Plant Physiology and Biochemistry 138 (2019) 9–16

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

The regulatory effects of *MeTCP4* on cold stress tolerance in *Arabidopsis thaliana:* A transcriptome analysis

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ARTICLE INFO

Keywords:
Cold stress
MeTCP4
Cassava
Arabidopsis
RNA-Seq

ABSTRACT

Cassava (*Manihot esculenta*), an important food crop in tropical areas, is well-adapted to drought conditions, but is sensitive to cold. The expression of *MeTCP4*, a transcription factor involved in the regulation of plant development and abiotic stresses responses, was altered under cold stress. However, its biological function under abiotic stress responses is still unclear. Here, we show that increased *MeTCP4* expression enhances cold stress tolerance in *Arabidopsis* (*Arabidopsis thaliana*). To better understand the biological role of *MeTCP4*, the mRNA from overexpression and wild-type (WT) plants was isolated for whole genome sequencing to identify *MeTCP4*-mediated cold-responsive genes. Our results identify 1341 and 797 differentially expressed genes (DEGs) affected by *MeTCP4* overexpression under normal and cold conditions, respectively. Gene ontology analysis revealed that a portion of the DEGs were involved in reactive oxygen species (ROS) metabolism process after cold treatment. qRT-PCR analysis revealed that the expression of cold-responsive genes and ROS-scavenging-related genes were increased in *MeTCP4* overexpression plant, which could be responsible for the reduced ROS levels and enhanced cold resistance observed in transgenic plant. The findings provide insight into mechanisms of *MeTCP4*-mediated cold stress response, and provide clues for development of low temperature-tolerant cassava cultivars.

1. Introduction

Cassava is an important tropical food crop for humans and animals, and also serves as raw material for biofuel (E1-Sharkawy, 2004). Cold stress severely limits global production of cassava, and significantly decreases yield (Huang et al., 2005). Since plants are sessile, a variety of mechanisms are necessary for them to respond and adapt to environmental changes, such as temperature variation. Extensive studies have been performed to understand how plants respond to cold stress. Physiological studies have shown that low temperatures contribute to growth defects in cassava plants, including reduced photosynthetic activity and increased presence of reactive oxygen species (Utsumi et al., 2012; Xu et al., 2013). Consistent with physiological results, transcriptomic profiling of cassava seedlings also reveal high expression levels of genes involved in transcription, protein degradation, osmotic adjustment and ROS detoxification (Zeng et al., 2014, 2017; An et al., 2016a; Li et al., 2017). ROS are by-products of aerobic metabolism, including hydrogen peroxide (H_2O_2), superoxide radical (O_2-), and hydroxyl radicals (.OH) (You and Chan, 2015). Despite control by the active oxygen scavenging system, overproduction of ROS in cold environments causes oxidative damage, and even cell death (Quan et al., 2008).

Recent research on the development of cold-tolerant cassava cultivars has focused on increasing cold response capacity by changing related gene expression levels. For example, transgenic cassava plants coexpressing cytosolic superoxide dismutase and ascorbate peroxidase, two ROS-scavenging enzymes, have shown enhanced tolerance to oxidation and chilling (Xu et al., 2013). Overexpression of the *C-repeat-binding factor* (*CBFs*) gene in cassava increases several types of abiotic stress tolerance (An et al., 2016b, 2017). In various plant species, CBF proteins have been shown to act as central regulators by binding to the CRT/DRE element of cold-inducible gene promoters (Zhong et al., 2006; Thomashow, 2010). Moreover, RNAi-driven repression of the myeloblastosis (MYB) transcription factor *MeMYB2* contributes to

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https://doi.org/10.1016/j.plaphy.2019.02.015
Received 29 October 2018; Received in revised form 15 February 2019; Accepted 18 February 2019

cassava tolerance to freeze and drought (Ruan et al., 2017). *MeMYB2* also shows effects on the expression of a number of stress-responsive genes under stress conditions (Ruan et al., 2017). The results show similar cold tolerance mechanisms in different plant species, suggesting the potential to develop cold-tolerant cassava by genetic modification.

MicroRNAs (miRNAs) are generally 21-24 nt non-coding RNAs that inhibit translation or cleaves mRNA (Zhang et al., 2006). Plant miRNAs has been reported to be involved in abiotic stress response (Zhang, 2015; Shriram et al., 2016). MiR319, one of the most conserved and ubiquitous miRNAs in plants, has been studied primarily for its role in controlling leaf development, including aging, cell proliferation and differentiation (Koyama et al., 2017; Bresso et al., 2018; Palatnik et al., 2003; Sun et al., 2017; Schommer et al., 2014; Ori et al., 2007). A plant-specific bHLH transcription factor family, *TEOSINTE BRANCHED/CYCLOIDEA/PCF* (*TCP*), has been identified as a target of miR319. This group of *TCP* genes belongs to *CINCINNATA* (*CIN*) subclass (Schommer et al., 2008). The bHLH motif of the *TCP* family is highly conserved, 59-residue-long and non-canonical. It mainly modulates DNA binding, protein nuclear localization and protein-protein interactions (Palatnik et al., 2003; Martin-Trillo and Cubas, 2010). TCP proteins play a critical role in the development of multiple plant species (Nicolas and Cubas, 2016), such as in lateral branching in rice (Takeda et al., 2003), internode length and leaf shape in *Arabidopsis* (*Arabidopsis thaliana*) (Kieffer et al., 2011), and fruit ripening in the strawberry (Wei et al., 2016). Previous studies have also shown the involvement of *TCPs* in cold stress response, such as significantly altered *TCP* expression in sugarcane roots and shoots (Thiebaut et al., 2012) and repressed miR319 targets (*OsPCF5* and *OsTCP21*) in rice (Wang et al., 2014; Yang et al., 2013).

Previous results have shown that a number of *MeTCP* genes isolated from cassava have exhibited different patterns of expression. For example, *MeTCP11a* and *MeTCP20e* are significantly induced by cold and drought stresses. However, *MeTCP3a* and *MeTCP4,* two *CIN* subclass *TCPs* of cassava, are strongly repressed by cold treatment, suggesting that *MeTCPs* have distinct functions in response to abiotic stresses (Lei et al., 2017). Although there has been some evaluation of the role of *TCP*s, such as Os*TCP*21 in rice (Wang et al., 2014), data on *MeTCPs* in cassava plants still remain scarce. Hence, in this study, we generated transgenic *Arabidopsis* with *MeTCP4* overexpressed, and tolerance to cold stress was measured. Genome-wide transcriptome analysis enabled identification of Me*TCP*4-regulated cold-responsive genes by using high-throughput sequencing. The putative function of these genes was annotated in the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Furthermore, transcript levels of the differentially expressed genes involved in *MeTCP4* overexpression were measured by quantitative real-time PCR (qRT-PCR).

2. Materials and methods

2.1. Plant materials

Stems of the cassava (*Manihot esculenta*) cultivar (60444) were cut into segments for growth on MS medium in a greenhouse (16 h light and 8 h dark at 26 ± 2 °C). Seedlings were then treated at 4 °C as cold stress for different time lengths (0/6/12/24/48/72 h). For RNA-seq, young leaves and shoot apexes were collected. *Arabidopsis* transgenic lines using the Columbia-0 (Col-0) ecotype were used as a comparison to cassava. For both the WT and transgenic lines, seeds were sterilized 1 min in 70% EtOH and 10 min in 1% $NaClO_3$ with a three-time wash in sterile dH_2O. Seeds were then grown into seedlings on MS medium with 1% sucrose, with incubation of 10 days at 22 °C with 8 h of light daily. The seedlings were then repotted in peat soil and grown in the greenhouse under the same temperature and light conditions. 10-day-old plants grown in soil were subjected to 24 h at −4 °C for cold stress treatment and phenotype observation.

2.2. Generation of transgenic arabidopsis plants overexpressing MeTCP4

The coding region of *MeTCP4* was isolated from cassava with primers listed in Supplementary Table S4. After cut with BamH I and Sal I, the sequence was inserted into binary vector 35S:pCambia1301. The vector was then transformed into *Agrobacterium tumefaciens* GV3101. *Arabidopsis* (*Arabidopsis thaliana*) ecotype Columbia (Col) was transformed via the floral dip method (Li et al., 2014). T3 homozygous *Arabidopsis* transgenic lines (5# and 9#) were used for further experiments.

2.3. Transcriptome sequence analysis of transgenic and WT plants

For RNA-seq, four separate cDNA libraries were prepared: 14-day-old WT seedlings treated with cold stress (cold, 24 h at 4 °C); untreated WT seedlings (normal); transgenic (*35S::rMeTCP4 5#*) seedlings treated with cold stress (cold, 24 h at 4 °C), and untreated transgenic seedlings (normal). There were two replicates for each library. RNA extraction, library preparation and deep sequencing were conducted at the Guangzhou Genedenovo Biotechnology Co., Ltd (Guangzhou, China). Initial sequencing was on a HiSeq 2500 platform, generating 150-nt paired-end reads. Adapter-linked and low-quality reads were removed from the raw reads for quality control. Contigs and transcripts in *de novo* assembly of the clean reads were generated using the Tophat 2.0 and Cufflinks programs, respectively (Trapnell et al., 2012). All unigenes were aligned to the TAIR 10 database for functional annotation. FPKM values were determined for the expression level of each unigene (Wagner et al., 2012). Differentially expressed genes between transgenic and WT plants was calculated using the cuffdiff program, filtering DEGs of significance (FDR < 0.05). The RNA-seq data was submitted to National Center for Biotechnology Information (NCBI) under the accession number PRJNA435849.

2.4. Annotation and functional analysis of the DEGs

AgriGO was used for functional analysis of the Me*TCP*4-regulated DEGs (Tian et al., 2017), detecting GO terms that show significant enrichment (p-value < 0.05). The KEGG (Kyoto Encyclopedia of Genes and Genomes) database (Kanehisa et al., 2008) was used to annotate pathways that also show significant enrichment (p-value < 0.05).

2.5. Validation of MeTCP expression levels and RNA-Seq data

Cassava and *Arabidopsis* transgenic seedlings treated/untreated with cold stress were used to exam the expression level of *MeTCPs* by qRT-PCR. Meanwhile, to test the accuracy of RNA-seq data of *Arabidopsis* transgenic plants, 14-day-old WT and transgenic seedlings under normal and cold conditions (24 h at 4 °C) were collected and frozen immediately in liquid nitrogen. Total RNA was isolated from 0.1 g young cassava leaves, as well as from the *Arabidopsis* WT and *35S::rMeTCP4* transgenic seedlings, using the Plant RNA kit (OMEGA). Second, 2 μg of each RNA sample was used for reverse transcription into cDNA templates. The SYBR Premix ExTaq™ II Kit (TaKaRa) was used to run qRT-PCR and detect the transcript levels of *MeTCPs*, with *AtACTIN* and *MeACTIN* as reference, respectively. Finally, transcript levels were computed according to $2^{-\Delta\Delta Ct}$ values. Primer sequences are in Table S4.

2.6. Histochemical detection of H_2O_2, malondialdehyde (MDA) and proline content

Four-week-old soil-grown *Arabidopsis* plants treated with cold stress (4 °C) for 24 h were used for detecting H_2O_2, MDA and proline contents. Accumulated H_2O_2 in the leaf was stained by 3, 3′-diaminobenzidine (DAB) for measurement. Immediately following treatment, leaves were immersed in a 1 mg/mL DAB solution (pH 3.8) for a 6-h incubation in light at 25 °C and bleached in 95% boiling EtOH for at least 10 min.

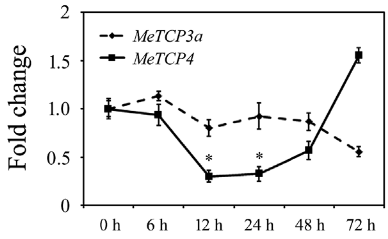

Fig. 1. Expression patterns of *MeTCP3a* and *MeTCP4* at 4 °C in young leaves of cassava seedlings at 0, 6, 12, 24, 48, 72 h after cold treatment.

Hydrogen peroxide (H_2O_2) levels (An et al., 2012) were determined as in previous studies. To measure MDA content and proline concentrations, thiobarbituric acid (TBA) reaction and sulfosalicylic acid-acid ninhydrin were applied, respectively (Fu et al., 2016).

3. Results

3.1. Expression pattern of MeTCP4 in response to cold stress

To understand how *MeTCPs* respond to cold stress, qRT-PCR was performed to determine relative expression levels of *MeTCP4* and *MeTCP3a* in cold-treated cassava leaves. RNA-seq results from a previous study showed strong repression of the two *MeTCPs* by cold stress (Lei et al., 2017). In this study, *MeTCP4* initially showed significant down-regulation in cold stress conditions after 12–24 h of cold treatment. This was followed by weaker repression, and was stably up-regulated after 48 h of treatment (Fig. 1). For *MeTCP3a*, cold stress initially increased its expression level after 6 h, but then decreased it from 12 to 72 h. The expression profile of *MeTCPs* suggests that these genes were involved in cold tolerance.

3.2. Molecular characterization and phenotype of MeTCP4 transgenic plants

To verify the role of *MeTCP4* genes responding to cold stress, *Arabidopsis* transgenic lines expressing *rMeTCP4* (miR319 resistant versions of *MeTCP4*) with the CaMV35S promoter were generated (Fig. 2A). In total, 22 independent transgenic lines with similar phenotypes were obtained. Among them, 6 single-copy insertion lines were chosen for further analysis. All transgenic lines showed significantly increased expression of *rMeTCP4* compared to WT plants, according to qRT-PCR results (Fig. 2B). Subsequent experimental results were obtained from the homozygous progeny (T3) of 5# and 9# lines. The phenotype of the *rMeTCP4*-overexpressing plants included delayed flowering time, fewer seeds and increased number of leaves per plant (Fig. 2C and D), indicating the role of *rMeTCP4* on plant development.

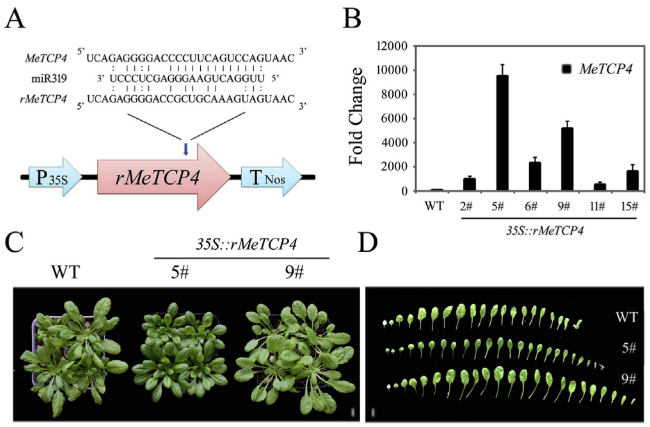

Fig. 2. Phenotype and qRT-PCR validation of Arabidopsis MeTCP4-overexpressing lines: (A) Schematic diagram illustrating the plasmid that contains miR319 resistant versions of MeTCP4 (rMeTCP4) driven by the CaMV 35S promoter. (B) Expression levels of MeTCP4 in wild-type plants and overexpressing lines. MeACTIN was the reference gene. Data was mean ± SD of three independent assays. (C) Phenotype of WT and selected transgenic lines (5# and 9#) at the vegetative stage. (D) Leaf number per plant in WT and transgenic lines. Bar = 1 cm.

3.3. Overexpression of MeTCP4 in arabidopsis improves cold tolerance

To investigate the effects of *rMeTCP4* overexpression on cold stress tolerance, we observed 10-day-old WT and overexpression (OE) plants that were subject to direct 1-day treatment at −4 °C before normal growth temperature was restored. Results showed sustained vigor of OE seedlings under cold stress (Fig. 3A). Nearly 86.1% and nearly 77.4% of seedlings survived in OE lines 5# and 9#, respectively, whereas only 37.2% of WT seedlings survived (Fig. 3B) ($P < 0.05$). Several studies report that proline content in plants is positively correlated with stress tolerance (An et al., 2016b; Wang et al., 2014). To demonstrate the relationship between the cold tolerance conferred by *MeTCP4* and proline accumulation, free proline contents were measured *in planta*. The contents between WT and OE plants were not significantly different at normal temperatures, but were clearly accumulated in both groups after treatment. However, OE plants experienced more rapid accumulation than did WT (Fig. 3C) ($P < 0.05$). In addition to proline, malondialdehyde (MDA), a key indicator of stress-responsive cell membrane degradation, also accumulates rapidly under cold stress (Chen and Murata, 2002). Similar to proline accumulation studies, both *MeTCP4* OE lines and WT plants only showed MDA accumulation after cold treatment. However, unlike proline accumulation, there was a significant increase in MDA levels in WT (Fig. 3D). These findings lead to the conclusion that plants gain tolerance to cold stress because overexpressed *MeTCP4* promotes proline accumulation and represses cell membrane damage under cold stress.

3.4. Overexpression of MeTCP4 enhances expression of cold-responsive genes

Several marker genes of cold stress response were also selected to measure their transcript levels in WT and OE plants. Well-studied genes that enhance cold tolerance in plants include *CBF1*, *COR15a* (*cold-responsive 15a)* and *RD29a* (*desiccation-responsive protein 29a*). After cold treatment, induction of these marker genes was clearly observed, and their transcript levels were significantly higher in OE plants ($P < 0.05$) (Fig. 4). Thus, alteration in gene expression levels also revealed the enhancement of cold stress tolerance by *MeTCP4*.

3.5. RNA-seq of the MeTCP4 OE and WT plant transcriptome

To generate the expression profile of *Arabidopsis* genes differentially regulated by *MeTCP4* in response to cold stress, four cDNA libraries (two replicates per library) were constructed from *MeTCP4* OE plants (*35S::rMeTCP4* Normal and Cold) and WT plants (WT Normal and Cold). RNA-seq of these libraries generated approximately 50–68 million clean reads per sample under quality control and raw read filtering. Most of the reads (> 95%) from the eight libraries were further analyzed by comparison to the *Arabidopsis* genome. As shown in the data summary, clean reads from every library showed a match rate of approximately 95% to the *Arabidopsis* genome (Table 1); the majority of them were uniquely mapped.

3.6. Transcriptome changes mediated by MeTCP4

Computation and normalization were performed on the unique clean read number for each differentially expressed gene to RPKM (Supplementary Fig. S1). Genes with a > 2.0-fold change in expression

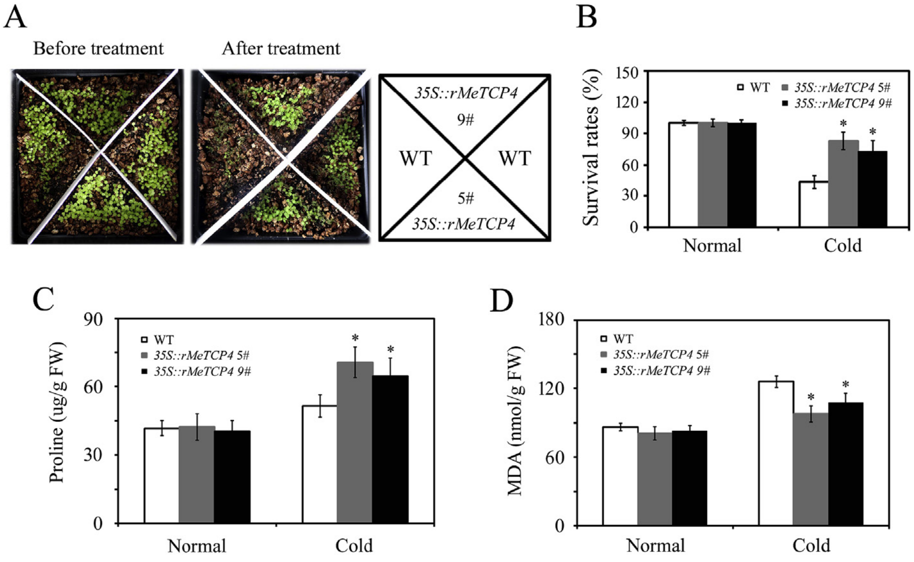

Fig. 3. WT and transgenic plants display different cold tolerance abilities. (A) Plant growth with or without cold treatment. WT and transgenic seedlings experienced a 2-day treatment at −4 °C and a 3-day recovery at 22 °C. (B) Survival rates of WT and OE plants with or without cold treatment. n > 30 (C) Proline concentration in *planta* at −4 °C for 24 h. (D) Leaf MDA contents after 24 h treatment at −4 °C. FW: fresh weight. Data was mean ± SD of three independent assays. Asterisks indicate differences of statistical significance between WT and OE plants (*$p < 0.05$).

were screened for significant up-regulation; genes with a < 0.5-fold change were screened for significant down-regulation; an FDR < 0.05 was applied to both screenings. By comparing the *MeTCP4* OE and WT, normal and cold libraries, a total of 1341 and 797 *MeTCP4*-responsive DEGs were identified using these criteria under normal and cold conditions, respectively (Fig. 5A, Supplementary Table S1). Among these DEGs, 191 genes were found to be induced and 223 genes were repressed by *MeTCP4* in both normal and cold conditions (Fig. 5B). Interestingly, we also observed that a large proportion of *MeTCP4*-regulated DEGs under normal conditions were not affected by cold treatment (Fig. 5B).

3.7. Functional annotation of DEGs

Gene ontology analysis was carried out for the *MeTCP4*-regulated DEGs, and assigned to three main categories. DEGs in the biological process category were commonly related to “response to oxygen-containing compound”, “response to stimulus” and “response to chemical” in both normal and cold conditions (Fig. 6A and B, Supplementary Table S2). Most DEGs assigned to the “molecular function” category function in transcription factor activity, DNA binding and quercetin 3-O-glucosyltransferase activity under normal conditions (Fig. 6A). However, after cold treatment, DEGs containing oxidoreductase activity, peroxidase activity and antioxidant activity were dominant (Fig. 6B). These results indicate the involvement of *MeTCP4* in stimulus and chemical response processes and that *MeTCP4* regulates distinct downstream genes under normal or cold conditions. Specifically, *MeTCP4*-responsive DEGs under normal conditions were involved in DNA binding and transcription regulation, such as transcription factors. However, under cold stress, the genes related to antioxidant enzymes were highly enriched. Thus, the enriched subcategories might offer clues for the screening of genes related to the putative function of *MeTCP4* in ROS scavenging during cold stress. In addition, annotation of DEGs into KEGG pathways showed that “biosynthesis of secondary metabolites” were the most enriched, followed by “metabolism of terpenoids and polyketides” (Supplementary Table S3). KEGG pathway analysis indicated the strong effects of *MeTCP4* on cell metabolism and biochemistry.

3.8. MeTCP4 Positively regulates expression of ROS-scavenging enzymes

Cold stress commonly induces plant cell damage and generates reactive oxygen species. To demonstrate the potential involvement of *MeTCP4* in ROS biosynthesis and metabolism, ROS accumulation was observed in cold-treated WT and OE seedlings by treating young leaves with 3, 3’ -diaminobenzidine (DAB). The WT leaf displayed more staining spots and contained much more H_2O_2 level than the leaf from *MeTCP4* OE plants, revealing the role of *MeTCP4* in ROS elimination (Fig. 7A and B). Subsequently, the transcript levels of ROS-responsive genes in both WT and *MeTCP4* OE plants, including two *glutathione transferases* (*GSTF7*: AT1G02920 and GSTU12: AT1G69920), one *ferric reduction oxidase 3* (*FRO3*: AT1G23020) and one *peroxidase* (*PRXCA*: AT3G49110), were determined. Consistent with the RNA-seq data, qRT-PCR showed that all ROS-scavenging-related genes were up-regulated under cold stress, while in *MeTCP4* OE plants, the expression was much higher than in WT after cold treatment (Fig. 7C–E). In contrast, the transcript level of *PRXCA*, which plays a significant role in generating H_2O_2 (Shigeto and Tsutsumi, 2016), was reduced in transgenic plants compared to WT plants under normal and cold conditions (Fig. 7F). Results show that plants gain tolerance to cold stress through repressed ROS generation and scavenging by *MeTCP4*.

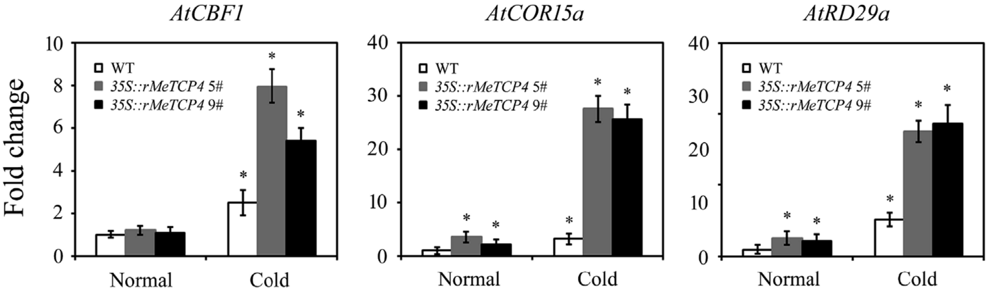

Fig. 4. Transcript abundance of marker genes in WT and OE plants. The expression levels of *AtCBF1*, *AtCOR15a* and *AtRD29a* are represented in transgenic lines compared to WT. The transcript levels of marker genes in WT without cold treatment were set as 1. Data was the mean ± SD of three independent assays. Asterisks indicate differences of statistical significance (*$P < 0.05$) between WT and OE.

Table 1
Summary of RNA-seq reads mapped to the *Arabidopsis* genome.

Sample	Total Reads	Unmapped Reads	Unique Mapped Reads	Multiple Mapped reads	Mapping Ratio
WT-Normal-1	50079538	2080695 (4.15%)	47738485 (95.33%)	260358 (0.52%)	95.85%
WT-Normal-2	62876428	2477543 (3.94%)	60068239 (95.53%)	330646 (0.53%)	96.06%
WT-Cold-1	63379782	2628155 (4.15%)	60373673 (95.26%)	377954 (0.60%)	95.85%
WT-Cold-2	50324142	2095658 (4.16%)	47954362 (95.29%)	274122 (0.54%)	95.84%
35S::rMeTCP4-Normal-1	51458382	2189986 (4.26%)	48996392 (95.22%)	272004 (0.53%)	95.74%
35S::rMeTCP4-Normal-2	58412636	2481584 (4.25%)	55555474 (95.11%)	375578 (0.64%)	95.75%
35S::rMeTCP4-Cold-1	67624538	3002804 (4.44%)	64262318 (95.03%)	359416 (0.53%)	95.56%
35S::rMeTCP4-Cold-2	57701952	2606008 (4.52%)	54808098 (94.98%)	287846 (0.50%)	95.48%

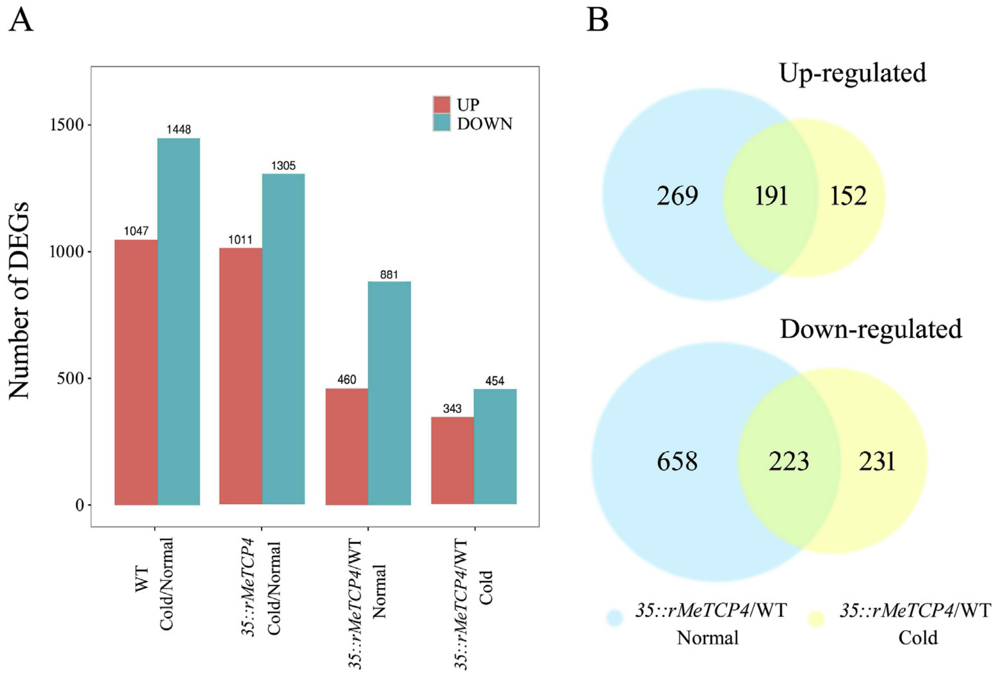

Fig. 5. Variation in gene expression of cold-treated WT and *MeTCP4* OE plants. (A) Comparison of DEG numbers between treatments and control, WT and transgenic plants. (B) Venn diagram shows overlap of up- and down-regulated DEGs that are modulated by *MeTCP4*.

4. Discussion

It has been suggested that in addition to the role that *TCP* genes play in plant growth and development, they are essential in adaptation to environmental stresses (Nicolas and Cubas, 2016; Danisman, 2016). *TCP* genes encode bHLH transcription factors in plants (Martin-Trillo and Cubas, 2010). These consist of 36 *TCP* family members in cassava; 5 members (*MeTCP2a*, *MeTCP2b*, *MeTCP3a*, *MeTCP3b* and *MeTCP4*)

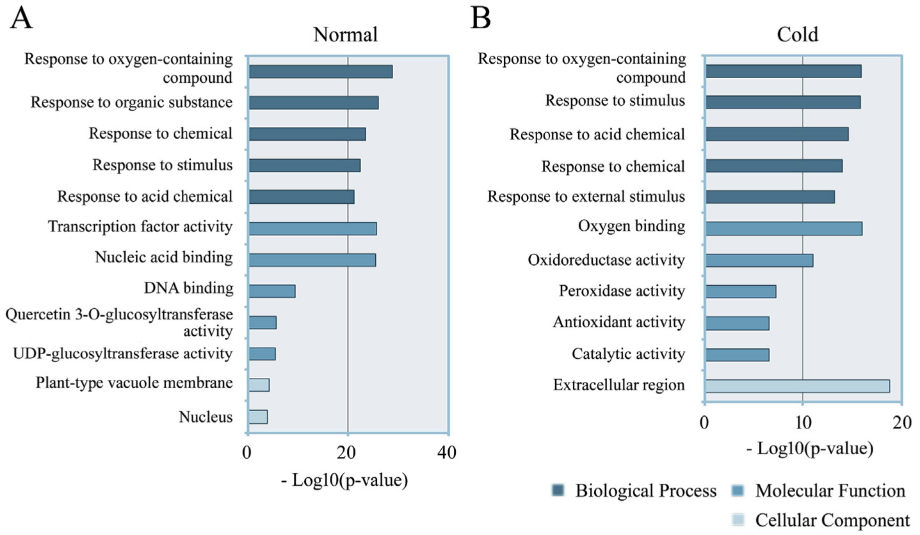

Fig. 6. Functional annotation of the DEGs. The subcategories represent biological processes, molecular functions and cellular components, respectively.

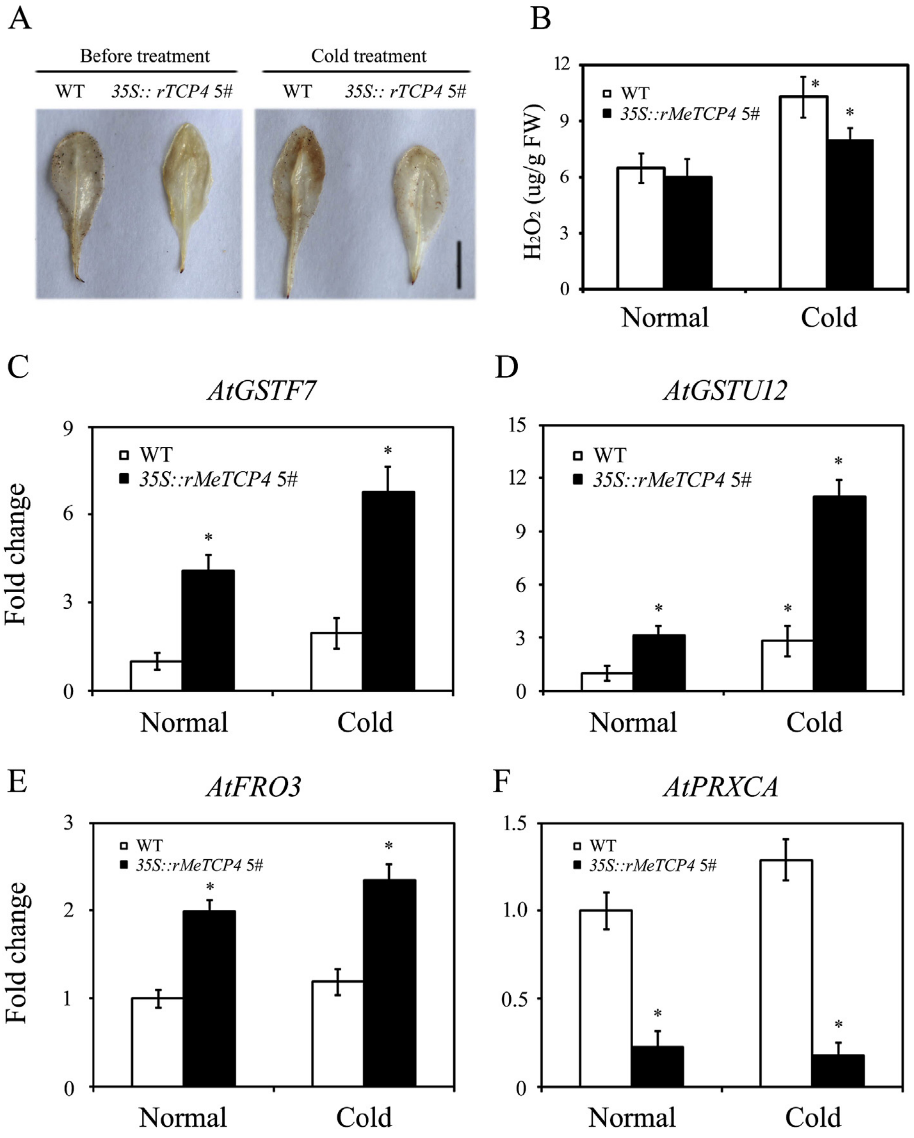

Fig. 7. ROS generation and transcript abundance of ROS-metabolism genes in WT and transgenic lines. (A–B) ROS amounts in WT and OE plants under normal and cold conditions. FW, fresh weight. (C–E) Transcript abundance of ROS-scavenging genes (*AtGSTF7/AtGSTU12/AtFRO3*) in WT and OE plants. (F) Expression levels of ROS-biosynthetic gene (*AtPRXCA*) in WT and transgenic plants. Asterisks indicate differences of statistical significance (*P $<$ 0.05) between WT and OE. Bar = 1 cm.

might be miR319-targeted (Lei et al., 2017). Recent research reveals increased expression of these target genes by cold stress (Wang et al., 2014). However, whereas overexpressed *Osa-miR319b* conferred cold tolerance in rice by enhancing survival and proline generation, its target gene, *OsTCP21*, was repressed (Wang et al., 2014). Here, we observed smaller and darker green leaves with smooth margins in *MeTCP4* overexpressing *Arabidopsis* seedlings, whereas those of the wild type had serrations in the margin. This result was largely consistent with the phenotype of the *mTCP3* plants in the previous reports (Schommer et al., 2008; Koyama et al., 2010; Li and Zachgo, 2013). However, *MeTCP4* overexpression in transgenic plants demonstrating increased tolerance to cold stress is the opposite of results observed in rice (Wang et al., 2014; Yang et al., 2013). This suggests that *MeTCP4* may play different roles in different plant species during cold stress. How *MeTCP4* affects leaf development and abiotic stress response in cassava plants requires further systematic investigation.

Proline contents were detected to elucidate the underlying mechanisms for enhanced freeze tolerance by *MeTCP4* overexpression. Free proline commonly accumulates under cold conditions (An et al., 2016b; Wang et al., 2014; Gilmour et al., 2000; Mahajan and Tuteja, 2005), and plant cell dehydration caused by cold stress can be prevented by proline (Mahajan and Tuteja, 2005). Greater contents of proline were detected in the *MeTCP4* transgenic lines, partially revealing the potential mechanism of accelerating proline accumulation by *MeTCP4* to confer cold tolerance. It is also noteworthy that marker genes responsive to cold stress, such as *AtCBF1*, *AtRD29a* and *AtCOR15a*, are highly expressed in the transgenic lines. By activating *COR* genes at the CRT/DRE motif (Baker et al., 1994; Wang and Hua, 2009), CBFs are central factors in freeze tolerance. While well studied in model plants, *CBF* genes also have a large number of homologs in other dicot and monocot species (Zhong et al., 2006; Akhtar et al., 2012). In cassava, *AtCBF3* overexpression may increase cold and drought tolerance. However, transgenic plants also exhibit serious developmental disorders, such as delayed plant growth, curled leaves and reduced storage root yield (An et al., 2016b). *AtRD29a* genes encode hydrophilic proteins, and are commonly induced by desiccation, freeze and high salinity (Narusaka et al., 2003; Nakashima et al., 2006; Msanne et al., 2011). In our study, *AtCBF1*, *AtRD29a* and *AtCOR15a* genes display upregulated expression in cold-treated *MeTCP4* transgenic lines. These results demonstrate the regulatory effect of *MeTCP4* on several cold-responsive genes.

Transcriptome analysis has been widely utilized to explain gene regulatory events in abiotic stress response of various plant species including *Arabidopsis*, rice, wheat and cotton (Zhu et al., 2013; Kreps et al., 2002; Gulick et al., 2005; Aprile et al., 2009; Nakashima et al., 2009; Mittal et al., 2012). We performed genome-wide transcript analysis using the WT and transgenic lines to find DEGs in *Arabidopsis*. The RNA-Seq results identified 1341 and 797 *MeTCP4*-responsive DEGs under normal and cold conditions, respectively. Among the DEGs, 414 were commonly regulated by *MeTCP4* in both conditions. Furthermore, through GO analysis, a number of genes were identified in response to environmental stimuli and chemical signals. These results indicated the potential of *MeTCP4* to change whole plant-cell omics by mediation of metabolic and cellular pathways. For the KEGG pathway annotation, DEGs were enriched into 22 and 21 different pathways under normal and cold conditions, respectively. Secondary metabolite biosynthesis and terpenoids/polyketides metabolism were the most enriched pathways. With further exploration of *MeTCP4*-specific genes, we observed that they exhibited distinct expression patterns under normal and cold conditions. Under cold treatment, *MeTCP4* was more likely to modulate the DEGs involved in ROS metabolism.

ROS are by-products of aerobic metabolism, i.e. respiration and photosynthesis in plant organelles such as mitochondria, chloroplasts and peroxisomes (You and Chan, 2015). The plant cell maintains intracellular ROS contents at low levels. Under normal growth conditions, ROS triggers various biotic/abiotic stress responses as a signaling molecule. However, high and low temperatures trigger ROS production. High ROS levels might cause oxidative damage and induce cell death. Our results show much lower leaf ROS levels in *MeTCP4* OE plants, suggesting the ability of *MeTCP4* to prevent *ROS* overaccumulation. In addition, overexpression of *MeTCP4* increased the transcript levels of *GSTF7*, *GSTU12* and *FRO3* under low temperature stress conditions. Glutathione S-transferases (GSTs) are ubiquitous and multifunctional conjugating proteins that prevent oxidative damage due to accumulation of ROS (Vijayakumar et al., 2016). Previous studies have revealed that GSTs have a wide spectrum of substrates in *Arabidopsis*, including those related to high tolerance against cold, drought, salt and oxidative stress (Dixon et al., 2009). In the plant cell, transition metal, such as Fe, is central in redox control and electron transport. Proteins that carry this metal ion (FROs) have been found to play an essential role in mediating diverse biochemical processes, including regulation of nucleic acids and ROS detoxification (Ravet and Pilon, 2013). A number of FROs that function to regulate Fe homeostasis under stress conditions, such as FRO2, have been characterized, and play a key role in glycine betaine-mediated chilling tolerance and ROS accumulation (Einset and Connolly, 2009). This may suggest that these genes are responsible for the reduced accumulation of ROS in *MeTCP4* OE plants after cold treatment.

Our study finds that increased expression of *MeTCP4* can effectively enhance cold tolerance in plants. Cold-treated OE plants exhibit more growth vigor and increased expression of cold-responsive genes than WT plants. Transcriptome of *MeTCP4* OE plants show significant upregulation of ROS scavenging genes, which may be responsible for the lower accumulation of ROS in OE plants. Overall, *MeTCP4* may regulate a network of ROS-signaling genes in response to cold conditions, and suggests an interesting area for future study.

Author contributions

Ming Peng and Shuxia Li designed research; Zhihao Cheng wrote the manuscript; Ning Lei, Jie Shen and Shuxia Li performed research; All authors discussed the results and commented on the manuscript. We acknowledge TopEdit LLC for the linguistic editing and proofreading during the preparation of this manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by the grants from the National Key R&D Program of China (2018YFD1000500), the National Natural Science Foundation of China (31701484, 31561143012 and 31701903), and the Program of Hainan Association for Science and Technology Plans to Youth R & D Innovation (HAST201627).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.02.015.

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