered in the barely genome. II: Instability Index, pl: Isoelectric point, GRAVY: Grand Average of Hydropathy, Pep: Peptide, Mw: Molecular Weight

Phytozome ID	Rename	PAC	MW	pI	II	GRAVY	Strand	CDS	Pep	no	Start	End	Length
HORVU0Hr1G016330	*HvPAL1*	PAC:38336220	77120.32	5.89	31.42	-0.073	R	2148	715	6	87346259	87350619	249774706
HORVU2Hr1G062750	*HvPAL2*	PAC:38347142	19615.19	9.38	43.54	-0.382	R	2688	184	2	423031718	423032845	768075024
HORVU2Hr1G089440	*HvPAL3*	PAC:38350661	77059.81	5.81	30.17	-0.139	F	2142	713	2	638986924	639231515	768075024
HORVU2Hr1G038140	*HvPAL4*	PAC:38359917	78012.73	6.14	30.27	-0.052	F	2172	723	2	177374427	177380781	768075024
HORVU2Hr1G089540	*HvPAL5*	PAC:38370457	49984.35	6.15	27.09	-0.075	F	1410	469	2	639339734	639342347	768075024
HORVU2Hr1G038120	*HvPAL6*	PAC:38375695	13647.51	9.97	61.39	-0.585	F	2142	127	2	176958153	176972622	768075024
HORVU3Hr1G005330	*HvPAL7*	PAC:38386390	74608.01	6.12	33.5	0.058	R	2073	690	3	13107104	13112423	699711114
HORVU1Hr1G022060	*HvPAL8*	PAC:38460212	76035.84	5.89	30.98	-0.078	R	2124	707	1	91840018	91842898	558535432
HORVU6Hr1G058840	*HvPAL9*	PAC:38501338	76716.88	5.77	36.31	-0.053	F	2139	712	6	386020333	386023043	583380513
HORVU6Hr1G058820	*HvPAL10*	PAC:38507371	75619.36	5.89	34.77	-0.063	R	2115	704	6	385750043	385753866	583380513

Analysis of the subcellular localization of the ten *HvPAL* genes revealed diverse distribution patterns. Notably, 36.75% of the proteins were identified in the chloroplast, 18.37% in the cytoplasm, and 11.31% in the endoplasmic reticulum. Additionally, 9.19% were associated with vacuoles, suggesting their potential role in cellular storage. Mitochondria and the nucleus accommodated 7.07% and 6.01% of the proteins, respectively, whereas 0.71% was found extracellularly. The Cysk_Nucl compartment constituted 2.83% of the total, indicating nuclear localization of cysteine-rich proteins. These results highlight the varied subcellular roles played by HvPAL proteins in plant cells (Figure 1).

Conserved Cis elements

Analysis of cis elements in the promoter regions of 10 *HvPAL* genes revealed a diverse array of regulatory motifs governing their transcriptional control. Notably, the TATA-box motif was consistently present across all the genes, constituting 19% of the identified motifs. CAAT-box, another prevalent motif, represented 23% of the total motifs. Distinct motifs, such as the MYB recognition site, MYC, and W box, exhibited varying frequencies, suggesting potential roles in gene regulation. A stress-responsive STRE motif was identified in 3% of the motifs. This comprehensive cis-element analysis revealed key motifs associated with stress responses and plant defence mechanisms in the *HvPAL* gene family. The CAAT-box, occurring 123 times, has emerged as a predominant cis-acting element, emphasizing its significance in regulating *HvPAL* expression during stress (Sami, et al., 2023). Additionally, the presence of elements such as the W box, implicated in responding to pathogen attacks, and the STRE motif further highlighted the involvement of genes in stress-responsive pathways. Noteworthy occurrences of Myb, MYB-like sequence, and WRE3 motifs, which are associated with transcriptional responses to drought and low oxygen, provided additional insights (Figure 2).

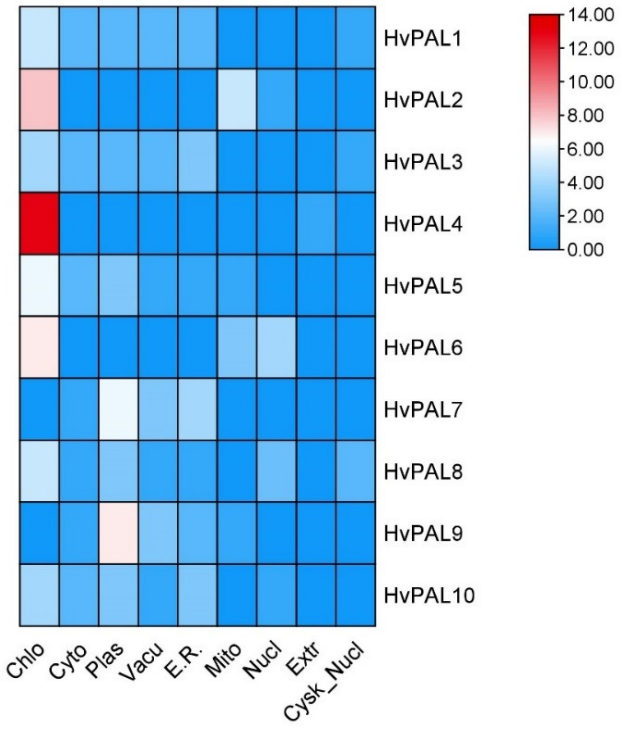

Figure 1. This heat map shows the distribution of 10 *HvPAL* genes, within parts of a plant cell, such as the nucleus, cytoplasm, chloroplast, Golgi apparatus, mitochondria, plasmid and peroxisomes. The intensity of color indicates a functional presence of the gene in that specific region while red color signifies a higher functional significance in that area

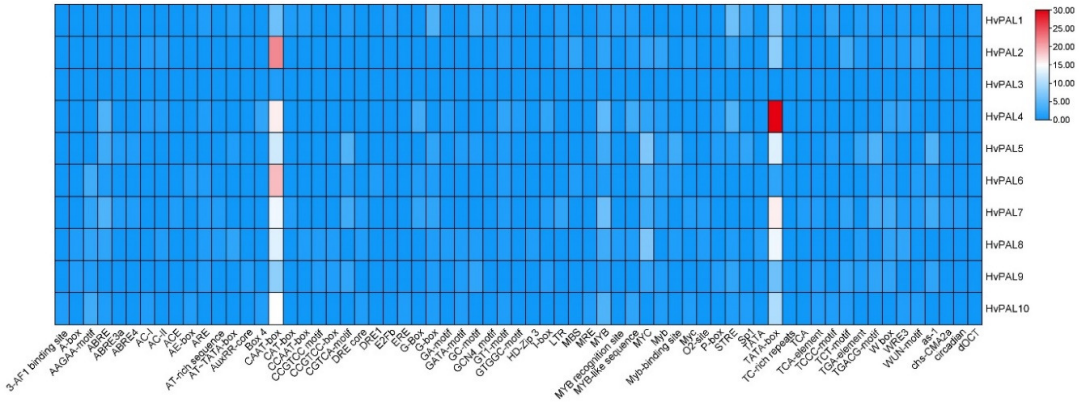

Figure 2. The visual depiction of the *HvPAL* gene's cis regulatory analysis along with the strength of each function. From red (highest) to the white (lowest) intensity is used to describe the biochemical and physiological activities happening in plants

Conserved motif analysis and domain prediction

During our analysis of conserved motifs among the 10 *HvPAL* genes, it was evident that motif 5 was uniformly conserved across the entire gene set. Notably, Motif 7 was conserved in all genes except *HvPAL4*. Remarkably, *HvPAL1*, *HvPAL3*, *HvPAL7*, *HvPAL8*, *HvPAL9*, and *HvPAL10* were conserved for all motifs (1-10), indicating a comprehensive shared motif pattern. In contrast, *HvPAL2* and *HvPAL6* displayed a more restricted motif profile, encompassing only Motifs 2 and 5. This distinctive motif composition in *HvPAL2* and

HvPAL6 implies the presence of unique regulatory elements or functional characteristics that set them apart from other *HvPAL* genes (Figure 3).

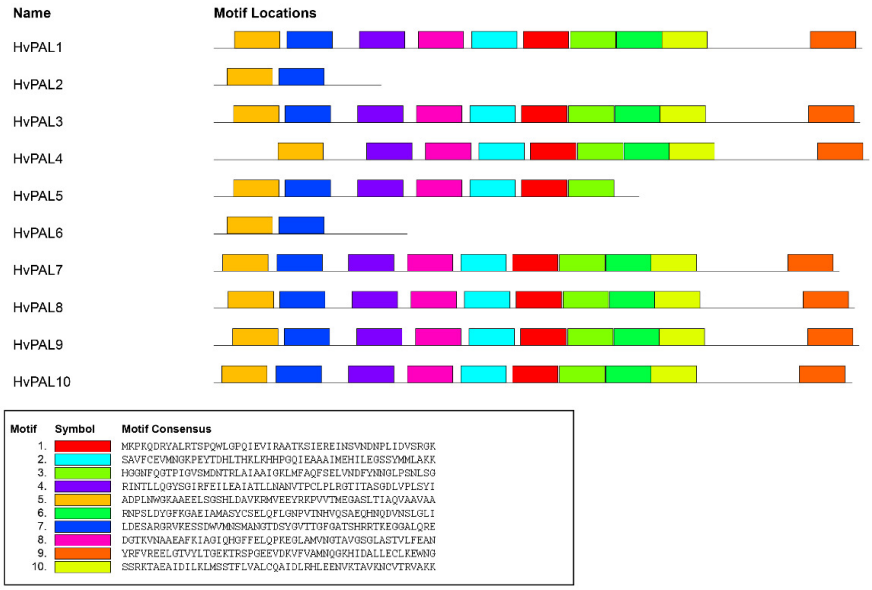

Figure 3. The distribution of 10 motifs along the 10 members of the barley *PAL* protein family

Our conserved domain analysis across the 10 *HvPAL* genes revealed remarkable consistency, as a singular domain, identified as the Lyase_I_like superfamily (Accession: cl00013), was present in all genes. Notably, *HvPAL1*, *HvPAL3*, *HvPAL4*, *HvPAL7*, *HvPAL8*, *HvPAL9*, and *HvPAL10* exhibited an additional conserved domain, PLN02457 (accession number: PLN02457), which belongs to the Lyase_I_like superfamily. This indicated a high degree of conservation of the Lyase_I_like superfamily domain across all 10 *HvPAL* genes (Figure 4).

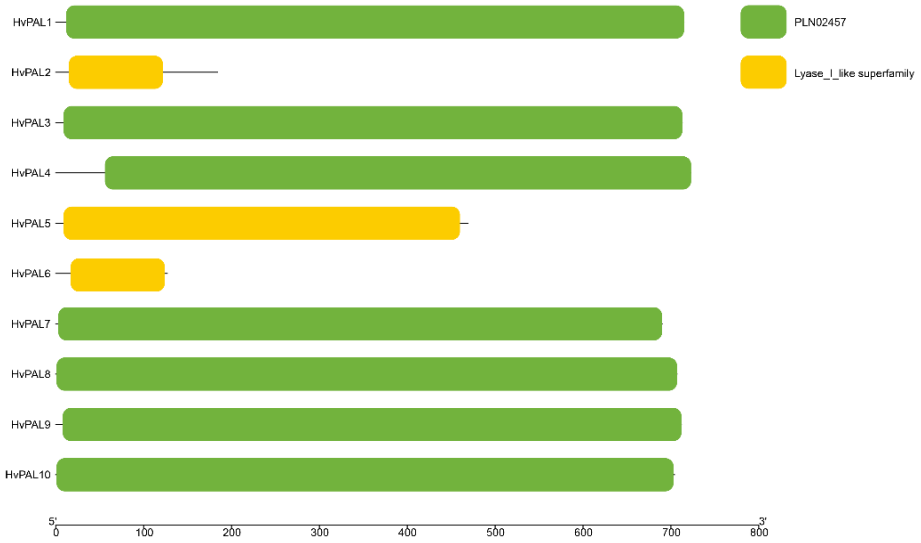

Figure 4. Illustration of the domain pattern of all *HvPAL* genes
The Lyase_I_like superfamily were conserved in all *HvPAL* genes

Exon-intron analysis

Distinct patterns emerged when the exon-intron structures of the *HvPAL* gene family were examined. *HvPAL2*, *HvPAL4*, *HvPAL8*, and *HvPAL9* had a unique profile featuring only a single exon and an absence of introns. In contrast, *HvPAL1*, *HvPAL3*, *HvPAL5*, *HvPAL7*, and *HvPAL10* shared a conserved structure marked by two exons and one intron. These observations highlight significant genomic variations within the *HvPAL* gene family, showing diversity in exon-intron organization (Figure 5).

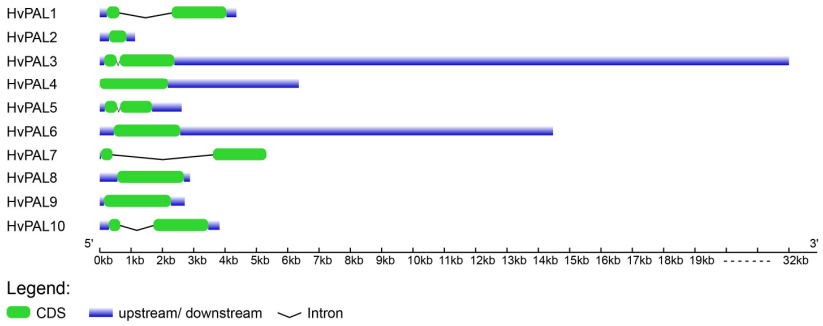

Figure 5. The exon-intron structure is phylogenetically represented, green color represents exons and black line show introns

Analysis of the HvPAL gene family's phylogeny

In the conserved phylogenetic analysis, PAL genes from four plant species, namely *O. sativa*, *H. vulgare* L, *A. thaliana*, and *S. bicolor*, were systematically categorized into four distinct clades labeled I-IV. The study included 34 PAL genes, including 10 from *O. sativa*, 10 from *H. vulgare*, 5 from *A. thaliana*, and 9 from *S. bicolor*. To enhance clarity and facilitate a comprehensive understanding of phylogenetic relationships, each clade is denoted by a specific color scheme. Clade I is represented by the color red, clade II by yellow, clade III by green, and clade IV by blue Figure 6.

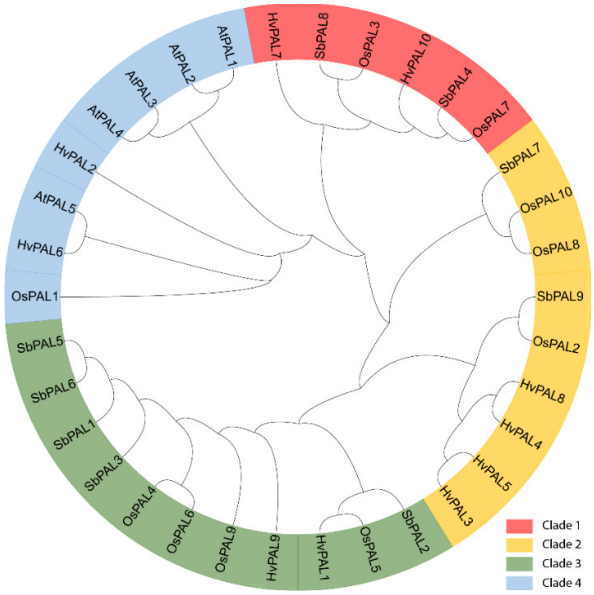

Figure 6: Phylogenetic analysis illustrates that PAL genes from four plant species, namely *Oryza sativa, Hordeum vulgare* L, *Arabidopsis thaliana*, and *Sorghum bicolor*, were systematically categorized into four distinct clades labeled I-IV

MicroRNA (miRNA) analysis

miRNA analysis of *HvPAL* genes uncovered a spectrum of interactions with distinct miRNAs, each characterized by varying lengths. The shortest miRNA, miRNA1436, spanned positions 1 to 21, while the longest, miR5048a, extended from positions 1 to 22. Notably, miR6210 targeting *HvPAL* 5 exhibited a minimum cleavage inhibition value of 4 and a UPE of -1. In contrast, a maximum cleavage inhibition expectation value of 5 was observed for multiple miRNAs targeting *HvPAL5*, *HvPAL8*, *HvPAL1*, *HvPAL4*, and *HvPAL9*. Translation inhibition events resulting in cleavage processes ranged from 1, indicating a single cleavage event. Specifically, miR6210 targeted *HvPAL5*, miR1436 targeted *HvPAL3*, miR6189 targeted *HvPAL4* and *HvPAL6*, miR5048a and miR5048b targeted *HvPAL9*, miR5050 targeted *HvPAL5*, miR6178 targeted *HvPAL8*, miR6189 targeted *HvPAL8* and *HvPAL1*, and miR6192 and miR6198 targeted *HvPAL4* and *HvPAL* 9, respectively.

PAL gene duplication and Synteny analysis

The chromosomal distribution of PAL genes indicates their dispersion across chromosomes. PAL genes were identified on chromosomes 1, 2, 3, and 6. Notably, the majority of PAL genes are located on chromosome 3, encompassing *HvPAL* 2, *HvPAL* 3, *HvPAL* 4, *HvPAL* 5, and *HvPAL* 6 (Irfan, et al., 2023). Chromosomes 1 and 3 harbored *HvPAL7* and *HvPAL8*, respectively. *HvPAL1*, *HvPAL9*, and *HvPAL10* were localized on chromosome 6 (Figure 7).

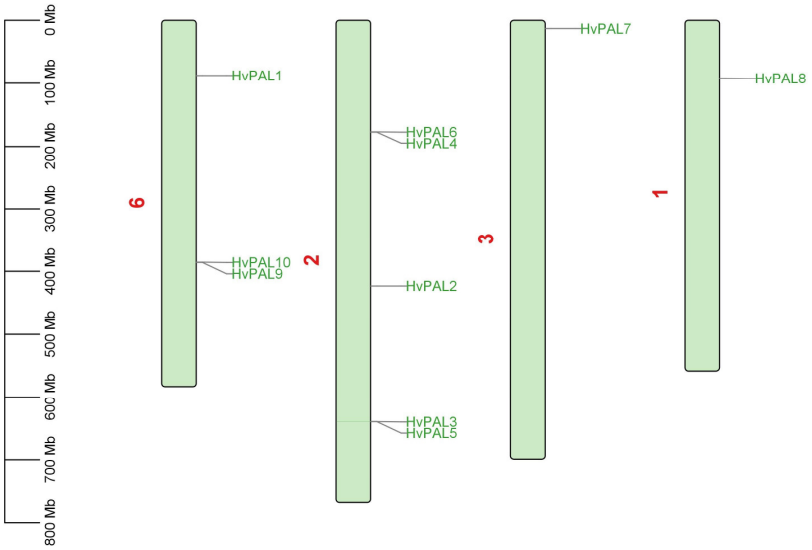

Figure 7. The chromosomal distribution of PAL genes indicates their dispersion across different chromosomes

Ka/Ks analysis identified *HvPAL1_HvPAL6* and *HvPAL6_HvPAL10* as having elevated values (4.497 and 3.032, respectively). In contrast, *HvPAL3_HvPAL5* and *HvPAL2_HvPAL4* had lower Ka/Ks values (0.014 and 0.037, respectively). The divergence time estimation, measured in MYA, supports these findings, with lower MYA values for *HvPAL3_HvPAL5* and *HvPAL2_HvPAL4*, implying more recent divergence and higher MYA values for *HvPAL* 1_*HvPAL6* and *HvPAL6_HvPAL10*, indicating ancient divergence from a common ancestor (Figure 8).

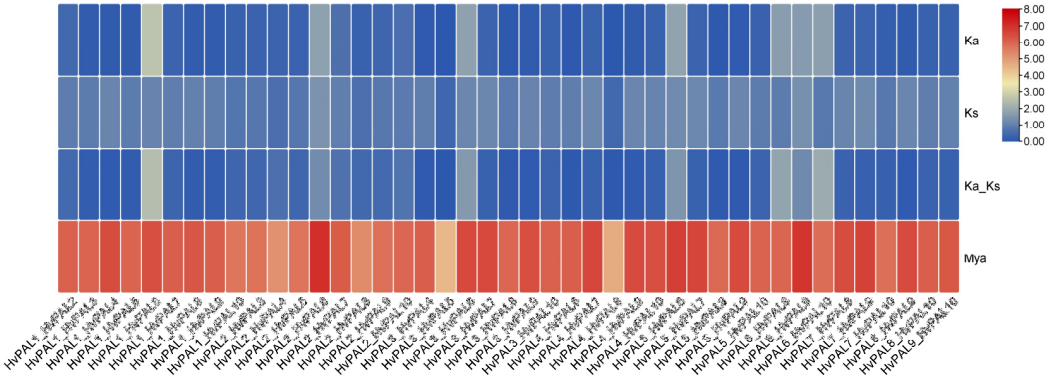

Figure 8. The expression ka/ks represents the ratio of mutations involving synonymous substitutions (ks) to mutations involving non-synonymous substitutions (ka). The gene duplication over selection and evolutionary pressure to paralogous pairings of barley's PAL genes were calculated based on ks and ka values

The syntenic analysis reveals segmental duplications among *HvPAL* genes, such as *HvPAL* 7 (chromosome 3) paralog with *HvPAL8* (chromosome 1), *HvPAL7* (chromosome 3) paralog with *HvPAL9* (chromosome 6), *HvPAL8* (chromosome 1) paralog with *HvPAL10* (chromosome 10), *HvPAL8* (chromosome 1) paralog with *HvPAL4* (chromosome 2), and *HvPAL6* (chromosome 2) and *HvPAL7* (chromosome 3) paralog with *HvPAL4* (chromosome 2) and *HvPAL6* (chromosome 2), indicating segmental duplications. Additionally, *HvPAL5* paralogs with *HvPAL3* and *HvPAL4* paralogs with *HvPAL6*, both exhibiting duplication on chromosome 2, suggest tandem duplication (Sharma *et al*., 2019). Furthermore, the *HvPAL9* and *HvPAL9* paralogs on chromosome 6 also indicated tandem duplication events (Figure 9A).

In the analysis of syntenic relationships between *Arabidopsis* and barley, a segment duplication occurred on chromosome 2, involving the *HvPAL2* and *HvPAL6* genes, with the AtPAL2 gene identified on chromosome 3. Similarly, in a comparison between maize and barley, the *HvPAL9* gene on chromosome 6 underwent duplication events with maize genes Zm00001d003015_T001.RefGen_V4 (chromosome 2), Zm00001d051163_T001.RefGen_V4 (chromosome 4) and Zm00001d017275_T001.RefGen_V4 (chromosome 5) Figure 9B.

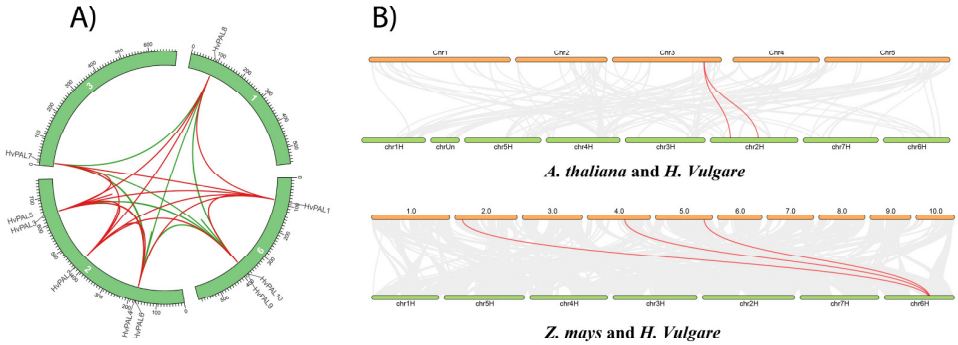

Figure 9A. Genes with structurally similar sequence share conserved areas, according to an in-depth study of genome-wide synteny in barley PAL genes. The technique gives a visual representation of the degree of similarity through genomic regions using pink and green shades. Gene duplication is a result of an array of dynamic mechanisms and genomic rearrangements that allow the genes to remain stable secure, adapt to their environment, and get new properties over time. **Figure 9B.** Illustrate the segmental duplication of *Arabidopsis* and barley, maize and barley

GO and orthologue analyses

The GO enrichment analysis performed in this study provides insights into the roles of the *HvPAL* genes. Specifically, these genes were found to play a role in acid biosynthesis (GO: 0009800), phenylalanine ammonia lyase (GO: 0045548), and the metabolic process of amino acids belonging to the erythrose 4 phosphate/phosphoenolpyruvate family (GO: 1902223) (Figure 10).

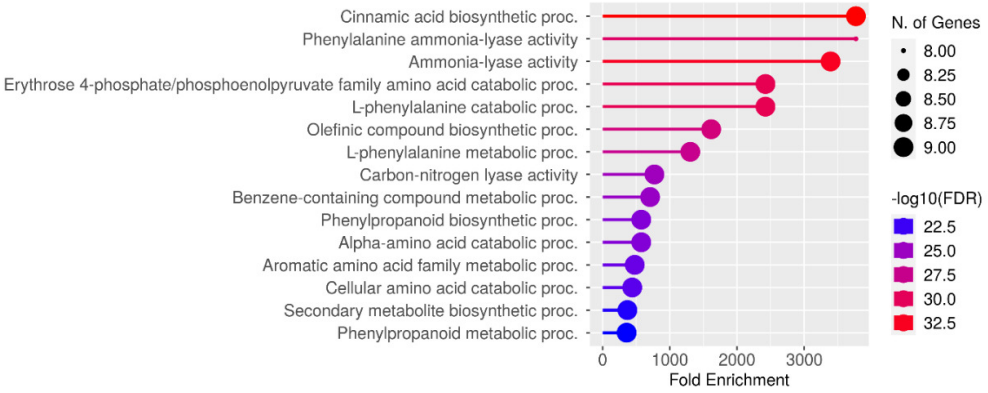

Figure 10. The conducted Go enrichment analysis in this study offered valuable insights into the functional roles of *HvPAL* genes, with red indicating high gene function values and blue representing low gene function values

Protein-protein interaction

During the protein-protein interaction investigation, a total of five nodes and one edge were observed. The calculated average node degree is 0.4, accompanied by an average local clustering coefficient of 0.4. Notably, the expected number of edges in this context was zero, and the p-value for protein-protein interaction enrichment was remarkably low at 0.0567, indicating a significant enrichment of interactions. A low confidence threshold of 0.150 was applied to satisfy the minimum required interaction score. Specifically, *HvPAL* was found to interact with *HvPAL10*, whereas the remaining proteins showed no association within themselves or with other proteins (Figure 11).

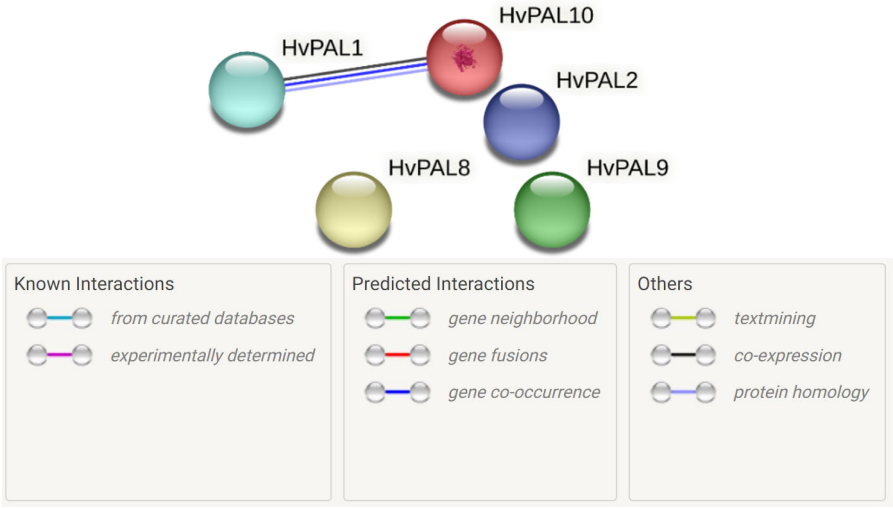

Figure 11. Illustration of Protein-Protein Interaction of *HvPAL* Genes. Only 2 *HvPAL* genes (*HvPAL1* and *HvPAL10*) show a significant interaction

Transcriptomic analysis of 23 races of Puccinia graminis f. sp. tritici

In a comprehensive analysis aimed at understanding the intricate interactions between barley and various races of *Puccinia graminis* f. sp. *tritici*, we examined the expression patterns of 10 PAL genes (phenylalanine ammonia lyase), designated as *HvPAL1* through *HvPAL10*. These genes were scrutinized in response to different races of the pathogen, including WM-1, R29M, R29JB, R29JA, R11c, QCCJ, P84-16, AC-12, A-5, A-48, A-21, A-15, A-14, A-12, 81AC46, 81AC34, 81AC28, 79_20, 79.2, 79-1, 72_00, 640C, and 370C. Notably, *HvPAL1* and *HvPAL10* showed robust upregulation across all tested races, indicating their pivotal role as universal responders in barley defense mechanisms. In contrast, *HvPAL7* remained unresponsive, showing no changes in expression levels compared with the control and tested races. *HvPAL4*, however, consistently exhibited downregulation in all races. Among the remaining PAL genes, we observed a different interplay between upregulation and downregulation. *HvPAL2*, *HvPAL3*, and HvNPR8 were also highly significant, whereas *HvPAL5*, *HvPAL6*, and *HvPAL9* were consistently downregulated (Figure 12).

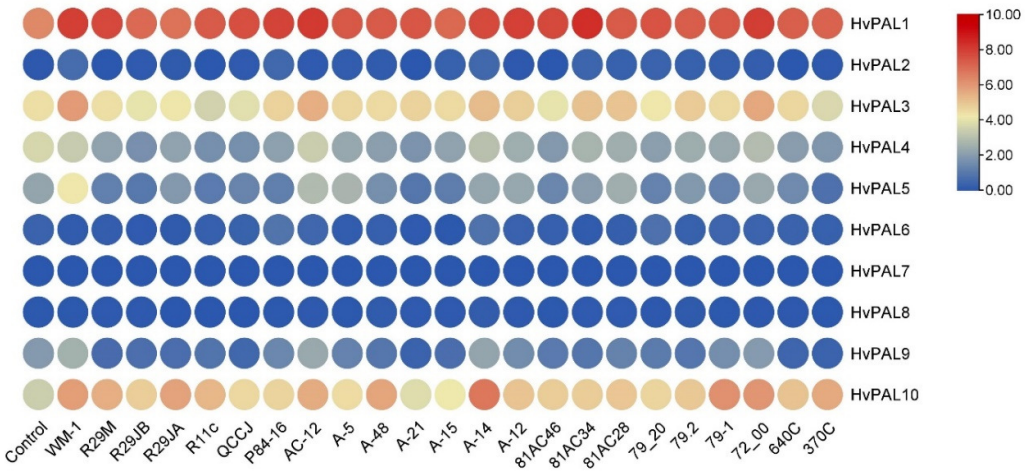

Figure 12. Expression patterns of 10 PAL genes (phenylalanine ammonia lyase), designated as *HvPAL1* through *HvPAL10*. These genes were scrutinized in response to different races of the pathogen, including WM-1, R29M, R29JB, R29JA, R11c, QCCJ, P84-16, AC-12, A-5, A-48, A-21, A-15, A-14, A-12, 81AC46, 81AC34, 81AC28, 79_20, 79.2, 79-1, 72_00, 640C, red indicating high gene expression and blue representing low gene expression

Discussion

Barley, which is a cereal crop worldwide, plays a vital role in both agricultural production and economic progress. When faced with pressures, such as metals, salinity, and drought, barley demonstrates increased sensitivity that leads to responses beyond the usual context of reactive oxygen species (ROS) production (Grando and Macpherson, 2005). To protect itself from attacks and other threats, barley relies on the activation of ammonia lyase (PAL) genes, which are triggered by stress (Patel *et al*., 2020). PAL, which is an enzyme, in the pathway plays a significant role in producing secondary metabolites, like phytoalexins and lignin precursors. These compounds are well-known for their properties (Yadav *et al*., 2020). This defensive arsenal showcases barley's adaptive strategies to combat biotic stressors, reflecting the intricate interplay between PAL gene expression and overall resistance of the plant, contributing to its survival and productivity (Abd El Moneim *et al*., 2023). A genome-wide investigation was conducted to identify and understand PAL genes in barley. The physicochemical properties of ten *HvPAL* genes in the barley genome were investigated to observe

their distinctions within a clade of proteins (Walker, 2012). All the *HvPAL* proteins that were identified showed hydrophilic properties, which means that they have a tendency to interact with water and their electrical charges depend on pl levels. This can be observed in the GRAVY values (Ju, 1994). Upon examination using the instability index, it was observed that eight distinct proteins possessed features suggestive of stability, and the remaining two proteins were suggestive of instability (Table 1).

Investigation into where *HvPAL* proteins are located within cells revealed that they can be found in organelles, including the chloroplast mitochondria, cytoplasm, cytosol, endoplasmic reticulum, nucleus, and plasma membrane. It is important to note that 36.75% of the proteins were in the chloroplast, 18.37% in the cytoplasm, and 11.31% in the endoplasmic reticulum. This suggests that *HvPAL* proteins may play vital roles within these organelles (Figure 1). Upon scrutiny of conserved motifs among *HvPAL* genes, a consistent presence of Motif 5 was identified throughout the entire gene set, underscoring its importance. The shared conservation of all motifs (1-10) among *HvPAL* 1, *HvPAL3*, *HvPAL7*, *HvPAL8*, *HvPAL9*, and *HvPAL10* is particularly noteworthy, reflecting a comprehensive motif pattern. In contrast, *HvPAL2* and *HvPAL6* exhibited a limited motif profile, with only Motif 2 and Motif 5. This distinctive motif composition in *HvPAL2* and *HvPAL6* implies unique regulatory elements or functional characteristics that potentially contribute to their specific roles in the defense mechanisms of barley (Owji and Hemmati, 2018) Figure 3. In the analysis of conserved domains among the ten *HvPAL* genes, a predominant Lyase_I_like superfamily domain (Accession: cl00013) was consistently identified in all genes. Additionally, *HvPAL1*, *HvPAL3*, *HvPAL4*, *HvPAL7*, *HvPAL8*, *HvPAL9*, and *HvPAL10* had an additional conserved domain, PLN02457 (Accession: PLN02457), within the Lyase_I_like superfamily. This high degree of conservation in the Lyase_I_like superfamily domain across all *HvPAL* genes suggests its integral role in defense mechanisms in barley (Zhang *et al*., 2023) Figure 4.

Through the examination of the genomes of diverse species, a profound understanding of gene history and arrangement has emerged. This analysis not only facilitates the transfer of genomic data from well-researched taxonomic groups to less-explored ones but also provides valuable insights into gene duplication within *HvPAL* (Adams and Wendel, 2005). The detection of 34 paralogous genes in this study signifies the process of gene replication through duplication events. Such duplications offer crucial knowledge on the expansion of gene families, a phenomenon prevalent in plants, through tandem and segmental duplications (Li *et al*., 2019). Comparative analysis of PAL proteins within similar clades provides significant insights into their functional behavior (Guo *et al*., 2019). In this study, 34 proteins known as PAL proteins were classified into four groups according to their sequence structures and evolutionary connections. Notably, *HvPAL2* and *HvPAL6* fell within clade IV alongside AtPAL, indicating functional similarities with AtPAL. In contrast, the remaining proteins in Arabidopsis exhibited functional deviations from their counterparts in (Figure 6). Previous research has proposed that the arrangement of exons and introns within gene families is significant in the context of evolution (Lam, 1996). The study found that the arrangement of exons, introns, and motifs within a population and clade was consistent with the tree structure (Haider *et al*., 2023). Notably, four PAL genes exclusively featured exons that lacked introns, whereas the remaining genes exhibited a more intricate gene structure with two exons and a solitary intron. The presence of two exons and one intron suggests a more complex gene architecture in which introns play pivotal roles in gene regulation, alternative splicing, and the emergence of novel genes (Mattick, 1994). A single intron between exons provides a platform for diverse regulatory elements and alternative splicing events, fostering the diversity of gene products. Moreover, insufficient exon length may affect the functionality of the encoded protein or RNA molecules, potentially imposing functional constraints. Adequate exon length is crucial for proper protein folding and functionality, and abbreviated exons may curtail a protein's capacity to fulfill its intended biological functions (Schluttenhofer, 2011; Figure. 5).

Cis-regulatory elements crucially dictate gene expression at the transcriptional level, often residing in the gene promoter region (Haberle and Lenhard, 2016). Analysis of 10 *HvPAL* genes revealed significant regulatory motifs, with TATA-box and CAAT-box being prevalent, constituting 19% and 23%, respectively. Noteworthy elements contributing to plant defense mechanisms include the CAAT-box, which recurred 123 times, suggesting its vital role in *HvPAL* regulation during stress. The W box, which is associated with plant gene induction in response to pathogen attacks, and the stress-responsive STRE motif occurred 12 and 17 times, respectively. Moreover, Myb, MYB-like sequence, and WRE3 motifs (Ain-Ali *et al*., 2021), linked to transcription binding sites activated during drought and low oxygen conditions, appeared eight times each. These findings underscore the potential involvement of *HvPAL* genes in the stress response and plant defense, revealing the intricate regulatory network orchestrating their expression under challenging environmental conditions (Figure 2). The results of GO enrichment analysis showed that *HvPAL* genes play a crucial functional role of particular significance because these genes were discovered to be key contributors in terms of the biosynthesis of cinnamic acid, demonstrating their direct involvement in this important metabolic pathway (Mavandad *et al*., 1990). Furthermore, because they also have a link to phenylalanine ammonia-lyase activity, it becomes clear that the three are equally important in providing catalytic power for sugar production (Rasool *et al*., 2021). The link to the erythrose-4-phosphate phosphoenolpyruvate family of amino acid metabolism brings home again their position in the larger overall system for metabolizing all substances (Calero and Nikel, 2019) Figure 10.

Genetic mapping shows how PAL genes are arranged along the barley genome, which shows that they occur on several different chromosomes (Li *et al*., 1999). Such a distribution reflects the complexity and diversity of PAL genes. PAL genes are concentrated on certain chromosomes, particularly on chromosome 3. Knowledge of the chromosomal localization of PAL genes is particularly important for understanding their functional value, regulatory mechanisms, and evolutionary processes in the barley genome (Zhan *et al*., 2022). This spatial arrangement might facilitate coordinated responses to a variety of environmental stimuli, helping one understand (Pervaiz *et al*., 2017) that gene mapping is important for understanding the organization and function of genes in barley (Figure 7). Ka/Ks analysis revealed higher nonsynonymous substitution rates for *HvPAL1_HvPAL6* and *HvPAL6_HvPAL* 10, suggesting potential positive selection or adaptive evolution. In contrast, *HvPAL* 3_*HvPAL5* and *HvPAL2_HvPAL4* had lower Ka/Ks values, indicating conservation or selective pressure. Divergence time estimation supported these findings, with lower MYA values for *HvPAL3_HvPAL5* and *HvPAL2_HvPAL4*, indicating recent divergence, and higher MYA values for *HvPAL1_HvPAL6* and *HvPAL6_HvPAL10*, suggesting ancient divergence from a common ancestor (Wang *et al*., 2019) (Figure 8). Syntenic analysis showed that *HvPAL* genes are involved not only in segmental duplications but also in tandem duplications. These data revealed on the evolutionary mechanisms that shape the barley PAL gene family (Rasool *et al*., 2021). For instance, the *HvPAL7* paralog with *HvPAL8* belongs to one type of segmental duplication in which the same family expands across different chromosomes, and there are other sets such as the *HvPAL7* paralog andHNPV4. Tandem duplications were found in *HvPAL5* and *HvPAL3* on chromosome 2; they share a progenitor but have undergone separate mutations. The two subgroups show opposite homology alignments, which is of critical importance for understanding the genomic structure, evolutionary environment, and functional characteristics of duplicated PAL-like genes. This study provides new insights into how barley adapts to abiotic stresses (Cass *et al*., 2015) Figure 9.

MicroRNAs (miRNAs) play a pivotal role in regulating diverse biological processes in plants, influencing growth, development, and responses to various stresses (Djami-Tchatchou *et al*., 2017). miRNA analysis of *HvPAL* genes revealed specific interactions with crucial miRNAs associated with plant defence mechanisms. Notably, miR6210 exhibited a minimum cleavage inhibition expectation value of 4 and targeted *HvPAL5*, indicating a significant regulatory role in defense responses. Multiple miRNAs, including miR5048a and miR5048b, with a maximum cleavage inhibition expectation value of 5, targeted *HvPAL* genes (*HvPAL5*,

HvPAL8, *HvPAL1*, *HvPAL4*, and *HvPAL* 9), emphasizing their involvement in fine-tuning defense-related pathways (Sun, 2012). In addition, miR6189 demonstrated significant regulatory activity by targeting *HvPAL4* and *HvPAL6*. This investigation highlights the intricate regulatory network orchestrated by miRNAs in modulating *HvPAL* genes, specifically influencing plant defense mechanisms against biotic challenges. In the protein interaction study involving five nodes and one edge, *HvPAL* exhibited a notable interaction with *HvPAL10*, suggesting a potential role in plant defense mechanisms. The remarkably low p-value of 0.0567 underscores the significant enrichment of interactions, supporting the relevance of these proteins in functional networks (Murale *et al*., 2016). However, the absence of associations among the remaining proteins or with other proteins raises questions about their direct involvement in plant defense mechanisms (De and Damme, 2015), emphasizing the unique importance of *HvPAL* and *HvPAL10* in the context of protein-protein interactions related to defense responses (Figure 11).

Gene expression analysis revealed distinct patterns in response to pathogenic races, highlighting the key players in barley defense mechanisms. *HvPAL1* and *HvPAL10* exhibited robust upregulation across all races, indicating their universal roles as responders to pathogenic threats. In contrast, *HvPAL7* remained unresponsive, suggesting its limited involvement in the tested conditions. *HvPAL4* consistently displayed downregulation in all races, indicating a potential regulatory role in negative defense responses (Sharma *et al*., 2019). Notably, *HvPAL2*, *HvPAL3*, and HvNPR8 were significantly upregulated, whereas *HvPAL5*, *HvPAL6*, and *HvPAL9* were consistently downregulated, suggesting a nuanced and diverse regulatory network governing the expression of PAL genes in response to pathogenic challenges (Chen *et al*., 2023) Figure 12. Enhancing the expression of *HvPAL5*, *HvPAL6*, and *HvPAL9* is a strategic approach for developing barley varieties resistant to *Puccinia graminis* f. sp. *tritici*. Gene expression analysis consistently showed downregulation of these genes across all tested races, highlighting their potential as pivotal components in the defense mechanism of barley against the pathogen. By manipulating the expression of *HvPAL5*, *HvPAL6*, and *HvPAL9*, barley plant resistance to *Puccinia graminis* f. sp. *tritici* can be improved, contributing to the creation of more robust and disease-resistant barley varieties. These discoveries do not enhance our knowledge of the functions and evolutionary aspects of the PAL gene family in plants, but they also offer valuable insights for future research. This will aid in gene cloning and examination. The extensive genome-wide identification and analysis performed in this study lays the groundwork for future investigations.

Conclusions

This study identified 10 PAL genes (*HvPAL*) in barley, shedding light on their conserved sequences, motifs, and regulatory elements. This investigation underscores their role in biotic stress response, hormone signaling, and developmental processes through promoter analysis. MicroRNAs emerge as crucial regulators. RNA-seq data revealed nuanced expression patterns, with *HvPAL2*, *HvPAL3*, and HvNPR8 upregulated, and *HvPAL5*, *HvPAL* 6, and *HvPAL9* downregulated, particularly in response to 23 species of *Puccinia graminis* f. sp. *tritici*. These findings present opportunities for enhancing agronomic traits and developing pathogen-resistant barley varieties by modulating the expression of *HvPAL5*, *HvPAL6*, and *HvPAL9*. This study contributes to a deeper understanding of PAL gene versatility and will guide future genetic advancements in barley.

Authors' Contributions

The author read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

This study is supported via funding from Prince sattam bin Abdulaziz University project number (PSAU/2024/R/1445)

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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