ORIGINAL ARTICLE

Role of the Arabidopsis calcineurin B-like protein-interacting protein kinase CIPK21 in plant cold stress tolerance

Wei Tang[1] · Wells A. Thompson[2]

Abstract

The Arabidopsis calcineurin B-like protein-interacting protein kinase CIPK21 gene (*AtCIPK21*) plays important roles in cell metabolism, plant development, and abiotic stress responses. However, the function of the *AtCIPK21* gene in cold stress tolerance in plant cells is not fully understood. In this study, we use cell cultures of three plant species including rice (*Oryza sativa* L.), cotton (*Gossypium hirsutum* L.), and white pine (*Pinus strobus* L.), as well as *Agrobacterium tumefaciens* strain GV3101 harboring pBI-*AtCIPK21* to generate transgenic cell lines. After confirmation of integration of the *AtCIPK21* gene into the genome by polymerase chain reaction (PCR), southern blotting, and northern blotting analyses, the rice, cotton, and pine *AtCIPK21* transgenic cell lines were used to examine cold stress tolerance. The experimental results demonstrated that overexpression of the *AtCIPK21* gene enhanced cold stress tolerance of transgenic cells by increasing cell viability and cell growth rate, decreasing lipid peroxidation and ion leakage, increasing the content of polyamines, as well as elevating the activity of antioxidative enzymes. In rice cells, *AtCIPK21* increases expression of Ca^{2+}-dependent protein kinase (CPK) genes and mitogen-activated protein kinase (MAPK) genes under cold stress. These results indicated that overexpression of the *AtCIPK21* gene in plant cells improved cold stress tolerance by elevating polyamines content, antioxidative enzyme activity, and expression of CPK genes and MAPK genes. Overexpression of the *AtCIPK21* gene may be a valuable approach for engineering plant cold stress tolerance.

Keywords *Agrobacterium*-mediated transformation · *AtCIPK21* gene · Cold stress · *Pinus* · Transgenic cell cultures

Introduction

Abiotic stresses such as low temperature, high NaCl, drought, and heavy metal exposure affect plant growth, development, and productivity (Kovacs et al. 2010; Zhang et al. 2010). Cold stress is one of these factors. Plants protect themselves from cold stress through different signal transduction pathways and molecular genetic regulation (Cui et al. 2005; Dekanski et al. 2009; Yabuta et al. 2002; Zur et al. 2009). To respond to cold stress, cells regulate their biological activities at transcriptomic and proteomic levels and genes related to ROS, transcription factors, hormones, and carbohydrate metabolisms play important roles

in keeping cell membrane integrity (Chan and Shi 2015). Calcium-related signaling play an important role in abiotic stress tolerance of diverse plants. Calcineurin B-like protein-interacting protein kinases (CIPKs) are associated with calcium-signaling processes of plant stress responses. Plant calcineurin B-like protein-type Ca^{2+} sensors interact with and activate CIPKs that phosphorylate downstream components to transduce Ca^{2+} signals (Kurusu et al. 2010; Pandey et al. 2007; Yu et al. 2011). Multiple CIPKs have been identified in different plant species (Chen et al. 2014; Deng et al. 2013a, b; Deng et al. 2013a, b; Kimura et al. 2013; Liu et al. 2013; Piao et al. 2010; Ruan et al. 2013; Zhou et al. 2014). CIPKs may be involved in particular types of signal that are related to stimulus–response process (Gonzalez Bosc et al. 2016; Pandey et al. 2015; Rodriguez-Medina et al. 2015). Several CIPK genes have been reported to function in plant responses to drought, salinity, and osmotic stress in Arabidopsis (*Arabidopsis thaliana*) (Kimura et al. 2013; Liu et al. 2013; Pandey et al. 2007), rice (*Oryza sativa*) (Piao et al. 2010), pea (*Pisum sativum*), and maize (*Zea mays*) (Chen

✉ Wei Tang
   wt10yu604@gmail.com

1 College of Horticulture and Gardening, Yangtze University, Jingzhou 434025, Hubei, China

2 CIMAS, 101 Science Drive, Duke University, Durham, NC 27708, USA

et al. 2014). The Arabidopsis calcineurin B-like protein-interacting protein kinase CIPK21 gene (*AtCIPK21*) has been reported to play important roles in cell metabolism and abiotic stress responses (Pandey et al. 2015).

Calcineurin B-like protein-interacting protein kinases (CIPKs) are featured with a group of typical Ser/Thr functional regions that mediate calcium signals (Chen et al. 2014; Pandey et al. 2015). Abiotic stresses including salt, cold, and drought stimulate CIPK21 expression and enhanced expression of CIPK21 contributes to stress tolerance in plants (Beier et al. 2018; Pandey et al. 2007). Among different members of CIPKs, CIPK1 and CIPK19 overexpression result in Ca^{2+} overaccumulation and affect the pollen tube growth in Arabidopsis. It has been reported that many calcium signatures activate CIPKs in Arabidopsis plants to protect plants from damage caused by abiotic stresses. In rice, shorter exposure to abiotic stress enhances OsCIPK31 expression and longer exposure to stresses reduces OsCIPK31 expression (Dirks-Hofmeister et al. 2013).

In this report, we examined the function of the *AtCIPK21* gene in cold stress response in rice (*Oryza sativa* L.), cotton (*Gossypium hirsutum* L.), and white pine (*Pinus strobus* L.) by examining the activity of antioxidative enzymes and the content of polyamines. In rice cells, the expression of Ca^{2+}-dependent protein kinase (CPK) genes and mitogen-activated protein kinase (MAPK) genes was evaluated in *AtCIPK21* transgenic cells under treatment of cold. Our findings suggest that *AtCIPK21* mediates responses to cold stress condition in *O. sativa*, *G. hirsutum*, and *P. strobus*, at least in part, by regulating multiple cell signaling pathways across several plant species. Overexpression of the *AtCIPK21* gene may be a valuable approach for engineering plant cold stress tolerance.

Materials and methods

Plasmid constructs

The full-length *AtCIPK21* coding sequence (1251 bp) was cloned into expression vector pBI121 according to the method previously described (Guan and Nikolau 2016). The vector of pBI121 and the cDNA of *AtCIPK21* were digested by restriction enzymes *Kpn*I and *Bam*HI (Promega, Madison, WI, USA) at 37 °C. The digested DNA was purified using QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA) and ligated to generate the expression vector (Tang and Newton 2005). The resulting expression vector was designated as pBI-*AtCIPK21* and the vector pBI-*AtCIPK21* was introduced into *Agrobacterium tumefaciens* strain LBA4404 using electroporation.

Transformation of suspension cells

AtCIPK21 transgenic cell lines of rice (*Oryza sativa* L.), cotton (*Gossypium hirsutum* L.), white pine (*Pinus strobus* L.), and Arabidopsis (*Arabidopsis thaliana*) were generated as described before (Tang and Page 2013), using *Agrobacterium tumefaciens* strain LBA4404 carrying pBI-*AtCIPK21* and cultured cells of *O. sativa*, *G. hirsutum*, and *P. strobus* (Tang, et al. 2007; Tang and Page 2013). After transformation, cell cultures of *O. sativa*, *G. hirsutum*, and *P. strobus* were grown for 7 weeks and then these cell cultures were used for further analysis.

Polymerase chain reaction analyses of transgenic cells

Polymerase chain reaction (PCR) analysis of transgenic cells was conducted as previously described (Tang, et al. 2007; Tang and Page 2013). Seven grams 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 neomycin phosphotransferase II gene (*NPTII*) are forward primer (nfp: 5′-GTCGACATG GCGGAGGAATTTGGAAGCATAG-3′) and the reverse primer (nrp: 5′-CCATGGTAGACTCCTGCTTCGACA TCATGG-3′). The primers used to amplify the transcription factor *AtCIPK21* DNA fragments are forward primer (cf: 5′-CTTTCAATGG CGGATTTGTT AAGAAAAGTG-3′) and the reverse primer (cr:5′-CTTCATTTTT CATCTT CCTA ATCAGTATCA GAAAG-3′). The PCR mixture, the PCR conditions, and gel electrophoresis were carried out as described previously (Tang and Page 2013). PCR was done in a PTC-100TM machine (MJ Research, San Francisco, CA, USA). A total of 285 ng of genomic DNA of rice, cotton, and pine was used as a template, respectively. Cell cultures of *O. sativa* (Os1, Os2, and Os3), *G. hirsutum* (Gh1, Gh2, and Gh3), and *P. strobus* (Ps1, Ps2, and Ps3) were used for further analysis.

Southern blot analysis of transgenic cells

Southern blotting analysis of *O. sativa*, *G. hirsutum*, and *P. strobus* transgenic cells was conducted as previously described (Tang et al. 2007; Tang and Page 2013). One gram of control cells and transgenic cells of *O. sativa* (Os1, Os2, and Os3), *G. hirsutum* (Gh1, Gh2, and Gh3), and *P. strobus* (Ps1, Ps2, and Ps3) was used to isolate genomic DNA, using a Genomic DNA Isolation Kit (Sigma). Twenty-eight micrograms of DNA were digested within 16 h with the enzyme *Xba* I (Boehringer Mannheim) at 37 °C. The molecular probes (1251 pb fragment of *AtCIPK21*) were

labeled using Digoxigenin (DIG) (Roche Diagnostics, Indianapolis, IN, USA).

RNA isolation and Northern blot analysis

Six grams of transgenic and control cells of rice, cotton, and pine were used to extract total RNA, using a RNeasy Mini Plant Kit (Germantown, MD, USA) by following the manual. Nine micrograms of total RNA was used for northern blotting which was performed as described before (Tang et al. 2007). The digoxigenin (DIG) labeling *AtCIPK21* DNA (1251 pb) (Roche Diagnostics) was used as the hybridization probe. The rRNA was used as the loading control of RNA samples.

Cold treatment

Cold treatment was carried out by incubation of *AtCIPK21* transgenic cells and control cells of Os1, Os2, and Os3 derived from *O. sativa*, Gh1, Gh2, and Gh3 were derived from *G. hirsutum*, and Ps1, Ps2, and Ps3 were derived from *P. strobus* at − 12, 0, 12, and 24 °C in the dark for 24 h in growth chambers. After 24 h of cold stress treatment, cells were moved to the normal growth environment. Cell images of *O. sativa*, *G. hirsutum*, and *P. strobus* were taken using an inverted microscope. Cell size was measured using ImageJ.

Determination of cell viability and growth rate

After the *AtCIPK21* transgenic cells and control cells of rice, cotton, and pine were treated by cold, cells were moved to the normal growth environment. The cell growth rate and the cell viability of *O. sativa* (Os1, Os2, and Os3), *G. hirsutum* (Gh1, Gh2, and Gh3), and *P. strobus* (Ps1, Ps2, and Ps3) were examined, as previously described (Tang and Page 2013). The cell growth rate was expressed as mg/g FW/day. The rate of cell growth was measured 7 days after treatment. Samples from both cold treated and control groups were collected with three biological replicates.

Measurement of thiobarbituric acid reactive substances

The amount of thiobarbituric acid reactive substances (TBARS) was determined using thiobarbituric acid (TBA) reaction as described previously (Tang and Page 2013; Tang, et al. 2005). Three grams of transgenic cells from each of *O. sativa* (Os1, Os2, and Os3), *G. hirsutum* (Gh1, Gh2, and Gh3), and *P. strobus* (Ps1, Ps2, and Ps3) and control cells were used for sampling. The sample mixture was heated at 95 °C for 30 min and centrifuged at 10,000×*g* for 15 min before the absorbance was measured at 532 nm. The content

of TBARS was calculated using the method previously described (Tang and Page 2013; Tang et al. 2005).

Measurement of ion leakage

The ion leakage was measured using transgenic and control cells of *O. sativa* (Os1, Os2, and Os3), *G. hirsutum* (Gh1, Gh2, and Gh3), and *P. strobus* (Ps1, Ps2, and Ps3), as previously described. Afterwards, the samples of rice, cotton, and pine were incubated in 1000 µl distilled water for 1 h respectively. The conductivity of transgenic and control cells was examined using a B-173 conductivity meter (Horiba, Kyoto, Japan).

Determination of APOX, CAT, PPO, POD activity and ROS level

The activities of ascorbate peroxidase (APOX), catalase (CAT), polyphenol oxidase (PPO), and peroxidase (POD) were determined as described previously (Tang, et al. 2007; Tang and Page 2013). Two grams of control and transgenic cells of *O. sativa* (Os1, Os2, and Os3), *G. hirsutum* (Gh1, Gh2, and Gh3), and *P. strobus* (Ps1, Ps2, and Ps3) were homogenized in 3 ml of extraction buffer. Extraction buffer consists of 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP, and 0.5% (v/v) Triton X-100 at 4 °C. The extracts were centrifuged at 10,000×*g* for 20 min and the supernatant was used to determine the enzyme activity, as described previously (Tang et al. 2007; Tang and Page 2013). The activity of APOX was determined using ascorbate as substrate and was expressed as mmol ascorbate oxidized mg protein-1 min-1. The activity of POD was determined using 4-methylcatechol as substrate. The increase in the absorption caused by oxidation of 4-methylcatechol by H_2O_2 was measured at 420 nm spectrophotometrically. The activity of CAT was measured spectrophotometrically at room temperature by monitoring the decrease in absorbance at 240 nm resulting from the decomposition of H_2O_2. The activity assay of PPO was carried out by measuring the increase in absorbance at 420 nm for 4-methylcatechol spectrophotometrically. Determination of reactive oxygen species (ROS) production was performed as previously described (Smith and Heese 2014).

Determination of polyamines

The concentration of putrescine (Put), spermidine (Spd), and spermine (Spm) in the control and transgenic cells of *O. sativa* (Os1, Os2, and Os3), *G. hirsutum* (Gh1, Gh2, and Gh3), and *P. strobus* (Ps1, Ps2, and Ps3) was determined as described previously (Tang and Newton 2005). The control samples and transgenic cells were characterized using a HPLC instrument and the Spector Monitor 3200 Detector by

following the instructions. The measured polyamines (PAs) are total PAs.

Expression of Ca²⁺-dependent protein kinase genes

Expression of CPK genes in the control and the transgenic cells of *O. sativa* (Os1, Os2, and Os3), *G. hirsutum* (Gh1, Gh2, and Gh3), and *P. strobus* (Ps1, Ps2, and Ps3) was determined using qPCR as previously described (Wan et al. 2007). Total RNA was extracted from frozen sample cells using TRIzol reagent by following the description in the manufacturer's protocol (Invitrogen). To synthesize the first-strand cDNA in a 50-mL reaction containing 2.5 mM oligo(dT) primers, 2.5 mM random hexamer, and 2.5 μg of total RNA, the PrimeScript™ RT reagent kit (TaKaRa Co., Ltd, Ohtsu, Japan) was used by following the instructions.

Samples were analyzed in triplicate on the Applied Biosystems 7900HT System by following the description in the manufacturer's manual. The primers for qPCR are listed in Table 1. The *U6* gene was used as an internal control. Gene expression was measured after transgenic cells of *O. sativa* (Os1, Os2, and Os3), *G. hirsutum* (Gh1, Gh2, and Gh3), and *P. strobus* (Ps1, Ps2, and Ps3) were treated at − 12, 0, 12, and 24 °C. The data were normalized to the internal control. The delta–delta Ct method was used to obtain the expression value. The U6 gene was used as internal reference.

Expression of mitogen-activated protein kinase genes, ICE genes, and CBF genes

Expression of mitogen-activated protein kinase (MAPK) genes in the control and the transgenic cells of *O. sativa*

Table 1 Primers used in this study

Gene	Forward primer	Reverse primer
OsCPK1	5′-CACTCGGTCAACCTCATC-3′	5′-GAAGTCAGTGGCCTTGAG-3′
OsCPK2	5′-GGAGCATCTACACCATCG-3′	5′-GGACTGCTTGTCCTCGTA-3′
OsCPK3	5′-CGTCAAGTCGTCTCACTTCC-3′	5′-GATCCATGCACAGGTACGTC-3′
OsCPK4	5′-AAACAACCTCCCTCCTCCTC-3′	5′-CTCTCGTACCCGAACTCCAC-3′
OsCPK5	5′-AAGCTCATCACCAAGGAGGA-3′	5′-GGGCTTCCAACAACATCAGT-3′
OsCPK6	5′-ATGGGCAACTACTACTCGTG-3′	5′-ACGTACAGGTTGTCCTCGT-3′
OsCPK7	5′-GGGTACGTTCAGGGGGATAG-3′	5′-GGCGCACATCTTCTACATCC-3′
OsCPK8	5′-AGCAGAAGAAGGCGAACC-3′	5′-GCTGACGATGTTCGGATG-3′
OsCPK9	5′-AAGTCGACACCGACAAGGAT-3′	5′-TCTCAAGCCTGAATCGACTG-3′
OsCPK10	5′-CCTGCAAGTCCATCCTGAA-3′	5′-AGAGGAAGTTCTCGGGCTT-3′
OsCPK11	5′-ATCCAGATCATGCACCAC-3′	5′-GCTTGAAGAAGATGGAGA-3′
OsCPK12	5′-CTCGTCATGGAGTTCTGC-3′	5′-AAGACGGAGAGTCCGAAG-3′
OsCPK13	5′-AAGCTCATCACCAAGGAG-3′	5′-GCAGGAAGTTTTCTGGCTTG-3′
OsCPK14	5′-ATCTACACCGTGGGCAAG-3′	5′-TTGCTGAGGAGGAGGAAG-3′
OsCPK15	5′-GAGGTCCACATCATGCAC-3′	5′-CTTGAAGAAGACGGAGAGG-3′
OsMAPK1	5′-GATGGCGGAG GCCGGCGGC-3'	5′-TGGCAGTGCTCCTCTGACAATG-3′
OsMAPK2	5′-CATGGCCATCACGGTGGCATCG-3′	5′-AATGTAGACATCATTGAAGT-3′
OsMAPK3	5′-GGAGCTTATGGGATTGTTTG T-3′	5′-TTTCAGGTCTCTGTGGAGTATC-3′
OsMAPK4	5′-GGGGCTTATGGAATAGTTTG C-3′	5′-CCTATACCACCGGGTCACGA-3′
OsMAPK5	5′-ACGGCGGCCGGTACCTGCTC-3′	5′-AGTGGATGTACTTGAGCCCC-3′
OsMAPK6	5′-CCTCCTCCTCCTACTCCCATCA-3′	5′-TCTCCGAGTTAACAGCCGCGC-3′
OsMAPK7	5′-GCTTCGTTTTGTTTCTCTGG-3′	5′-CCCGGCTCCCGCTGTGGTGAAC-3′
OsMAPK8	5′-CTCAAACTTCTGCAGAGGCT-3′	5′-AGGGCAAGCCCCTCGTCCTG-3′
OsMAPK9	5′-GGAAGAGAGAGAGCAACCTTTC-3′	5′-GATACCCTGAACTCATCACTG-3′
OsICE1	5′-GTGCCCAAGATCAGCAAGA-3′	5′-CTCGAGCTCATTCTGAAGATCA-3′
OsCBF1	5′-CCGGCGGGGAGGACCAAGTTCAG -3′	5′-GCCGCCGCCCATCCCGTCGTA-3′
OsCBF2	5′-CGGGCGGGGAGGACCAAGTTCA -3′	5′-ACCTCGCAGTCGTAGTCCTCCTC-3′
OsCBF3	5′-GCGGGCGGGGAGGACCAAGTTCA-3′	5′-GCTCATCAGGGCTGGTTCGGTTC-3′
OsCBF8	5′-GCGGGGCGGTGGGTGTG-3′	5′-CGCCGCCGCATCCTTCGTC-3′
OsCBF9	5′-AGCCGTGCCGCCGCCTCTCG-3′	5′-CGGTCGTGCTGCTGCTGCTGGTG-3′
OsCBF10	5′-ATGTGCGGGATCAAGCAGGAGA-3′	5′-ACTCAGGACGTCCAGTTCGAAC-3′
OsCBF11	5′-GGACCAAGTTCAGGGAGACGAG-3′	5′-CTGCGCCAAGCTCGCGTAGTA-3′
OsCBF12	5′-AGAGAGTCATCCATGGAGGTG -3′	5′-GGAGAATCAAAAGGTGTCCACA-3′

(Os1, Os2, and Os3), *G. hirsutum* (Gh1, Gh2, and Gh3), and *P. strobus* (Ps1, Ps2, and Ps3) was examined using qPCR as previously described (Wan, et al. 2007). Total RNA was extracted from frozen sample cells using TRIzol reagent by following the description in the manufacturer's protocol (Invitrogen). To synthesize the first-strand cDNA in a 50-mL reaction containing 2.5 mM oligo(dT) primers, 2.5 mM random hexamer, and 2.5 μg of total RNA, the PrimeScript™ RT reagent kit (TaKaRa Co., Ltd, Ohtsu, Japan) was used by following the instructions. Samples were analyzed in triplicate on the Applied Biosystems 7900HT System by following the description in the manufacturer's manual. The primers for qPCR are listed in Table 1. The *U6* gene was used as an internal control. Gene expression was measured after treatment with 10 μl *Pseudomonas syringae* (OD$_{600}$ = 0.5) per 1 million transgenic cells at different time points (0, 1, 3, 5, and 7 days). The data were normalized to the internal control. The regular qPCR was used. The delta–delta Ct method was used to obtain expression value. The U6 gene was used as internal reference.

Statistical analyses

Statistical analysis of the control and transgenic cells of rice, cotton, and pine was performed using the General Linear Model procedure of SAS (Cary, NC, USA), employing ANOVA models. The significant differences were made at 5% level of probability. Each value was presented as means and standard errors of the mean.

Results

Expression and integration of transgenes

Cold stress is one of the abiotic stresses that negatively affect plant growth, development, and yield. In rice, cold stress affects morphological development, decreases seed production, causes poor germination, and increases seedling injury, and reduces rice yield in the world (Shakiba et al. 2017). In cotton, cold stress impairs plant growth and restricts the productivity and spatial distribution of plants (Cai et al. 2019). In white pine, cold stress changes carbohydrate and compatible solute concentrations, membrane lipid composition, and proteins, causes proteomic and metabolomics changes (Strimbeck et al. 2015). To respond to cold stress, plants have developed capabilities to activate hormone signaling, enhance cold stress-related gene expression, and increase the activity of transcription factors to adapt to this extreme condition. Transcription factors are essential for plant cells to respond to stress signals.

To evaluate the effect of the *AtCIPK21* gene on cold stress tolerance, cell cultures of *O. sativa*, *G. hirsutum*, and
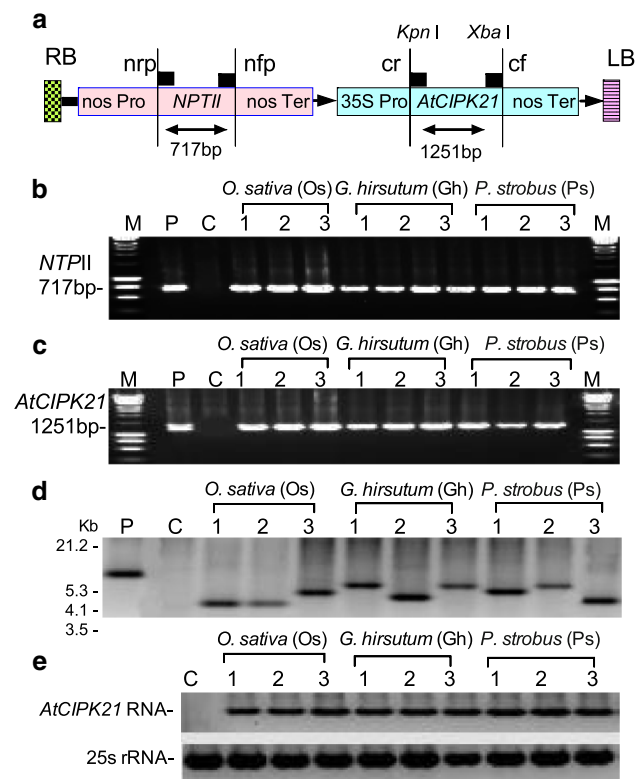

Fig. 1 Expression and integration of transgenes. **a** The T-DNA region that shows the right border (RB), left border (LB), the nos promoter (nos Pro), the *NPTII* gene (*NPTII*), the nos terminator (nos Ter), the 35 S promoter (35S Pro), and the *AtCIPK21* gene (*AtCIPK21*). **b** PCR amplification of the *NPTII* gene (717 bp) in *O. sativa*, *G. hirsutum*, and *P. strobus*. **c** PCR amplification of the *AtCIPK21* gene (1251 bp) in *O. sativa*, *G. hirsutum*, and *P. strobus*. **d** Southern blotting using the *AtCIPK21* fragment as a probe in *O. sativa*, *G. hirsutum*, and *P. strobus*. **e** Northern blotting using the *AtCIPK21* fragment as a probe in *O. sativa*, *G. hirsutum*, and *P. strobus*. The loading control is the tobacco 25S rRNA

P. strobus were transformed using *Agrobacterium tumefaciens* strain GV3101 carrying pBI-*AtCIPK21* (Fig. 1a) to produce *AtCIPK21* overexpression cell lines. One hundred and thirty-one *AtCIPK21* transgenic cell lines have been produced including 42 *O. sativa* lines, 51 *G. hirsutum* lines, and 38 *P. strobus* lines. Integration of transgenes in the genome of *O. sativa*, *G. hirsutum*, and *P. strobus* was confirmed by PCR amplification of the *NPTII* gene (Fig. 1b) and the *AtCIPK21* gene (Fig. 1c) in *O. sativa*, *G. hirsutum*, and *P. strobus*. Integration of transgenes into the genome of rice, cotton, and pine was also confirmed by southern blotting (Fig. 1d) and northern blotting (Fig. 1e) using the *AAE13.1* fragments as a probe in *O. sativa*, *G. hirsutum*, and *P . strobus*. After confirmation of the integration of transgene into the genome of rice, cotton, and pine, rice, cotton, and pine, cell lines Os1, Os2, and Os3 from *O. sativa*, from *G. hirsutum*, and Ps1, Ps2, and Ps3 from *P. strobus* were used for cold stress tolerance experiment.

Overexpression of AtCIPK21 increases the cell viability, growth rate, and decreases TBARS and ion leakage under cold stress

After confirmation of the *AtCIPK21* gene integration into the genome of transgenic cell lines by PCR, southern blotting, and northern blotting analysis in *O. sativa*, *G. hirsutum*, and *P. strobus* (Fig. 1d, e), respectively, cell viability, cell growth rate, TBARS, and ion leakage of transgenic cells under cold stress at − 12 °C, 0 °C, 12 °C, and 24 °C were

determined. The negative control is the transgenic cells that are transformed with the expression vector, which does not have the *AtCIPK21* gene. Overexpression of the *AtCIPK21* gene increases cell viability, cell growth rate, and reduces TBARS and ion leakage in transgenic cells of *O. sativa* (Fig. 2a, d, g, j), *G. hirsutum* (Fig. 2b, e, h, k), and *P. strobus* (Fig. 2c, f, i, l) after treatment with cold at − 12 °C and 0 °C, but does not change them at 12 °C and 24 °C, respectively, compared to the controls. Overexpression of the *AtCIPK21* gene protects cells from cold stress in monocotyledonous

Fig. 2 Overexpression of *AtCIPK21* increases the cell viability, growth rate, and decreases TBARS and ion leakage under cold stress. Overexpression of *AtCIPK21* increases the cell viability, the cell growth rate, and decreases TBARS and ion leakage in *O. sativa* (**a**, **d**, **g**, and **j**), *G. hirsutum* (**b**, **e**, **h**, and **k**), and *P. strobus* (**c**, **f**, **i**, and **l**), respectively. Overexpression of *AtCIPK21* changes phenotype of transgenic cells (**m**). The asterisk shows the significant differences, as assessed by a *t* test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant relative to control. *N.S.* no statistical significance
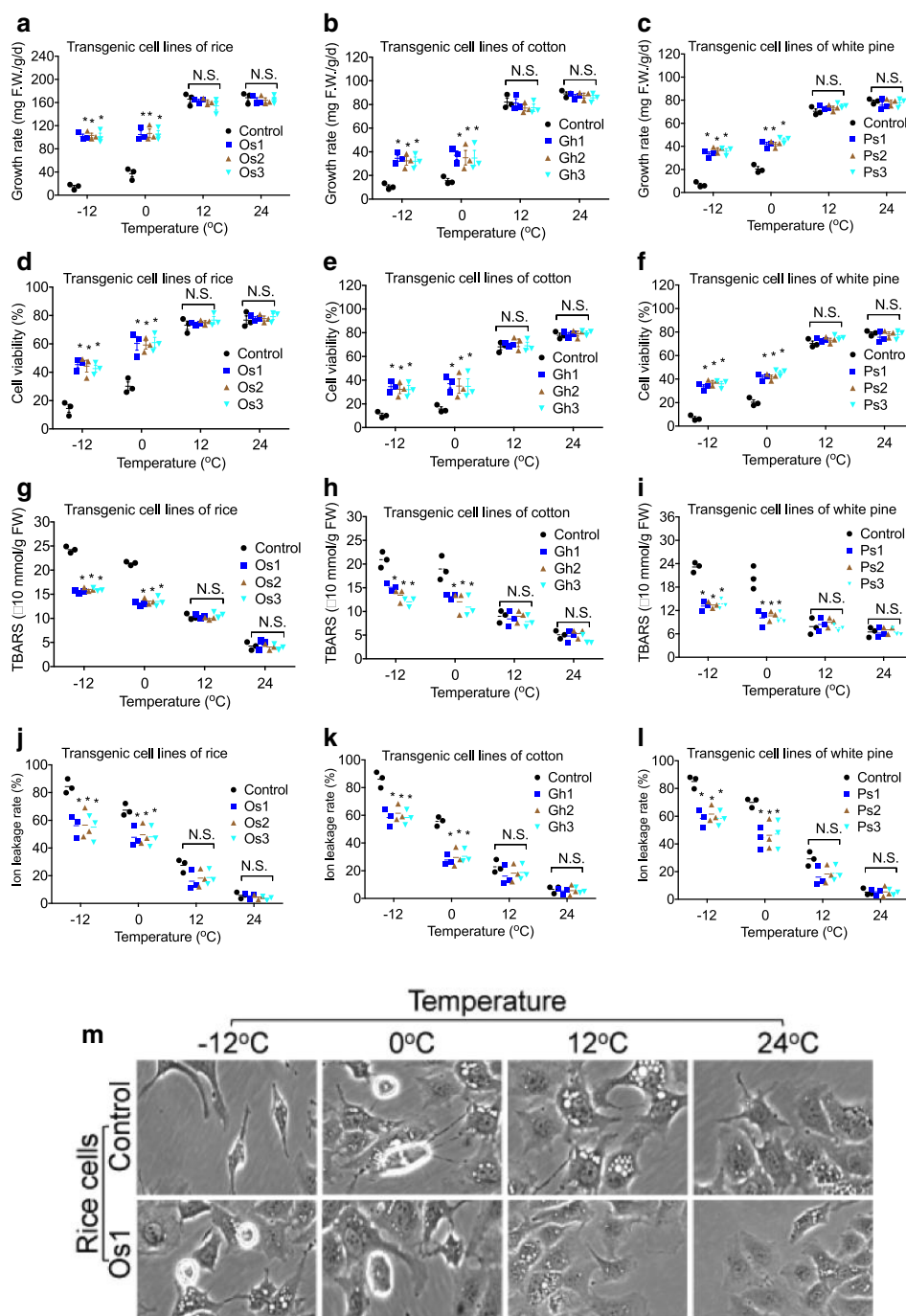
(*O. sativa*), dicotyledonous (*G. hirsutum*), and gymnosperm (*P. strobus*) plant cells by decreasing TBARS and ion leakage. Overexpression of the *AtCIPK21* gene cause phenotype changes (Fig. 2m) including cell death.

The activity of ascorbate peroxidase (APOX), catalase (CAT), polyphenol oxidase (PPO), peroxidase (POD), and ROS level

The activity of APOX, CAT, PPO, and POD was examined in *AtCIPK21* transgenic cell lines of *O. sativa*, *G. hirsutum*, and *P. strobus*, treated with different temperatures

at − 12 °C, 0 °C, 12 °C, and 24 °C, respectively. Overexpression of the *AtCIPK21* gene significantly increases the activity of APOX, CAT, PPO, and POD in transgenic cell lines of *O. sativa* (Fig. 3a, d, g, j), *G. hirsutum* (Fig. 3b, e, h, k), and *P. strobus* (Fig. 3c, f, i, l), treated at − 12 °C and 0 °C, respectively. The activity of APOX, CAT, PPO, and POD in transgenic cell lines of *O. sativa* (Fig. 3a, d, g, j), *G. hirsutum* (Fig. 3b, e, h, k), and *P. strobus* (Fig. 3c, f, i, l) was not significantly changed in the treatment at 12 °C and 24 °C. These results indicated that overexpression of the *AtCIPK21* gene increased cold stress tolerance which may be related to the increased activity of APOX, CAT, PPO, and POD in *O. sativa*, *G. hirsutum*, and *P. strobus*.

Fig. 3 Effect of the *AtCIPK21* gene on the activities of APOX, CAT, PPO, and POD in transgenic cells. Overexpression of the *AtCIPK21* gene increases the activities of APOX, CAT, PPO, and POD in transgenic cells of *O. sativa* (**a**, **d**, **g**, and **j**), *G. hirsutum* (**b**, **e**, **h**, and **k**), and *P. strobus* (**c**, **f**, **i**, and **l**), respectively. Overexpression of the *AtCIPK21* gene decreases the level of ROS in transgenic cells of *O. sativa* (**m**), *G. hirsutum* (**n**), and *P. strobus* (**o**), respectively. The asterisk indicates the significant differences, as assessed by a *t* test. *$P < 0.05$, significant relative to control
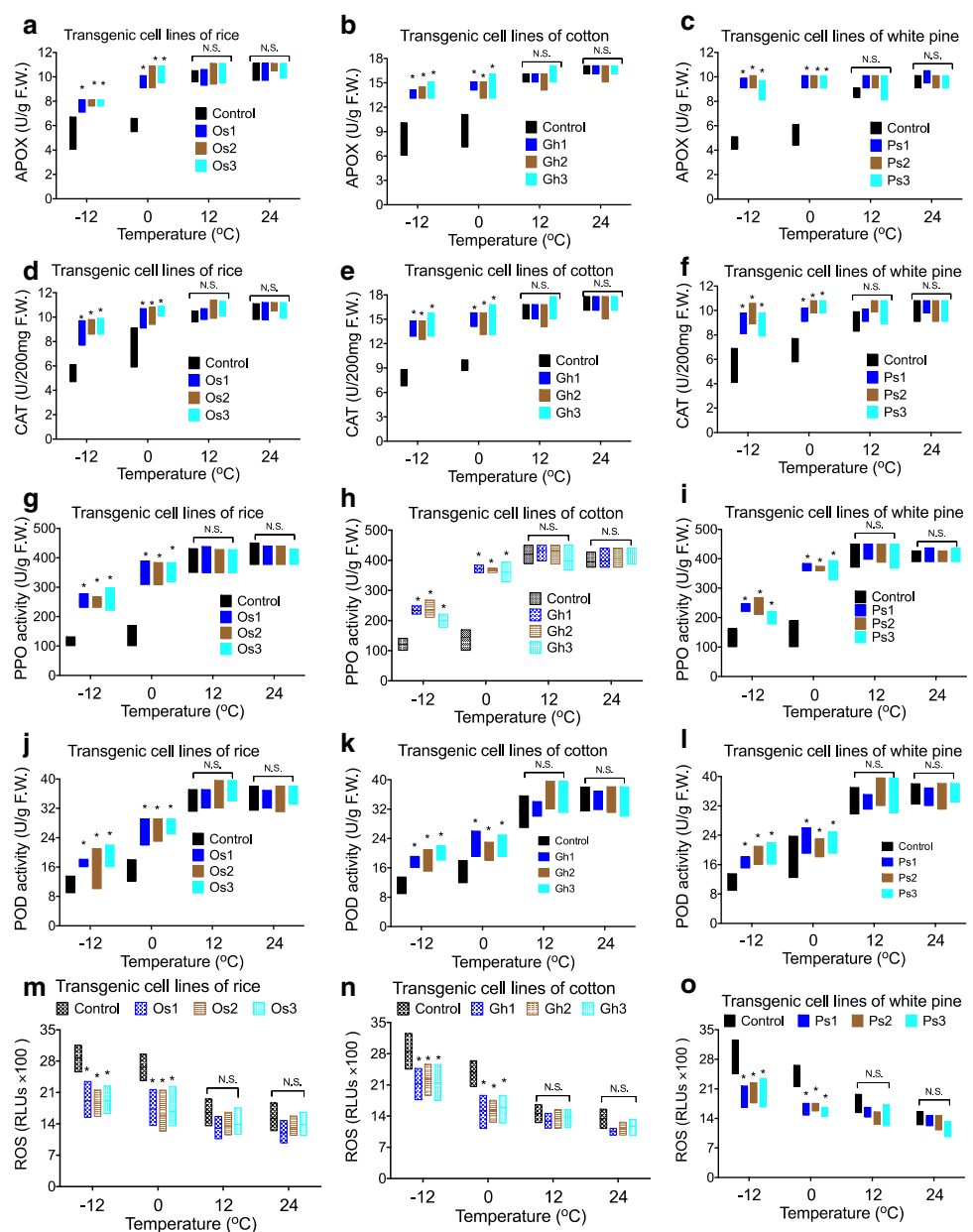
Overexpression of AtCIPK21 elevates the levels of polyamines

To examine if overexpression of the *AtCIPK21* 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 *AtCIPK21* gene significantly increases the levels of Put, Spd, and Spm in transgenic cells of *O. sativa* (Fig. 4a–c), *G. hirsutum* (Fig. 4d–f), and *P. strobus* (Fig. 4g–i), respectively, under stress at − 12 °C and 0 °C, respectively. Overexpression of the *AtCIPK21* gene does not significantly increase the levels of Put, Spd, and Spm in transgenic cells of *O. sativa* (Fig. 4a–c), *G. hirsutum* (Fig. 4d–f), and *P. strobus* (Fig. 4g–i), respectively, under stress at 12 °C and 24 °C.

Expression of CPK genes in AtCIPK21 transgenic rice cell

Ca^{2+}-dependent protein kinase (CPK) genes protect cells from cold stress tolerance. To evaluate whether overexpression of the *AtCIPK21* gene affects the expression of *CPK* genes in transgenic cells, expression of CPK protein kinase genes was examined by qRT-PCR in *O. sativa* transgenic cell lines under cold stress at − 12 °C, 0 °C, 12 °C, and 24 °C, respectively. Among 15 CPK genes examined,

overexpression of the *AtCIPK21* gene significantly increased expression of *OsCPK1* (Fig. 5a), *OsCPK5* (Fig. 5e). *OsCPK6* (Fig. 5f), *OsCPK7* (Fig. 5g), *OsCPK8* (Fig. 5h), *OsCPK9* (Fig. 5i), and *OsCPK11* (Fig. 5k) in transgenic cells treated at − 12 °C and 0 °C, but did not significantly affect expression of *OsCPK2* (Fig. 5b), *OsCPK3* (Fig. 5c), *OsCPK4* (Fig. 5d), *OsCPK10* (Fig. 5j), and *OsCPK12* (Fig. 5l) in *AtCIPK21* transgenic rice cell under treatment at 12 °C and 24 °C, respectively. These results indicated that overexpression of the *AtCIPK21* gene increased cold stress tolerance may be related to expression of *CPK* genes including *OsCPK1* (Fig. 5a), *OsCPK5* (Fig. 5e). *OsCPK6* (Fig. 5f), *OsCPK7* (Fig. 5g), *OsCPK8* (Fig. 5h), *OsCPK9* (Fig. 5i), and *OsCPK11* (Fig. 5k), and may be not related to expression of *CPK* genes including *OsCPK2* (Fig. 5B), *OsCPK3* (Fig. 5c), *OsCPK4* (Fig. 5d), *OsCPK10* (Fig. 5j), and *OsCPK12* (Fig. 5l) in *AtCIPK21* transgenic rice cell.

Expression of mitogen-activated protein kinase genes in transgenic cells

To investigate if the *AtCIPK21* gene has an effect on expression of mitogen-activated protein kinase (MAPK) genes in rice under treatment of low temperature, expression of MAPK genes including *OsMAPK1*, *OsMAPK2*, *OsMAPK3*, *OsMAPK4*, *OsMAPK5*, *OsMAPK6*, *OsMAPK7*, *OsMAPK8*, and *OsMAPK9* was examined in three *O. sativa* transgenic cell lines Os1, Os2, and Os3 under cold stress at − 12 °C,

Fig. 4 *AtCIPK21* elevates 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–c**), *G. hirsutum* (**d–f**), and *P. strobus* (G, H, and I), respectively. The asterisk indicates the significant differences, as assessed by a *t* test. *P < 0.05, significant relative to control. *N.S.* no statistical significance
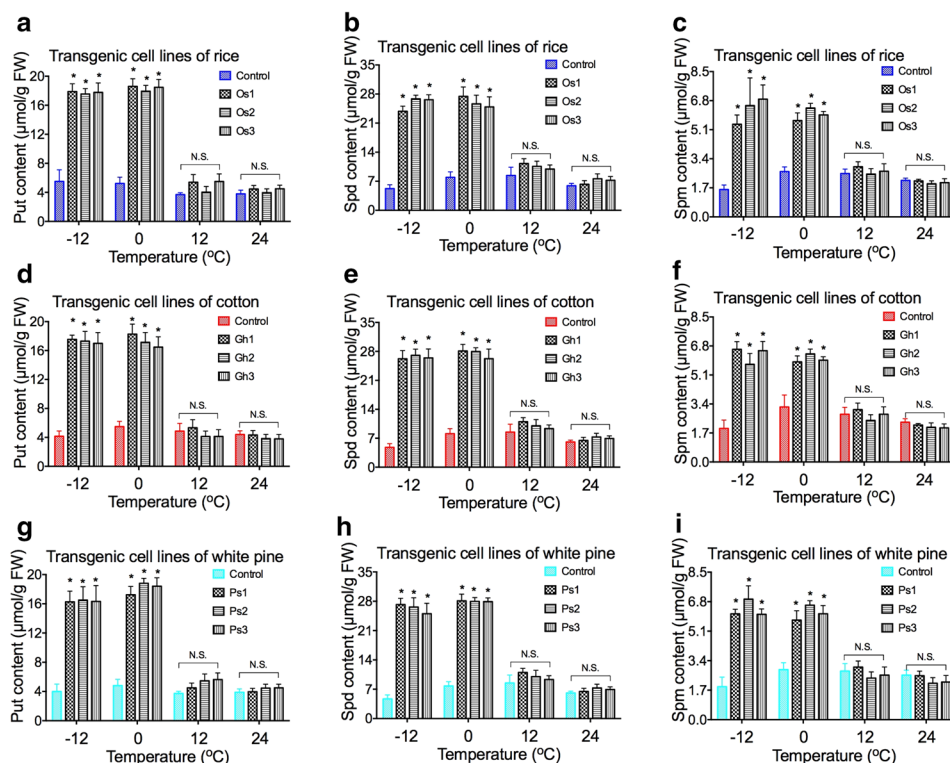
Fig. 5 Expression of CPK genes in *AtCIPK21* transgenic rice cell. Expression of *OsCPK1* (**a**), *OsCPK2* (**b**), *OsCPK3* (**c**), *OsCPK4* (**d**), *OsCPK5* (**e**), *OsCPK6* (**f**), *OsCPK7* (**g**), *OsCPK8* (**h**), *OsCPK9* (**i**), *OsCPK10* (**j**), *OsCPK11* (**k**), *OsCPK12* (**l**), *OsCPK13* (**m**), *OsCPK14* (**n**), and OsCPK15 (**o**) in *AtCIPK21* transgenic rice cell. The asterisk indicates significant differences, as assessed by a *t* test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant relative to control. *N.S.* no statistical significance
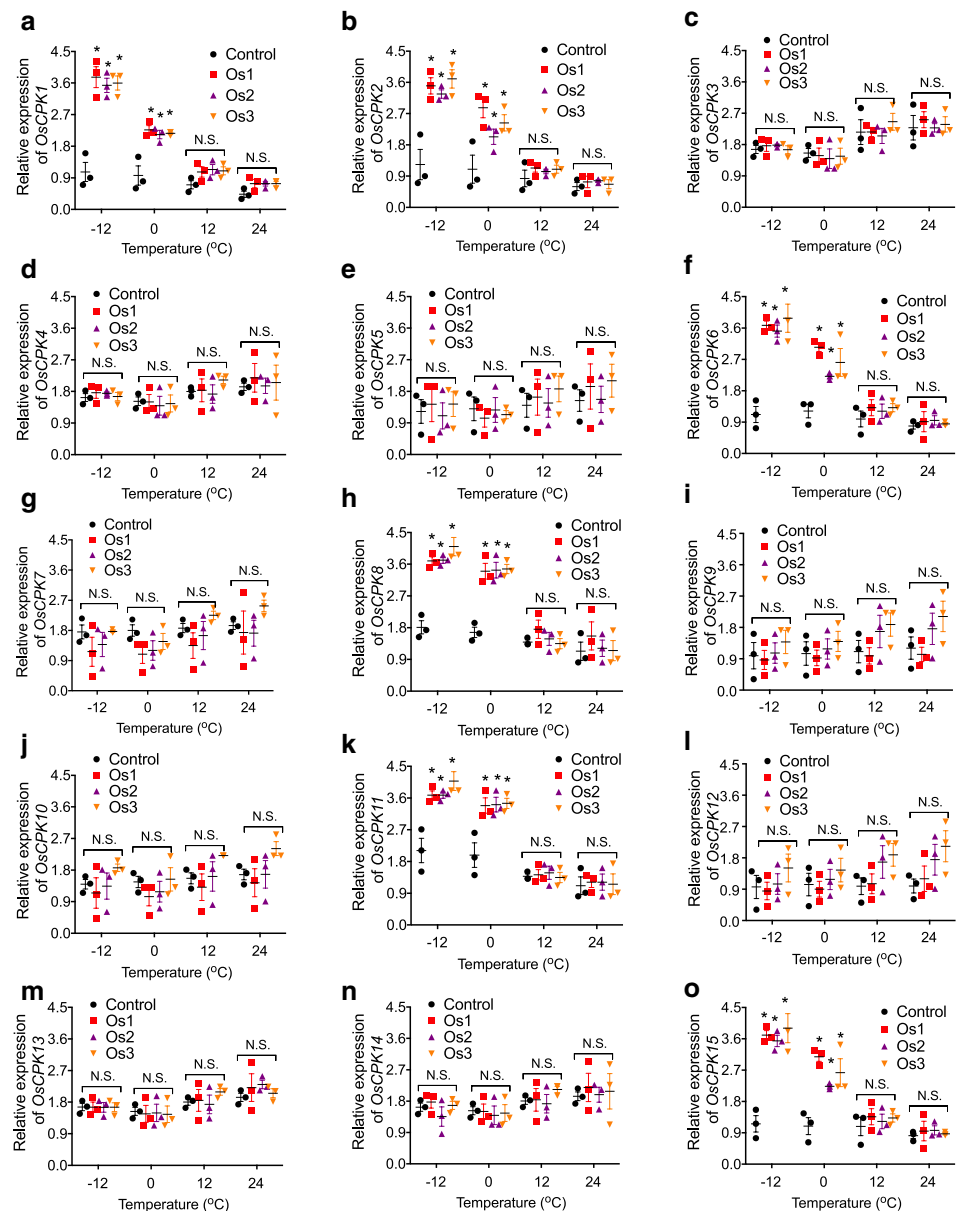
0 °C, 12 °C, and 24 °C. Compared to the control, the expression of MAPK genes *OsMAPK1* (Fig. 6a), *OsMAPK2* (Fig. 6b), *OsMAPK3* (Fig. 6c), *OsMAPK8* (Fig. 6h), and *OsMAPK9* (Fig. 6i) was increased significantly in transgenic rice cells with the treatment at − 12 °C and 0 °C, respectively. Expression of MAPK genes *OsMAPK1* (Fig. 6a), *OsMAPK2* (Fig. 6b), *OsMAPK3* (Fig. 6c), *OsMAPK8* (Fig. 6h), and *OsMAPK9* (Fig. 6i) was not significantly changed in transgenic rice with the treatment at 12 °C and 24 °C. Expression of MAPK genes *OsMAPK4* (Fig. 6d), *OsMAPK5* (Fig. 6e), *OsMAPK6* (Fig. 6f), and *OsMAPK7* (Fig. 6g) was not significantly changed in *O. sativa* transgenic cell lines Os1, Os2, and Os3 under cold stress at − 12 °C, 0 °C, 12 °C, and 24 °C.

We choose the Arabidopsis CIPK21, not the CIPK21 from cotton, rice, and pine, for the work presented in the manuscript because: (1) we are interested in investigating the function of CIPK21 that is derived from the popular model plant Arabidopsis in different economically plant species to see if the AtCIPK21 functions in the heterologous expression systems; (2) the biology of the Arabidopsis CIPK21 in abiotic stress has been well documented in Arabidopsis, but its function in cold stress has not been documented in cotton, rice, and pine; and (3) we hope to obtain insights into the mechanism of AtCIPK21-associated cold stress tolerance.

Fig. 6 Expression of MAPK genes in *AtCIPK21* transgenic rice cells. Overexpression of the *AtCIPK21* gene modulates expression of transcription factor genes *OsMAPK1* (**a**), *OsMAPK2* (**b**), *OsMAPK3* (**c**), *OsMAPK4* (**d**), *OsMAPK5* (**e**), *OsMAPK6* (**f**), *OsMAPK7* (**g**), *OsMAPK8* (**h**), and *OsMAPK9* (**i**) in transgenic rice cell lines. The asterisk indicates significant differences, as assessed by a *t* test. *$P < 0.05$, significant relative to control
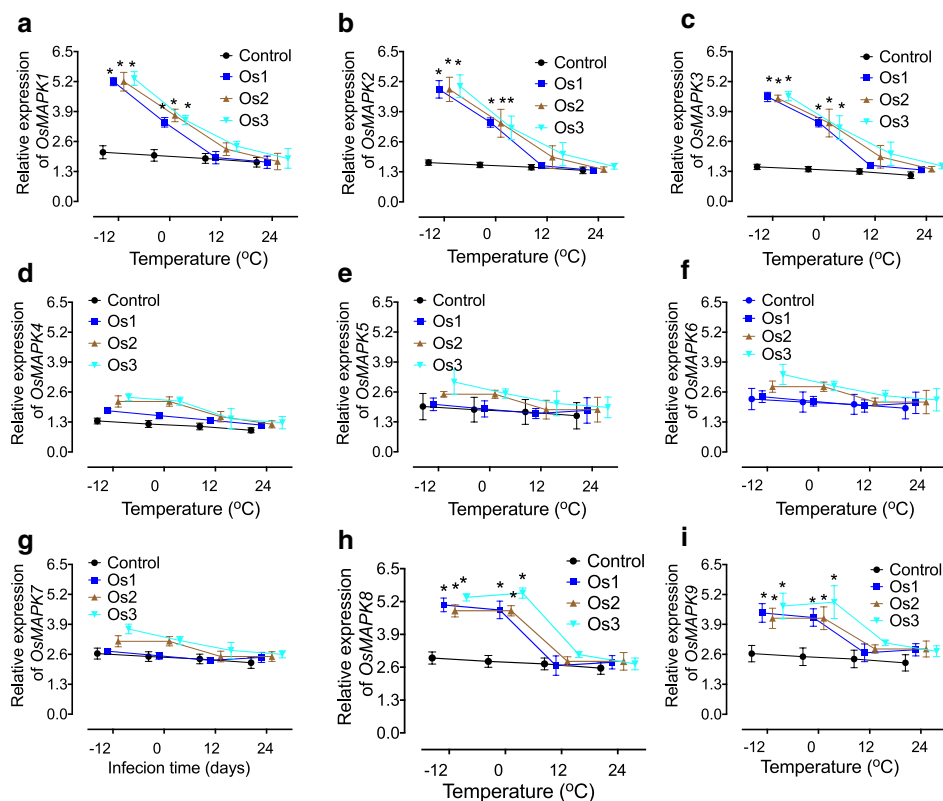
Overexpression of AtCIPK21 changes the cell morphology under cold stress

To examine whether overexpression of the *AtCIPK21* gene affects cell morphology of transgenic cell lines, images of cells were examined in *O. sativa*, *G. hirsutum*, and *P. strobus* transgenic cell lines under treatment at − 12 °C, 0 °C, 12 °C, and 24 °C, respectively. Overexpression of *AtCIPK21* changes the cell morphology of *O. sativa* (Fig. 7a–h), *G. hirsutum* (Fig. 7i–p), and *P. strobus* (Fig. 7q–x) under treatment at − 12 °C and 0 °C, respectively. Compared to the control, size of *O. sativa* (Fig. 7y), *G. hirsutum* (Fig. 7z), and *P. strobus* (Fig. 7za) transgenic cells was significantly increased under treatment at − 12 °C and 0 °C, respectively. These results indicated that overexpression of the *AtCIPK21* gene increased cell size under treatment at − 12 °C and 0 °C, respectively.

## Overexpression of AtCIPK21 enhances cold stress tolerance in Arabidopsis

The activity of APOX, CAT, PPO, POD, and ROS was examined in *AtCIPK21* transgenic cell lines of *A. thaliana*, treated with different temperatures − 12 °C, 0 °C, 12 °C, and 24 °C, respectively. Overexpression of the *AtCIPK21* gene significantly increases the activity of APOX, CAT, PPO, and POD, and decreases ROS level in transgenic cell lines

of *A. thaliana* (Fig. 8), treated at − 12 °C and 0 °C, respectively. Overexpression of the *AtCIPK21* gene significantly increases expression of *ICE1* gene and expression of *CBF* genes (Cramer et al. 2011; Yuan et al. 2018) in rice (Fig. 9). The *AtCIPK21* gene participates in the process of cold stress signaling pathway in plant cells (Fig. 10).

Discussion

CIPKs play important roles in abiotic stress responses in plants by forming complexes that regulate Ca^{2+} changes (Chen et al. 2014; Kurusu et al. 2010; Pandey et al. 2007, 2015; Wang et al. 2016). Although functions of some of CIPKs have been identified in Arabidopsis (Beier et al. 2018; Liu et al. 2019; Wang et al. 2016; Wu et al. 2019; Yin et al. 2017; Zhou et al. 2015), functions of many CIPKs have not been characterized. This investigation is focused on the function of *AtCIPK21* gene in cold stress tolerance in cells of rice (*Oryza sativa* L.), cotton (*Gossypium hirsutum* L.), and white pine (*Pinus strobus* L.). In our study, molecular analyses were performed to explore the effect of the *AtCIPK21* gene in response to cold stress in rice, cotton, and pine. Overexpression of the *AtCIPK21* gene increases cell viability and cell growth rate, as well as decreases TBARS and ion leakage in transgenic cells of *O. sativa*, *G. hirsutum*, and *P. strobus* after treatment at − 12, and 0 °C, respectively.

Fig. 7 Overexpression of *AtCIPK21* changes the cell morphology under cold stress. Overexpression of *AtCIPK21* changes the cell morphology under cold stress in *O. sativa* (**a–h**), *G. hirsutum* (**i–p**), and *P. strobus* (**q–x**), and the cell size (**y–za**), respectively. The asterisk indicates significant differences, as assessed by a *t* test. *$P < 0.05$, significant relative to control. *N.S.* no statistical significance
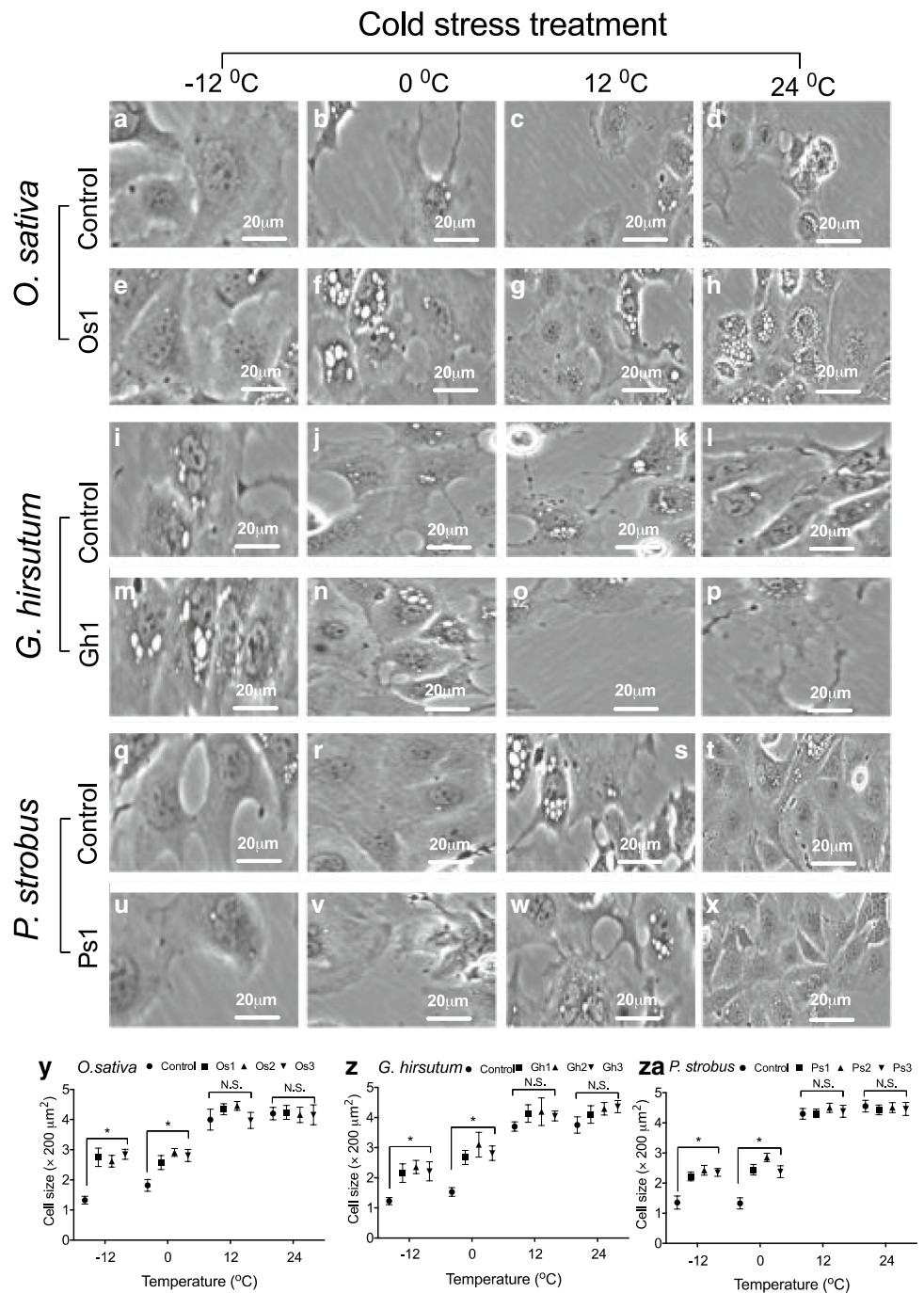
The overexpression of the *AtCIPK21* gene in different plant species demonstrated a similar pattern of decreasing TBARS and ion leakage in transgenic cells of *O. sativa* (Fig. 2a, d, g, j), *G. hirsutum* (Fig. 2b, e, h, k), and *P. strobus* (Fig. 2c, f, i, l) after treatment with cold at − 12 °C and 0 °C, respectively. These results showed that overexpression of the *AtCIPK21* gene-enhanced cold stress tolerance could be achieved in different plant species.

Increased activity of antioxidant enzymes is associated with the increased tolerance stress (Nejadsadeghi et al.
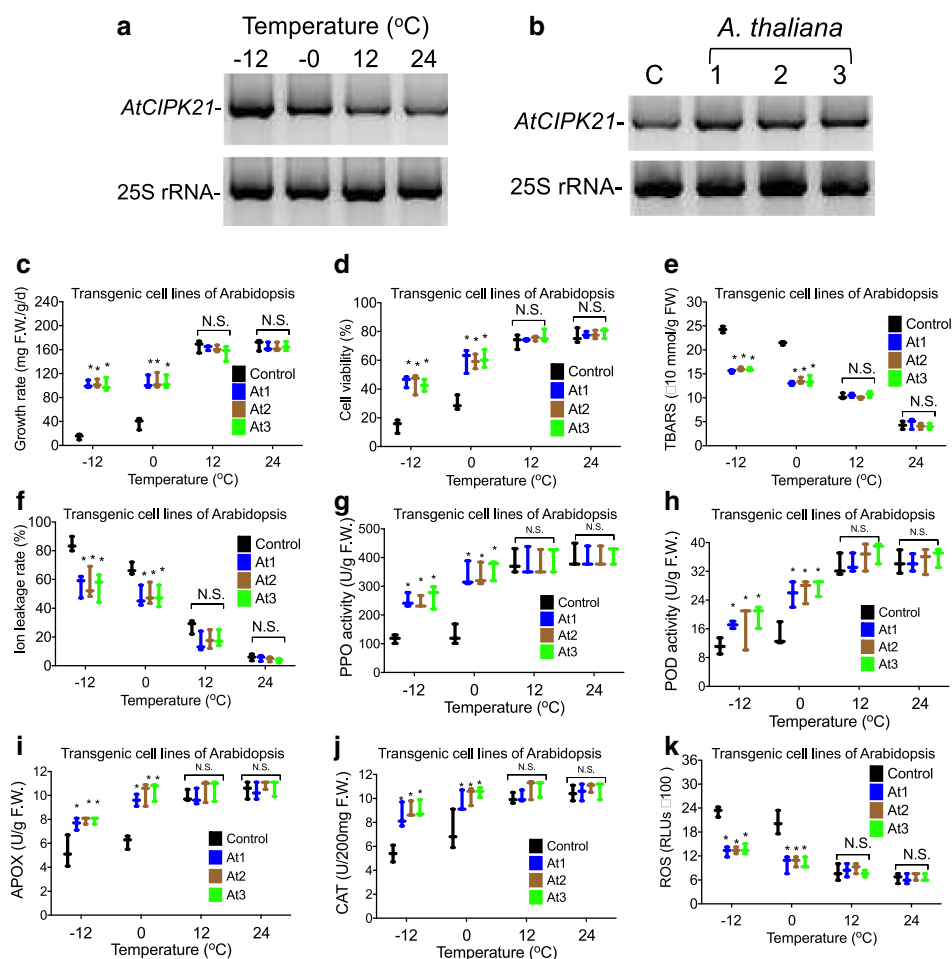
2014). In this investigation, responses of cells to cold stress were comparatively studied in *O. sativa*, *G. hirsutum*, and *P. strobus*. Increasing evidence showed that reactive oxygen species (ROS) are controlled by enzymatic antioxidant defense systems that are related to abiotic stress and homeostasis regulation of ROS in plants is involved in essential proteins that are related to abiotic stress tolerance (Cui et al. 2005; Guo et al. 2006; Kazemi Shahandashti et al. 2013; Kazemi-Shahandashti et al. 2014; You and Chan 2015; Zur et al. 2009). It has been reported that the activity of APOX,

CAT, PPO, and POD is related to cold stress tolerance in a large number of plant species (Adak and Datta 2005; Correa-Aragunde et al. 2015; Faltin et al. 2010; Gundinger and Spadiut 2017; Miller et al. 2007; Stajic et al. 2004; Yang et al. 2006). For example, to protect from enzymatic browning, a single polyphenol oxidase (PPO) can play a critical role and RP-UHPLC-MS analysis demonstrated that a mixture of phenolic compounds and the phenolic fraction was responsible for inhibition of PPO (Dirks-Hofmeister et al. 2013; Kampatsikas et al. 2017; Kuijpers et al. 2014; Molitor et al. 2016; Nautiyal et al. 2008; Poiatti et al. 2009). Our results demonstrated that *AtCIPK21* enhances cold stress tolerance by increasing the activity of APOX, CAT, PPO, and POD in different species including *O. sativa*, *G. hirsutum*, and *P. strobus*.

The involvement of polyamines as antioxidants against abiotic stress-derived oxidative damage has been reported (Basu et al. 2014; Groppa and Benavides 2008; Huang et al. 2015; Kasukabe et al. 2004; Kovacs et al. 2010; Sun et al. 2014). Polyamines have different effects that could help plants deal with abiotic stresses and they have also been proven to act as regulators of gene expression during

the development of stress responses (Agurla et al. 2018; Romero et al. 2018; Zhao et al. 2017). In this investigation, the experimental results demonstrated that overexpression of the *AtCIPK21* gene significantly increases the levels of Put, Spd, and Spm in transgenic cells of *O. sativa* (Fig. 4a–c), *G. hirsutum* (Fig. 4d–f), and *P. strobus* (Fig. 4g–i), respectively, under stress at − 12 °C and 0 °C, respectively. Overexpression of the *AtCIPK21* gene does not significantly increase the levels of Put, Spd, and Spm in transgenic cells of *O. sativa* (Fig. 4a–c), *G. hirsutum* (Fig. 4d–f), and *P. strobus* (Fig. 4g–i), respectively, under stress at 12 °C and 24 °C.

Plant Ca^{2+}-dependent protein kinase (CPK) signaling is important in protecting plant cells from abiotic stress including drought, salt and cold stress and has been demonstrated to function in mediating the signaling following Ca^{2+} influx after stress (Arimura and Sawasaki 2010). Calcium is an intracellular secondary messenger and CPKs are Ca^{2+} sensors that have both Ca^{2+} sensing function and kinase activity that play important roles in plant responses to various abiotic stresses. Characterization of *CPK* gene family members in plants provides an important foundation for crop improvement and improved understanding of

Fig. 9 Expression of ICE and CBF genes in *AtCIPK21* transgenic rice cells. Overexpression of the *AtCIPK21* gene modulates expression of *OsICE1* (**a**), *OsCBF1* (**b**), *OsCBF2* (**c**), *OsCBF3* (**d**), *OsCBF8* (**e**), *OsCBF9* (**f**), *OsCBF10* (**g**), *OsCBF11* (**h**), and *OsCBF12* (**i**) in transgenic rice cell lines. The asterisk indicates significant differences, as assessed by a *t* test. *$P < 0.05$, significant relative to control
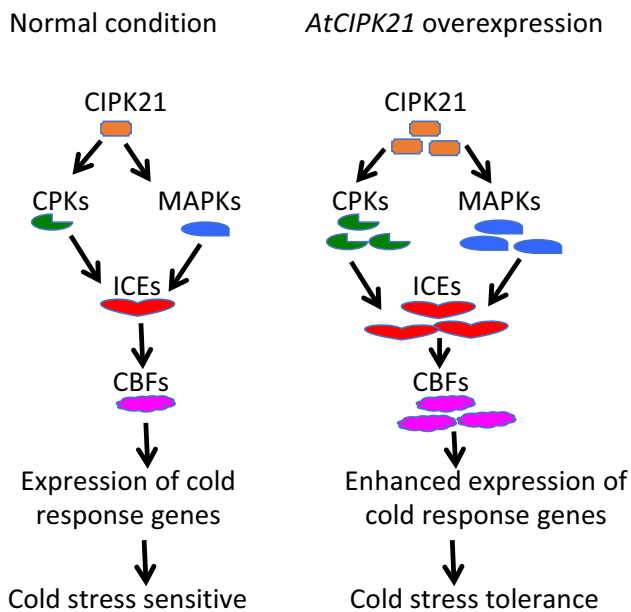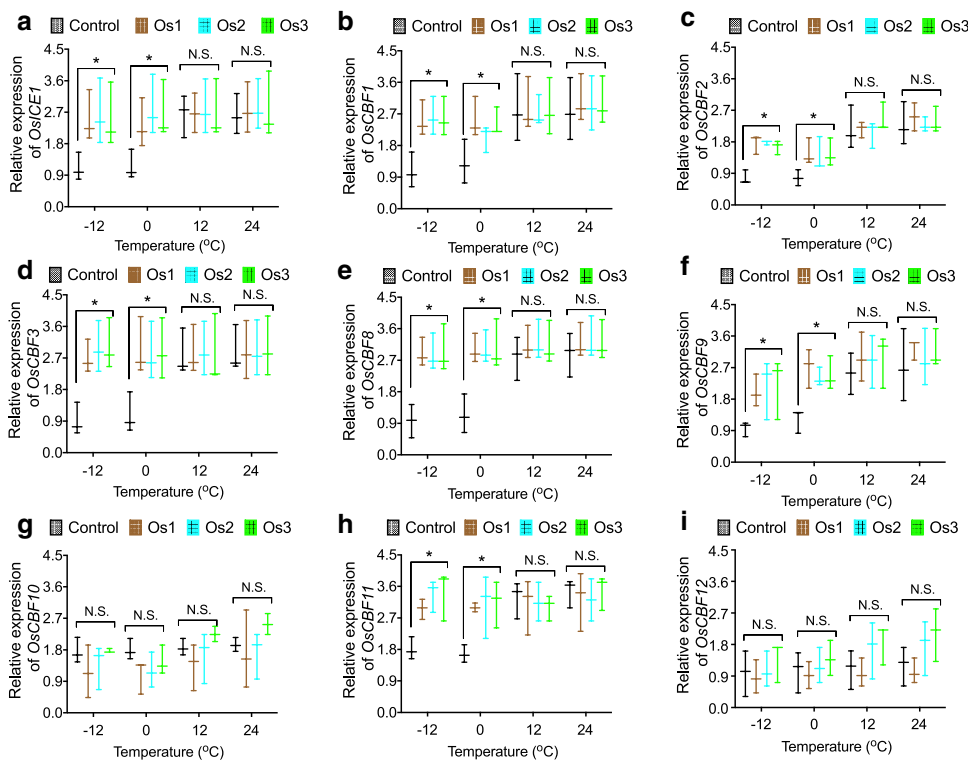

Fig. 10 Cold stress signaling pathway related to overexpression of *AtCIPK21* in plant cells

signal transduction in plants (Geng et al. 2013; Hu et al. 2015; Zhang et al. 2014). In this study, our results indicated that overexpression of the *AtCIPK21* gene increased cold stress tolerance may be related to expression of *CPK* genes including *OsCPK1* (Fig. 6a), *OsCPK5* (Fig. 6e), *OsCPK6* (Fig. 6f), *OsCPK7* (Fig. 6g), *OsCPK8* (Fig. 6h), *OsCPK9* (Fig. 6i), and *OsCPK11* (Fig. 6k), and may be not related to expression of *CPK* genes including *OsCPK2* (Fig. 6b), *OsCPK3* (Fig. 6c), *OsCPK4* (Fig. 6d), *OsCPK10* (Fig. 5j), and *OsCPK12* (Fig. 6l) in *AtCIPK21* transgenic rice cell.

Mitogen-activated protein kinase (MAPK) signaling plays critical roles in protecting plant cells from abiotic stresses and MAPK signaling regulates the response to cold stress through the Inducer of CBF expression 1 (ICE1)–CBF-cold-responsive (COR) transcriptional pathway in *Arabidopsis thaliana* (Liu and Zhou 2018). The involvement of MAP kinases in abiotic stresses including drought, cold, and salt stress is related to the complexity of signal transduction pathways, as well as the specificity of MAP kinase signaling modules (Li et al. 2014; Moustafa et al. 2014; Wang et al. 2015). In this study, our results demonstrated that mitogen-activated protein kinase genes play an important role in cold stress tolerance. Our results showed that the expression of mitogen-activated protein kinase genes *OsMAPK1* (Fig. 5a), *OsMAPK2* (Fig. 6b), *OsMAPK3* (Fig. 6c), *OsMAPK4* (Fig. 6d), *OsMAPK5* (Fig. 6e), and *OsMAPK6* (Fig. 6f) was increased significantly in transgenic rice cells with the treatment at − 12 °C and 0 °C.

Cell growth and proliferation are dependent on the environmental conditions and are affected by different stresses. Changes in cell growth environment could result in alteration in the cell cycle regulation, because stresses regulate cell cycle progression, leading to a shortened G2

period (de Marcia et al. 2017; Herranz and Medina 2014; Lee et al. 2017; Majda et al. 2017; Matia et al. 2010). Embryogenic cell suspensions of wheat (*Triticum aestivum* L.) have been used as sources to study their morphogenic capacity under stress and changed culture conditions. Stress caused an abnormal chromosome number and low viability (Ahmed and Sagi 1993). Cell viability and cell size are important for increased cold stress tolerance. The cell viability determined by the trypan blue is valid after 7 days of culture. Growth rate was significantly increased in high cell viability cultures. Increased sensitivity of the cells to stresses could result in a 35% increase in the cell size (Steward et al. 1999). In Arabidopsis, physiologically homogenous cell cultures could be subcultured over several months under cold stress and low culture temperature is robust for heterotrophic and semi-autotrophic cells (Boisson et al. 2012; Trexler et al. 2005; Wucherpfennig et al. 2014). In our study, overexpression of the *AtCIPK21* gene decreases the size of transgenic cells in rice (Fig. 5a–d), tobacco (Fig. 5e–h), and pine (Fig. 5i–l), respectively, under treatment of low temperature (− 10 °C and − 4 °C). These results will increase our understanding of the *AtCIPK21* gene in cold stress tolerance in monocotyledonous (rice), dicotyledonous (cotton), and gymnosperm (pine) plants. The findings are also valuable in plant molecular biotechnology.

Conclusion

We report that overexpression of the *AtCIPK21* gene increases cold stress tolerance by increasing both cell viability and growth rate and decreasing TBARS under cold stress in rice (*Oryza sativa* L.), cotton (*Gossypium hirsutum* L.), and white pine (*Pinus strobus* L.). *AtCIPK21* enhances cold stress tolerance by increasing the activity of APOX, CAT, PPO, and POD, as well as by elevating accumulation of Put, Spd, and Spm, respectively. The *AtCIPK21* gene increased cold stress tolerance may be related to expression of *OsCPK1, OsCPK5, OsCPK6, OsCPK7, OsCPK8, OsCPK9, and OsCPK11*. Among nine mitogen-activated protein kinase genes examined, *AtCIPK21* overexpression changed expression of MAPK transcription factors *OsMAPK1, OsMAPK2, OsMAPK3, OsMAPK4, OsMAPK5,* and *OsMAPK6* under cold stress. *AtCIPK21* counteracts cold stress by enhancing expression of stress response transcription factors. Our results indicate that overexpression of the *AtCIPK21* gene in plant cells improved cold stress tolerance by elevating polyamines content, antioxidative enzyme activity, and expression of *CPK* genes and *MAPK* genes. Overexpression of the *AtCIPK21* gene may be a valuable approach for engineering plant cold stress tolerance.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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