

Identification and fine mapping of a novel leaf stripe resistance locus, QY-156, in the Tibetan hulless barley landrace Pengcuolinzongqingke

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Abstract

Leaf stripe disease, caused by the seed-borne fungal pathogen *Pyrenophora graminea*, poses a major threat to hulless barley production on the Qinghai–Tibet Plateau. In this study, a novel resistance locus, *QY-156*, was identified in the Tibetan hulless barley landrace Pengcuolinzongqingke. Bulk segregant analysis coupled with whole-genome resequencing (BSA-WGRS), combined with KASP marker-based fine mapping and pangenome analysis, localized *QY-156* to a 101-kb interval on chromosome 6H (533.393–533.494 Mb). Genetic analysis demonstrated that resistance is controlled by a single dominant gene. Expression analysis of candidate genes revealed that *HORVU.MOREX.r3.6HG0622460.1* was strongly induced in the resistant genotype following *P. graminea* infection, whereas *HORVU.MOREX.r3.6HG0622470.1*, encoding a GLR2.8-like protein, was significantly upregulated in the susceptible parent. Bioinformatic and structural analyses indicated that the protein encoded by *HORVU.MOREX.r3.6HG0622470.1* is a glutamate receptor-like protein containing conserved PBP1_GABAb_receptor_plant, GluR_Plant, and Lig_chan domains, with predicted roles in signal transduction. These findings suggest that *QY-156* represents a previously unreported resistance locus and that multiple genes within this interval may coordinately contribute to the regulation of stress responses. The tightly linked molecular markers and candidate genes identified in this study provide valuable resources for marker-assisted selection and map-based cloning of resistance genes in hulless barley.

1. Introduction

Hulless barley (*Hordeum vulgare* var. *coeleste*), commonly known as naked barley, is the principal staple crop of the Qinghai–Tibet Plateau, accounting for approximately 95% of the region's barley cultivation area (Kang et al. 2024). Owing to its early maturity and exceptional adaptation to high-altitude environments, hulless barley plays a crucial role in regional food security. However, barley leaf stripe disease, caused by the seed-borne fungal pathogen *Pyrenophora graminea* (anamorph: *Drechslera graminea*), represents a major constraint to production. Under severe epidemic conditions, yield losses can reach 10–25% (Hu et al. 2020). Since its first report in eastern Tibet in the late 1970s (Cui 1984), the disease has spread extensively and continues to threaten agricultural productivity and food security across the Plateau.

Barley leaf stripe disease is primarily transmitted through fungal mycelia located between the pericarp and seed coat (Haegi et al. 2008), with infection initiated during seed germination. Although chemical seed treatments are widely used for disease management, their long-term application may adversely affect the fragile ecological environment of the Qinghai–Tibet Plateau. Developing resistant hulless barley cultivars therefore represents a more sustainable and environmentally friendly strategy. Host resistance can reduce dependence on fungicides, lower production costs, and improve yield stability (Yang et al. 2020), thereby contributing to both ecological sustainability and agricultural productivity.

Elucidating the genetic basis of resistance is fundamental for the development of resistant barley cultivars. To date, several major resistance genes have been identified. The first resistance gene, *Rdg1a*, was mapped to chromosome 2H in the cultivar Vada and co-localized with the powdery mildew resistance gene *MILa* derived from *Hordeum laevigatum* (Thomsen et al. 1997). Subsequently, *Rdg1a* was also identified in wild barley (*Hordeum spontaneum*) (Biselli et al. 2010). Another resistance gene, *Rdg2a*, was fine-mapped to chromosome 7H using high-resolution genetic mapping (Bulgarelli et al. 2004), while *Rdg3*, a distinct resistance gene located on chromosome 7HS, was later characterized by Si et al. (2019). These studies highlight the genetic diversity underlying barley resistance to leaf stripe disease and provide valuable resources for resistance breeding.

In addition to major resistance genes, numerous quantitative trait loci (QTLs) associated with leaf stripe resistance have been identified. Using biparental mapping populations, (Pecchioni et al. 1996) detected a partial resistance QTL on chromosome 7H, whereas (Arru et al. 2002, 2003) identified a major QTL on chromosome 2H, a minor QTL on chromosome 7H, and several isolate-specific QTLs on chromosomes 2H and 3H. More recently, genome-wide association studies (GWAS) have revealed additional markers and genomic regions linked to resistance (Si et al. 2015, 2019a). Collectively, these findings demonstrate the complex genetic architecture of leaf stripe resistance and provide a foundation for marker-assisted breeding.

Research on leaf stripe resistance in hulless barley remains relatively limited. Using BSR-seq analysis of a Kunlun 14 × Z1141 population, (Yang 2021) identified *HvnRGA* on chromosome 5H and *HvnWAK* on chromosome 6H as candidate defense-related genes. More recently, (Tan et al. 2025) integrated BSA-SNP and RNA-seq analyses in an F₃ population derived from Teliteqingke × Duli Huang and identified two candidate resistance genes, *HORVU.MOREX.r3.3HG0232110.1* and *HORVU.MOREX.r3.3HG0232410.1*, on chromosome 3H. These studies have provided important insights into the genetic mechanisms underlying leaf stripe resistance in hulless barley.

To identify additional resistance resources, we previously screened a diverse collection of hulless barley germplasm, including Tibetan landraces, wild accessions, and international cultivars (Hou et al. 2024; Kang et al. 2024). Among these materials, the landrace Pengcuolinzongqingke exhibited a high level of resistance to *P. graminea*. To investigate the genetic basis of this resistance, Pengcuolinzongqingke was crossed with the susceptible landrace Duli Huang to generate a segregating population for genetic analysis. In the present study, we analyzed the inheritance of leaf stripe resistance and employed bulk segregant analysis coupled with whole-genome resequencing (BSA-WGRS) to identify genomic regions associated with resistance. Kompetitive Allele-Specific PCR (KASP) markers were subsequently developed to construct a high-resolution genetic linkage map and refine the target interval. The physical position of the mapped region was validated through BLASTN alignment against the Barley Pan-genome v2. Candidate genes were further evaluated using reverse transcription quantitative PCR (RT-qPCR), and the candidate gene *HORVU.MOREX.r3.6HG0622470.1* was cloned for bioinformatic analyses, including protein structure and subcellular localization prediction.

The objectives of this study were to identify and map the resistance locus in Pengcuolinzongqingke, characterize candidate genes associated with resistance, and develop tightly linked molecular markers for breeding applications. The results provide new insights into the genetic basis of barley leaf stripe resistance and offer valuable genetic resources for map-based cloning, marker-assisted selection, and the development of resistant hulless barley cultivars. Given that only a few resistance genes, including *Rdg1a*, *Rdg2a*, and *Rdg3*, have been characterized to date, the identification of novel resistance loci remains essential for broadening the genetic basis of resistance and accelerating barley improvement.

2. Materials and Methods

2.1 Plant and Fungal Materials

The resistant hulless barley landrace Pengcuolinzongqingke (Qingyong 156), obtained from the National Crop Germplasm Resource Bank in Xining, Qinghai, China (36°43' N, 101°44' E), was crossed with the susceptible landrace Duli Huang from Gannan, Gansu Province, China (33°06'–36°10' N, 100°46'–104°44' E), to generate 140 F₃ lines. Duli Huang was used as the susceptible control in disease resistance evaluations.

The pathogenic strain B54 (GenBank accession no. PV444317.1), isolated from a barley field in Hualong County, Qinghai Province, China (36°9.66' N, 102°9.82' E) in 2023, was identified based on morphological characteristics, ITS phylogenetic analysis, and Koch's postulates, and was shown to be highly virulent. This isolate was subsequently used for inoculation of the mapping population and disease resistance assessments.

2.2 Assessment of Disease Resistance

Greenhouse resistance evaluations were conducted according to the method described by (Hou et al. 2025). The virulent isolate B54 was cultured in potato dextrose broth (PDB) at 25°C with continuous shaking until vigorous mycelial growth was obtained. Seeds of the parental lines and duplicate F₃ populations were inoculated by soaking them in 20 mL of fungal suspension for 48 h prior to sowing. The inoculated seeds were then transplanted into 5 × 5 cm pots and maintained under controlled greenhouse conditions with a 12 h light/20°C and 12 h dark/12°C photoperiod. Disease symptoms were assessed 25 days after sowing according to the disease rating scale described by Wang et al. (2024), where 0 = immune and 4 = highly susceptible. Disease incidence was calculated as: Disease incidence (%) was calculated as the percentage of infected plants relative to the total number of plants evaluated.

2.3 DNA Library Construction and Whole-Genome Re-Sequencing

Bulked segregant analysis (BSA) was performed to identify the genomic region associated with leaf stripe resistance in the hulless barley landrace Pengcuolinzongqingke. Genomic DNA was extracted from the parental lines and F₃ individuals using the CTAB method. Equal amounts of DNA from 15 resistant and 15 susceptible F₃ lines were pooled to construct the resistant (BulkR) and susceptible (BulkS) bulks, respectively. DNA libraries were prepared following fragmentation, end repair, A-tailing, adapter ligation, and size selection of 300–400 bp fragments. The libraries were sequenced on the Illumina HiSeq X Ten PE150 platform by GeneDenovo Biotechnology Co., Ltd. (Guangzhou, China; <https://www.genedenovo.com>, accessed on 15 August 2024). The barley reference genome MorexV3 was used for sequence alignment (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_904849725.1/).

Raw sequencing reads were quality-filtered using FASTP by removing reads containing ≥ 10% ambiguous bases (N), poly-A sequences, adapter contamination, or > 50% low-quality bases (Q ≤ 20). Clean reads were aligned to the MorexV3 reference genome using BWA-MEM (v0.7.16a-r1181) (Li and Durbin 2009). Alignment files were subsequently sorted and processed using SAMtools (v1.16.1) (Li and Durbin 2009), and sequencing coverage was assessed with BEDtools (v2.30.0) (Quinlan and Hall 2010). Single-nucleotide polymorphisms (SNPs) and insertion–deletion variants (InDels) were identified using GATK (v2.2.1) (DePristo et al. 2011) and functionally annotated with ANNOVAR (Wang et al. 2010).

2.4 BSA analysis

To identify genomic regions associated with leaf stripe resistance in the hulless barley landrace Pengcuolinzongqingke, bulked segregant analysis coupled with whole-genome resequencing (BSA-WGRS) was performed using four complementary statistical approaches: SNP-index, G-statistic (Gst), Euclidean distance (ED), and Fisher's exact test. The SNP-index method (Fekih et al. 2013) was used to identify genomic regions exhibiting significant differences in allele frequencies between the resistant and susceptible bulks. The G-statistic approach (Magwene et al. 2011) evaluated allele frequency divergence based on likelihood-ratio statistics, whereas the ED method (Hill et al. 2013) quantified genetic differentiation between bulks using Euclidean distance. Fisher's exact test was applied to assess the statistical significance of differences in allele depth distributions between the two bulks. The integration of these complementary analytical methods improved the accuracy, reliability, and resolution of candidate interval identification.

2.5 Marker development and gene mapping

To refine the genomic interval associated with QY-156, KASP markers were developed based on informative SNPs identified from the BSA-WGRS analysis. Flanking sequences (200 bp) containing informative SNPs were extracted using the Galaxy platform within the Triticeae Toolbox (<https://galaxy.triticeaetoolbox.org/>, accessed on 25 November 2024). Kompetitive Allele-Specific PCR (KASP) markers were designed using PolyMarker (Wang et al. 2024) (<https://www.polymarker.info/>, accessed on 27 November 2024) and Primer Premier (<https://www.premierbiosoft.com/primerdesign/>, accessed on 27 November 2024). The designed markers were first screened for polymorphism between the parental lines, Duli Huang and Pengcuolinzongqingke. Markers exhibiting clear and reproducible clustering patterns were retained and subsequently validated in the F₃ population. Polymorphic markers showing reliable segregation were used for genotyping and linkage analysis. Genetic mapping was conducted in the Duli Huang × Pengcuolinzongqingke F₃ population to refine the target interval associated with leaf stripe resistance.

Genetic distances were calculated from recombination frequencies using the Kosambi mapping function (Kosambi 2016). Linkage analysis was performed with MAPMAKER 3.0 using a logarithm of odds (LOD) threshold of 3.0, and the resulting genetic map was visualized using MapDraw V2.1 (Liu and Meng 2003).

2.6 Validation of the Flanking Markers against the Barley Pan-genome v2

To validate the genetic localization, we performed BLASTN analysis of the flanking markers for the target gene interval against the barley pan-genome v2 (available at <https://graingenes.org/GG3/content/barley-pangenome-v2-released>) (Yao et al. 2022), using the following parameters: task blastn, eval 1e-05,

sc-match 2, sc-mismatch -3, gap-open 5, gap-extend 2, and low-complexity filtering using 'filter L;m'. The BLAST results confirmed the physical positions of the flanking markers in the pan-genome, thereby verifying the accuracy of the mapped interval.

2.7 Synteny Analysis of the Target Interval

To further validate the fine-mapped interval of *QY-156*, the sequences of the flanking KASP markers were aligned to the barley pan-genome v2 (Yao et al. 2022) using BLASTN. Alignment was performed with the following parameters: task = blastn, e-value = 1×10^{-5} , match score = 2, mismatch penalty = -3, gap opening penalty = 5, gap extension penalty = 2, and low-complexity filtering set to "filter L;m". The physical positions of the flanking markers were consistent across the pan-genome assemblies, providing independent validation of the candidate interval identified through genetic mapping.

2.8 RT-qPCR Analysis of Gene Expression in Response to *P. graminea* Inoculation

To investigate the transcriptional responses of candidate genes to *P. graminea* infection, gene expression analyses were performed using RT-qPCR. Two-week-old seedlings of the resistant hulless barley landrace Pengcuolinzongqingke and the susceptible control Dulihuang were inoculated with the virulent isolate B54, while mock-inoculated plants served as controls. All plants were maintained under controlled growth conditions with a 12 h light/12 h dark photoperiod at 20°C/12°C (day/night). Three independent biological replicates were included for each treatment, and each biological replicate was analyzed with three technical replicates.

Total RNA was extracted from leaf tissues using TRIzol reagent according to the manufacturer's instructions. First-strand cDNA was synthesized from DNase-treated RNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Beijing, China). The resulting cDNA samples were stored at -20°C until further analysis.

Candidate genes located within the fine-mapped interval were selected for expression analysis. Gene-specific primers were designed using Primer Premier 5 (<https://www.premierbiosoft.com/primerdesign>, accessed on 11 April 2025) and synthesized by Qinghai Kemeiyi Biotechnology (Xining, China). RT-qPCR was performed using TB Green® Premix Ex Taq™ II (TaKaRa, Beijing, China), with 18S rRNA serving as the internal reference gene. Each 20 µL reaction mixture contained 10.0 µL of 2× TB Green Premix, 0.8 µL of each primer (10 µM), 0.4 µL of ROX Reference Dye (50×), 2.0 µL of cDNA template, and 6.0 µL of RNase-free water. Amplification was conducted under the following conditions: an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Relative transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Qi et al. 2015).

2.9 Gene Cloning and Vector Construction

Using hulless barley leaf cDNA as the template, gene-specific primers containing *SacI* and *XbaI* restriction sites as well as homologous sequences to the vector were designed based on the principle of homologous recombination. The target gene was amplified using a 2× Hieff Canace® Gold PCR Master Mix under the following conditions: pre-denaturation at 98°C for 3 min; 35 cycles of 98°C for 15 s, 60°C for 20 s, and 72°C for 1 min; followed by a final extension at 72°C for 5 min. The PCR product was subsequently purified using a gel extraction kit. Meanwhile, the pCAMBIA2300-GFP vector was digested with *SacI* and *XbaI*, and the linearized fragment was purified. The purified PCR product was then recombined with the linearized vector using a homologous recombination system and incubated at 50°C for 20 min to complete the ligation. The recombinant product was transformed into *Escherichia coli* DH5α competent cells. Positive clones were screened on kanamycin-containing medium and verified by colony PCR and sequencing. Confirmed recombinant plasmids were subsequently introduced into *Agrobacterium tumefaciens* GV3101. Transformants were selected using double antibiotic screening and further confirmed by PCR, thereby completing gene cloning and vector construction.

2.10 Bioinformatics Analysis

The coding sequence of *HORVU.MOREX.r3.6HG0622470.1* was identified using the NCBI ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>). The physicochemical properties of the encoded protein, including molecular weight, theoretical isoelectric point (pI), and hydropathicity, were predicted using the ProtParam tool available on the ExPASy server (Wilkins et al. 1999). Protein secondary structure elements, including α-helices, extended strands, β-turns, and random coils, were predicted using the SOPMA program with default parameters (Geourjon and Deléage 1995). The three-dimensional (3D) structure of the protein was modeled using the SWISS-MODEL server under default settings, and the reliability of the generated model was evaluated based on standard structural assessment criteria (Waterhouse et al. 2018). Subcellular localization was predicted using WoLF PSORT with the organism type set to plant (Horton et al. 2007). Transmembrane helices were identified using the TMHMM v2.0 server (<https://services.healthtech.dtu.dk/services/TMHMM-2.0/>) (Krogh et al. 2001). Conserved domains and functional motifs were identified using the SMART database and the NCBI Conserved Domain Database (CDD), with an E-value cutoff of < 0.05, to classify the protein into its corresponding gene family (Letunic et al. 2015; Marchler-Bauer et al. 2015).

2.11 Subcellular Localization

Agrobacterium tumefaciens strain GV3101 carrying the recombinant plasmid was cultured in Luria-Bertani (LB) medium supplemented with 50 mg L⁻¹ kanamycin and 25 mg L⁻¹ rifampicin at 28°C with shaking at 200 rpm for 16–20 h. The bacterial culture was pelleted by centrifugation at 3,000 × g for 10 min at room temperature, washed twice with infiltration buffer (10 mM 2-(N-morpholino)ethanesulfonic acid [MES], 10 mM MgCl₂, 150 µM acetosyringone, pH 5.6), and resuspended to an optical density at 600 nm (OD₆₀₀) of 0.6–0.8. The suspension was incubated at room temperature for 2–3 h without shaking prior to infiltration. *Nicotiana benthamiana* plants were grown in a greenhouse at 22–25°C under a 16 h light/8 h dark photoperiod. Four-week-old plants were used for agroinfiltration. Prior to infiltration, plants were placed under white fluorescent light for 1 h to promote stomatal opening. The third and fourth fully expanded leaves from the top were selected for infiltration. The abaxial leaf surface was gently abraded with a needleless 1 mL syringe to disrupt the cuticular wax layer. The bacterial suspension was infiltrated into the abaxial side of the leaf by gently pressing the syringe plunger. After infiltration, plants were maintained under high humidity by covering with a transparent plastic dome and incubated in the dark for 16–20 h. The dome was removed the following day, and plants were returned to normal growth conditions for 48–72 h before sampling. For confocal microscopy, leaf discs (approximately 1 cm²) were excised from the infiltrated regions and mounted on glass slides. Fluorescence signals were examined using an FV10-ASW laser scanning confocal

microscope (Olympus, Tokyo, Japan). GFP fluorescence was excited at 488 nm and detected at 500–530 nm. Chlorophyll autofluorescence was excited at 488 nm and detected at 650–750 nm. DAPI staining was performed by infiltrating 1 µg mL⁻¹ DAPI solution into the leaf tissue 30 min prior to observation, with excitation at 358 nm and detection at 430–470 nm. Bright-field images were captured using differential interference contrast (DIC) optics. Images were processed and merged using FV10-ASW software (version 4.2, Olympus), and scale bars were added. Final images were exported in TIFF format.

2.12 Statistical Analysis

Chi-square tests were used to assess the goodness-of-fit of observed segregation ratios to expected Mendelian ratios. One-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc test was applied to evaluate differences in relative gene expression levels among treatments. Significance was determined at $P < 0.05$ and $P < 0.01$, respectively. KASP marker genotyping was performed using the KlusterCaller software (LGC Biosearch Technologies, UK). All statistical analyses were conducted using GraphPad Prism version 8.4.3 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1 Genetic Analysis of Leaf Stripe Resistance in Pengcuolinzongqingke

Genetic analysis of leaf stripe resistance was conducted using two independent F₃ progeny sets derived from the cross between resistant landrace Pengcuolinzongqingke and susceptible Duli Huang. Following inoculation with *P. graminea* strain B54, Pengcuolinzongqingke exhibited complete resistance, whereas Duli Huang showed full susceptibility (Fig. 1A). Segregation analysis of the first evaluation revealed 104 resistant and 36 susceptible families, fitting a 3:1 ratio ($\chi^2 = 0.010$, $P = 0.845$) (Table 1). This ratio was confirmed in the second evaluation ($\chi^2 = 0.086$, $P = 0.696$), demonstrating monogenic dominant inheritance. The resistance locus was designated QY-156.

Table 1
Genetic analysis of leaf stripe resistance gene in hulless barley landrace Pengcuolinzongqingke.

Parents and cross	Generations	Total number of plants/lines	No. of resistant plants/lines	No. of susceptible plants/lines	Expected ratio	χ^2	p -value
Duli Huang	P1			15			
Pengcuolinzongqingke	P2		15				
P1 × P2	F ₃	140	104	36	3 : 1	< 0.010	0.845
P1 × P2	F ₃	140	107	33	3 : 1	0.086	0.696

3.2 Confirmation of candidate intervals

BSA-WGRS generated 132.78 Gb and 130.94 Gb of high-quality clean data from the resistant and susceptible bulks, respectively. The Q30 scores were > 93.99%, and the GC contents ranged from 44.62% to 45.74%. Over 96% of reads from both bulks were aligned to the MorexV3 reference genome. A total of 26,369,638 variants were identified, and four complementary algorithms (Δ SNP-index, Gst, ED, and Fisher's exact test) were applied to detect candidate regions associated with leaf stripe resistance. The SNP-index, Gst, ED, and Fisher's exact test methods identified 12, 95, 96, and 8 candidate intervals, respectively. Integration of these approaches refined the candidate regions to three genomic intervals: one on chromosome 1H (3,200,001–4,400,000 bp), one on 6H (532,500,001–534,400,000 bp), and another on 7H (16,900,001–17,800,000 bp) in Pengcuolinzongqingke (Fig. 1B).

3.3 Fine mapping of QY-156

To fine-map the candidate interval of QY-156, we developed 23, 42, and 38 KASP markers on chromosomes 1H (3,200,001–4,400,000 bp), 6H (532,500,001–534,400,000 bp), and 7H (16,900,001–17,800,000 bp), respectively (Table S1). Genotyping of the parental lines and the F₃ mapping population revealed that only four KASP markers (NC-533393406, NC-533488613, NC-533491944, and NC-533494407) co-segregated within the 6H region (532,500,001–534,400,000 bp) (Table 2). These results indicate that the resistance locus QY-156 is located within this interval (Fig. 1C).

Table 2
KASP markers developed for Chromosome 6H Interval (532,500,001–534,400,000)

Marker	Forward sequence A(FP1)	SNP site B(FP2)	Forward sequence C(R)
NC-533393406	GAAGGTGACCAAGTTCATGCTcgcagcagattttctctccatc	GAAGGTGGAGTCAACGGATTcgcagcagattttctctccatt	atcagttccctgccgga
NC-533488613	GAAGGTGACCAAGTTCATGCTccccGaccgcggtattcg	GAAGGTGGAGTCAACGGATTccccGaccgcggtattca	caaccgCttCcgcttaC
NC-533491944	GAAGGTGACCAAGTTCATGCTgcattcctgccccgttca	GAAGGTGGAGTCAACGGATTgcattcctgccccgttcg	ccagggttgacaaac
NC-533494407	GAAGGTGACCAAGTTCATGCTccccaatctgaattacaagtctcc	GAAGGTGGAGTCAACGGATTccccaatctgaattacaagtctcg	aatgacgcgtgcatcgt

Genetic linkage analysis, integrating phenotypic data from F₃ plants with the four co-segregating KASP markers, delineated the position of QY-156. The locus was flanked by markers NC-533488613 and NC-533393406, at genetic distances of 0.086 cM and 0.166 cM, respectively. Consequently, QY-156 was fine-

mapped to a 0.256-cM interval, corresponding to a physical distance of approximately 101 kb on chromosome 6H, delimited by markers NC-533393406 and NC-533494407 (Fig. 1C).

3.4 Validation of the Genetic Interval on the Barley Pan-genome v2

BLASTN analysis of the flanking markers against the barley pan-genome v2 revealed significant alignments with high sequence identity (> 95.5%) across all 76 accessions (Table S2). The genomic positions of the flanking markers were consistently localized to chromosome 6H. Furthermore, analysis of annotated genes within this interval identified a core set of conserved genes across nearly all accessions, including a diacylglycerol kinase 3-like protein, an uncharacterized protein, and a glutamate receptor 2.8-like protein.

3.5 Synteny Analysis of the Target Interval

Synteny analysis revealed a high degree of gene order conservation in the target interval among the four accessions, and clear syntenic relationships were observed (Fig. 2). Specifically, the gene arrangement in the corresponding chromosomal regions was largely consistent across accessions, indicating that this region has remained structurally stable during evolution. Additionally, several small-scale structural variations were detected, such as local sequence rearrangements or deletions in certain accessions, which may underlie phenotypic differences among accessions.

3.6 Expression of the Candidate Genes in Response to *P. graminea*

The expression of three disease resistance-related candidate genes within the fine-mapped interval was investigated by RT-qPCR (Table 3). Transcript levels of *HORVU.MOREX.r3.6HG0622450.1* (encoding a diacylglycerol kinase, DGK) remained stable following *P. graminea* inoculation, whereas the other two genes displayed differential expression patterns. *HORVU.MOREX.r3.6HG0622460.1* (encoding an uncharacterized protein) was strongly induced in the resistant genotype Pengcuolinzongqingke, and *HORVU.MOREX.r3.6HG0622470.1* (encoding a glutamate receptor, GLR) was specifically upregulated in the susceptible parent Dulihuang (Fig. 3).

Table 3
Genes associated with disease resistance in the candidate interval of QY-156 and primers used for RT-qPCR.

Gene	Physical genomic location	Functional annotation	Forward primer (5'–3')	Reverse primer (5'–3')
<i>HORVU.MOREX.r3.6HG0622450.1</i>	Chr6H: 533392734..533399928	diacylglycerol kinase 3-like	GCCAGAGGGGAAATACAGAG	CCCAACGCTAAGGTAGTTGTAG
<i>HORVU.MOREX.r3.6HG0622460.1</i>	Chr6H: 533405390..533405973	uncharacterized protein	TTGCCCTTGTGTCGATG	GTGGCGGCAAGTTCACC
<i>HORVU.MOREX.r3.6HG0622470.1</i>	Chr6H: 533485184..533491234	glutamate receptor 2.8-like	GTTCCGGCTCATCTTCTACTTC	CCATATGATCACCACGAACCTC

3.7 Cloning and Vector Construction of *HORVU.MOREX.r3.6HG0622470.1*

Based on the principle of homologous recombination, the pCambia2300-GFP vector was used as the backbone to clone the target gene *HORVU.MOREX.r3.6HG0622470.1*. Gene-specific primers containing *SacI* and *XbaI* restriction sites and homologous sequences were designed, and the target gene (3042 bp) was successfully amplified by high-fidelity PCR (Fig. S1 A). The primer sequences used in this study are listed in Table 4. The purified PCR product was recombined with the *SacI/XbaI*-digested linearized vector backbone. The recombinant product was transformed into *Escherichia coli* DH5α competent cells. Colony PCR analysis of kanamycin-resistant transformants showed that lanes 1–8 all exhibited specific bands corresponding to the expected size of the target gene (Fig. S1 B), indicating a high positive rate. The confirmed recombinant plasmids were further introduced into *Agrobacterium tumefaciens* GV3101 competent cells. Following selection on medium containing kanamycin and rifampicin, colony PCR verification showed that lanes 1–4 produced specific bands of the expected size (Fig. S1 C), confirming the successful generation of recombinant *Agrobacterium* strains for subsequent experiments. These results demonstrate that the *HORVU.MOREX.r3.6HG0622470.1* gene was successfully cloned and the fusion expression vector was constructed, providing a solid foundation for subsequent subcellular localization and bioinformatic analyses.

Table 4
Primers used for gene cloning.

Primer name	Forward primer sequence(5'→3')	Reverse primer sequence(5'→3')
<i>HORVU.MOREX.r3.6HG0622470.1</i>	GAACACGGGGGACGAGCTCATGGTGGTGC GCGCGCTC	TCACCATGTCGACTCTAGACTGAAGTTGAGAGGCAGTTGGC
MC1-2300	AGAACACGGGGGACGAGCTC	ACCATGGTGTGCTGACTCTAGA

3.8 Bioinformatic analysis of the *HORVU.MOREX.r3.6HG0622470.1*.

Gene structure analysis showed that *HORVU.MOREX.r3.6HG0622470.1* contains three exons and two introns, with a cDNA length of 3,298 bp and a coding sequence (CDS) of 3,042 bp (Fig. 4A). Physicochemical analysis using the ExPASy ProtParam tool indicated that the encoded protein comprises 1,013 amino acids, with a theoretical molecular weight of 110.51 kDa and an isoelectric point (pI) of 6.18, classifying it as an acidic protein. The molecular formula is C₄₉₅₄H₇₆₉₆N₁₃₅₆O₁₄₆₆S₂₅, with a total of 15,497 atoms. The instability index was 43.06 (values < 40 indicate stability), suggesting that the protein is unstable. The aliphatic index is 83.32, and the grand average of hydropathicity (GRAVY) is - 0.085, indicating a hydrophilic protein (Fig. 4F). Secondary structure prediction revealed that the protein contains 400 α-helices (Hh), accounting for 39.49% of the structure; 147 extended strands (Ee), accounting for 14.51%;

and 466 random coils (Cc), accounting for 46.00% (Fig. 4C and D). Subcellular localization prediction using WoLF PSORT indicated that the protein is localized exclusively to the plasma membrane. The tertiary structure model showed multiple tightly folded domains, with the core region consisting of α -helices interconnected by random coils, forming a relatively stable spatial conformation. The N- and C-termini contain extended flexible regions that may participate in intermolecular interactions or regulatory functions, whereas a central transmembrane helical segment suggests membrane-associated localization and function (Fig. 4G). TMHMM-2.0 analysis predicted a clear transmembrane helical signal in the region spanning amino acids 600–700, suggesting that the protein contains at least one stable transmembrane domain. The overall topology indicates that most of the sequence is located either outside or inside the membrane, with the transmembrane segment anchoring the protein to the membrane, implying a potential role in membrane-associated transport or signal transduction (Fig. 4G). Conserved domain analysis using the SMART and NCBI CDD databases revealed three typical domains: an N-terminal PBP1_GABAb_receptor_plant domain (amino acids 50–444), a middle GluR_Plant domain (amino acids 478–823), and a C-terminal Lig_chan domain (amino acids 602–857) (Fig. 4B and H).

3.9 Subcellular Localization of *HORVU.MOREX.r3.6HG0622470.1*

To determine the subcellular localization of the protein encoded by *HORVU.MOREX.r3.6HG0622470.1*, the *HORVU.MOREX.r3.6HG0622470.1*-pCambia2300-GFP fusion construct was introduced into *Agrobacterium tumefaciens* GV3101. Subcellular localization was analyzed by agroinfiltration-mediated transient expression in *Nicotiana benthamiana* leaves. Green fluorescent protein (GFP) signals were observed using an FV10-ASW laser scanning confocal microscope (Olympus, Tokyo, Japan). Chlorophyll autofluorescence, nuclear DAPI staining, and bright-field (DIC) images were simultaneously captured and merged. GFP and chlorophyll autofluorescence were excited at 488 nm, and DAPI was excited at 358 nm. The GFP-specific fluorescence signal of the *HORVU.MOREX.r3.6HG0622470.1*-pCambia2300-GFP fusion protein was detected in both the nucleus and plasma membrane of tobacco epidermal cells, whereas the empty vector control showed diffuse cytoplasmic and nuclear localization (Fig. 5). These results indicate that the encoded protein is localized to the nucleus and plasma membrane.

4. Discussion

Barley resistance to leaf stripe disease is governed by complex defense mechanisms shaped by long-term co-evolution with *P. graminea* (Zhao et al. 2021). However, the genetic basis of resistance in Tibetan hulless barley landraces remains largely unclear. To date, only a single resistance locus has been reported in the landrace Teliteqingke (Tan et al. 2025), and its underlying molecular mechanism has not been elucidated. In this context, our study on the distinct landrace Pengcuolinzongqingke provides novel genetic insights into leaf stripe resistance and valuable resources for future molecular breeding.

Genetic analysis demonstrated that resistance in Pengcuolinzongqingke is controlled by a single dominant gene, consistent with Mendelian inheritance expectations. Using a BSA-seq strategy, we successfully mapped a resistance-associated locus to chromosome 6H, which validated the predicted genetic model. The robustness of BSA-seq in trait mapping has been widely demonstrated in multiple crops, including rapeseed (*Brassica napus* L.) (Neik 2019), chili pepper (*Capsicum annuum* L.) (Ban GL et al. 2025), maize (*Zea mays* L.) (Wu et al. 2025), rice (*Oryza sativa* L.) (Chen et al. 2024), tomato (*Solanum lycopersicum* L.) (Kong et al. 2025), and wheat (*Triticum aestivum* L.) (Wu et al. 2018), supporting the reliability of our mapping results.

To further refine the candidate interval, KASP marker technology was employed to construct a high-resolution genetic linkage map. As a powerful genotyping platform, KASP markers offer high accuracy, efficiency, and cost-effectiveness and have been widely applied in fine mapping of agronomic traits in wheat (Liu et al. 2024), soybean (*Glycine max*) (Zhang et al. 2025), melon (*Cucumis melo*) (Sun 2024), chili pepper, and Chinese cabbage (*Brassica rapa subsp. pekinensis*) (Zhang et al. 2025).

In addition, pangenome analysis further validated the reliability of the mapped region. BLASTN alignment of flanking markers against a barley pangenome comprising 76 accessions confirmed that all markers localized to a conserved region on chromosome 6H (pangenome v2). Synteny analysis further revealed strong structural conservation of this interval across diverse barley accessions, while also indicating the presence of natural variation, providing a structural basis for functional and evolutionary interpretation of this region.

Expression analysis of candidate genes revealed distinct transcriptional responses following *P. graminea* infection. Among the three genes examined, *HORVU.MOREX.r3.6HG0622450.1* (DGK) showed no significant change in expression, suggesting a limited role in resistance. In contrast, *HORVU.MOREX.r3.6HG0622460.1* exhibited a strong transcriptional response, being markedly induced in the resistant genotype Pengcuolinzongqingke, indicating a potential positive role in defense. However, this gene currently lacks clear functional annotation and conserved domain information, making it difficult to construct a testable mechanistic model. Therefore, its biological role remains uncertain when evaluated within a comprehensive framework that also considers structural features, annotation completeness, and experimental verifiability. Conversely, *HORVU.MOREX.r3.6HG0622470.1* (GLR2.8-like), which contains a typical GLR-like domain architecture (including GluR_Plant and Lig_chan domains) and transmembrane structural features, was specifically upregulated in the susceptible parent Duli Huang. This contrasting expression pattern suggests that its induction may be associated with pathogen response rather than effective resistance, or may reflect susceptibility-related signaling processes, such as pathogen-mediated manipulation of host signaling pathways. Importantly, given its well-defined GLR-like structural features and the well-documented roles of GLR family members in calcium signaling and stress responses, *HORVU.MOREX.r3.6HG0622470.1* provides a more biologically interpretable mechanistic hypothesis. It was therefore selected for functional validation based on a "structure–function interpretability-first" candidate gene prioritization strategy, rather than expression magnitude alone.

The GLR gene family has been implicated in multiple aspects of plant growth, development, and stress responses. In banana (*Musa nana* Lour.), GLRs play important roles in growth regulation and stress tolerance (Luo et al. 2023). Functionally, GLRs are involved in seed germination (Kong et al. 2015), root development (Singh et al. 2016), stomatal movement (Kong et al. 2015), pollen tube growth (Wudick et al. 2018), phototropism (Roy and Mukherjee 2017), light signaling (Hebda et al. 2022), and regulation of carbon/nitrogen balance (Kang and Turano 2003) as well as water homeostasis (Kang et al. 2004). In

stress responses, GLRs contribute to aluminum detoxification (Sivaguru et al. 2003), long-distance wound signaling (Shao et al. 2020; Yu et al. 2022), and tolerance to cold, drought, salt, and pathogen stresses through modulation of calcium signaling and hormonal pathways (Lu et al. 2014; Zheng et al. 2018; Wang et al. 2019; Li et al. 2019; Liu et al. 2021). Notably, GLRs have been shown to participate in both positive and negative regulation of stress responses, depending on context, tissue specificity, and signaling dynamics.

Consistent with these functional characteristics, *HORVU.MOREX.r3.6HG0622470.1* encodes a multi-domain receptor-like membrane protein containing PBP1_GABAb_receptor_plant, GluR_Plant, and Lig_chan domains, suggesting a role in ligand-gated ion channel activity and signal perception. Its predicted transmembrane structure and dual localization in the plasma membrane and nucleus further support a role in signal transduction between cellular compartments.

Taken together, although *HORVU.MOREX.r3.6HG0622460.1* showed stronger transcriptional correlation with disease resistance, *HORVU.MOREX.r3.6HG0622470.1* was selected for functional validation due to its conserved domain structure and strong signaling potential. These findings suggest that multiple genes within the *QY-156* locus may coordinately contribute to resistance, with *HORVU.MOREX.r3.6HG0622470.1* likely acting as a signaling-related negative regulator within this network.

Finally, the *QY-156* locus was mapped to a novel region on chromosome 6H (533.393–533.494 Mb), distinct from previously reported resistance loci. This study identifies *QY-156* as a new genetic resource for barley improvement. Future work using stable transformation and genome editing will be required to validate gene function and elucidate regulatory mechanisms.

5. Conclusions

This study identified a novel leaf stripe resistance locus, *QY-156*, in the Tibetan hulless barley landrace Pengcuolinzongqingke. Genetic analysis indicated that resistance is controlled by a single dominant gene. Through integration of BSA-seq, KASP-based fine mapping, and pangenome analysis, the locus was precisely localized to chromosome 6H (533.393–533.494 Mb). Candidate gene analysis revealed distinct expression patterns under *P. graminea* infection: *HORVU.MOREX.r3.6HG0622460.1* was significantly induced in the resistant genotype, suggesting a potential role in disease resistance, whereas *HORVU.MOREX.r3.6HG0622470.1* (GLR2.8-like) was upregulated in the susceptible genotype. Overall, *QY-156* represents a novel resistance locus in barley, and multiple genes within this region may contribute to stress response regulation. This study provides new insights into the molecular basis of hulless barley leaf stripe resistance and offers valuable genetic resources for marker-assisted selection and crop improvement.

Declarations

Conflict of interest

The authors declare that have no competing interests.

Author Contribution

Shenghua Kang, Zemin Tan, Sai Zhang, Liling Jiang, conducted the experiments; Shenghua Kang wrote the manuscript; Shenghua Kang, Zemin Tan, Sai Zhang, Yixiong Bai, analyzed the data and prepared the figures; Lu Hou conceived the project and designed the experiments; Lu Hou, Yixiong Bai, revised the manuscript. All authors approved the final manuscript and agreed on and consented to the publication in the journal.

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Figures

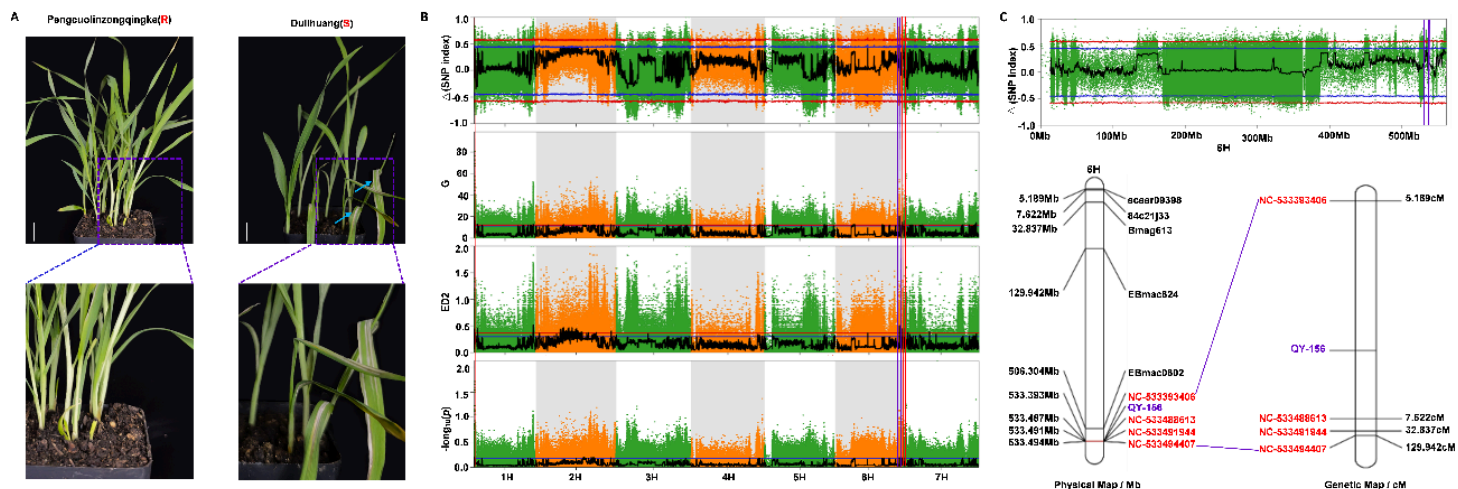


Figure 1 Genetic mapping of a leaf stripe resistance gene in hulless barley. (A) Phenotypic performance of the hulless barley landraces Pengcuolinzongqingke(R) and Duli Huang (S). Scale bars, 3 cm. (B) Genome-wide distribution of Δ SNP-index, G' value, ED value and Fisher test p -value across chromosomes. The positions marked on the graph represent three candidate intervals identified using the SNP-index, G-statistic, ED and Fisher methods. (C) Δ SNP-index distribution on chromosome 6H and mapping of QY-156 using F₃ families derived from the cross Pengcuolinzongqingke × Duli Huang.

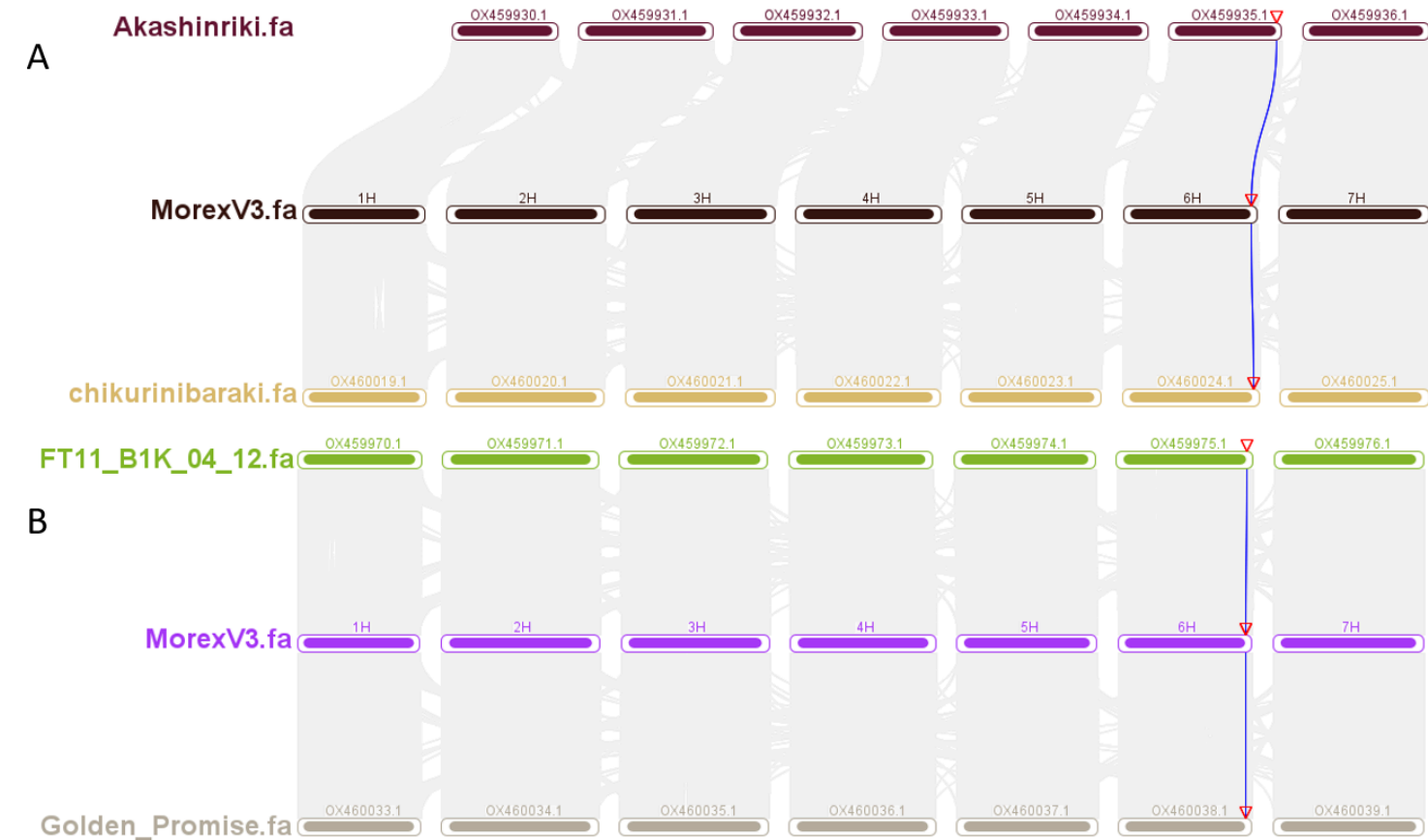


Figure 2 The collinearity analysis results between Morex V3 and a part of the Barley Pan-genome v2. **A:** chikurinibaraki and Akashinriki are landraces; **B:** Golden_Promise is a cultivated variety, and FT11 is a wild variety.

Note: The horizontal lines in different colors in the figure represent chromosomes, and the blue line indicates the synteny of genes within the related region.

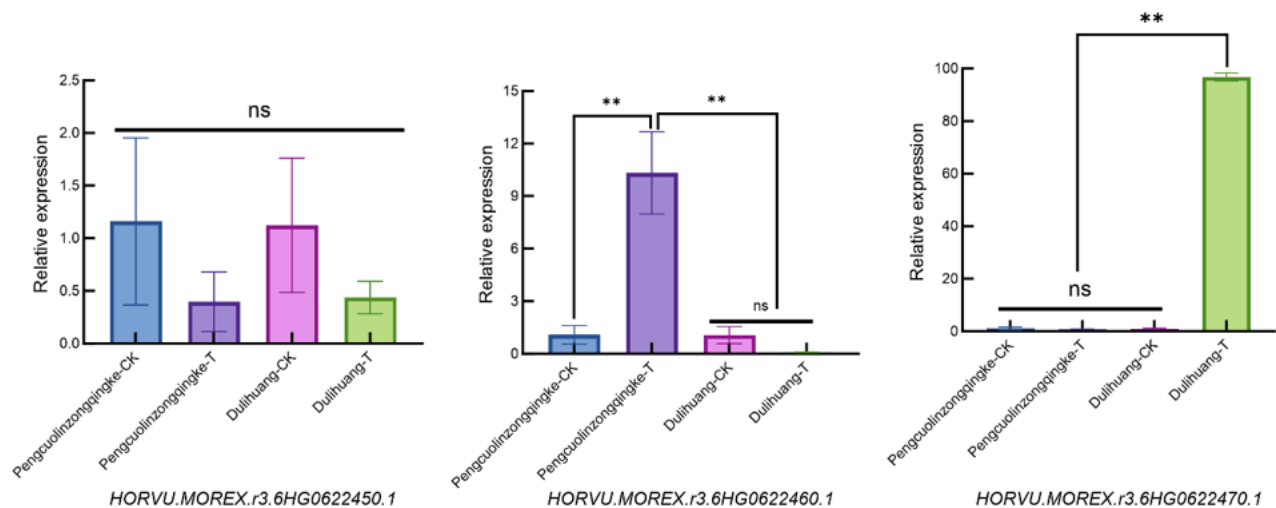


Figure 3

Relative expression of candidate genes in the landraces Pengcuolinzongqingke and Duli Huang.

Pengcuolinzongqingke-T and Pengcuolinzongqingke-CK represent the treatment and control groups for Pengcuolinzongqingke, while Duli Huang-T and Duli Huang-CK represent the treatment and control groups for Duli Huang, respectively. *: $P < 0.05$; **: $P < 0.01$; ns means no significant difference.

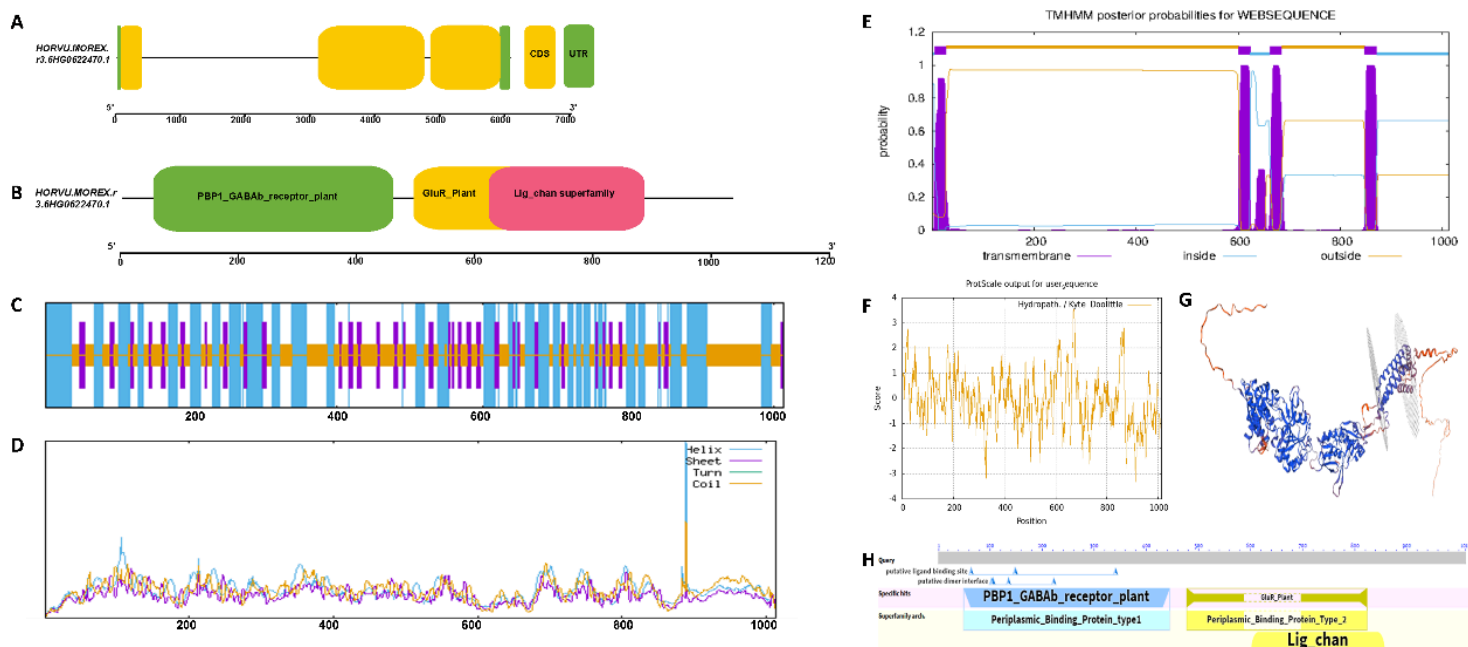


Figure 4

Bioinformatic characterization and structural prediction of *HORVU.MOREX.r3.6HG0622470.1*. The analysis includes gene structure (A), hydropathy profile (C), protein secondary structure prediction (D and E), three-dimensional structural model (F), transmembrane helix prediction (G), and conserved domain analysis (B and H). All analyses were performed using default parameters.

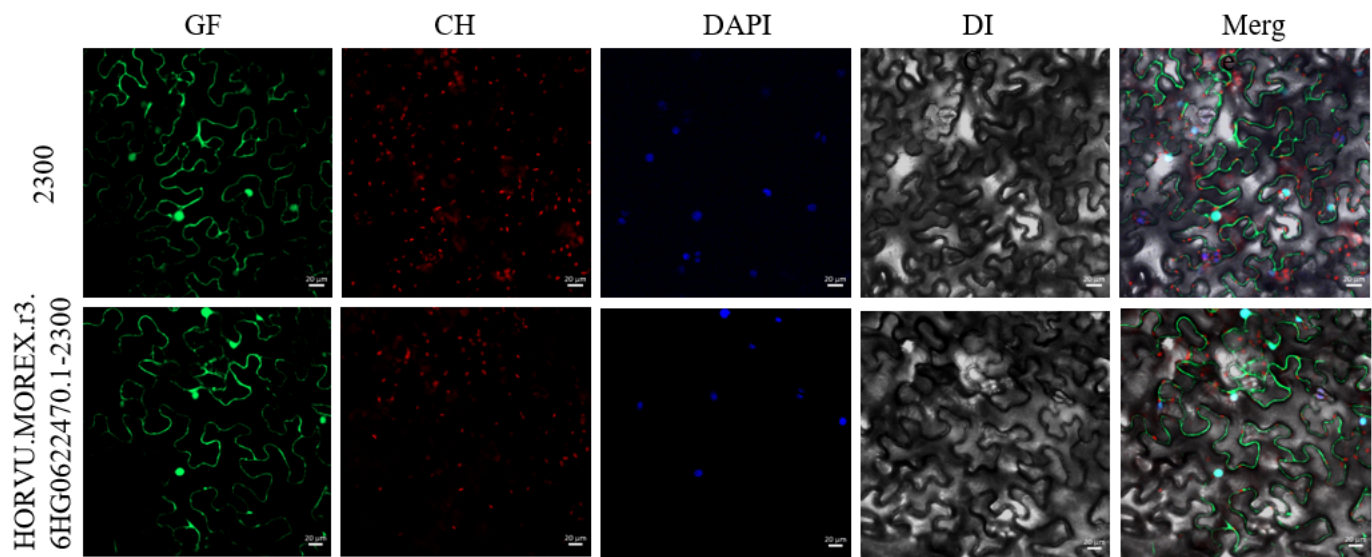


Figure 5

Subcellular localization of *HORVU.MOREX.r3.6HG0622470.1* in *Nicotiana benthamiana* leaves: Empty vector control (2300); *HORVU.MOREX.r3.6HG0622470.1-2300* fusion construct.

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