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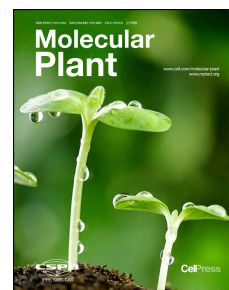
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Transcriptional and post-transcriptional regulation of OsNHX2 encoding a tonoplast-localized transporter controls intracellular K⁺ accumulation to regulate cold tolerance in rice

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Running title: Regulation of OsNHX2 modulates cold tolerance in rice

Short Summary: OsNHX2 is a tonoplast-localized K⁺ transporter that modulates intracellular K⁺ accumulation through transcriptional and post-transcriptional regulation. It maintains reactive oxygen species homeostasis under cold stress and promotes osmolyte accumulation, thereby enhancing cold tolerance in rice.

Abstract

Low-temperature stress significantly reduces rice yield and grain quality. Identifying major cold-tolerance genes and deciphering their regulatory networks is therefore essential for rice molecular breeding for cold tolerance. Here, through a genome-wide association study (GWAS) in a multi-parent advanced generation intercross (MAGIC) population, we identified *OsNHX2*, a tonoplast-localized K⁺ transporter that enhances cold tolerance at the seedling stage. *OsNHX2* enhances cold tolerance by promoting an increase in potassium ion content in shoots, which stimulates proline accumulation and reduces the levels of reactive oxygen species (ROS) and malondialdehyde (MDA). Natural variation in the coding region of *OsNHX2* underlies differences in K⁺ transport ability. We further demonstrated that *OsNHX2* expression is negatively regulated by the transcription factor *OsATH1*. One natural variant in the *OsNHX2* promoter, a 45-bp deletion, elevates *OsNHX2* expression by reducing *OsATH1* binding. Notably, we found that *OsNHX2* physically interacts with *OsGAPC3*, a cytosolic glyceraldehyde-3-phosphate dehydrogenase known to modulate responses to abiotic stresses via ROS signaling. This interaction masks the tonoplast-targeting signal of *OsNHX2*, altering its subcellular localization, impairing K⁺ transport, and promoting cytosolic K⁺ accumulation. Overexpression of *OsNHX2* significantly increased the seed-setting rate under cold stress at the booting stage without compromising grain yield under normal conditions. Haplotype analysis revealed that rice accessions carrying the cold-tolerant *OsNHX2* haplotype are predominantly distributed in higher-latitude regions, suggesting adaptive selection. Our findings demonstrate that *OsNHX2*, which is negatively regulated by *OsATH1* and *OsGAPC3* at both transcriptional and post-transcriptional levels, modulates intracellular K⁺ accumulation to control cold tolerance in rice.

Keywords: cold tolerance, potassium ion transport, natural variation, K⁺/H⁺ antiporter

Introduction

Rice (*Oryza sativa* L.) is a globally important staple crop cultivated across a wide range of regions (Langpoklakpam et al., 2023). As a crop originating in tropical and subtropical regions, rice is highly sensitive to low temperatures. Cold stress can inhibit seedling growth and reduce seed-setting rate, ultimately reducing grain yield (Pan et al., 2015; Zhao et al., 2015). Therefore, identifying key genes for cold tolerance and developing cold-tolerant rice varieties is crucial for ensuring food security and promoting sustainable agricultural development worldwide.

To date, 26 genes at the seedling stage and 13 genes at the booting stage were identified through a forward genetics approach, such as *COLD1*, *LTG1*, *qCTS9*, *bZIP73*, *HAN1*, *HAN2*, *CTB2*, *CTB3*, *CTB4a*, *CTB5*, *CTB6*, *COG1*, *COG3*, and *COG4* (Guo et al., 2025). Among them, the most cold-tolerant haplotype, Hap1 of *HAN1*, emerged concurrently with the northward expansion of temperate *japonica* (*geng*) rice and subsequently fixed through adaptive selection for high-latitude growth conditions (Mao et al., 2019). The cold-tolerant haplotype, Tej-Hap-KMXBG of *CTB4a*, represents a novel allele that arose from natural variation during rice domestication and was retained through artificial selection (Zhang et al., 2017). The cold-tolerant allele of *CTB3* is predominantly found in high-altitude and high-latitude regions and has undergone directional selection in temperate *geng* (Li et al., 2025). The favorable *CTB6* allele, directly derived from Or-III wild rice, underwent directional selection and has been retained in temperate *geng* rice from the Yunnan Plateau (Gao et al., 2025). The cold-tolerant *bZIP73*^{Jap} allele, directly derived from Or-III wild rice, is predominantly present in the *geng* rice (Liu et al., 2019). Given that these favorable alleles are already fixed in *geng* germplasm, their potential to further improve cold tolerance is limited. Consequently, the discovery and characterization of untapped cold-tolerance genes within the rice gene pool has become a research priority.

Potassium (K⁺) functions as an osmoprotectant and is translocated to various tissues, thereby alleviating multiple abiotic stresses, including salinity, drought, high temperature, and cold (Wang et al., 2013). Cold stress impairs plant growth by disrupting membrane fluidity, inducing lipid peroxidation, and triggering reactive

oxygen species (ROS) bursts (Iqbal et al., 2022). Field studies demonstrated that increasing potassium fertilization enhances cold tolerance in rice (Cao et al., 2017). Similarly, elevated potassium fertilizer application reduces ion leakage and increases the activity of ROS-scavenging enzymes in maize (Farooq et al., 2008). In grapevine, foliar application of K₂SO₄ decreases electrolyte leakage and ROS accumulation while promoting osmolyte accumulation (Karimi et al., 2017). In coconut, K⁺ supplementation under cold stress maintains hormonal and ROS homeostasis and reduces malondialdehyde content (Lu et al., 2024). Collectively, by sustaining osmotic balance and reducing ROS accumulation, K⁺ enhances plant cold tolerance.

NHX proteins constitute a family of transmembrane ion antiporters that are widely distributed in plants. They typically possess 10-12 transmembrane helices that form the functional domains responsible for ion antiport (Brett et al., 2005; Chanroj et al., 2012). In rice, the characterized NHX-type antiporters include OsNHX1, OsNHX2, OsNHX3, OsNHX4, OsNHX5, and OsSOS1 (Fukuda et al., 2004; Fukuda et al., 2011; Martínez-Atienza et al., 2007). Several NHX members have been reported to be involved in responses to abiotic stress. For example, OsNHX1 contributes to tolerance against salinity, drought, and osmotic stress, while OsSOS1 is specifically required for salt tolerance (Fukuda et al., 2004; Martínez-Atienza et al., 2007). However, it is still unclear whether the NHX family members confer cold tolerance in rice.

In this study, we identified a major quantitative trait locus (*qSR11.2*) associated with cold tolerance using a genome-wide association study (GWAS) in a large multi-parent advanced generation intercross (MAGIC) population. *OsNHX2*, encoding a tonoplast-localized K⁺ transporter, was considered the causal gene for *qSR11.2* and functions as a positive regulator of cold tolerance at both seedling and booting stages. Natural variations in both promoter and coding regions of *OsNHX2* underlie the differential cold tolerance between the two parent lines, CT16658 (cold-tolerant) and IR68 (cold-sensitive). Comprehensive analyses uncovered a regulatory cascade of *OsNHX2* involving transcriptional control by OsATH1. This module promotes K⁺ accumulation in shoots, enhancing cold tolerance in rice. These findings provide important insights into cold tolerance mechanism and new gene resources for rice

molecular breeding for cold tolerance.

Results

***qSR11.2* is a major QTL for cold tolerance in rice**

To identify key loci associated with cold tolerance in rice, we evaluated the leaf score of cold tolerance (SCT) and survival rate (SR) after cold treatment at seedling stage in a large MAGIC population comprising 1,346 lines derived from eight *indica* (*xian*) parental lines (named parents A-H; Supplemental Table 1, 2). Of the eight founders of the MAGIC population, parent F (CT16658-5-2-2SR-2-3-6MP, abbreviated to CT16658) exhibited the highest level of cold tolerance, with an SR of 100% and an SCT of 1. In contrast, parent G (IR68) was the most sensitive to cold, with an SR of 4.25% and an SCT of 9 (Supplemental Table 1). Both SR and SCT exhibited continuous distributions across the 1,346 MAGIC lines (Supplemental Figure 1A-F). We conducted a GWAS and detected six QTLs associated with SR and/or SCT. Of these, *qSR11.2* ($P = 1.36 \times 10^{-9}$) was the locus associated with both SR and SCT exhibiting the strongest association signal (Supplemental Table 3; Figure 1A; Supplemental Figure 2). Moreover, we identified the same genomic region of *qSR11.2* independently using linkage mapping in the 569 8way line population (Supplemental Table 4). Thus, *qSR11.2* is a major QTL for cold tolerance in rice.

To delimit the candidate gene underlying *qSR11.2*, we carried out fine-mapping using the BC₄F₂ population of 1,876 individuals derived from crossing IR68 (the recurrent parent) with CT16658 (the donor parent). Eventually, *qSR11.2* was confined to a 96.36-kb region (Figure 1B). A total of nine predicted open reading frames (ORFs) were located in this region, two of which encode functionally annotated proteins: *Os11g0648000* (*OsNHX2*) and *Os11g0648100* (Figure 1C). There are several previous studies supporting that the magnitude of cold-induced gene expression is a reliable indicator for screening candidate genes in rice (Jiang et al., 2026; Sun et al., 2024). Therefore, to identify candidate genes associated with *qSR11.2*, we used quantitative reverse-transcription PCR (qRT-PCR) to compare the expression patterns of the nine candidate genes in seedlings of CT16658 and IR68 before cold treatment (0 h) and at 4,

12, and 24 h after cold treatment. *Os11g0648200*, *Os11g0648500*, *Os11g0648800*, and *Os11g0649100* were not expressed, while *Os11g0648100*, *Os11g0648400*, *Os11g0648700*, and *Os11g0649000* were expressed, but did not show a strong response to cold. In contrast, *OsNHX2* was significantly upregulated at 12 h under cold stress, with higher expression in the tolerant parent CT16658 than in the sensitive parent IR68 (Supplemental Figure 3). Notably, sequence comparison between CT16658 and IR68 revealed 22 single nucleotide polymorphisms (SNPs), four insertions/deletions (InDels) in the promoter region and eight nonsynonymous SNPs within coding sequence (CDS) of *OsNHX2* (Supplemental Figure 4). Among these eight nonsynonymous mutations, H1-H4 are located in the cation/H⁺ exchanger domain, whereas H5-H8 reside in an unknown domain. Based on these findings, we identified *OsNHX2* as the most probable causal gene underlying *qSR11.2*.

***OsNHX2* is the causal gene of *qSR11.2*, positively regulating cold tolerance in rice**

To verify the biological function of *OsNHX2*, *nhx2* knockout mutants were generated by the CRISPR/Cas9 gene editing approach in the CT16658 background. Finally, two homozygous mutants were identified: *nhx2-1* harboring two single-base insertions, and *nhx2-2* with a 62-bp deletion (Figure 1D). The *nhx2-1* mutation caused premature translational termination of the protein at amino acid 110; *nhx2-2* didn't cause premature translational termination but instead resulted in a deletion of 7 amino acids at position 16 and a deletion of 9 amino acids at position 88, ultimately leading to the loss of *OsNHX2* function. Under normal growth conditions before cold treatment, no obvious morphological differences were observed among CT16658, *nhx2-1*, and *nhx2-2* (Figure 1E). However, after the cold treatment and recovery, the survival rates of *nhx2-1* and *nhx2-2* were significantly lower than that of CT16658 (Figure 1F), accompanied by remarkably higher electrolyte leakage rates (Figure 1G).

To further investigate the role of *OsNHX2* in cold tolerance, we created *OsNHX2*^{CT16658} overexpression lines in the IR68 background. Two homozygous transgenic lines, *OsNHX2-OE1* and *OsNHX2-OE2*, were randomly selected for analysis. The *OsNHX2* expression levels in the two OE lines were both significantly higher than in IR68 (Figure 1H). Although no significant growth differences were observed

between IR68 and *OsNHX2*-OE lines under normal conditions, the *OsNHX2*-OE lines exhibited significantly higher survival rates and lower ion leakage rates (Figure 1E, I, J).

Similarly, we double-checked the function of *OsNHX2* in *geng* rice Nip background. Two overexpression lines, *OsNHX2-OE3* and *OsNHX2-OE4*, which were confirmed to have substantially elevated *OsNHX2* expression levels, showed significantly higher survival rates than the wild-type Nip after cold treatment (Supplemental Figure 5A-C). Correspondingly, two *OsNHX2* knockout lines, *nhx2-3* and *nhx2-4*, in the Nip background exhibited significantly lower survival rates than the wild type after cold treatment (Supplemental Figure 5A, B, D). Collectively, these results demonstrate that *OsNHX2* positively regulates cold tolerance at the seedling stage and is the causal gene underlying *qSR11.2*.

OsATH1* negatively regulates the expression of *OsNHX2

To investigate the key variants of *OsNHX2* underlying cold tolerance, we examined the association strength between the SNPs in the promoter and CDS regions of *OsNHX2* and the cold tolerance at the seedling stage in our previous GWAS panel of 1,290 *xian* accessions (Shi et al., 2024). Among the SNPs identified in the *OsNHX2* promoter, three (V9: -233 G>A, V10: -234 A>C, and V22: -598 T>C) showed stronger associations with cold tolerance than other promoter SNPs (Figure 2A). In the coding region, two SNPs (H1: +109 A>G and H2: +970 G>A) exhibited stronger associations with cold tolerance than other CDS SNPs (Figure 2A). These findings suggest that the sequence variations in both the promoter and coding regions of *OsNHX2* likely contribute to the observed difference in cold tolerance between CT16658 and IR68.

OsNHX2 expression differed between CT16658 and IR68 (Supplemental Figure 3A). After cold treatment, the promoter activities of both *pOsNHX2^{IR68}* and *pOsNHX2^{CT16658}* increased significantly, and the activity of *pOsNHX2^{CT16658}* was significantly higher than that of *pOsNHX2^{IR68}* (Supplemental Figure 6A). Promoter fragments containing sequence variations were analyzed to predict transcription factor (TF) binding sites, which led to the identification of several TF families such as AT-hook, MYB, and bZIP (Supplemental Table 5). Among them, *OsATH1*, an AT-hook

family member, was predicted to bind with specificity to both the IR68-specific InDel V6 (-166 bp, designated V6) and the cis-element harboring SNP V22, implicating it as a potential upstream transcriptional regulator of *OsNHX2*. To determine whether *OsATH1* responds to cold stress, we analyzed its expression in Nip seedlings subjected to a cold treatment over a time course. *OsATH1* expression was significantly down-regulated upon prolonged cold exposure (Supplemental Figure 7A). Prediction of AT-hook binding sites in the *OsNHX2* promoters of CT16658 and IR68 using PlantPAN4.0 (<https://plantpan.itps.ncku.edu.tw/plantpan4/>) revealed three cis-elements (R1, R2 and R3) in *pOsNHX2^{CT16658}* and four cis-elements (R1, R2, R3 and R4) in *pOsNHX2^{IR68}*. R4 was located on an InDel V6 (Supplemental Figure 7B). EMSA analysis evaluating the binding of OsATH1 to the four cis-elements (R1, R2, R3 and R4) from both promoters demonstrated that markedly stronger binding to the R1 element (containing SNP V22) for the IR68 probe than for the CT16658 probe (Figure 2B). OsATH1 also bound to the R4 element present in the IR68 promoter (Figure 2C). However, there was no difference in its binding affinity for the R2 and R3 elements between the two promoters (Supplemental Figure 7C, D). Dual-luciferase assay showed that the *pOsNHX2^{CT16658}* promoter drove significantly stronger promoter activity than the *pOsNHX2^{IR68}*. Furthermore, it demonstrated that OsATH1 significantly suppressed the activity of both promoters (Figure 2D, E). Taken together, these results demonstrate that OsATH1 functions as a negative regulator of *OsNHX2*.

We found that deletion of the V6 in the *pOsNHX2^{IR68}* (*pOsNHX2^{IR68}* V6) eliminated the difference in OsATH1-mediated transcriptional repression between *pOsNHX2^{IR68}* V6 and *pOsNHX2^{CT16658}*, while significantly weakening repression compared with *pOsNHX2^{IR68}*. The LUC/REN ratio of *pOsNHX2^{IR68}* V6 was significantly higher than that of *pOsNHX2^{IR68}*, but comparable to that of *pOsNHX2^{CT16658}*. In contrast to V6, OsATH1-mediated transcriptional repression of *pOsNHX2^{IR68}* V6 was significantly weaker than that of *pOsNHX2^{IR68}* V22 (the SNP V22 in *pOsNHX2^{IR68}* was substituted with the *pOsNHX2^{CT16658}*), and the repression on *pOsNHX2^{IR68}* V22 was also significantly weaker than that on *pOsNHX2^{IR68}*. No significant difference in the LUC/REN ratio was detected when *pOsNHX2^{IR68}* V22 and

pOsNHX2^{IR68} were individually expressed in the tobacco (Supplemental Figure 6B-C). ChIP-qPCR confirmed that OsATH1 binds to the V6 region of *pOsNHX2^{IR68}*, and this interaction was attenuated by cold stress (Figure 2F). Collectively, these results indicate that the V6 is the key variation responsible for the differential promoter activity the key variation responsible for the differential promoter activity and OsATH1-mediated transcriptional repression of *pOsNHX2*.

We overexpressed *OsATH1* under control of the constitutive CaMV 35S promoter in the cold-sensitive parent IR68. Although no significant growth differences were observed between IR68 and the two overexpression lines (*OsATH1-OE1* and *OsATH1-OE2*) under normal conditions, these OE lines exhibited significantly lower survival rates under cold stress (Figure 2G-I). *OsNHX2* expression levels were significantly decreased in *OsATH1-OE1* and *OsATH1-OE2* (Figure 2H). To investigate the model in which OsATH1 negatively regulates cold tolerance by repressing *OsNHX2* expression, we generated targeted knockout mutations of the *OsATH1* gene in the CT16658 background using the CRISPR/Cas9 system. Transgenic lines with double knockout of both *OsATH1* and *OsNHX2* were also constructed. The results showed that knockout of *OsATH1* alone significantly increased survival rate, while simultaneous knockout of *OsATH1* and *OsNHX2* led to a survival rate significantly lower than that of the wild type (Figure 2G, J-K). These findings indicate that OsATH1 negatively regulates cold tolerance and is genetically upstream of *OsNHX2*.

OsNHX2 is a K⁺ antiporter

OsNHX2 encodes a 546-aa protein that belongs to the NHX family of cation/H⁺ antiporters (Supplemental Figure 8; Supplemental Table 6). To elucidate its molecular function, we analyzed its subcellular localization and expression pattern. Subcellular localization analysis confirmed that OsNHX2 is targeted to the tonoplast (Figure 3A). Expression pattern analysis showed that *OsNHX2* is expressed in roots and leaves at the seedling stage, and in roots, stems, flag leaves, panicles, and leaf sheaths at the booting stage (Supplemental Figure 9A). Histochemical GUS staining of *pOsNHX2::GUS* transgenic plants revealed *OsNHX2* expression in roots, stems, and leaves at the seedling stage, with a particularly strong signal in root tips (Supplemental

Figure 9B, C). Expression was also detected in panicles at the booting stage, specifically in the anthers (Supplemental Figure 9D, E). In situ hybridization further localized *OsNHX2* transcripts to the xylem, phloem, and cortical sclerenchyma of roots, as well as within the vascular bundles of seedling leaves (Figure 3B, C).

The *OsNHX2* alleles from CT16658 and IR68 differ by eight nonsynonymous mutations in their CDS (H1-H8) (Supplemental Figure 4). AlphaFold3 -based structural prediction of *OsNHX2*^{CT16658} and *OsNHX2*^{IR68} revealed that mutations H1 and H7 cause structural alterations (Supplemental Figure 10A, B), implying potential functional divergence between the two alleles. To experimentally validate the functional implications of these differences and compare the cation transport activity of *OsNHX2*^{CT16658} and *OsNHX2*^{IR68}, we performed a heterologous complementation assay in the K⁺-uptake defective yeast mutant R5421. Fusion constructs of pYES2-*OsNHX2*^{CT16658} and pYES2-*OsNHX2*^{IR68} were transformed into the yeast strain. The reported *Arabidopsis* Shaker family high-affinity K⁺ channel AtAKT1 (Rubio et al., 2008) was employed as the positive control, with the empty pYES2 vector as the negative control. *OsNHX2*^{CT16658} effectively rescued the growth of R5421 under low-K⁺ conditions (as low as 1 mM K⁺), demonstrating its ability to facilitate K⁺ uptake. In contrast, *OsNHX2*^{IR68} failed to promote detectable growth restoration even when the external K⁺ concentration was elevated to 3 mM K⁺, indicating a markedly attenuated ability of K⁺ uptake. Neither *OsNHX2*^{CT16658} nor *OsNHX2*^{IR68} sustained R5421 growth under 0.5 mM K⁺ conditions, confirming that their lower activity relative to AtAKT1 (Figure 3D). Measurement of cellular K⁺ content in yeast strains cultured in liquid media supplemented with 10 mM or 3 mM K⁺ further revealed that *OsNHX2*^{CT16658}-expressing cells accumulated significantly more K⁺ than *OsNHX2*^{IR68}-expressing cells under both conditions (Figure 3E, F). Because H1, H2 located in the cation/H⁺ exchanger domain and exhibited stronger associations with cold tolerance than the other CDS SNPs (Figure 2A), mutating these two residues in IR68 to CT16658 residues enhanced yeast growth on 1 mM K⁺, with H2 showing a greater effect than H1 (Supplemental Figure 11A), suggesting H2 may be the key SNP responsible for the functional difference between CT16658 and IR68. These results confirmed that

OsNHX2 possesses K^+ transport activity, and that the nonsynonymous SNPs distinguishing the CT16658 and IR68 alleles impair this function, resulting in a higher K^+ transport ability in OsNHX2^{CT16658} compared to OsNHX2^{IR68}.

Given the similar physicochemical properties of Na^+ and K^+ , numerous antiporters mediating K^+ transport are also capable of Na^+ antiport activity (Zhang et al., 2019). To test whether OsNHX2 transports Na^+ , we conducted a heterologous complementation assay using the Na^+ -sensitive yeast strain B31. The *Arabidopsis* gene *AtHKT1;1* (Xue et al., 2011), encoding a known Na^+ antiporter, was used as the positive control. The empty pYES2 vector was the negative control. Yeast transformants expressing pYES2-*AtHKT1;1* exhibited increased sensitivity and grew markedly poorer than those expressing either pYES2-OsNHX2 or the empty pYES2 vector under 50 mM and 200 mM Na^+ conditions (Supplemental Figure 11B). These observations indicated that OsNHX2 does not mediate significant Na^+ uptake.

We next investigated changes in K^+ content in *OsNHX2* transgenic plants under cold stress. Under normal conditions, the K^+ contents in both shoots and roots of CT16658 were significantly higher than those in the *nhx2-1* and *nhx2-2* mutants. Under cold stress, the K^+ contents in shoots and roots of both mutants were significantly lower than those in CT16658 and were also markedly reduced compared to their own levels under normal conditions (Figure 3G, H). In contrast, CT16658 exhibited increased K^+ accumulation in both shoots and roots under cold stress. Conversely, under both normal and cold stress conditions, the shoot and root K^+ contents in *OsNHX2-OE1* and *OsNHX2-OE2* were significantly higher than those in IR68. While IR68 showed a significant increase in shoot K^+ content but a decrease in root K^+ content under cold stress, both OE lines accumulated significantly more K^+ in shoots and roots under cold stress compared to their respective normal conditions (Figure 3I, J). These results indicated that loss-of-function of *OsNHX2* impairs K^+ uptake in rice, particularly under cold stress, while overexpression of *OsNHX2* enhances K^+ accumulation.

To examine the impact of exogenous K^+ availability on cold tolerance in *OsNHX2* transgenic lines, we assessed the phenotypic performance of IR68, *OsNHX2-OE1*, and *OsNHX2-OE2* under cold stress supplemented with either 1 mM or 0.1 mM K^+ (Figure

3L). Under cold stress with 1 mM K⁺, the survival rates of *OsNHX2-OE1* and *OsNHX2-OE2* were 94.44% and 97.22%, respectively. In contrast, under cold stress with 0.1 mM K⁺, the survival rates declined significantly to 61.90% and 64.22%, respectively. A statistical comparison confirmed that the survival rates under 0.1 mM K⁺ were significantly lower than those under 1 mM K⁺ conditions (Figure 3K). We also evaluated CT16658, *nhx2-1* and *nhx2-2* under cold stress supplemented with either 1 mM or 6 mM K⁺ (Supplemental Figure 12A-B). Under cold stress with 1 mM K⁺, the survival rate of CT16658 was 32.5%, while those of *nhx2-1* and *nhx2-2* were 0% (Supplemental Figure 12C). In contrast, under cold stress with 6 mM K⁺, the survival rate of CT16658 was 53.26%, and those of *nhx2-1* and *nhx2-2* were 31.88% and 33.30%, respectively (Supplemental Figure 12C). These results suggest that reducing the K⁺ concentration in the nutrient solution reduces the cold tolerance of the *OsNHX2*-OE lines, whereas increasing the K⁺ concentration in the nutrient solution improves the cold tolerance of *nhx2* mutants.

To investigate how potassium ion alterations affect cold tolerance, we measured physiological indicators in *OsNHX2* transgenic plants after cold treatment. We found that after 24 h of cold treatment, the proline (Pro) content in *nhx2-1* and *nhx2-2* mutants decreased significantly compared with CT16658, while the levels of malondialdehyde (MDA), H₂O₂, and O₂⁻ increased markedly (Figure 3M, N; Supplemental Figure 13A). In contrast, after 24 h of cold treatment, *OsNHX2-OE1* and *OsNHX2-OE2* exhibited a significant increase in Pro content and a notable reduction in MDA content relative to IR68. Moreover, the increase of tetraethylammonium chloride (TEA, a K⁺ channel blocker) suppressed the cold-induced elevation of Pro content (Supplemental Figure 13B, C). These results suggest that *OsNHX2* enhance cold tolerance in rice by promoting Pro accumulation and reducing the levels of ROS and MDA under cold stress.

OsGAPC3-OsNHX2 physical binding redirects OsNHX2 subcellular localization and loss of K⁺ transport function

To dissect the molecular mechanism underlying *OsNHX2*-mediated cold tolerance, we performed a yeast two-hybrid (Y2H) screen using a rice membrane yeast two-hybrid library, which identified 21 potential *OsNHX2*-interacting proteins (Supplemental

Table 7). Among these candidates, rice cytosolic glyceraldehyde-3-phosphate dehydrogenase, OsGAPC3 (Tien et al., 2012; Zhang et al., 2011), was identified as interacting with OsNHX2. The physical interaction between OsNHX2 and OsGAPC3 was validated by quantitative co-immunoprecipitation (Co-IP) and luciferase complementation assays (LCA) in tobacco leaves, and quantitative Co-IP further showed that the binding ability between OsGAPC3 and OsNHX2 significantly decreased after cold treatment (Figure 4A, Supplemental Figure 14A). To verify the specific region mediating OsNHX2 and OsGAPC3 interaction, we generated three truncated derivatives: OsNHX2-1 (amino acids 1-442), OsNHX2-2 (amino acids 27-442), and OsNHX2-3 (amino acids 27-546) (Figure 4B). Bimolecular fluorescence complementation (BiFC) assays in rice protoplasts further confirmed that the interaction between OsNHX2-1 and OsGAPC3 occurs in the cytoplasm, with no fluorescence signal observed for OsNHX2-2 or OsNHX2-3 (Figure 4C). Subsequent Y2H assays showed that OsNHX2-1 interacted with OsGAPC3, whereas no interaction was detected between OsGAPC3 and either OsNHX2-2 or OsNHX2-3 (Figure 4D). Through BiFC assays and Y2H assays, we verified that the 26-amino-acid N-terminal region of OsNHX2 interacts with OsGAPC3 in the cytoplasm (Supplemental Figure 14B-C). Taken together, these results indicate that OsGAPC3 specifically binds to the N-terminal 26-amino-acid region of OsNHX2 in the cytoplasm.

Interestingly, although OsNHX2 was localized to the tonoplast (Figure 3A), truncation of its N-terminal 26 amino acids (OsNHX2^{-26 aa}) resulted in altered subcellular localization of OsNHX2 to cytoplasm (Figure 4E), indicating that this N-terminal region contains a tonoplast-targeting signal of OsNHX2. Subcellular localization experiments showed that OsGAPC3 is localized in the cytoplasm (Figure 4E). Given the interaction between OsGAPC3 and OsNHX2 in the cytoplasm (Figure 4C), we hypothesized that OsGAPC3 binding interferes with the tonoplast-targeting signal of OsNHX2, thereby abrogating its tonoplast localization and retaining the protein in the cytoplasm.

To investigate the functional impact of OsGAPC3 interaction on OsNHX2-mediated K⁺ transport, we co-transformed pYES2-OsGAPC3 and pYES2-OsNHX2

into the K⁺ uptake-deficient yeast strain R5421. Under 3 mM K⁺ conditions, neither OsGAPC3 transformants nor the empty pYES2 vector controls supported yeast growth, but co-expression of OsGAPC3 with OsNHX2 significantly impaired yeast growth compared to OsNHX2 expression alone (Figure 4F). Measurement of cellular K⁺ content in yeast transformants expressing AtAKT1, empty pYES2, OsNHX2, OsGAPC3, or OsNHX2 + OsGAPC3 cultured in media supplemented with 10 mM or 3 mM K⁺ revealed that K⁺ accumulation was significantly reduced in OsNHX2 + OsGAPC3 co-expressing cells than in those expressing OsNHX2 alone (Figure 4G, H). These results demonstrate that OsGAPC3 acts as a negative regulator of the K⁺ transport activity of OsNHX2.

We next isolated 1×10^6 protoplasts from IR68, *OsNHX2-OE2*, *OsGAPC3-OE2*, and *OsNHX2/OsGAPC3-OE2* plants and incubated them with the cytosolic K⁺ fluorescent probe Enhanced Potassium Green-4 AM (EPG-4 AM) under normal conditions. Fluorescence intensity analysis showed that *OsNHX2-OE2* protoplasts exhibited significantly lower signal than those of *OsGAPC3-OE2* protoplasts (Figure 4I). The dual-overexpression line *OsNHX2/OsGAPC3-OE2* showed fluorescence intensity comparable to IR68 (Figure 4I). Inductively coupled plasma mass spectrometry (ICP-MS) analysis of K⁺ content in 1×10^6 protoplasts revealed the highest K⁺ levels in *OsNHX2-OE2* and the lowest in *OsGAPC3-OE2* (Figure 4J). Further, the ratio of cytosolic K⁺ fluorescence intensity to total cellular K⁺ content was significantly lower in *OsNHX2-OE2* than in *OsGAPC3-OE2* (Figure 4K). Taken together, these results suggest that OsGAPC3 antagonizes OsNHX2 function to promote cytoplasmic K⁺ retention by subcellular mislocalization-induced functional abrogation of K⁺ antiport of OsNHX2.

***OsGAPC3* and *OsNHX2* regulate rice cold tolerance via a shared genetic pathway**

We analyzed their expression dynamics in shoots and roots of Nip seedlings subjected to varying durations of cold treatment. In shoots, the expression levels of *OsNHX2* and *OsGAPC3* were both induced to a similar extent after 3 h and 12 h of cold exposure. However, at 6 h post-treatment, *OsGAPC3* was upregulated more strongly than *OsNHX2*, whereas by 9 h, the induction of *OsNHX2* surpassed that of *OsGAPC3*

(Figure 5C). In roots, *OsNHX2* was induced significantly more than *OsGAPC3* at 3 h, 6 h, and 9 h of cold treatment, with no notable difference at 12 h (Figure 5D). These results indicate a tissue-specific pattern: *OsNHX2* induction was consistently stronger in roots under cold stress, while in shoots the temporal dynamics differed, with *OsGAPC3* responding earlier.

To validate the function of *OsGAPC3* in cold tolerance, we generated *gapc3* knockout mutants by the CRISPR/Cas9 genome-editing system (Figure 5E) and *OsGAPC3*-OE lines driven by the constitutive CaMV 35S promoter (Figure 5F). Following cold treatment at 9°C during the seedling stage, the *gapc3* mutants exhibited significantly reduced cold tolerance compared to the wild type CT16658 (Figure 5A, G). In contrast, the two *OsGAPC3*-OE lines showed significantly enhanced cold tolerance relative to the wild type IR68, with survival rates of 97.16% and 98.33%, respectively (Figure 5B, H). These results indicate that *OsGAPC3* positively regulates cold tolerance in rice.

To further dissect the genetic relationship between *OsNHX2* and *OsGAPC3*, we created double-knockout mutants and double-overexpression lines in the CT16658 and IR68 backgrounds, respectively. After cold treatment, the seedling survival rates of the *nhx2/gapc3* double-knockout mutants were 2.72% and 0%, respectively, similar to the phenotype of the *OsNHX2* mutant (Figure 5A, G). Notably, the mutants *gapc3-1* and *gapc3-2* had higher survival rates (11.35% and 26.75%, respectively) than the *nhx2-1* and *nhx2-2* mutants (5.57% and 0%, respectively). However, the *OsGAPC3* mutants (*gapc3-1*, and *gapc3-2*) still maintained higher survival rates than the double-knockout lines (*nhx2/gapc3-1* and *nhx2/gapc3-2*) (Figure 5A, G). Conversely, simultaneous overexpression of both genes resulted in very high survival rates (97.92% and 100%), which were similar to those of the single-gene overexpression lines (Figure 5B, H). The K⁺ contents in the shoots and roots of *gapc3* knockout, *OsGAPC3*-OE, *nhx2/gapc3* double-knockout mutants, and *OsNHX2/OsGAPC3*-OE lines were measured under normal and cold treatment conditions. We found that under cold treatment, the K⁺ contents in both shoots and roots were significantly decreased in the *gapc3* knockout and the *nhx2/gapc3* double-knockout lines. In contrast, the *OsGAPC3*-OE line

exhibited a non-significant increase in shoot K^+ content but a significant increase in root K^+ content after cold treatment. Notably, the double overexpression line showed significantly increased K^+ contents in both shoots and roots following cold treatment (Supplemental Figure 15). Collectively, these genetic data suggest that *OsGAPC3* and *OsNHX2* may regulate cold tolerance in the same genetic pathway.

***OsNHX2* positively regulates cold tolerance at the booting stage**

To assess the role of *OsNHX2* in cold tolerance during the booting stage, we exposed transgenic plants to three cold stress conditions—controlled phytotron (CS-PT), deep-water pool (CS-DW), and high-altitude natural environment (CS-HAA)—and evaluated main panicle seed-setting rate and yield per plant.

In the CT16658 background, both *OsNHX2* knockout lines (*nhx2-1* and *nhx2-2*) exhibited significantly lower relative seed-setting rates than wild-type CT16658 under CS-PT and CS-DW (Figure 6A, C). Similarly, under CS-HAA, both mutants showed pronounced reductions in seed-setting rate and yield per plant (Figure 6C). Under normal conditions (NC), while *nhx2-1* maintained a seed-setting rate comparable to that of CT16658, *nhx2-2* displayed a significant decrease (Figure 6D). Moreover, both knockout mutants produced significantly less grain yield per plant than CT16658 under normal conditions (Figure 6D; Supplemental Figure 16A).

Conversely, *OsNHX2*-OE lines (*OsNHX2-OE1* and *OsNHX2-OE2*) in the IR68 background consistently showed significantly higher seed-setting rates than IR68 under all three cold stress treatments (Figure 6B, E). Their yield per plant was significantly increased under CS-HAA conditions (Figure 6E). Under normal conditions, although seed-setting rates of the OE lines were not altered, their yield per plant differed: *OsNHX2-OE1* produced significantly higher grain yield per plant, whereas *OsNHX2-OE2* showed no difference from IR68 (Figure 6F; Supplemental Figure 16B). These results indicate that *OsNHX2* positively regulates cold tolerance at the booting stage in the IR68 background.

To determine whether this function is conserved across genetic backgrounds, we evaluated *OsNHX2* transgenic lines in the Nip background under the same three cold stress regimens. Under cold stress, both Nip knockout lines (*nhx2-3* and *nhx2-4*)

consistently exhibited significantly lower seed-setting rates than wild-type Nip across all conditions (CS-PT, CS-DW, CS-HAA) (Figure 6G, I). In contrast, the overexpression lines (*OsNHX2-OE3* and *OsNHX2-OE4*) generally displayed enhanced cold tolerance, with significantly increased seed-setting rates (Figure 6H, I). Under CS-HAA, overexpression of *OsNHX2* increased yield per plant by 18.13-22.60% relative to Nip (Figure 6I). Under normal conditions, the knockout lines exhibited markedly lower seed-setting rates and grain yield per plant, whereas the OE lines maintained seed-setting rates comparable to Nip but produced significantly higher grain yield per plant (Figure 6J; Supplemental Figure 16C).

Collectively, these findings confirm that *OsNHX2* positively regulates cold tolerance at the booting stage in both *xian* and *geng* backgrounds without compromising grain yield under normal conditions.

Genetic diversity of *OsNHX2* in germplasm resources

To elucidate the genetic diversity of *OsNHX2* in rice germplasm resources and identify favorable alleles, we performed gene haplotype analysis using 1,992 rice accessions (Shi et al., 2024). We identified four major haplotypes: Hap1 (CT16658), Hap2, Hap3 (IR68), and Hap4. Among them, Hap3 and Hap4 were predominantly distributed in *xian* accessions, while Hap2 was more frequent in *geng* accessions (Supplemental Figure 17A). Association analysis showed that Hap1 and Hap2 as haplotypes significantly linked to superior cold tolerance, with significantly higher seedling survival rates under cold stress across the *geng* and *xian* subpopulations (Supplemental Figure 17B). Haplotype network was constructed using 412 wild rice accessions (data from RiceSuperPIRdb, <http://www.ricesuperpir.com/web/rspvm>) and the aforementioned 1,992 cultivated rice accessions (Supplemental Figure 17C). The results indicated that *OsNHX2* haplotypes diversified in wild rice. The favorable haplotype Hap2 was primarily distributed in the Or-I wild rice, and Hap1 was widely present across Or-I, Or-II, and Or-III wild rice. Cold-tolerant haplotype Hap1 is mainly enriched in Southeast Asia (Indonesia, Philippines, and Malaysia), which was decreased in the *geng* but increased in frequency in *xian* during domestication and improvement

(Supplemental Figure 17D, E). Hap2 is mainly distributed in East Asia, and was likely favored by selection for adaptation to high-latitude regions (Supplemental Figure 17D, E). Both Hap3 and Hap4 have decreased in frequency during modern breeding.

Discussion

Rice is highly susceptible to cold stress, which frequently constrains its growth and adversely affects grain yield in temperate and high-altitude rice-growing regions worldwide. Understanding the molecular mechanisms underlying cold tolerance is therefore paramount for developing rice cold-tolerant varieties. Although most cloned cold-tolerance related genes in rice confer substantial cold tolerance, their favorable alleles are often fixed in *geng* varieties, limiting potential their utility for further improvement for cold tolerance. Therefore, mining unexplored cold-tolerance genes from a broader genetic pool has become a priority for rice molecular breeding. In this study, we utilized a large MAGIC population to identify OsNHX2, a tonoplast-localized K^+/H^+ antiporter, as a novel and key positive regulator of cold tolerance in rice. Our findings reveal that transcriptional and post-transcriptional control of OsNHX2 controls intracellular K^+ accumulation to modulate cold tolerance in rice. This work provides the direct molecular evidence linking a K^+ transporter to cold tolerance in rice, and our findings advance the molecular understanding of cold stress mechanisms in rice.

Plant NHX antiporters are membrane proteins that transport protons (H^+) across membranes in exchange for Na^+ or K^+ . Studies have shown that they are critical for cellular ion homeostasis and pH regulation, playing significant roles in diverse processes such as intracellular pH and cation balance, vesicle trafficking, growth, development, and tolerance to salt and drought (Bassil et al., 2012; Reguera et al., 2014). In wheat, *TaNHX2*, the homolog of rice *OsNHX2*, is a tonoplast-localized K^+/H^+ antiporter that regulates drought tolerance (Li et al., 2024). Our study expands the understanding of the functional divergence within the OsNHX family, highlighting OsNHX2 primarily as a K^+ transporter in response to cold stress. The superior K^+ transport activity of the *OsNHX2* allele from the cold-tolerant parent CT16658 highlights the importance of natural variation in conferring adaptive traits. Functional differentiation of homologous genes may be influenced by their physiological

environment.

Based on the acquired data, we propose a model elucidating the functional mechanism of *OsNHX2* (Figure 7). Under cold stress, the expression of *OsATH1* is down-regulated, which releases its repression on *OsNHX2*. Consequently, *OsNHX2* is highly expressed in the root, thereby promoting root K^+ uptake. After K^+ is translocated to the shoots, OsGAPC3 interacts with the N-terminal tonoplast-targeting signal of *OsNHX2*, altering its subcellular localization and abolishing its K^+ transport activity. This results in the retention of a large amount of K^+ in the cytosol. Potassium ions have been shown to enhance plant cold tolerance by reducing ROS generation (Wang et al., 2013). Consistent with this, the loss of *OsNHX2* under cold stress leads to a significant increase in ROS levels. Furthermore, existing research indicates that deficiency of *OsATH1* promotes peroxidase (POD)-mediated scavenging of ROS (Liu et al., 2021). Therefore, we propose that *OsNHX2* enhances cold tolerance in rice mainly through the suppression of ROS accumulation. The allele *OsNHX2*^{CT16658} is less suppressed by *OsATH1* and exhibits stronger promoter activity and higher K^+ transport activity compared with *OsNHX2*^{IR68}, which may ultimately underlie the difference in cold tolerance between CT16658 and IR68 (Figure 7). Notably, NHX proteins are frequently subject to post-translational phosphorylation modifications. In Arabidopsis, the salt-induced Ca^{2+} signal activates the SOS3-SOS2 complex, which phosphorylates and activates the Na^+/H^+ antiporter SOS1 to mediate Na^+ efflux from the cytoplasm (Quintero et al., 2002; Qiu et al., 2002). Similarly, the tonoplast-localized Na^+/H^+ exchanger MdNHX1 is a target of the glucose-sensing kinase MdHXX1, and its phosphorylation by MdHXX1 affects MdNHX1 protein stability and exchange activity (Sun et al., 2018). Based on these observations, we speculate that *OsNHX2* may also be regulated by phosphorylation via OsGAPC3. This possibility will be an important focus of our future investigations.

K^+ plays a pivotal role in plant cold stress adaptation by boosting antioxidant levels and reducing ROS production (Wang et al., 2013). Additionally, K^+ mitigates cold injury, which damages cell membranes and leads to cellular dehydration, by increasing cytosolic K^+ concentration to lower the freezing point of cellular fluid and maintain

osmotic balance (Wang et al., 2013; Cakmak et al., 2005). Consistent with these physiological roles, field studies have reported a strong negative correlation between cold sensitivity and both soil K⁺ availability and plant K⁺ content (Römheld et al., 2010; Oosterhuis et al., 2013). In legume tree *Samanea saman*, the temperature-sensitive K⁺ channel SPORK2 triggers stomatal closure in response to cold stress (Muraoka et al., 2023). Although agronomic practices demonstrate that K⁺ supplementation enhances cold tolerance in crops, direct molecular evidence establishing a causal link between K⁺ transport and rice cold tolerance has remained elusive prior to this study. Here, we provide definitive evidence that cold stress upregulates *OsNHX2*, which drives substantial K⁺ uptake, elevates shoot K⁺ content, promotes Pro accumulation, decreases ROS accumulation, reduces MDA levels, maintains membrane fluidity, and prevents K⁺ leakage, thereby ultimately enhancing cold tolerance. Notably, when external K⁺ was limited, the survival advantage of *OsNHX2*-OE lines was significantly diminished. These findings establish that dynamic K⁺ allocation and transport are central to plant cold adaptation. Moreover, external K⁺ supplementation can compensate for the reduced transport activity of specific *OsNHX2* alleles, ensuring sufficient K⁺ availability during cold stress.

The identification of *OsNHX2* and its favorable haplotype offers a valuable genetic resource for safeguarding global rice production because it protects against cold stress at two key stages of rice development without compromising grain yield under normal condition. Our findings show that the N-terminal 26-amino-acid motif of *OsNHX2* is critical for tonoplast targeting (Figure 4E). This is a key regulatory point, which can be manipulated to change *OsNHX2* activity. Targeted modification of the OsATH1-binding site within the *OsNHX2* promoter (e.g. editing InDel V6) could also lead to cold-induced upregulation of *OsNHX2*, while maintaining other physiological processes. Such precision breeding minimizes pleiotropic effects, which is important for maintaining yield stability. Integrating marker-assisted selection with genetic engineering can develop varieties that maintain high yields under stress and normal conditions. For instance, through gene editing to knockout or reduce the activity of the *OsATH1* gene, the expression level of the *OsNHX2* gene can be increased, thereby

raising shoots K^+ content to enhance cold tolerance. Given that haplotype Hap2 of the *OsNHX2* gene accounts for a low proportion in *xian* and *geng* rice varieties, introgressing it into elite cultivated *xian* and *geng* varieties via marker-assisted selection, or pyramiding it with other cold tolerance genes, can further improve the cold tolerance of *xian* and *geng* varieties. The conservation of *OsNHX2* orthologs across cereals (Li et al., 2024) suggests that the mechanisms uncovered here may improve cold tolerance in other crops.

While this study represents a significant advance, several important questions remain unresolved. The upstream signaling events responsible for downregulating the repressor OsATH1 upon cold perception are still unknown. Future work should aim to identify the initial cold sensor and the pathway linking it to OsATH1. Although OsGAPC3 acted as a negative regulator of OsNHX2-mediated K^+ transport (Figure 4F-K), cold tolerance phenotype of *OsGAPC3* transgenic lines indicate that *OsGAPC3* positively regulates rice cold tolerance (Figure 5A-B, G-H). This discrepancy suggests OsGAPC3 may be involved in extra cold-responsive pathways separate from the OsATH1–OsNHX2–OsGAPC3 module. Future work should further dissect the comprehensive roles of OsGAPC3 in rice cold tolerance.

Materials and Methods

Plant materials

This study utilized plant materials from three rice multi-parent advanced generation inter-cross (MAGIC) populations developed at the International Rice Research Institute (IRRI), Philippines (Meng et al., 2016). The populations, designated DC1, DC2, and 8way, were derived from eight elite parental varieties/lines of diverse origins. Both DC1 and DC2 are four-way recombinant inbred line (RIL) populations, developed from the crosses $(A \times B) \times (C \times D)$ and $(E \times F) \times (G \times H)$, respectively, and advanced by single-seed descent (SSD). The 8way population was generated by intercrossing 100 F_1 plants each from the ABCD and EFGH four-way crosses, yielding an eight-way cross $[(A \times B) \times (C \times D)] \times [(E \times F) \times (G \times H)]$, and was subsequently advanced

via SSD from 1,000 F₁ plants to form an eight-parent RIL population (Chen et al., 2022). Phenotypic investigation was conducted using 392 DC1 lines, 385 DC2 lines, and 569 8way lines. Seeds of the three MAGIC populations mentioned above were kindly provided by Guoyou Ye's group at the International Rice Research Institute.

The following transgenic materials were generated:

In the CT16658 background: *OsNHX2* knockout lines (*nhx2-1*, *nhx2-2*); *OsGAPC3* knockout lines (*gapc3-1*, *gapc3-2*); *OsATH1* knockout lines (*ath1-1*, *ath1-2*); and double-knockout lines (*nhx2/gapc3-1*, *nhx2/gapc3-2*, *ath1/nhx2-1*, *ath1/nhx2-2*). The target sequences for knockout via the CRISPR-Cas9 system are provided in Supplemental Table 8.

In the IR68 background: *OsNHX2*-OE lines (*OsNHX2-OE1*, *OsNHX2-OE2*); *OsGAPC3*-OE lines (*OsGAPC3-OE1*, *OsGAPC3-OE2*); *OsATH1*-OE lines (*OsATH1-OE1*, *OsATH1-OE2*); and double-OE lines (*OsNHX2/OsGAPC3-OE1*, *OsNHX2/OsGAPC3-OE2*). The overexpression sequence of *OsNHX2/OsGAPC3/OsATH1* was derived from the parental line CT16658.

In the Nipponbare (Nip) background: *OsNHX2* knockout lines (*nhx2-3*, *nhx2-4*) and *OsNHX2* OE lines (*OsNHX2-OE3*, *OsNHX2-OE4*).

The preparation of both knockout and overexpression materials was completed by BIORUN BIOSCIENCES CO., LTD.

GWAS analysis and fine mapping

Genome-wide association study (GWAS) for survival rate (SR) and leaf score of cold tolerance (SCT) was performed using the Mixed Linear Model (MLM) in TASSEL version 5.2.3, incorporating principal component analysis (PCA) coefficients and a kinship matrix as covariates. A significance threshold of $P < 0.00001$ was set for marker-trait associations. Two adjacent QTLs within a 300 kb window were considered a single QTL. The coefficient of determination (R^2) was used to estimate the phenotypic variance explained by the associated loci.

For fine-mapping, a BC₄F₂ population comprising 1,876 individuals, derived from a cross between CT16658 and IR68, was cultivated in the summer of 2024 at the Changping Experimental Base of The Institute of Crop Sciences in Beijing. Open

Reading Frames (ORFs) were predicted using the RFGDB database (<https://v1.rmbreeding.cn/>). The primer sequences are provided in Supplemental Table 8.

Cold treatment at the seedling and booting stages

The seedling cold tolerance of MAGIC population materials was evaluated in controlled phytotron chambers at the Institute of Crop Cultivation and Tillage, Heilongjiang Academy of Agricultural Sciences. Seedlings at the three-leaf with one-heart stage were subjected to cold treatment at 9°C for five days, followed by a seven-day recovery period under normal temperature conditions. For each material, two replicates were performed, with 50 seedlings per replicate. Survival rate (SR) and leaf score for cold tolerance (SCT) have been used as two indicator traits to evaluate seedling-stage cold tolerance, as the extent of leaf wilting reflects the varying levels of cold tolerance among rice plants (Kim et al., 2011; Zhang et al., 2014). SR and SCT were then recorded. Then, the SR was calculated as: the survival rate (%) = No. of survival plants/total No. of plants × 100. The leaf score for cold tolerance (SCT) was assessed according to the Technical Code of Practice for Identification of Cold Tolerance in Rice (Chinese Agricultural Industry Standard, NY/T 4454-2025). Scores were assigned as follows: 1 (all leaves remain green or nearly green; highly tolerant), 3 (≤ 30% of leaf tips discolored or chlorotic; tolerant), 5 (> 30% of leaves chlorotic or < 50% of leaves withered; moderately tolerant), 7 (50%-90% of leaves withered; sensitive), and 9 (> 90% of leaves dead; highly sensitive). Detailed scoring criteria are provided in Supplemental Table 9.

In 2025, cold tolerance at the booting stage was evaluated using three methods: high-altitude area cold stress (CS-HAA), deep-water cold stress (CS-DW), and phytotron cold stress (CS-PT) (Guo et al., 2025). For CS-HAA, materials were transplanted in summer at the experimental fields of the Yunnan Academy of Agricultural Sciences in Songming County (elevation >1,974 m). According to the 2025 meteorological data from Songming County, the average daily minimum temperature from July to August ranged from 16.39°C to 15.25°C. Each material was planted in three rows of 10 plants per row, with three replications. The seed-setting rate

of the main panicle and grain yield per plant were measured from five representative plants per replication. For CS-DW and CS-PT, plants were grown during summer in the experimental fields at China Agricultural University, Beijing. At the booting stage, plants were transferred to pools irrigated with cold water (16-18°C, CS-DW) or to a phytotron (15-17°C, relative humidity of 60%, CS-PT) for treatment. Materials in the CT16658 and IR68 backgrounds were treated for 5 days, whereas those in the Nip background were treated for 7 days, after which all plants were returned to the field. Following harvest, the relative seed-setting rate was assessed, and at least nine plants per material were processed.

RNA extraction and quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Tiangen). We used 2 µg of RNA for first -strand cDNA synthesis with the FastingKing One -Step Genomic DNA Removal and cDNA First -Strand Synthesis Premix Reagent (Vazyme Biotech Co.,Ltd). Quantitative RT-PCR was conducted on an ABI 7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's protocol, using the 2× Taq Universal SYBR qPCR Master Mix (Vazyme Biotech Co.,Ltd). The rice ubiquitin gene (Ubq) were used as internal controls. The primer sequences are listed in Supplemental Table 8.

Dual-LUC assay

The promoters of *OsNHX2*^{CT16658} and *OsNHX2*^{IR68} were cloned into a vector containing the firefly luciferase (LUC) and Renilla luciferase (REN), the primer sequences are listed in Supplemental Table 8: LZ-*OsNHX2*-F/R. The coding sequence (CDS) of *OsATH1* was cloned into a stable overexpression vector, the primer sequences are listed in Supplemental Table 8: LC-*OsATH1*-F/R. The *pOsNHX2*^{CT16658} or *pOsNHX2*^{IR68} construct was co-infiltrated with the *OsATH1* overexpression construct into tobacco leaves. After 48 h, leaf discs were harvested and lysed, and luciferase activities were measured using the Promega Dual-Luciferase® Reporter Assay System. The LUC/REN ratio was calculated to determine promoter activity.

EMSA

Four pairs of Cy5-labeled probes were designed based on the *OsNHX2* promoter

sequence. The probes sequences are listed in Supplemental Table 8: OsNHX2-R1, R2, R3, and R4. The probes were synthesized by Beijing Tsingke Biotech Co., Ltd. The full-length CDS of OsATH1 was cloned into the BamHI and HindIII sites of the linearized pET28a-SUMO vector to generate a His-OsATH1 fusion construct via homologous recombination. The primer sequences are listed in Supplemental Table 8: pET28a-OsATH1-F/R. The resulting construct was transformed into *E. coli* BL21 (DE3) competent cells. A single colony was selected to inoculate LB medium supplemented with kanamycin. When the culture reached an OD₆₀₀ of 0.8, protein expression was induced by adding 0.5 mM IPTG. The pET28a-OsATH1 protein was then purified using the Beyotime His-tag Protein Purification Kit (Cat. No. P2226). Purified protein (2 µg) was incubated with the labeled probes, and the reaction mixtures were resolved on a native polyacrylamide gel. After electrophoresis, the gel was visualized using an Amersham Imager 600 RGB system to detect probe-protein complex formation and migration (O'Dwyer M et al., 2025).

ChIP-qPCR assays

ChIP-qPCR assays were performed using three-week-old seedlings of transgenic lines carrying an OsATH1-FLAG tag. Approximately 2 g of leaf tissue was ground into powder in liquid nitrogen, then crosslinked with 1% formaldehyde at 4°C for 30 minutes. The crosslinking reaction was terminated by adding 2 M glycine for 5 minutes. After nuclear lysis and chromatin isolation, genomic DNA was sonicated to obtain fragments of approximately 300 bp. The supernatant was incubated with anti-FLAG A/G agarose beads (Sigma, Cat. No. F1804-50UG) at 4°C overnight. A sample without antibody was used as a negative control. The precipitated DNA was purified and subsequently analyzed by quantitative PCR.

ICP-MS

A 0.1 g sample (dried and ground aboveground or underground parts of rice) or the precipitate from 50 mL of yeast culture was mixed with 1 mL of ultrapure water and 4 mL of nitric acid. Three replicates were set for each sample. After digestion using a digestion instrument (EXPEC 790S), the mixture was diluted with ultrapure water to a final volume of 50 mL. The potassium ion content in the digested solution was then

determined using an inductively coupled plasma mass spectrometer (SUPEC 7000). Following automatic tuning, the collision mode was selected, and the samples were then analyzed.

Determination of physiological indicators

Wild-type CT16658 and IR68, along with *OsNHX2* mutants (*nhx2-1* and *nhx2-2*) and *OsNHX2* overexpression lines (*OsNHX2-OE1* and *OsNHX2-OE2*), were cultivated until the three-leaf stage. Leaves were sampled after 0 h and 24 h of cold treatment, with three replicates per sample. The contents of proline (Pro), MDA, superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2) were measured using assay kits (Suzhou Geruisi Biological Technology Co., Ltd.). NBT staining and DAB staining were also performed using corresponding kits from the same company.

Yeast heterologous complementation assay

Potassium transport assay: The yeast potassium ion transport activity assay kit (Cat. No. YH5001-20T) purchased from Coolaber Company was used to detect the potassium transport activity of *OsNHX2* in the potassium-uptake-deficient yeast strain R5421 (MAT α , *ura3-52*, *leu2*, Δ *trk1*, *his3* Δ 200, *his4-15*, *trk2* Δ 1::pCK64). Various components were added according to the manufacturer's instructions to prepare 100 mL of APG/-Ura solid medium containing different KCl concentrations (10 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM, 0.5 mM). The target genes were ligated into the pYES2 vector, which had been double-digested with *EcoRI* and *XhoI*, via homologous recombination. The primer sequences are listed in Supplemental Table 8: pYES2-*OsNHX2*-F/R and pYES2-*OsGAPC3*-F/R. The recombinant vector was transformed into the potassium-deficient yeast strain R5421. Yeast transformants were cultured overnight in SD/-Ura Broth (containing 100 mM KCl) until the OD₆₀₀ reached approximately 1. The cultures were then serially diluted 10-fold, 100-fold, 1,000-fold, and 10,000-fold with ddH₂O (yielding OD₆₀₀ = 1, 0.1, 0.01, 0.001, and 0.0001). In sequence, 3 μ L of each dilution was spotted onto APG/-Ura solid plates with different potassium ion concentrations. The plates were incubated at 30°C for 2-5 days, and the growth status of each yeast transformant on the different selection plates was observed to determine whether the target protein possessed potassium ion transport activity.

Sodium transport assay: The sodium ion transport activity was assessed using the sodium-sensitive yeast strain B31 (MAT α , his3, leu2, ura3, trp1, ade2, ena1::HIS3::ena4, nha1::LEU2). APG/-Ura solid medium (100 mL) was prepared containing different NaCl concentrations (200 mM, 50 mM, and 0 mM). The primer sequences are listed in Supplemental Table 8: pYES2-OsNHX2-F/R. Yeast transformants were cultured overnight in SD/-Ura Broth liquid medium until the OD600 reached approximately 1. The cultures were then serially diluted 10-fold, 100-fold, 1,000-fold, and 10,000-fold with ddH₂O (resulting in OD600 values of 1, 0.1, 0.01, 0.001, and 0.0001, respectively). In order, 3 μ L of each dilution was spotted onto APG/-Ura solid plates with different sodium ion concentrations. The plates were incubated at 30°C for 2-5 days, and the growth status of each yeast transformant on the different selection plates was observed to determine whether the target protein possessed sodium ion transport activity. Yeast transformants with sodium transport activity failed to survive on high-sodium medium.

Y2H assay

The membrane yeast two-hybrid library was constructed by BIORUN BIOSCIENCES CO., LTD. Using pPR3-N-OsNHX2 as bait, we screened and identified 21 interacting proteins (Supplemental Table 7) (Hartley et al., 2000; Alberti et al., 2010). The CDS of *OsNHX2* and its truncated versions, *OsNHX2-1* (1-442 aa), *OsNHX2-2* (27-442 aa), and *OsNHX2-3* (27-546 aa), were cloned into the pBT3-STE vector. The CDS of *OsGAPC3* was cloned into the pPR3-N vector, the primer sequences are listed in Supplemental Table 8. Construct pairs were co-transformed into yeast strain NMY51. Transformants were spotted as 3 μ L onto SD/-Leu/-Trp (control) and SD/-Leu/-Trp/-His/-Ade (selection) media and incubated at 30°C for 3-5 days to monitor interaction-dependent growth.

Subcellular localization and BiFC

Subcellular localization: The CDS sequences of *OsNHX2*, *OsNHX2*^{-26aa}, and *OsGAPC3* were cloned into the pYBA1132-EGFP vector, the primer sequences are listed in Supplemental Table 8. Rice etiolated seedlings with healthy and robust stems were selected as experimental materials. The stems were cut into 0.5-1 mm segments using

a razor blade. The segments were then incubated in 20 mL of enzyme solution (containing 2.186 g mannitol, 2 mL of 100 mM MES (pH 5.7), 0.3 g Cellulose R-10 (SIGMA), 0.15 g Macerozyme R-10 (Yakult Honsha), 0.02 g BSA, and 20 μ L of 1 M CaCl_2 , with ddH₂O added to a final volume of 20 mL) on a shaker at 28°C in the dark for 4 hours to release protoplasts. An aliquot of 200 μ L of the protoplasts was transferred to a 2 mL round-bottom centrifuge tube, mixed gently with 10 μ g of plasmid DNA to ensure full contact between the plasmid and protoplasts. Subsequently, 210 μ L of PEG4000 solution was added to the mixture, and the transformation was carried out at 28°C in the dark for 15 minutes. After removing the supernatant, 1 mL of WI solution (prepared by dissolving 9.1 g mannitol, 2 mL of 200 mM MES (pH 5.7), and 2 mL of 2 M KCl in ddH₂O to a final volume of 100 mL) was added. The samples were then incubated in the dark at 28°C for 16 hours. After incubation, the morphology and fluorescence signals of the rice protoplasts were observed using a Zeiss LSM980 confocal laser scanning microscope. Images were captured in fields with clean background and intact protoplasts.

BiFC: The CDS of *OsNHX2* and its truncated versions (*OsNHX2-1*, *OsNHX2-2*, and *OsNHX2-3*) were cloned into the pRTV-mVenusN vector. The CDS of *OsGAPC3* was cloned into the pRTV-mVenusC vector, the primer sequences are listed in Supplemental Table 8. The method for protoplast preparation was the same as described above. The morphology and fluorescence signals of the rice protoplasts were observed using a Zeiss LSM980 confocal laser scanning microscope, and images were captured in fields with clean background and intact protoplasts.

Quantitative Co-immunoprecipitation (Co-IP) Assay

Agrobacterium infiltration solutions containing OsNHX2-MYC + GFP, or OsNHX2-MYC + OsGAPC3-GFP, were respectively injected into tobacco leaves. The injected plants were divided into a cold treatment group and a normal control group. For each protein construct, the same total amount of Agrobacterium suspension was infiltrated. After 36 h of expression, tobacco plants in the cold treatment group were placed in a growth chamber at 4°C for 12 h, while those in the normal control group were maintained under normal conditions for 12 h. Subsequently, 1 g of leaf tissue was

collected from both groups, ground into powder in liquid nitrogen, and mixed with 600 μ L of IP Lysis Buffer containing Protease Inhibitor Cocktail (added immediately before use). The mixture was vortexed to a homogeneous slurry, incubated on ice for 30 min, and centrifuged. The supernatant was collected as the total protein extract. Equal amounts of total protein lysate from the cold treatment and normal control groups were incubated with GFP-agarose beads overnight at 4°C. After incubation, the beads were washed, and the precipitated proteins were eluted by adding 45 μ L of IP Lysis Buffer and 15 μ L of SDS Sample Buffer, followed by heating at 100°C for 5 min. The supernatant was collected as the IP fraction. For the Input control, 45 μ L of total protein lysate was mixed with 15 μ L of SDS Sample Buffer and heated at 100°C for 5 min. The samples were then analyzed by Western blotting.

LCA

The CDS of *OsNHX2* was cloned into the nLUC vector, and the CDS of *OsGAPC3* was cloned into the cLUC vector, the primer sequences are listed in Supplemental Table 8. The nLUC and cLUC vectors are from Coolaber (each vector carries its own independent start and stop codons). These constructs were co-infiltrated into tobacco leaves. After two days, the intensity was measured with a Rocel multifunctional plant live imaging system.

Cytosolic K⁺ fluorescence intensity / Total cellular K⁺ content

Protoplasts were isolated from IR68, *OsNHX2-OE2*, *OsGAPC3-OE2*, and *OsNHX2/OsGAPC3-OE2* lines. A total of 1×10^6 protoplasts were incubated with the cytosolic potassium fluorescent probe Enhanced Potassium Green-4 AM (EPG-4 AM) at 37°C in the dark for 40-90 min. After incubation, an additional volume of the corresponding buffer without EPG-4 AM was added, and the mixture was incubated for another 60 min to ensure complete de-esterification of the probe by the cells. The cytosolic potassium fluorescence intensity was then measured. Separately, protoplasts were isolated from the same set of materials. A total of 1×10^6 protoplasts were digested with nitric acid, and the total potassium ion content was determined using ICP-MS. Three replicates were set for each sample. The ratio of cytosolic potassium fluorescence intensity (from 1×10^6 protoplasts) to total cellular potassium content (from 1×10^6

protoplasts) was calculated.

Haplotype analysis for *OsNHX2*

Haplotype classification of *OsNHX2* was performed using 1,992 cultivated rice accessions and their cold tolerance survival rate data (Shi et al., 2024), with each haplotype containing more than 30 accessions. A haplotype network was constructed with PopART software (version 1.7) using 412 wild rice accessions (data sourced from RiceSuperPIRdb, <http://www.ricesuperpir.com/web/rspvm>) and the 1,992 cultivated rice varieties.

Data and Code Availability

The data and materials that support the findings of this study are available upon request from the corresponding author. Sequence data used in this study are available in the RAP-DB database (<http://rice.plantbiology.msu.edu/>) under the following accession numbers: *OsNHX2* (*Os11g0648000*), *OsATH1* (*Os07g0184800*), *OsGAPC3* (*Os08g0126300*). The SNP datasets for the 3,000 rice genome project (3KRGP) are from the RFGB online website (<https://rfgb.rmbreeding.cn/index>). Information of a gene query is accessible by searching with either locus ID. This study did not generate any custom code or datasets that require deposition in a public database. All other data supporting the findings of this study are available within the article and its Supplemental Information files.

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Author Contributions

J.X. and F.Z. designed the study. H.S., K.S., Z.D., J.F., Z.Z., H.C. and W.Z. performed

the experiments. K.C., X.Z., T.Z., D.L. and W.W. provided assistance with experiments. H.S., J.X. and F.Z. analyzed the data and wrote the manuscript. J.X. and F.Z. co-supervised the study. All authors reviewed and approved the final manuscript.

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Declaration of Interests

All authors have no conflicts to declare.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used DeepSeek to assist with language editing and improve the clarity and readability of the text. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Supplemental Information

Supplemental Figures

Supplemental Figure 1 Frequency distributions of SR and SCT in the MAGIC population.

Supplemental Figure 2 Genome-wide association study (GWAS) of cold tolerance-related traits in the MAGIC RILs.

Supplemental Figure 3 qRT-PCR expression analysis of candidate genes.

Supplemental Figure 4 SNPs and InDels in the promoter and CDS of *OsNHX2* were identified in IR68 and CT16658, with Nipponbare as the reference.

Supplemental Figure 5 Functional validation of *OsNHX2* in regulating cold tolerance.

Supplemental Figure 6 The InDel V6 in the *OsNHX2* promoter is the key variation

responsible for differential promoter activity between CT16658 and IR68 and mediates OsATH1 binding.

Supplemental Figure 7 EMSA analysis of OsATH1 binding to R2 and R3 cis-elements.

Supplemental Figure 8 Phylogenetic tree of the NHX gene family.

Supplemental Figure 9 Expression profile of *OsNHX2*.

Supplemental Figure 10 Effects of OsNHX2 sequence variants on predicted protein structures.

Supplemental Figure 11 Yeast complementation assays for K⁺ and Na⁺ transport activities of OsNHX2.

Supplemental Figure 12 Cold tolerance of CT16658, *nhx2-1* and *nhx2-2* under different K⁺ concentrations.

Supplemental Figure 13 Determination of proline (Pro) and malondialdehyde (MDA) contents in *OsNHX2*-OE and *nhx2* lines.

Supplemental Figure 14 The N-terminal 26-amino-acid region of OsNHX2 mediates its interaction with OsGAPC3 in the cytoplasm.

Supplemental Figure 15 Determination of K⁺ content in *gapc3* knockout and *OsGAPC3*-OE lines, as well as in *nhx2/gapc3* double-knockout and *OsNHX2/OsGAPC3*-OE lines.

Supplemental Figure 16 Performance of panicle and yield of *OsNHX2* transgenic lines under normal and cold stress conditions.

Supplemental Figure 17 Genetic diversity of *OsNHX2* in germplasm resources.

Supplemental Tables

Supplemental Table 1 Pedigree, geographic origin, key agronomic traits, and cold tolerance of the eight founder accessions used to develop the rice MAGIC population.

Supplemental Table 2 Seedling-stage cold-tolerance phenotypes of 1,346 MAGIC lines.

Supplemental Table 3 QTL identified by genome-wide association study (GWAS).

Supplemental Table 4 QTLs for SR detected in the 8way population by linkage analysis.

Supplemental Table 5 Candidate upstream transcription factors predicted from promoter sequence variations in the *OsNHX2* gene between CT16658 and IR68.

Supplemental Table 6 Amino acid sequence alignment.

Supplemental Table 7 Potential interacting proteins of OsNHX2 screened through yeast library.

Supplemental Table 8 Primer sequences.

Supplemental Table 9 Leaf score for cold tolerance (SCT) for cold tolerance at the seedling stage in rice.

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Figure Legends

Figure 1 *OsNHX2* positively regulates cold tolerance in rice

(A) Manhattan plot and quantile-quantile (Q-Q) plot of GWAS for SR in the MAGIC population. The red dashed horizontal line indicates the genome-wide significance threshold ($P = 1 \times 10^{-5}$) in the Manhattan plot.

(B) Fine-mapping of *qSR11.2*. The *qSR11.2* locus was initially mapped to the long arm of chromosome 11 between the marker loci RM206 and RM144, then narrowed to a 96.36-kb region containing nine genes. The numbers of recombinant individuals are shown beneath the marker locus positions. Survival rate (SR) is shown on the right of each representative recombinant lines. Different lowercase letters indicate significant differences at $P < 0.05$ based on Duncan's multiple-range test; error bars represent mean $\pm$ SD ($n = 3$ biological replicates; each replicate contained is 24 plants).

(C) Candidate genes by fine-mapping of *qSR11.2*. Gene annotation was retrieved from the RAP-DB database (<http://rice.plantbiology.msu.edu/>).

(D) Mutation information for the gene-edited mutants (*nhx2-1* and *nhx2-2*). The corresponding sequence position in the CT16658 coding sequence is indicated; dashes represent deleted nucleotides.

(E) Transgenic rice plants (CT16658, *nhx2-1*, *nhx2-2*, IR68, *OsNHX2-OE1*, and *OsNHX2-OE2*) under normal (Before treatment) or recovery conditions (After treatment) for 7 days after cold stress (9°C, 5 days). Scale bar = 5 cm.

(F) SR of CT16658, *nhx2-1* and *nhx2-2* after cold treatment.

(G) Electrolyte leakage rates of CT16658, *nhx2-1* and *nhx2-2* after cold treatment.

(H) Relative expression levels of *OsNHX2* in IR68, *OsNHX2-OE1* and *OsNHX2-OE2*.

(I-J) SR (I) and electrolyte leakage rates (J) of IR68, *OsNHX2-OE1* and *OsNHX2-OE2* after cold treatment. In (F-J), Different lowercase letters indicate significant differences at $P < 0.05$ based on Duncan's multiple-range test; error bars represent mean $\pm$ SD ($n=3$ biological replicates; each replicate contained is 24 plants).

Figure 2 OsATH1 directly binds to *pOsNHX2* and inhibits its expression

(A) *OsNHX2*-based association mapping. Red dots indicate SNPs with $P < 0.05$ within the 700 bp promoter and 4,105 bp genomic sequence of *OsNHX2*. Data are from GWAS of seedling-stage cold tolerance involving 1,290 *indica* accessions (Shi et al., 2024). SNPs annotations V2-V23 and H1-H7 correspond to those in Supplemental Figure 4A.

(B-C) EMSA showing binding of recombinant His-*OsATH1* protein to the R1 (B) and R4 (C) probes of the *OsNHX2* promoter. The symbols “-” and “+” indicate the absence and presence of the corresponding proteins or probes. “Shift probes” represent protein-probe complexes; “Free probes” indicate unbound probes.

(D-E) Dual-LUC assays showing that *OsATH1* represses the activity of both the *OsNHX2*^{CT16658} and *OsNHX2*^{IR68} promoters in tobacco leaves. P values were calculated by two-tailed Student’s t -tests. Error bars indicate mean $\pm$ SD ($n = 3$).

(F) ChIP-qPCR analysis showed the enrichment of *OsATH1* in the V6 fragment of the *OsNHX2* gene promoter region. P values were determined by two-tailed Student’s t -tests (** $P < 0.01$; *** $P < 0.001$). Error bars represent mean $\pm$ SD ($n = 3$).

(G) Phenotypes of transgenic rice plants under normal conditions or after recovery from cold stress. Plants of the indicated genotypes were exposed to cold stress (IR68, *OsATH1-OE1*, *OsATH1-OE2*: 9°C for 3 days; CT16658, *ath1-1*, *ath1-2*, *ath1/nhx2-1*, *ath1/nhx2-2*: 9°C for 9 days) and photographed after a 7-day recovery period. Scale bar = 5 cm.

(H) Relative expression levels of *OsATH1* and *OsNHX2* in IR68, *OsATH1-OE1*, and *OsATH1-OE2* seedlings. P values were determined by two-tailed Student’s t -tests (* $P < 0.05$; *** $P < 0.001$). Error bars indicate mean $\pm$ SD ($n = 3$).

(I) Survival rates (SR) of IR68, *OsATH1-OE1*, and *OsATH1-OE2* after cold treatment. Different lowercase letters indicate significant differences at $P < 0.05$ based on Duncan's multiple-range test. Error bars represent mean $\pm$ SD ($n = 3$ biological replicates; each replicate comprised 24 plants).

(J) Mutation details for the CRISPR/Cas9-generated *OsATH1* mutants (*ath1-1*, *ath1-2*). The corresponding position in the CT16658 *OsATH1* coding sequence is indicated. Dashes represent deleted nucleotides.

(K) SR of CT16658, *ath1-1*, *ath1-2*, *ath1/nhx2-1*, and *ath1/nhx2-2* after cold treatment. Different lowercase letters indicate significant differences at $P < 0.05$ based on Duncan's multiple-range test. Error bars represent mean $\pm$ SD ($n = 3$ biological replicates; each replicate comprised 24 plants).

Figure 3 OsNHX2 mediates K⁺ antiport

(A) Subcellular localization of OsNHX2. Scale bar = 10 μ m. mCherry, a fluorescent tag for the *Arabidopsis* tonoplast protein AtINT1.

(B-C) In situ hybridization detecting *OsNHX2* transcripts in leaf (B) and root (C) cross-sections. Enlarged views of the vascular bundles (B) and the xylem, phloem, and sclerenchyma (C) are shown on the right. Scale bars = 20 μ m. X, xylem; P, phloem; Ep, epidermis; Scl, sclerenchyma.

(D) Complementation assay in the potassium uptake-defective yeast strain R5421. Yeast cells expressing the indicated constructs were serially diluted (ten-fold) and spotted onto APG/-Ura medium containing the specified K⁺ concentrations. AtAKT1 (an *Arabidopsis* K⁺ channel) served as the positive control, and the empty pYES2 vector was the negative control.

(E-F) K⁺ content of yeast transformants grown in 30 mL (3 day) of liquid APG/ -Ura medium containing 10 mM (E) or 3 mM (F) K⁺, measured by Inductively coupled plasma mass spectrometry (ICP -MS).

(G-J) K⁺ content in shoots (G, I) and roots (H, J) of the indicated rice lines under control or cold-stress (9°C, 48 h) conditions, measured by ICP-MS.

(K) SR of IR68, *OsNHX2-OE1*, and *OsNHX2-OE2* after cold treatment under nutrient solutions containing 1 mM or 0.1 mM K⁺.

(L) Phenotypes of IR68, *OsNHX2-OE1*, and *OsNHX2-OE2* under normal conditions or after cold treatment (9°C for 5 days followed by 7-day recovery) in nutrient solutions containing 1 mM or 0.1 mM K⁺. Scale bar = 5 cm.

(M) NBT staining and O₂⁻ content detection were performed on the leaves of CT16658, *nhx2-1*, and *nhx2-2* after 0 h and 24 h of cold treatment.

(N) DAB staining and H₂O₂ content detection were conducted on the leaves of

CT16658, *nhx2-1*, and *nhx2-2* after 0 h and 24 h of cold treatment. In E-K, M, N, different lowercase letters indicate significant differences at $P < 0.05$ based on Duncan's multiple-range test. Error bars represent mean $\pm$ SD ($n = 3$).

Figure 4 Interaction between OsGAPC3 and OsNHX2

(A) In tobacco leaves, the interaction between OsGAPC3 and OsNHX2 was verified by quantitative co-immunoprecipitation (Co-IP) under normal and cold treatment conditions (12 h). The symbols “-” and “+” indicate the absence or presence of the specified components, respectively. The band intensities of the Co-IP assay were quantified using ImageJ software. The gray values of Anti-MYC and Anti-GFP protein bands were measured separately for the normal and cold-treatment groups, and the interaction strength between the two groups was compared by calculating the ratio of Anti-MYC and Anti-GFP.

(B) Schematic diagram of the OsNHX2 protein domain architecture.

(C) Bimolecular fluorescence complementation (BiFC) assay confirming the interaction between OsGAPC3 and OsNHX2 in rice protoplasts. Scale bar = 5 μ m. pRTV-mVenusN and pRTV-mVenusC were both empty vectors. Besides the negative control of the empty pRTV-mVenusN and pRTV-mVenusC vectors, OsGAPC3 with pRTV-mVenusN, and OsNHX2 with pRTV-mVenusC were used as negative controls.

(D) Yeast two-hybrid assay showing that OsGAPC3 interacts with OsNHX2 -1 but not with OsNHX2-2 or OsNHX2-3. Serial ten-fold dilutions of yeast cultures were spotted onto SD/ -Leu/ -Trp or SD/ -Leu/ -Trp/ -Ade/ -His medium, as indicated by the black triangles.

(E) Subcellular localization of OsGAPC3 and the N-terminal deletion variant OsNHX2^{-26aa}. Scale bar = 5 μ m. The RFP was fused to UGPase (UDP-glucose pyrophosphorylase), a cytosolic marker protein, to visualize the cytoplasm.

(F) Complementation assay in the K⁺-uptake-defective yeast strain R5421. Yeast cells (pYES2-AtAKT1, pYES2, pYES2-OsNHX2, pYES2-OsGAPC3, and pYES2-OsNHX2 + pYES2-OsGAPC3) expressing the indicated constructs were serially diluted (ten-fold) and spotted onto APG/ -Ura solid medium containing the specified

K⁺ concentrations. AtAKT1 served as the positive control and the empty pYES2 vector as the negative control.

(G, H) K⁺ content of yeast transformants grown in 30 mL of liquid APG/-Ura medium containing 10 mM (G) or 3 mM (H) K⁺ for 3 days, as measured by ICP-MS.

(I-K) Protoplasts were isolated from IR68, *OsNHX2-OE2*, *OsGAPC3-OE2*, and *OsNHX2/OsGAPC3-OE2* lines under normal conditions and normalized to a quantity of 1×10^6 . Cytosolic fluorescence intensity in protoplasts was detected using the potassium ion fluorescent probe EPG-4 AM (I). Total potassium ion content in 1×10^6 protoplasts was measured by ICP-MS (J). The ratio of cytosolic fluorescence intensity to total cellular potassium ion content was calculated (K). In G-K, different lowercase letters indicate significant differences at $P < 0.05$ based on Duncan's multiple-range test; error bars represent mean $\pm$ SD ($n = 3$).

Figure 5 Upstream position of *OsGAPC3* relative to *OsNHX2*

(A) Phenotypes of CT16658, *nhx2 -1*, *nhx2 -2*, *gapc3 -1*, *gapc3 -2*, *nhx2/gapc3 -1*, and *nhx2/gapc3 -2* under control conditions or after cold treatment (9°C for 7 days followed by 7 -day recovery). Scale bar = 5 cm.

(B) Phenotypes of IR68, *OsNHX2-OE1*, *OsNHX2-OE2*, *OsGAPC3-OE1*, *OsGAPC3-OE2*, *OsNHX2/OsGAPC3-OE1*, and *OsNHX2/OsGAPC3-OE2* plants under normal conditions or after cold treatment (9°C for 5 days followed by 7-day recovery). Scale bar = 5 cm.

(C-D) Relative expression levels of *OsGAPC3* and *OsNHX2* in shoots (C) and roots (D) of Nip seedlings at the indicated time points during cold stress. P values were determined by two-tailed Student's t -tests (ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Error bars indicate mean $\pm$ SD ($n = 3$).

(E) Mutation details of the CRISPR/Cas9-generated *OsGAPC3* mutants (*gapc3-1* and *gapc3-2*). The corresponding sequence position in the wild-type (CT16658) *OsGAPC3* coding sequence is indicated; dashes represent deleted nucleotides.

(F) Expression levels of *OsGAPC3* in the wild-type (IR68) and overexpression lines (*OsGAPC3-OE1*, *OsGAPC3-OE2*), determined by qRT-PCR. P values were calculated

using a two-tailed Student's *t*-test (*** $P < 0.001$). Error bars represent mean $\pm$ SD ($n = 3$).

(G) SR of the lines shown in (A) after cold treatment.

(H) SR of the plant lines shown in (B) after cold treatment. In G-H, different lowercase letters indicate significant differences at $P < 0.05$ based on Duncan's multiple-range test. Error bars represent mean $\pm$ SD ($n = 3$ biological replicates, each with 24 plants).

Figure 6 Cold tolerance of *OsNHX2* at the booting stage

(A) Phenotypes of whole plants (left) and panicles (right) of CT16658, *nhx2-1*, and *nhx2-2* under high-altitude area cold stress (CS-HAA). Scale bars: 10 cm (plants), 5 cm (panicles).

(B) Phenotypes of whole plants (left) and panicles (right) of IR68, *OsNHX2-OE1*, and *OsNHX2-OE2* under CS-HAA. Scale bars: 10 cm (plants), 5 cm (panicles).

(C) Relative seed-setting rates of CT16658, *nhx2-1*, and *nhx2-2* under phytotron cold stress (CS-PT) and deep-water cold stress (CS-DW). Seed-setting rates and yield per plant under CS-HAA conditions are also shown.

(D) Yield per plant and seed-setting rates of CT16658, *nhx2-1*, and *nhx2-2* under normal conditions (NC).

(E) Relative seed-setting rates of IR68, *OsNHX2-OE1*, and *OsNHX2-OE2* under CS-PT and CS-DW. Seed-setting rates and yield per plant under CS-HAA conditions are also shown.

(F) Yield per plant and seed-setting rates of IR68, *OsNHX2-OE1*, and *OsNHX2-OE2* under NC. In C-F, different lowercase letters indicate significant differences at $P < 0.05$ based on Duncan's multiple-range test. Error bars represent mean $\pm$ SD ($n \geq 9$ plants).

(G-H) Phenotypes of whole plants (left) and panicles (right) under CS-HAA for Nip, *nhx2-3*, and *nhx2-4* (G), and for *OsNHX2-OE3* and *OsNHX2-OE4* (H). Scale bars: 10 cm (plants), 5 cm (panicles).

(I) Relative seed-setting rates of Nip, *nhx2-3*, *nhx2-4*, *OsNHX2-OE3*, and *OsNHX2-OE4* under CS-PT and CS-DW. Seed-setting rates and yield per plant under CS-HAA conditions are also shown.

(J) Yield per plant and seed-setting rate of *Nip*, *nhx2-3*, *nhx2-4*, *OsNHX2-OE3*, and *OsNHX2-OE4* under NC. In I-J, different lowercase letters indicate significant differences at $P < 0.05$ based on Duncan's multiple-range test. Error bars represent mean $\pm$ SD ($n \geq 9$ plants).

Figure 7 Model illustrating how OsNHX2 determines cold tolerance in rice.

Under cold stress, downregulated OsATH1 alleviates its repression of *OsNHX2*. This leads to elevated *OsNHX2* expression in roots and consequently enhances root K^+ uptake. After K^+ is translocated to shoots, OsGAPC3 interacts with the N-terminal of *OsNHX2*, which alters the subcellular localization of *OsNHX2* and suppresses its K^+ transport activity. This facilitates substantial K^+ retention in the cytosol. Compared with *OsNHX2*^{IR68}, *OsNHX2*^{CT16658} shows stronger promoter activity and higher K^+ transport activity, resulting in increased proline accumulation, reduced ROS levels, and improved cold tolerance. Black arrows indicate positive regulation, and black lines with blunt ends indicate negative regulation. Solid and dashed lines indicate direct and indirect regulation, respectively. Line thickness represents the strength of regulation.

