Rice

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# Natural Variation of *PH8* Allele Improves Architecture and Cold Tolerance in Rice

Cheng Chen[1,2†], Xia Zhang[1,3†], Jialin Chen[4†], Mingjia Xu[1], Weiying Zhao[1], Yangkai Wang[1], Zhuo Chen[4], Jiawei Xiong[1], Hua Yuan[1], Weilan Chen[1], Bin Tu[1], Ting Li[1], Liangzhu Kang[1], Shiwen Tang[1], Yuping Wang[1], Bingtian Ma[1], Shigui Li[1*] and Peng Qin[1*]

## Abstract

Empirical breeding efforts targeting cold tolerance and ideal plant architecture have significantly improved yield and facilitated the geographic expansion of *japonica* rice cultivation. However, the genetic drivers and underlying molecular mechanisms of these traits remain insufficiently understood. Here, we identify *Plant Height 8* (*PH8*) as a key gene regulating both plant stature and cold stress response in rice. Genome wide association analysis (GWAS), supported by functional validation, shows that loss of *PH8* reduces plant height without affecting other agronomic traits. Notably, we found that *PH8* also negatively regulates cold tolerance. A prevalent haplotype, $PH8^{Hap.0}$, exhibits reduced *PH8* expression due to natural variation in its promoter region, resulting in shorter plants and enhanced cold tolerance. Selective sweep and geographic distribution analyses indicate that $PH8^{Hap.0}$ originated in high-latitude regions and underwent strong directional selection during modern *japonica* improvement. Functional assays demonstrate that *PH8* enhances cold tolerance via improved reactive oxygen species (ROS) scavenging by repressing *APX2*, an antioxidant gene involved in ROS detoxification. Our findings reveal *PH8* as a dual regulator of plant architecture and cold stress adaptation, and highlight $PH8^{Hap.0}$ as a historically selected allele that contributed to the climatic adaptation and geographical expansion of *japonica* rice.

**Keywords** Rice, Plant height, Cold tolerance, GWAS, Selection

## Introduction

As one of the most crucial staple crops globally, rice (*Oryza sativa* L.) has undergone continuous improvement to meet human needs for over 10,000 years (Kovach et al. 2007; Zhang et al. 2024). During the mutual selection process between the environment and humans, diverse region-specific landraces have emerged, forming the genetic foundation for modern cultivated rice (Ma et al. 2025). In recent decades, advancements in breeding strategies, particularly hybrid breeding techniques, have greatly improved the agronomic traits of rice, improving yield and stress resistance (Li et al. 2020; Chen et al. 2023). However, traditional empirical breeding techniques are constrained by lengthy breeding cycles, significant uncertainties, and limited efficiency (Gao et al. 2024). Although these breeding programs have achieved agronomic success, the molecular basis for

†Cheng Chen, Xia Zhang and Jialin Chen have contributed equally to this work.

*Correspondence:
Shigui Li
lishigui@sicau.edu.cn
Peng Qin
qinpeng@sicau.edu.cn
[1] Rice Research Institute, Sichuan Agricultural University, Chengdu 611130, China
[2] Industrial Crop Research Institute, Sichuan Academy of Agricultural Science, Chengdu 610300, China
[3] Hainan Seed Industry Laboratory, Sanya 572025, China
[4] College of Horticulture, Center for Plant Metabolomics, Haixia Institute of Science and Technology, Fujian Agriculture and Forestry University, Fuzhou 350002, China

key traits such as cold tolerance and plant architecture, among others, remains underexplored. Elucidating the mechanisms that have historically shaped such traits will be critical for enabling more targeted and efficient advances in molecular breeding.

Temperature is a critical factor for crop growth and development, and the failure to adapt to low temperatures significantly limits both growth and yield (Ding et al. 2020; Wang et al. 2024). Exposure to low temperatures during the reproductive stage reduces rice grain protein content by 6% and decreases yield by up to 34% (Ma et al. 2024), resulting in substantial economic losses. Geng/*japonica* (GJ) rice, a subspecies of Asian cultivated rice, exhibits the widest distribution and has developed cold tolerance during its northward migration (Sang & Ge 2007). The alleles associated with rice cold tolerance have undergone natural and artificial selection for thousands of years, which has promoted agricultural development. A single-nucleotide mutation at *COLD1*, derived from the Chinese wild populations of *Oryza rufipogon*, confers cold tolerance in GJ rice and enables its growth in colder regions (Ma et al. 2015a, b). During the domestication of rice, *HAN1* diverged a specific allele in the promoter region, refining the conversion of biologically active jasmonic acid (JA) and modulating JA-mediated chilling responses (Mao et al. 2019). Additionally, *CTB4a*, *qPSR10*, *bZIP73*, *MPK3*, and *LEA6* were also proven to be involved in cold acclimation evolution of GJ rice (Liu et al. 2018, 2019; Xiao et al. 2018; Li et al. 2021; Lou et al. 2022) (Table S1). These genes contribute to cold resistance through mechanisms such as cytosolic $Ca^{2+}$ influx regulation, MAPK cascade activation, osmoprotectant accumulation (e.g., soluble sugars and proline), and ROS scavenging.

Exposure to low temperatures leads to excessive ROS accumulation, which can inhibit growth and induce cell damage or mortality (Vogel et al. 2005; Suzuki & Mittler 2006; Wang et al. 2022; Shen et al. 2024). Studies have demonstrated that genes involved in ROS scavenging contribute significantly to cold tolerance. For instance, *OsAPX2* and *OsAPXa*, encoding ascorbate peroxidases (APX), mitigate cold-induced oxidative stress by scavenging ROS (Sato et al. 2011; Zhang et al. 2013). Mutations in *SOP10*, encoding a mitochondrial pentatricopeptide repeat protein, reduce ROS accumulation and enhance cold tolerance under stress (Zu et al. 2023). Similarly, OsCYP20-2 interacts with OsFSD2 homodimers to enhance their enzymatic activity, thereby reducing ROS accumulation under low-temperature conditions (Ge et al. 2020).

In this study, we performed GWAS on plant height with heading date as a covariate and identified a plant height regulator *PH8*, which encodes a 2-oxoglutarate and Fe(II)-dependent dioxygenase (2OGD). Further analysis revealed that *PH8* plays a dual role in regulating both plant height and cold tolerance. The $PH8^{Hap.0}$ allele associated with reduced plant height and improved cold tolerance, originates from northern regions. It was unintentionally selected over the past few decades due to these advantageous traits. Our findings elucidate the molecular basis underlying *PH8* allele selection during rice empirical breeding, providing valuable genetic resources for coordinated optimization of plant height and cold tolerance in cultivated rice.

## Results

### GWAS Identifies *qPH8* as a Key Locus Influencing Plant Height

In a panel of 450 GJ rice accessions (Table S2) from Northeast China, the agronomic traits of plant height (PH) and heading date (HD) were investigated (Li et al. 2020). GWAS analysis for PH using General Linear Model (GLM) with HD as a covariate revealed a significant locus *qPH8* (Chr8: 27.9–28.1 Mb), which showed strongest signals across different years and locations (Fig. 1A and Fig. S1). Twenty-five candidate genes were identified in a 136 kb interval of *qPH8* (Fig. 1B and Table S3). Among them, *SPINDLY* (*SPY*, *LOC_Os08 g44510*) was found to be involved in rice architecture regulation through gibberellin (GA) signaling (Yano et al. 2019). Additionally, *LOC_Os08 g44590*, hereafter referred to as *PH8*, encodes a 2OGD with transcriptional activation activity in its N-terminal region and plays an essential role in rice shoot development (Zhang et al. 2020). The variation of *PH8* SNPs mainly lies in the promoter region, which contains two main alleles, $PH8^{Hap.0}$ and $PH8^{Hap.1}$ by haplotype analysis (Fig. 1C). Previous data (Li et al. 2020; Chen et al. 2023) show that Hap.0 accessions have significantly lower plant height than Hap.1 accessions (Fig. 1D). Additionally, Hap.0 accessions showed significantly lower transcript levels relative to Hap.1 accessions (Fig. S2 A & B), suggesting that *PH8* improves plant height.

### CRISPR Knockouts Confirm Functional Role of *PH8* in Growth Regulation

To identify whether *PH8* participates in plant height regulation, we generated knockout mutant lines *ph8-1* and *ph8-2*. Both mutants carry a 1 bp T insertion, while mutant *ph8-1* carries a 1 bp A insertion and mutant *ph8-2* carries a 1 bp C deletion, leading to frameshift mutations and premature termination of PH8 protein translation (Fig. 1E). Phenotypic evaluation revealed that both mutant lines exhibited a significant reduction in plant height compared to wild-type Daohuaxiang 2 (DHX2), whereas other agronomic traits, such as tiller number, panicle length, and seed-setting rate, remained

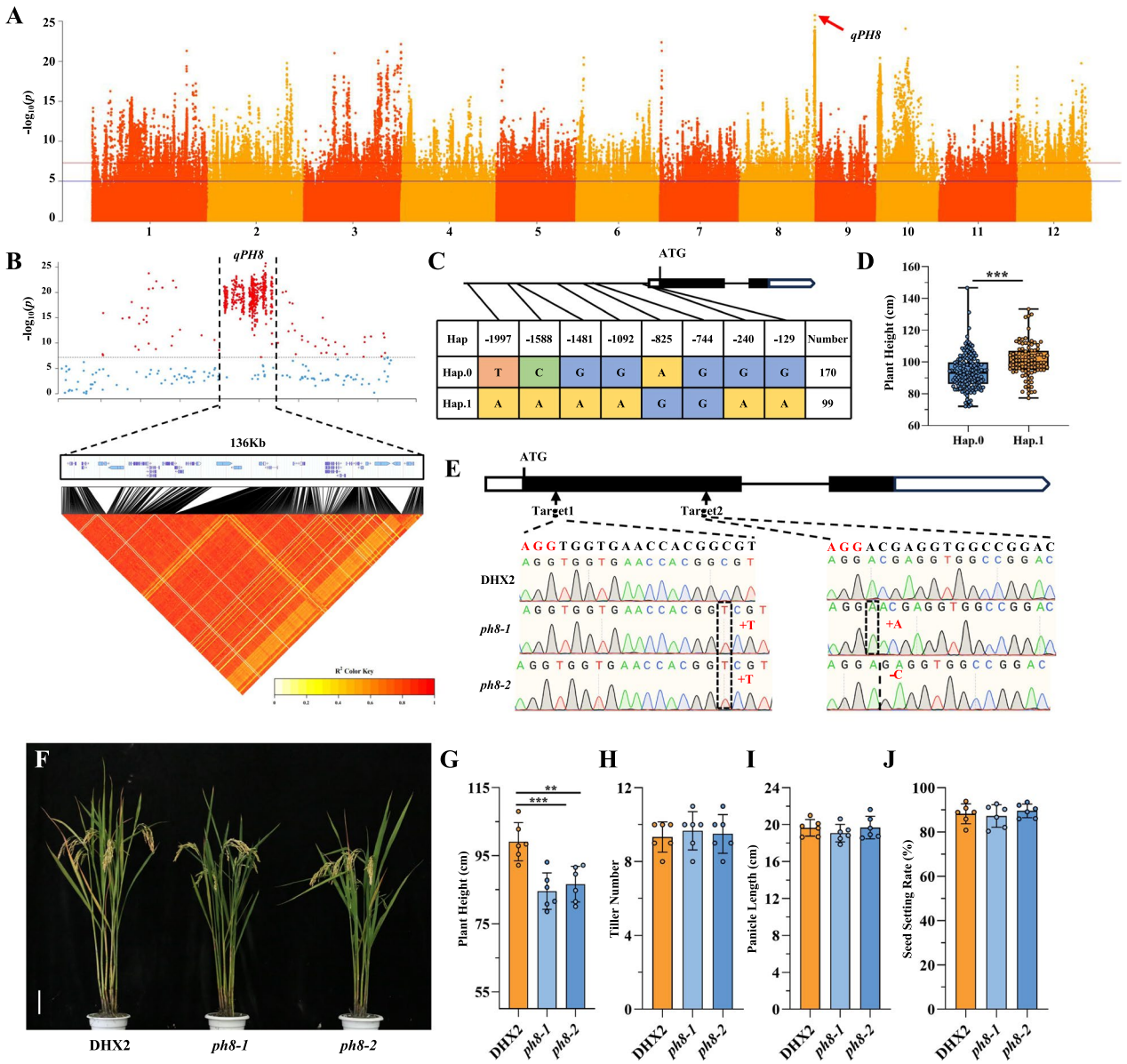


**Fig. 1** Identification of *PH8* for plant height by GWAS. **A** The Manhattan plot displays the GWAS results for plant height in WuChang in 2015. **B** Regional Manhattan plot of *qPH8* and pairwise LD analysis. Significant SNPs (-log10(*P*) ≥ 7.5) are presented as red dots. Blue boxes indicate annotated genes. **C** Major haplotypes (Hap) of *PH8* based on promoter and CDS region SNPs. **D** Plant height of Hap.0 and Hap.1 accessions. **E** CRISPR knockout targets of *ph8-1* and *ph8-2* mutant lines. The red font indicates the PAM sequence. **F** Phenotypes of DHX2 and *ph8* mutant lines at the reproductive stage. Scale bar = 10 cm. **G**–**J** Agronomic traits in DHX2 and *ph8* mutant lines. The plant height (cm) is represented by (**G**), tiller number is shown in (**H**), panicle length (cm) in (**I**), and Seed Setting Rate (%) in (**J**). The significance of difference was derived using two-tailed Student's t-test (***P* < 0.01, ****P* < 0.001)

unaffected (Fig. 1H–J). These findings establish *PH8* as a positive regulator of plant height in rice.

### *PH8*$^{Hap.0}$ is a Selected Allele Enhancing Cold Tolerance in *Japonica*

A total of 557 GJ rice accessions, including representative landraces and modern cultivars from major GJ cultivation regions, were used to investigate natural variations of *PH8* (Table S4) (Li et al. 2020). Haplotype analysis revealed that *PH8*$^{Hap.0}$ was predominantly present in modern cultivars, whereas *PH8*$^{Hap.1}$ was primarily found in landraces (Fig. 2A). To assess the adaptive relevance of these haplotypes, we examined previously reported seed-setting data under cold stress (Chen et al.

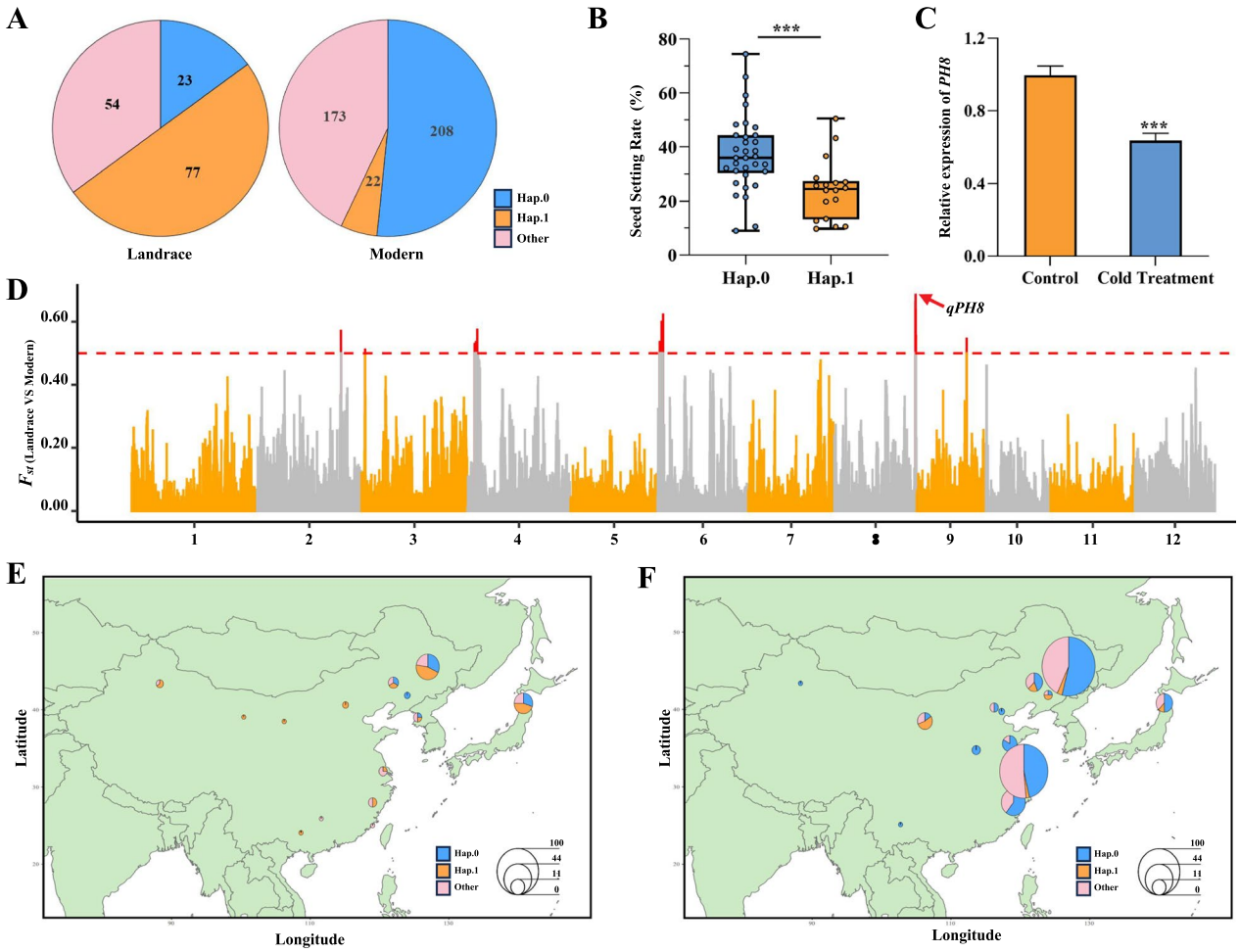


**Fig. 2** Geographic origins and selection of *PH8* in breeding. **A** The proportion of each haplotype in landraces and modern cultivars. **B** The seed setting rate after 5 days of cold retreatment in Hap.0 (n = 33) and Hap.1 (n = 18). **C** The expression level of *PH8* under cold treatment in DHX2 (n = 3). **D** Genome-wide distribution of selective-sweep signals (sliding windows = 100 kb) identified through comparisons between landraces and modern cultivars. The red dashed lines represent the thresholds (top 1% of $F_{ST}$ values). **E**, **F** Geographic distributions of Hap.0 and Hap.1 accessions in landraces and modern cultivars, respectively. Each pie chart represents the planting area of these accessions; the area size represents the number of accessions

2023). Accessions carrying $PH8^{Hap.0}$ exhibited a significantly higher seed setting rate compared to those harboring $PH8^{Hap.1}$ (Fig. 2B), suggesting a positive association between $PH8^{Hap.0}$ and enhanced cold tolerance. To further dissect the functional relevance of *PH8* under cold stress, we analyzed transcriptomic data from the rice RNA database (https://plantrnadb.com/ricerna/). Among the 25 candidate genes at the *qPH8* locus, 14 exhibited differential expression in Nipponbare (NIP) leaves under low-temperature stress conditions (Fig. S2 C). Notably, *PH8* expression was significantly downregulated following cold treatment. This observation was validated by similar downregulation patterns in DHX2 after cold stress (Fig. 2C). Based on these findings, we suppose that *PH8* is involved in the regulation of cold tolerance.

To investigate the selection dynamics underlying PH8 variation, selective sweep analysis was conducted using population fixation statistics ($F_{ST}$ values with 100 kb sliding window). A total of 37 regions with the top 1% of $F_{ST}$ values were identified as candidate divergence sweeps distinguishing landraces and modern cultivars (Table S5). The strongest signal region Chr8: 28.0–28.1Mb harbors *PH8* and overlaps with the previously identified GWAS candidate region *qPH8* (Fig. 2D). These results indicate that PH8 has undergone strong directional selection during *japonica* improvement. Geographic distribution analyses further revealed distinct spatial patterns associated with *PH8* haplotypes. In landrace accessions, Hap.0 accessions were exclusively present in high latitude northern regions, whereas Hap.1 distributed

broadly across multiple agroecological zones (Fig. 2E). In contrast, modern cultivars exhibited widespread distribution, with $PH8^{Hap.0}$ emerging as the predominant haplotype (Fig. 2F). Taken together, these results indicate that $PH8^{Hap.0}$ originated as a cold-adaptive allele in northern regions before modern breeding. Its prevalence in modern cultivars reflects natural selection in cold climates and unintentional selection for improved cold tolerance and plant architecture.

### Expression Patterns and Subcellular Localization of PH8 in Rice

The expression patterns of *PH8* were examined in various tissues of DHX2. PH8 was highly expressed in young roots, leaf sheaths, and elongating stems, with lower expression in reproductive tissues, such as young panicles, anthers, and caryopses (Fig. S3 A). Histochemical β-glucuronidase (GUS) staining of $promoter^{PH8}$::*GUS* transgenic lines corroborated these results, showing strong GUS signals in young roots, leaf sheaths, developing stems, and spikes, but no apparent GUS signals were observed in young panicles, anthers, and caryopses during development. These findings suggest that PH8 predominantly expresses in vegetative tissues. To determine the subcellular localization of PH8, a PH8-eGFP fusion protein was transiently expressed in rice protoplasts, revealing dual localization in both the cytoplasm and nucleus (Fig. S3P).

### *PH8* Negatively Regulates Cold Tolerance by Promoting ROS Accumulation

To investigate the effect of *PH8* on cold tolerance, we conducted cold stress treatment experiments (4℃ for six days). The *ph8* mutants exhibited significantly higher survival rates than wild-type plants, indicating enhanced cold tolerance (Fig. 3A, B). Since ROS accumulation is an early hallmark of cold stress responses (Suzuki and Mittler 2006; Wang et al. 2022; Zu et al. 2023), we evaluated ROS levels using Nitro blue tetrazolium (NBT, for $O_2^-$ detection) and 3,3'-diaminobenzidine (DAB, for $H_2O_2$ detection) staining. After 48 h of cold treatment at 4 °C, wild-type leaves exhibited strong NBT and DAB staining, indicative of high $O_2^-$ and $H_2O_2$ accumulation, respectively, whereas *ph8* mutants displayed significantly reduced ROS levels (Fig. 3C and D). Furthermore, peroxidase (POD) and superoxide dismutase (SOD) activities were markedly higher in *ph8* mutants compared to wild-type plants following 12 h of cold stress (Fig. 3E and F). Taken together, these results suggest that PH8 negatively regulates ROS scavenging under cold stress.

Previous studies proposed that PH8 possesses transcriptional activity (Zhang et al. 2020), which we validated using the yeast two-hybrid system. Yeast co-transformed with pGBKT7-*PH8* and pGADT7 survived and activated reporter expression on selective medium, supporting PH8's role as a transcriptional regulator (Fig. S4). To identify downstream targets of PH8-mediated regulation, we focused on *APX2*, which encodes a type of POD enzyme. After cold treatment, *APX2* expression levels were significantly higher in *ph8* mutant lines compared to wild-type plants (Fig. 3G), consistent with the previously observed increase in POD enzyme activity (Fig. 3E). We next employed a dual-luciferase reporter assay to examine the regulatory effect of PH8 on *APX2* promoter activity in rice protoplasts (Fig. 3H). Under normal temperatures, luciferase activity remained unchanged across constructs. Under cold stress, constructs co-expressing PH8 with *APX2* promoter exhibited significantly reduced luciferase activity, suggesting that PH8 suppresses *APX2* transcription in a temperature-dependent manner (Fig. 3I). These results suggest that PH8 represses the expression of *APX2* and impairs ROS-scavenging enzyme activity, thereby acting as a negative regulator of cold tolerance.

## Discussion

In this study, we identified the locus *qPH8*, through plant height-associated GWAS, using heading date as a covariate (Fig. 1A). This locus encompasses two tightly linked genes, *SPY* and *PH8* (Table S3; Fig. 1B), which exhibit similar haplotype distributions (Table S2), suggesting a potential history of co-selection during rice breeding. *SPY* encodes a known repressor of GA signaling (Yano et al. 2019). Its expression inversely correlates with plant height: low *SPY* expression leads to spindly plants, while high expression results in semi-dwarfism. In contrast, *PH8* (previously *SLC1*) does not participate in GA signaling; its knockout reduces plant height and overexpression induces a slender phenotype (Zhang et al. 2020; Fig. 1F, G). Notably, *ph8* mutant lines exhibit reduced plant height without affecting other key agronomic traits (Fig. 1F–J), making *PH8* a promising target for fine-tuning plant architecture. Despite their divergent molecular mechanisms, both *PH8* and *SPY* impact plant height, highlighting their convergence on the same agronomic trait and supporting the hypothesis of their co-selection during breeding.

At the biochemical level, both *PH8* and the classic semi-dwarf gene *SD1* encode enzymes belonging to the 2OGD superfamily. This enzyme family plays a central role in the oxidation and hydroxylation of plant hormones, including GA (Hedden and Sponsel 2015; Hedden and Thomas 2012; Li et al. 2018), auxin (Zhang et al. 2016), and salicylic acid (SA) (Zhang et al. 2017). *SD1* governs a key step in GA biosynthesis by converting $GA_{53}$ to $GA_{20}$, thus influencing seed dormancy and

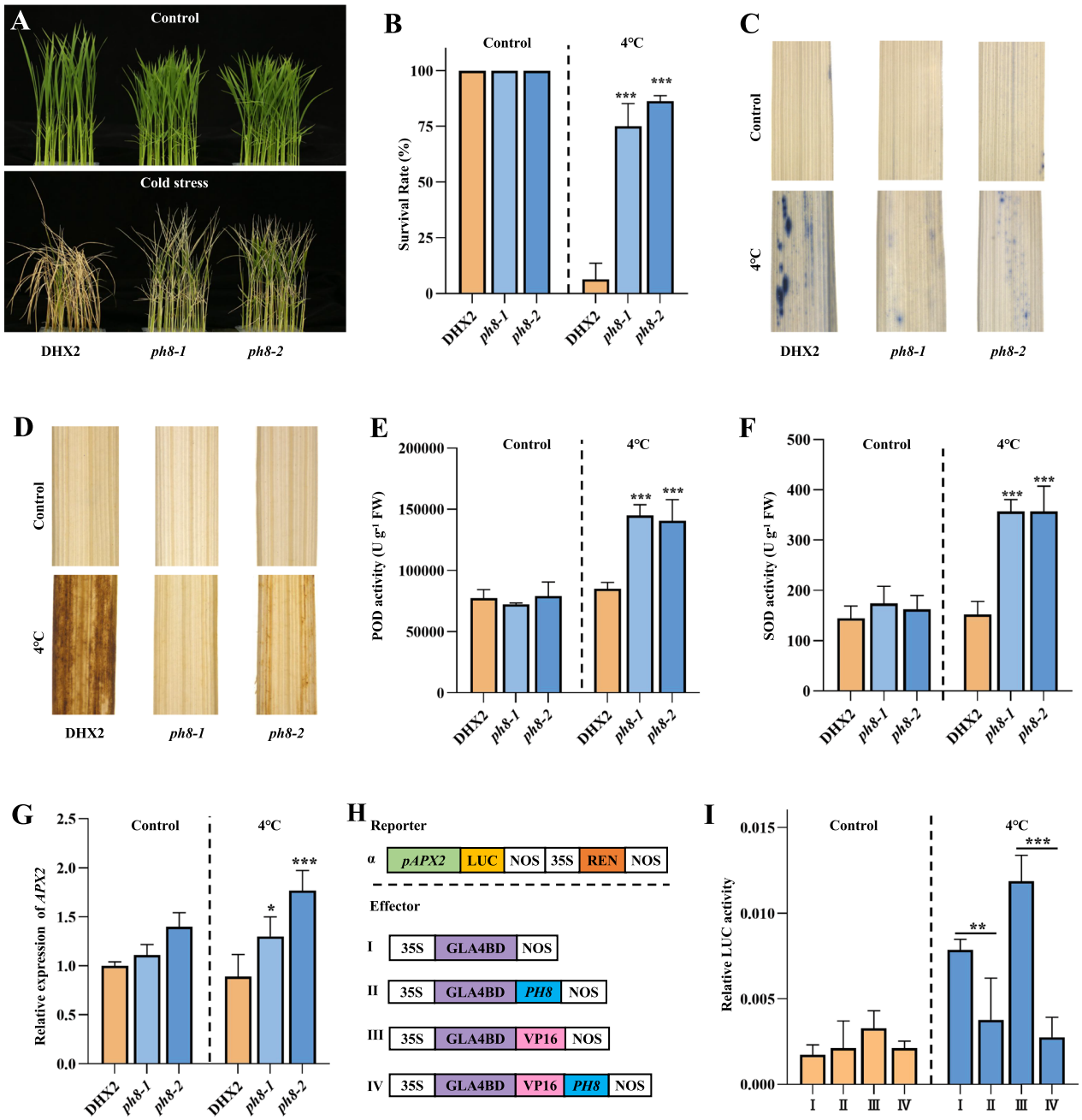


**Fig. 3** PH8 negatively regulates cold tolerance by promoting ROS accumulation. **A**, **B** The phenotype (**A**) and survival rates (**B**) of DHX2 and *ph8* mutant lines under room temperature and cold stress conditions (n = 40). **C**, **D** DAB (**C**) and NBT (**D**) staining showing ROS accumulation in detached leaves of three-leaf-stage seedlings from DHX2 and *ph8* mutant lines. **E**, **F** Activity of POD (**E**) and SOD (**F**) enzymes in DHX2 and *ph8* mutant lines (n = 3). **G** Relative expression levels of *APX2* in DHX2 and *ph8* mutant lines under room temperature and cold stress conditions (n = 3). **H** Schematic diagram of the reporter and effector used for transient dual-luciferase assays in rice protoplasts. The reporter is the fusion of the 2 Kb *APX2* promoter and the firefly luciferase gene, respectively. NOS, transcriptional terminator of the nopaline synthase gene. VP16, a transcriptional activation domain as a positive control. **I** Transcriptional activity of *APX2* inhibited by PH8 was analyzed in rice protoplasts under room temperature and cold stress conditions. The significance of difference was derived with two-tailed Student's t-test (***P* < 0.01, ****P* < 0.001)

plant height (Ye et al. 2015). In contrast, *PH8* regulates salicylic acid metabolism and exerts additional functions as a transcriptional regulator, affecting both plant architecture and cold tolerance (Zhang et al. 2020; Fig. S3P & S4; Fig. 3H–I). Compared to *SD1*, *PH8* exerts a more moderate effect on plant height (Fig. 1F–G).

Furthermore, to date, there are no available research reports addressing the cold resistance functions of the 2OGD superfamily. Despite both *PH8* and *SD1* belonging to the 2OGD superfamily, their functions exhibit significant differences. The dual functionality of *PH8* in both metabolic and transcriptional regulation highlights its broader physiological significance and potential utility in breeding programs for optimizing plant architecture.

A key finding of this study is the identification of the $PH8^{Hap.0}$ allele, which is characterized by reduced *PH8* expression and is positively correlated with improved cold tolerance (Fig. 2B, C). Selective sweep analysis indicates strong selection on *PH8* during the transition from landraces to modern cultivars (Fig. 2D). The $PH8^{Hap.0}$ allele was originally present only in landraces from high latitude regions (Fig. 2E), suggesting its initial formation was due to adaptation to cold climates. However, in recent decades, this allele has become increasingly common among modern accessions (Fig. 2E). Over time, this allele became increasingly prevalent among modern accessions, likely due to its pleiotropic contributions to desirable traits such as moderate plant height and cold resilience. As contemporary breeding strategies aim to integrate multiple favorable traits, alleles like the $PH8^{Hap.0}$ allele have likely been unintentionally retained and disseminated through the recurrent use of backbone parent lines (Gao et al. 2024; Chen et al. 2023). Thus, we hypothesize that $PH8^{Hap.0}$ originated in high-latitude environments under natural selection and was subsequently utilized by breeding programs owing to its significant agronomic benefits.

From our experimental results, it can be seen that PH8 mediates cold tolerance by regulating ROS homeostasis (Fig. 3C–F). Plants utilize a variety of antioxidant enzymes, such as POD, SOD, glutathione peroxidases, and peroxiredoxins, to mitigate oxidative stress and maintain redox balance under abiotic stress (Mittler et al. 2004). Among these, *APX2* has been shown to enhance cold tolerance (Zhang et al. 2013). *APX2* expression level was significant upregulated in *ph8* mutant lines (Fig. 3G), implicating PH8 as a potential repressor of *APX*. Consistent with this hypothesis, PH8 exhibits cold-specific transcriptional repression of *APX2*, as shown in our transcriptional activity assay (Fig. 3H–I). Interestingly, this regulatory effect was absent under normal temperature conditions, suggesting a temperature-dependent transcriptional switch. Given its ability to interact with other nuclear proteins (Zhang et al. 2020), we propose that PH8 may collaborate with cold-responsive transcription factors to modulate ROS scavenging gene expression under stress conditions. Indeed, recent studies have identified phosphorylated OsbZIP20 (Di et al. 2024) and SpBBX18 (Li et al. 2024) as direct regulators of *APX* family genes. Whether PH8 physically or functionally interacts with these factors to orchestrate cold-responsive gene expression remains an open question warranting further investigation.

## Conclusion

In conclusion, *PH8*, which encodes a 2OGD, exerts a distinct dual role in regulating both plant architecture and cold tolerance. Uniquely, *PH8* integrates the regulation of plant architecture and cold tolerance by independently modulating plant height and facilitating ROS scavenging under cold stress. The advantageous $PH8^{Hap.0}$ allele, characterized by cold tolerance and optimized plant architecture, has become increasingly prevalent in modern cultivars. Our findings uncover the molecular logic behind *PH8* allele selection in empirical breeding, providing valuable genetic resources to further optimize both plant height and cold tolerance in cultivated rice.

## Materials and Methods

### Plant Materials, Growth Conditions, Phenotyping, and Cold Treatment

Data on plant height and heading date were collected from 450 rice accessions across Northeast China, Japan, Russia, and Korea. Measurements were taken in Wuchang, LingShui, HaErBin, HeiHe, and JiaMuSi during the 2015 and 2016 growing seasons (Li et al. 2020) (Table S2). The field measurements were conducted in experimental fields at the Rice Research Institute of Sichuan Agricultural University. The cold treatment and seedling cultivation were conducted in a growth chamber with a photoperiod of 16 h of light and 8 h of darkness. The cold treatment was set at 4 °C, while the room temperature condition was maintained at 25 °C as a control.

### GWAS, Candidate Genes and Haplotype Analysis

Sequencing data and agronomic traits data were derived from a previous study (Li et al. 2020). MMAX model was used for GWAS analysis, and for running GWAS with HD as a covariate, we used the equation: $Y = \alpha X + \beta P + \beta HD + \mu + \varepsilon$ (Crowell et al. 2016). The threshold of $P$ ($P < 1 \times 10^{-7.5}$) was defined as the threshold for genome-wide significance. The candidate region was defined as a genomic region containing at least three clustered significant SNPs within a distance of < 136 kb. The linkage disequilibriums (LD) analysis was conducted using PopLDdecay v1.29 (https://github.com/BGI-shenzhen/PopLDdecay). Haplotype analysis was performed by geneHapR (Zhang et al. 2023).

### RNA Extraction and Gene Expression Analysis

Total RNA was extracted from various tissues of DHX2 (Sample_Y255, $PH8^{Hap.1}$) and three-leaf-stage seedlings

subjected to a 4 °C treatment for 0 and 12 h using the Plant Total RNA Isolation Kit (FOREGENE, RE-05014, China). For cDNA synthesis, RT Easy™ Mix (FOREGENE, RT-01012, China) was used according to the manufacturer's protocol. The qRT-PCR was performed following the protocol of PerfectStart® Green qPCR SuperMix (TransGen Biotech, AQ601, China). *UBQ5* and *FhaB* were used as internal controls (Zhao et al. 2020). Each analysis was conducted with three independent biological replicates. The primers used for qPCR are listed in Table S6.

#### Generation of Knocked-out Mutant Lines

The CRISPR/Cas9 vector was constructed by selecting two sgRNA targets, following the method described in Ma et al. (2015). Subsequently, the resulting CRISPR/Cas9 construct was introduced into DHX2 through *Agrobacterium tumefaciens*-mediated transformation. Detailed primer sequences can be found in Table S6.

#### Selection Analysis of *PH8*

We used Vcftools to calculate population fixation statistics ($F_{st}$) with 100 Kb windows (Danecek et al. 2011). Regions with the top 1% of $F_{st}$ values (> 0.441) were recognized as candidate regions (Zhang et al. 2021; Wang et al. 2023).

#### β-glucuronidase Staining Analysis

For GUS staining analysis, the *promoter*$^{PH8}$::*GUS* construct was generated using the vector DX2181 (Tu et al. 2025) and transformed into DHX2. Tissue samples from various developmental stages were collected and subjected to GUS staining in accordance with the manufacturer's protocol (Huayueyang, GT0391, China). Images were captured using a Leica S6 D stereomicroscope and a ScanMaker i800 Plus scanner. All primer sequences are given in Table S6.

#### Subcellular Localization

The full-length coding sequence of *PH8* lacking a stop codon was fused with eGFP and inserted into the pCAMBIA1302-eGFP vector to generate a *35S*::*PH8-eGFP* construct. Additionally, the nuclear protein AtH2B (Sridhar et al. 2007) was cloned and incorporated into the pCAMBIA1302-eRFP vector as the nuclear localization marker. The *35S*::*H2B-eRFP* and *35S*::*PH8-eGFP* were transiently expressed in rice protoplasts to visualize the subcellular localization of PH8. The *35S*::*H2B-eRFP* and *35S*::*eGFP* constructs were transiently expressed in rice protoplasts as a positive control. Following a culture period of 16 h, images were acquired using a Leica laser confocal microscope. All primer sequences are given in Table S6.

#### Measurement of POD and SOD Activity

Three-leaf-stage seedlings treated at 4 °C for 0 h and 24 h were used for POD and SOD activity measurement in accordance with the manufacturer's protocols of the POD activity test kit (Solarbio, BC0090, China) and the SOD activity test kit (Solarbio, BC5165, China). Each analysis was conducted with three independent biological replicates. Four individual seedlings were used for each biological replicate.

#### Histochemical Staining for H2O2 and $O_2^-$

The leaves of three-leaf seedlings treated at 4 °C for 0 and 48 h were subjected to staining with a solution of DAB (Coolaber, SL1805, China) and a 1% NBT solution (Coolaber, SL18061, China), following the manufacturer's protocol, respectively, to visualize the accumulation of $H_2O_2$ and $O_2^-$. At least 5 leaves from 5 independent seedlings for each line were stained.

#### Transactivation Activity Assay

Yeast two-hybrid system was used for transcription activation ability identification. The full-length CDS of *PH8* was cloned into pGBKT7 vector. The pGBKT7-*PH8* fusion vector and pGADT7 were co-transformed into the Y2H gold strain. Plasmid pGBKT7-Lam and pGADT7-T, as well as pGBKT7 and pGADT7, were used as negative controls. Plasmids pGBKT7-53 and pGADT7-T were positive controls. The transformants were screened on the screening medium (SD/-LW). Positive cells were cultured in selective solid medium (SD/-LWHA +20 mg/L x-α-gal) and self-activation assays were performed on selective solid medium supplemented with 20 mM 3 AT and 125 ng/mL AbA. All yeasts were incubated at 30 °C for 3 days.

The 2 Kb promoters of *APX2* were cloned into pGreenII 0800-LUC reporter vector. Full length of *PH8* and *VP16* were cloned into pGreenII 62 SK acting as the effectors The *VP16* recombinant plasmid served as positive control, while empty pGreenII 62 SK vector was used as negative control. The recombinant plasmid was co-transfected into rice protoplasts and incubated overnight at 28 °C. Subsequently, half of the protoplasts were transferred to 4 °C for 1 h, while the remaining half were maintained at room temperature for post-experimental analysis. The Dual Luciferase Reporter Gene Assay Kit (Beyotime, RG027, China) was used to measure Firefly luciferase (LUC) and Renilla luciferase (REN) activity according to manufacturer's protocol. Values represent the mean ± SE of three repeated experiments. All primer sequences are given in Table S6.

**Abbreviations**

| | |
|---|---|
| GJ | Geng/*japonica* |
| GWAS | Genome-wide association study |
| JA | Jasmonic acid |
| ROS | Reactive oxygen species |
| PH | Plant height |
| HD | Heading date |
| GA | Gibberellin |
| DHX2 | Daohuaxiang 2 |
| NIP | Nipponbare |
| GLM | General Linear Model |
| $F_{ST}$ | Fixation statistics |
| eGFP | Enhanced green fluorescent protein |
| DAB | 3,3′-Diaminobenzidine |
| NBT | Nitroblue tetrazolium |
| POD | Peroxidase |
| SOD | Dismutase superoxide |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12284-025-00793-5.

Supplementary material 1: Table S1 List of major cold tolerance genes. Table S2 List of the 450 GJ rice accessions used in GWAS. Table S3 The candidate gene in *qPH8*. Table S4 List of the 557 GJ rice accessions used in Selection Analysis. Table S5 Selective sweeps between landrace and modern cultivated. Table S6 Primers used in this study.

Supplementary material 2: Fig. S1 Manhattan plots of GWAS results on plant height across different years and locations. Fig. S2 Relative expression of *PH8* in various accessions.Relative expression of *PH8* in the leaves of 20 Hap.0 and 20 Hap.1 accession seedlings.The average relative expression of *PH8* in Hap.0 and Hap.1 accessions.Expression patterns of *qPH8* region genes under cold treatment in the NIP background.The seedling height in DHX2 and *ph8* mutant lines. The significance of difference was derived with two-tailed Student's t-test. Fig. S3 Expression patterns and subcellular localization of PH8.Expression profile analysis of *PH8* was conducted in the *japonica* rice variety DHX2, with samples taken from different tissues at various developmental stages.The $promoter^{PH8}$::*GUS* staining in various tissue parts of transgenic plants, including the bud sheath and young roots during development, leaves at tillering stage, 1.5 cm young panicle and stem, stem with a panicle length of 7 cm, stem with a panicle length of 9 cm, node in the elongation stage, cross section of stem, 0.5 cm glume, 0.9 cm glume, developed and mature spikelet, mature anthers and pistil, 5 day caryopsis, 10 day caryopsis,and grated caryopsis after 20 days; B-H, bars = 10 mm; I-O, bars = 1 mm.Subcellular localization of PH8-eGFP fusion protein. The nuclear localization indicated by the use of AtH2B-eRFP; bars = 10 μm. Fig. S4 Transcriptional activation validation in yeast. Positive control: AD-T + BD-P53; negative controls: AD + BD and AD-T + BD-Lam. The letters L, W, and H represent Leu, Trp, and His respectively.

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**Author Contributions**
C.C., X.Z. and JL.C. contributed equally to this work. P.Q. and SG.L. managed the project. C.C., JL.C. and Z.C. performed the data analyses. C.C., X.Z., MJ.X., WY.Z. and LZ.K. performed part of the experiments. YK.W. and SW.T. conducted field management. C.C. wrote and finalized the manuscript, with advice from JW.X., H.Y., WL.C., B.T., T.L., YP.W., BT.M., SG.L. and P.Q. All authors read and approved the manuscript.

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**Data Availability**
No datasets were generated or analysed during the current study.

## Declarations

**Ethics Approval and Consent to Participate**
Not applicable.

**Consent for Publication**
Not applicable.

**Competing Interests**
The authors declare no competing interests.

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