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AtCIPK19 aggrandizes polyamines-involved cold stress tolerance in plant cells

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Abstract: The CALCINEURIN B-LIKE PROTEIN-INTERACTING PROTEIN KINASEs (CIPKs) play important roles in regulating ion homeostasis and stress responses. However, molecular mechanism of the Arabidopsis (*Arabidopsis thaliana*) CALCINEURIN B-LIKE PROTEIN-INTERACTING PROTEIN KINASE19 (CIPK19) enhanced cold stress tolerance is not fully understood. Here, we report that overexpression of the Arabidopsis *CIPK19* gene (*AtCIPK19*) results in increasing cell viability and growth rate under cold stress in *O. sativa*, *G. hirsutum*, and *P. strobus*. *AtCIPK19* increases cold stress tolerance by decreases the thiobarbituric acid reactive substances (TBARS) and increases the levels of putrescine (Put), spermidine (Spd), and spermine (Spm). In *AtCIPK19* transgenic rice cell lines, the transcript levels of genes associated with biosynthesis of Put, Spd, and Spm include *OsADC1*, *OsADC2*, *OsADC3*, *OsODC1*, *OsODC2*, *OsODC3*, *OsCPA1*, *OsCPA2*, *OsCPA3*, *OsAIH*, *OsSAMDC1*, *OsSAMDC2*, *OsSAMDC3*, *OsSAMDC4*, *OsSAMDC5*, *OsSAMDC6*, *OsSPD/SPM1*, *OsSPD/SPM2*, *OsSPD/SPM3*, and *OsSPD/SPM4* are increased under cold stress. These results will increase our understanding of *AtCIPK19*-related cold stress tolerance in different plant species and are valuable in plant molecular breeding application.

Keywords: CALCINEURIN B-LIKE PROTEIN; Cold stress; *Pinus;* Putrescine; Spermidine; Spermine

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Introduction

Cold stress reduces plant growth and crop yield. To counteract the damage caused by cold stress, plants have developed capabilities to adapt to this extreme environment, including activation of hormone signaling and alterations of expression of cold stress-related genes. In order to fully characterize the adaptive responses of plants to cold stress, it is important to determine the final levels of mRNAs and proteins in order to reveal the precise novel regulatory mechanisms which specifically function in the response to cold stress [1]. Transcription factors participate in biological processes of cold stress responses. In *Prunus mume*, increased expression of 15 putative PmNACs transcription factors was observed to tolerate freezing-stress [2]. In rice, OsmiR156 enhances cold stress tolerance by regulating the expression of transcription factor genes [3]. Membrane-to-nucleus signals modulate plant cold tolerance [4]. A yeast one-hybrid assay demonstrated that *AaDREB1* encodes a transcription activator and specifically binds to DRE/CRT to enhance tolerance to low temperature [5]. In cucumber, *CsWRKY46* was up-regulated in response to cold stress and its overexpression increased the expression of stress-inducible genes, including *RD29A* and *COR47* [5]. In *K. obovata*, mRNA expression analysis indicated that the *KoHSP70* was increased significantly after 48 h cold stress, and reached the highest level at 168 h after cold treatment [6]. In wheat and barley, high transcription level of dehydrin was observed after plants exposed to cold stress [7]. In *Glyycine soja*, over-expression of *GsZFP1* increased the expression of stress-response marker genes, including *CBF1*, *CBF2*, *CBF3*, *NCED3*, *COR*47, and *RD29A* in response to cold stress [8].

Polyamines including putrescine, spermidine, and spermine might help plants to deal with cold stress by interacting with negatively charged macromolecules and regulate their functions [9]. In apple (*Malus domestica*), genome-wide investigation showed that 18 S-adenosylmethionine decarboxylase and spermidine synthase-related sequences were involved in polyamines biosynthesis in response to cold stressed condition [10]. In rubber tree, S-Adenosylmethionine decarboxylase (SAMDC), a key rate-limiting enzyme involved in polyamines biosynthesis, plays important roles in cold stress response [11]. In bamboo, the endogenous polyamines significantly increased to avoid injury during cold stress [12]. In trifoliate orange [*Poncirus trifoliata* (L.) Raf.], miRNA396b (ptr-miR396b) positively regulates cold tolerance through reducing *ACO* transcript levels and simultaneously promoting polyamine synthesis [13]. In Banana, the chilling tolerance induced by NO treatment might be ascribed to the enhanced catabolism of polyamine [14]. In tea (*Camellia sinensis*) subjected to low-temperature stress, the *CsSPMS* gene is quickly induced by cold stress [15]. In rice, SamDC, a key enzyme in the polyamine biosynthesis pathway, functions in response to the abiotic stress treatments of cold [16,17]. In Siberian spruce (*Picea obovata*), the accumulation of polyamines may act as compatible solutes or cryoprotectants to act on freezing tolerance development [18,19].

Calcineurin B-like protein interacting protein kinases (CIPKs) are vital elements in plant abiotic stress signaling pathways. Calcineurin B-like protein interacting protein kinases (CIPKs) are key regulators of pre-transcriptional and post-translational responses to cold stress. In *Arabidopsis thaliana*, CIPK16 (AtCIPK16) was identified as a regulator of cold stress tolerance [20]. In roots of rice seedling, the serine/threonine/tyrosine protein kinase gene, *OsACTPK1*, accumulated under abiotic stress [21]. In *Brachypodium distachyon*, *BdCIPK31* was downregulated by polyethylene glycol, NaCl, H2O2, and abscisic acid (ABA) treatments. Overexpression of *BdCIPK31* could elevate several stress-

associated gene expressions under stress conditions [22]. In *Arabidopsis thaliana*, the calcineurin B-like protein-interacting protein kinase-7 (AtCIPK7) could negatively regulate TuYV export from infected cells [23]. In wheat, TaCIPK29 was identified as a regulator of stress tolerance. Over-expression of TaCIPK29 in tobacco resulted in increased salt tolerance [24]. In rice, OsCIPK14 and OsCIPK15, are rapidly induced by stress [25]. In Arabidopsis, the calcineurin B-like protein-interacting protein kinase 9, AtCIPK9, acted as a critical regulator of abiotic stress [26].

It has been reported that the Arabidopsis (*Arabidopsis thaliana*) CALCINEURIN B-LIKE PROTEIN-INTERACTING PROTEIN KINASE19 (*AtCIPK19*) acts as an important element to pollen tube growth [27]. Overexpression of *AtCIPK19* was associated with elevated cytosolic Ca^{2+} throughout the bulging tip, indicating that CIPK19 may be involved in maintaining Ca^{2+} homeostasis through its potential function in the modulation of Ca^{2+} influx [28]. Although CIPKs play important role in cold stress tolerance, how *AtCIPK19* regulates cell response to cold stress is not fully understood. In this investigation, we demonstrated that *AtCIPK19* increases cold stress tolerance in *O. sativa*, *G. hirsutum*, and *P. strobus*. In rice, *AtCIPK19* enhanced cold stress tolerance by expression of *OsADC1*, *OsADC2*, *OsADC3*, *OsODC1*, *OsODC2*, *OsODC3*, *OsCPA1*, *OsCPA2*, *OsCPA3*, *OsAIH*, *OsSAMDC1*, *OsSAMDC2*, *OsSAMDC3*, *OsSAMDC4*, *OsSAMDC5*, *OsSAMDC6*, *OsSPD/SPM1*, *OsSPD/SPM2*, *OsSPD/SPM3*, and *OsSPD/SPM4*. To our best knowledge, this is the first report that describes a detailed interaction between *AtCIPK19* and genes associated with biosynthesis of Put, Spd, and Spm in improving cold stress tolerance in different plant species.

Materials and Methods

Plasmid constructs

The cDNA sequence of *AtCIPK19* was amplified from the Arabidopsis genome and cloned into expression vector pCAMBIA1301 as previously described [27]. DNA of pCAMBIA1301 and *AtCIPK19* were digested by *Kpn*I and *Bam*HI (Promega, Madison, WI, USA) at 37ºC. DNA was purified using QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA) and ligated to generate the expression vector pCAMBIA1301-*AtCIPK19*. Expression vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. The neomycin phosphotransferase II (*NPT II*) gene was used as a select marker in the transformation.

Transformation of *O. sativa*, *G. hirsutum*, and *P. strobus* cells

AtCIPK19 transgenic cell lines of *O. sativa*, *G. hirsutum*, and *P. strobus* were generated as described before [28], using *Agrobacterium tumefaciens* strain GV3101 carrying pCAMBIA1301-*AtCIPK19* to transform cultured cells. After transformation, cell cultures of different cell lines were growing for 4-5 week, and then these cell cultures were used for further analysis including cold stress and gene expression.

Polymerase chain reaction analyses of transgenic cells

Polymerase chain reaction (PCR) analysis of transgenic cells of *O. sativa*, *G. hirsutum*, and *P. strobus* was conducted as previously described [28]. One gram of control cells and transgenic cells were used to isolate genomic DNA, using a Genomic DNA Isolation Kit (Sigma). The primers used to amplify the *NPTII* gene are forward primer (nfp) 5'-GTCGACATGGCGGAGGAATTTGGAAGC ATAG-3' and the reverse primer (nrp) 5'-

CCATGGTAGACTCCTGCTTCGACATCATGG-3'. The primers used to amplify the *AtCIPK19* gene are forward primer (ckr) 5'-CTTTCAATGG CGGATTTGTT AAGAAAAGTG -3' and the reverse primer (ckf) 5'- CTTCATTTTT CATCTTCCTA ATCAGTATCA GAAAG- 3'. The PCR mixture, the PCR conditions, and gel electrophoresis were carried out as described previously [28].

Southern blot analysis of transgenic cells

Southern blotting analysis of transgenic cells of *O. sativa*, *G. hirsutum*, and *P. strobus* was conducted as previously described [28]. Five grams of control cells and transgenic cells were used to isolate genomic DNA, using a Genomic DNA Isolation Kit (Sigma). Twenty-five micrograms of DNA were digested 16 hours with the enzymes *Xba* I (Boehringer Mannheim) at 37ºC. The molecular probes (1452 pb fragment of *AtCIPK19*) were labeled by Digoxigenin (DIG) (Roche Diagnostics, Indianapolis, IN, USA).

RNA isolation and Northern blot analysis

Five grams of fresh cultures of transgenic and control cells of *O. sativa*, *G. hirsutum*, and *P. strobus* were used to isolate total RNA, using a RNeasy Mini Plant Kit (Germantown, MD, USA) by following the manual. Six µg of total RNA was separated by agarose-gel electrophoresis. Electrophoresis of RNAs and northern blotting were performed as described before [28]. The hybridization probe is the Digoxigenin (DIG)-labelling *AtCIPk19* DNA (1452 pb) (Roche Diagnostics). The rRNA was used as the loading control of RNA samples.

Cold treatment and determination of cell viability and cell growth rate

Cold treatment was performed by incubation of *AtCIPK19* transgenic cells (at the age of 4-5 weeks) and control cells of *O. sativa*, *G. hirsutum*, and *P. strobus* at -10, 4, 12, and 24ºC in the dark for 24 hours in growth chambers (Beijing ZNYT, China). Control seedlings were grown under the same conditions. After 24 h of chilling treatment, cells were moved to the normal growth environment. The influences of cold stress on cell growth and cell viability of *O. sativa*, *G. hirsutum*, and *P. strobus* were determined, as previously described [29]. The average growth was expressed as mg/g FW/day. The rate of cell growth and the cell viability were measured 7 days after treatment. Samples from both chilled and control samples were collected with 3 biological replicates.

Measurement of thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation was determined as the amount of thiobarbituric acid reactive substances (TBARS) measured by the thiobarbituric acid (TBA) reaction as described previously (Tang and Page 2013). Transgenic and control cells (1 g) were homogenized in 3 ml of 20 % (w/v) trichloroacetic acid (TCA), then centrifuged at 5,000 rpm for 20 min, following by mixing with 20 % TCA containing 0.5 % (w/v) TBA and100 µl 4 % BHT in ethanol at 1:1. The absorbance of the extracts of different cell lines was measured at 532 nm. The value of TBARS was calculated using the method described previously [28,29].

Determination of polyamines

Determination of polyamines putrescine (Put), spermidine (Spd), and spermine (Spm) from tissues of *P. strobus* was carried out as described previously [30]. Samples were examined using a HPLC and Spector Monitor 3200 Detector by following the manual of the facility. The measured polyamines are total PAs.

Expression of genes associated with biosynthesis of putrescine, spermidine, and spermine

Expression of genes associated with biosynthesis of putrescine (Put), spermidine (Spd), and spermine (Spm) in different cell lines of *O. sativa*, *G. hirsutum*, and *P. strobus* was examined using *qRT–PCR* by the method of [16,31,32]. Genes associated with biosynthesis of Put, Spd, and Spm include *OsADC1*, *OsADC2*, *OsADC3*, *OsODC1*, *OsODC2*, *OsODC3*, *OsCPA1*, *OsCPA2*, *OsCPA3*, *OsAIH*, *OsSAMDC1*, *OsSAMDC2*, *OsSAMDC3*, *OsSAMDC4*, *OsSAMDC5*, *OsSAMDC6*, *OsSPD/SPM1*, *OsSPD/SPM2*, *OsSPD/SPM3*, and *OsSPD/SPM4* in *AtCIPK19* transgenic cell lines have examined. The U6 gene was used as internal reference of qRT-PCR because U6 is one of most suitable reference gene under salinity and cold stresses [16,31-34]. The qRT-PCR data were normalized to the U6 internal control. The regular qRT-PCR was used. The delta-delta Ct method was used to obtain expression value.

RNA isolation and qRT-PCR

Total RNA was isolated using the regents of Sepasol RNA I Super (Nacalai Tesque, Kyoto, Japan). To synthesize the first-strand cDNA in a 50-mL reaction containing 2.5 mM oligo(dT) primers, 2.5 mM random heximer, and 2.5 mg of total RNA, the PrimeScript™ RT reagent kit (TaKaRa Co., Ltd, Ohtsu, Japan) was used by following the instruction. The qRT–PCR was performed using 1Å~ SYBR Premix Ex Taq II (TaKaRa). Five microliters of each reaction mixture was used as a template for PCR amplification in a 25-mL mixture containing 1.5 mM MgCl2, 200 mM. The primer sequences used for genes of *OsADC1*, *OsADC2*, *OsADC3*, *OsODC1*, *OsODC2*, *OsODC3*, *OsCPA1*, *OsCPA2*, *OsCPA3*, *OsAIH*, *OsSAMDC1*, *OsSAMDC2*, *OsSAMDC3*, *OsSAMDC4*, *OsSAMDC5*, *OsSAMDC6*, *OsSPD/SPM1*, *OsSPD/SPM2*, *OsSPD/SPM3*, and *OsSPD/SPM4* are as described previously (Do, et al. 2013). The U6 gene was used as internal reference [16,31-34]. The data were normalized to the internal control. The delta-delta Ct method was used to obtain expression value.

Statistical analyses

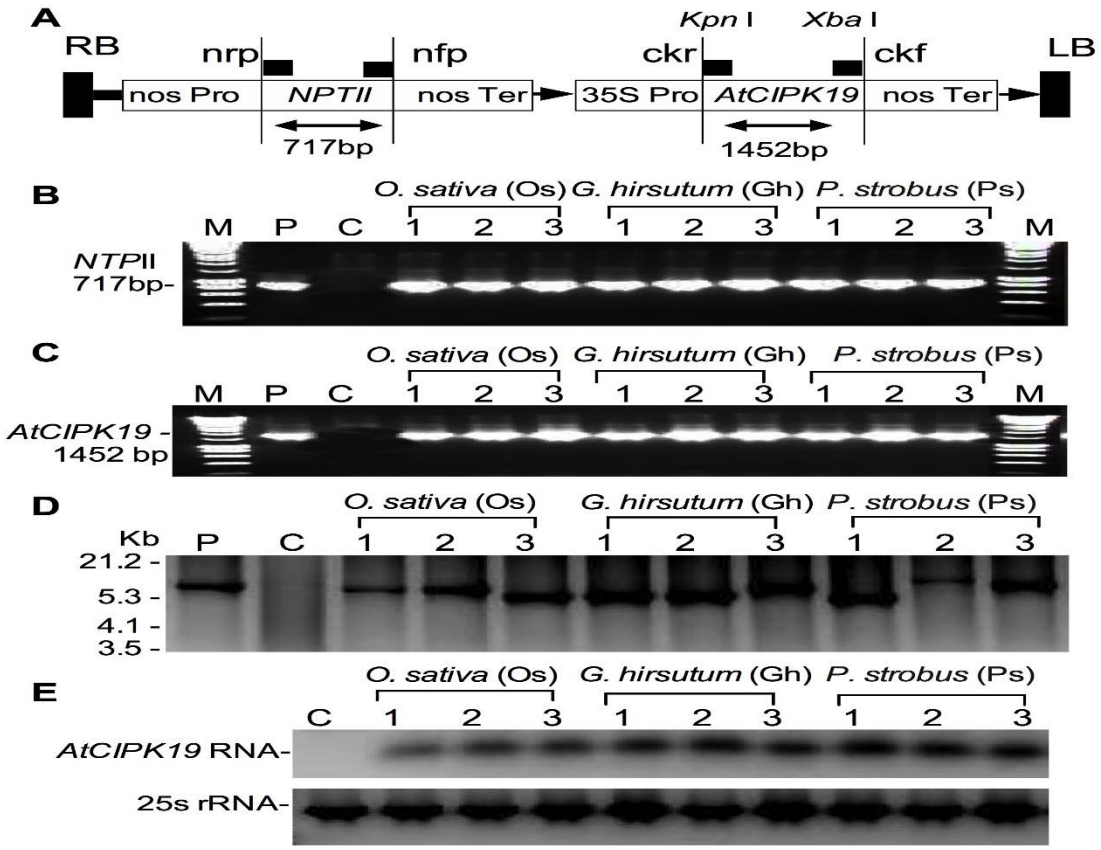
Statistical analysis was performed using the General Linear Model procedure of SAS (Cary, NC, USA), employing ANOVA models. The significant differences between mean values of different cell lines derived from *O. sativa*, *G. hirsutum*, and *P. strobus* were made at 5 % level of probability, using the T-test. Each value of different cell lines derived from *O. sativa*, *G. hirsutum*, and *P. strobus* was presented as means standard errors of the mean.

Results

Generation of transgenic cell lines in *O. sativa*, *G. hirsutum*, and *P. strobus*

AtCIPK19 transgenic cell lines of *O. sativa*, *G. hirsutum*, and *P. strobus* were generated using *Agrobacterium tumefaciens* strain GV3101 carrying pCAMBIA1301-*AtCIPK19* (Figure 1A) to transform cultured cells. After transformation, a total of 29 transgenic cell lines of *O. sativa*, a total of 32 transgenic cell lines of *G. hirsutum*, and a total of 36 transgenic cell lines of *P. strobus* were produced. After transgenic cell lines of *O. sativa*, *G. hirsutum*, and *P. strobus* were confirmed by PCR (Figure 1B,C), Southern blotting (Figure 1D), and northern blotting analyses (Figure 1E), nine cell lines (Os1, Os2, and Os3 from *O. sativa*, Gh1, Gh2, and Gh3 from *G. hirsutum*, and Ps1, Ps2, and Ps3 from *P. strobus*) from each of *O. sativa*, *G. hirsutum*, and *P. strobus* were selected and analyzed in this study.

Figure 1: Expression vector and generation of transgenic cells.



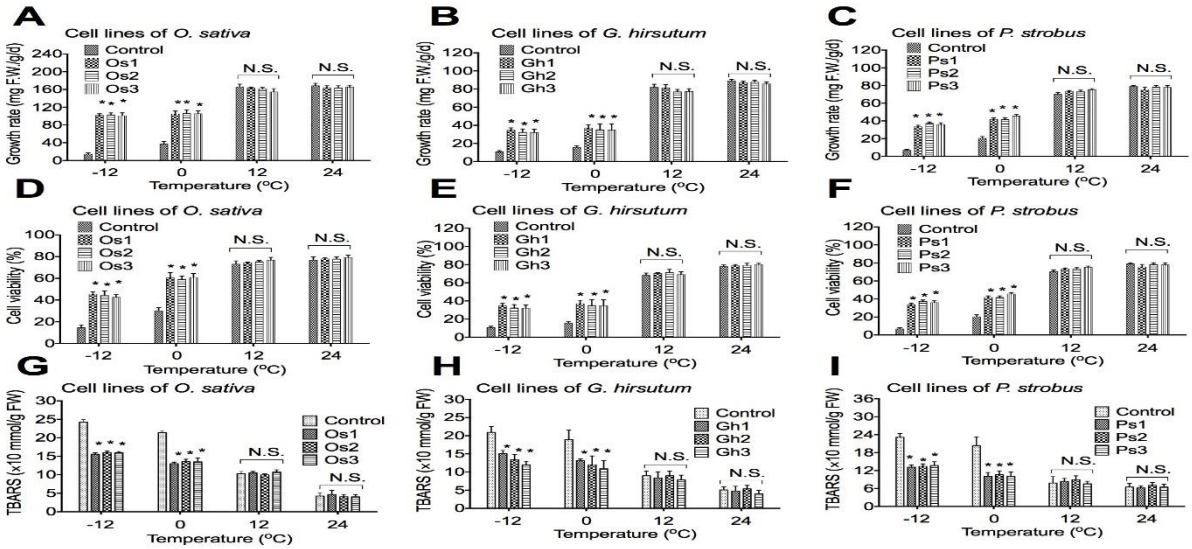
Overexpression of the *AtCIPK19* gene increases the levels of putrescine (Put), spermidine (Spd), and spermine (Spm) in transgenic cells of *O. sativa* (A, B, and C), *G. hirsutum* (D, E, and F), and *P. strobus* (G, H, and I), respectively. The statistically significant difference between groups was determined by one-way ANOVA. Data are presented as means of three independent experiments. Error bars represent standard error. The asterisk indicates significant differences compared to the control, as assessed by a *t*-test. *P<0.05, significant relative to control. N.S., no statistical significance.

Overexpression of *AtCIPK19* increases cell viability, cell growth rate, and decreases TBARS

To determine if overexpression of the *AtCIPK19* gene increases cold stress tolerance, the cell growth rate, the cell viability, and the TBARS in transgenic cell lines of *O. sativa*, *G. hirsutum*, and *P. strobus* were examined. The cell growth rate and the cell viability were significantly increased in transgenic cell lines of *O. sativa* (Figure 2A,D), *G. hirsutum* (Figure 2B,E), and *P. strobus* (Figure 2C,F), respectively, under stress at -10°C and -4°C. The cell growth rate and the cell viability were not significantly increased in transgenic cell lines of *O. sativa* (Figure 2A,D), *G. hirsutum* (Figure 2B,E), and *P. strobus* (Figure 2C,F), respectively, under stress at 10°C and 24°C. The amount of TBARS was significantly increased in transgenic cell lines of *O. sativa* (Fig. 2G), *G. hirsutum* (Figure 2H), and *P. strobus* (Figure 2I), respectively, under stress at -10°C and -4°C. The amount of TBARS was not

significantly increased in transgenic cell lines of *O. sativa* (Figure 2G), *G. hirsutum* (Fig. 2H), and *P. strobus* (Figure 2I), respectively, under stress at 10ºC and 24ºC.

Figure 2: Overexpression of *AtCIPK19* increases cell viability, cell growth rate, and decreases TBARS.

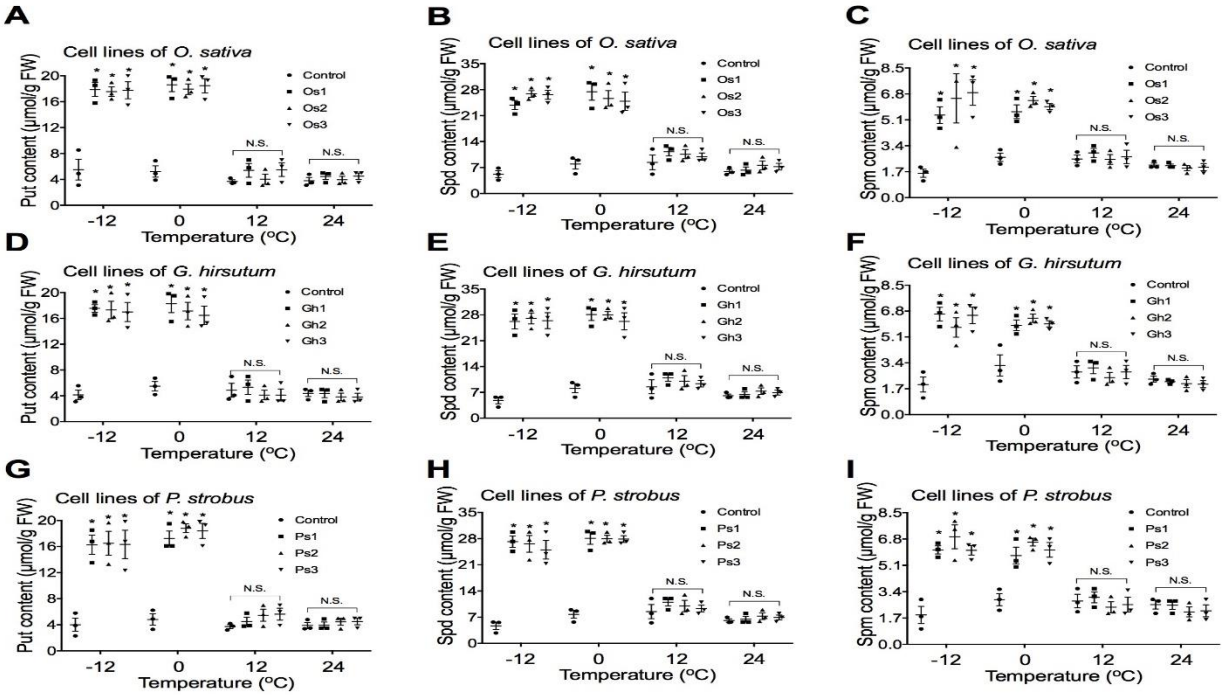


Overexpression of the *AtCIPK19* gene increases the cell growth rate and the cell viability, but decreases the TBARS in transgenic cells of *O. sativa* (A, D, and G), *G. hirsutum* (B, E, and H), and *P. strobus* (C, F, and I), respectively. The statistically significant difference between groups was determined by one-way ANOVA. Data are presented as means of three independent experiments. Error bars represent standard deviation. The asterisk indicates significant differences compared to the control, as assessed by a *t*-test. *P<0.05, significant relative to control. N.S., no statistical significance.

Overexpression of *AtCIPK19* increases contents of Put, Spd, and Spm

To examine if overexpression of the *AtCIPK19* gene-enhanced cold stress tolerance is related to the biosynthesis of putrescine (Put), spermidine (Spd), and spermine (Spm), the contents of Put, Spd, and Spm were analyzed in transgenic cell lines of *O. sativa*, *G. hirsutum*, and *P. strobus*. Overexpression of the *AtCIPK19* gene significantly increased the levels of putrescine (Put), spermidine (Spd), and spermine (Spm) in transgenic cells of *O. sativa* (Figure 3A,B and C), *G. hirsutum* (Figure 3D,E, and F), and *P. strobus* (Fig. 3G, H, and I), respectively, under stress at -10ºC and -4ºC. Overexpression of the *AtCIPK19* gene did not significantly increase the levels of putrescine (Put), spermidine (Spd), and spermine (Spm) in transgenic cells of *O. sativa* (Fig. 3A, B, and C), *G. hirsutum* (Figure 3D,E, and F), and *P. strobus* (Figure 3G,H, and I), respectively, under stress at 10ºC and 24ºC.

Figure 3: Overexpression of *AtCIPK19* increases contents of Put, Spd, and Spm.

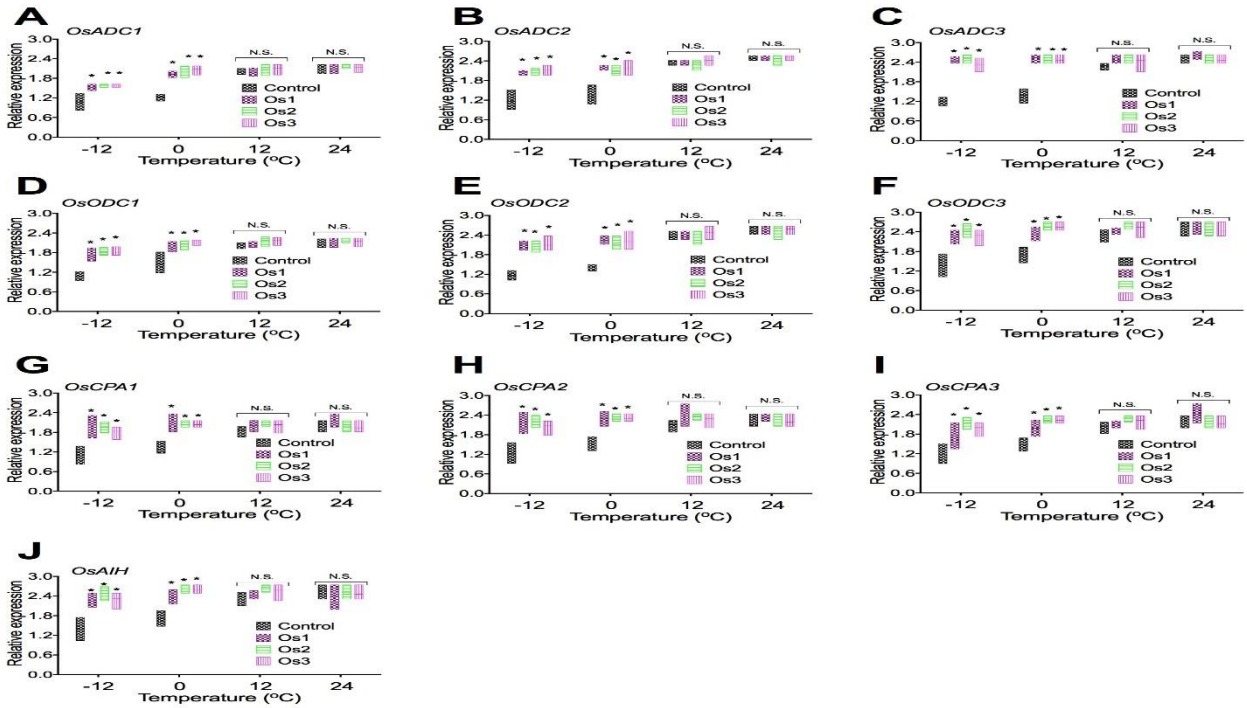


Overexpression of the *AtCIPK19* gene increases the levels of putrescine (Put), spermidine (Spd), and spermine (Spm) in transgenic cells of *O. sativa* (A, B, and C), *G. hirsutum* (D, E, and F), and *P. strobus* (G, H, and I), respectively. The statistically significant difference between groups was determined by one-way ANOVA. Data are presented as means of three independent experiments. Error bars represent standard error. The asterisk indicates significant differences compared to the control, as assessed by a *t*-test. *P<0.05, significant relative to control. N.S., no statistical significance.

Expression of genes associated with putrescine biosynthesis in transgenic rice cells

To examine if overexpression of the *AtCIPK19* gene-increased contents of Put, Spd, and Spm was related to the expression of genes associated with putrescine biosynthesis, expression of *OsADC1* (Figure 4A), *OsADC2* (Figure 4B), *OsADC3* (Figure 4C), *OsODC1* (Figure 4D), *OsODC2* (Figure 4E), *OsODC3* (Figure 4F), *OsCPA1* (Figure 4G), *OsCPA2* (Figure 4H), *OsCPA3* (Figure 4I), and *OsAIH* (Figure 4J) were analyzed in transgenic cell lines (Os1, Os2, and Os3) of *O. sativa*. Overexpression of the *AtCIPK19* gene significantly increases expression of putrescine biosynthesis enzymes genes (Figure 4) under stress at -10°C and -4°C. Overexpression of the *AtCIPK19* gene does not significantly increases expression of putrescine biosynthesis enzymes genes (Figure 4) under stress at 10°C and 24°C.

Figure 4: Expression of genes associated with putrescine biosynthesis in transgenic rice cells**.**

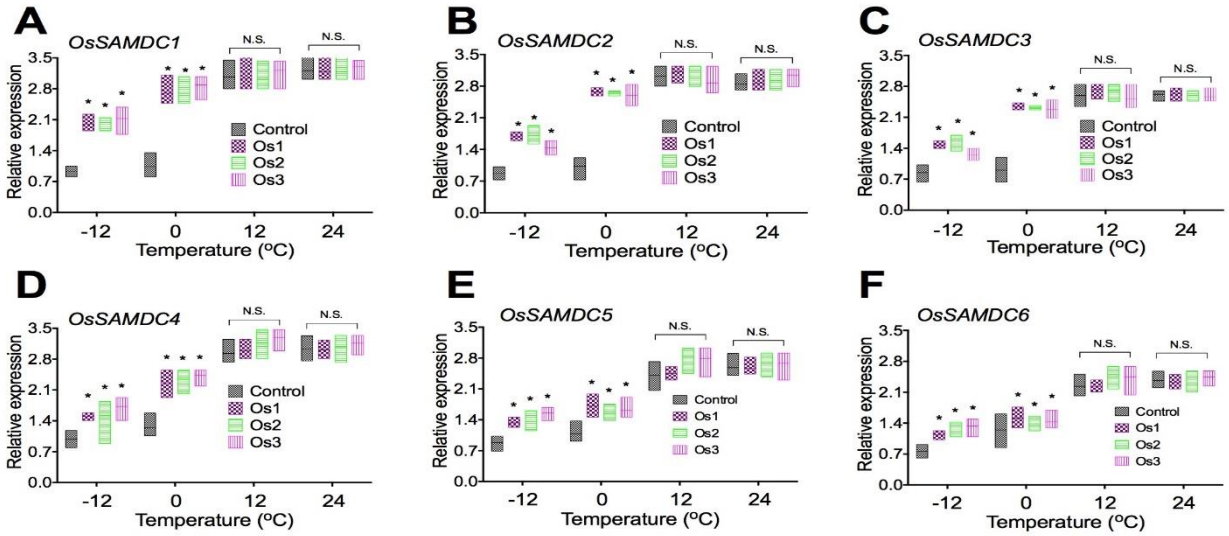


Overexpression of the *AtCIPK19* gene increases expression of putrescine biosynthesis enzymes genes *OsADC1* (A), *OsADC2* (B), *OsADC3* (C), *OsODC1* (D), *OsODC2* (E), *OsODC3* (F), *OsCPA1* (G), *OsCPA2* (H), *OsCPA3* (I), and *OsAIH* (J). The statistically significant difference between groups was determined by one-way ANOVA. Data are presented as means of three independent experiments. Error bars represent standard error. The asterisk indicates significant differences compared to the control, as assessed by a *t*-test. *P<0.05, significant relative to control. N.S., no statistical significance.

Expression of genes associated with decarboxylated Sadenosylmethionine biosynthesis in transgenic rice cells

To examine if overexpression of the *AtCIPK19* gene-increased contents of Put, Spd, and Spm was related to the expression of genes associated with decarboxylated Sadenosylmethionine biosynthesis, expression of *OsSAMDC1* (Figure 5A), *OsSAMDC2* (Figure 5B), *OsSAMDC3* (Figure 5C), *OsSAMDC4* (Figure 5D), *OsSAMDC5* (Figure 5E), and *OsSAMDC6* (Figure 5F) were analyzed in transgenic cell lines (Os1, Os2, and Os3) of *O. sativa*. Overexpression of the *AtCIPK19* gene significantly increased expression of decarboxylated Sadenosylmethionine (dsSAM) biosynthesis enzymes genes *OsSAMDC1* (Figure 5A), *OsSAMDC2* (Figure 5B), *OsSAMDC3* (Figure 5C), *OsSAMDC4* (Figure 5D), *OsSAMDC5* (Figure 5E), and *OsSAMDC6* (Figure 5F) under stress at -10°C and -4°C**.** Overexpression of the *AtCIPK19* gene did not significantly increase expression of decarboxylated Sadenosylmethionine (dsSAM) biosynthesis enzymes genes *OsSAMDC1* (Figure 5A), *OsSAMDC2* (Figure 5B), *OsSAMDC3* (Figure 5C), *OsSAMDC4* (Figure 5D), *OsSAMDC5* (Figure 5E), and *OsSAMDC6* (Figure 5F) under stress at 10°C and 24°C**.**

Figure 5: Expression of genes associated with decarboxylated Sadenosylmethionine biosynthesis in transgenic rice cells.

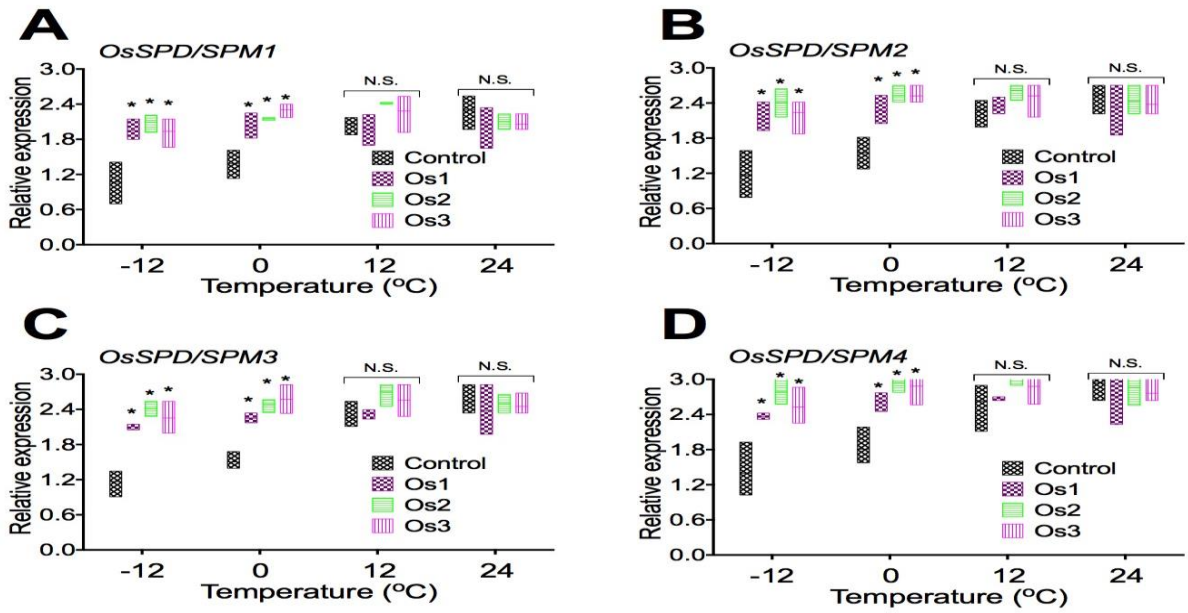


Overexpression of the *AtCIPK19* gene increases expression of decarboxylated Sadenosylmethionine (dsSAM) biosynthesis enzymes genes *OsSAMDC1* (A), *OsSAMDC2* (B), *OsSAMDC3* (C), *OsSAMDC4* (D), *OsSAMDC5* (E), and *OsSAMDC6* (F)**.** The statistically significant difference between groups was determined by one-way ANOVA. Data are presented as means of three independent experiments. Error bars represent standard error. The asterisk indicates significant differences compared to the control, as assessed by a *t*-test. *P<0.05, significant relative to control. N.S., no statistical significance.

Expression of genes associated with spermidine and spermine biosynthesis in transgenic rice cells.

To examine if overexpression of the *AtCIPK19* gene-increased contents of Put, Spd, and Spm was related to the expression of genes associated with spermidine and spermine biosynthesis, expression of *OsSPD/SPM1* (Figure 6A), *OsSPD/SPM2* (Figure 6B), *OsSPD/SPM3* (Figure 6C), and *OsSPD/SPM4* (Figure 6D) were analyzed in transgenic cell lines (Os1, Os2, and Os3) of *O. sativa*. Overexpression of the *AtCIPK19* gene significantly increased expression of spermidine and spermine biosynthesis enzymes genes *OsSPD/SPM1* (Figure 6A), *OsSPD/SPM2* (Figure 6B), *OsSPD/SPM3* (Figure 6C), and *OsSPD/SPM4* (Figure 6D) under stress at -10ºC and -4ºC**.** Overexpression of the *AtCIPK19* gene did not significantly increase expression of spermidine and spermine biosynthesis enzymes genes *OsSPD/SPM1* (Figure 6A), *OsSPD/SPM2* (Figure 6B), *OsSPD/SPM3* (Figure 6C), and *OsSPD/SPM4* (Figure 6D) under stress at 10ºC and 24ºC**.**

Figure 6: Expression of genes associated with spermidine and spermine biosynthesis in transgenic rice cells.

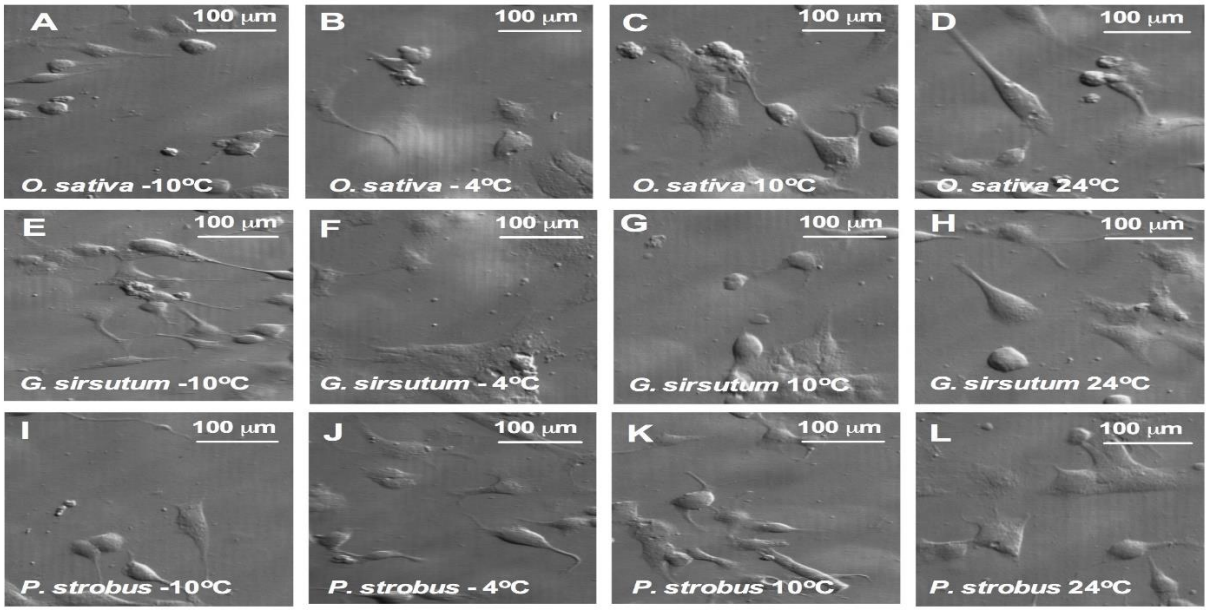


Overexpression of the *AtCIPK19* gene increases expression of spermidine (Spd), and spermine (Spm) biosynthesis enzymes genes *OsSPD/SPM1* (A), *OsSPD/SPM2* (B), *OsSPD/SPM3* (C), and *OsSPD/SPM4* (D). The statistically significant difference between groups was determined by one-way ANOVA. Data are presented as means of three independent experiments. Error bars represent standard error. The asterisk indicates significant differences compared to the control, as assessed by a *t*-test. *P<0.05, significant relative to control. N.S., no statistical significance.

Morphological changes of transgenic cell lines.

To examine if overexpression of the *AtCIPK19* gene-increased contents of Put, Spd, and Spm caused morphological changes of transgenic cell lines, cell morphology was analyzed in transgenic cells in *O. sativa* (Figure 7A-D), *G. hirsutum* (Figure 6 7E-H), and *P. strobus* (Figure 7I-L), respectively, under treatment of -10ºC -4ºC, 10ºC, and 24ºC. Cold stress causes cell morphological change 3 days after treatment of different temperature at -10ºC -4ºC, 10ºC, and 24ºC.

Figure 7: Morphological changes of transgenic cell lines.



(a-d) Cell morphology of transgenic cells in *O. sativa* (A, B, C, and D), *G. hirsutum* (E, F, G, and H), and *P. strobus* (I, J, K, and L), respectively, under treatment of -10°C -4°C, 10°C, and 24°C. Cell images were taken 3 days after treatment of different temperature at -10°C -4°C, 10°C, and 24°C.

Discussion

Cold stress, which causes dehydration damage to the plant cell, is one of the most common abiotic stresses that adversely affect plant growth and crop productivity. Cold stress response is mediated by multiple signaling pathways and is regulated by many factors, among which CIPKs and polyamines may play a role. In Scots pine (*Pinus sylvestris* L.), temperature was a more effective signal than day-length for dehardening [36]. Phosphorylation-mediated signaling transduction plays a crucial role in the regulation of plant defense mechanisms against cold stress. In tomato, about 5500 phosphoproteins were identified to be involved in cold tolerant signaling [37]. In *Carpobrotus edulis*, cold stress reduced melatonin contents and increased salicylic acid contents [38]. *GhHyPRP4* gene was isolated from cotton genome. Overexpression of *GhHyPRP4* in yeast (*Schizosaccharomyces pombe*) significantly enhanced the cell survival rate upon treatment under -20 degrees C for 60 h, indicating that *GhHyPRP4* may be involved in plant response to cold stress [39]. Expression of dehydrins is upregulated under cold stress, suggesting their involvement in membrane stabilization [40]. In *Brassica napus*, *BnCOR25* was expressed at high levels in hypocotyls, cotyledons, stems, and flowers, and *BnCOR25* transcripts were significantly induced by cold stress treatment [41].

The signal transduction of the plant hormone abscisic acid (ABA) has been studied extensively. ABA plays an important role in improving plant tolerance to cold via integrating sugars and reactive oxygen species signaling pathways [42-43]. Plants counteract cold stress through signal transduction and molecular genetic regulation. In our study, genetic and biochemical assays were performed to explore the effect of *AtCIPK19* in response

to cold stress in different plant species. Overexpression of *AtCIPK19* increased the cell viability, the cell growth rate, and decreases the TBARS in cells of *O. sativa*, *G. hirsutum*, and *P. strobus* after treatment at -10, and 4°C, respectively. These results demonstrated that overexpression of *AtCIPK19* enhanced cold stress tolerance could be achieved in different plant species [27,49,50].

Polyamines (PAs) are vital signals in modulating plant response to abiotic stress. Polyamines have been globally associated to plant responses to abiotic stress. Particularly, putrescine has been related to a better response to cold and dehydration stresses [51,52]. It is known that this polyamine is involved in cold tolerance. Cold temperature inhibits stomatal opening and causes stomatal closure. Cold-acclimated plants often exhibit marked changes in their lipid composition, particularly of the membranes. Cold stress often leads to the accumulation of ABA, glycine betaine, polyamines, and proline [51]. In *Leymus chinensis*, the S-adenosylmethionine decarboxylase gene, *LcSAMDC1*, was up-regulated by cold stress. Overexpression of *LcSAMDC1* in transgenic Arabidopsis promoted increased tolerance to cold stress, indicating that *LcSAMDC1* could be used to improve the abiotic resistance of crops [52]. Studies on the relationship between NO and PAs in response to cold stress in tomato showed that NO induced by Spd plays a crucial role in tomato's response to chilling stress [53]. In Arabidopsis, the level of putrescine increased substantially under cold stress [54]. In *Arabidopsis thaliana*, the increment in putrescine upon cold treatment correlated with the induction of known stress-responsive genes, and putrescine may be directly or indirectly involved in ABA metabolism and gene expression [55]. The levels of endogenous polyamines have been shown to increase in plant cells challenged with low temperature. The accumulation of putrescine under cold stress is essential for proper cold acclimation and survival at freezing temperatures [56-58]. Cold stress has been the subject of intense investigation to unravel the complex mechanisms responsible for cold tolerance. In this study, we foud that *AtCIPK19* increases cold stress tolerance by regulating expression of *OsADC1*, *OsADC2*, *OsADC3*, *OsODC1*, *OsODC2*, *OsODC3*, *OsCPA1*, *OsCPA2*, *OsCPA3*, *OsAIH*, *OsSAMDC1*, *OsSAMDC2*, *OsSAMDC3*, *OsSAMDC4*, *OsSAMDC5*, *OsSAMDC6*, *OsSPD/SPM1*, *OsSPD/SPM2*, *OsSPD/SPM3*, and *OsSPD/SPM4*.

Calcineurin B-like proteins interacting protein kinases (CIPKs) play important roles in diverse plant stress responses. In *Arabidopsis thaliana*, *CIPK21* ubiquitously expressed in tissues and up-regulated under multiple abiotic stress conditions. *CIPK21* mediated responses to cold stress is associated with ion and water homeostasis [49]. In maize, *ZmCIPK21* was primarily localized in the cytoplasm and the nucleus of cells and displayed enhanced expression under stress. Over-expression of *ZmCIPK21* in wild-type Arabidopsis plants increased their tolerance to salt stress [59]. In Arabidopsis, overexpressing of CIPK9 resulted in a low-K(+)-sensitive phenotype [60]. In wheat, *TaCIPK14* was upregulated under cold conditions. Transgenic tobaccos overexpressing *TaCIPK14* exhibited higher contents of chlorophyll and sugar under cold stress [61]. In rice (*Oryza sativa*), *OsCIPK14/15* play a crucial role in the microbe-associated molecular functions of stress tolerance [62]. Three CIPK genes (*OsCIPK03*, *OsCIPK12*, and *OsCIPK15*) were overexpressed in japonica rice and transgenic plants overexpressing the transgenes *OsCIPK03*, *OsCIPK12*, and *OsCIPK15* showed significantly improved tolerance to cold stress [63]. In this investigation, our results demonstrated that *AtCIPK19* increased cold stress tolerance by regulating expression of genes associated with putrescine biosynthesis including *OsADC1*, *OsADC2*, *OsADC3*,

OsODC1, *OsODC2*, *OsODC3*, *OsCPA1*, *OsCPA2*, *OsCPA3*, *OsAIH*.

Conclusion

In this investigation, we identified a mechanism of *AtCIPK19* enhanced cold stress tolerance. We found that overexpression of *AtCIPK19* increased cell viability and growth rate under cold stress. *AtCIPK19* increased cold stress tolerance by regulating expression of genes associated with putrescine biosynthesis including *OsADC1*, *OsADC2*, *OsADC3*, *OsODC1*, *OsODC2*, *OsODC3*, *OsCPA1*, *OsCPA2*, *OsCPA3*, *OsAIH*. In AtCIPK19 transgenic rice cell lines, the transcript levels of *OsSAMDC1*, *OsSAMDC2*, *OsSAMDC3*, *OsSAMDC4*, *OsSAMDC5*, *OsSAMDC6*, *OsSPD/SPM1*, *OsSPD/SPM2*, *OsSPD/SPM3*, and *OsSPD/SPM4* were increased, indicating that *AtCIPK19* enhances cold stress tolerance by regulating expression of these genes in plant cells. These results will increase our understanding of *AtCIPK19*-related cold stress tolerance in different plant species including monocotyledonous, dicotyledonous, and gymnosperm plants.

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Author contributions

WT conceived and designed the experiments. WT wrote the paper. WT performed the experiment and analyzed the data. All authors read and approved the final manuscript.

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