Overexpression of *MdCPK1a* gene, a calcium dependent protein kinase in apple, increase tobacco cold tolerance via scavenging ROS accumulation

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Abstract

Calcium-dependent protein kinases (CDPKs) are important calcium receptors, which play a crucial part in the process of sensing and decoding intracellular calcium signals during plant development and adaptation to various environmental stresses. In this study, a CDPK gene *MdCPK1a*, was isolated from apple (*Malus* × *domestica*) which contains 1701bp nucleotide and encodes a protein of 566 amino acid residues, and contains the conserved domain of CDPKs. The transient expression and western blot experiment showed that MdCPK1a protein was localized in the nucleus and cell plasma membrane. Ectopic expression of *MdCPK1a* in *Nicotiana benthamiana* increased the resistance of the tobacco plants to salt and cold stresses. The mechanism of *MdCPK1a* regulating cold resistance was further investigated. The overexpressed *MdCPK1a* tobacco plants had higher survival rates and longer root length than wild type (WT) plants under cold stress, and the electrolyte leakages (EL), the content of malondialdehyde (MDA) and reactive oxygen species (ROS) were lower, and accordingly, antioxidant enzyme activities, such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were higher, suggesting the transgenic plants suffered less chilling injury than WT plants. Moreover, the transcript levels of ROS-scavenging and stress-related genes were higher in the transgenic plants than those in WT plants whether under normal conditions or cold stress. The above results suggest that the improvement of cold tolerance in *MdCPK1a*-overexpressed plants was due to scavenging ROS accumulation and modulating the expression of stress-related genes.

Introduction

Abiotic stresses, such as drought, high salinity, cold or submergence, are serious threats to crop productivity. Plants have evolved fine signaling strategies enabling them to overcome these stresses and other harmful conditions. Among the strategies adopted by plants, calcium signals are important regulators in many crucial and sophisticated cellular processes [1].
When plants are subjected to various stresses, they rapidly release calcium ions (Ca\(^{2+}\)) from storage compartments (such as vacuole, endoplasmic reticulum) into the cytosol. Transient increases of free Ca\(^{2+}\) in cytosolic are perceived and decoded through different Ca\(^{2+}\) sensors and Ca\(^{2+}\) binding proteins, such as calcium-dependent protein kinases (CDPKs), calmodulin-like proteins, calmodulins and calcineurin B-like proteins. CDPKs distinguished from other calcium-sensing proteins, as they not only can decode and translate the increase of Ca\(^{2+}\) concentration into improvement of protein kinase activity but also can activate downstream effectors [2].

CDPKs exist in protists, oomycetes, green algae and plants, but not in animals [3]. Genome-wide analysis of different plant species showed that they are encoded by a large multi-gene family. For example, Oryza sativa, Zea mays, Malus domestica, Populus trichocarpa, and Arabidopsis thaliana were identified 31, 35, 37, 30 and 34 CDPK genes in their genomes, respectively [4–11]. CDPKs have a conserved modular structure including a variable N-terminal domain, a kinase domain, an auto-inhibitory domain or junction domain and a regulatory domain or CaM-like domain, which canonically contains four EF-hands [12, 13]. In the absence or low concentration of cytoplasmic Ca\(^{2+}\), auto-inhibitory domain blocks the kinase domain and inhibits its activity [14, 15]. When plants perceive stimuli, an immediate increase of the concentration of Ca\(^{2+}\) in plant intracellular promotes Ca\(^{2+}\) binding to EF-hand motifs, which will induce molecular conformation changes and activate enzyme activities, leading to phosphorylation of the targeted substrates as well as CDPK autophosphorylations [16–18]. The phosphorylated proteins probably participate in plant defense reactions, ethylene synthesis, cytoskeleton organization, carbon and nitrogen metabolism, and stress responses [5, 17, 19–22]. Knowledge about CDPK functions and mechanisms of the responses to environmental stress is increasing. Substantial experimental evidences indicate CDPKs play important roles in response to abiotic/biotic stress. For example, Arabidopsis CPK28 acts as a positive regulator in response to osmotic stress [23]. OsCPK9 in rice plays a positive role in drought, osmotic, and dehydration stress responses [24]. Overexpressing of OsCPK4, OsCPK12 in rice exhibited increased salt/drought stress tolerance and rice blast disease resistance [25–27]. CaCDPK15 in pepper (Capsicum annum) positively regulates response to Ralstonia solanacearum [28]. In Arabidopsis, overexpression of SiCDPK24 enhanced drought tolerance [29]. OsCDPK1 positively regulates salt and drought tolerance in rice [30], meanwhile it acts as a positive regulator of OsPR10a participating in the defense signaling pathway [31]. Conversely, some CDPKs are negative regulators of stress response because transgenic plants overexpressing them are more sensitive to abiotic/biotic stresses. Arabidopsis thaliana cpk23 mutant increased endurance to drought and salt stresses, while AtCPK23 overexpressing plants reduced the resistance to drought and salt stresses [32]. Overexpression of ZmCPK1 in maize mesophyll protoplasts suppressed the expression of the cold-induced marker gene Zmnetf3, and ectopic expression of ZmCPK1 in Arabidopsis reduces plants adaption to the cold tolerance, suggesting ZmCPK1 act as a negative regulator of cold stress signalling in maize [33]. The Arabidopsis CPK28 plays as a negative regulator of immune signaling that continually buffers immune signaling by controlling the turnover of BIK1, an important convergent substrate of multiple pattern recognition receptor (PRR) complexes [34]. Thus, CDPKs are implicated in both positive and negative regulation of plant abiotic/biotic stress adaptation.

However, the research on function of CDPKs in apple has been rarely reported. This study focused on the function of MdCPK1a, a CDPK gene from M. domestica, in response to abiotic stresses. MdCPK1a-overexpressed N.benthamiana plants were investigated to different abiotic stress conditions. Experimental results showed that overexpression of MdCPK1a in N. benthamiana confers it resistance to salt and cold stresses. Furthermore, the mechanism of enhancement of cold tolerance in the transgenic plants was disclosed in this research.
Materials and methods

Cloning, sequencing and phylogenetic analysis of MdCPK1a

The fourth and fifth young leaves were taken from the annual branches of the *Malus domestica* cv. ‘Jonathan’ growing in the greenhouse. Total RNA was extracted by using CTAB method [35]. Based on the released sequence of *MdCPK1a* (MDP0000153100) from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html), a pair of primers GSP1 was designed for gene amplification by RT-PCR (S1 Table). The PCR amplification was performed in a total 50 μL reaction volume containing 300 ng cDNA, 1×TransStart Fast Pfu buffer, 0.25 mM dNTPs, 0.4 mM of each primer and 2.5 units of TransStart Fast Pfu DNA polymerase. PCR conditions were set as follows: initial denaturation at 95˚C for 2 min; 40 cycles of 95˚C for 20 s, 55˚C for 20 s, and 72˚C for 60 s, and followed by a final extension at 72˚C for 5 min. The construction of PCR products ligation with pMD19-T vector were named pMD19T-MdCPK1a, and sequenced by Invitrogen (Shanghai, China).

The domain was identified through PROSITE and Smart™ databases (http://smart.embl-heidelberg.de/); Molecular weight and theoretical isoelectric point (pI) were calculated by ExPASy software (http://www.expasy.org/); The position of S-Palmitoylation and N-Myristoylation were predicted using the online tool GPS-Lipid (http://lipid.biocuckoo.org/presult.php) [36–38]. The homologous proteins were searched by BLASTp program (http://www.ncbi.nlm.nih.gov/) using the deduced amino acid sequence of *MdCPK1a*. Multialignment was performed by DNAMAN software (http://www.lynnon.com/). A phylogenetic tree was built through the neighbor-joining (NJ) method under the MEGA 6.0 program with Poisson-corrected distances with 500 bootstrap replicates.

Subcellular localization analysis

The coding sequence of *MdCPK1a* with termination codon removal was amplified from pMD19T-MdCPK1a using primer GSP3 (S1 Table). Amplification products were digested with Xba I and BamH I, and cloned into the downstream of CaMV 35S promoter in pCAMBIA1300 vector resulting in 35S::MdCPK1a-GFP. The 35S::MdCPK1a-GFP and 35S::GFP (control) constructs were transiently transformed into *N. benthamiana* leaves described by Sheludko [39]. GFP fluorescence was imaged under a laser confocal fluorescence microscopy (Zeiss TCS SP8) with an excitation wavelength of 488 nm and a 505–530 nm bandpass filter.

Protein extraction and western blot

Tobacco leaves that transiently expressed 35S::MdCPK1a-GFP and 35S::GFP (control) were homogenized in liquid nitrogen. The nuclear proteins, cytoplasmic proteins and plasma membrane (PM) proteins were extracted with the Plant Nuclear, Cytoplasmic and Membrane Proteins Extraction Kit (BestBio, Shanghai, China) from plant tissues, respectively. Total proteins were extracted with extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1%SDS, 1%Triton X-100), and followed by centrifugation at 12000 rpm for 15min, and the supernatants were collected.

Following standardization of protein concentrations using BCA Protein Assay kit (BestBio, Shanghai, China), Equal amounts of protein were employed in 10% SDS-PAGE and transferred to the NC membrane. After blocking with 5% skimmed milk powder in PBST (0.5% Tween in PBS) at room temperature for 2 h, the membrane was incubated with Anti-GFP rabbit polyclonal antibody (Sangon Biotech, Shanghai, China) at 4˚C overnight. After this, the membrane was rinsed three times with PBST for 5 min and then incubated with the HRP-
conjugated Goat Anti-Rabbit IgG (Sangon Biotech, Shanghai, China) for 1 h. Subsequently, the membrane was washed with PBST, visualized by enhanced chemiluminescence and then detected in the Tanon 2500 chemiluminescence imaging system (Shanghai, China).

**Overexpression of MdCPK1a in N. benthamiana**

The full length ORF of MdCPK1a flanking BamHI and SacI at 5’ and 3’ respectively was amplified by the primers GSP2 (S1 Table). The PCR products were double-digested with BamHI and SacI, then ligated into the pYH455 vector at downstream of CaMV35S promoter (S2 Fig), generating a plasmid pYH455-MdCPK1a. Subsequently, it was transferred into EHA105. Tobacco transformation was conducted using leaf disk method [40]. Transgenic tobacco plants were selected on MS medium supplement 50 mg L\(^{-1}\) kanamycin. The kanamycin-resistance plants were further confirmed by PCR and RT-PCR respectively with the control of non-transformed tobacco plants cultured on MS medium.

**Abiotic tolerance analysis of the transgenic tobacco plants**

Three independent lines (A2, A4 and A36) and wild type (WT) plants were used to analyze abiotic tolerance. After being surface disinfected, the seeds of A2, A4, A36 and WT were sown on MS medium (for transgenic lines MS medium supplemented 50 mg L\(^{-1}\) kanamycin). *N. benthamiana* were cultured under long day conditions 16 h light at 23–25˚C and 8 h dark at 18–20˚C.

To assess cold resistance, we grew the seedlings under cold stress (4˚C) for 10 d after seeds germinating on MS medium and measured the root length after cold treatment. Meanwhile, four-week-old plants growing in medium were stressed at 4˚C for 10 d, and then the survival rates were calculated after recovering at 25˚C for 14 d according to the number of green plants.

For salt or drought tolerance assays, 4-week-old plants were transplanted into soil with sufficient water under a normal environmental chamber at 25˚C for 14 d. They were then watered with 200 mM NaCl solution in soil for salt stress analysis, or cultured without irrigation for 25 d, and then recovered by re-watering for 10 d for drought stress analysis. The biomass and phenotype were investigated after the treatments.

**Physiological measurements and histochemical staining**

Sixty-day-old plants treated at 4˚C for 48 h were used as material. Malondialdehyde (MDA) contents were measured using the thiobarbituric acid (TBA)-based colorimetric method [41]. Leaf samples (0.5 g) were homogenized in 2 mL 20% trichloroacetic acid with the aid of some sand, and then the homogenate was centrifuged at 16,000 g for 20 min at 4˚C. The supernatant (1 mL) was mixed with equal volume of 0.5% (w/v) TBA. The mixture was heated at 95˚C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000 g for 10 min, absorbancy was measured at 532 nm corrected for nonspecific turbidity by subtracting the absorbancy at 600 nm. The MDA content was calculated using its molar extinction coefficient (155 mM\(^{-1}\) cm\(^{-1}\)), and the value was expressed as μmol MDA·mg\(^{-1}\) fresh weight (FW). Electrolyte leakages (EL) were detected using the protocol according to [42]. The leaf segments from at least three plants of each line were placed in deionized water for 2 h at 25˚C. Total electrolyte content was measured after autoclaving the leaf segments for 15 min and taken as 100% leakage. Activities of catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) were analyzed according to [43]. Leaf samples (0.2 g) were homogenized in liquid nitrogen adding 2 mL precooled 50 mM pH 7.8 phosphate buffer (containing 0.1 mM EDTA and 1% PVP) and ground into homogenate in an ice bath. Add extraction medium to rinse the mortar for 2–3
times and make the final volume 8.0 mL. The supernatant was centrifuged at 12 000 × g for 15 min at 4˚C and stored in the ice bath for the detection of SOD, POD, CAT activities. The accumulation of H₂O₂ and O₂⁻ was tested by histochemical staining with nitroblue tetrazolium (NBT) and 3, 3'-diaminobenzidine (DAB) respectively. Leaves were incubated in the NBT solution (0.1 mg·mL⁻¹) and DAB solution (1.0 mg·mL⁻¹, pH 3.8) for 24 h at 25˚C in the dark. Then, the leaves were soaked in 95% ethanol overnight to remove the chlorophyll [44, 45]. DAB/NBT-stained leaves were scanned, and the pixel intensity of the DAB/NBT stain was quantified using Adobe PHOTOSHOP CS4 software.

**Quantitative RT-PCR analysis of gene expression in transgenic plants**

The expression level of stress-related genes was monitored by quantitative RT-PCR (qPCR) on an ABI7300 Detection System using SYBR® Premix ExTaq™ qRT-PCR kits (TaKaRa, Dalian, China). Gene-specific primers were designed by Primer 5.0 (S1 Table). PCR mixtures contained 10.0 μL of 2×SYBR Premix, 1.0 μL of cDNA template, 200 nM of each primer, then added ddH₂O up to a total volume of 20.0 μL. PCR reaction was performed as follows: denaturation at 94˚C for 3 min followed by 40 cycles at 94˚C for 20 s, 60˚C for 20 s, and 72˚C for 40 s. After that, melting curves were determined as follows: 95˚C for 15 s, 60˚C for 1 min, and 95˚C for 15 s. qPCR was performed three independent biological repeats for each sample and three technical repeats for each reaction. Expression values were normalized with *NtTubulin* gene (Accession No: EF051136). The relative expression of a gene was calculated by using 2^−ΔΔCt method 

\[ \Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{tubulin gene}})_{\text{treatment}} - (Ct_{\text{target gene}} - Ct_{\text{tubulin gene}})_{\text{control}} \]

**Statistical analysis**

Every experiment was repeated three times, and the value was got from an average from three independent replicates and shown with error bar representing with standard error (SE). All statistical analyses were performed using SPSS software and based on Duncan’s multiple range tests, statistical differences were compared and p values <0.05 or <0.01 were used as the thresholds for significant or extremely significant differences, respectively.

**Results**

**Cloning and bioinformatics analysis**

The full length open reading frame (ORF) of *MdCPK1a* was isolated from apple, which consisted of 1701 nucleotides encoding a 566-amino acid polypeptide with the predicted molecular weight 62.86 kDa and the isoelectric point 5.16. *MdCPK1a* protein possesses the characteristics as other plant CDPKs: an N-terminal variable domain (107aa) preceding a Ser/Thr protein kinase catalytic domain (259 aa), a junction domain (42 aa), a CaM-like domain containing four EF hand Ca²⁺-binding motifs (142 aa) and a C-terminal variable domain (16 aa). A possible ATP-binding site and active site in the N-terminal region and 15 invariant amino acid residues for eukaryotic Ser/Thr protein kinase in the N-terminal of kinase domain were shown in Fig 1. The putative post-translational modifications of *MdCPK1a* protein was predicted by the software GPS-Lipid, showing that there are one myristoylation (Gly at the 2nd residue from the N-terminus) and two palmitoylation (Cys at the 5th and 136th residues from the N-terminus) in the protein (Fig 1).

Multiple sequence alignments showed the deduced amino acid sequence of *MdCPK1a* with 72.73% similarity to *OsCDPK7* (BAB16888), 76% to *AtCPK1* (NP_196107), and 71.75% to *ZmCPK1* (BAA12338). The phylogenetic relationships between *MdCPK1a* and several stress-related CDPKs are presented in S1 Fig. The selected CDPK proteins were clustered into three
subgroups including I, II, III. *MdCPK1a* along with *OsCDPK7*, *AtCPK1*, and *ZmCPK1* which were reported to regulate abiotic and biotic stress tolerances [33, 46, 47], belongs to the subgroups I, which hints that *MdCPK1a* may participate in stress responses in apple.
Subcellular localization

The subcellular location of a protein determines or is closely correlated with its function. To investigate the subcellular location of MdCPK1a protein, we cloned the full-length ORF sequence of MdCPK1a into pCAMBIA1300 vector under CaMV35S promoter, constructing an in-frame fusion protein plasmid 35S::MdCPK1a-GFP (Fig 2A). The construct was transformed to N. benthamiana leaves by agro-infiltration for transient expression analysis. The subcellular location of MdCPK1a-GFP was detected by laser scanning confocal microscopy, with the leaves transiently transformed 35S::GFP as the control. The tobacco cells expressing the 35S::MdCPK1a-GFP emitted fluorescence both in nucleus and plasma membrane, whereas in expressing the 35S::GFP tobacco cells, the fluorescence filled the entire cytoplasm, plasma membrane, and nucleus (Fig 2B). We further verified the subcellular localization by western blot by immunoblotting with anti-GFP antibody. MdCPK1a-GFP was detected exclusively in the fractions of plasma membrane and cell nucleus but not in the fraction of cytosol (Fig 2C). These results indicated that MdCPK1a protein was localized to the nucleus and cell plasma membrane.

Overexpression of MdCPK1a gene in tobacco

The overexpression construct of MdCPK1a (pYH455-MdCPK1a) was introduced into N. benthamiana by A. tumefaciens-mediated transformation. Ten transgenic lines were obtained and further identified by PCR using gene-specific primers (GSP1). Six lines were randomly selected for gene transcription analysis. The result showed MdCPK1a was expressed constitutively in these lines, among which three independent lines (A4, A36, and A2) were used for analysis of the resistance to abiotic stresses. The levels of MdCPK1a mRNA in the three transgenic lines were quantified by qPCR. MdCPK1a mRNA displayed the highest level in A4 and the lowest level in A2 (S2 Fig).

Fig 2. Subcellular location of MdCPK1a. (a) Schematic representations of the vector constructs of 35S::GFP and 35S::MdCPK1a-GFP. (b) Subcellular localization of MdCPK1a-GFP fusion protein was conducted by transient expression experiment in N. benthamiana cells. Images were taken by using Leica confocal microscopy at 72 hours post agroinfiltration (GFP: fluorescence, green; Bright: visible light image; Merge: merged images of above two images). Bars = 20 μm. (c) Subcellular localization of MdCPK1a-GFP fusion protein was detected by western blot. Total protein extract (T), cell membrane fraction (Cm), cytosolic fraction (Cy), and cell nucleus fraction (Cn) isolated from 35S::GFP-expressing (left) 35S::MdCPK1a-GFP-expressing (right) tobacco cells were immunoblotted with anti-GFP antibody.
Stress tolerance of MdCPK1a-overexpressed N. benthamiana plants

For salt stress analysis, 6-week-old of WT and T₂ plants of A4, A36 and A2 were irrigated with 200 mM NaCl solution in the soil once a week. After suffering from salt stress for 25 d, WT plant leaves turn yellow, the MdCPK1a-overexpressed (MdCPK1a-OX) transgenic tobacco plants grew better and more vigorously compared with WT, (Fig 3A). The dry weight of shoots and roots of the transgenic lines, except A2, was significant higher than that of WT (Fig 3B), which suggests that MdCPK1a-OX tobacco plants increased the tolerance to salt stress. However, the similar symptoms between WT and the transgenic lines were observed under drought stress. The transgenic plants and WT displayed slow-growing, rolled and wilted leaves without irrigation for 25 d and showed no significant difference after re-watering for 10 d (Fig 4), suggesting that there were no obvious differences of drought resistance between WT and the transgenic lines.

The cold tolerance of seedlings of WT and the transgenic plants was monitored on MS medium at 4°C. Both of them showed severe growth inhibition, however, the root length of WT was significantly shorter than that of the transgenic plants (Fig 5A and 5C). Meanwhile, we also analyzed the cold resistance of them at 4 weeks old. They were stressed in 4°C for 10 d then recovered in normal conditions (25°C) for 14 d. WT plants suffered from chilling injury more severely than A4, A36, and A2. Only 17% of WT plants survived after cold treatment, while 60–96% of the transgenic lines survived (Fig 5B and 5D). Physiological analysis showed that the transgenic plants had lower MDA content and less electrolyte leakage (EL) than WT plants under cold stress (Fig 5E and 5F), indicating that the transgenic plants were less injured compared with WT plants.

Fig 3. Phenotype and stress tolerance of the MdCPK1a-OX plants under salt stress. (a) Photographs of wild-type and MdCPK1a-OX plants (line A4, A36, and A2) under 200 mM NaCl. The left shows the phenotype of aerial part of tobacco plants, the right shows the phenotype of shoot and root. (b) Shoot and root dry weight of MdCPK1a-OX plants after the salt-stress treatment. Error bars indicate the standard error of the mean (SEM) of three independent experiments. Significant differences between the WT and transgenic plants are indicated by asterisks (*p < 0.05, **p < 0.01).

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Analysis of ROS levels and antioxidant enzyme activities in transgenic *N. benthamiana*

Under abiotic stresses, reactive oxygen species (ROS) such as hydroxyl radical (\(\cdot\)HO), superoxide radical (\(O_2^-\)) or hydrogen peroxide (\(H_2O_2\)), are excessively accumulated in plants, which

![Fig 4. Photographs of wild-type and MdCPK1a-OX plants under drought stress.](https://doi.org/10.1371/journal.pone.0242139.g004)

![Fig 5. Overexpression of MdCPK1a enhances cold tolerance in transgenic tobacco.](https://doi.org/10.1371/journal.pone.0242139.g005)
act as important signal molecules and also are toxic by-products leading to oxidative damage [48]. To reduce the damage of excessive production of ROS, plants have developed a scavenging mechanism allowed them to overcome ROS toxicity. To know whether MdCPK1a regulates ROS levels in cold response, we compared with the ROS levels in the overexpressing tobacco lines and WT plants after suffering cold stress. NBT and DAB staining were used to detect the accumulation of $O_2^-$ or $H_2O_2$ in leaves, respectively. Before the cold treatment, the leaves had similar dyeing degree in the transgenic plants and WT, indicating $O_2^-$ or $H_2O_2$ accumulation was similar in both plants. However, lower dyeing degree was detected in the transgenic plants whether by NBT (Fig 6A and 6C) or DAB (Fig 6B and 6D) staining under cold stress, suggesting less ROS accumulated in the transgenic plants than that in WT. Furthermore, compared with WT, the enzyme activities of CAT, POD and SOD of the transgenic lines were higher before treatment and significantly higher after 48h cold treatment. POD activity was still significantly higher in the transgenic plants while there was no significant difference in CAT and SOD activities after 72h cold treatment (Fig 7).

**Expression analysis of the stress related genes in transgenic *N. benthamiana***

The transcriptional levels of stress-responsive and ROS-related genes (*NtSOD*, *NtGPX*, *NtCAT*, and one ROS-producing NADPH oxidase gene, *NtrbohD*) were analyzed by qPCR in transgenic *N. benthamiana*.
WT and the transgenic plants before and after cold treatments. The gene transcriptional levels of \textit{NtSOD}, \textit{NtGPX} and \textit{NtCAT} were remarkably higher in transgenic tobacco whether under normal condition or cold stress, except \textit{NtbohD} which was lower under normal condition and significantly lower after cold stress in the transgenic tobacco. The mRNA levels of cold-responsive genes (\textit{NtLEA5}, \textit{NtSPS}, \textit{NtDREB3}, except \textit{NtERD10C}) were higher in the transgenic tobacco plants than those of WT plants under normal and cold stress condition. The expression of \textit{NtERD10C} in the transgenic tobacco plants was similar with that in WT under normal condition, but higher under cold stress (Fig 8).

**Discussion**

Calcium-dependent protein kinases respond to abiotic stress and play important roles in calcium signaling pathways. In apple, CDPKs are encoded by a multigene family consisting of 37 genes [9], however, the biological functions of which mostly remain unclear. In this study, \textit{MdCPK1a} was identified in apple and characterized in transgenic tobacco. The sequence alignment of \textit{MdCPK1a} with different plant CDPKs shows high similarity with stress-responsive CDPK genes such as \textit{AtCPK1} [47, 49], \textit{OsCDPK7} [46] and \textit{ZmCPK1} [50]. It suggests that \textit{MdCPK1a} might be involved in stress tolerance.

**Apple \textit{MdCPK1a} protein localization**

CDPK function is dependent on specific subcellular localization. Previous research has shown that CDPK proteins are found in cytoplasm, nucleus, the plasma membrane, oil bodies, mitochondrial outer membrane, peroxisome, and endoplasmic reticulum [6], suggesting their different functions. The N-terminal domain of CDPKs is important to subcellular localization. It is reported that membrane association is mediated by N-terminal acylation. The membrane localized CDPKs harbour a predicted N-myristoylation site and cysteine residues which would allow further palmitoylation in their N-terminus [51]. A recent study
Fig 8. The expression of the ROS-related and cold-responsive genes in WT and MdCPK1a-OX plants. Data represent the means ± SE of at least three replicates. The significant differences between the WT and MdCPK1a-OX plants are indicated by asterisks (*p < 0.05, **p < 0.01).

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revealed that OsCPK17 has five alternative splicing (AS) forms with different subcellular localization [52]. In our experiment, MdCPK1a protein has been proved to be localized in the plasma membrane and nucleus, which is conformity with the prediction that MdCPK1a is putatively myristoylated and palmitoylated at its N-terminal (Fig 1). It indicates the post-translational modifications might allow targeting MdCPK1a protein to the plasma membrane. The location of MdCPK1a protein suggests it might participate in early signaling pathways of environmental stress by phosphorylation and activation of downstream genes [53]. The plasma membrane- or nucleus- localized CDPKs involved in abiotic stress response were also reported in other plants, such as ZoCDPK1 in Zingiber officinale [54], SiCDPK24 [29] in Setaria italica response to drought stress. Our results further indicate that CDPKs might have multiple subcellular localizations and involved in multiple signal transduction pathways.

Apple MdCPK1a involves in response to abiotic stresses

To further understand MdCPK1a function, the T2 plants of MdCPK1a overexpressing tobacco were used to study their responses to abiotic stresses. After 200 mM NaCl treatment, transgenic tobacco A4 and A36 lines showed more tolerant to salt stress, while the dry weight of shoots and roots of the transgenic lines A2 was comparable to that of the wild type, indicating that the tolerance of transgenic lines to salt stress is positive correlated to the ectopic expression levels of MdCPK1a in tobacco (Figs 3 and S2).

One of early responses to low temperature or other abiotic stresses in plant cell is a transient increase in cytosolic Ca$^{2+}$ derived from influx from the apoplastic space and release from internal stores [55]. Ca$^{2+}$ binding proteins can sense the transient increases of cytosolic Ca$^{2+}$, and then transmit signals to its target protein. CDPKs are the main responders in combining calcium signal with particular protein phosphorylation cascades. Although some studies showed that several CDPK mRNA are responsive to cold stress [8, 56–58], only a few of them were made further functional identification. In plants, as far as we know, OsCPK7, OsCPK13, OsCPK17 and OsCPK24 in rice and AtCPK1 in Arabidopsis have been reported that participated in the response to cold stress [46, 59–62]. Furthermore, the transgenic Arabidopsis plants overexpressing VaCPK20, a CDPK from Vitis amurensis and PeCPK10 from Populus euphratica improved freezing resistance [63, 64], while in Zea mays, ZmCPK1 negatively regulate cold tolerance [33]. In our research, ectopic expression of MdCPK1a improved tobacco cold tolerance and also exhibit slightly increased salt tolerance, but no obvious improvement of drought tolerance. The cold-responsive genes, such as NtDREB3(dehydration-responsive element binding protein), NtERD10C (early response to dehydration 10C), NtLEA5 (late embryogenesis abundant protein) and NtSPS (Suc-P synthase) were significantly up-regulated in the overexpressing MdCPK1a transgenic tobacco plants compared with the WT plants. It is known that DREBs are important transcription factors by regulating the expression of stress-responsive genes, including ERD10C, LEA and SPS, and so on [65, 66]. Overexpression of MdCPK1a increased cold-responsive genes in tobacco suggest that MdCPK1a may function upstream of DREBs as a positive regulator participated in the response to cold stress. Additionally, the root length of transgenic plants A4 and A36 is longer than that of WT when the seedlings of them cultured at 25˚C for 10 d on MS medium (Fig 5A). The aerial part of WT plants is little lower than that of the transgenic plants under normal condition (Fig 4). We speculated that MdCPK1a might also participate in the regulation of plant development. Our previous research showed that MdCPK1a was also induced by biotic stress [9]. These results suggest that apple MdCPK1a like CDPK genes in other species has overlapping functions [33, 67, 68].
Overexpressing *MdCPK1a* enhanced tolerance to cold stress in the transgenic tobacco by scavenging ROS

Plants produced less ROS in organelles under optimal growth conditions, but under abiotic stress, the rate of ROS production is significantly elevated. ROS was produced by two major sources under abiotic stress: one is as a consequence of disruptions in metabolic activity and another is as signaling ROS which produced by NADPH oxidase [69]. ROS accumulation is a double-edged sword for plants response to abiotic stress: on the one hand, they are signaling molecules of the abiotic stress–response signal transduction network [70], on the other hand, they are also toxic byproducts that can cause oxidative destruction of cell [48, 71]. In general, CDPKs seem to function as positive regulators of ROS production in biotic stress signaling [49, 72–75], while some researches showed that CDPKs decrease ROS accumulation in abiotic stress by increasing the expression of ROS scavenging enzymes such as ascorbate peroxidase (APX), superoxide dismutase(SOD), catalase(CAT), and glutathione peroxidase(GPX) [76, 77]. For example, overexpression of the constitutively active form of oilseed rape *BnaCPK2* induces ROS accumulation and cell death through interacting with NADPH oxidase-like respiratory burst oxidase homolog D (RbohD) [78]. However, overexpression *OsCPK12* decreases ROS accumulation by increasing the expression of *OsAPx2, OsAPx8* and *Osrboh1* and confers increased tolerance to salt stress in rice [25]. Overexpression of *OsCPK4* in rice confers salt and drought tolerance by preventing cellular membranes from stress-induced oxidative damage [26]. In this study, overexpression of *MdCPK1a* in tobacco promoted the tolerance to cold stress by decreasing the expression of *NtRbohD* and increasing the expression of *NtSOD, NtCAT* and *NtGPX*. Compared with WT plants, the enzyme activities of CAT, POD, and SOD is higher and the accumulation of ROS was less in transgenic tobacco plants under cold stress. Collectively, these results suggest that *MdCPK1a* plays roles in abiotic stresses, and ectopic expression of *MdCPK1a* gene in tobacco enhances the tolerance to cold stress, which contributes to increasing the transcription levels of stress-relative genes and regulating the expression of APX, CAT, SOD and rbohD to reduce the damage to plants caused by ROS accumulation.

**Conclusion**

In this research, a CDPK gene *MdCPK1a* from apple was characterized. *MdCPK1a* protein was found to localize the plasma membrane and the nucleus. Overexpression *MdCPK1a* in tobacco plants showed significantly improved their cold and salt stress tolerance than the wild type. Furthermore, Tobacco plants transfected with *MdCPK1a* showed increased resistance to cold stress by scavenging ROS accumulation and modulating the expression of stress-related genes. These results will be useful to further explore the function of *MdCPK1a* in apple.

**Supporting information**

S1 Table. Primer sequences used for cloning, subcellular localization, vector construction, transgenic confirmation and expression analysis. (TIF)

S1 Fig. Phylogenetic relationship between *MdCPK1a* and other CDPK proteins. The unrooted tree was generated using MEGA 6.0 program (http://www.megasoftware.net/) by the neighbor-joining method. Bootstrap supports from 500 replicates are indicated at each branch. (TIF)
S2 Fig. The identification of transgenic tobacco. (a) Schematic representations of the vector constructs of pYH455-MdCPK1a. (b) Ten T1 lines of transgenic tobacco were confirmed by PCR with specific primers (PST1). (c) Six T1 lines of transgenic tobacco were confirmed by RT-PCR with specific primers (PST1). (d) Three T2 lines of transgenic tobacco were confirmed by RT-PCR with specific primers. (e) Quantification the expression of MdCPK1a mRNAs in the transgenic tobacco plants performed by real-time RT-PCR. RNA was extracted from the leaves of WT and MdCPK1a-transformed tobacco plant lines (A4, A36, and A2). Transcript abundance was normalized against the Nttubulin gene expression level. Data represent means and standard errors of three replicates. Significant differences between the WT and transgenic plants are indicated by asterisks (*p < 0.05, **p < 0.01).

(TIF)

S1 Raw images.

(ZIP)

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References

1. Sanders D, Brownlee C, Harper JF. Communicating with calcium. Plant Cell. 1999; 11: 691–706. https://doi.org/10.1105/tpc.11.4.691 PMID: 10213787

2. Harmon AC, Gribskov M, Harper JF. CDPKs—a kinase for every Ca²⁺ signal? Trends Plant Sci. 2000; 5: 154–159. https://doi.org/10.1016/s1360-1385(00)01577-6 PMID: 10740296

3. Valmonte GR, Arthur K, Higgins CM, MacDiarmid RM. Calcium-Dependent Protein Kinases in Plants: Evolution, Expression and Function. Plant Cell Physiol. 2014; 55: 551–569. https://doi.org/10.1093/pcp/pct200 PMID: 24363288

4. Harmon AC, Gribskov M, Gubrium E, Harper JF. The CDPK superfamily of protein kinases. New Phytol. 2001; 151: 175–183.

5. Boudsocq M, Sheen J. CDPKs in immune and stress signaling. Trends Plant Sci. 2013; 18: 30–40. https://doi.org/10.1016/j.tplants.2012.08.008 PMID: 22974587
6. Harper JE, Breton G, Harmon A. Decoding Ca\textsuperscript{2+} signals through plant protein kinases. Annu Rev Plant Biol. 2004; 55: 263–288. https://doi.org/10.1146/annurev.arplant.55.031903.141627 PMID: 15377221

7. Ma P, Liu J, Yang X, Ma R. Genome-wide identification of the maize calcium-dependent protein kinase gene family. Appl Biochem Biotechnol. 2013; 169: 2111–2125. https://doi.org/10.1007/s12010-013-0125-2 PMID: 23397323

8. Ray S, Agarwal P, Arora R, Kapoor S, Tyagi AK. Expression analysis of calcium-dependent protein kinase gene family during reproductive development and abiotic stress conditions in rice (Oryza sativa L, ssp. indica). Mol Genet Genomics. 2007; 278: 493–505. https://doi.org/10.1007/s00438-007-0267-4 PMID: 17636330

9. Wei M, Wang S, Dong H, Cai B, Tao J. Characterization and comparison of the CPK gene family in the apple (Malus domestica) and other Rosaceae species and its response to Alternaria alternata infection. PLoS One. 2016; 11: e0155590. https://doi.org/10.1371/journal.pone.0155590 PMID: 27186637

10. Xu X, Liu M, Lu L, He M, Qu W, Xu Q, et al. Genome-wide analysis and expression of the calcium-dependent protein kinase gene family in cucumber. Mol Genet Genomics. 2015; 290: 1403–1414. https://doi.org/10.1007/s00438-015-1002-1 PMID: 25687625

11. Zuo R, Hu R, Chai G, Xu M, Qi G, Kong Y, et al. Genome-wide identification, classification, and expression analysis of CDPK and its closely related gene families in poplar (Populus trichocarpa). Mol Biol Rep. 2013; 40: 2645–2662. https://doi.org/10.1007/s11033-012-2351-z PMID: 23242656

12. Cheng SH, Willmann MR, Chen HC, Sheen J. Calcium signaling through protein kinases. The Arabidopsis calcium-dependent protein kinase gene family. Plant Physiol 2002; 129: 469–485. https://doi.org/10.1104/pp.005645 PMID: 12068094

13. Hrabak EM, Chan CWM, Gribkov M, Harper JF, Choi JH, Halford N, et al. The Arabidopsis CDPK- SnRK superfamily of protein kinases. Plant Physiol 2003; 132: 666–680. https://doi.org/10.1104/pp.102.011999 PMID: 12805596

14. Harper J, Huang J, Lloyd S. Genetic identification of an autoinhibitor in CDPK, a protein-kinase with a calmodulin-like domain. Biochemistry 1994; 33: 7267–7277. https://doi.org/10.1021/bi00189a031 PMID: 8003490

15. Ishino T, Orito Y, Chinzei Y, Yuda M. A calcium-dependent protein kinase regulates Plasmodium ookinetes access to the midgut epithelial cell. Mol Microbiol 2006; 59: 1175–1184. https://doi.org/10.1111/j.1365-313X.2006.03501.x PMID: 16430692

16. Liese A, Romeis T. Biochemical regulation of in vivo function of plant calcium-dependent protein kinases (CDPK). BBA-Mol Cell Res. 2013; 1833: 1582–1589.

17. Schulz P, Herde M, Romeis T. Calcium-dependent protein kinases: Hubs in plant stress signaling and development. Plant Physiol 2013; 163: 523–530. https://doi.org/10.1104/pp.113.222539 PMID: 24014579

18. Wernimont A, Artz J, Finerty P, Lin Y, Amani M, Allali-Hassani A, et al. Structures of apicomplexan calcium-dependent protein kinases reveal mechanism of activation by calcium. Nat Struct Mol Biol 2010; 17: 596–595. https://doi.org/10.1038/nsmb.1795 PMID: 20436473

19. Sebastia CH, Hardin SC, Clouse SD, Kieber JJ, Huber SC. Identification of a new motif for CDPK phosphorylation in vitro that suggests ACC synthase may be a CDPK substrate. Arch Biochem Biophys 2004; 428: 81–91. https://doi.org/10.1016/j.abb.2004.04.025 PMID: 15234272

20. Allwood EG, Smertenko AP, Hussey PJ. Phosphorylation of plant actin-depolymerising factor by calmodulin-like domain protein kinase. FEBS letters 2001; 499: 97–100.

21. Xing Y, Guo S, Chen X, Du D, Liu M, Xiao Y, et al. Nitrogen metabolism is affected in the nitrogen-deficient mutant esl4 with a calcium-dependent protein kinase gene mutation. Plant Cell Physiol. 2018; 59(12), 2512–2525. https://doi.org/10.1093/pcp/pcy169 PMID: 30165687

22. Klimecka M, Muszynska G. Structure and functions of plant calcium-dependent protein kinases. Acta Biochim Pol 2007; 54: 219–233. PMID: 17446936

23. Gao A, Wu Q, Zhang Y, Miao Y, Song C. Arabidopsis calcium-dependent protein kinase CPK28 is potentially involved in the response to osmotic stress. Chinese Sci Bull. 2014; 59: 1113–1122.

24. Wei S, Hu W, Deng X, Zhang Y, Liu X, Zhao X, et al. A rice calcium-dependent protein kinase OsCPK9 positively regulates drought stress tolerance and spikelet fertility. BMC Plant Biol. 2014; 14: 133. https://doi.org/10.1186/1471-2229-14-133 PMID: 24884869

25. Asano T, Hayashi N, Kobayashi M, Aoki N, Miyao A, Mitsuhara I, et al. A rice calcium-dependent protein kinase OsCPK12 oppositely modulates salt-stress tolerance and blast disease resistance. Plant J. 2012; 69: 26–36. https://doi.org/10.1111/j.1365-313X.2011.04766.x PMID: 21883553

26. Campo S, Baldrich P, Messegue J, Lalanne E, Coca M, San Segundo B. Overexpression of a calcium-dependent protein kinase confers salt and drought tolerance in rice by preventing membrane lipid peroxidation. Plant Physiol. 2014; 165: 688–704. https://doi.org/10.1104/pp.113.230268 PMID: 24784760
27. Bundo M, Coca M. Enhancing blast disease resistance by overexpression of the calcium-dependent protein kinase OsCPK4 in rice. Plant Biotech J. 2016; 14: 1357–1367. https://doi.org/10.1111/pbi.12500 PMID: 26578239

28. Shen L, Yang S, Yang T, Liang J, Cheng W, Wen J, et al. CaCDPK15 positively regulates pepper responses toRalstonia solanacearum inoculation and forms a positive-feedback loop with CaWRKY40 to amplify defense signaling. Sci Rep. 2016; 6. https://doi.org/10.1038/srep22439 PMID: 26928570

29. Yu T, Zhao W, Fu J, Liu Y W, Chen M, Zhou Y, et al. Genome-wide analysis of CDPK family in foxtail millet and determination of SiCDPK24 functions in drought stress. Front Plant Sci. 2018; 9. https://doi.org/10.3389/fpls.2018.00651 PMID: 30093908

30. Ho SL, Huang LF, Lu CA, He SL, Wang CC, Yu SP, et al. Sugar starvation- and GA-inducible calcium-dependent protein kinase 1 feedback regulates GA biosynthesis and activates a 14-3-3 protein to confer drought tolerance in rice seedlings. Plant Mol Biol. 2013; 81: 347–361. https://doi.org/10.1007/s11103-012-0606-z PMID: 23329372

31. He SL, Jiang JZ, Chen BH, Kuo CH, Ho SL. Overexpression of a constitutively active truncated form of OsCDPK1 confers disease resistance by affecting OsPR10a expression in rice. Sci Rep. 2018; 8: 403. https://doi.org/10.1038/s41598-017-18629-2 PMID: 29321675

32. Ma S, Wu W. AtCPK23 functions in Arabidopsis responses to drought and salt stresses. Plant Mol Biol. 2007; 65:511–518. https://doi.org/10.1007/s11103-007-9187-2 PMID: 17541706

33. Weckwerth P, Ehler B, Romeis T. ZmCPK1, a calcium-independent kinase member of the Zea mays CDPK gene family, functions as a negative regulator in cold stress signalling. Plant Cell Environ. 2015; 38: 544–558. https://doi.org/10.1111/j.1365-3141.2014.25059212

34. Monaghan J, Matschi S, Shorinola O, Rovenich H, Matei A, Segonzac C, et al. The calcium-dependent protein kinase CPK28 buffers plant immunity and regulates BIK1 turnover. Cell Host Microbe. 2014; 16: 605–615. https://doi.org/10.1016/j.chom.2014.10.007 PMID: 25525792

35. Chang S, Puryear J, Cairney J A. Simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep. 1993; 11: 113–116.

36. Ren J, Wen L, Gao X, Jin C, Xue Y, Yao X. CSS-palm 2.0: an updated software for palmitoylation sites prediction, Protein Eng Des Sel. 2008; 21:639–644. https://doi.org/10.1093/protein/gzn039 PMID: 18753194

37. Xie Y, Zheng Y, Li H, Luo X, He Z, Cao S, et al. GPS-lipid: a robust tool for the prediction of multiple lipid modification sites, Sci Rep. 2016; 6:28249 https://doi.org/10.1038/srep28249 PMID: 27306108

38. Pan Z, Liu Z, Cheng H, Wang Y, Gao T, Ullah S, et al. Systematic analysis of the in situ crosstalk of tyrosine modifications reveals no additional natural selection on multiply modified residues, Sci Rep. 2014; 4:7331 https://doi.org/10.1038/srep07331 PMID: 25476580

39. Sheludko YV, Sindarovska Y R, Gerasymenko IM, Bannikova M A, Kuchuk NV. Comparison of several Nicotiana species as hosts for high-scale Agrobacterium-mediated transient expression. Biotechnol Bioeng, 2007; 96(3), 608–614. https://doi.org/10.1002/bit.21075 PMID: 16983697

40. Gallois P, Marinho P. Leaf disk transformation using Agrobacterium tumefaciens- expression of heterologous genes in tobacco. In Plant gene transfer and expression protocols, pp. 1995; 39–48. Springer.

41. Heath RL, Packer L. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys. 1968; 125: 189–198. https://doi.org/10.1016/0003-9861(68)90654-1 PMID: 5655425

42. Liu JH, Nada K, Honda C, Kitashiba H, Wen XP, Pang XM, et al. Polyamine biosynthesis of apple callus under salt stress: importance of the arginine decarboxylase pathway in stress response. J Exp Bot. 2006; 57: 2589–2599. https://doi.org/10.1093/jxb/erl018 PMID: 16825316

43. Paolotti F, Aldinucci D, Mocali A, Caparrini A. A sensitive spectrophotometric method for the determination of superoxide dismutase activity in tissue extracts. Anal Biochem. 1986; 154: 536–541. https://doi.org/10.1016/0003-2697(86)90026-6 PMID: 3089061

44. Jabs T, Dietrich RA, Dangi JL. Initiation of runaway cell death in an Arabidopsis mutant by extracellular superoxide. Science. 1996; 273: 1853–1856. https://doi.org/10.1126/science.273.5283.1853 PMID: 8791589

45. Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB. Subcellular localization of H$_2$O$_2$ in plants. H$_2$O$_2$ accumulation in papillae and hypersensitive response during the barley—powdery mildew interaction. Plant J, 1997; 11(6): 1187–1194.

46. Saijo Y, Hata S, Kyozuka J, Shimamoto K, Izui K. Over-expression of a single Ca$^{2+}$-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J. 2000; 23: 319–327. https://doi.org/10.1046/j.1365-313x.2000.00787.x PMID: 10929125

47. Coca M, Segundo B. AtCPK1 calcium-dependent protein kinase mediates pathogen resistance in Arabidopsis. Plant J. 2010; 63: 526–540. https://doi.org/10.1111/j.1365-313X.2010.04255.x PMID: 20497373
48. Das K, Roychoudhury A. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Front Environ Sci. 2014; 2 (53).

49. Cheng SH, Sheen J, Gerrish C, Bolwell GP. Molecular identification of phenylalanine ammonia-lyase as a substrate of a specific constitutively active Arabidopsis CDPK expressed in maize protoplasts. FEBS letters. 2001; 503: 185–188. https://doi.org/10.1016/s0014-5793(01)02732-6 PMID: 11513879

50. Berberich T, Kusano T. Cycloheximide induces a subset of low temperature-inducible genes in maize. Mol Gen Genet. 1997; 254: 275–283 https://doi.org/10.1007/s004380050416 PMID: 9150261

51. Simeunovic A, Mxair A, Wurzinger B, Teige M. Know where your clients are: subcellular localization and targets of calcium-dependent protein kinases, J Exp Bot. 2016; 67: 3855–3872. https://doi.org/10.1093/jxb/erw157 PMID: 27117335

52. Almadanim MC, Gonçalves NM, Rosa MTG, Alexandre BM, Cordeiro AM, Rodrigues M, et al. The rice cold-responsive calcium-dependent protein kinase OsCPK17 is regulated by alternative splicing and post-translational modifications. BBA-Mol Cell Res, 2018, 1865(2): 231–246. https://doi.org/10.1016/j.bbamcr.2017.01.010 PMID: 29100789

53. Seybold H, Trempel F, Ranf S, Scheel D, Romeis T, Lee J. Ca$^{2+}$ signalling in plant immune response: from pattern recognition receptors to Ca$^{2+}$ decoding mechanisms. New Phytol. 2014; 204: 782–790. https://doi.org/10.1111/nph.13031 PMID: 25539002

54. Vivek PJ, Tuteja N, Soniya EV. CDPK1 from ginger promotes salinity and drought stress tolerance with- out yield penalty by improving growth and photosynthesis in Nicotiana Tabacum. Plos One, 2013; 8. https://doi.org/10.1371/journal.pone.0076392 PMID: 24194837

55. Knight H. Calcium signaling during abiotic stress in plants. International Review of Cytology—a Survey of Cell Biology, Vol 195. 2000; 195: 269–324. https://doi.org/10.1016/s0074-7696(08)62707-2 PMID: 10603578

56. Kong X, Lv W, Jiang S, Zhang D, Cai G, Pan J, et al. Genome-wide identification and expression analy- sis of calcium-dependent protein kinase in maize. BMC genomics. 2013; 14. https://doi.org/10.1186/1471-2164-14-433 PMID: 23815483

57. Martin ML, Busconi L. A rice membrane-bound calcium-dependent protein kinase is activated in re- sponse to low temperature. Plant Physiol. 2001; 125: 1442–1449. https://doi.org/10.1104/pp.125.3. 1442 PMID: 11244123

58. Wan B, Lin Y, Mou T. Expression of rice Ca2+-dependent protein kinases (CDPKs) genes under differ- ent environmental stresses. FEBS letters. 2007; 581: 1179–1189. https://doi.org/10.1016/j.febslet. 2007.02.030 PMID: 17336300

59. Abbasi F, Onodera H, Toki S, Tanaka H, Komatsu S. OsCDPK13, a calcium-dependent protein kinase gene from rice, is induced by cold and gibberellin in rice leaf sheath. Plant Mol Biol. 2004; 55: 541–552. https://doi.org/10.1007/s11103-004-1178-y PMID: 15604699

60. Almadanim MC, Alexandre BM, Rosa MT, Sapeta H, Leitão AE, Ramalho JC, et al. Rice calcium-depen- dent protein kinase gene OsCPK17 targets plasma membrane intrinsic protein and sucrose-phosphate synthase and is required for a proper cold stress response. Plant Cell Environ. 2017; 40(7): 1197–1213. https://doi.org/10.1111/pce.12916 PMID: 28102545

61. Komatsu S, Yang G, Khan M, Onodera H, Toki S, Yamaguchi M. Over-expression of calcium-depen- dent protein kinase 13 and calreticulin interacting protein 1 confers cold tolerance on rice plants. Mol Genet Genomics. 2007; 277: 713–723. https://doi.org/10.1007/s00438-007-0220-6 PMID: 17318583

62. Liu Y, Xu C, Zhu Y, Zhang L, Chen T, Zhou F, et al. The calcium-dependent kinase OsCPK24 functions in cold stress responses in rice: J Integr Plant Biol, 2018; 60(2):173–188. https://doi.org/10.1111/jipb. 12164 PMID: 29193704

63. Dubrovin AS, Kiselev KV, Khristenko VS, Aleynova OA. VaCPK20, a calcium-dependent protein kinase gene of wild grapevine Vitis amurensis Rupr., mediates cold and drought stress tolerance. J Plant Physiol. 2015, 185: 1–12. https://doi.org/10.1016/j.jplph.2015.05.020 PMID: 26264965

64. Chen J, Xue B, Xia X, Yin W. A novel calcium-dependent protein kinase gene from Populus euphratica, confers both drought and cold stress tolerance. Biochem Biophs Res Co. 2013; 441: 630–636. https://doi.org/10.1016/j.bbrc.2013.10.103 PMID: 24177011

65. Cook D, Fowler S, Fiehn O, Thomashow MF. A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis. Proc Natl Acad Sci USA. 2004; 101: 15243–15248. https://doi.org/10.1073/pnas.0406069101 PMID: 15383661

66. Wu L, Zhang Z, Zhang H, Wang X, Huang R. Transcriptional modulation of ethylene response factor protein JERF3 in the oxidative stress response enhances tolerance of tobacco seedlings to salt, drought, and freezing. Plant Physiol. 2008; 148:1953–1963. https://doi.org/10.1104/pp.108.126813 PMID: 18945933
67. Matschi S, Werner S, Schulze WX, Legen J, Hilger HH, Romeis T. Function of calcium-dependent protein kinase CPK28 of Arabidopsis thaliana in plant stem elongation and vascular development. Plant J. 2013; 73: 883–896. https://doi.org/10.1111/tpj.12090 PMID: 23252373

68. Myers C, Romanowski SM, Barron YD, Garg S, Azuse CL, Curran A, et al. Calcium-dependent protein kinases regulate polarized tip growth in pollen tubes. Plant J. 2009; 59: 528–539. https://doi.org/10.1111/j.1365-313X.2009.03894.x PMID: 19392698

69. Leshem Y, Seri L, Levine A. Induction of phosphatidylinositol 3-kinase-mediated endocytosis by salt stress leads to intracellular production of reactive oxygen species and salt tolerance. Plant J. 2007; 51: 185–197. https://doi.org/10.1111/j.1365-313X.2007.03134.x PMID: 17521408

70. Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ. 2010; 33: 453–467. https://doi.org/10.1111/j.1365-3040.2009.02041.x PMID: 19712065

71. Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol. 2004; 55: 373–399. https://doi.org/10.1146/annurev.arplant.55.031903.141701 PMID: 15377225

72. Kobayashi M, Ohura I, Kawakita K, Yokota N, Fujiwara M, Shimamoto K, et al. Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. Plant Cell. 2007; 19:1065–80. https://doi.org/10.1105/tpc.106.048884 PMID: 17400895

73. Boudsocq M, Willmann MR, McCormack M, Lee H, Shan L, He P, et al. Differential innate immune signalling via Ca++ sensor protein kinases. Nature, 2010; 464(7287):418–422. https://doi.org/10.1038/nature08794 PMID: 20164835

74. Dubiella U, Seybold H, Durian G, et al. Calcium-dependent protein kinase / NADPH oxidase activation circuit is required for rapid defense signal propagation. Proc Natl Acad Sci USA. 2013; 110(21):1–6. https://doi.org/10.1073/pnas.1221294110 PMID: 23650383

75. Kadota Y, Sklenar J, Derbyshire P, et al. Direct regulation of the NADPH oxidase RBOHD by the PRR-associated kinase BIK1 during plant immunity. Mol Cell, 2014, 54(1):43–55. https://doi.org/10.1016/j.molcel.2014.02.021 PMID: 24630626

76. Iba K. Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. Annu Rev Plant Biol. 2002; 53: 225–245. https://doi.org/10.1146/annurev.arplant.53.100201.160729 PMID: 12221974

77. Mittler R, Vanderauwera S, Gollery M, Van Breusegem F. Reactive oxygen gene network of plants. Trends Plant Sci. 2004; 9: 490–498. https://doi.org/10.1016/j.tplants.2004.08.009 PMID: 15465684

78. Wang W, Zhang H, Wei X, Yang L, Yang B, Zhang L, et al. Functional characterization of calcium-dependent protein kinase (CPK) 2 gene from oilseed rape (Brassica napus L.) in regulating reactive oxygen species signaling and cell death control. Gene. 2018; 651, 49–56. https://doi.org/10.1016/j.gene.2018.02.006 PMID: 29408396