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Jiao Pan

Gansu Agricultural University

Ming Guo

Gansu Agricultural University

Wenjuan Yang

Gansu Agricultural University

Chengdao Li

Murdoch University

Yaxiong Meng

Gansu Agricultural University

Xiaole Ma

Gansu Agricultural University

Baochun Li

Gansu Agricultural University

Lirong Yao

Gansu Agricultural University

Hong Zhang

Gansu Agricultural University

Ke Yang

Gansu Agricultural University

Erjing Si

siej@gsau.edu.cn

Gansu Agricultural University

Huajun Wang

Gansu Agricultural University

Juncheng Wang

Gansu Agricultural University

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Functional analysis of *HvRHF9870* in barley resistance to *Pyrenophora graminea*

Jiao Pan^{1, 2}, Ming Guo^{1, 2}, Wenjuan Yang^{1, 2}, Chengdao Li³, Yaxiong Meng^{1, 2}, Xiaole Ma^{1, 2}, Baochun Li^{2, 4}, Lirong Yao^{1, 2}, Hong Zhang^{1, 2}, Ke Yang¹, Erjing Si^{1, 2*}, Huajun Wang^{1, 2*}, Juncheng Wang^{1, 2*}

¹ *Department of Crop Genetics and Breeding, College of Agronomy, Gansu Agricultural University, Lanzhou 730070, China*

² *Seed Industry Research Institute of Gansu Provincial University, Lanzhou 730070, China*

³ *Western Barley Genetics Alliance, College of Science, Health, Engineering and Education, Murdoch University, Murdoch, Australia*

⁴ *Department of Botany, College of Life Sciences and Technology, Gansu Agricultural University, Lanzhou 730070, China*

*Correspondence Erjing Si, E-mail: siej@gsau.edu.cn; Huajun Wang, E-mail: wanghj@gsau.edu.cn; Juncheng Wang, E-mail: wangjc@gsau.edu.cn

Abstract

Key message

HvRHF9870 is a novel RING-H2 finger E3 ligase upregulated in barley resistant to *Pyrenophora graminea*.

Abstract Barley leaf stripe, caused by *Pyrenophora graminea*, is a major disease of barley globally. The role of RING-H2 finger (RHF) E3 ubiquitin ligase enzyme in barley resistance to *P. graminea* remains unclear. In this study, we conducted comparative transcriptomic analysis between highly resistant and susceptible barley accessions following pathogen inoculation across 14 days post-infection. RNA-seq analysis demonstrated that a new resistant gene *HvRHF9870* showed 13-fold higher expression in resistant plants at 14 days post-inoculation. The protein encoded by *HvRHF9870* was a typical RING-H2 finger domain and localized in nucleus. Silencing *HvRHF9870* by virus induced gene silencing (VIGS) resulted in impaired superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities, leading to compromised resistance of *P. graminea*, indicating that *HvRHF9870* plays a crucial role in enhancing barley resistance against *P. graminea*. Furthermore, yeast two-hybrid screening identified POD as an interacting partner of *HvRHF9870*, revealing a mechanistic link between E3 ligase-mediated regulation and antioxidant enzyme activity. Our results establish a foundation for uncovering the molecular mechanism of *HvRHF9870* to barley diseases and provides novel genetic resources for barley breeding.

Introduction

Barley (*Hordeum vulgare* L.) is one of the most extensively grown cereal grains worldwide, plays a pivotal role as a primary source of livestock feed and a fundamental raw material for the malting and brewing industries (Astrand et al. 2024; Jayakodi et al. 2020). Concurrently, barley has attracted growing interest as a functional ingredient in human foods because of its well-recognized nutritional and physiological benefits (Suman et al. 2019). Studies have shown that barley contains abundant protein and carbohydrates, including β -glucan, as well as lipids, vitamins, and minerals. Owing to its distinctive chemical composition and associated health benefits, barley has received

growing attention from both agricultural and food researchers (Raffo et al. 2025). Moreover, barley has been reported to possess antioxidant and anti-inflammatory activities, which may contribute to its potential anticancer effects. In addition, barley and barley-derived products are widely used across multiple industries, including food, beverages, bakery products, animal feed, and cosmetics (Sidhu et al. 2007; Maddirala et al. 2025). Being immobile, barley plants are inherently vulnerable to a wide range of biotic and abiotic stress factors that significantly disrupt their normal growth and development, resulting in significant losses in barley production worldwide (Panahi and Hamid 2025). Barley leaf stripe, a worldwide epidemic disease caused by *Pyrenophora graminea*. It has occurred in barley producing areas in Europe, North America, North Africa, Russia, India, and China (Liang et al. 2019). A 1% increase in disease incidence typically results in a 1% reduction in yield (Porta-Puglia et al. 1986). When the disease incidence is high, yield losses of up to 70% have been observed in barley cultivars (Hu et al. 2022). Thus, elucidating the molecular mechanisms underlying disease resistance and identifying key resistance genes are essential for developing durable and effective barley breeding strategies.

Plants have developed a two-tiered innate defense system to counteract various pathogens, including pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Chai et al. 2024; Ngou et al. 2022; Jones et al. 2024). The first layer of defense, known as PTI, is activated when cell surface-localized pattern recognition receptors (PRRs) perceive conserved pathogen-associated molecular patterns (PAMPs) (Fu et al. 2024), whereas ETI, the second layer of defense, is initiated upon direct or indirect recognition of pathogen-delivered effectors by intracellular nucleotide-binding leucine-rich repeat (NLR) receptor proteins (Nabi et al. 2024). Studies have shown that these pathways result in numerous overlapping downstream effects, including mitogen-activated protein kinase (MAPK) cascades, calcium flux, reactive oxygen species (ROS) bursts, and plant hormone signaling (Yuan et al. 2021; Park et al. 2017). Hydrogen peroxide (H₂O₂) is a relatively stable ROS that can diffuse into subcellular compartments. H₂O₂ can be generated and cleared by many enzymes, such as superoxide dismutase (SOD),

peroxidase (POD), and catalase (CAT) (Wu et al. 2020). The role of H₂O₂ in the extracellular matrix is not limited to participating in a defense response but is also involved in regulating the synthesis of cell wall components (Cheng et al. 2020). Overaccumulation of H₂O₂ impairs the integrity of plant biomacromolecules and disturbs normal cellular metabolism and physiological functions (Li et al. 2019). Therefore, plants maintain cellular H₂O₂ homeostasis by modulating the activities of antioxidant enzymes, thereby enhancing resistance to pathogen invasion. However, pathogens have co-evolved sophisticated countermeasures, notably by secreting effector proteins that actively disrupt host signaling pathways and suppress immune responses to facilitate successful colonization (Hou et al. 2025). In this dynamic molecular arms race, the stability and spatiotemporal regulation of key immune-related proteins have emerged as pivotal determinants governing both the amplitude and specificity of the plant immune output.

During pathogen infection, the ubiquitin-26S proteasome system (UPS) plays a crucial role in inactivating numerous regulatory proteins, thereby enabling both rapid activation and sustained activity of immune pathways in the context of the highly intertwined PTI and ETI responses (Yan et al. 2024). The UPS is primarily composed of ubiquitin (Ubi), ubiquitin-activating enzyme (E1), ubiquitin-binding enzyme (E2), ubiquitin-binding protein ligase (E3), and the 26S proteasome (Qi et al. 2025). In plants, E3 ubiquitin ligases function as crucial mediators of plant immune regulation, and both PTI and ETI related immune receptors also be regulated by UPS (Yan et al. 2024; Zheng et al. 2023). For example, rice Spl11 is an E3 ligase and functions as a negative regulator of programmed cell death and disease resistance (Zeng et al. 2004). The rice E3 ubiquitin ligases APIP6 and APIP10 ubiquitinate the *Magnaporthe oryzae* effector AvrPiz-t, leading to its proteasomal degradation (Bai et al. 2019). The novel E3 ligases OsPUB41 from PUB-mediated immune mechanism in rice as a potential target for genome editing, which may facilitate the development of blast-resistant rice (Xu et al. 2025). Therefore, an optimal balance between disease resistance and growth can be achieved by harnessing ubiquitination-mediated regulatory mechanisms.

Recent studies have indicated that E3 ligases have been categorized into four classes

based on their structures: really interesting new gene (RING), cullin-RING ligases (CRLs), homologous to E6-associated protein C-terminal (HECT), and U-Box (Huang et al. 2026). RING finger proteins harbor typical zinc finger protein domains that are classified into $C_3H_2C_3$ (RING-H2) and C_3HC_4 (RING-HC), and modified RING domain proteins based on the fifth conserved amino acid (Jackson et al. 2000). RING-H2 finger (RHF) E3 ligases contain a specific sequence that Cys-X₂-Cys-X₍₉₋₃₉₎-Cys-X₍₁₋₃₎-His-X₍₂₋₃₎-Cys/His-X₂-Cys-X₍₄₋₄₈₎-Cys-X₂-Cys, where X denotes any amino acid. RING finger proteins have been demonstrated to mediate protein-protein interactions and formation of multi-protein complexes. At least 477 RING proteins have been found in *Arabidopsis* (Vierstra 2009). Previous studies have indicated that many plant RING proteins are identified as E3 ubiquitin ligases, which are essential for plant metabolism and development (Bi et al. 2025). For instance, the RING-type ubiquitin ligase protein APIP10 interacts with OsVOZ1 and OsVOZ2, playing a positive role in rice immunity through the 26S proteasome pathway (Wang et al. 2021). In rice, OsRFPH2-6 and OsRFPH2-10 act as negative regulators of immunity to *Magnaporthe oryzae* and rice dwarf virus, respectively (Bi et al. 2022). The RING-finger E3 ligase APIP6 positively regulates basal resistance to *M. oryzae* by interacting with AvrPiz-t (Park et al. 2012). RHF E3 ubiquitin ligase GhDIRP1 acts as a negative regulator of resistance to *Verticillium dahlia* (Miao et al. 2024). The effector protein AvrPtoB from the plant pathogen *Pseudomonas syringae* pv. *tomato* can target a host protein kinase, Fen, and degrades NPR1 to disrupt plant immunity through RING E3 ligase activity (Wei et al. 2015). Additionally, RING-finger E3 ligase XB3 interacts with and stabilises XA21, resulting in promoted XA21-mediated ETI resistance after *Xanthomonas oryzae* pv. *oryzae* infection (Wang et al. 2006). However, the regulatory functions of the RHF E3 ligase protein family in barley remain poorly characterized.

In this study, we identified that the expression of the *HvRHF9870* gene was significantly increased upon initial inoculation of the highly resistant variety with the *P. graminea* through transcriptome analysis. To further investigate the role of the *HvRHF9870* gene in the resistance to *P. graminea* in barley, we employed virus-induced gene silencing (VIGS) to investigate the role of *HvRHF9870* and reveal its

regulatory function in disease resistance. This study aimed to elucidate the role of *HvRHF9870* in the response to *P. graminea*, providing a theoretical basis for the development of resistant barley varieties.

Materials and methods

Fungal, plant and culture conditions

The strain QWC (wild type, WT) was isolated *P. graminea* was plated on potato dextrose agar (PDA) at 23°C for seven days. Barley cultivars ZDM02422 (resistant) and ZDM04908 (susceptible) were grown in an artificial climate chamber at 22 °C with a 16 h/8 h day/night cycle in this study.

Bioinformatic characterization of *HvRHF9870*

The full-length gene sequences and protein sequences of *HvRHF9870* were downloaded from Ensembl Plants (<https://plants.ensembl.org/index.html>). The number of amino acids, molecular weight (MW), theoretical isoelectric point (pI), instability index, aliphatic index, grand average of hydropathicity of the *HvRHF9870* were predicted using ExPASy ProtParam tool (<https://web.expasy.org/protparam/>). The signal peptides and transmembrane structures were predicted using SignalP 5.0 (<https://services.healthtech.dtu.dk/services/SignalP-5.0/>) and TMHMM Server v.2.0 (<https://services.healthtech.dtu.dk/services/TMHMM-2.0/>), respectively. The potential phosphorylation site prediction analysis was predicted using the NetPhosv 3.1 (<https://services.healthtech.dtu.dk/services/NetPhos-3.1/>). The secondary structure composition of the *HvRHF9870* was predicted using the SOPMA (https://npsa.lyon.inserm.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html). Three-dimensional structure modeling was performed using the SWISS-MODEL (<https://swissmodel.expasy.org/>).

Sequence alignment and phylogenetic analysis

To construct the phylogenetic tree of RHF proteins, full-length RHF amino acid sequences were downloaded from NCBI based on conserved regions common to RHF in barley, rice, wheat, and *Arabidopsis*. The tree was generated using the Neighbor-Joining method in MEGA-X software, and the phylogenetic tree was constructed using the maximum likelihood with 1000 bootstraps replicates. The tree was visualized via

the iTOL web server (<https://itol.embl.de/>).

Plant treatments and quantitative real-time PCR analysis

The improved sandwich method was employed for inoculation experiments in gene expression profiling analysis. Sterilized seeds of the barley variety ZDM02422 and ZDM04908 were placed on QWC and SPg14 (CK) that had been precultured for seven days. The assemblies were then incubated at 4°C. Germ buds were aseptically isolated from infected seeds at 14 days post-inoculation (dpi) and immediately flash-frozen in liquid nitrogen for RNA preservation. Mycelia cultured on sterile medium without seeds served as control. Total RNA was extracted using TRIzol® RNA Extraction Reagent (Beijing China), followed by reverse transcription and cDNA synthesis with the FastKing One-Step qRT-PCR Kit (Beijing China). Gene-specific primers for qRT-PCR are listed in Table 1. Transcript abundance was determined using the $2^{-\Delta\Delta C_t}$ method. All qPCR experiments included three independent biological replicates.

Gene cloning

The *HvRHF9870* gene was identified from an RNA-Seq database and found to be upregulated following inoculation with *P. graminea*. Primers were designed based on the sequences of *HvRHF9870* (Table 1). A total of 1 µg of RNA was reverse transcribed into cDNA using the SMART RACE cDNA Amplification Kit (Dalian, China). The amplification program was employed according to the kit instructions. PCR products were purified and recovered via agarose gel electrophoresis. Subsequently, the PCR products were cloned into the pMD19-T simple cloning vector (Dalian China) and verified by sequencing.

Subcellular localization analysis of *HvRHF9870*

The coding sequence of *HvRHF9870*, excluding the stop codon, was amplified via PCR using gene-specific primers pCAMBIA1300: 35S: EGFP: *HvRHF9870*-F and pCAMBIA1300: 35S: EGFP: *HvRHF9870*-R (Table 1), which incorporated *Kpn* I and *Xba* I restriction sites at their 5' ends. The amplified fragment cloned into a T-vector and the binary vector pCAMBIA1300: EGFP were separately digested with *Kpn* I and *Xba* I. The digested DNA fragments were purified from agarose gels using the SanPrep Column DNA Gel Extraction Kit (Shanghai, China). Ligation was carried out using T4-

DNA ligase was applied to generate the *HvRHF9870*-EGFP fusion expression vector pCAMBIA1300:35S: EGFP: *HvRHF9870*. The recombinant vector was then introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation or freeze-thaw transformation. Transformed *Agrobacteria* were cultured to mid-log phase and delivered into *Nicotiana benthamiana* leaves using infiltration protocols. Subcellular localization of the fusion protein was visualized by detecting EGFP fluorescence with a confocal laser scanning microscope.

Barley stripe mosaic virus (BSMV)-mediated gene silencing

To silence *HvRHF9870*, a 358 bp fragment was cloned and recombined into the BSMV vector using primers of BSMV: *HvRHF9870*-F and BSMV: *HvRHF9870*-R (Table 1), resulting in BSMV: *HvRHF9870*. A mixture of GV3101 carrying BSMV: α , BSMV: β , BSMV: *PDS*, BSMV: γ , and BSMV: *HvRHF9870* were infiltrated into two primary leaves of two-week-old *N. benthamiana*. Subsequently, the leaves of seven-day-old barley were inoculated using rub inoculation with a gloved finger. Successful gene silencing was confirmed when BSMV: *PDS*-inoculated leaves exhibited a bleaching phenotype. The silencing efficiency of *HvRHF9870* was then determined by qRT-PCR. After the barley leaves developed systemic viral symptoms, they were inoculated with *P. graminea*. Subsequently, leaves were harvested at 3 dpi, 5 dpi, and 7 dpi after *P. graminea* inoculated for qRT-PCR and phenotypic observations (Table 1). To assess the biomass of *P. graminea* in barley leaves, the first true leaves were harvested at 3, 5, and 7 dpi for DNA extraction. qPCR was used to quantify the relative amount of fungal DNA (Table 1). Each experiment was performed with at least three replicated measurements.

Physiological and biochemical measurements

Antioxidant enzyme activities of peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) were determined with a micro assay kit (Beijing, China) following the manufacturer's instructions. Similarly, H₂O₂ content was determined using a micro assay kit (Beijing, China). In addition, callose deposition (Suzhou, China) and ion leakage were determined. The relative chlorophyll content was measured in vivo as the Soil and Plant Analyzer Development (SPAD) value using a SPAD-502 plus

chlorophyll meter (Tokyo, Japan). All treatments were performed simultaneously, and the experiment was repeated three times.

Trypan blue and 3,3-Diaminobenzidine (DAB) staining assays

The leaves were fully immersed in the staining solution (10 mL glycerol, 10 mL lactic acid, 10 mL phenol, and 10 mL sterile water with 20 mg of trypan blue) and heated in a boiling water bath for 10 min. Then, the staining solution was removed, and the leaf samples were immersed in chloral hydrate solution (2.5 mg/mL) until the chlorophyll was completely cleared. For DAB staining, the leaves were completely immersed in the DAB working solution (1 mg/mL) in the dark at room temperature for 5-6 h and then transferred to anhydrous ethanol until chlorophyll was eliminated. Subsequently, the samples were observed under a microscope, and the lesion areas were measured and quantified using ImageJ software (Java 1.8.0_345, National Institutes of Health, Bethesda, MD, USA). Images were calibrated with a reference scale bar, and lesion areas were recorded in cm². The built-in tools of ImageJ were used to accurately outline and quantify lesion areas.

Yeast two-hybrid assays

The CDS of *HvRHF9870* was introduced into the pGBKT7 as bait plasmids to screen for host interacting proteins, while the coding sequence of HvPOD was cloned into pGADT7 as prey plasmids (Table 1). The recombinant bait and prey plasmids were co-transformed into the yeast Y2H Gold strain using PEG/LiAc method. Transformants were serially diluted to 10 ×, 100 ×, and 1000×, then cultured at 30°C on selective media (SD/-Leu-Trp, and SD/-Leu-Trp-His).

Molecular Docking

Molecular docking was performed to characterize the binding mode between the ligand and the receptor. The receptor structure was preprocessed using AutoDockTools by adding polar hydrogens and removing crystallographic water molecules and other non-essential components. The ligand structure was obtained from a chemical database, converted from SDF to PDBQT format, and prepared with MGLTools. A docking grid was defined to cover the putative active site of the receptor, and AutoDock Vina was used to predict ligand binding poses and affinities. The top-ranked pose was selected,

converted to PDB format, and visualized in PyMOL to inspect key protein–ligand interactions, including hydrogen bonds and other contact features, together with the residues lining the binding pocket.

Statistical analysis

The data were analyzed using Student's t-test and treated followed by Tukey's multiple comparison test ($P < 0.05$) in SPSS 26.0.

Results

HvRHF9870* shows strong induction in resistant barley after inoculation with *P. graminea

In this study, we analyzed FPKM values to assess the expression of *HORVU.MOREX.r3.5HG0499870* (*HvRHF9870*) in both resistant and susceptible barley 14 dpi after inoculation with *P. graminea* based on transcriptome sequencing data. The expression of *HvRHF9870* was significantly increased in resistant varieties after infection with *P. graminea* (Fig. 1A, 1B). The qRT-PCR results were in strong agreement with the transcriptomic data, exhibiting a high correlation and thereby validating the reliability of the RNA-seq results. (Fig. 1C, 1D). Notably, *HvRHF9870* exhibited strong upregulation specifically at 14 dpi, suggesting its potential involvement in the defense response against *P. graminea*.

Bioinformatic characterization of *HvRHF9870*

Physicochemical analysis showed that the full-length sequence of *HvRHF9870* contains a 675 bp open reading frame region and encodes 224 amino acids. The molecular weight of the protein is 23.96 kDa, an isoelectric point (pI) is 5.34, an instability index is 58.22, an aliphatic index is 79.87, an average hydrophilicity of -0.175, a negatively charged residues (Asp + Glu) is 31, and a positively charged residue (Arg + Lys) is 23. The results of the ProtScale online software analysis revealed that the protein had a distinct hydrophilic region, indicating that the *HvRHF9870* protein is hydrophilic (Fig. 2A). Phosphorylation site analysis revealed that *HvRHF9870* contains potential phosphorylation sites at tyrosine (Tyr), serine (Ser), and threonine (Thr) (Fig. 2B). SignalP 5.0 analysis indicated that *HvRHF9870* did not contain a signal peptide, suggesting that it is a non-secreted protein (Fig. 2C). In addition, *HvRHF9870* lacks

transmembrane domains, indicating it is a non-membrane protein (Fig. 2D). Furthermore, the conserved domain analysis indicated that HvRHF9870 possesses a conserved RING-H2 finger domain by NCBI-CDD. Secondary structure analysis predicted that HvRHF9870 is composed mainly of random coils (71.43%), followed by α -helices (19.64%) and extended strands (8.93%), while no β -turns, β -bridges, π -helices, or other structural states were detected using SOPMA (Fig. 3A). Tertiary structure prediction showed that HvRHF9870 adopts an irregular folded conformation with multiple loop regions, suggesting a flexible protein architecture (Fig. 3B).

Phylogenetic analysis of the *RHF* gene family

To further understand the evolutionary relationships between HvRHF and other plant RHF proteins, a phylogenetic tree was constructed using the neighbor-joining (NJ) method in MEGA-X. The tree resolved the RHF proteins into five well-supported subfamilies, designated Clade I-V (Fig. 4). These clades were clearly separated and were highlighted with distinct colors, indicating a robust phylogenetic structure within the *RHF* family. *RHF* members from different species were distributed across multiple clades rather than forming strictly species-specific clusters, suggesting that the major diversification of this family likely occurred before species divergence. Furthermore, the local clustering of members from the same species in several branches indicated that lineage-specific duplication events may have contributed to the expansion of the *RHF* family during evolution. The number of members varied among subfamilies, with some clades containing more members than others, reflecting different degrees of expansion and diversification during evolution. Overall, the *RHF* family has formed a relatively conserved subfamily framework during long-term evolution, accompanied by a certain degree of functional divergence, thus providing a phylogenetic basis for subsequent analyses of gene structure, conserved motifs, and potential biological functions.

Subcellular localization of *HvRHF9870*

We transiently expressed a pCAMBIA1300-35S-EGFP: *HvRHF9870* fusion protein in *N. benthamiana* using agroinfiltration. The subcellular localization result showed that the green fluorescence signal generated by *HvRHF9870* overlapped with the

fluorescence signals of nucleus and membrane (Fig. 6). The findings indicate that *HvRHF9870* was a nucleus-and plasma membrane-localized protein, suggesting that it may function in both compartments.

Silencing *HvRHF9870* in barley attenuates the plant's resistance to *P. graminea*

To further explore the role of *HvRHF9870* in resistance to *P. graminea* infection in barley, we performed BSMV-mediated VIGS technology. The results indicated that the system induced photobleaching symptoms and BSMV: γ and BSMV: *HvRHF9870* displayed mild chlorotic mosaic symptoms on the third or fourth leaves at 14 dpi after inoculation (Fig. 7A). qRT-PCR analysis demonstrated that the expression of *HvRHF9870* was significantly reduced compared with BSMV: γ plants (Fig. 7B). After silencing *HvRHF9870* in ZDM02422 and inoculation with *P. graminea*, the leaves of WT and BSMV: γ plants only developed slight lesions. In contrast, the BSMV: *HvRHF9870* plants showed significant lesions development, and the gene expression level was also significantly decreased (Fig. 7C). As expected, the BSMV: *HvRHF9870* plants showed markedly increased disease lesion area compared with WT and BSMV: γ plants at 7 dpi (Fig. 7D). Consistent with this, fungal biomass was markedly increased in silenced plants (Fig. 7G). Histochemical staining analysis revealed that cell death and H₂O₂ accumulation were significantly increased in BSMV: *HvRHF9870* plants compared with the WT and BSMV: γ plants (Fig. 7E). The SPAD values in BSMV: *HvRHF9870* plants were significantly lower than that in WT and BSMV: γ at all time points ($P < 0.05$) (Fig. 7F). These findings demonstrate that silencing *HvRHF9870* compromises disease resistance and exacerbates oxidative damage.

Silencing of *HvRHF9870* reduced the activity of protective enzymes in barley under the infection of *P. graminea*

To determine whether the silencing of *HvRHF9870* will affect the activities of antioxidant enzymes in barley under the infection of *P. graminea*, the activities of SOD, POD, CAT, the content of H₂O₂ and callose deposition, and electrolyte leakage were measured in the WT, BSMV: γ , and BSMV: *HvRHF9870* plants at 3, 5, and 7dpi. The results indicated that the enzyme activities of SOD, POD, and CAT in BSMV: *HvRHF9870* plants significantly decreased compared with WT and BSMV: γ plants

(Fig. 8A, B, C). Consistent with the impaired antioxidant defense, the callose accumulation in BSMV: *HvRHF9870* plant was significantly lower than that in WT and BSMV: γ ($P < 0.05$) (Fig. 8E). In contrast, the content of H_2O_2 and electrolyte leakage in BSMV: *HvRHF9870* plants increased continuously with time, which were significantly higher than those in WT and BSMV: γ plants at the same point ($P < 0.05$) (Fig. 8D, F). Taken together, these results demonstrate that silencing *HvRHF9870* compromises both the antioxidant enzyme system and callose-mediated physical defense, leading to excessive H_2O_2 accumulation, aggravated oxidative damage, and increased electrolyte leakage, thereby reducing the disease resistance of barley under pathogen infection.

Identification of *HvRHF9870*-interacting proteins by yeast two-hybrid screening

To better elucidate the regulation mechanism of *HvRHF9870* in disease resistance, we performed Y2H screens using a cDNA library derived from *P. graminea*-infected barley. A total of eight candidate proteins were identified to interact with *HvRHF9870*, including ubiquitin-conjugating enzyme UBC28 (NCBI Reference Sequence: XM_045100444), chaperonin HSP60 (NCBI Refseq: XM_045100115), nucleoside diphosphate kinase (NDPK, NCBI Refseq: XM_045115948), NADP-malic enzyme (NADP_ME, NCBI Refseq: XM_045111252), deubiquitinating enzymes (DUB, NCBI Refseq: XR_006611033), POD (NCBI Refseq: XM_045114035), UDP-glucuronate decarboxylase (UGD, NCBI Refseq: XM_045126063), and histone H2B (NCBI Refseq: XM_045107366).

To investigate the potential interaction modes of *HvRHF9870*, molecular docking was performed with the eight proteins identified through the screening procedure as its putative interacting partners (Fig. 9). The docking results suggested that the eight candidate proteins differed substantially in their ability to form stable complexes with *HvRHF9870*, and this variability was closely associated with the degree of interfacial complementarity in the predicted docking poses. Among all tested proteins, *HvPOD* exhibited the strongest binding capacity toward *HvRHF9870*, with a docking energy of -14.9 kcal/mol, indicating the formation of a highly stable complex. In addition, *HvUBC28* and *HvNDPK* also showed favorable binding to *HvRHF9870*, with

predicted energies of -5.1 and -5.2 kcal/mol, respectively, supporting their potential as credible HvRHF9870-interacting proteins. By comparison, HvHSP60, HvNADP_ME, and HvDUB displayed much weaker docking scores (-0.5, -0.4, and -1.6 kcal/mol, respectively), suggesting relatively unstable associations. However, HvUGD and HvH2B yielded positive binding energies, indicating that these proteins are unlikely to bind HvRHF9870 efficiently.

Consistent with the docking scores, the predicted interaction interfaces further supported the differential binding behavior among the candidate proteins. HvPOD, HvUBC28, and HvNDPK formed observable interfacial contact regions with HvRHF9870, whereas HvHSP60 and HvUGD did not display clear interaction sites in the predicted complexes. The remaining proteins showed either weakly supported or energetically unfavorable docking conformations. Together, these findings indicate that HvPOD is the most likely binding partner of HvRHF9870 among the proteins tested, while HvUBC28 and HvNDPK may also interact with HvRHF9870 with moderate affinity.

To validate the pairwise interactions identified by Y2H, the full-length coding sequences of *HvRHF9870* and each candidate interactor were cloned into the pGBKT7 and pGADT7 vectors, respectively, and co-transformed into the yeast strain Y2HGold. All co-transformants grew on SD/-Leu/-Trp media. Crucially, only yeast cells co-expressing *HvRHF9870* and a candidate protein were able to grow on SD/-Leu/-Trp/-His medium (Fig. 10). Furthermore, VIGS-mediated silencing of *HvRHF9870* led to a significant reduction in POD activity in barley leaves following pathogen challenge. This finding suggests that *HvRHF9870* may positively regulate POD activity, at least in part, through direct interaction with these candidate proteins.

Discussion

Gene expression regulation and post-translational modification are widely recognized as crucial mechanisms enabling multicellular organisms to adapt evolutionarily to changing environments (Meng et al. 2023). The UPS is an evolutionarily conserved regulatory mechanism for intracellular protein proteolysis, and E3 is a crucial component in the ubiquitination pathway (Lyzenga and Stone 2012). RING-type E3

ubiquitin ligase is defined by a cysteine-and histidine-rich domain that can form two zinc-binding domains. RING H2 finger proteins constitute a broadly distributed eukaryotic family whose members act as E3 ubiquitin ligases, catalyzing the ubiquitin modification of specific proteins to route them to 26S proteasomal degradation (Mao et al. 2024). Previous research has shown that RING-type E3 ubiquitin ligase plays a role in modulating plant responses to disease resistance (Zhou and Zeng 2017). For example, OsBBI1, OsRING80, OsRING113, OsRGLG5, OsRGLG6, and OsZFP1 positively regulate rice immunity against *M. oryzae* or *X. oryzae* (Li et al. 2011; Zhang et al. 2024; Liu et al. 2023; Chen et al. 2025; Yang et al. 2025), however, OsDjA6, OsRFPH2-6, OsRFPH2-10, OseIF5A4, OsATL32, and OsJMJ715 negatively regulate rice immunity against *M. oryzae* (Zhong et al. 2018; Wang et al. 2022; Liu et al. 2014; Wang et al. 2025; Yan et al. 2024; Zhang et al. 2021). Moreover, the RING finger genes were widely distributed among plants, including *Arabidopsis* (Stong et al. 2005), *Brassica rapa* (Alam et al. 2017), soybean (Aguilar-Hernandez et al. 2011), cotton (Ghulam et al. 2018), *Ostreococcus tauri* (Gao et al. 2016), and wheat (Zhang et al. 2024). However, a comprehensive analysis of RING-H2 finger genes has not been reported in barley. In this study, *HvRHF9870* was identified from the transcriptome and functionally analyzed using VIGS. The results indicated that *HvRHF9870* plays an important role in the defense response to *P. graminea* infection, thereby deepening our understanding of the molecular mechanisms underlying barley disease resistance.

In recent years, an increasing number of *RHF* genes have been reported to function in many plants, including rice (Bai et al. 2019), poplar (Johnny et al. 2010), *Arabidopsis* (Kim et al. 2024), pepper (Lee et al. 2011), cotton (Miao et al. 2024), and soybean (Fan et al. 2025). In this study, *HvRHF9870* exhibited distinct expression patterns during infection with *P. graminea*, suggesting that *HvRHF9870* plays a crucial role in defense against *P. graminea*. Our results indicated that *HvRHF9870* exhibited the highest relative expression at 14 dpi, and subcellular localization analysis confirmed its localization to the nucleus and plasma membrane, which are key sites for immune signaling transduction (Wang et al. 2006). We determined that *HvRHF9870* had a RING-H2 finger domain. We also showed that *HvRHF9870* had an E3 ubiquitin ligase

activity and the conserved RING domain was necessary for its activity and localization. These observations align with studies in cotton where *RH2FE3* genes were strongly upregulated in response to phytohormones and abiotic stresses (Ghulam et al. 2018), Similarly, the RING-H2 E3 ligases APIP6 and APIP10 mediate resistance to *M. oryzae* (Park et al. 2012; Park et al. 2016).

The incompatible interaction between plants and pathogens triggers a hypersensitive reaction (HR) to limit the growth of the pathogen, which is a local defense, caused primarily by the production and accumulation of ROS (Ge et al. 2018). H₂O₂ can diffuse across membranes and act as a signal during cell wall synthesis and fortification associated with disease resistance (Jiang et al. 2020). Previous research showed that *TaWRKY31* gene silencing (BSMV: *TaWRKY31-Ias* and BSMV: *TaWRKY31-2as*) resulted in decreased activities of SOD, POD, and CAT (Ge et al. 2024). Silencing *TaCDPK27* induced excess contents of ROS and decreased the activities of SOD, POD, and catalase CAT (Yue et al. 2022). *TaAnn12*-silenced plants can accelerate the removal of ROS and lead to a decrease in disease resistance level of silent plants (Shi et al. 2023). Silencing *TaGPX3.2A* by VIGS led to reduced resistance of wheat to *Fusarium graminearum*, indicating that *TaGPX3.2A* plays a crucial role in enhancing wheat resistance against *F. graminearum* (Jiang et al. 2023). BSMV: *OsMED16*- and BSMV: *OsMED25*-silenced plants reduced the resistance to *Magnaporthe grisea* by modulating the accumulation of H₂O₂ and expression of defense-related genes (Zhang et al. 2023). *SlPti5* silencing can induce accumulation of ROS by repressing the expression of genes involved in ROS scavenging and reducing their enzyme activity which led to impairing disease resistance to *Botrytis cinerea* (Tang et al. 2022). In this study, we observed accumulation of H₂O₂ in the *HvRHF9870*-silenced barley plants after *P. graminea* infection. Excessive H₂O₂ accumulation led to severe membrane damage, as evidenced by the elevated electrolyte leakage and reduced SPAD values, indicating accelerated chlorophyll degradation and photosynthetic dysfunction. However, no significant differences in ROS accumulation were seen between the WT and BSMV: γ -silenced plants. Further study showed that SOD, POD, and CAT activities of WT and BSMV: γ -silenced plants were significantly higher than those of

BSMV: *HvRHF9870*-silenced plants within 3-7 dpi after inoculation, thereby impairing disease resistance to *P. graminea*. Furthermore, callose deposition in BSMV: *HvRHF9870*-silenced plants was drastically attenuated, suggesting impaired physical defense. Therefore, we speculated that the silencing of *HvRHF9870* delayed the response of antioxidant enzymes to the infection of pathogens. Previous work has also revealed that higher POD activity is more conducive to oxygen free radical scavenging under stress conditions. In addition, POD can participate in a variety of physiological metabolism and activity of peroxidase enzyme was inhibited in susceptible species. Here, the lower POD activity in *HvRHF9870*-silenced plants than WT and BSMV: γ -silenced plants might lead to the cell wall weakening which in turn promotes the pathogen penetration.

To further elucidate the molecular mechanism of *HvRHF9870*, we performed a Y2H screening and identified HvPOD as a direct interacting protein of *HvRHF9870*. Notably, this molecular interaction provides a mechanistic explanation for the remarkably reduced POD activity observed in *HvRHF9870*-silenced plants. As an important antioxidant enzyme, POD not only participates in ROS detoxification but also contributes to cell wall reinforcement and pathogen restriction. Therefore, the weakened interaction caused by *HvRHF9870* silencing may directly reduce POD stability or activity, leading to excessive ROS accumulation, aggravated oxidative damage, and ultimately compromised resistance in barley.

In summary, we identified *HvRHF9870* as a novel gene conferring resistance to leaf stripe in barley using transcriptome. Our VIGS data demonstrated that silencing *HvRHF9870* significantly reduced barley resistance to stripe disease. In addition, yeast two-hybrid screening identified several candidate *HvRHF9870*-interacting proteins, suggesting that *HvRHF9870* functions through a broader regulatory network rather than acting alone. Collectively, these findings indicate that *HvRHF9870* is an important positive regulator of barley defense and provide a foundation for further elucidating how its interaction network modulates immunity and disease susceptibility.

Author contributions

JW, HW and ES designed the experiments. JP wrote the first draft of the manuscript.

ES and CL revised and edited the manuscript. JP, MG and WY conducted the comparative transcriptomic analysis and identified *HvRHF9870*. JP, YM, XM and BL conducted the VIGS, subcellular localization, and enzyme activity assays. JP, XM, LY, HZ and KY performed the yeast two-hybrid screening. All authors read, revised, and approved the manuscript.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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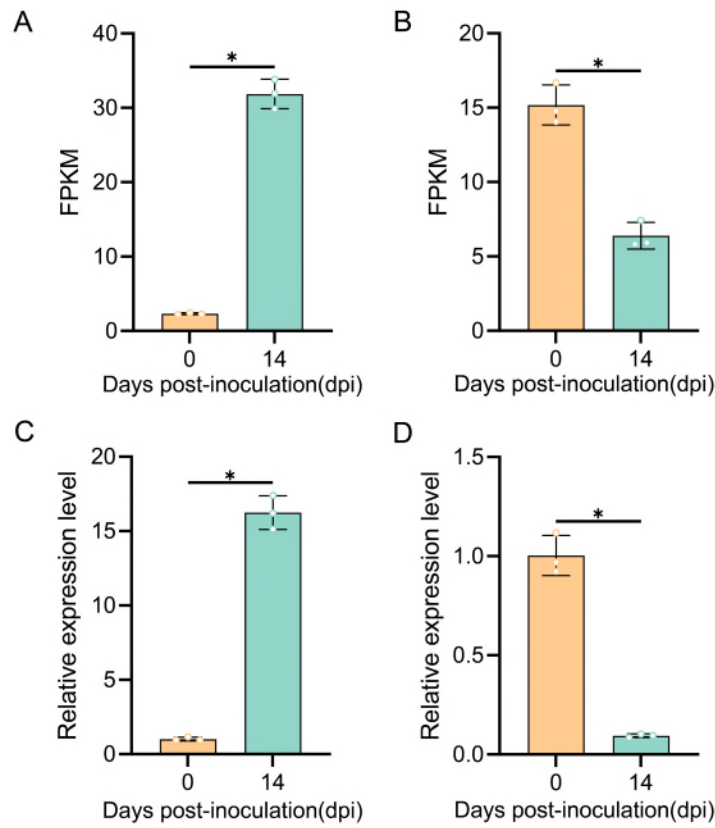


Figure 1. Validation of RNA-seq data by RT-qPCR in resistant and susceptible barley cultivars inoculated with *P. graminea* at 14 days post-inoculation (dpi). (A-B) The FPKM value of *HvRHF9870* in barley at 14 dpi in resistant (ZDM02422) and susceptible (ZDM04908) barley. (C-D) The relative expression levels were calculated by the $2^{-\Delta\Delta C_t}$ method in resistant (ZDM02422) and susceptible (ZDM04908) barley. Values are means $\pm$ SE of three independent experiments. Asterisks indicate significant differences from CK (0 h) ($P < 0.05$).

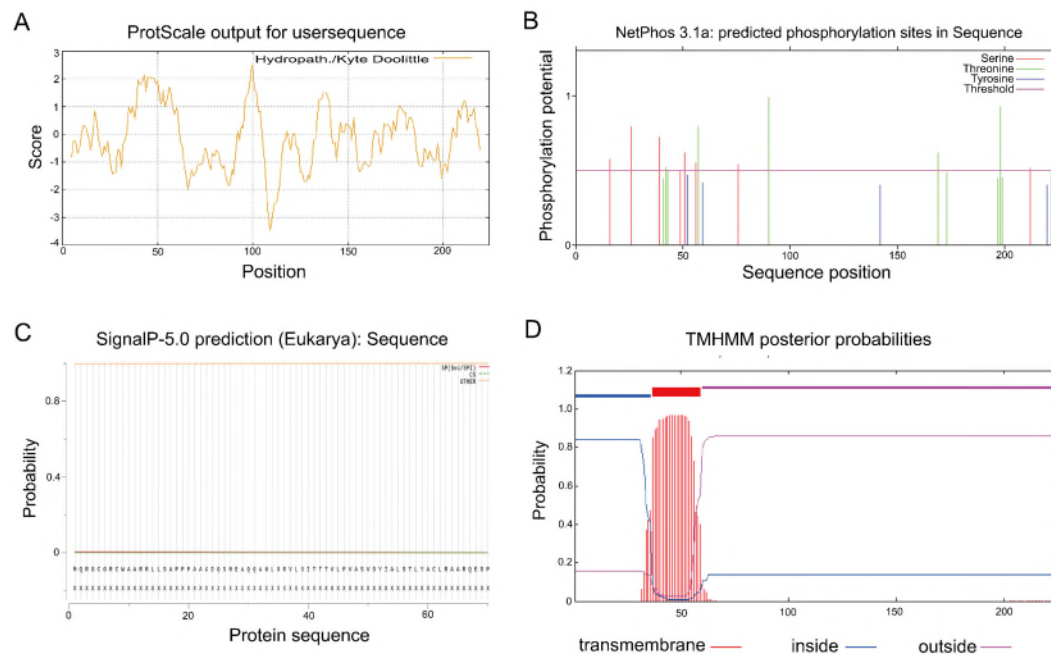


Figure 2. Prediction of hydrophobicity, phosphorylation, signal peptide, and transmembrane of the HvRHF9870 protein. (A) Prediction of hydrophobicity or hydrophilicity. (B) Phosphorylation site prediction of HvRHF9870. (C) Signal peptides and site prediction of HvRHF9870. (D) Prediction of transmembrane structural domains of HvRHF9870.

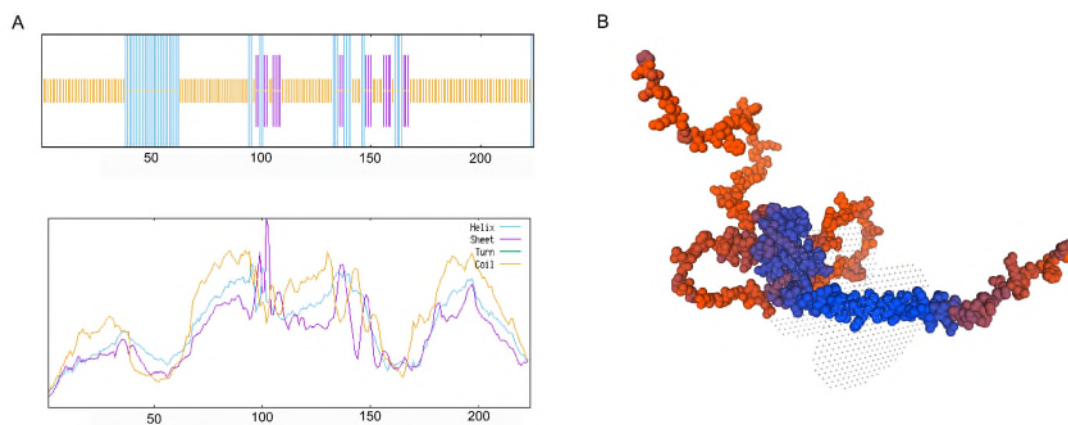


Figure 3. Secondary and tertiary structure predictions of the HvRHF9870 protein. (A) Secondary structure prediction of HvRHF9870, including alpha helix (blue), random coil (purple), beta turn (green), and extended strand (orange). (B) Three-dimensional structure prediction of HvRHF9870. The structural model was generated by homology modeling using SWISS-MODEL.

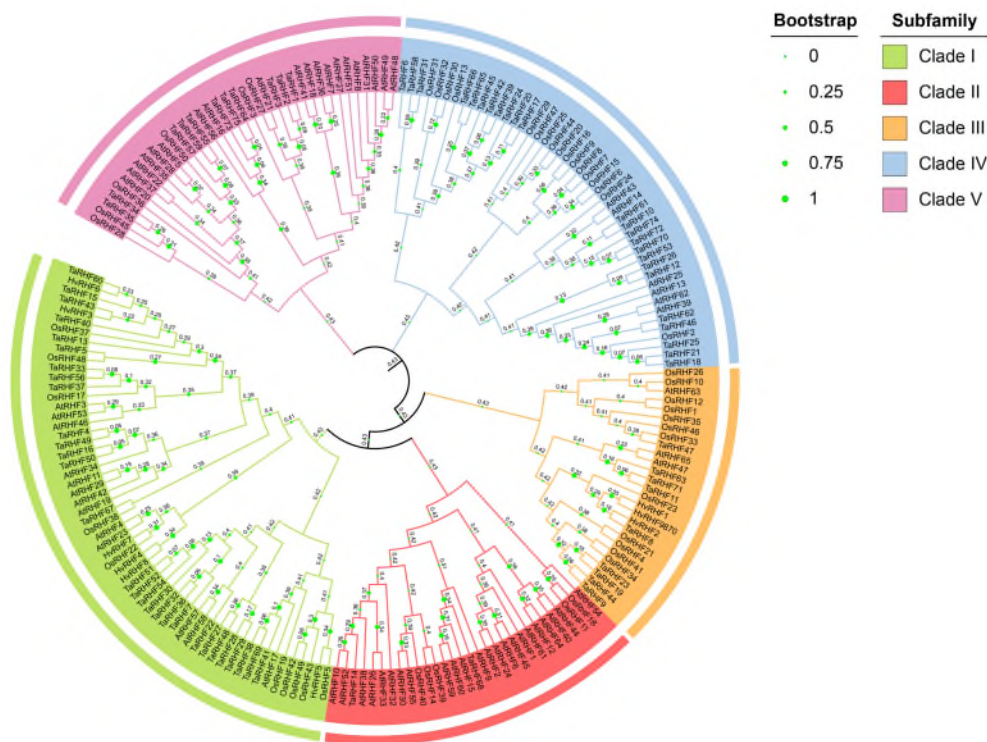


Figure 4. Phylogenetic tree of RHF proteins in *Hordeum vulgare*, *Triticum aestivum*, *Oryza sativa*, and *Arabidopsis thaliana*. Different subgroups correspond to different regional colors which are divided into ten groups. The phylogenetic tree was constructed by MEGA-X using the maximum likelihood method (1000 bootstraps).

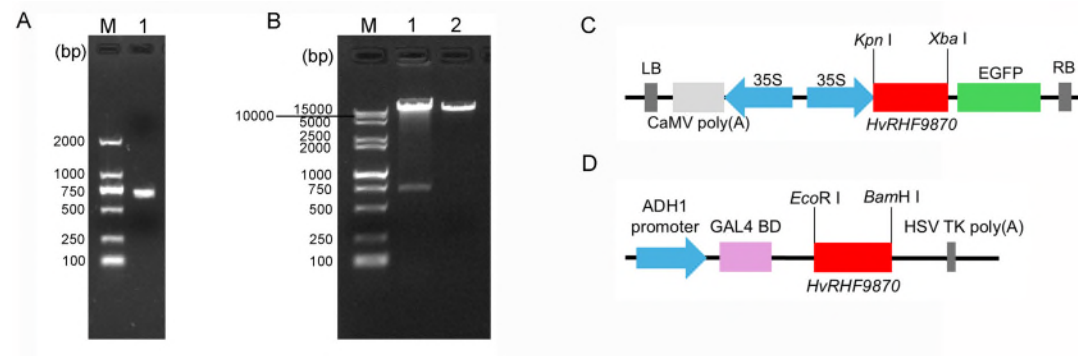


Figure 5. Cloning of the *HvRHF9870* gene, restriction enzyme digestion analysis, and construction of recombinant vectors. (A) PCR amplification of the *HvRHF9870* gene. M is DNA marker; lane 1 is amplified *HvRHF9870* fragment. (B) Restriction enzyme digestion analysis of the recombinant plasmid. M is DNA marker; lane 1 is recombinant plasmid digested with restriction enzymes; lane 2 is recombinant plasmid before digestion. (C) Schematic diagram of the plant expression vector pCambia1300: 35S: EGFP : *HvRHF9870*, which *HvRHF9870* was inserted into the vector between the *Kpn* I and *Xba* I sites under the control of the CaMV 35S promoter. (D) Schematic diagram of the bait vector construct for yeast two-hybrid analysis, which *HvRHF9870* was cloned into the pGBKT7 vector between the *Eco*R I and *Bam*H I sites to generate the pGBKT7: *HvRHF9870* fusion construct.

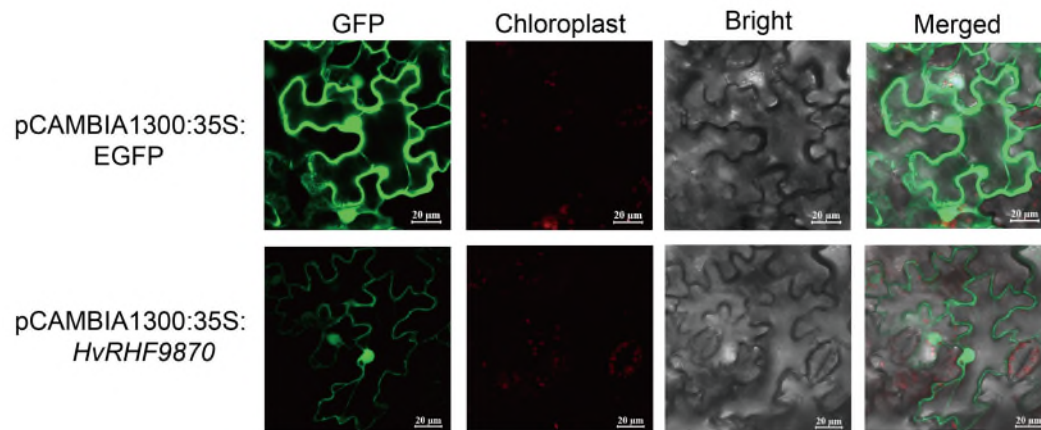


Figure 6. Subcellular localization of *HvRHF9870*. *Agrobacterium tumefaciens* carrying EGFP: *HvRHF9870* were infiltrated into *N. benthamiana* leaves of 3-4-week-old. Scale bars 20 μ m.

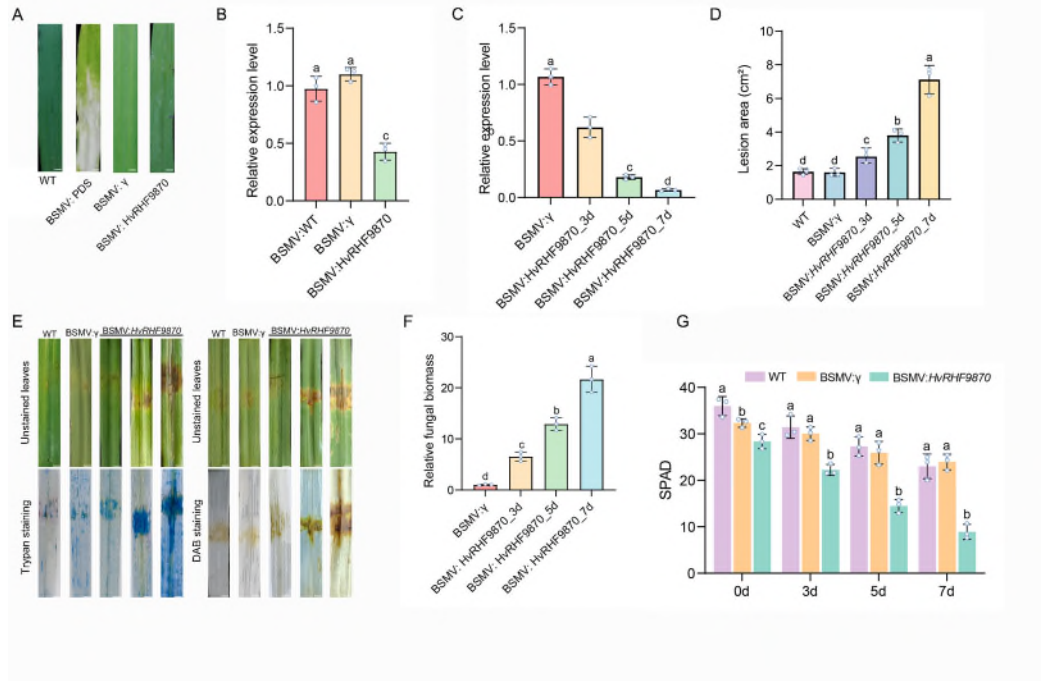


Figure 7. Effects of silencing the *HvRHF9870* gene using the virus-induced gene-silencing (VIGS) technique. (A) Mild chlorotic mosaic symptoms were observed on leaves inoculated with BSMV: PDS at 14 days post-inoculation (dpi). Bars = 0.5 cm. (B) The relative transcript levels of WT, BSMV: γ , and BSMV: *HvRHF9870* at 0 dpi. Data are means $\pm$ SE of three independent biological replicates. Different lowercase letters above the columns indicate significant differences ($P < 0.05$). (C) The relative transcript levels of WT, BSMV: γ , and BSMV: *HvRHF9870* at 3, 5, and 7dpi. Data are means $\pm$ SE of three independent biological replicates. Different lowercase letters above the columns indicate significant differences ($P < 0.05$). (D) The lesion area of WT, BSMV: γ , and BSMV: *HvRHF9870* plants after *P. graminea* inoculation at 3, 5, and 7dpi. Data are means $\pm$ SE of three independent biological replicates. Different lowercase letters above the columns indicate significant differences ($P < 0.05$). (E) Pathogenic phenotypic analysis of uninfected leaves (CK), wild-type (WT), BSMV: γ , and BSMV: *HvRHF9870* on barley leaves at 3, 5, and 7dpi by trypan and DAB staining. Bars = 0.5 cm. (F) The soil and plant analyzer development (SPAD) of WT, BSMV: γ , and BSMV: *HvRHF9870* at 0 dpi. Data are the means $\pm$ SE of three independent biological replicates. Different lowercase letters represent significant differences ($P < 0.05$).

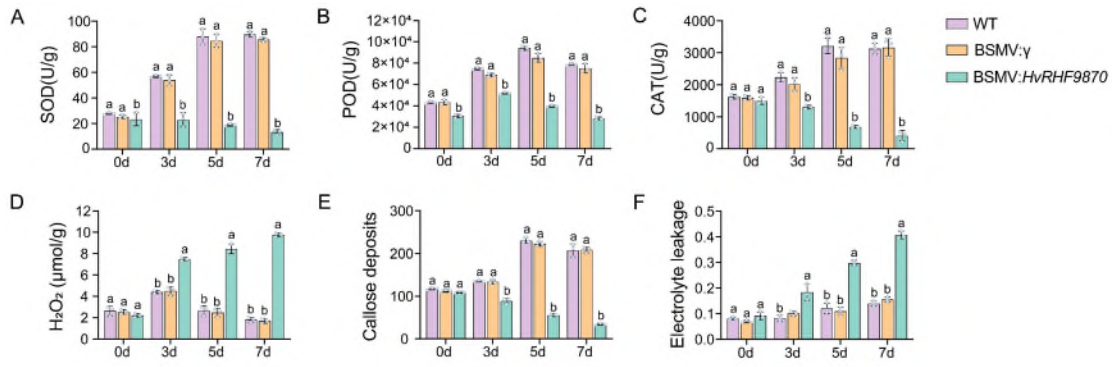


Figure 8. Changes in antioxidant enzyme activity after *P. graminea* inoculation at 3, 5, and 7dpi. (A-F) SOD, POD, and CAT activities, content of H₂O₂ and callose, and ion permeability in WT, BSMV: γ, and BSMV: *HvRHF9870* plants after *P. graminea* inoculation at 3, 5, and 7dpi. Data are the means ± SE of three independent biological replicates. Different lowercase letters represent significant differences (P < 0.05).

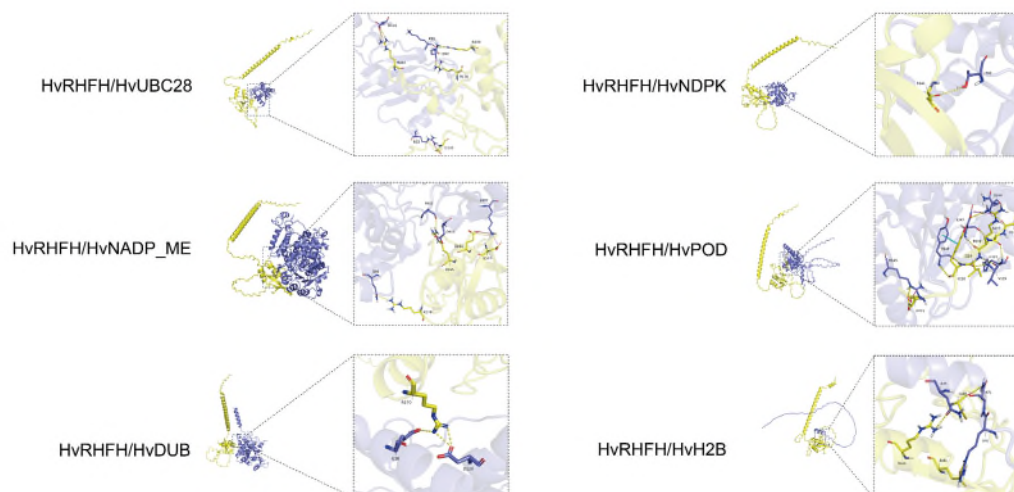


Figure 9. Molecular docking analysis of HvRHF9870 with candidate interacting proteins. Predicted molecular docking models are shown of HvRHF9870 with HvUBC28, HvNADP_ME, HvPOD, HvNDPK, HvDUB, and HvH2B. The overall docking conformations are displayed on the left, and enlarged views of the interaction interfaces are shown on the right. HvRHF9870 and its interacting partners are represented in different colors. The enlarged panels highlight the putative binding sites and key amino acid residues involved in the interactions.

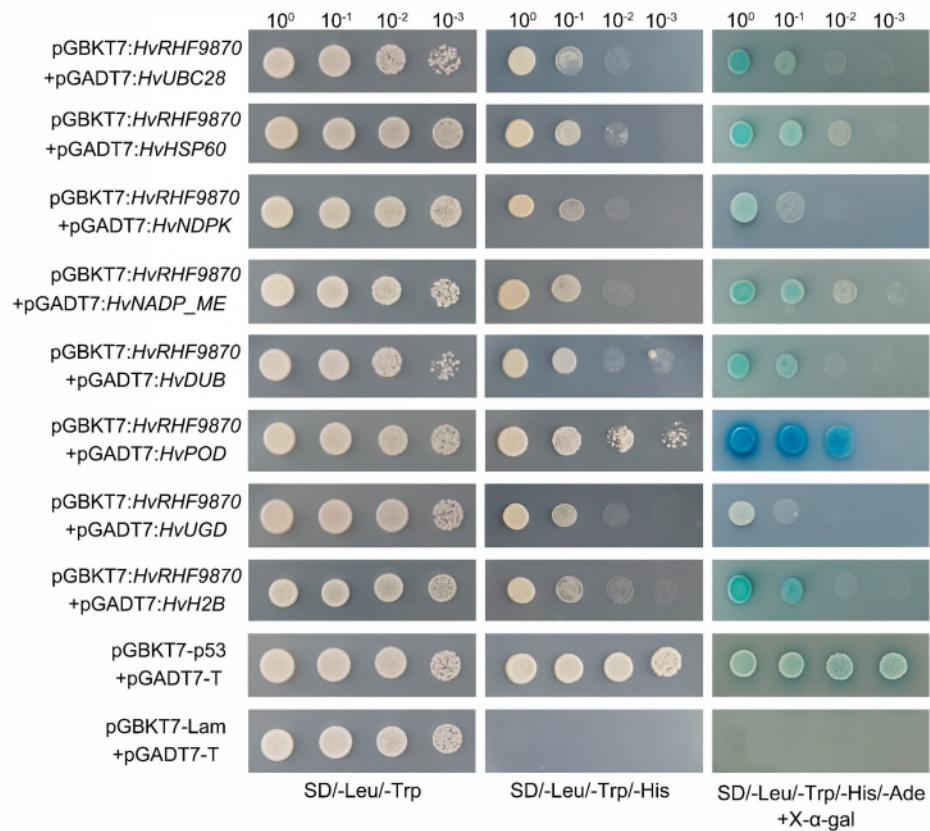


Figure 10. Interactions were evaluated on SD/-Trp/-Leu and SD/-Trp/-Leu/-His media. pGBKT7: *HvRHF9870* and pGADT7-candidate proteins were cotransformed into Y2H Gold to perform one-to-one verification. pGBKT7-p53+pGADT7-T served as the positive control, pGBKT7-Lam/pGADT7-T empty served as the negative controls.

Table 1 Primers used in this research

Primer name	Primer sequence (5'–3')	Application
<i>HvRHF-F</i>	ATGCAGCGAGGGTGCGGCAG	clone
<i>HvRHF-R</i>	TCACGGGTCCGTCATATATA	clone
<i>EGFP: HvRHF-Xba I-F</i>	CGGGGTACCATGCAGCGAGGGTGCG	Construction of the subcellular localization vector
<i>EGFP: HvRHF-Hind III-R</i>	GCTCTAGACGGGTCCGTCATATATATCC	Construction of the subcellular localization vector
<i>BSMV: HvRHF-F</i>	TTAACCACCACCACCGTCCTCTACGCCTGC CTCCG	Construction of the VIGS vector
<i>BSMV: HvRHF-R</i>	TCCGTTTCTAAGGAAGTTTAACGTGAGCAG CCAGCGGTC	Construction of the VIGS vector
<i>BD: HvRHF-EcoR I-F</i>	ATCTCAGAGGAGGACCTGATGGTTATGGTC GAACACCT	Construction of the Yeast two-hybrid assays
<i>BD: HvRHF-BamH I-R</i>	CCCAAGGGGTTATGCTAGCTAGCTTGGGCC CACGACAC	Construction of the Yeast two-hybrid assays
<i>BSMV: HvRHF-F</i>	GCCAAGAGAGGCCACCAA	qRT-PCR
<i>BSMV: HvRHF-R</i>	GTAGTCCTTGGCCCTCCAC	qRT-PCR
<i>HvActin-F</i>	GCTGACCGTATGAGCAAGGA	qRT-PCR
<i>HvActin-R</i>	GGAAAGTGCTGAGTGAGGCT	qRT-PCR

Note: The restriction enzyme sites included in the primers are listed underlined in the corresponding sequences.