J. Plant Biol. (2017) 60:539-547
DOI 10.1007/s12374-016-0400-1

ORIGINAL ARTICLE

# Knock Out of the Annexin Gene *OsAnn3* via CRISPR/Cas9-mediated Genome Editing Decreased Cold Tolerance in Rice

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Received: August 3, 2016 / Accepted: July 5, 2017


**Abstract** Plant annexins are $Ca^{2+}$-dependent phospholipid-binding proteins and exist as multigene families in plants. They are implicated in the regulation of plant development as well as protection from environmental stresses. In this study, the rice annexin gene *OsAnn3* knockout was performed via the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated proteins) mediated genome editing. Thus, mutant plantlets were successfully obtained. We identified cold tolerance phenotype of $T_1$ mutant lines from $T_0$ biallelic mutants using the 4~6°C for 3 days cold treatment. The results showed that REC (the relative electrical conductivity) of $T_1$ mutant lines was increased, and the survival ratio of $T_1$ mutant lines was decreased dramatically compared with the wild type after the exposure to cold treatment. It was suggested that *OsAnn3* was involved in cold tolerance of rice.

**Keywords:** Annexins, Cold tolerance, CRISPR/Cas9, Rice

## Introduction

Rice (*Oryza sativa* L.) is a main food crop that feeds more than half of the world's population (Sasaki and Burr, 2000). It has evolved in tropical and subtropical areas and is sensitive to cold stress (Kovach et al. 2007; Saito et al. 2001; Sang and Ge 2007). Hybrid rice cultivars have developed high yields in tropical or subtropical climates but are constantly harmed by cold. Low temperature stress has become one of the major environmental stresses that affect rice yield, quality, and distribution. Transgenic techniques have already been frequently applied to improve cold tolerance in rice. A key step for transgenic improvement is to identify and characterize candidate genes that have the potential to further increase cold tolerance.

Thus far, according to statistics from the China rice data center, a total of 60 genes related to cold tolerance have been cloned in rice. These can be separated into four groups. The first group is comprised of transcription factors, including *Osmyb4* (Candida et al. 2004), *MYBS3* (Su et al. 2010), *OsDREB1F* (Wang et al. 2008) and *OsbZIP52* (Liu et al. 2012) et al. The total of 23 genes account for 38.3% of 60 cloned genes. The second group is protein kinase including *OsMEK1* (Wen et al. 2002), *OsMAP1*(Wen et al. 2002), *OsCDPK13* (Yang et al. 2003) and *OsCDPK7* (Yusuke et al. 2000). The total of 4 genes account for 6.7%. The third group is microRNA including *Osa-miR319a* (Yang et al. 2013) and *Osa-miR319b* (Yang et al. 2013). The total of 2 genes account for 3.3%. The fouth group is other types of protein including *COLD1* (Ma et al. 2015), *OsLEA4* (Hu et al. 2012), *OsbHLH1* (Wang et al. 2003), and *OsFAD8*. (Prakash et al. 2009) et al. The total of 31 genes account for 51.7%.

Plant annexins are $Ca^{2+}$-dependent phospholipid-binding proteins, which exist as multigene families in various plants and are involved in the regulation of plant development as well as in the protection from abiotic stresses. It is explicit that certain plant annexin genes are transcriptionally activated in answer to all kinds of abiotic stresses. Since the first report that the annexin gene *AnnMs2* was activated by osmotic stress, drought, and ABA in alfalfa (Kovacs et al. 1998), regulation of plant annexin genes by abiotic stress or

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phytohormones was subsequently described for many species including Arabidopsis (Gidrol et al. 1996; Lee et al. 2004; Cantero et al. 2006; Konopka-Postupolska et al. 2009), Brassica (Jami et al. 2008; Yan et al. 2015), tobacco (Vandeputte et al. 2007), Mimosa (Hoshino et al. 2004), wheat (Breton et al. 2000), Nelumbo nucifera (Chu et al. 2012), tomato (Loukehaich et al. 2012) and loblolly pine (Watkinson et al. 2003). These evidence indicates that plant annexins may participate in response to environmental challenges, as was demonstrated by the fact that *AnnAt1* was found to be drought-tolerant in Arabidopsis (Konopka-Postupolska et al. 2009). Nucleotide sequence analysis showed the presence of several stress-responsive *cis*-elements in different annexin promoters (Rhee et al. 2000; Jami et al. 2009, 2012a). This indicated that the activity of annexin was regulated by transcription factors when plant encountered specific abiotic stress conditions. In contrast, the role of annexins in rice under abiotic stresses has not been extensively evaluated. Making use of the released genome sequence information, Jami et al. (2012a) first identified ten members of the rice annexin gene family and determined the shoot and root tissue expression pattern of the rice annexin gene family in response to various environmental stress treatments (salinity, drought, cold, and heat) at seedling stage. Subsequently, Qiao et al. (2015) reported that the rice annexin gene *OsANN1* enhanced drought stress tolerance by modulating antioxidant accumulation based on the analysis from RNA interference and overexpression of *OsANN*. Thus far, this is the only report on functional analyses of rice annexins under abiotic stresses. To date, although as many as 60 cold-tolerant genes in rice have been cloned, no annexin genes have been reported in these cloned genes. The role of rice annexins during cold stress remains unclear, and the answer to this relies on functional analyses in transgenic rice. Exploration of genotype influence on phenotype and description of the function of a single gene or a gene family are routine strategies for most biology researchers. Knock-out the targeted gene as well as gene overexpression is effective tools for achieving this objective. However, the development of of gene function research is restricted due to the off-target effect and the incompleteness of regulation via RNA interference. Therefore, new gene knock-out technologies with higher specificity and lower off-target rate are necessary for the research group. CRISPR/Cas9 is a new genome editing tool that meets expectations. The CRISPR/Cas9 system has great advantages such as its specificity, simplicity, and diversity, as well as by the fast and cost-effective procedure in comparison with other genome editing tools. Since the emergence of CRISPR/Cas9 technology, its use has become widespread. Many studies, which were conducted in vitro (Bikard et al. 2013), in bacteria (Jiang et al. 2013), human cells (Jinek et al. 2013; Mali et al. 2013; Cong et al. 2013), Drosophila (Gratz et al. 2013), Nicotiana benthamiana (Nekrasov et al. 2013), Arabidopsis (Li et al. 2013), rice (Shan et al. 2013) and in other organisms, were regularly featured.

In the present study, it was demonstrated that the expression of rice annexin gene *LOC_Os05g31750.1* can be induced by cold treatment (4~6°C for 3 days), which was designated as *OsAnn3* [consistent with the nomenclature of Singh et al. (Singh et al. 2014)]. Then, we knocked down *OsAnn3* gene in japonica rice variety Taipei309 via the CRISPR/Cas9-mediated genome editing and successfully obtained mutant plantlets after the genetic transformation. Finally, we showed that REC of $T_1$ mutant lines was significantly increased, and that the survival ratio of $T_1$ mutant lines was dramatically decreased compared with the wild type after the exposure to cold treatment.

## Results

### Expression of *OsAnn3* Under Cold Stress Conditions

Extensive evidence from various plant species has shown the increased expression of annexin genes in response to abiotic stress conditions (Laohavisit and Davies 2011; Jami et al. 2012a). In this study, we used quantitative reverse-transcription (qRT)-PCR to evaluate the expression of annexin gene *OsAnn3* in the wild type rice variety Taipei309 leaf tissue under normal conditions (28°C) or after the 4~6°C for 3 days treatment. Our results showed that the transcript levels of *OsAnn3* in Taipei309 were 9.8 fold up-regulated following cold treatment as compared with normal conditions (Fig. 1). Therefore, it was suggested that *OsAnn3* was regulated by cold stresses and was possibly involved in cold tolerance in

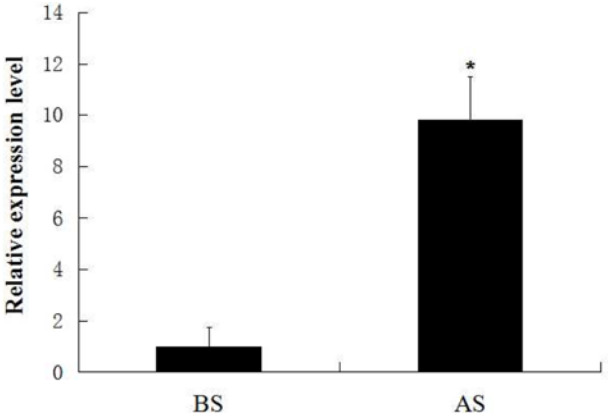


**Fig. 1.** Expression of *OsAnn3* under normal (28ºC) conditions (control) or after exposure to cold stress (4~6°C and 12 h light/12 h dark cycle for 3 days). The expression levels of genes were determined via qRT-PCR using ubiquitin as an internal control, and were calculated from three independent experiments. One asterisk indicates significant difference ($P < 0.05$) in comparison with wild type. Error bars represent the s.e.m. BS: before stress, AS: after stress.

rice. Previous studies have shown the up-regulated expression of Arabidopsis annexin genes in response to cold treatments (Cantero et al. 2006). In addition, annexin proteins were detected in rice root (Hashimoto et al. 2009) and wheat shoots (Breton et al. 2000) where increased levels of annexin protein were observed in the plasma membrane fraction in response to cold.

## CRISPR/Cas9 Binary Vector Set

To construct binary vectors, two types of backbones were utilized. The first type was based on cloning of the psgR-Cas9-Os vector carrying CRISPR/Cas9 gene, while the second type was based on the plant expression vector pSK51. The advantage of psgR-Cas9-Os vectors is their relatively small size, which allows them to be used to clone sgRNA of the target sites. pSK51, with a hygromycin-resistance gene as a selectable marker, was derived from pCAMBIA1300. First, two oligomeric primers carrying 018-Oligo1 and 018-Oligo2 were synthesized according to the targeted site sequence located in the second exon of *OsAnn3*. The joint sequence was complemented with a sticky end of the vector digested by the *Bbs*I enzyme. Two oligomeric primers were annealed and then ligated into *BbsI* sites of the cloning vector psgR-Cas9-Os. Ligation products were transformed into *E. coli* competent cells. PCR identification of positive clones was performed using the M13-F and 018-Oligo2 primers. The results showed that all positive clones were amplified out of the 253 bp band. Only the PsgR-Cas9-Os empty vector as a template (negative control) was not amplified out (Fig. 2A). This suggested that the Cas9 clone vector of the target loci was successfully constructed. Subsequent plasmid sequencing also confirmed the results. Then, the recombinant plasmid was digested with *Eco*RI and *Hind*III, and approximately 5.6 Kb fragments containing Cas9 and sgRNA were recycled. Then, the fragments were ligated into *Hind*III/*Eco*RI sites of the plant expression vector pSK51 and transformed into *E. coli* competent cells. The identification of positive clones was performed using *Eco*RI and *Hind*III. The result showed that the Cas9 knockout plant expression vector of *OsAnn3* gene was constructed successfully (Fig. 2B).

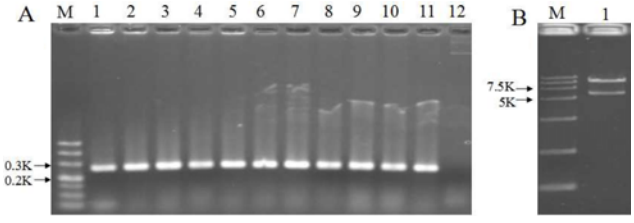


**Fig. 2.** (A) PCR identification of Cas9-018 recombinant clones. M:DL500; lane 1-11, Recombined clones; lane 12, Negative control. (B) Digestion identification of pSK51-Cas9-018. M, DL15000; 1, Double digestion of recombinant plasmid pSK51-Cas9-018 by *Eco*RI and H*ind*III.

## Pattern of Targeted Gene Modifications

*Agrobacterium*-mediated transformation of rice (*Oryza sativa* L. cv. Taipei309) using scutellum-derived calli was performed as described previously (Toki 1997; Toki et al. 2006). One-month-cultured rice calli were infected with *Agrobacterium* carrying the pSK51-cas9 expression vector that contained the Cas9 gene and a single-stranded guide RNA (sgRNA) targeting the *OsAnn3* gene. In total, 19 individual rice transgenic $T_0$ lines were subjected to mutation detection using Sanger sequencing of PCR products amplified with primers flanking the sgRNA target sites from leaf. The total of 6 mutants were detected and then subjected to zygosity analysis by cloning PCR products into the T vector for DNA sequencing. The examination of a few leaves from early $T_0$ seedlings revealed four types of NHEJ (Non-homologous end joining) mutations: +1 (1-bp insertion), -1 (1-bp deletion), -3 (3-bp deletion), and -4 (4-bp deletion) (Fig. 3). Out of the 6 mutants, two of them were monoallelic mutants and accounted for 33.3%. Three of them were homozygous biallelic mutants and accounted for 50%. One of them was heterozygous biallelic mutant and accounted for 16.7%.

## Identification of Cold Tolerance Phenotype of $T_1$ Mutant Lines

Three $T_1$ mutant lines from the $T_0$ biallelic mutant of the *OsAnn3* gene (018-TP-8, 018-TP-12 and 018-TP-18) were selected for the identification of cold tolerance phenotype,

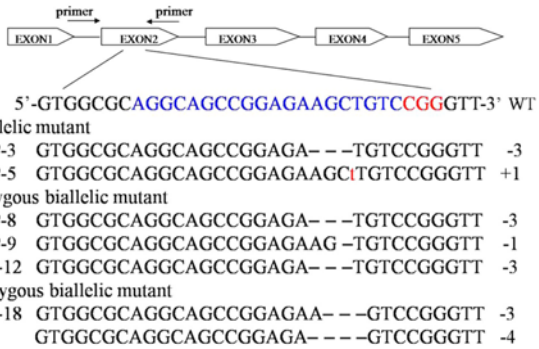


**Fig. 3.** sgRNA:Cas9-induced *OsAnn*$_3$ mutations in transgenic rice plants. Blue color indicates the sgRNA:Cas9 targets, and red (CGG) color indicates the corresponding PAMs. WT: wild type. DNA samples from independent transgenic rice seedlings were analyzed for mutations using PCR product sequencing and T-vector clone sequencing. 018-TP-3 and 018-TP-5 are monoallelic mutants of *OsAnn*$_3$, 018-TP-8, 018-TP-9 and 18-TP-12 are homozygous biallelic mutants of *OsAnn*$_3$. 018-TP-18 are heterozygous biallelic mutants of *OsAnn*$_3$.

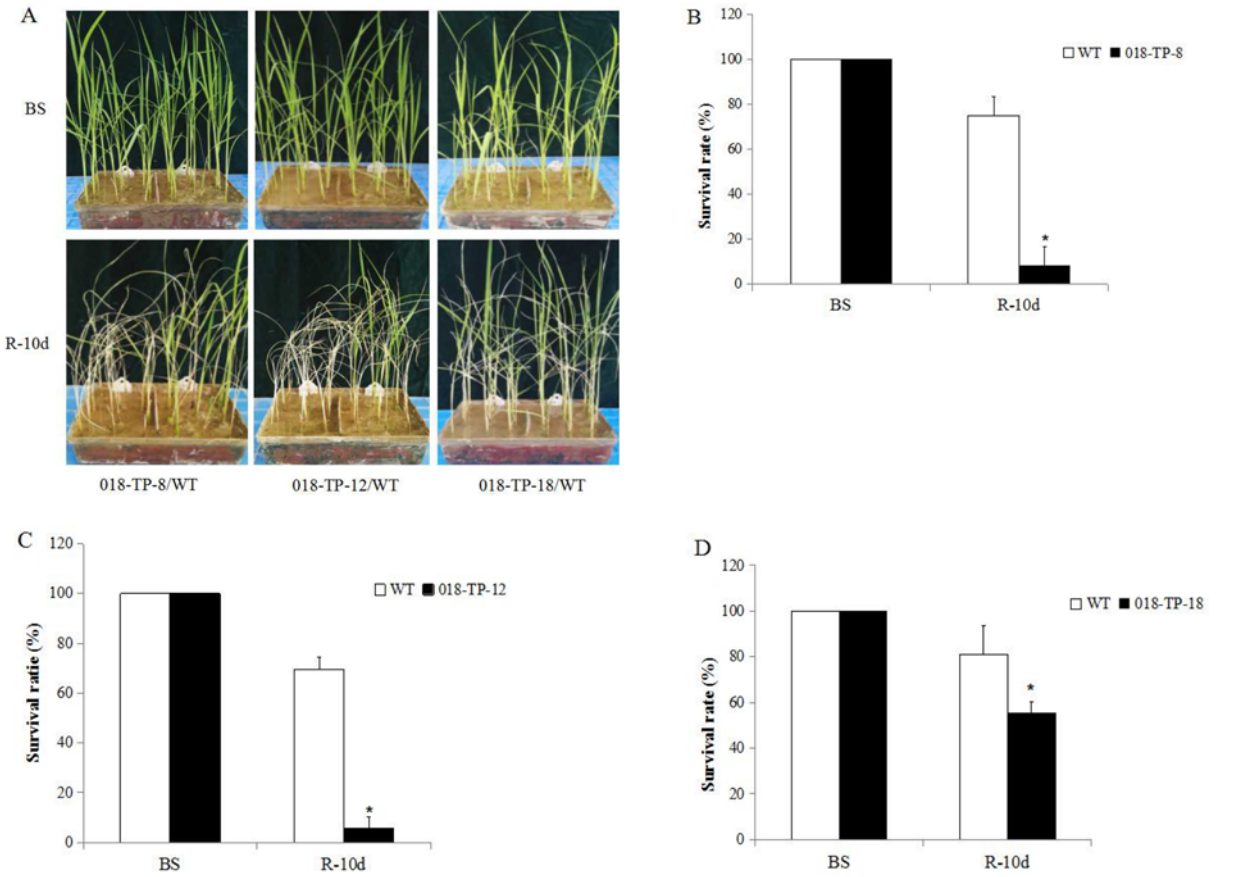


**Fig. 4.** Three biallelic mutant lines of the *OsAnn3* gene showed decreased cold tolerance. (A) Growth performance of biallelic mutant lines and wild type seedlings during the three-four leaf stage in the same barrel (left, mutant plants; right, wild type) before and after stress (4~6°C for 3 d). BS, before stress; R-10d, recovery for 10 days after stress. The experiment was repeated three times. (B) Survival rate of the biallelic mutant line 018-TP-8 after stress. (C) Survival rate of the biallelic mutant line 018-TP-12 after stress. (D) Survival rate of the biallelic mutant line 018-TP-18 after stress. One asterisk indicates significant difference ($P < 0.05$) in comparison with wild type. Error bars represent the s.e.m.

because the $T_0$ biallelic mutant progeny were all mutant. Because the $T_0$ biallelic mutant 018-TP-9 did not produce $T_1$ seeds, it was not used in our subsequent experiment. To examine the effect of the *OsAnn3* gene knockout on cold tolerance, the three-four leaf stage old rice seedlings of both the wild type and the *OsAnn3* gene knockout rice ($T_1$ generation) were exposed to cold stress conditions (4~6°C for 3 days), and then returned to the normal growth conditions to recover. After 10 days in the greenhouse, three $T_1$ mutant lines of *OsAnn3* exposed to cold treatment re-grew 8.3, 5.5, and 55.5%, respectively, while the survival ratio of the corresponding wild type rice under the same conditions reached 75, 69.5, and 81.1% respectively (Fig. 4). Using the same cold treatment, we further measured the relative electrical conductivity of leaves, which is a well-recognized parameter that reflects the damage by cold stress. Our results showed that the relative electrical conductivity levels in three $T_1$ mutant lines were significantly increased after cold treatment compared with the levels in the wild type rice, although they

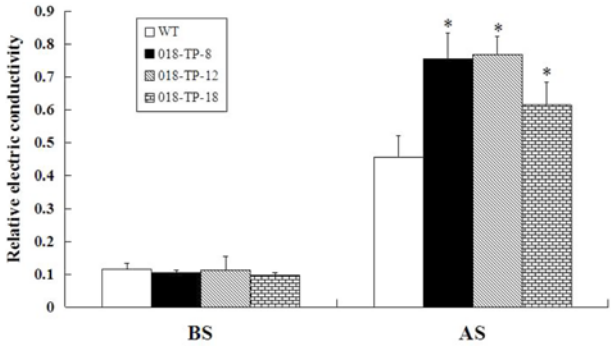


**Fig. 5.** Relative electrical conductivity of rice seedling leaves (three-four leaf stage) before and after cold treatment (4~6°C for 3 d). BS, before stress; AS, after stress. WT, wild type TP309. 018-TP-8, 018-TP-12, and 018-TP-18 are the three biallelic mutant lines. One asterisk indicates significant difference ($P < 0.05$) in comparison with wild type. Error bars represent the s.e.m.

remained at approximately the same level in both the three $T_1$ mutant lines and wild type rice in the absence of any stress

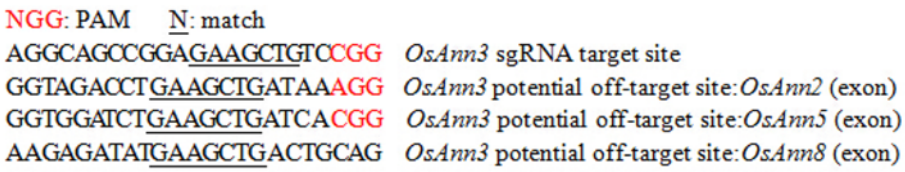


**Fig. 6.** Potential off targets at the *OsAnn2*, *OsAnn5* and *OsAnn8* locus. Matches between the potential off targets and the targeted region are underscored. The PAM sequences are indicated in red.

treatment (Fig. 5). These results showed that the *OsAnn3* gene knockout significantly decreased cold tolerance of rice.

Potential Off-targets were Analyzed at the *OsAnn2*, *OsAnn5* and *OsAnn8* Locus

Because rice annexin gene family include ten members, we assessed the potential off-targets at other nine rice annexin gene. The result showed that three candidate sites which had 7-bp out of 20-bp identity and existed in exon of the targeted *OsAnn2* (*LOC_Os02g51750*), *OsAnn5* (*LOC_Os06g11800*) and *OsAnn8* (*LOC_Os09g20330*) (Fig. 6). The genomic regions containing the potential off-target site were amplified in wild type and three $T_0$ biallelic mutants (018-TP-8, 018-TP-12 and 018-TP-18), and the PCR products were sequenced. However, no overlapped signals and indels were detected in our three $T_0$ biallelic mutants (Fig. S1). These results indicated that off-targeting did not occur in the candidate sites. It was suggested that the CRISPR/Cas9 system was highly specific for targeted mutagenesis in rice.

## Discussion

In plants, apart from three major classes of calcium transporters [i.e., channels, pumps (ATPases), and exchangers] (Smyth et al. 2006; Goel et al. 2011), other protein groups, such as annexins, were identified that non-specifically transport $Ca^{2+}$ (Jami et al. 2012b). Annexins can bind to phospholipids with a $Ca^{2+}$-dependent manner. Annexins have important motifs for the ATPase/GTPase and calcium channel activities (Gerke et al. 2002; Moss et al. 2004). Also, annexins contain various post-translational modification sites, which may be potential regulators because of their $Ca^{2+}$-dependent activity (Konopka-Postupolska et al. 2011; Laohavisit et al. 2011). Rice annexin OsAnn3 was reported to interact with various protein kinases such as receptor-like kinase (*Os01g02580*), sterile-20 (Ste20)-like kinase (*Os10g37480*), calcium/calmodulin-dependent protein kinase (*Os01g64970*), and caesin kinase (*Os01g38950*) (Rohila et al. 2006). The Ste20 kinase of yeast is a MAPKKK (mitogen-activated protein kinase kinase kinase) and was described to be involved in mating and osmotic stress responses (Dan et al. 2001; Ramezani-Rad 2003). The association of rice Ste20-like kinase with *OsAnn3* and MAPKK suggests that these proteins may participated a membrane-associated $Ca^{2+}$-dependent MAPK signal pathway. MAPKs are key components of MAPK cascades that mediate stress signal transduction (Teige et al. 2004). Calcium signal transduction and MAPK cascades that mediate stress signal transduction are two main classes of plant cold stress signal transduction. This research suggests a possible role of *OsAnn3* in signaling, which is triggered by cold stress conditions in rice.

Interestingly, previous studies demonstrated that the rice annexin gene *OsAnn3* did not contain any reported cis-elements, such as ABRE(ACGTG), DRE/CRT (G/ACCGCC), and LTRE (CCGAC) motifs, in silico sequence analysis (Jami et al. 2012a). Although, 8 out of 10 rice annexin genes contained at least one of the three putative cis-elements. However, our results showed that the transcript levels of *OsAnn3* in leaves during the three-four leaf stage from the Taipei309 rice variety significantly increased following the cold treatment (4~6°C for 3 days), as compared with normal conditions. This implies that the genes that do not contain any reported cis-motif may actually show up-regulation and also may contain a novel type of cis-motif. In addition, the results are not commensurate with previous studies that reported no increase in the transcript abundance of rice annexin gene *OsAnn3* in seedlings exposed to cold stress (4°C for 2 h or 4±1°C for 3 h) (Jami et al. 2012a; Singh et al. 2014). Because a potentially different induction kinetics exists, it can be speculated that the expression of *OsAnn3* was not triggered in rice because the cold treatment time was too short in previous studies. *OsAnn3* may function only during the late stage of cold response rather than during the initial stages under cold stress.

It is important for the genome editing technology to obtain more mutations in the process of application. Compared with a 4%~9.4% mutation frequency in the first reported application of the CRISPR/Cas9 genome editing technology in rice (Shan et al. 2013), our results demonstrate that the CRISPR/Cas9 system is highly efficient for targeted mutations of first generation of transgenic rice plants. In this study, mutations in *OsAnn3* were identified in 6 out of 19 independent transgenic plants, which is 31.5%. Moreover, four plants were found to harbor biallelic mutations. We believe that this

was attributed to using an appropriate vector psgR-Cas9-Os whose Cas9 codon was optimized for rice, designing a specific target site and prolonging culture time of calli on the selection medium with hygromycin resistance. In addition, previous studies demonstrated that the use of Cas9 codon, target sequences, and culture period of Cas9 and gRNA-transformed calli significantly affected mutation frequency (Mikami et al. 2015a; Mikami et al. 2015b). Overall, our research demonstrated that the CRISPR/Cas9 system could induce mutations effectively in stable transgenic rice. Using this method, we can obtain monoallelic and biallelic mutants in the $T_0$ generation. Thus, the CRISPR/Cas9 system is an effective tool for promoting functional studies of uncharacterized genes in rice. Woo et al even reported that the overall mutation frequency reached 46% in lettuce calli using CRISPR/Cas9 system. With continuous develpment and improvement of CRISPR/Cas9 technology, it is foreseeable that the higher mutation frequency will be reported in plant genome editing (Woo et al. 2015).

In this study, based on the electrical conductivity or survival ratio tests, $T_1$ mutant lines from three $T_0$ biallelic mutants showed increased cold sensitivity compared with the Taipei309 wild type rice variety under cold stress. However, compared with $T_1$ mutant lines from 018-TP-8 and 018-TP-12, $T_1$ mutant lines from 018-TP-18 showed higher cold tolerance but still were more sensitive to cold than the wild type. This was possibly due to different encoding amino acids, which resulted from different mutations in the target site, leaded to the difference of protein function of the target gene. These results indicated that the knockout of *OsAnn3* in rice significantly decreased plant tolerance to cold stress. This was the first report indicating that annexin genes were involved in cold tolerance of rice seedlings. These results expand our understanding of the complex mechanisms involved in cold tolerance in rice.

## Materials and Methods

### Plant Materials and Stress Treatment

The Japonica rice variety Taipei309 was used as a transgenic acceptor in this study. The seeds of Taipei309 and $T_1$ mutant lines from the $T_0$ biallelic mutant were sterilized and germinated at 37°C in darkness for 2 days. Then, the seeds were sown in a filled with soil plastic container (19 × 13 × 12 cm) in a greenhouse at the state key laboratory of hybrid rice of China. The average day/night temperature was 28°C/22°C. The seeds were watered daily until the three-four leaf stage. Rice seedlings received daily natural sunlight, and no additional artificial light was supplemented. When rice seedlings grew to the three-four leaf stage, the seedlings were transferred to 4~6°C for 3 days of cold treatment.

### Real-time PCR

Total RNA was extracted from three Taipei309 seedlings grown under normal conditions or under cold treatment (4~6°C for 3 days) using RNAiso plus (Takara, Japanese). RNA was subsequently treated by use of DNase I (Promega, USA), and synthesized of first strand cDNA was performed with a purified total RNA by use of a PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Japanese). Real-time PCR was performed with 018-F and 018-R gene-specific primers (Table 1) as described (Shen et al. 2014). The relative expression analysis was evaluated by three biological replicates with three technical replicates and the amplification of ubiquitin was used as an internal control to normalize all data.

**Table 1.** Primers used in this study

| Primer name | Primer sequence (5'- 3') |
|---|---|
| 018-F | CACGAGGAGACACTGAGGGAG |
| 018-R | TGTAGTAGCCGTGCGCCTTC |
| 018-Oligo1 | TGGCAGGCAGCCGGAGAAGCT |
| 018-Oligo2 | AAACAGCTTCTCCGGCTGCCT |
| TB-018F | GTGTGTGCATTTATGCTGCTG |
| TB-018R | GCCATTGAAGCTCTTCCTG |

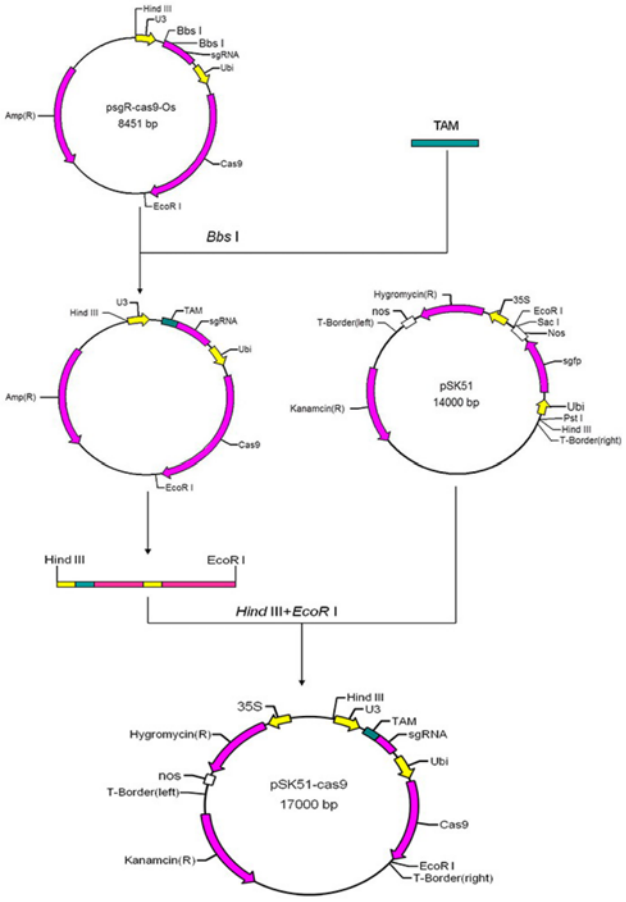


**Fig. 7.** Construction of plant knock-out expression vector pSK51-Cas9. TAM: target adapter molecule.

### Plasmid Construction

For endogenous gene modification, two 21-bp DNA oligonucleotides (018-Oligo1 and 018-Oligo2) were synthesized for the targeted site,

as listed in Table 1, and inserted into *Bbs*I sites of the cloning vector psgR-Cas9-Os after annealing and phosphorylation, according to the online protocol (http://www.genome-engineering.org/crispr/). Then, the resulting plasmid with targeting sgRNA cassettes and Cas9 was digested with *Hind*III and *Eco*RI, and the fragment was ligated into *Hind*III/*Eco*RI sites of the plant expression vector pSK51 (Fig. 7). Finally, the target site knock-out expression vector pSK51-cas9 plasmids were obtained. PCR and enzyme digestion were used to identify positive clones.

Detection of Targeted Gene Mutations

DNA was extracted from the wild-type controls and transgenic plants by use of customary CTAB method. Genomic regions surrounding the CRISPR target sites were amplified by use of rice leaf with primer pairs TB-018F and TB-018R (Table 1). Targeted gene mutations were detected by aligning sequencing chromatograms of these PCR products with the wild-type controls. All $T_0$ individual lines were analyzed for the target site of *OsAnn3*. Then, their mutants were subjected to zygosity analysis by cloning PCR products into the pEASY-Blunt Zero Cloning Kit vector (TransGen Biotech, Beijing, China) for DNA sequencing.

Measurement of Electrical Conductivity and Survival Ratio

To measure the relative electrical conductivity after the exposure to cold treatment, 0.1 g of leaves from each sample material was collected and placed into a tube containing 20 mL of distilled water. The electric conductivity was measured and the REC was calculated as described (Shen et al. 2014). The survival ratio experiments were conducted by transferring the three-four leaf stage old plants to a chamber with a temperature of 4~6°C for 3 days, and then, returning the plants to 28°C for 10 days. Thus, the survival rate was observed. The survival rate was calculated, as follows: the survival ratio = the number of surviving plants / the total number of plants. The test of the survival rate for every mutant line was repeated for three times. 12 wild type and 12 mutants plants from the same mutant line were used for every time experiment. The mean values of the survival rate were calculated from three independent experiments.

Off-target Sequence Identification

Potential off-target sites were identified by comparing the 20-nt gRNA target sequences in *OsAnn3* with gene sequence from other nine members of the rice annexin gene family using BLASTN (optimize for somewhat similar sequences) respectively. Setting the e-value threshold to 6 since the query sequence (sgRNA) is only 20 nt. The loci were all considered for analysis no matter with or without the required protospacer-adjacent motif (PAM) NGG motif at the 3′ end of the sequence. Specific primers of the off-target loci in this study are listed in Table S1.

## Acknowledgements

This research was financially supported by the National Key Technology R&D Program (2014AA10A603), the National Program of Transgenic Variety Development of China (2011ZX08001-001, 2011ZX08001-004), the Key Science and Technology Program of Hunan Province (2014FJ2014), the Open Research Fund of the State Key Laboratory of Hybrid Rice (Hunan Hybrid Rice Research Center) (No. 2014KF04), National Natural Science Foundation of China (Grant No. 31660379) and Science and Technology Program of Jiangxi Province Education Department (GJJ151039).

## Author's Contributions

MC conceived and designed the experiments. CS performed the experiments. CS analyzed the data. CS wrote the paper, and all authors read and approved the final manuscript.

## Supporting Information

**Fig. S1.** Off-target analysis of three $T_0$ biallelic mutants (018-TP-8, 018-TP-12 and 018-TP-18) at the *OsAnn2*, *OsAnn5* and *OsAnn8* locus by sequencing.
**Table S1**. Specific primers of the off-target loci in this study.

## References

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