

# The rice annexin gene *OsAnn5* is a positive regulator of 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. In this study, we report on the cloning and functional characterization of the rice annexin gene *OsAnn5*. We found that the expression of *OsAnn5* was induced by cold stress treatment at the seedling stage of rice. 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, overexpression of *OsAnn5* significantly increased survival rates at the seedling stage under cold stress, while 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 a positive regulator of cold stress tolerance at the seedling stage.

## Background

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 (Bohnert et al., 1995; Browse and Xin, 2001; Chinnusamy et al., 2007). 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 (Thomashow 1999; Shinozaki et al., 2003; Nakashima et al., 2009). Transcriptome analysis of four rice genotypes demonstrated that an average of 5975 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).

Annexins are an evolutionarily conserved multigene family of Ca<sup>2+</sup>-dependent phospholipid-binding proteins that occur widely in plants and animals (Rescher and Gerke, 2004; Mortimer et al., 2008; Jami et al., 2012). Sequence analysis has demonstrated that plant annexins harbor motifs or residues related to peroxidase and ATPase/GTPase activity, as well as calcium channel activity (Mortimer et al., 2008), which has also been well demonstrated in subsequent research (Gorecka et al., 2005; 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 (Calvert et al., 1996; Mortimer et al., 2008; Qiao et al., 2015; Wang et al., 2018). Plant annexins play a role in diverse aspects of plant growth and development, and they are expressed in many tissues from different development stages (Clark et al., 2012). Moreover, previous evidence suggests that annexin genes from a range of plant species are transcriptionally activated in response to abiotic stresses. An initial report suggested that the alfalfa annexin gene (*AnnMs2*) is activated by drought stress, osmotic stress, and ABA treatment (Kovacs 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*, such that *AnnAt1* and *AnnAt4* regulated salt and drought stress tolerance by interacting with each other in a light-dependent manner (Lee et al., 2004; 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 (Zhou et al., 2011; Zhang et al., 2015).

Genome sequencing revealed that there are ten 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., 2014). 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). We have recently demonstrated that the rice annexin gene *OsAnn3* (*Os05g0382600*) is relevant for cold stress tolerance, with more sensitivity to cold stress when *OsAnn3* was knocked out by CRISPR/Cas9-mediated gene modification (Shen et al., 2017). This study was 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.

In the present study we isolate and characterized a putative annexin protein family gene in rice, designated as *OsAnn5* (*Os06g0221200*) [consistent with the nomenclature of Singh et al (Singh et al., 2014)]. We demonstrate that the expression of *OsAnn5* increased following low temperature treatment (4 ~ 6 °C for 4 days). We directly tested the role of *OsAnn5* by constructing a series of transgenic rice plants; we used CRISPR/Cas9-mediated genome editing to create an *OsAnn5* knock out (KO) line and also constructed OE, *OsAnn5*pro::GUS and *OsAnn5*-GFP lines. We found that the OE lines enhanced cold tolerance in rice, whereas the KO lines were more sensitive to cold stress.

## Methods

### 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<sub>1</sub> KO lines from the T<sub>0</sub> biallelic mutant were sterilized and germinated at 37 °C in darkness for 2 days. Then, the seeds were sown in a plastic pot (22 × 17.5 × 7.5 cm) filled with soil in a light incubator, with a 12/12 light/dark cycle at temperatures of 28/25 °C (day/night). The seeds were watered daily using sterile water until the cold stress treatment. When rice seedlings were four weeks old they were transferred to 4 ~ 6 °C for 3 days of cold

treatment. With the same light and temperature conditions but not soil and sterile water, the seeds of WT and the T<sub>3</sub> generation OE lines were sown in 9 cm diameter glass petri dishes, and rice seedlings were watered daily using Yoshida solution until four weeks old (until cold treatment). The survival rate and relative electric conductivity were measured as described previously (Shen et al., 2017).

## RNA extraction and quantitative Real-Time PCR (qRT-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). SuperReal PreMix Plus (SYBR Green) Kit (TIANGEN, China) was used for qRT-PCR analysis and carried out on a StepOne Real-Time PCR System (Applied Biosystems, USA). 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.

## β-glucuronidase (GUS) staining

In order to characterize the expression patterns of *OsAnn5*, we generated *OsAnn5* promoter::GUS transgenic rice plants. 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.

## Subcellular localization of *OsAnn5* protein

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.

### Construction of *OsAnn5* expression vectors

To overexpress *OsAnn5*, the full-length cDNA was amplified from Taipei309 and inserted into the pCAMBIA1301-35S::*OsAnn5* vector. 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 *BbsI* sites of the cloning vector psgR-Cas9-Os (Fig. S1). Then, the targeting single-stranded guide RNA (sgRNA) cassettes and Cas9 in the cloning vector were digested with *HindIII* and *EcoRI*, 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 *BsaI* and *Eco31I* 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 *Ann5pro-F/R* and inserted into the *KpnI* and *BglI* 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.

## Detection of Targeted Gene Mutations

Rice leaf genomic DNA was extracted from the WT rice cultivar Taipei309 and all T<sub>0</sub> transgenic lines modified with the CRISPR/Cas9 expression vector using the CTAB method. The sequence segments surrounding the two target sites were amplified using high 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<sub>0</sub> 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 6–8 positive clones from every mutant DNA sample were sent for DNA sequencing.

## 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 0.09, 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.

## Results

### Expression patterns of *OsAnn5*

The promoter sequence of *OsAnn5* was characterized with PlantCARE software (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). We analyzed a 2082 bp DNA sequence upstream of the start codon for *OsAnn5* and found that there were several cis-acting elements, including two DRE cores, one MYB recognition site, one MYB-binding site, and one CCAAT-box (MYBHv1 binding site) that have been previously reported to be associated with stress responses

(Table S1). To directly evaluate the effect of *OsAnn5* in responding to cold stress, qRT-PCR was performed using four week old WT rice seedling leaves under normal conditions (28 °C) or after 4 ~ 6 °C cold treatment for 4 days. Results showed that the transcription levels of *OsAnn5* in WT rice followed a low-high-high-low-low change rule before and after cold stress (Fig. 1). *OsAnn5* expression reached the highest level (1.84-fold up-regulated) following 2 days of cold treatment compared to the normal condition (Fig. 1). These findings suggest that *OsAnn5* expression is regulated by cold stress and may be involved in cold tolerance. Additionally, we evaluated the expression patterns of *OsAnn5* in various rice tissues using the GUS reporter gene. 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 (Fig. 2).

### Overexpression of *OsAnn5* results in enhanced rice cold tolerance

To evaluate the function of *OsAnn5* in responding to cold stress, T<sub>3</sub> generation OE and WT lines were grown in the same batch of Yoshida solution in 9 cm diameter glass petri dishes for about four weeks, then treated with 4 ~ 6 °C for 3 days, and then returned to the normal growth conditions to recover. After approximately 10 days in the greenhouse following cold treatment, OE lines plants showed better growth, while WT plants had severe wilting and rolling leaves (Fig. 3A). Additionally, OE lines had a higher average survival rate of 39.97% (OE-18, 29.30%; OE-23, 46%; OE-24 44.60%) compared to 6.67% in the WT rice (Fig. 3B). Under the same cold treatment conditions, experiments were carried out to measure the relative electric conductivity of leaves. Results revealed that the relative electric conductivity levels in the three T<sub>3</sub> OE lines were significantly lower after cold treatment compared to the WT condition, while they were similar in the three T<sub>3</sub> OE lines and WT rice in non-stress condition (Fig. 3C). These results indicate that overexpression of *OsAnn5* in rice can enhance tolerance to cold stress.

### *OsAnn5*-GFP probably localizes to the endoplasmic reticulum apparatus

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 more pronounced in endoplasmic reticulum locations compared to the cytosol-localized GFP with empty vector (Fig. 4). We further confirmed the subcellular location of *OsAnn5*-GFP by expressing it together with the endoplasmic reticulum mcherry marker, with results indicating that the fusion protein was mainly localized to the endoplasmic reticulum apparatus (Fig. 4). Therefore, we concluded that in rice, *OsAnn5* is most likely localized to the endoplasmic reticulum apparatus.

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

One month-cultured rice calli was infected using the Agrobacterium-mediated transformation of rice (*Oryza sativa* L. cv. Taipei309) method, with an Agrobacterium clone carrying the CRISPR/Cas9 expression vector containing the Cas9 gene and a sgRNA targeting the *OsAnn5* gene. For the expression vector corresponding to the targeted site in the fifth exon of *OsAnn5*, 18 individual rice transgenic T<sub>0</sub> lines were obtained and were subjected to mutation detection by sequencing the PCR products harboring the sgRNA target sites. Only one mutant was identified and subjected to zygosity analysis by cloning PCR products into the T vector for DNA sequencing. The examination revealed the mutant was a homozygous biallelic mutant resulting from a 2-bp deletion (Fig. 5). For the expression vector corresponding to the targeted site in the third exon of *OsAnn5*, 34 individual rice transgenic T<sub>0</sub> 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) (Fig. 6). Out of the 3 mutants, two were monoallelic mutants and one of them was a heterozygous biallelic mutant. In view of the finding that the T<sub>0</sub> biallelic mutant progeny were all mutant, two T<sub>1</sub> mutant lines from the T<sub>0</sub> biallelic mutant (B1-KO-8 and B2-KO-21) were used for the identification of the cold tolerance phenotype. To examine the effect of the *OsAnn5* gene knockout on cold tolerance, the four week old rice seedlings of the WT and KO lines were exposed to cold stress treatment (4 ~ 6 °C for 3 days), and then returned to the normal growth conditions to recover. After approximately 10 days in the greenhouse following cold treatment, the two T<sub>1</sub> KO lines re-grew 37.5% and 34.7%, respectively, while the survival ratio of the corresponding WT lines reached 80.5% and 78.2% respectively (Fig. 7). Under the same cold treatment conditions, the relative electric conductivity of leaves was measured. Results revealed that the relative electric conductivity levels in the two T<sub>1</sub> mutant lines were significantly increased after cold treatment in comparison to the WT, while they were similar in the two T<sub>1</sub> KO lines and WT non-stress conditions (Fig. 7). These results showed that the knockout of the *OsAnn5* gene significantly decreased cold tolerance of rice at the seedling stage.

## 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). For the targeted site in the fifth exon of *OsAnn5*, off-target loci prediction revealed three candidate sites which had 16-bp out of 20-bp identity and existed in exon regions of the targets *Os07g0275475*, *Os11g0682300* and *Os07g0598300* (Fig. 8). For the targeted site in the third exon of *OsAnn5*, there were two candidate sites which 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 (Fig. 9). The genomic sequence harboring the potential off-target site was amplified from WT rice and two T<sub>0</sub> biallelic mutants (B1-KO-8 and B2-KO-21), and the PCR products were then sequenced. Overlapping signals and indels were not detected in our two T<sub>0</sub> biallelic mutants (Fig. S2). These results suggest that off-targeting did not take place in the evaluated candidate sites.

## Discussions

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. Many of these are common promoter elements, including thirty-seven CAAT-box (a common cis-acting element in promoter and enhancer regions) and twenty-seven TATA-box (a core promoter element located around - 30 bp from the transcription onset). Some are 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 - 2082 bp, including plant hormone regulatory elements involved in methyl jasmonate, gibberellin and salicylic acid responsiveness, and several elements involved in light responsiveness. These findings suggest that *OsAnn5* may be regulated by both common and specific transcription factors in rice.

We also demonstrated that *OsAnn5*-GFP fusion protein was mainly 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 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 (Deora et al., 2004; Rescher et al., 2008). *AtAnn1* was found to exist widely in the plasma membrane, mitochondria, cytoplasm, thylakoid and glyoxylate cycle (Laohavisit and 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. In this study, in order to enhance mutation efficiency and ensure that mutations can result in the loss of the target gene function, we designed two target sites in different exons of *OsAnn5*, and mutant plantlets were successfully obtained in both sites. However, for one of the two target sites, only one mutant was detected in 18 individual rice transgenic T<sub>0</sub> lines, providing mutation efficiency of only 5.6%. Although we used the same CRISPR/Cas9-mediated expression vector backbones, this mutation efficiency is notably lower than the 31.6% mutation efficiency in our previous study (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; Mikami et al., 2015b). In view of the higher mutation efficiency obtained using the same vector backbones in our 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; Mikami et al., 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 (Nekrasov et al., 2013; Shan et al., 2013; Li et al., 2013; Xie and Yang. 2013). Experimental evidence of off-target activity was detected in rice (Shan et al., 2013; Xie and Yang. 2013). For the study of gene function using CRISPR/Cas9-mediated gene editing, off-target activity can affect the final phenotypic determination: once off-target activity takes place, it becomes difficult to determine whether phenotypic change is due to target gene knockout or off-target activity. Therefore, it is necessary to evaluate potential off-target loci when we carry out gene knockout using a CRISPR/Cas9-mediated 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/>) is a convenient and integrated toolkit by which we can expedite all experimental designs and analyses of mutations for CRISPR/Cas9 genome editing in plants, and it provides a set of powerful tools for prediction of off-target sites. 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 our identification of the cold tolerance phenotypes in mutants.

Finally, our results demonstrated that based on the electrical conductivity and survival ratio tests, T<sub>1</sub> mutant lines from two T<sub>0</sub> biallelic mutants showed decreased cold tolerance compared with the Taipei309 WT variety. Additionally, overexpression of *OsAnn5* improved tolerance to cold stress in rice. These results indicate that the rice annexin gene *OsAnn5* is a positive regulator of 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.

Table 1  
cis-regulatory elements predicted in *OsA*

Site name	position
CAAT-box	-820,-857,-939,-961,-1006,-1025,-1052,-1086,-1183,-1205,-1250,-1286,-1310,-1319,-1387,-1559,-1643,-1851,-1855,-1889,-1897,+349,+448,+1268,+1
TATA-box	-131,-144,-1048,-1049,-1504,-1505,-1506,-1507,-1583,-1584,-1585,-1586,-1610,-1661,-1663,-1706,+24,+25,+133,+143,+145,+726,+1030,+1031,+1
GC-motif	+ 1938, + 2003, +2027, + 2075
CGTCA-motif	-495, -1690,+1300
TGACG-motif	+ 495, + 1690,-1300
CAT-box	-532, -1907
A-box	-113, + 1450
DRE core	+ 730, + 2008
CARE	+ 422
CCAAT-box	+ 598
G-box	-510
GATA-motif	+ 555
I-box	-411
LAMP-element	+ 413
MYB recognition site	-598

Site name	position
MYB-binding site	-949
O2-site	+ 697
TATC-box	-704
TCA-element	-886

## Supplementary Information

Table S1. Primers used in this study.

Table S2. Specific primers of the off-target loci in this study

Fig. S1. Construction of expression vector pSK51-Cas9.

Fig. S2. Off-target analysis of two  $T_0$  biallelic mutants (B1-KO-8 and B2-KO-21) at each potential off-target site.

File 1. Promoter sequence of *OsAnn5* from rice variety Taipei309

File 2. Full-length cDNA sequence of *OsAnn5* from rice variety Taipei309

## Abbreviations

CRISPR/Cas9: clustered regularly interspaced short palindromic repeats/CRISPR associated proteins; WT: wild type; OE: overexpression; KO: Knock out; qRT-PCR; Real-Time PCR; sgRNA: single-stranded guide RNA; NHEJ: Non-homologous end joining; GFP: green fluorescent protein.

## Declarations

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### Authors' Contributions

CS conceived and designed the experiments. ZQ performed the experiments. QL, TL, SL, JZ, GC and CS analyzed the data. ZQ and CS wrote the manuscript. All authors read and approved the final manuscript.

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### Availability of Data and Materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

## Ethics Approval and Consent to Participate

Not applicable.

## Consent for Publication

Not applicable.

## Competing Interests

The authors declare that they have no competing interests.

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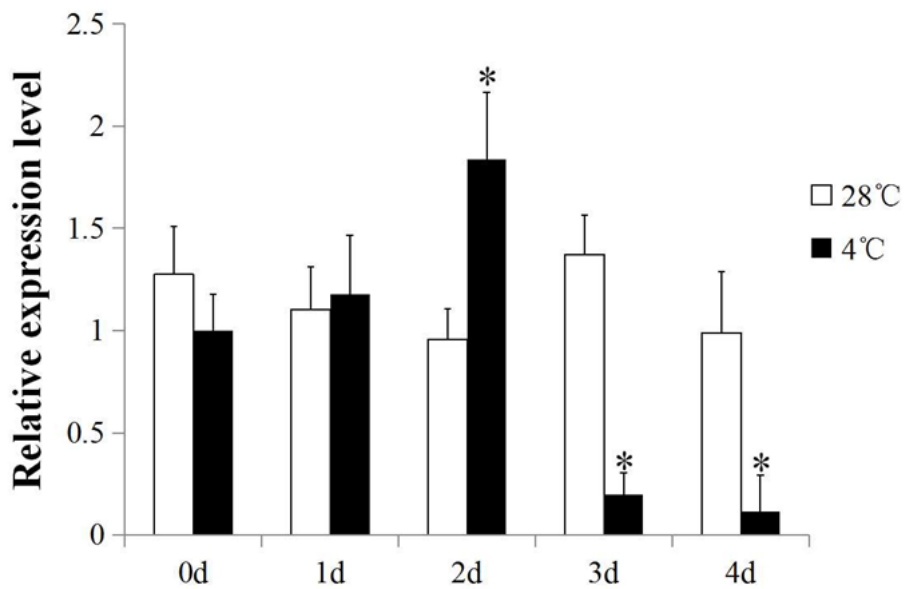
## References

1. Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to environmental stresses. *Plant Cell* 7:1099–1111
2. Browse J, Xin Z (2001) Temperature sensing and cold acclimation. *Curr Opin Plant Biol* 4:241–246
3. Calvert C, Gant S, Bowles D (1996) Tomato annexins *p34* and *p35* bind to F-actin and display nucleotide phosphodiesterase activity inhibited by phospholipid binding. *Plant Cell* 8:333–342
4. Chen S, Tao L, Zeng L, Vega-Sanchez ME, Umemura K, Wang GL (2010) A highly efficient transient protoplast system for analyzing defense gene expression and protein-protein interactions in rice. *Mol Plant Pathol* 7:417–427
5. Chinnusamy V, Zhu J, Zhu JK (2007) Cold stress regulation of gene expression in plants. *Trends Plant Sci* 12:444–451
6. Clark GB, Morgan RO, Fernandez MP, Roux SJ (2012) Evolutionary adaptation of plant annexins has diversified their molecular structures, interactions and functional roles. *New Phytol* 196:695–712
7. Deora AB, Kreitzer G, Jacovina AT, Hajjar KA (2004) An annexin 2 phosphorylation switch mediates p11-dependent translocation of annexin 2 to the cell surface. *J Biol Chem* 279:43411–43418
8. Gorecka KM, Konopka-Postupolska D, Hennig J, Buchet R, Pikula S (2005) Peroxidase activity of annexin 1 from *Arabidopsis thaliana*. *Biochem Biophys Res Comm* 336:868–875
9. Huang GT, Ma SL, Bai LP, Zhang L, Ma H, Jia P, Liu J, Zhong M, Guo ZF (2012) Signal transduction during cold, salt, and drought stresses in plants. *Mol Biol Rep* 39:969–987
10. Huh SM, Noh EK, Kim HG, Jeon BW, Bae K, Hu HC, Kwak JM, Park OK (2010) Arabidopsis annexins *AnnAt1* and *AnnAt4* interact with each other and regulate drought and salt stress responses. *Plant Cell Physiol* 51:1499–1514
11. Ijaz R, Ejaz J, Gao S, Liu T, Imtiaz M, Ye Z, Wang T (2017) Overexpression of annexin gene *AnnSp2*, enhances drought and salt tolerance through modulation of ABA synthesis and scavenging ROS in tomato. *Sci Rep* 7:1–14
12. Jami SK, Clark GB, Ayele BT, Ashe P, Kirti PB (2012) Genome wide comparative analysis of annexin superfamily in plants. *PLoS One* 7:e47801
13. Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions:  $\beta$ -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 6:3901–3907
14. 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 Physiol* 150:1394–1410
15. Kovács I, Ayaydin F, Oberschall A, Ipacs I, Bottka S, Pongor S, Dudits D, Toth EC (1998) Immunolocalization of a novel annexin-like protein encoded by a stress and abscisic acid responsive gene in alfalfa. *Plant J* 15:185–197
16. Laohavisit A, Davies JM (2011) Annexins. *New Phytol* 189:40–53
17. Laohavisit A, Richards SL, Shabala L, Chen C, Colaço RD, Swarbreck SM, Shaw E, Dark A, Shabala S, Shang Z, Davies JM (2013) Salinity-induced calcium signaling and root adaptation in Arabidopsis require the calcium regulatory protein annexin1. *Plant Physiol* 163:253–262
18. Laohavisit A, Shang Z, Rubio L, Cuin TA, Véry AA, Wang A, Mortimer JC, Macpherson N, Coxon KM, Battey NH, Brownlee C, Park OK, Sentenac H, Shabala S, Webb AAR, Davies JM (2012) Arabidopsis annexin1 mediates the radical-activated plasma membrane  $Ca^{2+}$ - and  $K^{+}$ -permeable conductance in root cells. *Plant Cell* 24:1522–1533
19. Lee S, Lee EJ, Yang EJ, Lee JE, Park AR, Song WH, Park OK (2004) Proteomic identification of annexins, calcium-dependent membrane binding proteins that mediate osmotic stress and abscisic acid signal transduction in Arabidopsis. *Plant Cell* 16:1378–1391
20. Li JF, Norville JE, Aach J, McCormack M, Zhang D, Bush J, Church GM, Sheen J (2013) Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. *Nat Biotechnol* 31:688–691
21. 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 Sci* 284:212–220
22. Mikami M, Toki S, Endo M (2015a) Comparison of CRISPR/Cas9 expression constructs for efficient targeted mutagenesis in rice. *Plant mol Biol* 88:561–572



23. Mikami M, Toki S, Endo M (2015b) Parameters affecting frequency of CRISPR/Cas9 mediated targeted mutagenesis in rice. *Plant Cell Rep* 34:1807–1815
24. Mortimer JC, Laohavisit A, Macpherson N, Webb A, Brownlee C, Battey NH, Davies JM (2008) Annexins: multifunctional components of growth and adaptation. *J Exp Bot* 59:533–544
25. Nakashima K, Ito Y, Yamaguchi-Shinozaki K (2009) Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses. *Plant Physiol* 149:88–95
26. Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S (2013) Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nat Biotechnol* 31:691–693
27. 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 *OsaANN1*, enhances heat stress tolerance by modulating the production of H<sub>2</sub>O<sub>2</sub>. *J Exp Bot* 66:5853–5866
28. Rengel D, Arribat S, Maury P, Martin-Magniette ML, Hourlier T, Laporte M, Varès D, Carrère S, Grieu P, Balzergue S, Gouzy J, Vincourt P, Langlade NB (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
29. Rescher U, Gerke V (2004) Annexins-unique membrane binding proteins with diverse functions. *J Cell Sci* 117:2631–2639
30. Rescher U, Ludwig C, Konietzko V, Kharitonov A, Gerke V (2008) Tyrosine phosphorylation of annexin A2 regulates Rho-mediated actin rearrangement and cell adhesion. *J Cell Sci* 121:2177–2185
31. Richards SL, Laohavisit A, Mortimer JC, Shabala L, Swarbreck SM, Shabala S, Davies JM (2014) *Annexin 1* regulates the H<sub>2</sub>O<sub>2</sub>-induced calcium signature in *Arabidopsis thaliana* roots. *Plant J* 77:136–145
32. Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu JX, Xi JJ, Qiu JL, Gao CX (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat Biotechnol* 31:686–688
33. Shen CX, Que ZQ, Xia YM, Tang N, Li D, He RH, Cao ML (2017) Knock out of the annexin gene *OsaAnn3* via CRISPR/Cas9-mediated genome editing decreased cold tolerance in rice. *J Plant Biol* 60:539–547
34. Shen CX, Li D, He RH, Fang Z, Xia YM, Gao J, Shen H, Cao ML (2014) Comparative transcriptome analysis of RNA-Seq data for cold-tolerant and cold-sensitive rice genotypes under normal and cold stress. *J Plant Biol* 57:337–348
35. Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. *Curr Opin Plant Biol* 6:410–417
36. Singh A, Kanwar P, Yadav AK, Mishra M, Jha SK, Baranwal V, Pandey A, Kapoor S, Tyagi AK, Pandey GK (2014) Genome-wide expressional and functional analysis of calcium transport elements during abiotic stress and development in rice. *FEBS J* 281:894–915
37. Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu Rev Plant Biol* 50:571–599
38. Wang J, Song J, Clark G, Roux SJ (2018) *ANN1* and *ANN2* function in post-phloem sugar transport in root tips to affect primary root growth. *Plant Physiol* 178:390–401
39. Xie K, Yang Y (2013) RNA-guided genome editing in plants using a CRISPR-Cas system. *Mol Plant* 6:1975–1983
40. Xie X, Ma X, Zhu Q, Zeng D, Li G, Liu YG (2017) CRISPR-GE: A Convenient Software Toolkit for CRISPR-Based Genome Editing. *Mol Plant* 10:1246–1249
41. Yadav D, Ahmed I, Shukla P, Boyidi P, Kirti PB (2016) Overexpression of Arabidopsis *AnnAt8* alleviates abiotic stress in transgenic Arabidopsis and tobacco. *Plants* 5:18
42. 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 Mol Biol* 87:47–67
43. Zhou L, Duan J, Wang XM, Zhang HM, Duan MX, Liu JY (2011) Characterization of a novel annexin gene from cotton (*Gossypium 290 hirsutum* cv CRI 35) and antioxidative role of its recombinant protein. *J Integr Plant Biol* 53:347–357

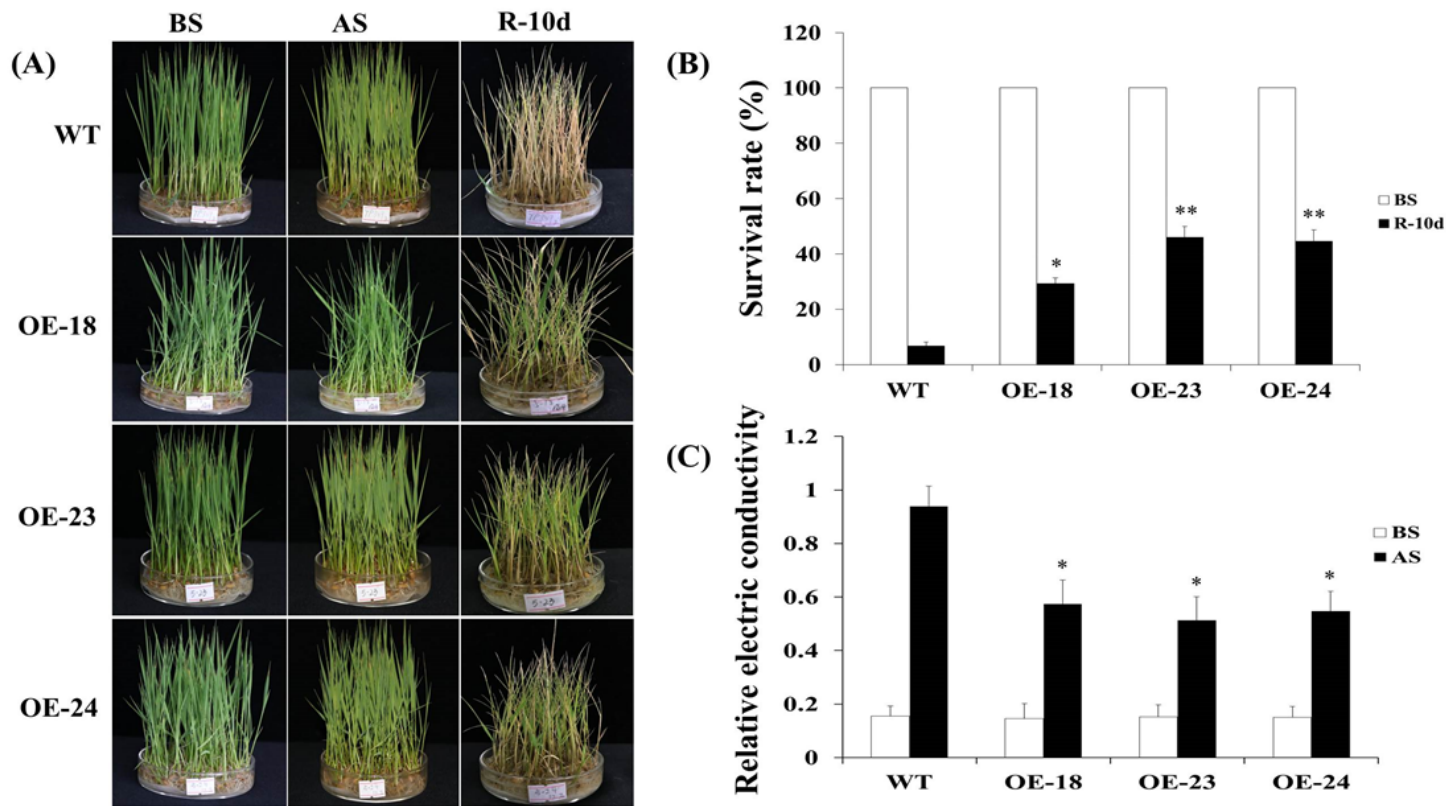
## Figures



**Figure 1**  
 Expression of *OsAnn5* under control (28°C) conditions or after exposure to cold stress (4~6°C and 14 h light/10 h dark cycle for 4 d). 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 ( $P < 0.05$ ) in comparison with WT. Error bars represent the s.e.m. WT, wild type.

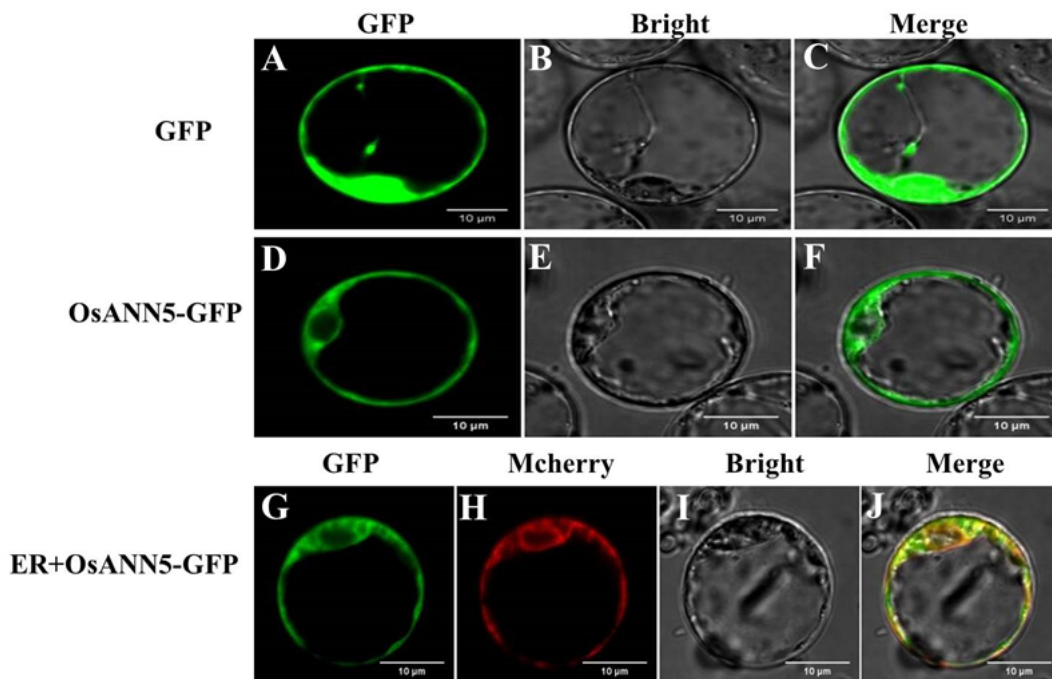


**Figure 4**  
 Histochemical analysis of *OsAnn5pro::GUS* transgenic rice plants. (a) Germinating seeds; (b) stem; (c) transverse section of a node; (d) flower; (e) anther and other sections of the flower.



**Figure 6**

Overexpression of OsAnn5 resulted in increased cold tolerance. (A) Growth performance of OE and WT seedlings that were four weeks old, in 9 cm diameter glass petri dishes before and after stress (4~6 °C for 3 d). WT, wild type; OE, overexpression; BS, before stress; R-10d, recovery for 10 d after stress. The experiment was repeated three times. (B) Survival rate of OE and WT seedlings after stress. (C) Relative electrical conductivity of OE and WT seedling leaves before and after cold treatment. OE seedlings (OE-18, OE-23 and OE-24) showed higher survival rate, lower relative electrical conductivity and better growth than WT seedlings. One asterisk indicates significant difference ( $P < 0.05$ ) in comparison with WT. Error bars represent the s.e.m.



**Figure 8**

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  $\mu$ m; WT, wild type.

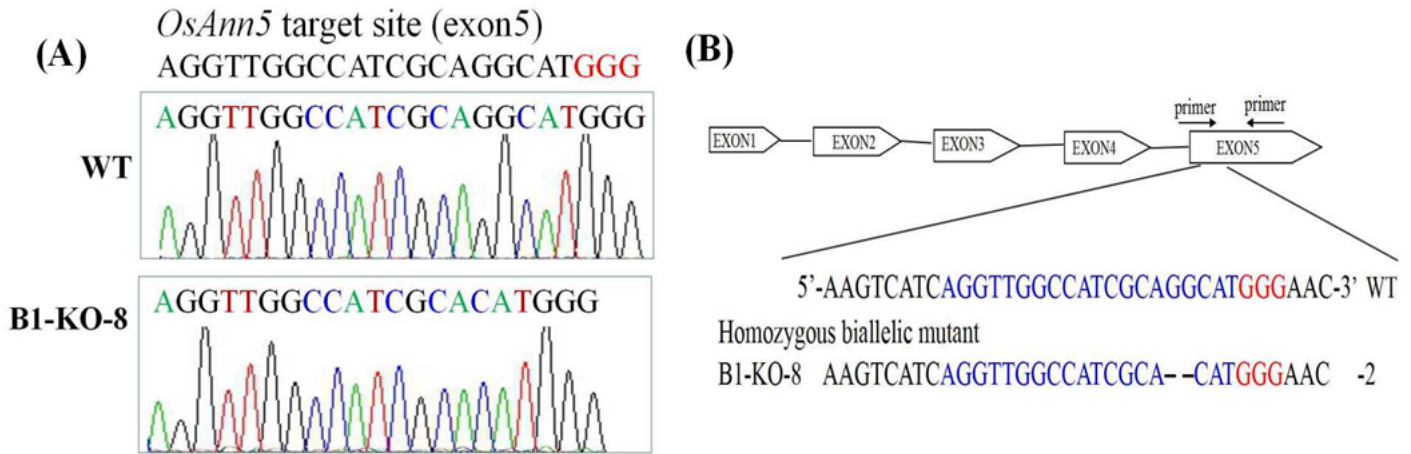


Figure 10

Mutation detection in the targeted site in the fifth exon of *OsAnn5*. (A) Sequencing chromatogram of PCR products from WT (Taipei309) and T0 mutant B1-KO-8 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-8 is a homozygous biallelic mutant of the targeted site in the fifth exon of *OsAnn5*. WT, wild type.

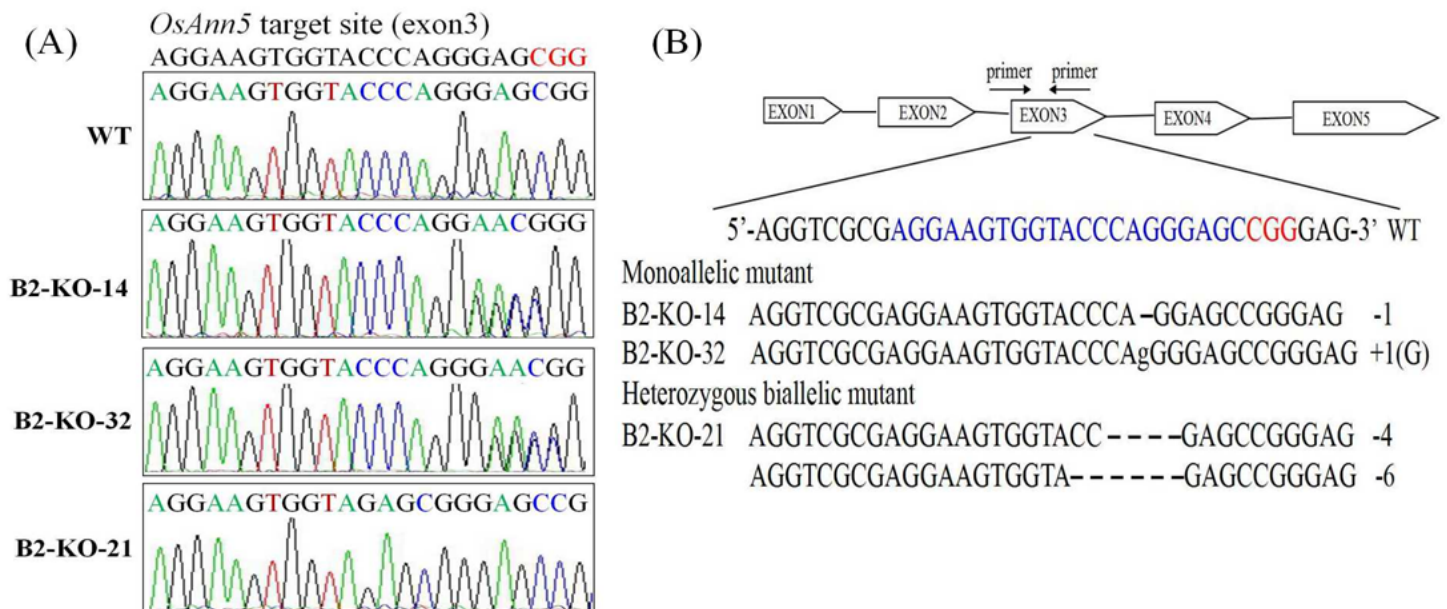


Figure 12

Mutation detection in the targeted site in the third exon of *OsAnn5*. (A) Sequencing chromatogram of PCR products from WT (Taipei309) and three T0 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.



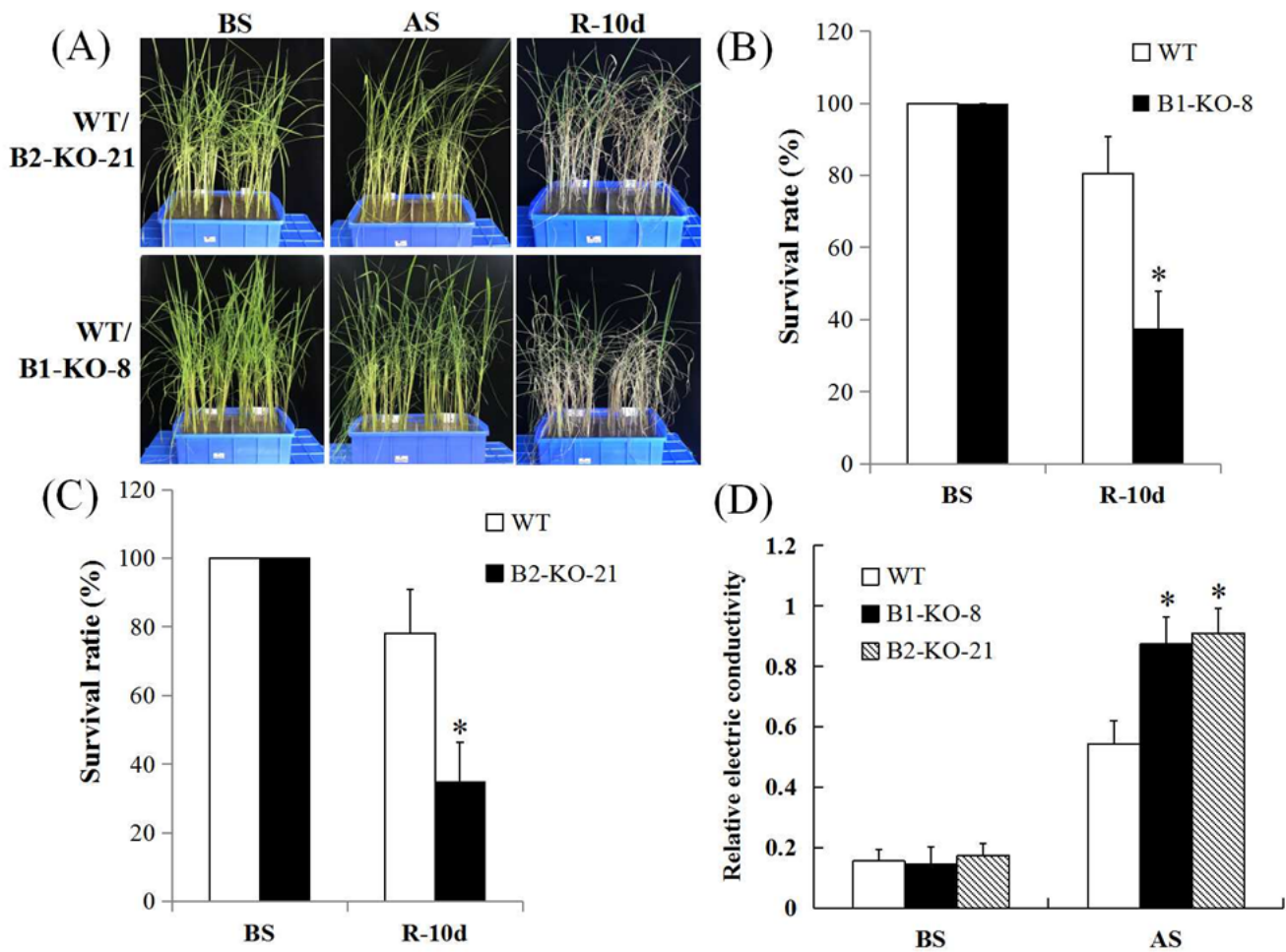


Figure 14

Two biallelic mutant lines of the *OsAnn5* gene showed decreased cold tolerance. (A) 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 3 d). BS, before stress; R-10d, recovery for 10 d after stress. The experiment was repeated three times. (B) Survival rate of the biallelic mutant line B1-KO-8 after stress. (C) Survival rate of the biallelic mutant line B2-KO-21 after stress. (D) Relative electrical conductivity of rice seedling leaves before and after cold treatment (4~6°C for 3 d). One asterisk indicates significant difference (P<0.05) in comparison with WT. Error bars represent the s.e.m.

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)

Figure 15

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.

AGGAAGTGGTACCCAGGGAGCGG *OsAnn5* sgRNA target site (exon3)  
 AAGAAGAGGTACCAAGGGTTCGG *OsAnn5* potential off-target site: *Os03g0753500* (exon)  
 AGGATGTGCTAACCAAGGAAGCGG *OsAnn5* potential off-target site: *Os02g0654400* (intron)

Figure 18

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.

## Supplementary Files

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