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Genome-Wide Identification and Characterization of HvNPR1-like Genes Reveal HvNPR1 Interacting with HvWRKY70 in Barley Resistance to Leaf Stripe

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Abstract: Background: Barley leaf stripe disease, caused by the fungal pathogen Pyrenophora graminea, significantly reduces both grain yield and quality. Previous studies have demonstrated that NPR1-like proteins, as key regulators of salicylic acid (SA) signaling, play crucial roles in plant defense against fungal pathogens. However, systematic research on NPR1-like genes involved in barley leaf stripe resistance remains limited.Results: In this study, five HvNPR1-like family members were identified at the genome-wide level in barley. These genes were unevenly distributed across three chromosomes. Phylogenetic analysis based on Arabidopsis NPR1-like proteins classified the HvNPR1-Like proteins into three major groups (I, II, and III), which exhibited conserved features in terms of intron–exon organization. Synteny analysis revealed relatively few orthologous gene pairs between barley and Arabidopsis (one pair), whereas substantially more pairs were identified between barley and rice (four pairs), between barley and maize (five pairs), and between barley and wheat (twelve pairs), consistent with the phylogenetic relationships among these species.Transcriptome sequencing (RNA-seq) of barley varieties resistant and susceptible to leaf stripe disease identified three differentially expressed HvNPR1-like genes associated with the stress response. Among these, the Group I gene HvNPR1 was significantly up-regulated upon pathogen infection. Further analysis indicated that HvNPR1 played a pivotal role in the response to leaf stripe stress. Quantitative real-time PCR (qRT-PCR) analysis validated the expression patterns observed in RNA-seq data, with significantly higher transcript levels in the resistant cultivar compared to the susceptible cultivar. Protein–protein interaction assays demonstrated that HvNPR1 specifically interacted with HvWRKY70 in the nucleus, suggesting a potential regulatory module involved in defense signaling. These findings suggest strong regulatory interactions among these genes during the barley leaf stripe response. Conclusion: This study systematically identified and characterized HvNPR1-like family members in the barley genome through comprehensive analyses of gene structures, physicochemical properties, chromosomal localization, and evolutionary relationships. A key gene associated with leaf stripe resistance, HvNPR1, was identified, and a protein-protein interaction between HvNPR1 and the transcription factor HvWRKY70 was discovered. These findings provide a foundation for elucidating the roles of HvNPR1-likes in barley resistance to leaf stripe and offer potential targets for the genetic improvement of disease-resistant barley cultivars.

Keywords: NPR1; hulless barley ; Pyrenophora graminea

1 INTRODUCTION

Qingke (Tibetan hulless barley, Hordeum vulgare L. var. nudum Hook. f.), belonging to the genus Hordeum of the tribe Triticeae in the family Gramineae, is a variety of common barley. Due to the natural separation of its lemma and palea from the caryopsis at maturity, it is also known as hulless barley or naked barley,

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and is commonly referred to as "qingke" on the Qinghai-Tibetan Plateau (Liang, Peng, & Liu, 2011). Qingke is remarkably adapted to the extreme environmental conditions of the Qinghai-Tibetan Plateau, including high altitude, hypoxia, low temperature, drought, and intense ultraviolet radiation, and serves as a symbol of Tibetan agricultural civilization (Zeng et al., 2018). As a vital food, economic, and fodder crop on the plateau, qingke has been cultivated for approximately 3,500 years (Chen et al., 2015).

Barley leaf stripe, caused by the seed-borne fungus Pyrenophora graminea, is a systemic fungal disease and one of the most devastating diseases affecting qingke production (Guo et al., 2025). The disease is prevalent in major barley-growing regions worldwide, including Europe, Australia, the United States, Canada, as well as Northwest and Northeast China. In severe epidemic years, field incidence can exceed 60%, leading to yield losses of up to 73% (Arabi, & Jawhar, 2010). Infected plants exhibit pronounced symptoms on leaves, leaf sheaths, and stems, severely compromising both grain yield and quality (Yao et al., 2021b). To date, the molecular mechanisms underlying pathogen-host interactions during leaf stripe infection remain poorly understood. Through quantitative trait locus (QTL) analysis of resistant and susceptible crosses, Italian researchers mapped two resistance-related genes, Rdg1a and Rdg2a, to chromosomes 2HL and 7HS, respectively (Arru et al., 2002; Arru et al., 2003). Functional studies demonstrated that overexpression of these two genes significantly enhanced resistance to P. graminea in Mediterranean barley cultivars (Biselli et al., 2010; Biselli et al., 2013). Our previous transcriptome sequencing identified several additional genes associated with qingke leaf stripe resistance, including HvAGO1 and HvAGO2 (Yao et al., 2021a), and miRNA sequencing in the early stage identified hvu-miRNA-168-3p and others (Yao et al., 2021b).

NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1) is a master regulator of systemic acquired resistance (SAR) in plants, and its homologs (NPR1-like proteins) play pivotal roles in basal immunity and defense signal transduction (Dong, 2004; Pajerowska-Mukhtar et al., 2013). A defining structural feature of NPR1-like proteins is the presence of a highly conserved BTB/POZ domain and an hankering repeat domain, both of which mediate protein–protein interactions and regulate the expression of downstream pathogenesis-related (PR) genes (Cao et al., 1997; Kinkema, Fan, & Dong, 2000).Since the first NPR1 gene was isolated from Arabidopsis thaliana three decades ago, NPR1-like family members have been identified in a wide range of plant species (Cao et al., 1997; Liu et al., 2019; Maier et al., 2011). Accumulating evidence has demonstrated that NPR1-like proteins are crucial components of plant defense regulatory networks, particularly in response to biotic stresses such as fungal, bacterial, and viral infections (Backer et al., 2019; Kinkema, Fan, & Dong, 2000). In Arabidopsis, over-expression of AtNPR1 significantly enhances resistance to downy mildew (Hyaloperonospora parasitica) and black spot (Alternaria brassicicola) (Friedrich et al., 2001). AtNPR3 and AtNPR4 function as SA receptors that negatively regulate immune responses by modulating NPR1 protein stability (Fu et al., 2012). In rice, overexpression of OsNPR1/NH1 confers enhanced resistance to bacterial blight (Xanthomonas oryzae pv. oryzae) (Chern et al., 2005). In wheat, overexpression of TaNPR1 improves resistance to Fusarium head blight (Fusarium graminearum) (Makandar et al., 2006).With the completion of the Arabidopsis genome sequence, 5 AtNPR1 homologs were identified in the Arabidopsis genome, designated as AtNPR2, AtNPR3, AtNPR4, AtBOP2 (also known as AtNPR5), and AtBOP1 (also known as AtNPR6) (Khan et al., 2014; Liu et al., 2019). Phylogenetic analysis classified the AtNPR1-like gene family into three functionally distinct clades, each containing two members with functional redundancy. In the first clade, AtNPR1 and AtNPR2 function as SA receptors and act as transcriptional coactivators in plant immunity (Cao et al., 1997; Fu et al., 2012). In the second clade, AtNPR3 and AtNPR4 also serve as SA receptors but function as transcriptional corepressors in plant defense (Fu et al., 2012; Ding et al., 2018). In the third clade, AtBOP1 and AtBOP2 are involved in plant growth and development (Khan et al., 2014). Subsequently, an increasing number of NPR1-like members have been identified across various plant species. For example, 5 NPR1-like genes have been reported in rice, seven in maize, and 17 in bread wheat (Triticum aestivum), along with 5 in Triticum urartu, 12 in Triticum dicoccoides, and six in Aegilops tauschii through bioinformatics approaches (Liu et al., 2019).

With the completion of barley reference genome sequencing and annotation, an increasing number of genes responsive to both biotic and abiotic stresses have been identified (Wang et al., 2023; He et al., 2024). However, research on the NPR1 gene family in barley has primarily focused on individual members such as NPR1(Zhao et al., 2025), while a systematic investigation of the entire NPR1-like gene family remains lacking. Furthermore, the involvement of NPR1 genes in resistance to leaf stripe disease has not yet been reported. Therefore, systematic investigations into the NPR1-like gene family in barley leaf stripe resistance remain limited, and the underlying molecular mechanisms have yet to be elucidated. In this study, we performed a genome-wide identification of NPR1-like family members in barley and comprehensively analyzed their phylogeny relationships, gene structures, synteny, and expression profiles using bio-informatics approaches. Combined with RNA-seq and qRT-PCR validation, we screened for key genes responsive to leaf stripe stress, with HvNPR1 emerging as a candidate. Bimolecular fluorescence complementation (BiFC) assays confirmed the interaction between HvNPR1 and HvWRKY70, providing preliminary insights into the potential molecular mechanism by which HvNPR1 participates in defense regulation. These findings lay a theoretical foundation for further functional dissection of barley NPR1-like genes in leaf stripe resistance and offer new candidate gene resources for genetic improvement of disease-resistant barley cultivars.

2 Materials & Methods

2.1 Plant Materials and Barley Leaf Stripe Treatment

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The disease-resistant barley varieties 'Kunlun 14' and 'THIBAUT', along with the disease-susceptible barley variety 'Z1141', were provided by the Qinghai Academy of Agricultural and Forestry Sciences (Qinghai, China). Inoculation with P. graminea and subsequent sample collection were performed following the method described by Yao et al. (2021b).

2.2 Identification of Gene Family Members and Sequence Retrieval

The hidden Markov model (HMM) profile of the NPR1-like family (accession number: PF12313) was downloaded from the Pfam database (available online: https://www.ebi.ac.uk/interpro/entry/pfam/#table). Candidate barley sequences containing both BTB_POZ and ANKYR domains, along with their corresponding GTF annotation files, were retrieved from the Ensembl Plants database (available online: https://plants.ensembl.org/index.html). Structural domain validation was performed using the NCBI Conserved Domain Database (CDD; available online: https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). To further identify NPR1-like homologs in barley, BLAST searches (E-value ≤ $1e^{-4}$) were conducted against the whole-genome sequences of rice (available online: http://rice.uga.edu/), Arabidopsis thaliana (available online: https://www.arabidopsis.org/), wheat (available online: https://plants.ensembl.org/Triticum_aestivum/Info/Index), and maize (available online: https://plants.ensembl.org/Zea_mays/Info/Index). Sequences with severe domain deletions or redundant entries were removed.

2.3 Physicochemical Property and Subcellular Localization Analysis

Physicochemical properties of the five HvNPR1-like proteins, including amino acid length, molecular weight, theoretical isoelectric point (pI), instability index, and grand average of hydropathicity (GRAVY), were analyzed using the Expasy ProtParam online tool (available online: https://web.expasy.org/protparam/). Subcellular localization was predicted using the CELLO server (available online: http://cello.life.nctu.edu.tw/).

2.4 Phylogenetic Analysis

Phylogenetic analysis was conducted based on multiple sequence alignments generated by MUSCLE. A maximum-likelihood (ML) tree was constructed using MEGA 11 software with 1,000 bootstrap replicates to assess branch support.

2.5 Synteny Analysis and Chromosomal Localization

Synteny analysis between barley and Arabidopsis, rice, wheat, and maize was performed using MCscanX software to identify orthologous relationships of NPR1-like genes. Chromosomal positions of the five HvNPR1-like genes were extracted from the GTF annotation files, and chromosomal distribution maps were visualized using TBtools.

2.6 Gene Structure and Conserved Motif Analysis

Exon–intron structures of the 5 HvNPR1-like genes were extracted and visualized using TBtools based on GTF annotation files. Conserved motif prediction was performed using the MEME online suite (available online: https://meme-suite.org/meme/), with the number of motifs set to 10 and an optimal motif width ranging from 6 to 50 amino acids.

2.7 Promoter Cis-Acting Element Analysis

The 2,000 bp upstream sequences from the start codon of each HvNPR1-like gene were extracted as promoter regions. Cis-acting regulatory elements within these promoter sequences were predicted using the PlantCARE database (available online: http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), and the results were visualized using TBtools.

2.8 Screening of HvNPR1-Likes Associated with Barley Leaf Stripe

The relative expression of 5 HvNPR1-likes was obtained through transcriptome sequencing of Kunlun14 and Z1141 under leaf stripe stress and mapped using TBtools.

2.9 Expression of HvNPR1 Gene under Leaf Stripe Stress

Primer 5.0 was used to design primers for the HvNPR1 candidate gene, and TC139057 was used as the internal reference for qRT-PCR analysis (Yao et al., 2021a). Primer sequences are shown in Data S1. The P. graminea infestation treatment, RNA extraction, PCR reaction system, amplification conditions, and relative gene expression were calculated with reference to Yao et al. (2021a).

2.10 BiFC Assay for Validation of the HvNPR1–HvWRKY70 Interaction

Bimolecular fluorescence complementation (BiFC) assays were performed to validate the protein–protein interaction between HvNPR1 and HvWRKY70. The coding sequences of HvNPR1 and HvWRKY70 were cloned into the pSPYCE-35S and pSPYNE-35S vectors, respectively, generating HvNPR1-YFPC and HvWRKY70-YFPN fusion constructs. The recombinant plasmids were co-transformed into epidermal cells of Nicotiana benthamiana leaves via Agrobacterium tumefaciens-mediated infiltration. Yellow fluorescent protein (YFP) signals were observed using a laser scanning confocal microscope at 48–72 hours post-co-infiltration. Samples expressing YFPN or YFPC fusion proteins alone served as negative controls.

3 Results

3.1 Identification, Chromosomal Localization and Characterization of HvNPR1-Likes

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In this study, 5 candidate HvNPR1-likes were identified through genome-wide analysis, and they were named and classified based on the phylogenetic relationships within the Arabidopsis NPR1-like family. The physicochemical properties of the family members were analyzed (Table 1). The results showed that the five proteins ranged from 491 (HvNPR6) to 609 (HvNPR3) amino acids in length. The predicted molecular weights (MWs) ranged from 51.52 (HvNPR6) to 66.46 (HvNPR3) kDa, and the isoelectric points (pIs) ranged from 5.49 (HvNPR1) to 6.12 (HvNPR5). The predicted instability index (II) ranged from 43.42 (HvNPR5) to 47.07 (HvNPR3), and the grand average of hydropathicity (gravy) ranged from -0.007 (HvNPR5) to -0.226 (HvNPR3). Subcellular localization prediction results indicated that three of the NPR1-likes were localized in the nucleus. None of the proteins contained a signal peptide. Chromosomal localization analysis revealed that the 5 HvNPR1-likes were distributed on three chromosomes of barley (Figure 1). Among them, chromosome 3 harbored the highest number of HvNPR1-likes (3), while chromosomes 4 and 5 each contained only one (the lowest).

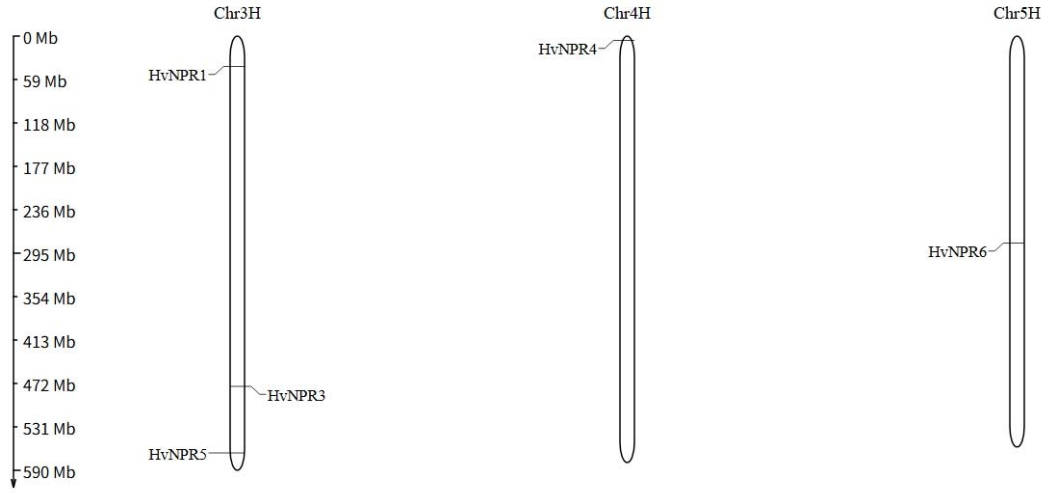

FIGURE 1 CHROMOSOME MAPPING OF CHROMOSOMAL LOCATION OF THE 5 HVNPR1-LIKES. THE GENES ARE LOCATED OVER THE THREE CHROMOSOMES.

TABLE 1 5 HVNPR1-LIKES GENE PHYSICAL AND CHEMICAL PROPERTIES.

Gene Name	Gene ID	Chromosome	Length (aa)	Mol. Weight (kDa)	pI	GRAVY	Instability Index	Signal Peptide	Subcellular Localization
NPR1	HORVU.MOREX.r3.3HG0236240.1	Chr3H	576	63.29	5.49	-0.160	47.00	No	Nuclear
NPR3	HORVU.MOREX.r3.3HG0291620.1	Chr3H	609	66.46	5.60	-0.266	47.07	No	Nuclear
NPR4	HORVU.MOREX.r3.4HG0333690.1	Chr4H	589	65.50	5.82	-0.216	45.41	No	Nuclear
NPR5	HORVU.MOREX.r3.3HG0319900.1	Chr3H	513	54.14	6.12	-0.007	43.42	No	Cytoplasmic
NPR6	HORVU.MOREX.r3.5HG0460940.1	Chr5H	491	51.52	6.11	-0.029	43.49	No	Cytoplasmic

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3.2 PHYLOGENETIC TREE CONSTRUCTION OF HVNPR1-LIKES PROTEINS

To investigate the evolutionary relationships among the HvNPR1-like proteins, a phylogenetic tree was constructed using 6 Arabidopsis NPR1-like proteins and a total of 17, 5, 5, and 7 NPR1-like proteins from wheat, barley, rice, and maize, respectively (Data S2; Figure 2). Based on the classification of the AtNPR1-like family, the 5 barley HvNPR1-like proteins were grouped into 3 clades: Group I, Group II, and Group III. Among them, one HvNPR1-like protein belonged to Group I, while the remaining four were evenly distributed between Group II and Group III.

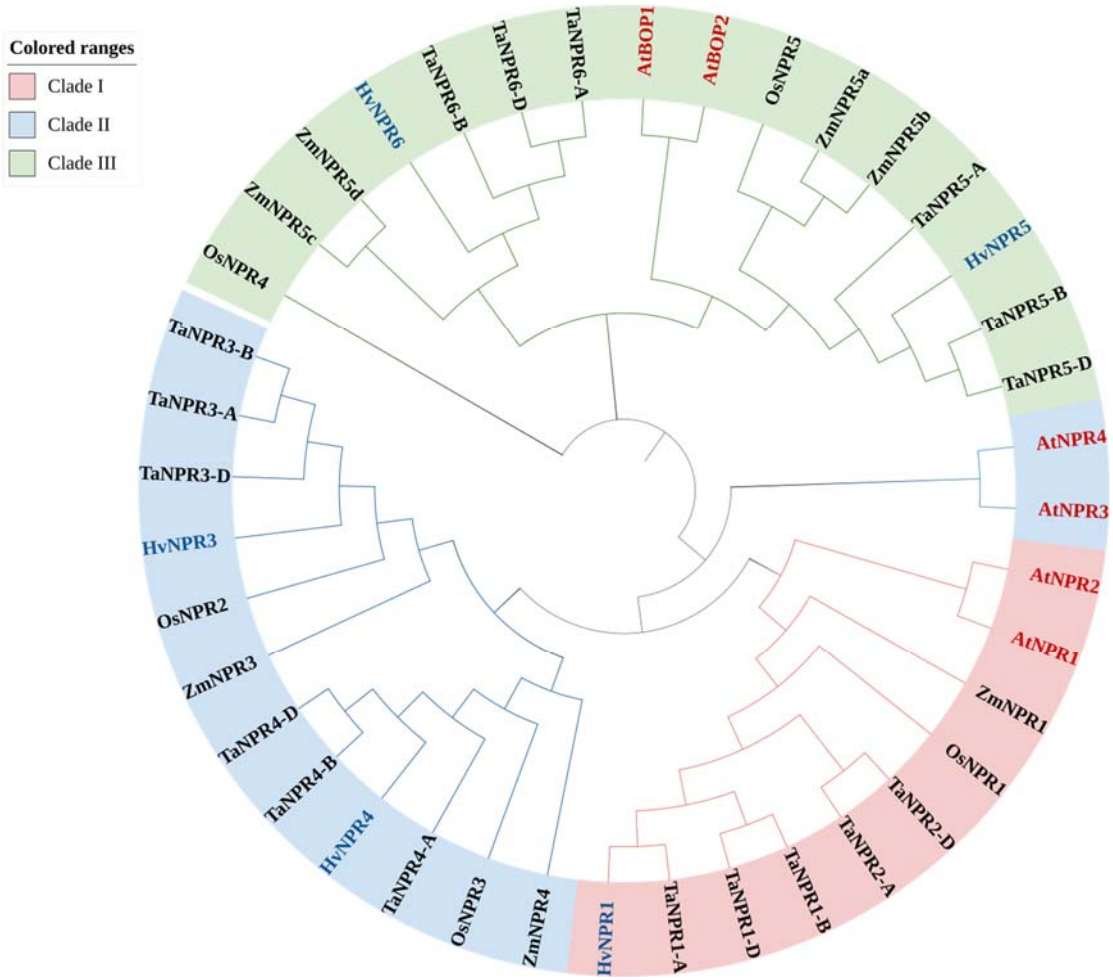

FIGURE 2 PHYLOGENETIC RELATIONSHIPS OF BARLEY, ARABIDOPSIS AND THREE GRASS FAMILY NPR1-LIKE PROTEINS. THE GENES IN BLUE INDICATE THAT THE NPR1-LIKE PROTEIN COMES FROM BARLEY; THE GENES IN RED INDICATE THAT THE NPR1-LIKE PROTEIN COMES FROM ARABIDOPSIS. PHYLOGENETIC TREE WAS CONSTRUCTED BY MEGA 11.0 USING THE NEIGHBOR JOINING (NJ) METHOD WITH 1000 BOOTSTRAP REPLICATIONS.

3.3 ANALYSIS OF COVARIANCE BETWEEN BARLEY AND ARABIDOPSIS, RICE, WHEAT, AND MAIZE NPR1-LIKE FAMILIES

To explore the evolutionary history of NPR1-like genes, we performed synteny analysis across the genomes of barley, Arabidopsis, rice, wheat, and maize (Figure 3). The results revealed only one pair of segmentally duplicated barley NPR1-like genes in the Arabidopsis genome, identified as HvNPR6 from Group III (Figure 3A). In contrast, 4, 12, and 5 pairs were identified in the rice, wheat, and maize genomes, respectively, all corresponding to HvNPR1, HvNPR3, HvNPR4, and HvNPR5 from barley (Figures 3B – 3D). These findings indicate that the number of syntenic gene pairs among monocot species (barley, rice, and maize) is substantially higher than that between barley and Arabidopsis, suggesting a low level of synteny for NPR1-like genes between Arabidopsis and barley. The retention of duplicated copies of these genes over long evolutionary timescales implies that they may have maintained conserved functions.

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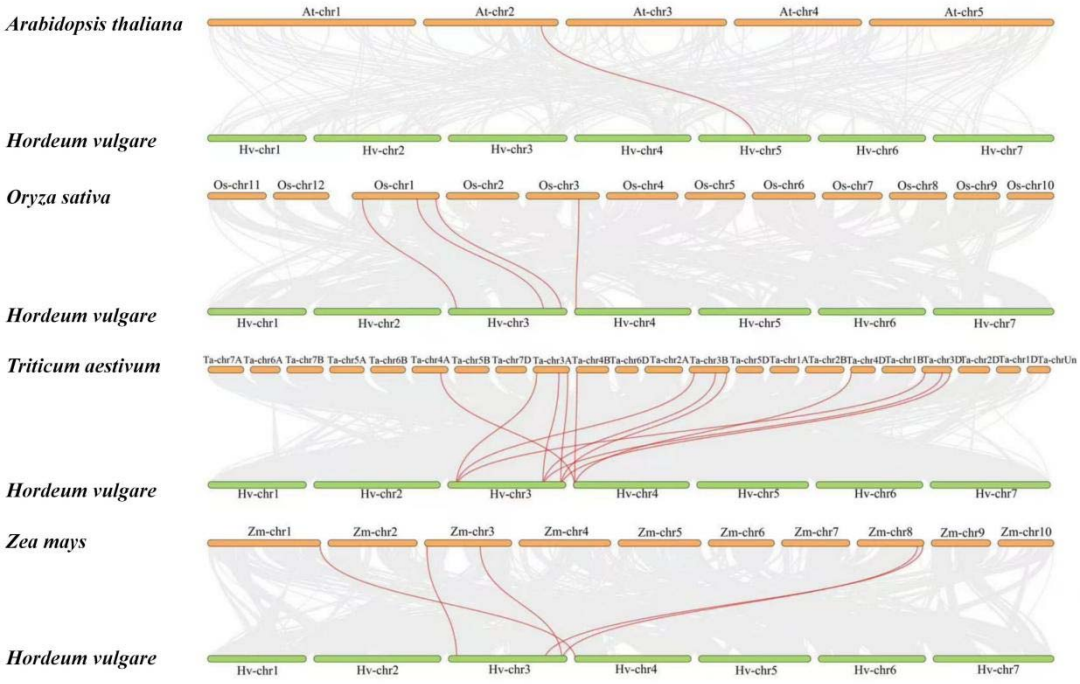

FIGURE 3 COLLINEARITY ANALYSIS OF NPR1-LIKES BETWEEN BARLEY AND ARABIDOPSIS, BARLEY AND RICE, BARLEY AND WHEAT, BARLEY AND MAIZE. EACH HORIZONTAL BAR REPRESENTS A CHROMOSOME. THE ORTHOLOGOUS NPR1-LIKE GENES WERE LINKED USING RED CURVES.

3.4 *DISTRIBUTION OF CONSERVED HVNPR1-LIKE MOTIFS AND DOMAINS*

To investigate the structural characteristics of HvNPR1-like family members, we analyzed their conserved motifs using the MEME suite (Data S3; Figure 4). A total of ten motifs were identified across the 5 HvNPR1-like proteins. Members within the same phylogenetic group exhibited similar motif compositions and orders, whereas notable differences in motif numbers were observed between groups. Specifically, Group III members contained only 8 motifs, whereas Groups I and II each harbored all 10 motifs.Functional annotation revealed that Motif 1 and Motif 7 correspond to ANKYR domains, while Motif 3 and Motif 8 were annotated as BTB/POZ domains. These two domains represent the hallmark features of NPR1-like proteins. Notably, with the exception of the Group III members HvNPR5 and HvNPR6, all other HvNPR1-like proteins contained Motif 5 and Motif 10.

Based on the presence or absence of the NPR1_like_C domain, the HvNPR1-like family could be further divided into two distinct categories (Figure 4). Groups I and II, which are associated with SA signaling, possess the NPR1_like_C domain. In contrast, Group III members, implicated in growth and development, lack this domain. Regardless of the presence of the NPR1_like_C domain, all HvNPR1-like proteins contain both the ANKYR and BTB/POZ domains, confirming their identity as bona fide NPR1-like family members.

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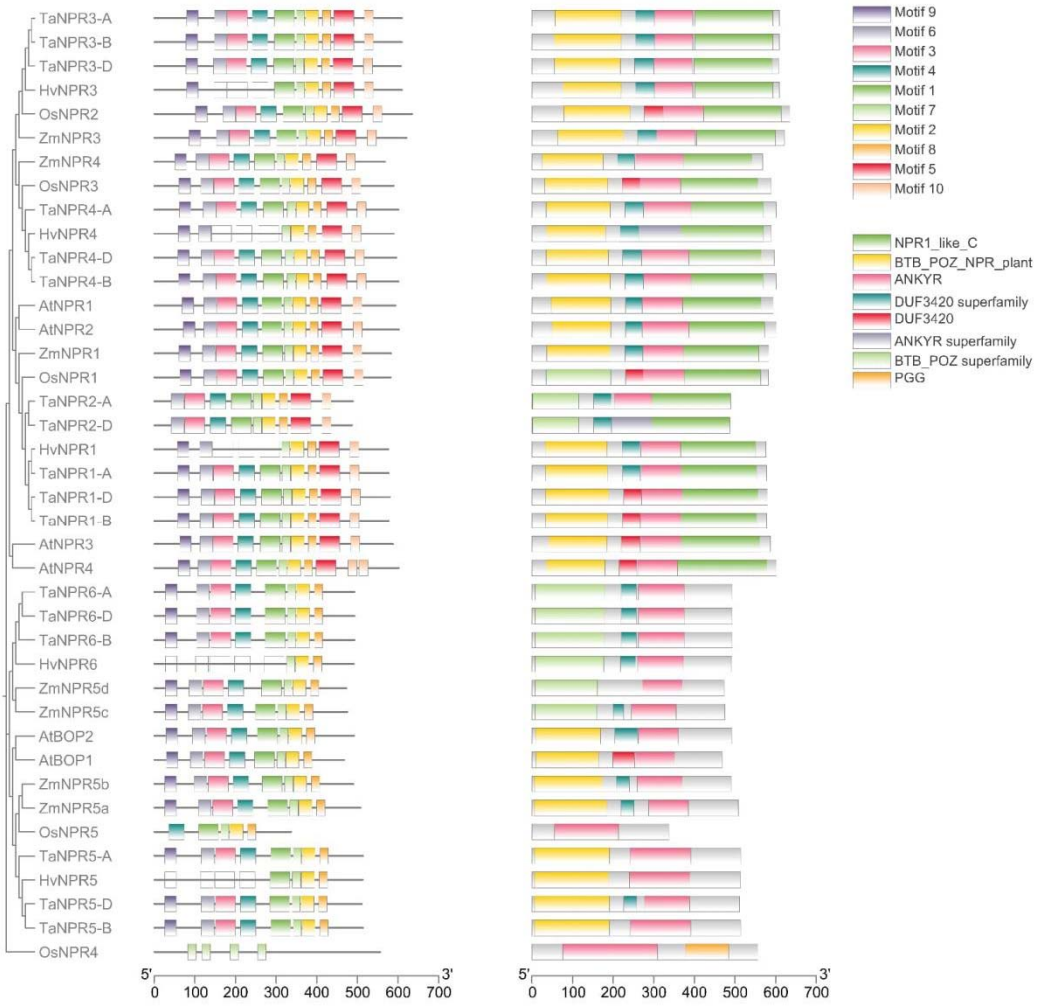

FIGURE 4 DOMAINS AND MOTIFS IN EACH GROUP OF HVNPR1-LIKE PROTEINS. TEN MOTIFS WERE PREDICTED BY MEME AND ARE SHOWN IN DIFFERENT COLORS, WITH GREEN, YELLOW, PINK, DARK GREEN, RED, GRAY, LIGHT GREEN, ORANGE, PURPLE, AND NUDE REPRESENTING MOTIF 1 TO 10, RESPECTIVELY. IN THE BACKGROUND, THE GREEN, YELLOW, PINK, DARK GREEN, RED, PURPLE, LIGHT GREEN, AND ORANGE REGIONS CORRESPOND TO THE NPR1_LIKE_C DOMAIN, BTB_POZ_NPR_PLANT DOMAIN, ANKYR, DUF3420 SUPERFAMILY, DUF3420, ANKYR SUPERFAMILY, BTB_POZ SUPERFAMILY, AND PGG DOMAIN.

3.5 *DIVERSITY OF HVNPR1-LIKE GENE STRUCTURES AND PROMOTERS CIS-ELEMENT REGULATOR ANALYSIS*

To investigate the structural characteristics of the 5 identified HvNPR1-like genes, we analyzed their exon – intron organization and cis-regulatory elements. Considerable variation in gene size was observed among the HvNPR1-like members, with genomic sequences ranging from 1,444 bp (HvNPR3) to 3,092 bp (HvNPR4). The coding sequence (CDS) lengths varied from 1,107 bp (HvNPR3) to 1,728 bp (HvNPR1), indicating substantial diversity in intron size (Figure 5). With the exception of the Group III HvNPR1-like gene, which contained a single intron, all other members from Groups I and II harbored three introns.

To gain insight into the potential responses of HvWRKYs under abiotic or biotic stress conditions, we further analyzed cis-acting regulatory elements in the promoter regions of the five HvNPR1-like genes (Data S4; Figure 5). A total of eight classes of stress- and hormone-related cis-elements were identified, including abscisic acid (ABA)-responsive elements (16 elements), defense and stress-responsive elements (1 element), WRKY transcription factor binding sites (5 elements), drought-responsive elements (3 elements), light-responsive elements (47

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elements), low-temperature-responsive elements (6 elements), jasmonic acid (JA)-responsive elements (24 elements), and SA-responsive elements (3 elements) (Data S5; Figure 5). All five HvNPR1-like genes contained between 16 (HvNPR1 and HvNPR4) and 28 (HvNPR5) elements associated with stress or hormone responses (Data S5; Figure 5). Nearly all HvNPR1-like promoters harbored ABA-, light-, and JA-responsive elements, with HvNPR5 containing up to 15 light-responsive elements (Data S5; Figure 5). Notably, defense and stress-responsive elements were exclusively found in HvNPR6, the sole member of Group III (Data S5; Figure 5).

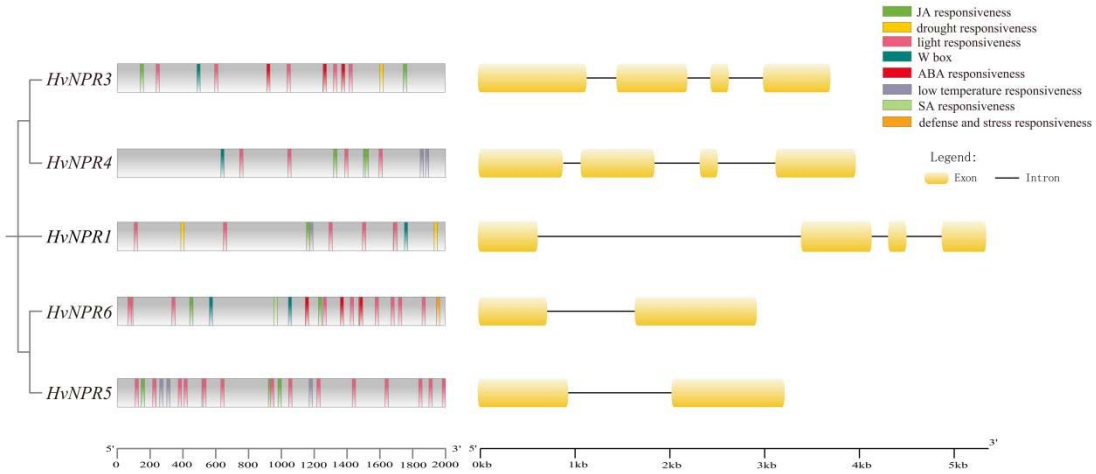

FIGURE 5 PREDICTION OF CIS-REGULATORY ELEMENTS IN THE PROMOTER REGIONS OF HVNPR1-LIKES AND EXON-INTRON STRUCTURE OF HVNPR1-LIKES. THE GREEN, YELLOW, PINK, DARK GREEN, RED, GRAY, LIGHT GREEN, AND ORANGE BLOCKS REPRESENT JA RESPONSIVENESS, DROUGHT RESPONSIVENESS, LIGHT RESPONSIVENESS, W-BOX, ABA RESPONSIVENESS, LOW TEMPERATURE RESPONSIVENESS, SA ROSPONSIVENOSS, DEFENSE AND STRESS RESPONSIVENESS, RESPECTIVELY; THE YELLOW BLOCK REPRESENTS THE CODING SEQUENCE (CDS), AND THE BLACK LINE REPRESENTS INTRON.

3.6 SCREENING OF DIFFERENTIALLY EXPRESSED GENES BY TRANSCRIPTOME AND RT-QPCR VALIDATION OF A TARGET GENE IN RESPONSE TO BARLEY LEAF STRIPE INFECTION

To investigate the transcriptional response of HvNPR1-like genes to leaf stripe disease, the resistant cultivar 'Kunlun 14' and the susceptible cultivar 'Z1141' were inoculated with the pathogen Pyrenophora graminea (Ito et Kurib.). Transcriptome sequencing was subsequently performed on both infected and healthy leaves from the two cultivars (Data S6). Among the 5 HvNPR1-like genes, three were identified as differentially expressed genes (Data S7; Figure 6A). Notably, all genes belonging to Groups I and II exhibited a significant response to leaf stripe stress. Further analysis revealed that only the Group I gene, HvNPR1, was upregulated upon pathogen infection, whereas the Group II genes HvNPR4 were downregulated in the susceptible cultivar following infection. Based on these findings, HvNPR1 was prioritized for subsequent investigation.

To validate the expression pattern of HvNPR1, the resistant cultivar 'THIBAUT' and the susceptible cultivar 'Z1141' were inoculated with P. graminea, and gene expression levels in both infected and healthy leaves were assessed using qRT-PCR. The results showed that HvNPR1 expression was significantly upregulated in both cultivars following pathogen infection, with significantly higher transcript levels observed in 'THIBAUT' compared to 'Z1141' ($P < 0.05$; Figure 6B). These findings were consistent with the RNA-seq data. In the resistant cultivar, HvNPR1 expression increased over time post-inoculation, and transcript levels were markedly higher in infected leaves than in healthy controls ($P < 0.05$). Moreover, at 5 weeks post-inoculation, the expression level in the resistant cultivar remained significantly higher than that in the susceptible cultivar ($P < 0.05$).

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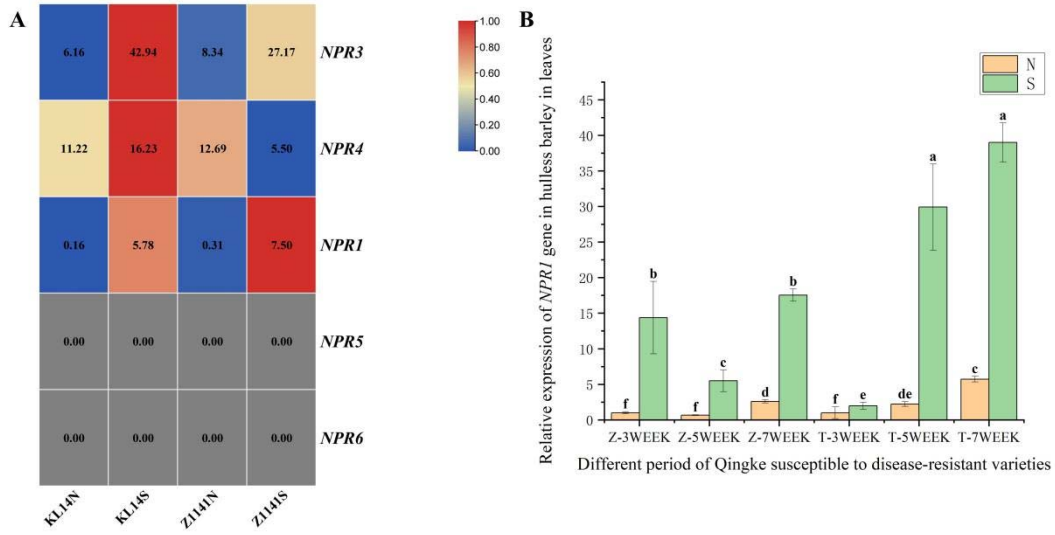

FIGURE 6. EXPRESSION PATTERNS OF HVNPR1-LIKE GENES IN RESPONSE TO BARLEY LEAF STRIPE DISEASE AND SPATIOTEMPORAL EXPRESSION PROFILES OF HVNPR1 IN RESISTANT AND SUSCEPTIBLE CULTIVARS.

(A) Heatmap showing the expression levels (FPKM values) of 5 HvNPR1-like genes in resistant cultivar 'Kunlun14' and susceptible cultivar 'Z1141' under mock and Pyrenophora graminea-inoculated conditions. KL14N, normal leaves of Kunlun14; KL14S, barley leaf stripe-infected leaves of 'Kunlun14'; ZN, normal leaves of Z1141; ZS, barley leaf stripe-infected leaves of 'Z1141'. The color scale represents normalized FPKM values, with blue indicating low expression and red indicating high expression. (B) qRT-PCR validation of HvNPR1 expression in resistant cultivar 'THIBAUT' and susceptible cultivar 'Z1141' at different time points post-inoculation (3, 5, and 7 weeks). Data are presented as mean ± SD (n=3). Different letters above the bars indicate statistically significant differences among treatments and time points according to Duncan's multiple range test ($P < 0.05$).

3.7 *VALIDATION OF THE INTERACTION BETWEEN HVNPR1 AND HVWRKY70 BY BIMOLECULAR FLUORESCENCE COMPLEMENTATION (BIFC) ASSAY*

Confocal microscopy observation revealed that strong yellow fluorescent signals, localized to the nucleus, were detected exclusively in tobacco epidermal cells co-expressing HvNPR1-YFPC and HvWRKY70-YFPN, whereas no fluorescent signals were observed in the control groups, indicating a specific interaction between these two proteins in vivo (Figure 7).

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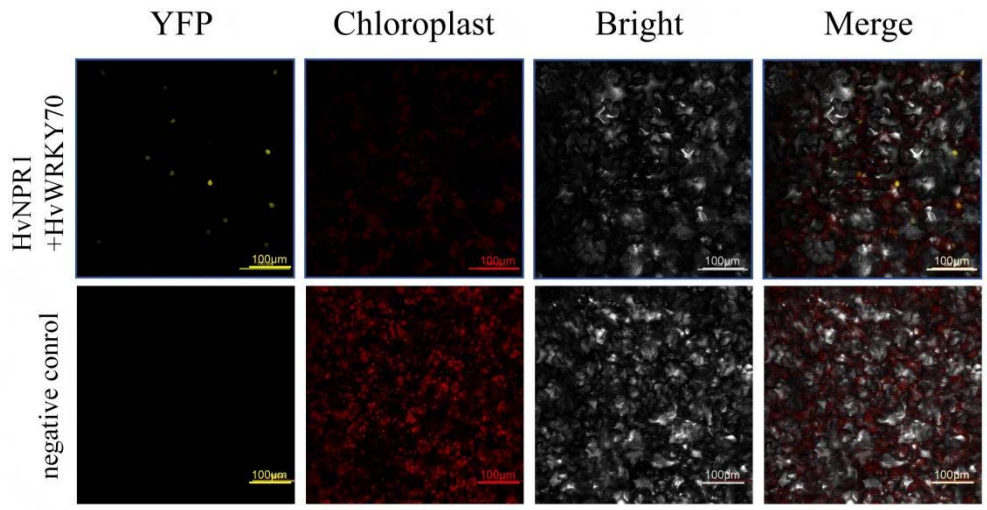

FIGURE 7. BIFC ASSAY VALIDATES THE INTERACTION BETWEEN HVNPR1 AND HVWRKY70

BiFC microscopy images showing yellow fluorescent protein (YFP) signals in Nicotiana benthamiana leaf epidermal cells co-expressing different fusion protein combinations. Scale bars = 100 μm. Upper panel: Co-expression of HvNPR1-YFP^{c} and HvWRKY70-YFP^{N}; Lower panel: Co-expression of HvNPR1-YFP^{c} and empty YFP^{N} vector (negative control).C assay validates the interaction between HvNPR1 and HvWRKY70

4 DISCUSSION

In this study, 5 NPR1-like genes were successfully isolated from barley through a genome-wide approach. The NPR1-like family constitutes a small gene family, and its distribution in barley is similar to that observed in other grass species. For instance, Oryza sativa contains 5 members (Yuan et al., 2007), Arabidopsis thaliana has 6 (Hepworth et al., 2005), maize possesses 7 (Zhang et al., 2024), and hexaploid wheat (Triticum aestivum) harbors 17 members (Liu et al., 2019). Beyond the grasses, 5 NPR1-like genes have been identified in Persea americana (Backer et al., 2015), 6 in Populus trichocarpa, 3 in Vitis vinifera, and 4 in Medicago truncatula (Kugler et al., 2013). Phylogenetic analysis across multiple species revealed that the NPR1-like genes identified in barley cluster into three distinct clades, with members evenly distributed among these groups.

Domain analysis revealed that all NPR1-like family members contain conserved protein domains: an N-terminal BTB/POZ domain and a central ankyrin repeat (ANK) domain. Based on the presence or absence of the NPR1_like_C domain, the NPR1-like family can be divided into two subfamilies: NPR and BOP (Shia et al., 2013). Subsequently, during long-term evolution involving duplication and divergence, the NPR subfamily further diversified into two distinct clades, forming the NPR1/2 and NPR3/4 groups, each fulfilling different functional roles. Following the classification system established in Arabidopsis, the barley NPR1-like gene family was also divided into three clades. Among them, Clade I contains one member, Clade II contains two members, and Clade III contains two members, the latter lacking the NPR1_like_C domain.

Synteny analysis and chromosomal localization revealed that the NPR1-like family is relatively conserved in Poaceae. Notably, four of the barley NPR1-like genes exhibit syntenic relationships with those in other grass species. The conserved synteny between NPR1 genes in barley and related cereal crops further supports their evolutionary conservation and potential functional similarity across species. This finding provides an evolutionary basis for leveraging NPR1-related research resources from model cereal crops, such as rice, to elucidate NPR1-like gene functions in barley. Interestingly, Clade I in barley contains only a single member (HvNPR1). It is hypothesized that functionally redundant genes within this clade may have undergone gradual pseudogenization or gene loss during evolution.

Plants have evolved a highly sophisticated and effective innate immune system to defend against a wide range of pathogens, including bacteria, fungi, viruses, and oomycetes (Jones & Dangl, 2006; Spoel & Dong, 2012; Chisholm et al., 2006). Upon pathogen attack, the first layer of defense is activated on the plant cell surface and is referred to as pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Bigeard et al., 2015; Couto & Zipfel, 2016). During PTI, pattern-recognition receptors (PRRs) localized at the plasma membrane recognize conserved PAMPs—such as fungal chitin, bacterial flagellin (flg22), and lipopolysaccharides (LPS)—thereby initiating downstream immune responses (Zipfel et al., 2004; Livajaa et al., 2008; Boutrot & Zipfel, 2017). However, many adapted pathogens deliver effector proteins into plant cells to suppress PTI, leading to a state known as effector-triggered

susceptibility (ETS) (Macho et al., 2014; Chen et al., 2017; Qi et al., 2018).

In response, plants have evolved a second layer of immunity, termed effector-triggered immunity (ETI), which is activated upon specific recognition of pathogen effectors by intracellular receptors encoded by resistance (R) genes (Wu et al., 2014; Cui et al., 2015). ETI often culminates in a robust defense response, including localized programmed cell death at the infection site, known as the hypersensitive response (HR), which effectively restricts pathogen proliferation (Wu et al., 2014). The HR is accompanied by the biosynthesis and accumulation of the defense hormone SA, both locally and in distal uninfected tissues (Ryals et al., 1996). This systemic signal ultimately establishes systemic acquired resistance (SAR), a broad-spectrum and long-lasting immune state that protects the whole plant against secondary infections (Durrant & Dong, 2004; Fu & Dong, 2013). A hallmark of SAR is the coordinated expression of pathogenesis-related (PR) genes throughout plant tissues, which encode antimicrobial proteins that contribute to enhanced resistance (Ryals et al., 1996; Durrant & Dong, 2004; Fu & Dong, 2013).

In this study, we identified members of the NPR1-like gene family in barley and found that HvNPR1 was significantly upregulated in response to Pyrenophora graminea infection, suggesting its potential involvement in regulating PTI or ETI signaling pathways. Previous studies have demonstrated that AtNPR1, a master regulator of SAR in Arabidopsis, enhances plant disease resistance by activating PR gene expression (Spoel & Dong, 2012; Durrant & Dong, 2004). NPR1 is a key regulator of SA signaling, and its paralogs in Arabidopsis also play roles in SA perception (Castelló et al., 2018). Our bimolecular fluorescence complementation (BiFC) assays confirmed a specific interaction between HvNPR1 and HvWRKY70, consistent with the established mechanism by which WRKY transcription factors function downstream of NPR1 to regulate PR gene expression (Fu & Dong, 2013). WRKY70 has been identified as a key transcription factor in the SA signaling pathway, acting as a node of convergence for SA- and jasmonate-mediated signals in plant defense (Li et al., 2004). This finding is also consistent with the study by Li et al. (2020), which demonstrated that WRKY transcription factors are shared by BTH-induced resistance and NPR1-mediated acquired resistance, improving broad-spectrum disease resistance in wheat. Therefore, we hypothesize that HvNPR1 may modulate defense gene expression through interaction with HvWRKY70 via the SA pathway, thereby contributing to barley resistance against leaf stripe disease.

Notably, the Group II members HvNPR4 were downregulated in the susceptible cultivar upon infection, a pattern reminiscent of the negative regulatory roles of AtNPR3 and AtNPR4 as SA receptors in Arabidopsis immunity (Chen et al., 2017; Fu & Dong, 2013). This suggests that the NPR1-like gene family may have retained conserved immune regulatory mechanisms between monocotyledonous and dicotyledonous plants. However, whether HvNPR3 and HvNPR4 function similarly as SA receptors, and their precise regulatory roles in leaf stripe resistance, require further functional validation.

In summary, this study provides preliminary insights into the potential role of the NPR1-like family member HvNPR1 in barley resistance to leaf stripe disease, laying a foundation for further dissection of its regulatory network. Future studies employing gene editing technologies to generate HvNPR1 overexpression or knockout lines, combined with transcriptomic and proteomic analyses, will help elucidate the molecular pathways through which HvNPR1 mediates defense signaling. Such efforts will provide both theoretical support and genetic resources for the breeding of disease-resistant barley cultivars.

5 CONCLUSION

In this study, five HvNPR1-like genes were identified in barley through a genome-wide approach and classified into three phylogenetic clades. All members contain conserved BTB/POZ and ankyrin repeat domains, with the NPR1_like_C domain present only in Clade I and II. Synteny analysis revealed that four barley NPR1-like genes are conserved in Poaceae, providing an evolutionary basis for functional studies. Expression analysis showed that HvNPR1 (Clade I) was significantly upregulated in resistant cultivars, while HvNPR3 and HvNPR4 (Clade II) were downregulated in susceptible cultivars, suggesting their involvement in leaf stripe resistance. qRT-PCR validation demonstrated that HvNPR1 expression levels were significantly higher in the resistant cultivar Thibaut than in the susceptible cultivar Z1141 after P. graminea inoculation ($P < 0.05$), with expression increasing over time and infected leaves showing significantly higher transcript levels than healthy controls ($P < 0.05$), consistent with RNA-seq data. Protein interaction assays confirmed that HvNPR1 specifically interacts with HvWRKY70, supporting the conserved NPR1-WRKY regulatory module in SA-mediated defense signaling. These findings identify HvNPR1 as a key candidate for leaf stripe resistance and provide valuable gene resources for breeding disease-resistant barley cultivars through genetic improvement strategies.

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