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OsCIPK7 point-mutation leads to conformation and kinase-activity change for sensing cold response

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Breakthrough Report

Summary Calcineurin B-like interacting protein kinases (CIPKs) play important roles via environmental stress. However, less is known how to sense the stress in molecular structure conformation level. Here, an *OsCIPK7* mutant via TILLING procedure with a point mutation in the kinase domain showed increased chilling tolerance, which could be potentially used in the molecular breeding. We found that this point mutation of OsCIPK7 led to a conformational change in the activation loop of the kinase domain, subsequently with an increase of protein kinase activity, thus conferred an increased tolerance to chilling stress.

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INTRODUCTION

Sensing cold is a fundamental instinct for plant adaptation to chilling stress. As known, cold tolerance is triggered by a sensing mechanism, leading to signaling transduction in plants. Rice is thermophilic and sensitive to cold temperature during its development and growth (Zhu 2016; Guo et al. 2018; Liu et al. 2018). The cold signal perceived by complexes such as COLD1/RGA1 triggers calcium signaling to elicit downstream responses (for example transcription factors ICE1 and CBFs) with their modification of phosphorylation on key factors, such as OST1 and MAPK3 on ICE1/OsbHLH002 (Ding et al. 2015; Ma et al. 2015; Zhang et al. 2017). A changed pattern of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) caused by stimuli is specific to the response such as plant hormones, biotic stresses and abiotic stresses (including drought, salt and cold) (Claus et al. 2018; Li et al. 2018; Qi et al. 2018; Yang and Guo 2018; Xu and Chong 2018). A unique group of calcium sensors of calcineurin B-like protein (CBL) and CBL-interacting protein kinases (CIPKs) functions in decoding calcium transients by specific stress signaling (Kolukisaoglu et al. 2004; Weinl and Kudla 2009). The CBL-CIPK complex is identified in plants in responses to various environmental stresses such as salt and drought (Zhu et al. 1998; Yang and Guo 2018). However, the mechanism of how the complex mediates the cold response remains elusive.

RESULTS AND DISCUSSION

Bioinformatic analyses of a genomic database revealed that there were 30 *CIPK* homologs in rice (Kolukisaoglu et al. 2004). We found that the expression of one homolog *OsCIPK7* was induced by cold, earlier than salt and drought (Figure S1A). *OsCIPK7* was constitutively expressed in all tissues, and the transcription level of *OsCIPK7* was increased by 6.8 folds after 3 h cold

treatment (Figure S1B). *OsCIPK7* over-expression transgenic lines exhibited increased tolerance to cold stress than the wild type (Figures 1A, B, S1C). We also obtained 43 allelic mutants with a point mutation in *OsCIPK7* through TILLING (Targeting-Induced Local Lesions IN Genomes), which were obtained from an EMS-mutagenized population in rice (*Oryza sativa* L., ssp. *japonica*, var. Zhonghua11, ZH11) (Table S1) (www.croptilling.org). Three mutants with mutations distributed throughout the encoded protein in the N-terminal kinase domain (*oscipk7-1* and *oscipk7-2*) and the C-terminal regulatory domain (*oscipk7-3*) were selected for further analysis. In *oscipk7-1*, the 422nd nucleotide G counted from the initiation codon ATG was mutated to A, leading to the change of the amino acid in the kinase domain from Arg^{141} to Gln^{141}. In *oscipk7-2*, a point mutation of C to T at the 506th nucleotide leads to the result that the Ala^{169} in the activation loop of the kinase

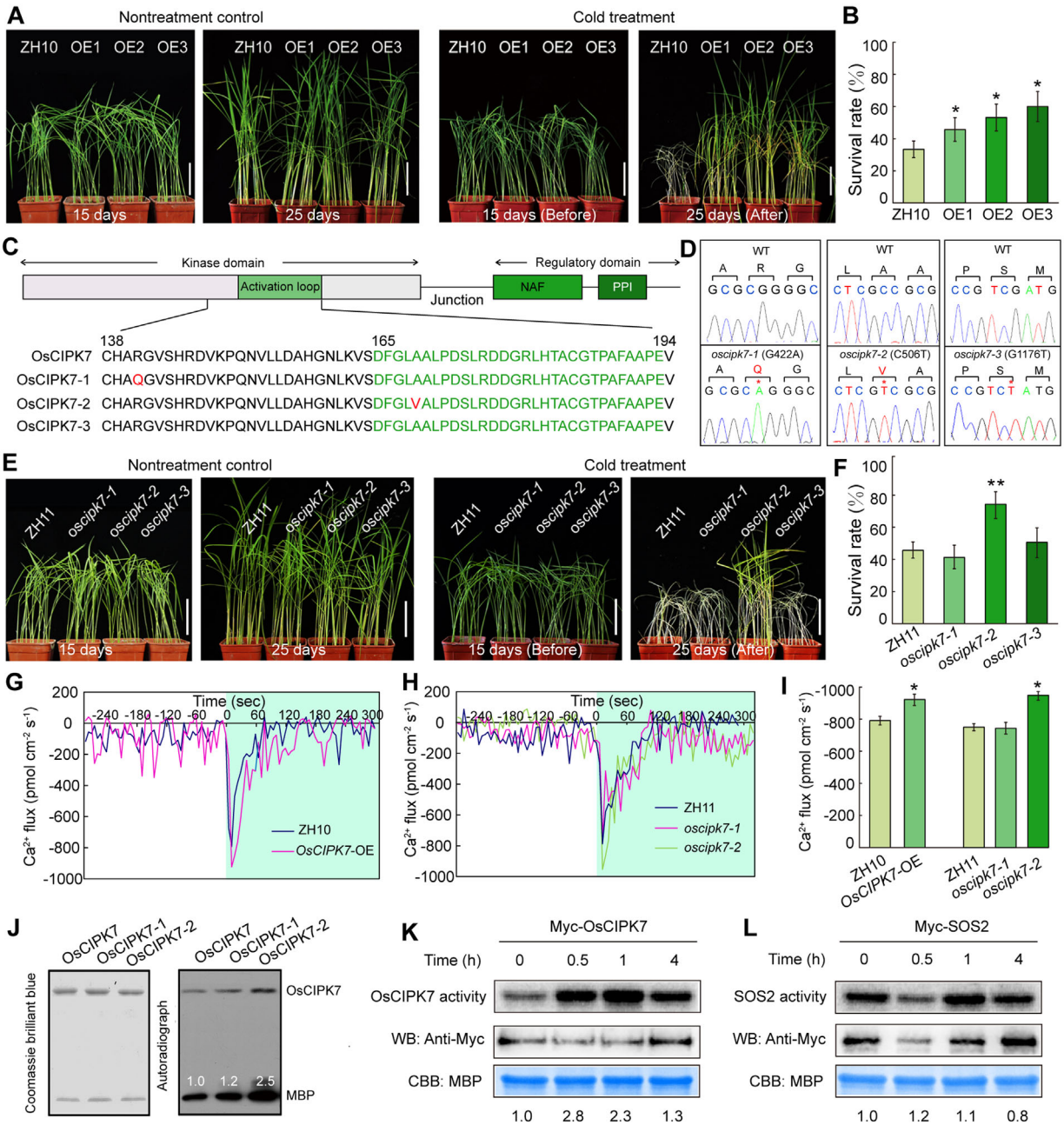

Figure 1. Continued

domain mutated to Val^{169}. The *oscipk7-3*, with a nonsense mutation located at the 1,176th nucleotide, was used as a control (Figure 1C, D). Phenotypic analyses showed that the survival rate of *oscipk7-2* under chilling treatment was significantly increased compared with the wild type. However, neither *oscipk7-1* nor *oscipk7-3* showed significant differences (Figure 1E, F). It hinted that the point mutation in *oscipk7-2* conferred chilling tolerance in rice, which was a gain-of-function variant. In order to further verify the genetic function of the 506th C to T point mutation, we have obtained the *OsCIPK7-2* over-expression lines (ZH11 background) to test the expression of *DREBs* in response of chilling stress (Figure S2). The basal *DREBs* expression in *OsCIPK7-2* over-expression lines was higher than that in wild type ZH11 and *OsCIPK7* over-expression lines, and the cold-induced expression of *DREBs* was much stronger than that of ZH11 and OsCIPK7-overexpression lines. These results hinted the fact that the *OsCIPK7-2* (C506T) point mutation really contributes to the chilling tolerance.

The calcium influx under cold stress was detected by Non-invasive Micro-test Technology (NMT) (Ludewig et al. 2003; Ma et al. 2015). There was an influx of extracellular Ca^{2+} with a shocked minus peak in wild type ZH10 roots upon cold stimulation, while the peak in *OsCIPK7*-overexpression line was bigger than that in ZH10 (Figure 1G, I). The *oscipk7-2* mutant exhibited stronger Ca^{2+} influx peak than the wild type and *oscipk7-1*, which is consistent with the cold tolerant phenotype (Figure 1H, I). These results suggested that the enhanced cold tolerant phenotype of the *oscipk7-2* mutant is related to the increased Ca^{2+} influx ability.

OsCIPK7 is a Ser/Thr protein kinase, and the kinase activation loop is important for its activity. So, we first tested whether these mutations affect the kinase activity of the purified OsCIPK7. Results showed that compared with the wild type, the purified OsCIPK7-2 protein, with a mutation occurred in *oscipk7-2*, exhibited enhanced autophosphorylation and phosphorylation activity. A general substrate myelin basic protein (MBP) was used. In contrast, OsCIPK7-1 showed no significant change in kinase activity (Figure 1J). These data suggested that the kinase activity is involved in the chilling tolerance of rice, and the hyper-activated OsCIPK7-2 protein may result in the increased chilling tolerance of plants. We then examined the kinase

Figure 1. OsCIPK7 with a point mutation at the activation loop exhibits enhanced kinase activity and confers chilling tolerance in rice

(A) Phenotype of *OsCIPK7* over-expressing lines under cold treatment. 15-d-old seedlings were treated under 4°C for 96 h, and then transferred back to the normal condition for recovery. Seedlings without cold treatment were used as a control. Pictures were taken at indicated time. Bar = 5 cm. **(B)** Survival rate of seedlings in **(A)** after cold treatment. Data are means ± *SD* ($n > 15$), Student's *t*-test, $^{*}P < 0.05$. **(C)** The amino acid sequence of the OsCIPK7 protein. Positions of the *oscipk7-1, oscipk7-2* and *oscipk7-3* point mutations are indicated. Sequences shown in blue represent the kinase activation loop. The mutated amino acids are shown in red. **(D)** Mutation validation of *oscipk7-1, oscipk7-2* and *oscipk7-3* by Sanger sequence analysis. Mutated nucleotides are indicated by asterisks. **(E)** Phenotype of *OsCIPK7* TILLING mutants under cold treatment. 15-day old seedlings were treated under 4°C for 96 h, and then transferred back to the normal condition for recovery. Seedlings with no cold treatment were used as a control. Pictures were taken at indicated time. Bar = 5 cm. **(F)** Survival rate of seedlings in **(E)** after cold treatment. Data are means ± *SD* ($n > 15$), Student's *t*-test, $^{**}P < 0.01$. **(G, H)** NMT measurements show dynamic change of extracellular Ca^{2+} influx in live roots of various genetic backgrounds responding to cold shock. The blue background represents the duration of cold treatment. The number was the cold treatment time on the abscissa. The minus numbers were the time before the treatment. **(I)** Quantitative statistics of the mean maximal Ca^{2+} influx after cold treatment in **(G)** and **(H).** Values are expressed as mean ± SD, $n > 6$, Student's *t*-test, $^{*}P < 0.05$. *OsCIPK7-OE* is compared with ZH10, and *oscipk7-1*, *oscipk7-2* are compared with ZH11, respectively. **(J)** Kinase activity of OsCIPK7, OsCIPK7-1 and OsCIPK7-2. Left panel, Coomassie brilliant blue-stained SDS-PAGE gel. Right panel, autoradiograph of kinase activity assays shown in the left panel. γ-^{32}P labeled Myelin Basic Protein (MBP) was used as a basal substrate to indicate the activity of OsCIPK7. **(K, L)** Kinase activity of OsCIPK7 and SOS2 under cold stress. Myc-OsCIPK7 and Myc-SOS2 were expressed and immunoprecipitated from the tobacco leaves which were treated at 4°C for the indicated times. Relative kinase activity is normalized to the Activity/WB which calculated by Image J software that relative to the value for tobacco leaves with cold treatment, respectively.

activity of OsCIPK7 under cold stress. Total proteins were extracted from tobacco leaves in which Myc-OsCIPK7 was transiently expressed and cold treated for 0, 0.5, 1 and 4 h. The phosphorylation level of MBP was used as an indicator of the OsCIPK7 kinase activity, which was increased during the cold stress (Figure 1K). An *Arabidopsis* homolog SOS2 (AtCIPK24) which responds to salt stress was used as a control (Lin et al. 2009; Ma et al. 2019). The activity of SOS2 showed no significant change under cold treatment (Figure 1L). This result indicated that OsCIPK7 is a cold-activated protein kinase, and the OsCIPK7 activity positively correlates with cold signaling.

CIPKs have conserved domains consisting of an N-terminal serine/threonine protein kinase domain, a junction domain and a C-terminal regulatory domain (Shi et al. 1999). The C domain includes the autoregulatory NAF domain and the phosphatase interaction domain (PPI) (Ohta et al. 2003). It was reported that CIPKs underwent large conformation changes to target the substrate (Nolen et al. 2004; Chaves-Sanjuan et al. 2014), which inspired us to investigate whether OsCIPK7 has a conformation change under different temperatures. First, we made alignment of the conserved amino acid sequence in the activation loop between OsCIPK7 and CIPK23. The mutation site A169 in *oscipk7-2* is sequentially similar to Ser176 of CIPK23, which was identified as a crucial residue to maintain a hydrophobic pocket of the activation loop. We modified the crystal structure of CIPK23 to highlight the activation loop domain (Figure 2B) (Chaves-Sanjuan et al. 2014). The simulation structure of OsCIPK7 from the Phyre2 website shows great similarity to that of CIPK23, where the A169-F166 link makes the local loop more stable at the activation region, which also prevents the exposure of hydrophobic sites (Figure 2C) (Kelley et al. 2015). The conformation changes of the OsCIPK7 and OsCIPK7-2 (A169V) proteins were monitored by Fourier-transform infrared (FTIR) spectroscopy (Figure 2D–F) (Zhang et al. 2013). Secondary structures are labeled at different wave numbers as shown in the figures. Substitution at site 169 by Val gives rise to a significant reduction of loop structure at 1,652 cm^{-1}, which is overlapped with α-helix in the same region. Along with this reduction, an increase of β-strand can be easily recognized around 1,615 cm^{-1}, which indicates a prominent transition of loop to anti-parallel β-strand. It is notable that the vanishment of hydrophilic β-sheet at 1,624 cm^{-1} is also involved under the single point mutation, while the hydrophobic β-sheet at 1,683 cm^{-1} remains stable (Figure 2D).

For OsCIPK7, cold-induced conformational changes show that the absorption peak at 1,652 cm^{-1} due to α-helix/loop is red-shifted, as the reason of stronger hydrogen bonds after cooling, and the blue-shifted β-sheet indicates this hydrophilic structure becomes more hydrophobic, which can be explained as the shrinkage of the local structure (Figure 2E). Without the influence of the hydrophilic β-sheet, the mutant protein OsCIPK7-2 exhibits a red-shifted β-strand after temperature decreasing, and no content variation can be distinguished (Figure 2F). It hints the mutant protein OsCIPK7-2 exhibits increased kinase activity and stable structure conformation, which is insensitive to cooling.

Considering the phenotypic difference between the OsCIPK7 and the mutant protein OsCIPK7-2, it can be concluded that both the loop and hydrophilic β-sheet at the vicinity of site 169 are crucial in suppressing kinase activity. The point mutation of OsCIPK7-2 may lead to an open form of the protein kinase, thus the binding/interaction between OsCIPK7-2 and its substrate might be stronger, which might be the reason why the kinase activity of OsCIPK7-2 was increased. Together with the results of FTIR, we believe that this point mutation may have enhanced the binding ability of OsCIPK7 to the substrate, thus leading to increased kinase activity.

In summary, we showed that the point mutation in the kinase domain of OsCIPK7 leads to a conformational change in the activation loop, results in an increase of kinase activity, therefore confers the chilling tolerance of rice. And the calcium influx was increased after the chilling treatment in the *oscipk7-2* compared with wild type. Based on the temperature signaling perception concept in animal cells, it is likely that OsCIPK7 as a sensor senses cold signaling through structure conformation change for increasing activity of the kinase in rice cells (Ma et al. 2015; Shi and Gong 2015; Shi and Yang 2015; Guo et al. 2018). The interesting questions such as the relation of OsCIPK7 to COLD1 and CBLs, as well as the substrate of OsCIPK7 for sensing will be addressed in the future. An allelic point mutation obtained by TILLING exhibited increased tolerance to chilling stress, suggesting its potential to be used in the molecular breeding for cold tolerance rice.

The Materials and Methods are to be presented as Supporting Information.

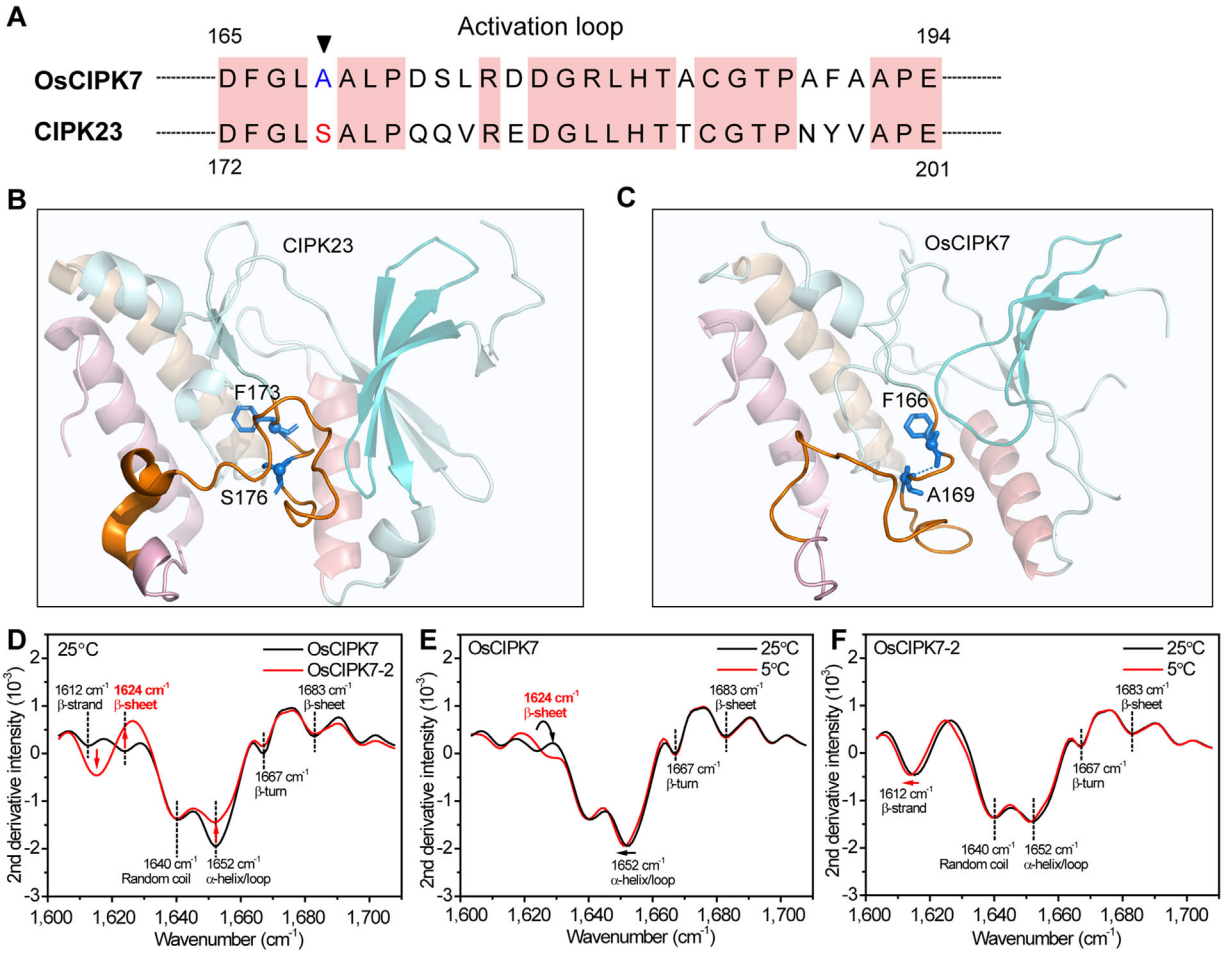

Figure 2. Conformational change of OsCIPK7 under cold stress detected by Fourier-transform infrared (FTIR) spectroscopy

(A) Alignment of sequences in the activation loop between OsCIPK7 and CIPK23. V169 of OsCIPK7 is equivalent to Ser176 of CIPK23 based on the conserved domain sequence alignment. **(B)** The crystal structure of CIPK23 based on X-ray diffraction. The crystal structure of CIPK23 from the Protein Data Bank was monitored by Pymol software. Ser176 is an important and conserved site for the activation of CIPK23 (Chaves-Sanjuan et al. 2014). **(C)** The simulation structure of CIPK7 from Phyre2 website. F166-A169 link makes the local loop more stable at the activation region, which also prevents the exposure of hydrophobic sites (Kelley et al. 2015). **(D)** The secondary derivative FTIR spectra of OsCIPK7 (black line) and OsCIPK7-2 (red line) at 25°C. Secondary structures are labeled at different wavenumbers as shown in the figure. Changes at different structures are pointed by narrows. **(E, F)** The secondary derivative FTIR spectra of OsCIPK7 and OsCIPK7-2 at 25°C (black line) and 5°C (red line), respectively. Secondary structures are labeled at different wavenumbers as shown in the figure. Changes at different structures are pointed by narrows.

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AUTHOR CONTRIBUTIONS

K.C. and Y.X. designed the study and revised the manuscript; D.Z. and X.G. conceived all the experiments and wrote the manuscript; X.G. performed the FTIR assay with the help of H.L. and the cold induced kinase activity detection with the help of L.M.; X.Y. performed the TILLING mutants screen and sequence; Y.G., Y.W.

and C.M.L. helped to interpret the data and revised the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.12800/suppinfo

Materials and Methods

Figure S1. Expression pattern analysis of *OsCIPK7*

(**A**) qPCR analysis of expression level of *OsCIPK7* under stresses including cold, NaCl and drought treatment with different time courses. Data are means ± SD ($n = 3$). (**B**) qPCR analysis of expression level of OsCIPK7 at different tissues. Data are means ± SD ($n = 3$).

(**C**) Expression level of OsCIPK7 in ZH10 and over-expression lines.

Figure S2. Expression pattern analysis of *DREBs* under cold stress

(**A**) Expression level of *OsCIPK7* in ZH11, *OsCIPK7*-OE and *OsCIPK7*-2-OE lines. *OsCIPK7*-OE represents *OsCIPK7* over-expression line, and *OsCIPK7*-2-OE indicates *OsCIPK7*-2 (C506T) over-expression line. (**B–D**) qPCR analysis of expression level of *DREB1A*, *DREB1B* and *DREB1C* under cold treatment for 3h. Data are means ± *SD* ($n = 3$). 2-week-old seedlings were used for the cold treatment.

Table S1. *OsCIPK7* mutants identified by TILLING technology

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