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Research article

The involvements of calcium-dependent protein kinases and catechins in tea plant [*Camellia sinensis* (L.) O. Kuntze] cold responses

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ABSTRACT

Temperature is one of the most important environmental factors limiting tea plant growth and tea production. Previously we reported that both Ca^{2+} and ROS signals play important roles in tea plant cold acclimation. Here, we identified 26 *CsCPK* transcripts, analyzed their phylogenetic and sequence characters, and detected their transcriptions to monitor Ca^{2+} signaling status. Tissue-specific expression profiles indicated that most *CsCPK* genes were constitutively expressed in tested tissues, suggesting their possible roles in development. Cold along with calcium inhibitor assays suggested that *CsCPKs* are important cold regulators and *CsCPK30/5/4/9* maybe the key members. Moreover, $LaCl_3$ or EGTA pre-treatment could result in impaired Ca^{2+} signaling and compromised cold-responding network, but higher catechins accumulation revealed their potential positive roles in cold responses. Those findings indicated that catechins and other secondary metabolites in tea plant may form an alternative cold-responding network that closely correlated with Ca^{2+} signaling status.

1. Introduction

Plants grown in constantly changing environments are confronted with various unfavorable conditions, including abiotic stresses, i.e., cold, heat, salinity, drought, and nutrient deficiency, and biotic stresses, i.e., pathogen infection and herbivore attacks. To adapt to these stressful environments, plants have developed several signaling systems such as Ca^{2+}, ROS, hormones to perceive and transmit stress signals to their cells. Among these, calcium signaling is the most important one that usually acts as a fundamental signaling system (Kudla et al., 2010; Valmonte et al., 2014). Generally, environmental stresses are first recognized by membrane receptors that cause cytosolic calcium (Ca^{2+}) ion level changes and generate calcium signals (McAinsh and Hetherington, 1998). Spatiotemporal parameters of the cytoplasmic Ca^{2+} level such as the amplitude, duration and frequency are accepted as Ca^{2+} signatures that encode signal information (Batistič and Kudla, 2012; Kudla et al., 2018). These Ca^{2+} signatures are sensed and transported by a vast array of calcium sensors that are usually EF-hand-containing proteins, i.e., calcium-dependent protein kinases (CPKs, also called CDPKs) (Sheen, 1996), calcineurin B-like proteins (CBLs) (Batistič and Kudla, 2009), calmodulins (CaMs) and CaM-like proteins (CMLs) (Snedden and Fromm, 1998). These calcium sensors can decode Ca^{2+} signatures, transduce them into specific protein-protein interactions, and lead to further physiological changes to adapt to environmental changes (Batistič and Kudla, 2012; Boudsocq and Sheen, 2013; Edel and Kudla, 2016).

Among these calcium sensors, CPKs are the best characterized and are unique for their direct sensing, decoding and transduction of Ca^{2+} signals. Typically, plant CPKs are serine/threonine (Ser/Thr) protein kinases (PKs) that contain four conserved domains: an N-terminal variable domain (N-VD), a PK domain, an autoinhibitory junction (AJ) and a C-terminal CaM-like domain (also called the calcium-binding domain, CBD) (Harmon et al., 2001; Boudsocq et al., 2012; Schulz et al., 2013; Valmonte et al., 2014). Under low Ca^{2+} concentrations or

Abbreviations: CPK, Calcium-dependent protein kinase; EGTA, Ethylene glycol tetraacetic acid; PK, protein kinases domain; ROS, Reactive oxygen species

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without Ca^{2+} stimulation, the AJ keeps CPK reside in an inactive state via a pseudosubstrate mechanism (Harmon et al., 2001). Under stress conditions, the Ca^{2+} level increases and causes Ca^{2+} ions to bind to the CBD, triggering protein conformational changes and the release of autoinhibition, resulting in the activation of CPKs (Christodoulou et al., 2004). This working model allows CPKs to recognize specific Ca^{2+} signatures and transduce them into downstream protein phosphorylation cascades, then to further responses like transcriptional changes.

In higher plants, CPKs are encoded by large multigene families (Hamel et al., 2014). The CPK family consists of 34 members in the model eudicot *Arabidopsis thaliana* (Cheng et al., 2002), 31 members in the model monocot rice (Ray et al., 2007), and 30 members in the model tree poplar (Zuo et al., 2013). Studies have shown that CPKs are involved in both of the development and stress response of plants (Boudsocq and Sheen, 2013). In *A. thaliana*, *AtCPK2/17/20/34* regulate pollen tube growth and elongation (Myers et al., 2010; Shi et al., 2018), and *AtCPK6/33* (Kawamoto et al., 2015) are involved in flower development. In addition, *AtCPK1/10/21* (Xu et al., 2010; Franz et al., 2011; Huang et al., 2018), *OsCPK4/12* (Asano et al., 2012; Campo et al., 2014) and *ZmCDPK4* (Jiang et al., 2013) are proposed play important roles in response to drought and salt stresses. Rice *OsCPK7/17/24* (Abbasi et al., 2004; Almadanim et al., 2018; Liu et al., 2018), maize *ZmCDPK1* (Weckwerth et al., 2015) and grape *VaCDPK20* (Dubrovina et al., 2015) are found involved in cold responses. *AtCPK5* (Liu et al., 2017) and *OsCPK4/12* (Asano et al., 2012; Wang et al., 2018a) are proposed positive modulators of biotic stress responses. Moreover, most of these CPKs mediate stress responses that are found correlated with other signaling pathways such as reactive oxygen species (ROS) and abscisic acid. In Arabidopsis, *AtCPK28/16/10/11* are proposed as important regulators of hormone generation (Abo-El-Saad and Wu, 1995; Rietz et al., 2010; Zhao et al., 2011). Nowadays, the expansion of plant genome sequences has facilitated studies of CPK function and evolution. However, the comprehensive analysis of algal and higher-plant CPKs conducted by Valmonte et al. suggested that CPKs grouping pattern and sequence conservation are only slightly correlated with gene functional diversity (Valmonte et al., 2014), suggesting studies refer to individual CPK in a particular species becomes more complicate.

Tea plant, an evergreen woody plant species that originated in the warm area in Southwest China, is one of the most important economic crops in Asia, Africa and Latin American developing countries (Taniguchi et al., 2014). Temperature is one of the most important environmental factors affecting tea plant growth and tea production, especially the sharp temperature decrease occurring in the spring seasons, which has caused considerable economic loss (Wang et al. 2012, 2013). In comparison to most crops, tea plant is special for the rich bioactive compounds in its leaves (Namita et al., 2012). Among these metabolites, catechins are one of the most characteristic compounds in both of fresh leaves and various tea beverages. Research findings suggest that catechins function as effective antioxidants in preventing oxidative stresses and some human diseases (Higdon and Frei, 2003). We previously proposed that catechins were involved in tea plant cold responses through unknown mechanisms (Ding et al., 2018). As well known, Ca^{2+} signaling plays a fundamental role in plants stress responses. In this study, our main purpose is trying to figure out whether the content changes of catechins in tea plant young leaves under cold condition is related to Ca^{2+} signaling. Therefore, we conducted gnome-wide identification of *CsCPK* genes, analyzed their sequence characters, and used their expression profiles as an indicator of Ca^{2+} signaling status. Moreover, we also detected the catechin content changes with or without of Ca^{2+} signal transduction inhibitors under cold conditions. These results provided basic information for the function studies of *CsCPK* family and catechins in tea plant cold tolerance regulation, and also provided a way to identify the function of other bioactive metabolites in tea plant leaves.

2. Materials and methods

2.1. Plant materials and cold and chemical double treatments

Five-year-old tea plants (*Camellia sinensis* (L.) O. Kuntze *cv Zhongcha108* (ZC108)) grown in the greenhouse of the Tea Research Institute (TRI), Chinese Academy of Agricultural Sciences (CAAS, N30°10′, E120°5′), Hangzhou, China, under 14 h light (25 °C)/10 h dark (20 °C), 12, 000 lx light intensity and with a relative humidity of 70%–75% were used in this study. For cold and Ca^{2+} inhibitor dual-treatments, 500 ml of distilled water (control) or 5 mM $LaCl_3$ or 5 mM ethylene glycol tetraacetic acid (EGTA) (all with 0.2% [*v/v*] Tween-20) was first spread evenly on each plant (Almadanim et al., 2017; Shi et al., 2012). The growth chamber conditions were set to 14 h light at 4 °C/10 h dark at 2 °C (it took approximately 10–15 min to cool down to 4 °C). The top bud and first two leaves (young shoots) were collected immediately after chemical treatments (defined as 0 h) and 3 h, 6 h, 24 h, 48 h, 72 h, 96 h after the chamber temperature had reached 4 °C and after 72 h (R72 h) of recovery from cold treatments. Samples were frozen in liquid nitrogen before being stored at −80 °C for later use.

2.2. Genome-wide retrieval, conserved domain, gene structure and promoter analysis

The Arabidopsis and rice CPK protein sequences were first obtained from The Arabidopsis Information Resource (*TAIR*, https://www.arabidopsis.org) and the Rice Genome Annotation Project (RGAP, http://rice.plantbiology.msu.edu), respectively. The genome sequence of tea cultivar Shuchazao were download from Tea Plant Information Archive (TPIA, http://tpia.teaplant.org). To identify the *CPK* genes in tea plant, a local blast search was performed using AtCPK and OsCPK protein sequences as queries, genomic sequences of the tea cultivar Shuchazao (Wei et al., 2018) and the published transcriptomic sequences (including those for the Longjing 43, Zhongcha108, Zhenong 12, Damianbai, and Zhenong 113 cultivars) were used as databases. The blast results were first analyzed, reannotated, and then verified for the presence of the PK domain using the PFAM (http://pfam.xfam.org), SMART (http://smart.embl-heidelberg.de/help/smart_glossary.shtml) and ScanProsite (https://prosite.expasy.org) online tools.

For *cis*-acting elements analysis, sequences of *CsCPKs* promoter regions in the tea plant (defined as the 2000 bp upstream sequences from the translational start site) were used in the plantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) online tool and analyzed with default parameters (Lescot et al., 2002).

2.3. Phylogenetic analysis

Full-length CPK protein sequences retrieved from TAIR, RGAP and Phytozome 13.0 database were used for phylogenetic analysis. An unrooted phylogenetic tree was constructed with MEGA 7.0 software using the neighbor-joining (NJ) method with 1000 bootstrap replicates (Kumar et al., 2016).

2.4. Gene structure and motif analysis

The exon/intron organization of each *CsCPK* gene was illustrated using the GSDS2.0 (http://gsds.cbi.pku.edu.cn) online tool by alignment of the coding sequences with their corresponding DNA sequences retrieved from the Shuchazao genome (Hu et al., 2015; Singh et al., 2017). Conserved motifs were analyzed using the Multiple Em for Motif Elicitation (MEME) suite (http://meme-suite.org/tools/meme) with the following search parameters: a maximum of five motifs with a width of 6–50 amino acids and 2 to 26 sites per motif.

Table 1
Bioinformatic information of *CsCPK* transcripts identified from tea plant.

Gene name	Genome ID (Shuchazao)	Scaffold location	Strand (−/+)	Arabidopsis homologs	Protein length (amino acid)	Gene size (bp)	No. of EF-hand	Protein kinase domain
CsCPK1a	TEA011744.1	Scaffold3778	–	AtCPK1	575	5828	4	Y
CsCPK1b	TEA030551.1	Scaffold3449	–	AtCPK1	574	18996	4	Y
CsCPK2	TEA026895.1	Scaffold2232	–	AtCPK2	609	5004	4	Y
CsCPK4	TEA005283.1	Scaffold434	–	AtCPK4	561	4019	4	Y
CsCPK5	TEA023488.1	Scaffold3964	–	AtCPK5	565	5296	4	Y
CsCPK6	TEA023488.1	Scaffold268	+	AtCPK6 (AtCDPK3)	487	5685	4	Y
CsCPK11	TEA005283.1	Scaffold1672	+	AtCPK11 (AtCDPK2)	506	17254	4	Y
CsCPK20	TEA000145.1	Scaffold3096	+	AtCPK20	608	9746	4	Y
CsCPK3a	TEA007308.1	Scaffold1963	–	AtCPK3 (AtCDPK6)	505	7253	4	Y
CsCPK3b	TEA007308.1	Scaffold763	–	AtCPK3 (AtCDPK6)	516	8670	4	Y
CsCPK9	TEA017145.1	Scaffold206	+	AtCPK9	528	4828	4	Y
CsCPK17a	TEA009176.1	Scaffold1816	–	AtCPK17	538	3814	4	Y
CsCPK17b	TEA017320.1	Scaffold432	+	AtCPK17	536	3696	4	Y
CsCPK21a	TEA017145.1	Scaffold2400	–	AtCPK21	586	9838	4	Y
CsCPK21b	TEA017393.1	Scaffold93	–	AtCPK21	537	5545	4	Y
CsCPK29.1	TEA009842.1	Scaffold1591	+	AtCPK29	528	5004	4	Y
CsCPK29.2	TEA009842.1	Scaffold1591	+	AtCPK29	488	5004	3	Y
CsCPK7	TEA023547.1	Scaffold604	–	AtCPK7	535	5999	4	Y
CsCPK8	TEA031082.1	Scaffold3474	+	AtCPK8 (AtCDPK19)	531	10157	4	Y
CsCPK10	TEA004560.1	Scaffold1878	–	AtCPK10 (AtCDPK1)	545	8103	4	Y
CsCPK13	TEA025829.1	Scaffold2727	+	AtCPK13	532	14305	4	Y
CsCPK24	TEA028945.1	Scaffold3581	+	AtCPK24	534	7447	4	Y
CsCPK30	TEA019714.1	Scaffold820	+	AtCPK30 (AtCDPK1a)	541	8768	4	Y
CsCPK32	TEA013665.1	Scaffold942	–	AtCPK32	534	3798	4	Y
CsCPK28a	TEA008785.1	Scaffold305	+	AtCPK28	565	5114	4	Y
CsCPK28b	TEA019257.1	Scaffold441	+	AtCPK28	578	6106	4	Y

2.5. RNA preparation and quantitative PCR

Total RNA was isolated using an RNAprep Pure kit (Tiangen, China) from approximately 100-mg fresh samples. Complementary DNA (cDNA) template for quantitative RT-PCR (qRT-PCR) was prepared using a PrimeScript™ RT reagent kit (TakaRa, Japan) with 1 μg of total RNA in a 20-μl reaction volume. The qRT-PCR assays were performed on a Light Cycler 480 (Roche, Switzerland) machine, and the qRT-PCR mixture contained 1 μl of 10-fold-diluted cDNA template, 5 μl of SYBR Green I Master Mix (Roche, Switzerland) and 0.4 μM forward and reverse gene-specific primers. Two technical and three biological replicates were performed. Relative expression was calculated using the comparative *Ct* value method, and the polypyrimidine tract-binding protein *CsPTB* (GenBank accession number GAAC01052498.1) was chosen as the endogenous gene reference (Livak and Schmittgen, 2001; Hao et al., 2014). The heatmap was produced using the 'pheatmap' package in R (v3.5.1) with centering and scaling in the row direction. Sequences of *CsCBF* (C-repeat/dream binding factor, *TEA011105.1*), *CsSOD1* (superoxide dismutase, *TEA012288.1*), *CsCAT2* (catalase, *TEA002986.1*) and *CsAPX2* (ascorbate peroxidase, *TEA026035.1*) were retrieved from TPIA database. Primers used in this study are listed in Table S1.

2.6. Total polyphenol and catechin content measurement

The total polyphenol and catechin contents were determined according to the method described in our previous work (Ding et al., 2018). First, 0.2-g fresh samples stored at −80 °C were ground in liquid nitrogen and extracted with 10 ml of 70% (v/v) methanol for 30 min at 70 °C. Then, the sample was centrifuged at 4000 *g* for 15 min, and the supernatants were collected and filtered through a 0.22-μm membrane.

Gallic acid was used as the standard as described in ISO 14502-1. One milliliter of extract or standard solution with a set amount of gallic acid was transferred to new tubes, and 5 ml of 10% (v/v) Folin-Ciocalteu reagent and 4 ml of sodium carbonate solution (7.5% w/v Na_2CO_3) were then added to each tube. After the tubes were kept at room temperature for 60 min, the absorbance was measured at 765 nm against water.

A high-performance liquid chromatography (HPLC) system (Waters e2695 Separation Module, USA) equipped with a 2998 PDA Detector at 280 nm was used for catechin component analysis. The analysis of data obtained with the HPLC assay was as described in our previous work (Ding et al., 2018).

2.7. Ion leakage

For ion leakage assay, young shoots were put into 50-ml tubes containing 20-ml distilled water, which were shaken at 25 °C for 2 h and the conductivity was measured as E1. Then, the tubes were put into boiled water at 100 °C for 15 min and shaken at 25 °C for 1 h, then E2 was measured. The conductivity of distilled water was measured as E0. The formula (E1-E0)/(E2-E0) was used to calculate ion leakage (Wang et al., 2019).

3. Results

3.1. Genome-wide identification and gene duplication

To identify the CPK homologs in tea plant, 34 Arabidopsis and 31 rice CPK protein sequences downloaded from TAIR and the RGAP database were used as queries against genome sequences of the Shuchazao cultivar (Wei et al., 2018) and published transcriptome sequences. The Basic Local Alignment Search Tool was used to conduct a 'tblastn' search, and the parameter 'max_target_seqs 1', with an e-value cutoff of '$1e^{-10}$', was used to control the number of output sequences. After removing redundant sequences, a total of 34 protein-coding sequences were identified. Subsequently, reannotation and manual analysis were performed by a Blastp search against the *A. thaliana* genome database to eliminate genes closely related to CPK such as CPK-related kinases (CRKs), phosphoenolpyruvate carboxylase kinases (PPCKs), and phosphoenolpyruvate carboxylase kinase-related kinases (PEPRKs). Finally, a total of 26 transcripts were confirmed belongs to the CPK family, while the other 8 sequences were considered CRKs, PPCKs and PEPRKs. Further domain analysis revealed that all 26 putative CPK proteins possessed a PK domain and an EF-hand structure (Table 1), suggesting their inclusion in the CPK family. The length of these proteins ranged

from 487 to 609 amino acids, which was similar to the lengths reported for CPK proteins in various species. Unlike most crops, tea plant is cross-pollinated and highly heterozygous, and polymorphisms always exist among different lineages, even among different cultivars (Taniguchi et al., 2014). Shuchazao and the cultivars from which transcriptomic data were obtained belong to the *Camellia sinensis var. sinensis* (CSS, Chinese type) lineage, while 'Yunkang 10' belongs to the *Camellia sinensis var. assamica* (CSA, Assam type) lineage. To minimize potential polymorphisms, the Shuchazao genome was chosen for further genome mapping and gene structural analysis. However, some identified CPK protein sequences showed relatively low identities (only 70 %–80%) with the annotated Shuchazao proteins (Table S2). When performing sequence alignments, polymorphisms that naturally existed would be detected as mismatches and result in lower sequence identities. Thus, we conducted another sequence alignment at the nucleotide level, and distinctly different results were obtained. As shown in Tables S3 and S4, mismatches and gaps were found at very low levels, suggesting that low protein similarities were probably caused by protein mispredictions. Finally, 26 CsCPK proteins and their coding sequences were isolated from tea plants of the CSS type (Table 1). In the case of *A. thaliana* and rice, *CPK* gene expansion and functional divergence were mainly caused by gene duplication events that occurred during evolution (Cheng et al., 2002; Ray et al., 2007). As reported earlier, two rounds of whole-genome duplications (WGDs) occurred during tea plant evolution (Wei et al., 2018), which may also have led to tandem duplication events in the *CsCPK* family. The genome distributions of 26 *CsCPKs* were analyzed to investigate the possible duplication event of *CsCPK* transcripts. The results showed that the coding sequences of these 26 CsCPK proteins were distributed on 25 scaffolds (Table 1). Transcripts CsCPK29.1 and CsCPK29.2 were mapped to a same region of scaffold 1591, suggesting that an alternative splicing event occurred in *CsCPK29* (Table 1 and Table S4). Furthermore, homology analysis revealed that 26 CsCPK proteins were homologous to only 20 members of the Arabidopsis AtCPK family (Table S2), which along with their genome distributions, suggests that gene duplications occurred in the tea plant CsCPK family among the *CsCPK1a/b*, *CsCPK3a/b*, *CsCPK17a/b*, *CsCPK21a/b* and *CsCPK28a/b* members. Finally, 25 *CsCPK* genes encoding 26 CsCPK proteins were identified from tea plant and *CsCPK29* was proposed having a splicing variant.

3.2. Phylogenetic analysis

To better understand the evolutionary relationships between these CsCPKs, an NJ tree was constructed with full-length amino acid sequences from tea plant, Arabidopsis, rice and poplar using MEGA 7.0 software based on the following parameters: the bootstrap value was tested with 1000 replicates, and evolutionary distances were computed using the p-distance. As shown in Fig. 1, the phylogenetic tree was divided into four distinct groups, similar to previously reported patterns in *A. thaliana* and many other species (Valmonte et al., 2014; Shi et al., 2018). Within these four species, their CPKs distribution pattern in each group were different. Most rice and poplar CPKs were clustered into group I, while for tea plant and *A. thaliana*, group II possess the largest number of CPKs in those two species (Fig. 1). In tea plant, 26 CsCPK proteins were also clustered into four groups, and each group contained 8, 9, 7 or 2 members (Fig. 1). Protein sequence alignment and phylogenetic analysis revealed a much smaller CPK family in tea plant, as 26 CsCPKs were homologous to only 20 AtCPKs, 11 OsCPKs, 17 PtCPKs. The *CsCPK* transcripts we identified from tea plant were named according to their homologs in *A. thaliana*. In addition, four previously reported *CsCDPKs*, namely, *CsCDPK1/3/20/26* (Wang et al., 2018b), were found to be the same as *CsCPK1a/3a/20/6*, respectively, based on sequence alignments.

3.3. Structural divergence of CsCPKs

Intron/exon organizations and intron types and numbers can indicate some kinds of evolutionary history within gene families (Long et al., 1995; Zhang et al., 2016). To achieve further insights into the evolution and structural diversity of the *CsCPK* family in tea plant, the gene structures and conserved motifs of all 26 members were analyzed. The gene structures were analyzed by comparing the coding and genomic sequences of each gene with the GSDS 2.0 online tool (Fig. 2). As shown in Fig. 2a, the exon numbers varied among *CsCPK* transcripts, ranging from 7 to 12. Moreover, *CsCPK* genes within the same groups shared very similar intron/exon features such as exon number and length. All members in group I possess seven exons and six introns, while all group II genes except for *CsCPK29.2*, an alternative splicing isoform of *CsCPK29.1*, contain eight exons and seven introns (Fig. 2a). In tea plant, group IV consists of only two members: *CsCPK28*a and *CsCPK28b*. Although both genes were homologous to AtCPK28, but their gene structure, gene size and mapped scaffold were different (Fig. 2a). *CsCPK28*a possesses 12 exons and encodes a protein with 565 amino acids, while *CsCPK28*b has fewer exons but encodes a slightly larger protein (Table 1 and Fig. 2a). As mentioned above, all 26 CsCPK proteins were confirmed to have the PK domain and four EF-hand structures, except CsCPK29.2, which possessed only three EF-hands (Table 1). A motif represents an approximate sequence pattern that occurs repeatedly in a group of related sequences. For sequences characterization, the top five conserved motifs were detected and analyzed using the MEME suite (Fig. 2b). Motif 1,3 and 5 were found localized in the PK domain region, motif 2 was localized in the EF-hand region, while function of motif 4 was unknown, sequences and the relative position of these five motifs are shown in Fig. S1. As illustrated in Fig. 2b and 22 members of 26 CsCPKs possessed all five motifs, and CsCPK4 in group I and CsCPK24 in group II contained four motifs, missing motif 3 and motif 5, respectively. Members in group IV, which was the most divergent cluster, possess only three motifs, lacking motif 2 and motif 5. Taken together, the similar gene structures and motif composition patterns within each group provide significant evidence for their close evolutionary relationship.

3.4. Tissue-specific expression profiles of CsCPKs in tea plants

To investigate the possible link between the evolutionary and functional divergences of the tea plant *CsCPK* family, the expression profiles of 26 *CsCPK* transcripts in the apical buds, 1st leaves, 2nd leaves, 3rd leaves, mature leaves, stems and roots collected in early April and flowers and seeds collected in middle October were determined by qRT-PCR analysis. To narrow down the dynamic range of transcription levels and improve inference, the expression values relative to those of an endogenous reference gene (*CsPTB*) were centered and scaled in the row direction. As shown in Fig. 3, *CsCPK* genes showed a broad expression pattern among different tissues, and their expression could be divided into five clusters (I–V). Most of the cluster I genes expressed higher in apical buds and 1st leaves, lower in roots and flowers. All cluster II genes were expressed more higher in seeds except for *CsCPK17a,* which showed the highest expression in mature leaves. Members of cluster III also exhibited the highest expression in seeds but the lowest expression in flowers. Cluster IV genes had the highest expression in roots, while cluster V genes had the highest expression in stems and lowest expression in flowers and seeds. Notably, members from different evolutionary groups showed similar expression patterns. For example, the genes that showed the highest expression in apical buds and 1st leaves were *CsCPK4* and *CsCPK20*, which belonged to group I, *CsCPK29.1/2* and *CsCPK21a/b,* which belonged to group II, and *CsCPK13,* which belonged to group III (Fig. 3). Moreover, *CsCPK1a/b*, *CsCPK6* and *CsCPK11* from group I, *CsCPK3a*, *CsCPK9* and *CsCPK17b* from group II, and *CsCPK32* from group III all displayed seed-specific expression. The fourth group of the tea plant CPK family

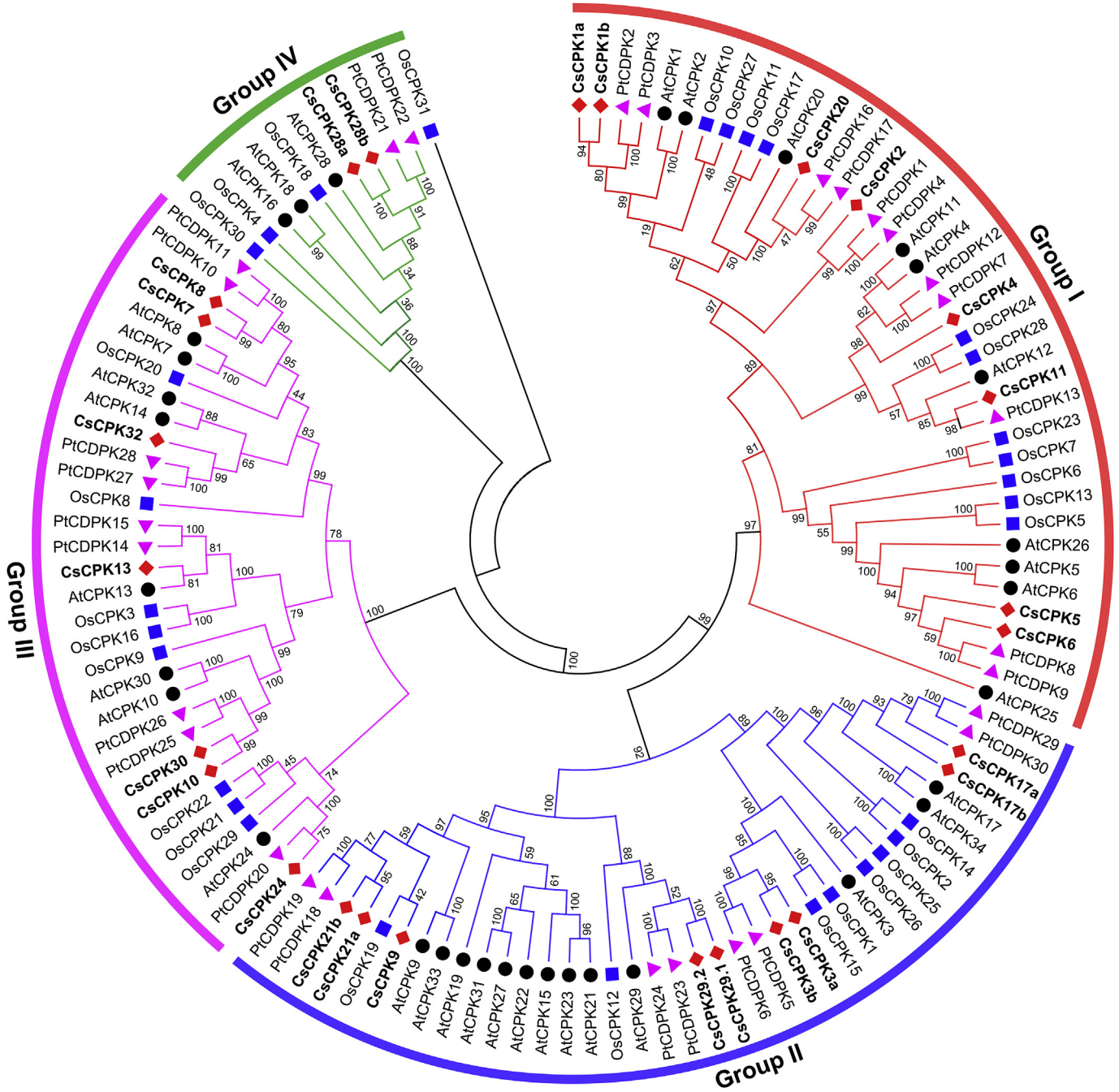

Fig. 1. Phylogenetic analysis of tea plant CsCPK proteins. The phylogenetic tree was constructed using full-length CPK amino acid sequences retrieved from the Arabidopsis and rice genome databases. The neighbor-joining (NJ) method with the default values was used in MEGA 7.0 (Kumar et al., 2016). The 26 CsCPK proteins identified from tea plants were clustered into four major phylogenetic subgroups.

consisted of only two members (Fig. 3), namely, *CsCPK28a* and *CsCPK28b*; both members had the highest expression in roots and lowest expression in other tissues.

3.5. The CsCPKs expression changes in responses to Ca^{2+} inhibitor treatments under cold conditions

In order to block Ca^{2+} signaling pathway, two Ca^{2+} inhibitors, one specific extracellular free Ca^{2+} chelator EGTA and one plasma membrane Ca^{2+} channel blocker $LaCl_3$ were chose, and distilled water (ddH_2O) was used as control. As mentioned earlier, CPKs are special for their direct sensing, decoding and transduction of Ca^{2+} signals. Thus, to evaluate the effect of inhibiting effect on Ca^{2+} signaling, expression changes of all *CsCPKs* were monitored under cold condition. In addition, the *CsCBF* (TEA011105.1) expression was analyzed to confirm the effect of low temperature treatment. As shown in Fig. 4, *CsCBF* was induced in a very short period of time (1 h) and showing the highest expression at 24 h in control assay, suggested that the tea plants had suffered from low temperature. However, remarkable *CsCBF* inhibition was observed in both of $LaCl_3$ and EGTA assays (Fig. 4b & c), demonstrated that the upstream signaling pathways of CBF-dependent cold responding network maybe impaired in these two assays. While for *CsCPKs*, their expression profiles were divided into two distinct clusters. Cluster I consisted of 12 *CsCPK* genes, and 10 of them showed down-regulation under cold stress. All 14 cluster II members were dramatically induced in control assay in at least one stage of cold treatment.

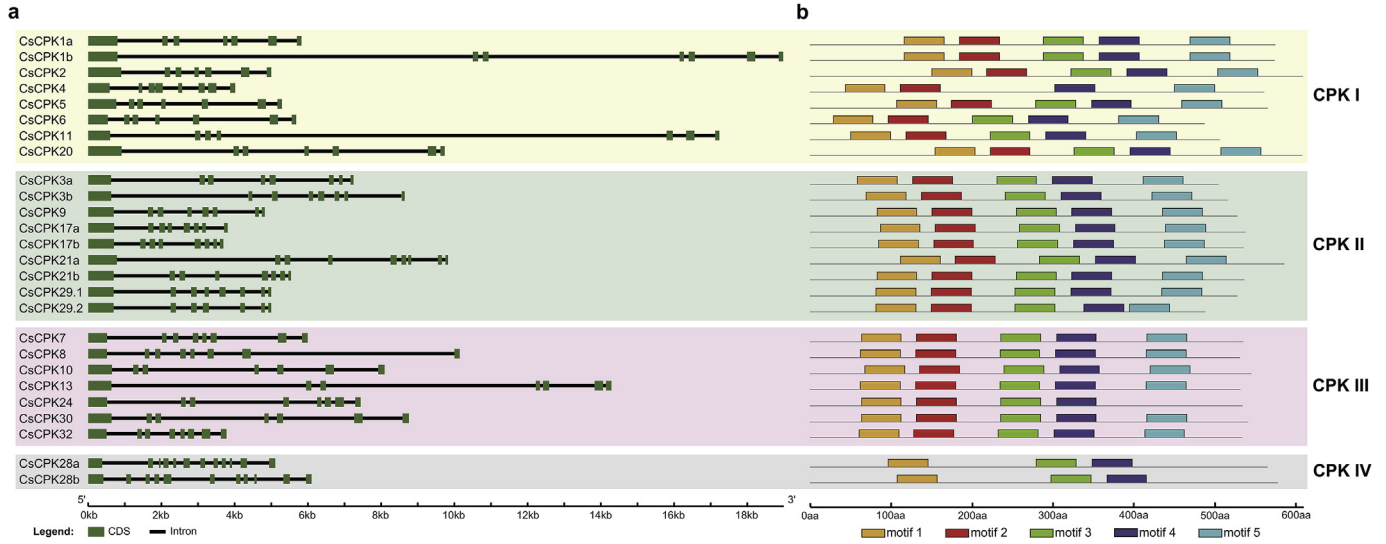

Fig. 2. Comparative gene structure and motif analysis of *CsCPK* transcripts. **a** Exon/intron organization of tea plant *CsCPKs*. CsCPK coding sequences were mapped onto reported genome sequences of the Shuchazao cultivar. Gene structures were constructed using the GSDS2.0 online tool. The exons and introns are represented by green boxes and solid lines, respectively. **b** Top five motifs identified from tea plant proteins obtained by MEME analysis. Each color represents a specific motif. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Among these cold-induced members, *CsCPK28a* and *CsCPK28b* showed the highest expression within a short period (1–3 h), while the highest expression level of the other 12 *CsCPKs* was observed mainly after 24 h–96 h (Fig. 4a). In addition, the relative expression of all 26 *CsCPKs* except *CsCPK29.1/2* and *CsCPK24* was restored to a level similar to that in untreated leaves after recovery for another 72 h under normal conditions, suggesting that cold stress have leaded to dramatically expression changes of *CsCPKs* in young shoots. For inhibitor assays, $LaCl_3$ inhibited all 26 *CsCPK* genes after 96 h of cold treatment, and the inhibition was much stronger for cluster 2 genes than for cluster 1 members (Fig. 4b). EGTA showed a different effect compared with $LaCl_3$, eight *CsCPKs* (*CsCPK13/20/21b/8/29.1/29.2/24/7*) in cluster 1 showed stronger inductions in EGTA assay, and this activation in leaves lasted for 72 h (R72 h) of recovery under normal conditions. While for cluster 2 members, their expression was also inhibited but with a lesser extent (Fig. 4c). Overall, the above results suggested that Ca^{2+} signals and *CsCPKs* played important role in tea plant cold responses, and Ca^{2+} inhibitor ($LaCl_3$ or EGTA) treatments could lead to Ca^{2+} signaling blockage and Ca^{2+} signal related cold responding network impairment.

3.6. Changes of catechin metabolism caused by Ca^{2+} signaling impairment and their potential function in tea plant cold responses

Compared to most higher plants, tea plant is special for the high level of polyphenols in its leaves, among which catechin is a major type that accounts for 12%–24% of dry weight in young leaves (Wei et al., 2018). The above *CsCBF* and *CsCPKs* expression profiles demonstrated that both of $LaCl_3$ and EGTA treatments have leaded to obviously Ca^{2+} signaling impairment. Therefore, we analyzed total polyphenols and catechins content changes trying to figure out their possible roles in tea plant cold responses. The results showed that no significant total polyphenols content change was detected in all three assays (Fig. 5a). As shown in Fig. 5b, total catechins did not show significant changes in the control assay, while were significantly accumulated at 96 h ($p < 0.01$) in the $LaCl_3$ assay and at 48 h ($p < 0.05$), 96 h ($p < 0.01$), and R72 h ($p < 0.01$) in the EGTA assay (compared with 0 h) suggested their involvement in tea plant cold response after Ca^{2+} signaling impairments. As well known, catechins in tea leaves consist of a mixture of epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC), epicatechin (EC), gallocatechin (GC), and catechins (C) (Balentine et al., 1997). Among all isomers, EGCG accounts for up to 70% of total catechins. Further analysis revealed significant EGCG increases in leaves pretreated with $LaCl_3$ (96 h and R72 h) or EGTA (48 h, 96 h and R72 h), but not in the control assay (Fig. 5c), suggested its dominant role in total catechins content changes. In addition, the EGC content was significantly increased in the $LaCl_3$ assay at 48 h and the EGTA assay at 96 h (Fig. 5d), and the EC content was increased in all three assays at 96 h (Fig. 5e). In contrast to those three isomers, GC decreased significantly in response to cold stress. However, pretreatments with $LaCl_3$ or EGTA inhibited this decrease to different degrees (Fig. 5f). For the other two isomers, ECG showed significant changes only in the control assay at R72 h and in the EGTA assay at 96 h and R72 h (Fig. 5g), while no significant changes were observed in any samples (Fig. 5h). Consequently, total catechin changes caused by Ca^{2+} signaling impairment were mainly attributed to the increase in EGCG, EGC, EC and GC isomers. To further elucidate the effect of Ca^{2+} signaling impairment on tea plant, we analyzed the expression changes of three representative antioxidant enzyme-encoding genes, namely, *CsSOD1* (superoxide dismutase, TEA012288.1), *CsCAT2* (catalase, TEA002986.1) and *CsAPX2* (ascorbate peroxidase, TEA026035.1) and detected the ion leakage in those three assays. The qRT-PCR data showed that *CsSOD1* was significantly upregulated at 24 h ($p < 0.01$) and 48 h ($p < 0.05$), *CsCAT2* and *CsAPX2* showed significantly induction at 24 h ($p < 0.05$) and 48 h ($p < 0.05$) respectively in the control assay (Fig. 6a–c). While in inhibitor assays, their cold-induction was severely inhibited, suggested that Ca^{2+} signaling impairments have leaded to significant changes in enzymatic-dependent antioxidant system. Ion leakage reflects low temperature-induced membrane injury and is usually chosen as an indicator of cold injury. In control assay, the ion leakage was gradually increased with cold stress and reached the highest level at 96 h, then restored to normal level after 72 h of recovery (R72 h) (compared with the corresponding 0 h value), demonstrated potential cold injuries have caused by low temperature (Fig. 6d). While, in $LaCl_3$ and EGTA assays, the ion leakage was higher at 96 h compared to the control assay, but no significant difference was detected, indicating Ca^{2+} signaling impairment did not leaded to sever cold injuries. Taken together, utilization of Ca^{2+} inhibitors under cold conditions provided important evidence for the involvement of catechins as important non-enzymatic antioxidants in tea plant cold responses.

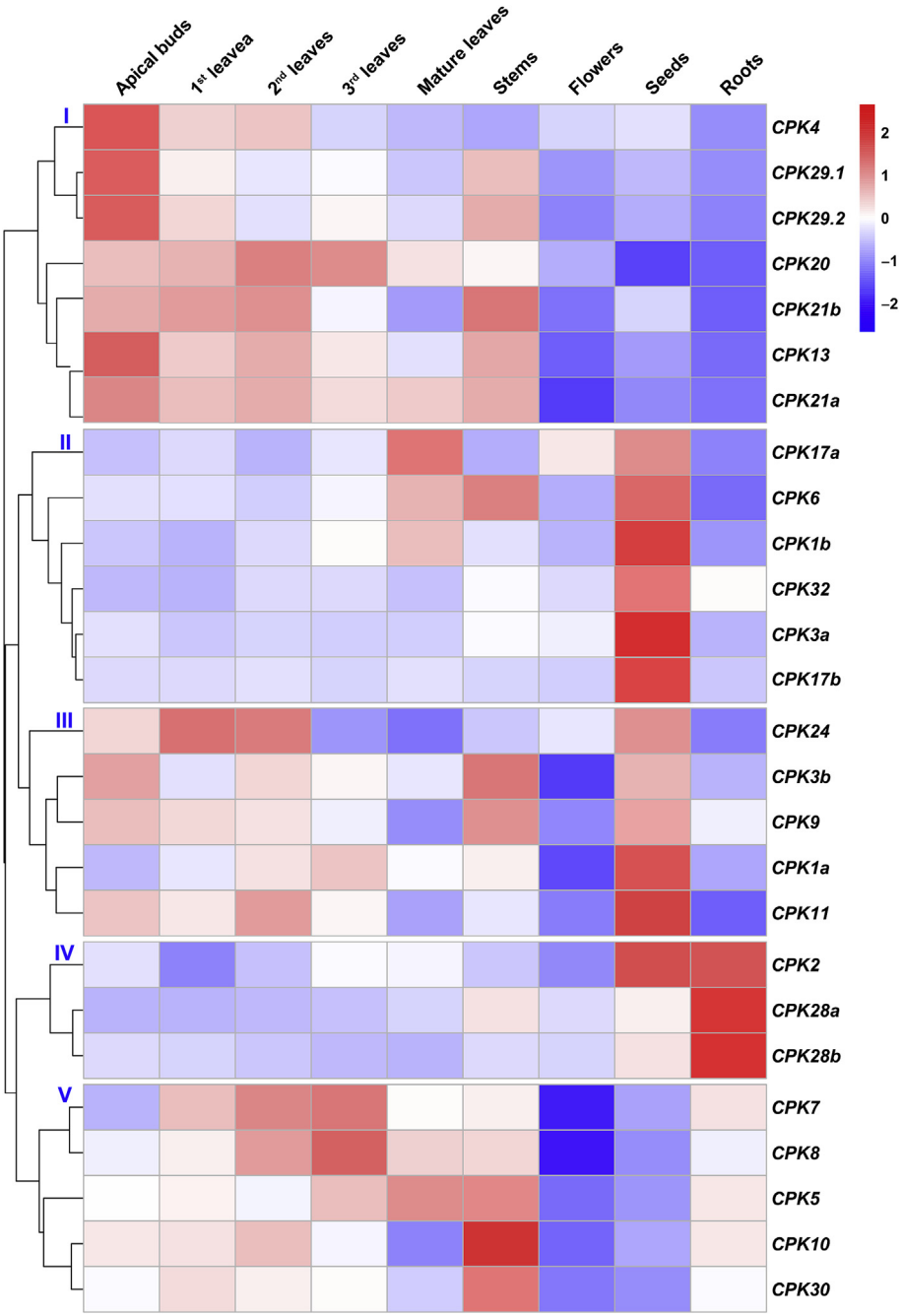

Fig. 3. Expression of tea plant *CsCPK* transcripts among various tissues. The relative expression was quantified using qRT-PCR by normalizing against *CsPTB* and calculated using the comparative Ct value method (Hao et al., 2014; Livak and Schmittgen, 2001). The heatmap was normalized in the row direction based on the mean expression value (n = 3) of each gene. Various tissues are displayed above each column, and gene names are displayed to the right of each row. *CsCPKs* were hierarchically clustered based on the 'Complete' distance. The color scheme blue to white to red represents expression levels. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

The activity of most of the known calcium-stimulated PKs in plants are associated with CPKs. In various species of higher plants, CPKs are evolutionarily conserved and encoded by multigene families (Harmon et al., 2001). Whole-genome sequencing has greatly facilitated CPK functional studies and has led to an increase in the availability of CPK sequences in various plant species such as rice, wheat and cucumber (Asano et al., 2005; Li et al., 2008; Xu et al., 2015). Except for genome sequencing of two tea plant lineages, namely, *Camellia sinensis var. assamica* (Xia et al., 2017) and *Camellia sinensis var. sinensis* (Wei et al., 2018), comprehensive genome-wide identification and putative function analysis of the CPK family in tea plants have not been undertaken. In this study, we conducted a genome-wide study that led to the identification of 26 CsCPK protein-coding sequences in tea plant (Table 1). Whole-genome duplication events have been reported in many species, and the gene duplication caused by whole-genome duplication events is one of the main drivers of multigene family expansion (Hughes, 1994). The CPK family of land plant ancestors usually consists of four genes, while that of extant green algae contains fewer than 10 members and that of angiosperms contains approximately 30–40 members (Valmonte et al., 2014), suggesting potential gene duplication during the evolution of the higher-plant CPK family. Among the 34 Arabidopsis *AtCPKs*, five (*AtCPK21/22/13/27/31*) underwent tandem duplication events (Hrabak et al., 2003). In grape, two duplication pairs (*VvCPK5* and *VvCPK11*, *VvCPK12* and *VvCPK17*) have been identified (Zhang et al., 2015). Wei et al. reported that two rounds of whole-genome duplication (~30–40 and ~90 to 100 mya) may have occurred during tea plant evolution (Wei et al., 2018). Genome mapping along with homology analysis demonstrated that duplication events may also have happened among *CsCPK* genes, as *CsCPK1a/b*, *CsCPK3a/b*, *CsCPK17a/b*, *CsCPK21a/b* and *CsCPK28a/b* showed high

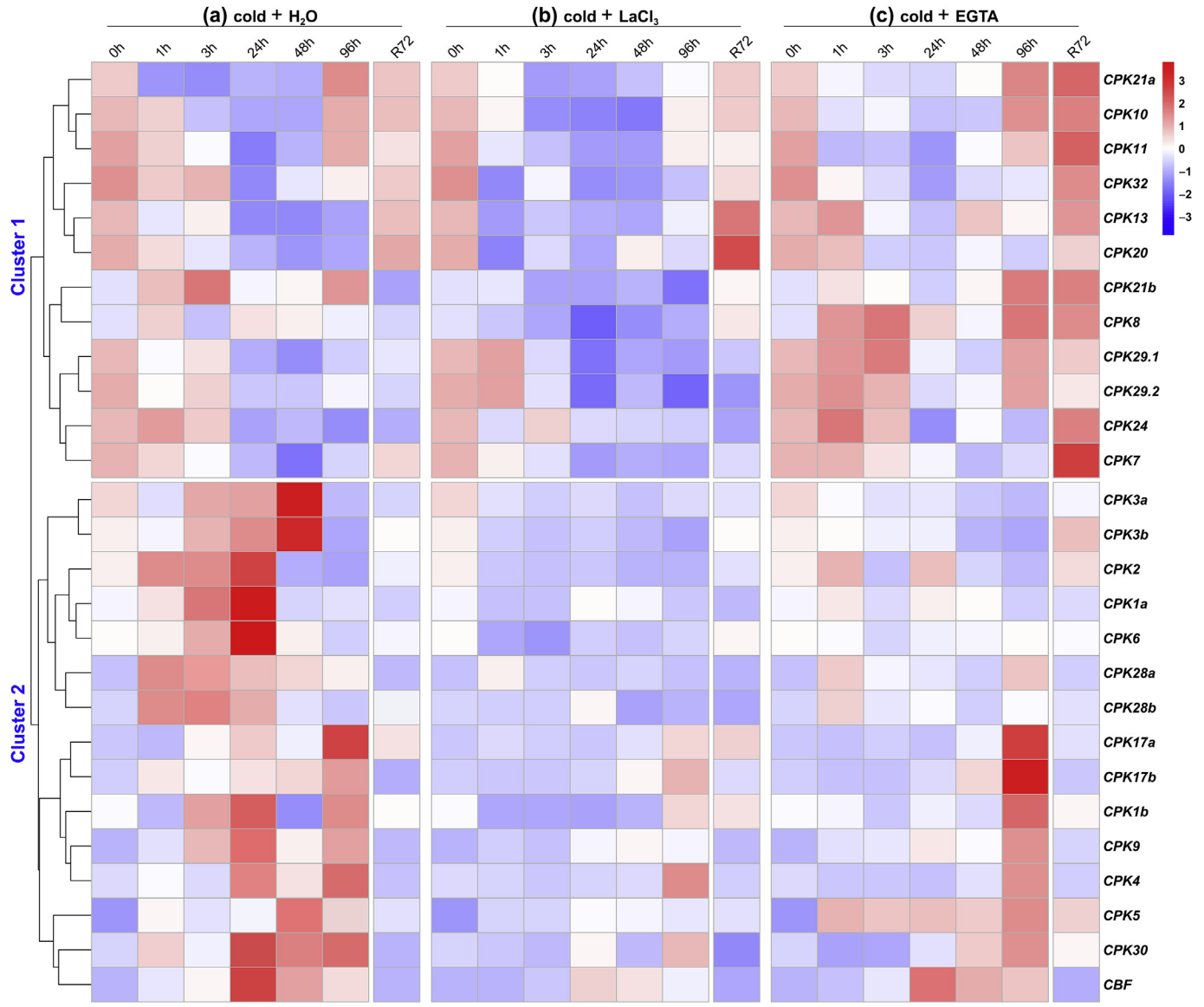

Fig. 4. Expression of tea plant *CsCPK* transcripts in response to cold stress. **a** Plants pretreated with ddH_2O. **b** Plants pretreated with the plasma membrane Ca^{2+} channel blocker $LaCl_3$. **c** Plants pretreated with the free Ca^{2+} chelator EGTA. The relative expression was quantified using qRT-PCR by normalizing against *CsPTB* and calculated using the comparative Ct value method (Livak and Schmittgen, 2001). The heatmap was normalized in the row direction based on the mean expression value (n = 3) of each gene. Treatments and time points are displayed above each column, and gene names are displayed to the right of each row. *CsCPKs* were hierarchically clustered based on the 'Complete' distance. The color scheme blue to white to red represents expression levels. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

protein similarity but were distributed on different scaffolds (Table 1). In addition to gene duplication, alternative splicing is another common phenomenon in eukaryotes that greatly increases the diversity of proteins encoded by the genome (Marquez et al., 2012). Several *CPK* genes within various species have undergone alternative splicing, e.g., *OsCPK17* in rice (Almadanim et al., 2018) and *VaCPK9* in *Vitis amurensis* (Dubrovina et al., 2016). In the case of *CsCPK29*, we also detected one alternatively spliced isoform, consisting of *CsCPK29*.1 and *CsCPK29*.2, located in the same region on scaffold 1591 (Table S4). Compared to model plants such as the dicot *A. thaliana* (34) (Cheng et al., 2002), the monocot rice (31) (Asano et al., 2005) and the tree poplar (30) (Zuo et al., 2013), tea plant possesses fewer CPK proteins (26). One plausible reason for the smaller size of the CPK family in tea plant could be that 26 CsCPK proteins are sufficient for Ca^{2+} signaling; thus, some *CsCPKs* might have been lost during the evolutionary process due to functional redundancy.

Phylogenetic analysis and sequence characterizations, i.e., intron/exon organization and conserved domain analysis, are crucial ways to predict gene functions. Another NJ tree constructed using 383 CPK protein sequences retrieved from various species also showed a four-groups pattern, and the distribution of CsCPKs in each group was same as previous analysis (Fig. 1, Fig. S2). Moreover, these possible duplication events were mainly clustered in group II, which was similar to the pattern observed in *A. thaliana* (Hrabak et al., 2003). Eukaryotic protein-coding genes are typically interrupted by multiple introns, and intron/exon organization is considered to be an important feature reflecting the evolutionary conservation of gene structures (Sverdlov et al., 2005). The *CPK* genes in *A. thaliana*, rice, maize and poplar typically consist of six to eight exons (Hrabak et al., 2003; Asano et al., 2005; Ma et al., 2013; Zuo et al., 2013), while members with fewer or more exons have also been reported, e.g., *MdCPK11* in apple has only one exon (Kanchiswamy et al., 2013), *ZmCPK18* in maize possesses only two exons, and *ZmCPK8* and *ZmCPK16* contain 12 exons (Ma et al., 2013). Tea plant *CsCPKs* have two main intron/exon organization

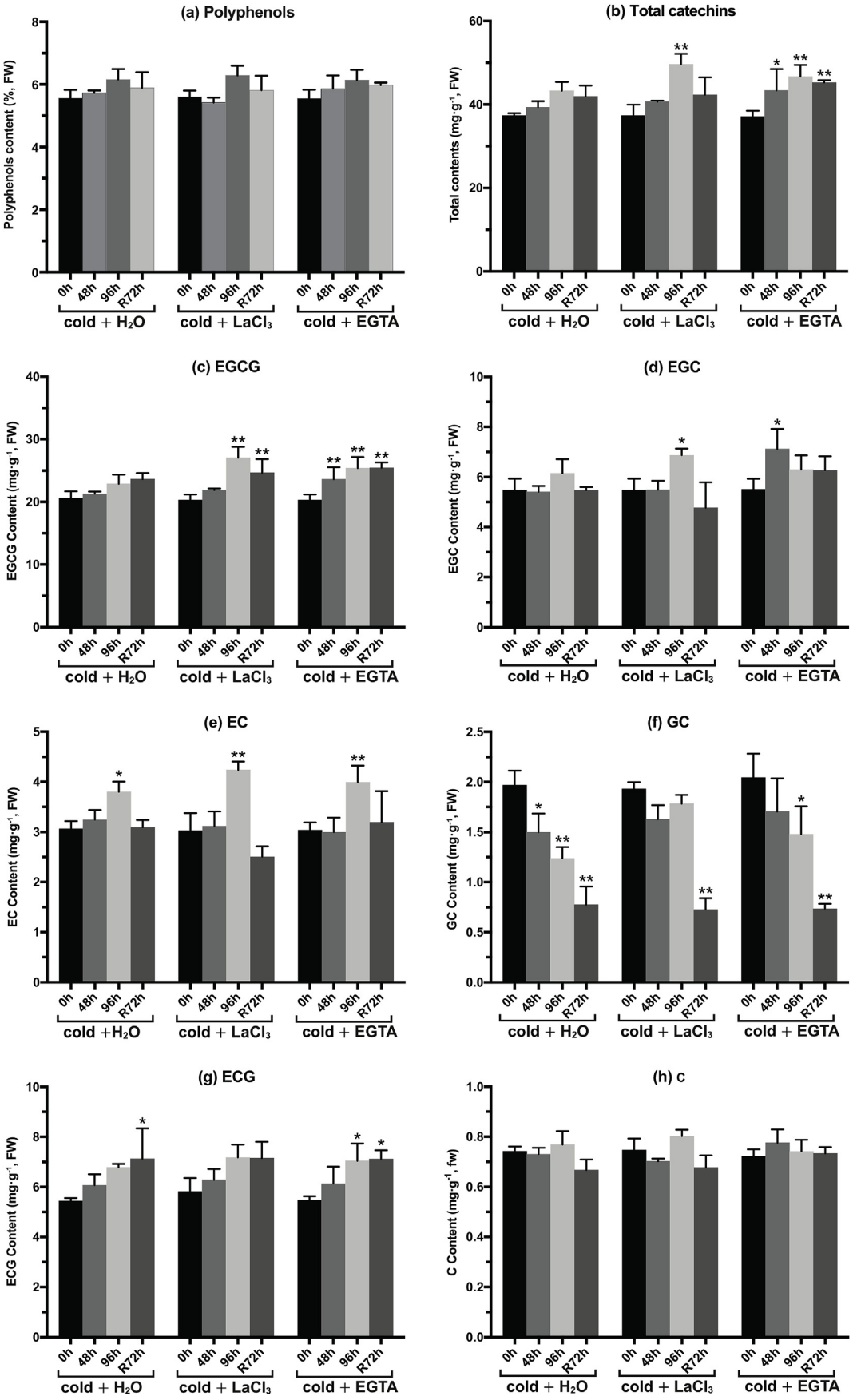

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Fig. 5. Changes in polyphenols, total catechins and each isomer in young shoots under cold stress. **a** Polyphenol content. **b** Total catechin content. **c** EGCG content. **d** EGC content. **e** EC content. **f** GC content. **g** ECG content. **h** C content. Young shoots collected from each assay and stored at - 80 °C were used to quantify the polyphenol and catechin contents. Gallic acid was used as a standard in polyphenol determination. Concentrations of each catechin component were quantified by comparing the peak areas of the component to those of standards prepared from authentic compounds. $^{*}P < 0.05$, $^{**}P < 0.01$, two-tailed *t*-test. Bars represent the mean ± SE (n = 3).

patterns: all members in groups I, II and III contain seven to eight exons, while the group IV members *CsCPK28a* and *CsCPK28b* have 12 and 11 exons, respectively (Fig. 2). The intron/exon patterns showed much higher conservation among CPKs that belong to the same evolutionary group; for instance, all group I members contain seven exons, while group II members have eight exons except for *CsCPK29.2*, an alternatively spliced product of *CsCPK29* (Fig. 2). Generally, CPKs recognize Ca^{2+} signals via the EF-hand structure and phosphorylate downstream proteins through the PK domain (Harmon et al., 2001). Based on the sequence analysis, we speculated that 21 out of 26 CsCPK proteins were activated by Ca^{2+} signals. The activity of another five proteins was hard to predict. CsCPK29.2 possesses both a PK domain and the five best-identified motifs, but only three EF-hands usually exist as pairs. Alternatively, CsCPK4 in group I, CsCPK24 in group III and CsCPK28a and CsCPK28b in group IV have four EF-hand structures and a PK domain but are missing one to two conserved motifs (Table 1 and Fig. 2).

CPKs are exclusively Ca^{2+} sensors that are distributed widely in higher plants and expressed ubiquitously in various tissues, including leaves, flowers, stems and roots; some are expressed in most tissues, while others are tissue specific. In tea plant, a high characteristic metabolite content is mainly distributed in green tissues (Ashihara et al., 2010). Interestingly, the expression of CsCPK family genes also differed among tissues. For instance, seven *CsCPKs* (*CsCPK4/29.1/29.2/20/21b/13/21a*) exhibited higher expression in green tissues (apical bud, leaves and stems) than in non-green tissues (flowers, seeds and root) (Fig. 3). Moreover, the expression profiles of *CsCPK* genes were different between young shoots (apical buds and 1st and 2nd leaves) and mature leaves, as at least 12 members were found to have higher expression in young shoots than in mature leaves (Fig. 3). This specificity may result in different regulatory networks being mediated by Ca^{2+} signals, such as different cold-sensitivity networks (Li et al., 2018). In tea plant, catechins exist ubiquitously in young leaves (Liu et al., 2009), mainly in the chloroplasts of mesophyll cells and in the vessel wall (Yang et al., 2012). The potential relationship between catechins accumulation and tissue-specific expression of those 12 *CsCPKs* is hard to know and need more efforts. To our knowledge, cold-responsive *CsCPKs* may be involved in catechins metabolism through phosphorylating some plasma membrane and chloroplast dual-localized proteins. Among the 30 *CPKs* in the model tree poplar, 13 members show preferential expression in roots (Zuo et al., 2013), while in tea plant we found only three. Among these three genes, *CsCPK28a* and *CsCPK28b* are a putative duplicated pair. Ca^{2+} and Ca^{2+} signaling usually play crucial roles in flower development and pollen tube elongation (Valmonte et al., 2014). In rice, based on the expression profile of *OsCPKs* in different panicle (P1 – P6) and seed developmental (S1 – S5) stages, at least seven genes (*OsCPK3/6/14/22/25/26/29*) are specifically expressed in the P6 stage, which corresponds to pollen development (Ray et al., 2007). Furthermore, in *A. thaliana*, 12 *AtCPKs* are

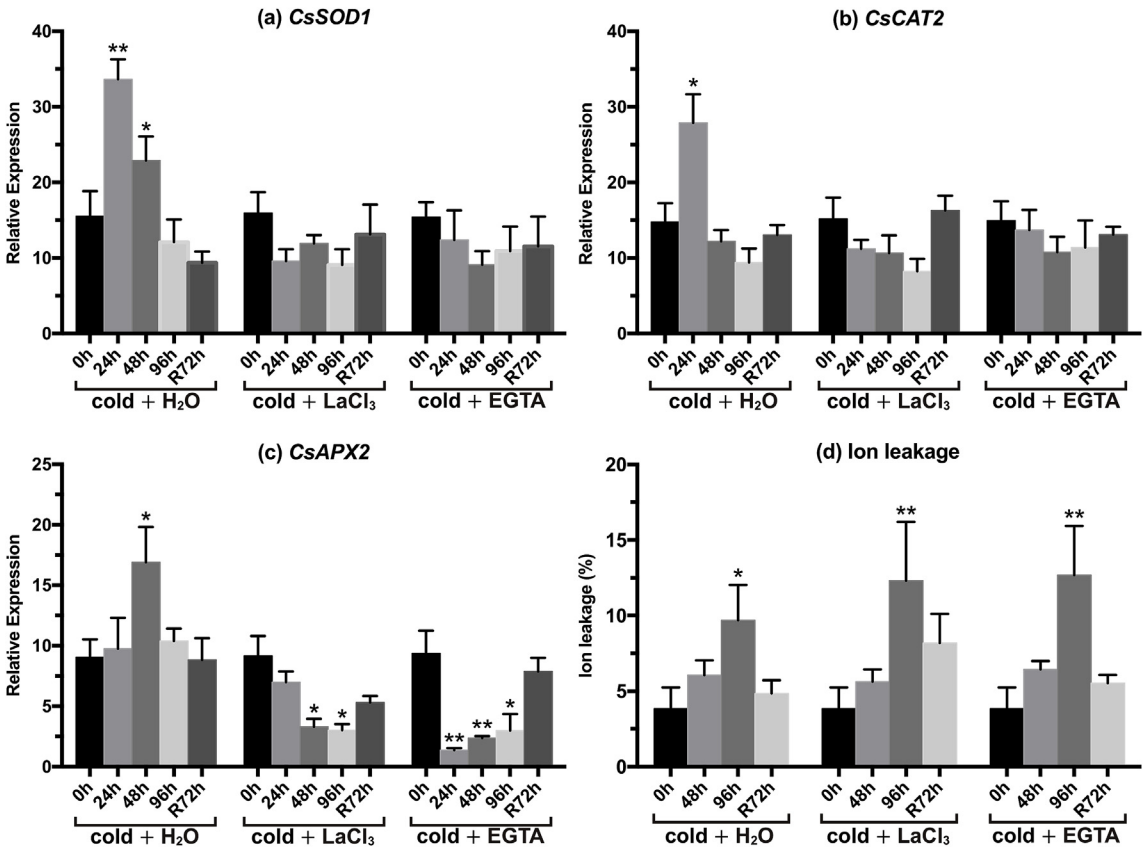

Fig. 6. Changes in *CsSOD1*, *CsCAT2*, and *CsAPX2* relative expression and ion leakage in response to cold stress. **a** Relative expression changes of *CsSOD1*. **b** Relative expression changes of *CsCAT2*. **c** Relative expression of *CsAPX2*. **d** Changes in ion leakage. The relative expression was quantified using qRT-PCR by normalizing against *CsPTB*. The values were calculated using the comparative Ct value method (Livak and Schmittgen, 2001). $^{*}P < 0.05$, $^{**}P < 0.01$, two-tailed *t*-test. Bars represent the mean ± SE (n = 3).

preferentially expressed in pollen, and *atcpk34atcpk17* double mutants show impaired pollen tube growth (Myers et al., 2009), suggesting crucial roles of these genes in flower development. However, we did not find any *CsCPKs* that were predominately expressed in flowers. Whether the low fertility and self-incompatibility of tea plant are correlated with the abovementioned phenomenon is still unknown. Overall, the tissue-specific expression of *CsCPKs* was evident, and the *CsCPKs* that showed similar expression profiles may have similar functions because gene expression can be connected to specific cell types.

There is ample evidence that CPKs play vital roles in cold responses in various species. For example, in rice, both *OsCPK13* and *OsCPK24* are induced by cold stress, and overexpression of *OsCPK13* or *OsCPK24* confers cold tolerance in transgenic plants (Saijo et al., 2000; Liu et al., 2018). Transgenic *A. thaliana* plants overexpressing poplar *PeCPK10* showed enhanced freezing tolerance (Chen et al., 2013). Some *CPKs* such as *OsCPK17* and *ZmCPK*1 were found negatively regulating plant cold tolerance, overexpression of *OsCPK17* (Almadanim et al., 2017) or *ZmCPK*1 (Weckwerth et al., 2015), leading to compromised cold tolerance of transgenic rice or *A. thaliana*. In tea plant, we previously reported that Ca^{2+} signaling pathway played crucial roles in mature leaves cold acclimation process (Wang et al., 2013). Here, the *CsCPKs* expression profiles under cold or cold and Ca^{2+} inhibition ($LaCl_3$ and EGTA) dual-treatment condition implied their important functions in tea plant cold tolerance regulation, as 16 *CsCPKs* were up-regulated and 10 were down-regulated (Fig. 4). In addition, *cis*-acting element analysis revealed that promoter of 9 *CsCPKs* possess cold-responsive element, 18 *CsCPKs* possess salicylic acid-responsive element, 20 members possess abscisic acid-responsive element (Table S5), which also provided important information for their function studies. Although, it may be hard to determine the most important sensor, however, these *CsCPKs* such as *CsCPK30*, *CsCPK5* (homolog of *OsCPK13*), *CsCPK4* (homolog of *OsCPK24*) and *CsCPK9* that showed similar expression patterns with *CsCBF* may play key roles in tea plant cold tolerance regulation.

Cold stress triggers physiological and biochemical changes in plants, including a decrease in cell membrane fluidity, the accumulation of metabolites, a change in redox state and reprogramming of gene expression (Guo et al., 2018). According to the current plant cold response model, the Ca^{2+} signaling pathway acts as the core upstream signaling pathway. Thus, the utilization of Ca^{2+} inhibitors could be useful for studies on cold-related metabolites changes. As mentioned above, the expression changes of *CsCPKs* in responding to Ca^{2+} inhibitors suggested that $LaCl_3$ or EGTA pre-treatment could block Ca^{2+} signaling pathway under cold condition (Fig. 4). Meanwhile, the inhibition of cold-induced *CsCBF*, *CsSOD1*, *CsCAT2* and *CsAPX2* expression (Fig. 6), revealed that the Ca^{2+} signaling related cold responding network also was severely impaired in those two Ca^{2+} inhibitor assays. However, the ion leakage which is a well-known cold injury indicator that is strongly correlated with cold tolerance (Murata and Tatsumi, 1979), did not show distinct differences in those three assays suggested the involvement of other mechanisms in response to cold stress in tea plant (Fig. 6 d). Similar to most abiotic stresses, low temperature can cause a burden of ROS, thus evoking antioxidant defenses. Antioxidant enzymes, i.e., SOD, CAT, APX, and nonenzymatic antioxidants, i.e., ascorbic acid, glutathione and phenolic compounds, provide an important line of defense against the toxic effects of elevated levels of ROS (Ahmad et al., 2010). Compared to most other higher plants, tea plant is special for the high content of polyphenols in its leaves. And the health benefits associated with tea consumption are mainly attributed to the antioxidative property of tea polyphenols (Murase et al., 2002). Among which, catechins are the most characteristic bio-active compounds that contribute to bitterness of tea and are credited with mediating the potential health benefits of tea drinking (Zhang et al., 2018). In fact, tea plant is thought to have evolved such characteristics for survival in adverse environments. Cheruiyot et al. (2007) proposed that polyphenols could be an indicator of drought tolerance in tea plant. In wheat leaves, the expression of flavonoid biosynthesis-related genes, i.e., *TaCHS*, *TaCHI*, *TaF3H*, *TaFNS* and *TaFLS*, was upregulated rapidly after drought (Ma et al., 2014). These studies suggested that characteristic metabolites, especially catechins (a subgroup of flavan-3-ols), may have vital functions in tea plant stress responses. Accordingly, the lost function of enzymatic-antioxidant system and disruption of ROS metabolism caused by Ca^{2+} inhibitor pre-treatment maybe the main reason of catechins accumulation, and that is consistent with our previous studies (Ding et al., 2018). Taken together, we postulated that catechins and other secondary metabolites may form an alternative line of antioxidant defense that closely correlated with the status of Ca^{2+} signaling in tea plant.

5. Conclusion

In present study, we identified 26 CsCPK transcripts from tea plant, conducted phylogenetic, domain, conserved motif and gene structure analyses, detected their expression profiles, and proposed that tea plant possess a typical plant CPK family that play critical roles in development and stress responses. Low temperature treatment along with the calcium inhibitor assays demonstrated that $LaCl_3$ or EGTA pre-treatment resulted in Ca^{2+} signaling blocking and cold response network impairment, while leaded to significantly accumulation of catechins including total content and EGCG, EGC, and EC isomers. Overall, we speculate that those characteristic metabolites may form an alternative line in response to various stress conditions. Therefore, more extensive and comprehensive investigations are critically needed in this new research area.

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Conflicts of interest

Authors declare no competing financial interest.

Author contribution statement

CD, XW and YY conceived and designed research. CD wrote the manuscript. LL, LY, LW, XH and NL performed the bioinformatic analysis and experiments. PY, YW and GG analyzed the data. XW and YY gave the approval for publication. All authors read and approved the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.005.

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