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The rice annexin gene *OsAnn5* is involved in cold stress tolerance at the seedling stage

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Abstract

Annexins exist widely in plants as multigene families and play critical roles in stress responses and a range of cellular processes. This study provides a comprehensive account of the cloning and functional characterization of the rice annexin gene *OsAnn5*. The findings reveal that a cold stress treatment at the seedling stage of rice induced *OsAnn5* expression. GUS staining assay indicated that the expression of *OsAnn5* was non tissue-specific and was detected in almost all rice tissues. Subcellular localization indicated that *OsAnn5*-GFP (green fluorescent protein) signals were found in the endoplasmic reticulum apparatus. Compared with wild type rice, knocking out *OsAnn5* using the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated proteins) mediated genome editing resulted in sensitivity to cold treatments. These results indicate that *OsAnn5* is involved in cold stress tolerance at the seedling stage.

KEYWORDS
annexin, cold stress tolerance, CRISPR/Cas9, rice

1 | INTRODUCTION

Abiotic stresses in the environment can disadvantageously affect the normal growth, development, and yield of crops. Because of frequent climate abnormalities and inappropriate agricultural management strategies, abiotic stresses have become a major challenge threatening global agricultural production and development. Plant damage from abiotic stresses is mainly caused by the loss of cell homeostasis leading to cell death (Huang et al., 2012; Rengel et al., 2012). In order to maintain the stability of the cell structure and function and survive under adverse conditions, plants have evolved a number of adaptative physiological, biochemical, cellular, and molecular responses to abiotic stresses (Chaudhary et al., 2023; Sardar et al., 2023). Plants respond to abiotic stresses by regulating the expression of a number of stress-induced genes that may be associated with stress tolerance, transcription regulation, or signal transduction (Chaudhary et al., 2023; Li et al., 2019; Zhou et al., 2011). Transcriptome analysis of four rice genotypes demonstrated that an average of 5,975 genes in every genotype, accounting for about 18% of the annotated genes, were differentially expressed under cold stress (Shen et al., 2014). To date, a number of genes have been identified that are associated with mechanisms of abiotic stress defense, and annexin genes are an important category of relevant genes (Clark et al., 2012). It is worth noting that rice fields are increasingly challenged by abiotic stresses brought about by climate change and polluted irrigation waters (Al-Huqail et al., 2022).

Annexins are an evolutionarily conserved multigene family of Ca^{2+}-dependent phospholipid-binding proteins that occur widely in plants and animals (Jami et al., 2012; Qiao et al., 2015). Previous studies have employed sequence analysis to demonstrate how plant annexins harbor motifs or residues related to peroxidase, ATPase/GTPase activity, and calcium channel activity (Feng et al., 2013;

Laohavisit et al., 2012, 2013; Richards et al., 2014). A number of annexin genes have been characterized successively in monocot and dicot plants since the first plant annexin protein was isolated successfully in tomato (Qiao et al., 2015; Wang et al., 2018; Zhu et al., 2014). Plant annexins play a role in diverse aspects of plant growth and development and are expressed in many tissues from different development stages (Clark et al., 2012). Moreover, previous evidence suggested that annexin genes from a range of plant species are transcriptionally activated in response to abiotic stresses (Gao et al., 2020; Li et al., 2019; Zhang et al., 2021; Zhou et al., 2013). An initial report suggested that the alfalfa annexin gene (AnnMs2) is activated by drought stress, osmotic stress, and ABA treatment (Kovács et al., 1998). Subsequent evidence suggests that annexins play an important role in other plant abiotic stress responses. For example, *AnnAt1* was found to be associated with drought tolerance in *Arabidopsis*, with more sensitivity to drought stresses in loss-of-function *AnnAt1* mutants and improved drought tolerance in gain-of-function mutants (Konopka-Postupolska et al., 2009). *AnnAt1* was also found to interact with *AnnAt4* in a light-dependent manner to regulate salt and drought stress tolerance (Huh et al., 2010). Overexpression of the annexin gene *AtANN8* enhanced salt and dehydration stress tolerance in *Arabidopsis* (Yadav et al., 2016). In tomato (*Solanum pennellii*), the annexin gene *SpANN2* was found to be involved in drought and salt stress tolerance, with improved growth in *SpANN2*-overexpression (OE) lines (Ijaz et al., 2017). The cotton annexin gene *GhANN1* was also found to be involved in drought and salt stress tolerance (Zhang et al., 2015; Zhou et al., 2011).

Genome sequencing revealed that there are 10 annexin genes in rice (Singh et al., 2014), and the functional roles of several of these genes in responding to abiotic stresses have been characterized. The rice annexin gene *OsANN1* (*Os02g51750*) was found to be associated with heat and drought stress response, with more sensitivity to heat and drought stress in RNA interference plants and improved growth in *OsANN1*-OE lines (Qiao et al., 2015). Similarly, *OsANN3* (*Os07g0659600*) was also confirmed to be a positive regulator of drought stress tolerance in rice in an ABA-dependent manner (Li et al., 2019). Recent studies have revealed the significance of rice annexin gene *OsAnn3* (*Os05g0382600*) on cold stress tolerance, as evidenced by an increase in cold sensitivity following a CRISPR/Cas9-mediated knockout (KO) of the gene (Shen et al., 2017). This study presents the first report of an annexin gene involved in cold tolerance in rice, despite the fact that low temperature is a common type of stress in the life cycle of rice. In general, the functional and physiological roles of rice annexin genes in responding to cold stress remain unknown.

The present study characterizes a putative annexin protein family gene in rice, designated as *OsAnn5* (*Os06g0221200*) (consistent with the nomenclature of Singh et al., 2014). The results of this experiment reveal a rise in the expression of *OsAnn5* following low temperature treatment (4~6°C for 24 h). The role of this gene was directly tested by constructing a series of transgenic rice plants. *OsAnn5*pro::GUS, OsAnn5-GFP, and *OsAnn5* KO lines were created, the latter of which displayed more sensitivity to cold stress at the seedling stage.

2 | MATERIALS AND METHODS

2.1 | Plant materials and stress treatment

The rice (*Oryza sativa* subsp. Japonica) cultivar Taipei309 was used in this experiment and was considered as the wild type (WT) control in all experiments. The seeds of Taipei309 and T_2 biallelic KO lines from the T_0 mutant were sterilized using a 1,000-fold dilution of 50% carbendazim. The seeds were then soaked in distilled water for 24 h and germinated at 37°C in darkness for 2 days. Then, the seeds were sown in a 96-well plant hydroponic box (127 × 114 × 87 mm), and rice seedlings were watered daily using Yoshida solution until 3 weeks old (until cold treatment) in a growth chamber, with a 12/12 light/dark cycle at temperatures of 28/25°C (day/night). When rice seedlings were 3 weeks old, they were transferred to 4~6°C for 24 h of cold treatment in the same growth chamber. The survival rate, relative electric conductivity, and malondialdehyde (MDA) content were measured as described previously (Shen et al., 2017). Relative electric conductivity and MDA content were calculated using means from three biological samples with three technical replicates. The test of the survival rate for every line was repeated thrice. Each experiment was performed using forty WT and 40 KO line plants from the same mutant line. The mean values of the survival rate were calculated from three independent experiments.

2.2 | RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from Taipei309 seedling leaves grown under normal (control) conditions or under cold treatment (4~6°C for 1~4 days) using a *TransZol* Up Reagent Kit according to the manufacturer's protocol (TransGen Biotech, China). The first stand cDNA synthesis was performed using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, China). Super-Real PreMix Plus (SYBR Green) Kit (TIANGEN, China) was used for quantitative real-time PCR (qRT-PCR) analysis and carried out on a StepOne Real-Time PCR System (Applied Biosystems, United States). Real-time PCR was finished with OsAnn5-F and OsAnn5-R gene-specific primers (Table S1) as described previously (Shen et al., 2014). The relative expression level was evaluated using means from three biological samples with three technical replicates, and the amplification of the ubiquitin gene (*Os03g0234200*) was used as an internal control for normalizing all data.

2.3 | β-glucuronidase staining

To characterize the expression patterns of *OsAnn5*, an OsAnn5 promoter::GUS transgenic rice plant line was created. β-glucuronidase (GUS) reporter staining was measured using histochemical GUS staining (Jefferson et al., 1987). Three positive transgenic rice lines were incubated in 5-bromo-4-chloro-3-indolyl-β-glucuronic acid buffer at

37°C without any light. After staining, the plant tissues were soaked in 75% ethanol until the chlorophyll ingredient was completely decolorized. Finally, the sample tissues were rinsed with distilled water to remove surface dyes and chlorophyll before being photographed.

2.4 | Subcellular localization

The *OsAnn5* full-length coding region without stop codon was amplified using the primers Ann5-GFP-F/R (Table S1). The PCR product of *OsAnn5* was then fused to the GFP N-terminus, and its expression was driven by the CaMV 35S promoter located in the transient expression vector pBWA(V)HS-ccdb-GLosgfp to generate a new construct, pBWA(V)HS-Ann5-GLosgfp. This construct was then co-transformed in rice protoplasts with the marker plasmid harbor red fluorescence protein (RFP), and transfected protoplasts were incubated as described previously (Chen et al., 2010). The GFP fluorescence was observed using a Nikon C2-ER confocal laser scanning microscope (Nikon, Japan) after 48 h of infiltration.

2.5 | Construction of *OsAnn5* expression vectors

To produce a CRISPR/Cas9 expression vector for use in plant gene editing, two targeted sites were designed. DNA oligonucleotides OsAnn5-Oligo1 (24-bp) and OsAnn5-Oligo2 (24-bp) were synthesized according to the targeted site in the third exon of OsAnn5, and DNA oligonucleotides OsAnn5-Oligo3 (24-bp) and OsAnn5-Oligo4 (24-bp) were synthesized on the basis of the targeted site sequence in the fifth exon of *OsAnn5*. After annealing and phosphorylation, they were inserted into *Bbs*I sites of the cloning vector psgR-Cas9-Os (Figure S1). Then, the targeting single-stranded guide RNA (sgRNA) cassettes and Cas9 in the cloning vector were digested with *Hind*III and *Eco*RI, and the fragments were ligated into the same sites of the plant expression vector pSK51 as previously described (Shen et al., 2017). To generate OsAnn5-GFP construct, *OsAnn5* full length cDNA was digested by *Bsa*I and *Eco31*I and then ligated into the pBWA(V)HS-ccdb-GLosgfp vector digested with the same enzymes. To obtain *OsAnn5* promoter::GUS construct, about 2 Kb upstream of the *OsAnn5* ATG start codon was amplified with the primer OsAnn5 promoter-1F/1R and inserted into the *Kpn*I and *Bgl*II cloning sites of the vector pCAMBIA1304. The primers used for constructing plasmids are listed in Table S1. The plant expressing vectors were transformed into Taipei309 using *agrobacterium tumefaciens*-mediated transformation.

2.6 | Detection of targeted gene mutations

Rice leaf genomic DNA was extracted from the WT rice cultivar Taipei309, and all T_0 transgenic lines were modified with the CRISPR/Cas9 expression vector using the CTAB method. The sequence segments surrounding the two target sites were amplified using high
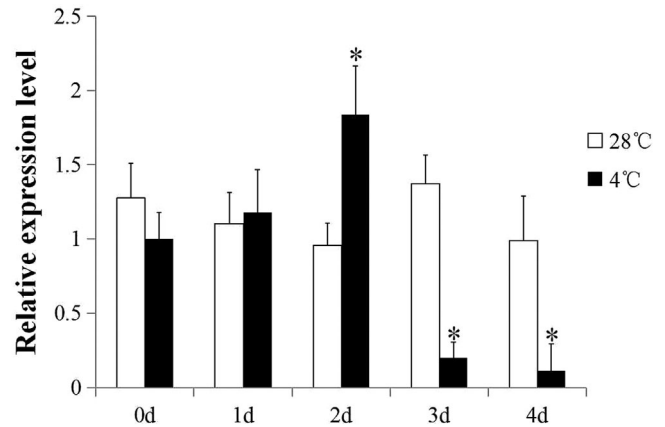

FIGURE 1 Expression of *OsAnn5* under cold stress (4∼6°C and 12 h light/12 h dark cycle for 24 h). The expression levels of genes were measured via qRT-PCR using ubiquitin as an internal control and were calculated from three independent experiments. One asterisk indicates a significant difference ($P < .05$) in comparison with normal condition. Error bars represent the s.e.m.

fidelity DNA polymerase with primer pairs TB-B1-Ann5F/R or TB-B2-Ann5F/R (Table S1). The target site mutations were evaluated by aligning sequencing chromatograms of the T_0 transgenic plants' PCR products with those of the WT rice cultivars. All mutants identified by PCR were then subjected to zygosity analysis by means of cloning corresponding PCR products into the pEASY-Blunt Zero Cloning Kit vector (TransGen Biotech, Beijing, China), and six to eight positive clones from every mutant DNA sample were sent for DNA sequencing.

2.7 | Off-target sequence identification

Possible off-target sites were evaluated by comparing the 20-nt gRNA target sequences in *OsAnn5* with the whole genomic sequences using a web-based software package, CRISPR-GE (Genome Editing) (http://skl.scau.edu.cn/) (Xie et al., 2017). The e-value threshold was set to 8 automatically because the query sequence (sgRNA) is only 20 nt. When the off-score value is equal or greater than .09, sites with the protospacer-adjacent motif (PAM) NGG motif were all considered for analysis regardless of whether they were in exons, introns, or intergenic regions. Specific primers of possible off-target loci in this experiment are listed in Table S2.

3 | RESULTS

3.1 | Expression patterns of *OsAnn5*

The promoter sequence of *OsAnn5* was characterized with PlantCARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). A 2,082 bp DNA sequence upstream of the start codon for *OsAnn5* was analyzed, revealing several cis-acting elements including
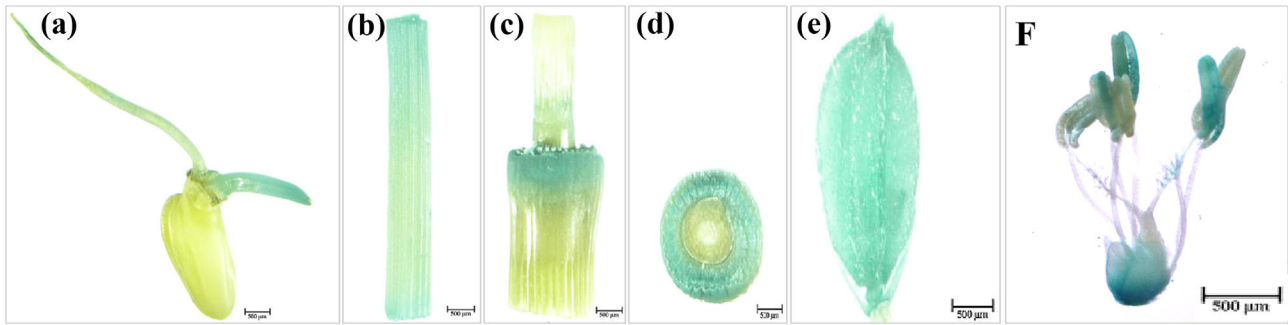
FIGURE 2 Histochemical analysis of *OsAnn5*pro::GUS transgenic rice plants. (a) Germinating seeds; (b) stem; (c) node; (d) transverse section of a node; (e) flower; and (f) anther and other sections of the flower. Scale bars = 500 μm.
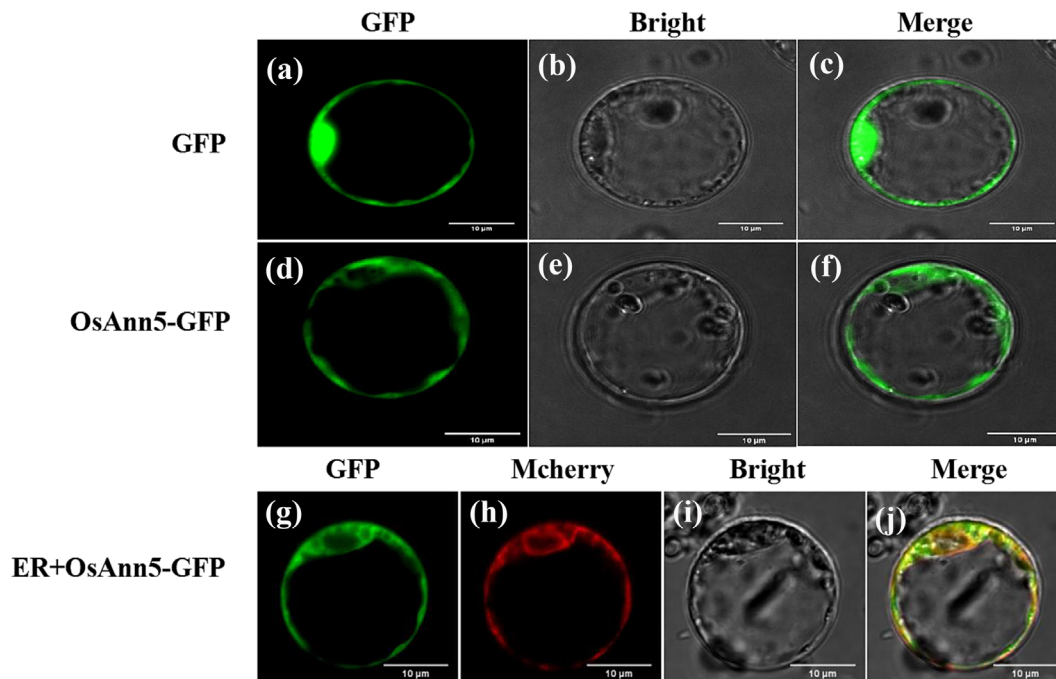

FIGURE 3 Subcellular location of OsAnn5-GFP in rice protoplasts. (a–f) The WT GFP and OsAnn5-GFP are separately transformed into rice protoplasts; (g–j) Co-localization of OsAnn5-GFP with mcherry marker at endoplasmic reticulum (ER). Scale bars = 10 μm.

two DRE cores, one MYB recognition site, one MYB-binding site, and one CCAAT-box (MYBHv1 binding site). Previous reports have documented an association between these elements and various stress responses (Table S1). To directly evaluate the effect of *OsAnn5* in responding to cold stress, qRT-PCR was performed using three-week-old WT rice seedling leaves under normal conditions (28°C) or after 4~6°C cold treatment for 24 h. Results showed that the transcription levels of *OsAnn5* in WT rice followed a low-high-low-high change rule before and after cold stress (Figure 1). *OsAnn5* expression reached the highest level (19.11-fold up-regulated) following 24 h of cold treatment compared to the normal condition (Figure 1). These findings suggest that *OsAnn5* expression is regulated by cold stress and may be involved in cold tolerance. Additionally, a GUS reporter gene was utilized to evaluate the expression patterns of *OsAnn5* in various rice tissues. Results demonstrated the presence of

28 independent positive transgenic rice lines expressing *OsAnn5*pro:: GUS, from which three independent lines were selected to carry out GUS staining experiments. The results of staining indicated that *OsAnn5* is expressed in multiple tissues, with the strongest signals found in the node, weaker signals found in the lemma, and staining also found in the embryo, roots, stems, and floral parts (Figure 2).

3.2 | Subcellular localization of OsAnn5 protein

To determine the specific subcellular localization of the OsAnn5 protein, rice protoplasts were transformed with the OsAnn5-GFP construct via PEG-mediated transient expression. When OsAnn5-GFP and pBWA(V)HS-ccdb-GLosgfp empty vectors were introduced into the rice protoplasts separately, the distribution of OsAnn5-GFP was
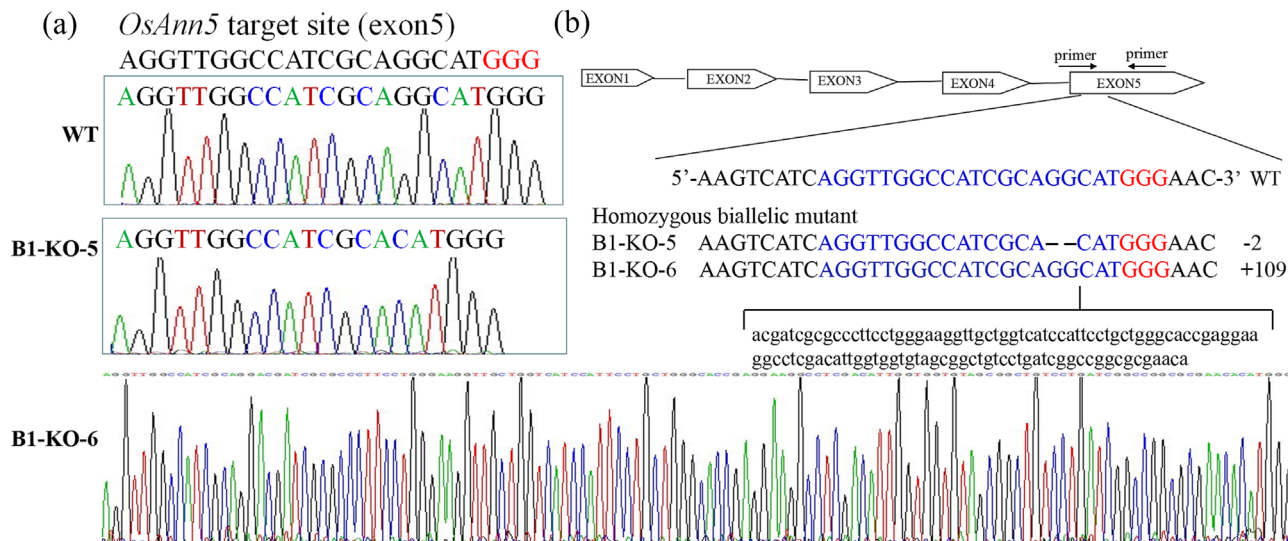
FIGURE 4 Mutation detection in the targeted site in the fifth exon of *OsAnn5*. (a) Sequencing chromatogram of PCR products from WT (Taipei309) and T_0 mutant B1-KO-5 and B1-KO-6 at the targeted site in the fifth exon of *OsAnn5*. Representative sequencing results of the region spanning the target sites are shown. (b) sgRNA:Cas9-induced mutations at the targeted site in the fifth exon of *OsAnn5* in transgenic rice plants. Blue color indicates the sgRNA:Cas9 targets, and red (GGG) color indicates the corresponding PAM. DNA samples from independent transgenic rice seedlings were analyzed for mutations using PCR product sequencing and T-vector clone sequencing. B1-KO-5 and B1-KO-6 are homozygous biallelic mutants of the targeted site in the fifth exon of *OsAnn5*. WT, wild type.

more pronounced in endoplasmic reticulum locations compared to the cytosol-localized GFP with empty vector (Figure 3). The subcellular location of OsAnn5-GFP was further confirmed via co-expression with the endoplasmic reticulum mCherry marker, the results of which displayed primary localization of the fusion protein to the endoplasmic reticulum apparatus (Figure 3). Therefore, we concluded that, in rice, OsAnn5 is most likely localized to the endoplasmic reticulum apparatus.

3.3 | Knocking out *OsAnn5* resulted in transgenic plants sensitivity to cold stress

In order to obtain mutant plants, a 1 month-cultured calli of rice (*O. sativa* L. cv. Taipei309) was transformed using an Agrobacterium clone carrying a CRISPR/Cas9 expression vector consisting of the Cas9 gene and a sgRNA targeting *OsAnn5*. For the expression vector corresponding to the targeted site in the fifth exon of *OsAnn5*, 37 individual rice transgenic T_0 lines were obtained and were subjected to mutation detection by sequencing the PCR products harboring the sgRNA target sites. Only two mutants were identified and subjected to zygosity analysis by cloning PCR products into the T vector for DNA sequencing. The examination revealed the two mutants were homozygous biallelic mutant resulting from a 2-bp deletion and 109-bp insertion, respectively (Figure 4). For the expression vector corresponding to the targeted site in the third exon of *OsAnn5*, 34 individual rice transgenic T_0 lines were obtained. The sequence analysis revealed four types of non-homologous end joining (NHEJ) mutations: +1 (1-bp insertion), −1 (1-bp deletion), −4 (4-bp deletion), and −6 (6-bp deletion) (Figure 5). Out of the three mutants, two were

monoallelic mutants, and one of them was a heterozygous biallelic mutant. In view of the finding that the T_0 biallelic mutant progeny were all mutant, three T_1 mutant lines from the T_0 biallelic mutants (B1-KO-5, B1-KO-6, and B2-KO-21) and T_2 biallelic mutant lines from the T_0 monoallelic mutant B2-KO-14 were used for the identification of the cold tolerance phenotype (Figure 6). To examine the effect of the *OsAnn5* gene KO on cold tolerance, the 3-week-old rice seedlings of the WT and KO lines were exposed to cold stress treatment (4~6°C for 1 day) and then returned to the normal growth conditions to recover. After approximately 5 days in a growth chamber, the four KO lines re-grew at rates of 6.6%, 11.7%, 26.7%, and 15.0%, while the corresponding WT lines reached survival ratios of 55.8%, 67.5%, 85.8%, and 76.6% (Figure 7). Under the same cold treatment conditions, the relative electric conductivity and MDA of leaves were measured. The findings revealed that the relative electric conductivity and MDA levels in the four KO lines were significantly increased after cold treatment in comparison with the WT. After cold treatment, the levels of electrical conductivity and MDA of each KO line exceeded those in the WT by more than 2 and 1.8 times, respectively. However, during non-stress conditions, the four KO lines and WT showed comparable levels of both factors (Figures 8 and 9). These results showed that the KO of the *OsAnn5* gene significantly decreased cold tolerance of rice at the seedling stage.

3.4 | Potential off-target loci analysis

In this study, potential off-target loci were analyzed using the CRISPR-GE software package (http://skl.scau.edu.cn/) (Xie et al., 2017). The off-target loci prediction of the fifth exon of *OsAnn5*
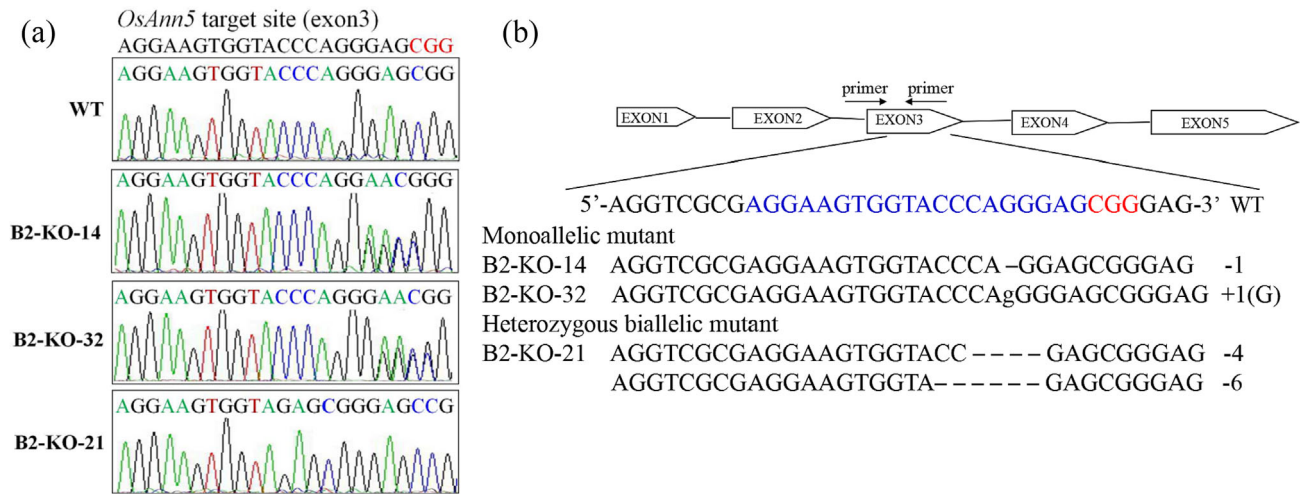
FIGURE 5 Mutation detection in the targeted site in the third exon of *OsAnn5*. (a) Sequencing chromatogram of PCR products from WT (Taipei309) and three T_0 mutants (B2-KO-14, B2-KO-32, and B2-KO-21) at the targeted site in the third exon of *OsAnn5*. Representative sequencing results of the region spanning the target sites are shown. (b) sgRNA:Cas9-induced mutations at the targeted site in the third exon of *OsAnn5* in transgenic rice plants. Blue color indicates the sgRNA:Cas9 targets, and red (CGG) color indicates the corresponding PAMs. DNA samples from independent transgenic rice seedlings were analyzed for mutations using PCR product sequencing and T-vector clone sequencing. B2-KO-14 and B2-KO-32 are monoallelic mutants of the targeted site in the third exon of *OsAnn5*. B2-KO-21 is a heterozygous biallelic mutant of the targeted site in the third exon of *OsAnn5*. WT, wild type.
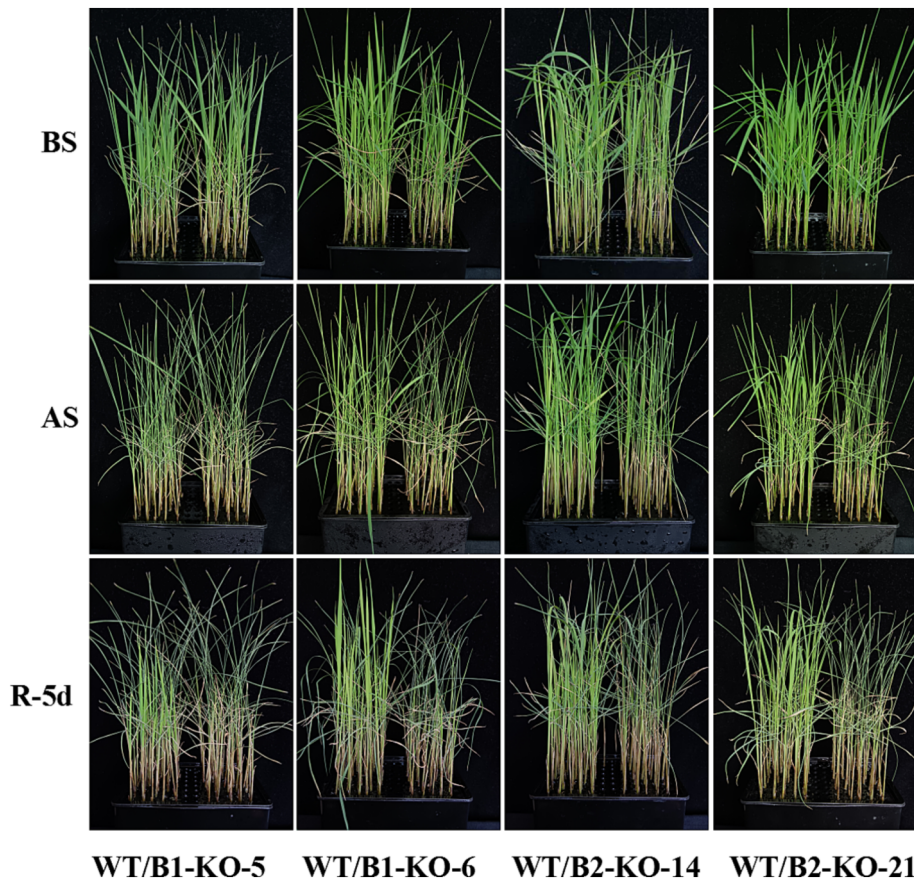

FIGURE 6 Four biallelic mutant lines of the *OsAnn5* gene showed decreased cold tolerance. Growth performance of biallelic mutant lines and WT seedlings in the same barrel (left, WT; right, mutant plants) before and after cold stress (4~6°C for 24 h). AS, after stress; BS, before stress; R-5d, recovery for 5 days after stress. The experiment was repeated three times.
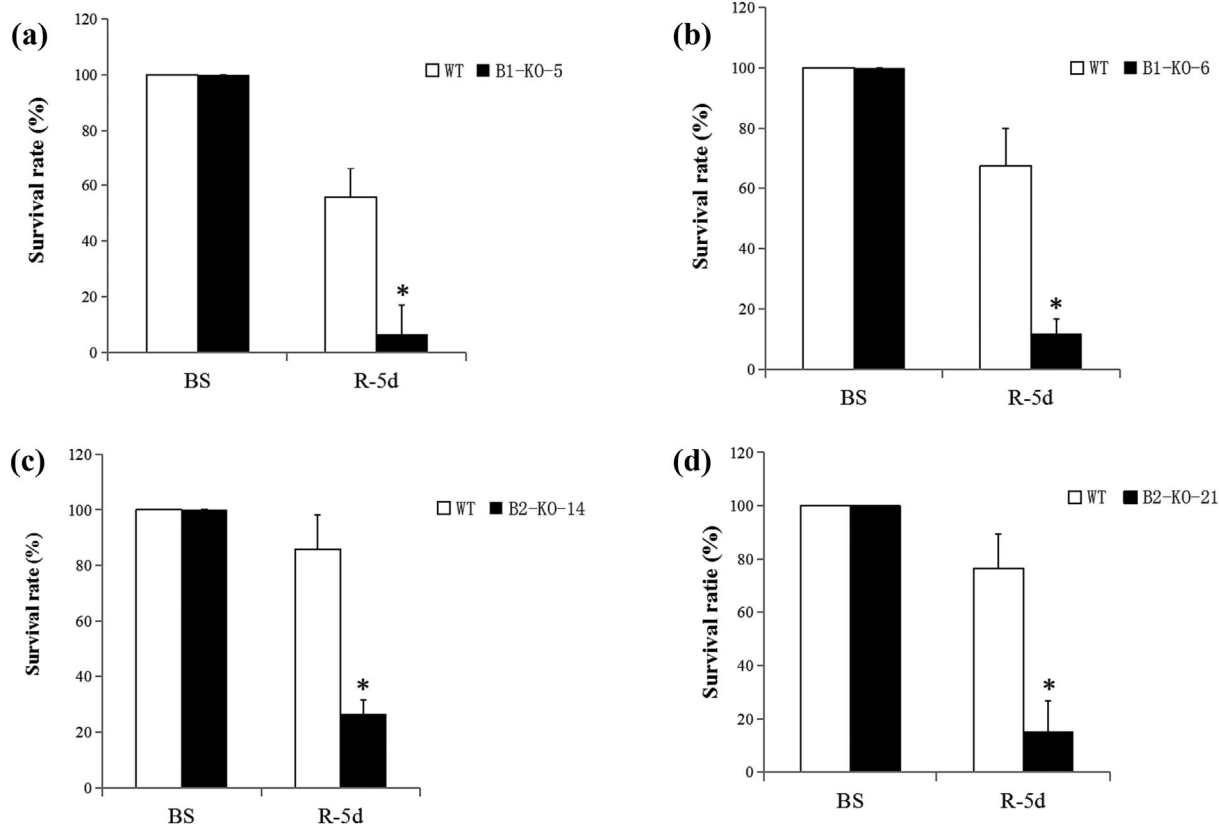
FIGURE 7 Survival rate of four biallelic mutant lines of the *OsAnn5* gene. (a) Survival rate of the biallelic mutant line B1-KO-5 after stress. (b) Survival rate of the biallelic mutant line B1-KO-6 after stress. (c) Survival rate of the biallelic mutant line B2-KO-14 after stress. (d) Survival rate of the biallelic mutant line B2-KO-21 after stress.

FIGURE 8 Relative electrical conductivity of rice seedling leaves from WT and four biallelic mutant lines of the *OsAnn5* gene before and after cold treatment (4~6°C for 24 h). One asterisk indicates significant difference ($P < .05$) in comparison with WT. Error bars represent the s.e.m.
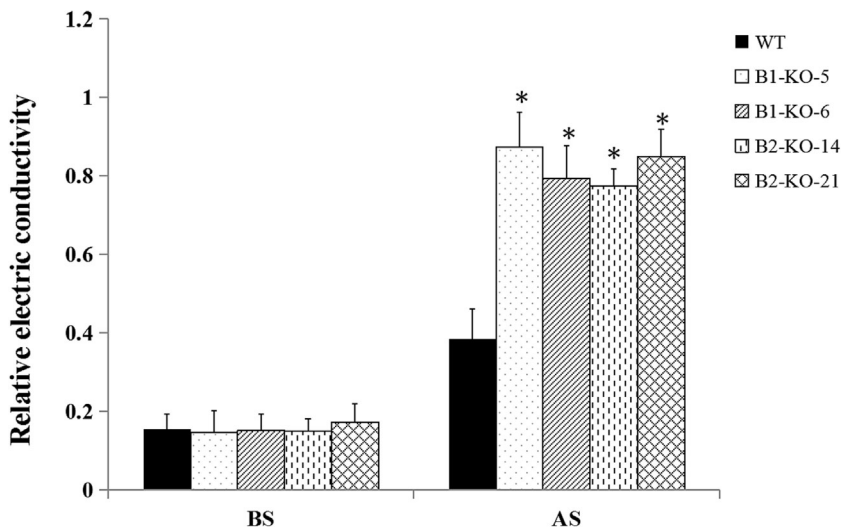
revealed three candidate sites in exon regions of the targets *Os07g0275475*, *Os11g0682300*, and *Os07g0598300*, all of which contained a 16-bp out of 20-bp identity (Figure 10). For the targeted site in the third exon of *OsAnn5*, there were two candidate sites that also had 16-bp out of 20-bp identity and existed in the exon region of the targeted *Os03g0753500* and the intron of the targeted *Os02g0654400*, respectively (Figure 11). The genomic sequence harboring the potential off-target site was amplified from WT rice and two T$_0$ biallelic mutants (B1-KO-5 and B2-KO-21), and the PCR products were then sequenced. Overlapping signals and indels were not

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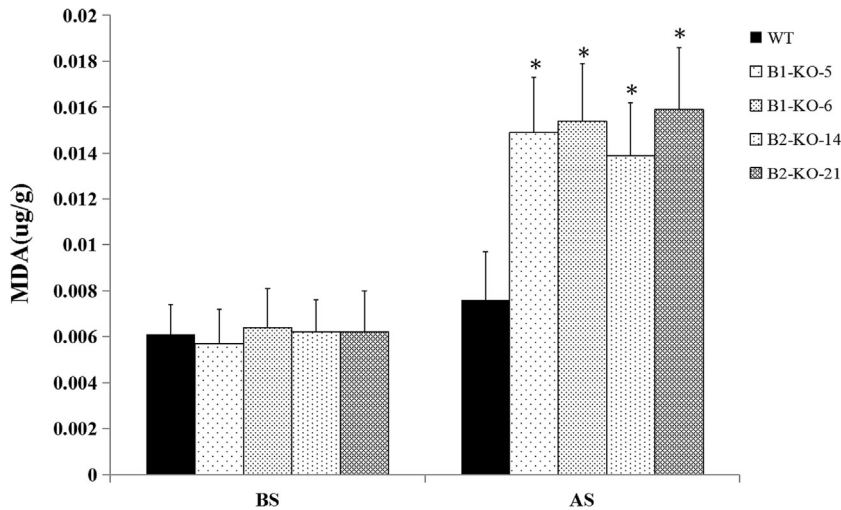

FIGURE 9 MDA of rice seedling leaves from WT and four biallelic mutant lines of the *OsAnn5* gene before and after cold treatment (4∼6°C for 24 h). One asterisk indicates significant difference ($P < .05$) in comparison with WT. Error bars represent the s.e.m.

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AGGTTGGCCATCGCAGGCATGGG    OsAnn5 sgRNA target site (exon5)
GCGTTGGCCATCACCGGCATGGG    OsAnn5 potential off-target site: Os07g0275475 (exon)
GGGATGGGCGTCGCAGGCATCGG    OsAnn5 potential off-target site: Os11g0682300 (exon)
AGTTTGGGCTTCGAAGGCATGGG    OsAnn5 potential off-target site: Os07g0598300 (exon)
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FIGURE 10 Potential off-targets at the *Os07g0275475*, *Os11g0682300*, and *Os07g0598300* loci. Mismatches between potential off-target sites and the targeted region are indicated in red. The PAM sequences are underscored.

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AGGAAGTGGTACCCAGGGAGCGG    OsAnn5 sgRNA target site (exon3)
AAGAAGAGGTACCAAGGGTGCGG    OsAnn5 potential off-target site: Os03g0753500 (exon)
AGGATGTGCTAACCAGGAAGCGG    OsAnn5 potential off-target site: Os02g0654400 (intron)
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FIGURE 11 Potential off-targets at the *Os03g0753500* and *Os02g0654400* loci. Mismatches between potential off-target sites and the targeted region are indicated in red. The PAM sequences are underscored.

detected in the two T0 biallelic mutants (Figure S2). These results suggest that off-targeting did not take place in the evaluated candidate sites of the two T0 biallelic mutants.

4 | DISCUSSION

With the increasing availability of genome sequencing, identification of rice annexin genes will continue to become easier. The role of rice annexins in responding to abiotic stress will also continue to be revealed. According to bioinformatics analyses, more than 20 putative cis-regulatory elements were identified in the OsAnn5 promoter region (Table 1). Many of these were common promoter elements, including 37 CAAT-box a (common cis-acting element in promoter and enhancer regions) and 27 TATA-box (a core promoter element located around −30 bp from the transcription onset). Some were unique to OsAnn5, including DRE core (a cis-acting element involved in CBF-mediated cold responsiveness) and MYB recognition sites. Additional cis-regulatory elements specific to OsAnn5 were identified in the region between the start codon ATG and −2,082 bp, including plant hormone regulatory elements involved in methyl jasmonate, gibberellin, and salicylic acid responsiveness, and several elements

involved in light responsiveness. This study concludes that OsAnn5 may be regulated by both common and specific transcription factors in rice. Additionally, no increase of expression in the rice annexin gene *OsAnn3* was recorded in seedlings exposed to cold stress (4°C for 2 h or 4 ± 1°C for 3 h) (Jami et al., 2012; Singh et al., 2014). Due to potential differences in induction kinetics, it can be speculated that inconsistencies in cold treatment time may be responsible for the lack of *OsAnn3* expression documented in previous studies. In this study, the transcription levels of *OsAnn5* increased rapidly, reaching a 7.32-fold up-regulation compared to the normal condition after 7 h of 4∼6°C cold treatment. Expression of the gene peaked following 24 h of cold treatment at a 19.11-fold increase in up-regulation (Figure 1). This experiment demonstrated that *OsAnn5* may function during both the initial and late stages of the cold stress response.

The results of this study demonstrated that OsAnn5-GFP fusion protein was primarily localized to the endoplasmic reticulum apparatus. Localization results were different in a recent analysis of the rice annexin gene *OsANN1*, whose subcellular localization was reported to be in the cytoplasm and cell periphery in the meristematic zone, and in the cell periphery in cells of the elongation zone (Qiao et al., 2015). Our findings were also different to those related to the rice annexin gene *OsANN3*. OsANN3-GFP fluorescence was observed in both the

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TABLE 1 Cis-regulatory elements predicted in *OsAnn5* promoter region.

Site name	Position	Signal sequence	Function of site
CAAT-box	−820, −857, −939, −961, −1,006, −1,025, −1,052, −1,086, −1,183, −1,205, −1,250, −1,286, −1,310, −1,319, −1,387, −1,559, −1,643, −1,851, −1,855, −1,889, −1,897, +349, +448, +1,268, +1,272, +1,292, +1,328, +1,432, +1,468, +1,525, +1,736, +1,842, +1,880, +1,884, +1,893, +1,894, +1,895	CAAAT/CAAT/ CCCAATTT	Common cis-acting element in promoter and enhancer regions
TATA-box	−131, −144, −1,048, −1,049, −1,504, −1,505, −1,506, −1,507, −1,583, −1,584, −1,585, −1,586, −1,610, −1,661, −1,663, −1,706, +24, +25, +133, +143, +145, +726, +1,030, +1,031, +1,032, +1,662, +1,705	ATATAA/TATA/ TATAA	Core promoter element located around −30 from transcription onset
GC-motif	+1,938, +2,003, +2,027, +2,075	CCCCCG	Enhancer-like element involved in anoxic specific inducibility
CGTCA-motif	−495, −1,690, +1,300	CGTCA	Cis-acting regulatory element involved in MeJA-responsiveness
TGACG-motif	+495, +1,690, −1,300	TGACG	Cis-acting regulatory element involved in MeJA-responsiveness
CAT-box	−532, −1,907	GCCACT	Cis-acting regulatory element related to meristem expression
A-box	−113, +1,450	CCGTCC	Cis-acting regulatory element
DRE core	+730, +2,008	GCCGAC	Cis-acting element involved in CBF mediated cold responsiveness
CARE	+422	CAACTCCC	Gibberellin-responsive element
CCAAT-box	+598	CAACGG	MYBHv1 binding site
G-box	−510	CACGAC	Cis-acting regulatory element involved in light responsiveness
GATA-motif	+555	GATAGGG	Part of a light responsive element
I-box	−411	GGATAAGGTG	Part of a light responsive element
LAMP-element	+413	CCTTATCCA	Part of a light responsive element
MYB recognition site	−598	CCGTTG	Cis-acting element involved in MYB mediated stress responsiveness
MYB-binding site	−949	CAACAG	Cis-acting element involved in Myb mediated stress responsiveness
O2-site	+697	GATGATGTGG	Cis-acting regulatory element involved in zein metabolism regulation
TATC-box	−704	TATCCCA	Cis-acting element involved in gibberellin-responsiveness
TCA-element	−886	CCATCTTTTT	Cis-acting element involved in salicylic acid responsiveness

plasma membrane and cell periphery of rice root tip cells (Li et al., 2019). The variable subcellular localization patterns among different rice annexin genes may reflect the need for diverse functions. These differences may also be caused by other factors, such as phosphorylation of proteins and the internal and external environment of the cell and so on. Phosphorylation of AnxA2 protein leads to its translocation to the plasma membrane. It was suggested that phosphorylation processes might regulate annexin distribution between cellular compartments (Gao et al., 2020; Zhang et al., 2021). AtAnn1

was found to exist widely in the plasma membrane, mitochondria, cytoplasm, thylakoid, and glyoxylate cycle (Laohavisit & Davies, 2011). The implication is that some plant annexins could be in different locations within the cell at the same time.

CRISPR/Cas9 technology has demonstrated enormous potential as an effective genome editing tool for basic and applied research in plants. To enhance mutation efficiency and ensure the loss of target gene function, two target sites in different exons of *OsAnn5* were designated and used to create and mutant plantlets. However, for

one of the two target sites, only two mutants were detected in 37 individual rice transgenic T_0 lines, providing mutation efficiency of only 5.4%. The observed mutation efficiency is notably lower compared to previous research employing identical CRISPR/Cas9-mediated expression vector backbones (Shen et al., 2017). The Cas9 gene codon usage and target site sequences all have a significant impact on mutation frequency (Mikami et al., 2015a, 2015b). In view of the higher mutation efficiency obtained using the same vector backbones in previous study (Shen et al., 2017), low mutation efficiency in the current study may reflect the choice of inappropriate target site sequences. Selection of promising target sequences by in vitro DNA cleavage assay may improve the success of in vivo CRISPR/Cas9-mediated targeted mutagenesis. In addition, prolonged culture of Cas9- and gRNA-transformed calli may enhance mutation frequency (Mikami et al., 2015a, 2015b). It may be useful for future research to combine the appropriate Cas9/gRNA expression construct with optimization of the culture period in developing more efficient targeted mutagenesis. Target specificity is an important issue for researchers to make effective use of genome editing technologies, including CRISPR/Cas. A number of previous studies have examined the specificity of the CRISPR/Cas system systematically (Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013; Xie & Yang, 2013). Experimental evidence of off-target activity was detected in rice (Shan et al., 2013; Xie & Yang, 2013). For the study of gene function using CRISPR/Cas9-mediated gene editing, off-target activity can affect the final phenotypic determination (Xie et al., 2017). Once off-target activity takes place, it becomes difficult to determine whether phenotypic change is due to target gene KO or off-target activity. Therefore, it is necessary to evaluate potential off-target loci when utilizing a CRISPR/Cas9-mediated KO approach. Off-target mutations caused by the CRISPR system can be minimized by choosing target sequences that have reduced numbers of off-targets. CRISPR-GE (http://skl.scau.edu.cn/) presented a convenient and integrated toolkit by which we could expedite all experimental designs and analyses of mutations for CRISPR/Cas9 genome editing in plants, and it provided a set of powerful tools for prediction of off-target sites (Xie et al., 2017). In this study, we evaluated five candidate off-targets, including two in the exons of *OsAnn5* using the CRISPR-GE software package. We found no evidence for off-targeting phenomena in the candidate sites. These results support the reliability of the identification of the cold tolerance phenotypes in mutants.

Finally, our results demonstrated that four KO lines showed decreased cold tolerance compared with the Taipei309 WT variety. This means that the rice annexin gene *OsAnn5* is involved in cold stress tolerance at the seedling stage. *OsAnn5* thus becomes only the second rice annexin gene reported to be involved in cold tolerance at the seedling stage, following previous reports of a similar role for the annexin gene *OsAnn3*. These results expand our understanding of the complex mechanisms of annexin response to cold stress in rice. Genetic engineering using annexin genes might offer a new and excellent platform to develop rice cold resistance breeding.

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REFERENCES

Al-Huqail, A. A., Kumar, P., Eid, E. M., Adelodun, B., Abou Fayssal, S., Singh, J., Arya, A. K., Goala, M., Kumar, V., & Širić, I. (2022). Risk assessment of heavy metals contamination in soil and two Rice (Oryza sativa L.) varieties irrigated with paper mill effluent. *Agriculture*, *12*, 1864.

Chaudhary, P., Xu, M., Ahamad, L., Chaudhary, A., Kumar, G., Adeleke, B. S., Verma, K. K., Hu, D.-M., Širić, I., Kumar, P., Popescu, S. M., & Abou Fayssal, S. (2023). Application of synthetic consortia for improvement of soil fertility, pollution remediation, and agricultural productivity: A review. *Agronomy*, *643*, 0643. https://doi.org/10.3390/agronomy13030643

Chen, S., Tao, L., Zeng, L., Vega-Sanchez, M. E., Umemura, K., & Wang, G. L. (2010). A highly efficient transient protoplast system for analyzing defense gene expression and protein-protein interactions in rice. *Molecular Plant Pathology*, *7*, 417–427. https://doi.org/10.1111/j.1364-3703.2006.00346.x

Clark, G. B., Morgan, R. O., Fernandez, M. P., & Roux, S. J. (2012). Evolutionary adaptation of plant annexins has diversified their molecular structures, interactions and functional roles. *The New Phytologist*, *196*, 695–712. https://doi.org/10.1111/j.1469-8137.2012.04308.x

Feng, Y. M., Wei, X. K., Liao, W. X., Huang, L. H., Zhang, H., Liang, S. C., & Peng, H. (2013). Molecular analysis of the annexin gene family in soybean. *Biologia Plantarum*, *57*, 655–662. https://doi.org/10.1007/s10535-013-0334-0

Gao, S., Song, T., Han, J., He, M., Zhang, Q., Zhu, Y., & Zhu, Z. (2020). A calcium-dependent lipid binding protein, OsANN10, is a negative regulator of osmotic stress tolerance in rice. *Plant Science*, *293*, 110420. https://doi.org/10.1016/j.plantsci.2020.110420

Huang, G. T., Ma, S. L., Bai, L. P., Zhang, L., Ma, H., Jia, P., Liu, J., Zhong, M., & Guo, Z. F. (2012). Signal transduction during cold, salt, and drought stresses in plants. *Molecular Biology Reports*, *39*, 969–987. https://doi.org/10.1007/s11033-011-0823-1

Huh, S. M., Noh, E. K., Kim, H. G., Jeon, B. W., Bae, K., Hu, H. C., Kwak, J. M., & Park, O. K. (2010). Arabidopsis annexins *AnnAt1* and *AnnAt4* interact with each other and regulate drought and salt stress responses. *Plant & Cell Physiology*, *51*, 1499–1514. https://doi.org/10.1093/pcp/pcq111

Ijaz, R., Ejaz, J., Gao, S., Liu, T., Imtiaz, M., Ye, Z., & Wang, T. (2017). Over-expression of annexin gene *AnnSp2*, enhances drought and salt tolerance through modulation of ABA synthesis and scavenging ROS in tomato. *Scientific Reports*, *7*, 12087. https://doi.org/10.1038/s41598-017-11168-2

Jami, S. K., Clark, G. B., Ayele, B. T., Ashe, P., & Kirti, P. B. (2012). Genome wide comparative analysis of annexin superfamily in plants. *PLoS ONE*, *7*, e47801. https://doi.org/10.1371/journal.pone.0047801

Jami, S. K., Clark, G. B., Ayele, B. T., Ayele, B. T., Roux, S. J., & Kirti, P. B. (2012). Identification and characterization of annexin gene family in rice. *Plant Cell Reports*, *31*, 813–825. https://doi.org/10.1007/s00299-011-1201-0

Jefferson, R. A., Kavanagh, T. A., & Bevan, M. W. (1987). GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal*, *6*, 3901–3907. https://doi.org/10.1002/j.1460-2075.1987.tb02730.x

Konopka-Postupolska, D., Clark, G., Goch, G., Debski, J., Floras, K., Cantero, A., Fijolek, B., Roux, S., & Hennig, J. (2009). The role of *annexin 1* in drought stress in Arabidopsis. *Plant Physiology*, *150*, 1394–1410. https://doi.org/10.1104/pp.109.135228

Kovács, I., Ayaydin, F., Oberschall, A., Ipacs, I., Bottka, S., Pongor, S., Dudits, D., & Tóth, É. C. (1998). Immunolocalization of a novel annexin-like protein encoded by a stress and abscisic acid responsive gene in alfalfa. *The Plant Journal*, *15*, 185–197. https://doi.org/10.1046/j.1365-313X.1998.00194.x

Laohavisit, A., & Davies, J. M. (2011). Annexins. *The New Phytologist*, *189*, 40–53. https://doi.org/10.1111/j.1469-8137.2010.03533.x

Laohavisit, A., Richards, S. L., Shabala, L., Chen, C., Colaço, R. D., Swarbreck, S. M., Shaw, E., Dark, A., Shabala, S., Shang, Z., & Davies, J. M. (2013). Salinity-induced calcium signaling and root adaptation in Arabidopsis require the calcium regulatory protein annexin1. *Plant Physiology*, *163*, 253–262. https://doi.org/10.1104/pp.113.217810

Laohavisit, A., Shang, Z., Rubio, L., Cuin, T. A., Véry, A. A., Wang, A., Mortimer, J. C., Macpherson, N., Coxon, K. M., Battey, N. H., Brownlee, C., Park, O. K., Sentenac, H., Shabala, S., Webb, A. A. R., & Davies, J. M. (2012). *Arabidopsis* annexin1 mediates the radical-activated plasma membrane Ca^{2+}- and K^+-permeable conductance in root cells. *The Plant Cell*, *24*, 1522–1533. https://doi.org/10.1105/tpc.112.097881

Li, J. F., Norville, J. E., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G. M., & Sheen, J. (2013). Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. *Nature Biotechnology*, *31*, 688–691. https://doi.org/10.1038/nbt2654

Li, X., Zhang, Q., Yang, X., Han, J., & Zhu, Z. (2019). *OsANN3*, a calcium-dependent lipid binding annexin is a positive regulator of ABA-dependent stress tolerance in rice. *Plant Science*, *284*, 212–220. https://doi.org/10.1016/j.plantsci.2019.04.019

Mikami, M., Toki, S., & Endo, M. (2015a). Comparison of CRISPR/Cas9 expression constructs for efficient targeted mutagenesis in rice. *Plant Molecular Biology*, *88*, 561–572. https://doi.org/10.1007/s11103-015-0342-x

Mikami, M., Toki, S., & Endo, M. (2015b). Parameters affecting frequency of CRISPR/Cas9 mediated targeted mutagenesis in rice. *Plant Cell Reports*, *34*, 1807–1815. https://doi.org/10.1007/s00299-015-1826-5

Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J. D., & Kamoun, S. (2013). Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. *Nature Biotechnology*, *31*, 691–693. https://doi.org/10.1038/nbt.2655

Qiao, B., Zhang, Q., Liu, D., Wang, H., Yin, J., Wang, R., He, M., Cui, M., Shang, Z., Wang, D., & Zhu, Z. (2015). A calcium-binding protein, rice annexin *OsANN1*, enhances heat stress tolerance by modulating the production of H_2O_2. *Journal of Experimental Botany*, *66*, 5853–5866. https://doi.org/10.1093/jxb/erv294

Rengel, D., Arribat, S., Maury, P., Martin-Magniette, M. L., Hourlier, T., Laporte, M., Varès, D., Carrère, S., Grieu, P., Balzergue, S., Gouzy, J., Vincourt, P., & Langlade, N. B. (2012). A gene-phenotype network based on genetic variability for drought responses reveals key physiological processes in controlled and natural environments. *PLoS ONE*, *7*, e45249. https://doi.org/10.1371/journal.pone.0045249

Richards, S. L., Laohavisit, A., Mortimer, J. C., Shabala, L., Swarbreck, S. M., Shabala, S., & Davies, J. M. (2014). *Annexin 1* regulates the H_2O_2-induced calcium signature in *Arabidopsis thaliana* roots. *The Plant Journal*, *77*, 136–145. https://doi.org/10.1111/tpj.12372

Sardar, H., Khalid, Z., Ahsan, M., Naz, S., Nawaz, A., Ahmad, R., Razzaq, K., Wabaidur, S. M., Jacquard, C., Širić, I., Kumar, P., & Abou Fayssal, S. (2023). Enhancement of salinity stress tolerance in lettuce (Lactuca sativa L.) via foliar application of nitric oxide. *Plants*, *12*, 1115. https://doi.org/10.3390/plants12051115

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, K., Liu, J., Xi, J. J., Qiu, J.-L., & Gao, C. (2013). Targeted genome modification of crop plants using a CRISPR-Cas system. *Nature Biotechnology*, *31*, 686–688. https://doi.org/10.1038/nbt.2650

Shen, C. X., Li, D., He, R. H., Fang, Z., Xia, Y. M., Gao, J., & Cao, M. (2014). Comparative transcriptome analysis of RNA-Seq data for cold-tolerant and cold-sensitive rice genotypes under normal and cold stress. *Journal of Plant Biology*, *57*, 337–348. https://doi.org/10.1007/s12374-014-0183-1

Shen, C., Que, Z., Xia, Y., Tang, N., Li, D., He, R., & Cao, M. (2017). Knock out of the annexin gene *OsAnn3* via CRISPR/Cas9-mediated genome editing decreased cold tolerance in rice. *Journal of Plant Biology*, *60*, 539–547. https://doi.org/10.1007/s12374-016-0400-1

Singh, A., Kanwar, P., Yadav, A. K., Mishra, M., Jha, S. K., Baranwal, V., Pandey, A., Kapoor, S., Tyagi, A. K., & Pandey, G. K. (2014). Genome-wide expressional and functional analysis of calcium transport elements during abiotic stress and development in rice. *The FEBS Journal*, *281*, 894–915. https://doi.org/10.1111/febs.12656

Wang, J., Song, J., Clark, G., & Roux, S. J. (2018). *ANN1* and *ANN2* function in post-phloem sugar transport in root tips to affect primary root growth. *Plant Physiology*, *178*, 390–401. https://doi.org/10.1104/pp.18.00713

Xie, X., Ma, X., Zhu, Q., Zeng, D., Li, G., & Liu, Y. G. (2017). CRISPR-GE: A convenient software toolkit for CRISPR-based genome editing. *Molecular Plant*, *10*, 1246–1249. https://doi.org/10.1016/j.molp.2017.06.004

Xie, K., & Yang, Y. (2013). RNA-guided genome editing in plants using a CRISPR-Cas system. *Molecular Plant*, *6*, 1975–1983. https://doi.org/10.1093/mp/sst119

Yadav, D., Ahmed, I., Shukla, P., Boyidi, P., & Kirti, P. B. (2016). Overexpression of Arabidopsis *AnnAt8* alleviates abiotic stress in transgenic Arabidopsis and tobacco. *Plants*, *5*, 18. https://doi.org/10.3390/plants5020018

Zhang, F., Li, S., Yang, S., Wang, L., & Guo, W. (2015). Overexpression of a cotton annexin gene, GhAnn1, enhances drought and salt stress tolerance in transgenic cotton. *Plant Molecular Biology*, *87*, 47–67. https://doi.org/10.1007/s11103-014-0260-3

Zhang, Q., Song, T., Guan, C., Gao, Y., Ma, J., Gu, X., Qi, Z., Wang, X., & Zhu, Z. (2021). OsANN4 modulates ROS production and mediates ca 2+ influx in response to ABA. *BMC Plant Biology*, *21*, 474. https://doi.org/10.1186/s12870-021-03248-3

Zhou, L., Duan, J., Wang, X. M., Zhang, H. M., Duan, M. X., & Liu, J. Y. (2011). Characterization of a novel annexin gene from cotton

(Gossypium 290 hirsutum cv CRI 35) and antioxidative role of its recombinant protein. *Journal of Integrative Plant Biology*, *53*, 347–357. https://doi.org/10.1111/j.1744-7909.2011.01034.x

Zhou, M. L., Yang, X. B., Zhang, Q., Zhou, M., Zhao, E. Z., Tang, Y. X., Zhu, X.-M., Shao, J.-R., & Wu, Y. M. (2013). Induction of annexin by heavy metals and jasmonic acid in Zea mays. *Functional & Integrative Genomics*, *13*, 241–251. https://doi.org/10.1007/s10142-013-0316-5

Zhu, J., Wu, X., Yuan, S., Qian, D., Nan, Q., An, L., & Xiang, Y. (2014). Annexin5 plays a vital role in arabidopsis pollen development via ca2+−dependent membrane trafficking. *PLoS ONE*, *9*, e102407. https://doi.org/10.1371/journal.pone.0102407

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