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Identification of a novel bZIP transcription factor in *Camellia sinensis* as a negative regulator of freezing tolerance in transgenic arabidopsis

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• **Background and Aims** Basic region/leucine zipper (bZIP) transcription factors play vital roles in the abiotic stress response of plants. However, little is known about the function of *bZIP* genes in *Camellia sinensis*.
• **Methods** *CsbZIP6* was overexpressed in *Arabidopsis thaliana*. Effects of *CsbZIP6* overexpression on abscisic acid (ABA) sensitivity, freezing tolerance and the expression of cold-responsive genes in arabidopsis were studied.
• **Key Results** *CsbZIP6* was induced during cold acclimation in tea plant. Constitutive overexpression of *CsbZIP6* in arabidopsis lowered the plants' tolerance to freezing stress and ABA exposure during seedling growth. Compared with wild-type (WT) plants, *CsbZIP6* overexpression (OE) lines exhibited increased levels of electrolyte leakage (EL) and malondialdehyde (MDA) contents, and reduced levels of total soluble sugars (TSS) under cold stress conditions. Microarray analysis of transgenic arabidopsis revealed that many differentially expressed genes (DEGs) between OE lines and WT plants could be mapped to 'response to cold' and 'response to water deprivation' terms based on Gene Ontology analysis. Interestingly, *CsbZIP6* overexpression repressed most of the cold- and drought-responsive genes as well as starch metabolism under cold stress conditions.
• **Conclusions** The data suggest that CsbZIP6 functions as a negative regulator of the cold stress response in *A. thaliana*, potentially by down-regulating cold-responsive genes.

Key words: CsbZIP6, cold stress, transgenic arabidopsis, transcription factor, tea plant (*Camellia sinensis*).

INTRODUCTION

The tea plant *Camellia sinensis* (L.) Kuntze grows best in temperate climates, and its natural distribution, development and growth are in large part shaped by the plant's low cold tolerance. Exposure to low temperatures, including chilling and frost, drastically reduces the yield and quality of tea and is associated with significant economic losses. Plants have evolved diverse mechanisms to adapt to adverse conditions such as cold temperatures, and regulatory proteins such as transcription factors (TFs) play important roles in these process.

A major advance in understanding the cold response in plants was the discovery of the ICE1 (inducer of CBF expression 1)–CBF/DREB (C-repeat-binding factor/dehydration-responsive element-binding factor)–COR (cold-regulated genes) signalling pathway (Thomashow, 1999). This transcriptional cascade has since been identified in many plants as an important regulator of cold signalling and acclimation. *ICE1* encodes a MYC-type basic helix–loop–helix (bHLH) TF that regulates the expression of *CBF3*/*DREB1A* in response to cold stress (Chinnusamy *et al*., 2003), while its homologue ICE2 activates the expression of *CBF1*/*DREB1B* and promotes freezing tolerance (Fursova *et al*., 2009). In arabidopsis, three CBF TFs bind C-repeat/dehydration-responsive elements (CRT/DREs; TACCGACAT) in the promoter regions of *COR* genes to activate the expression of the CBF regulon (Yamaguchi and Shinozaki, 1994; Jaglo-Ottosen *et al*., 1998; Liu *et al*., 1998; Thomashow, 2010). CBF1 and CBF3 co-ordinately induce *COR* genes, whereas CBF2/DREB1C negatively regulates the expression of *CBF1* and *CBF3* during cold acclimation (Novillo *et al*., 2004). In addition, the TF AtMYB15 binds MYB recognition *cis*-elements in the promoter regions of CBFs to regulate their expression negatively (Agarwal *et al*., 2006). *COR* gene expression has been shown to affect metabolism, protein stability and cell structure (Lee *et al*., 2005). Recent studies have isolated *CsICE1*, *CsCBF1* and *CsCOR* genes from tea plant and demonstrated not only that CsCBF1 can specifically bind the conserved CRT/DRE *cis*-element, but also that CsICE1 functions as a positive regulator in the tea plant cold response (Wang *et al*., 2009; Li *et al*., 2010; Wang *et al*., 2012; Ding *et al*., 2015).

In addition to the ICE1–CBF cold-response pathway, basic region/leucine zipper (bZIP) TFs have also been implicated in the regulation of signalling networks during cold stress in plants. bZIP proteins comprise a large and highly conserved group of eukaryotic TFs and are classified into 13 groups (designated A–L and S) (Correa *et al*., 2008). Members of group A, for example, the ABRE-binding factors (ABFs), have been extensively

studied and are thought to function in abscisic acid (ABA) and abiotic stress signalling in arabidopsis (Choi *et al*., 2000; Uno *et al*., 2000; Jakoby *et al*., 2002; Kang *et al*., 2002; Kim *et al*., 2004; Correa *et al*., 2008). AtbZIP1 and AtbZIP24, which belong to groups S and F, respectively, are also positive regulators of plant tolerance to abiotic stressors, and a group C bZIP, AtbZIP63, was found to be central to the glucose–ABA interaction network (Yang *et al*., 2009; Matiolli *et al*., 2011; Sun *et al*., 2012). In rice, OsABF1/OsbZIP12, OsABF2/OsbZIP46, OsbZIP23, OsbZIP71 and OsbZIP72 also play important roles in the abiotic stress response (Xiang *et al*., 2008; Lu *et al*., 2009; Amir Hossain *et al*., 2010; Hossain *et al*., 2010; Liu *et al*., 2014) Furthermore, the maize bZIP TF ZmbZIP72 has been shown to confer drought and salt tolerance in transgenic arabidopsis (Ying *et al*., 2012). Notably, a number of additional *bZIP* genes have been implicated in the regulation of the arabidopsis, rice and soybean cold response. For example, overexpression of *AtABF3* conferred increased tolerance to chilling, freezing, high temperature, oxidative stress and drought conditions, and in transgenic arabidopsis, overexpression of *GmbZIP1* improved the tolerance to several abiotic stresses including salinity, cold temperatures and drought (Kim *et al*., 2004; Gao *et al*., 2011). *OsbZIP52*, a member of the group C *bZIP* genes, participates in abiotic stress signalling and negatively regulates cold and drought responses in rice (Liu *et al*., 2012). The soybean C2H2-type zinc finger protein GmSCOF1 positively regulates *COR* gene expression mediated by GmSGBF1 (GmbZIP116) by enhancing its DNA-binding activity via protein–protein interactions and can thus enhance the cold tolerance of plants (Kim *et al*., 2001). Furthermore, GmbZIP44, GmbZIP62 and GmbZIP78 function as negative regulators of ABA signalling and confer salt and freezing tolerance in transgenic arabidopsis (Liao *et al*., 2008).

Our laboratory has previously cloned 18 *CsbZIP* genes in the tea plant and demonstrated that the exposure to cold, drought, salinity and ABA stress conditions resulted in the up- or down-regulation of different *CsbZIP* genes (Cao *et al*., 2015). Even though the expression of several *CsbZIP* genes has been reported to be regulated by abiotic stresses, few members of this multigene family have been functionally characterized in the tea plant. In this study, we report the functional characterization of CsbZIP6, a member of the group C bZIP family in the tea plant. *CsbZIP6* is upregulated during cold acclimation in the tea plant and localizes to the nuclei in arabidopsis roots. The overexpression of *CsbZIP6* in arabidopsis resulted in hypersensitivity to freezing and ABA treatment. Microarray analysis revealed the downregulation of many genes involved in the cold and water deprivation response in the leaves of *CsbZIP6* overexpression lines. A better understanding of the cold signalling mechanisms may ultimately allow the targeted engineering of hardier tea plants with an enhanced cold tolerance.

MATERIALS AND METHODS

Plant materials

Six different tea plant [*Camellia sinensis* (L.) Kuntze] cultivars, Damianbai (DMB), Hanlv (HL), Longjing 43 (LJ43), Zhenong 12 (ZN12), Zhenong 113 (ZN113) and Zhenong 21 (ZN21), were used in this study. The plants had been grown for 15 years in a field at the Tea Research Institute of the Chinese Academy of Agricultural Sciences, Hangzhou, China (TRI, CAAS, 3010′N, 1205′E). In the natural cold acclimation assay, the first two apical mature leaves from select healthy tea bushes in the same farm were sampled at 09·30 to 10·30 h. For quantitative RT-PCR (qRT-PCR) analysis, three independent biological replicates were performed. Each replicate was collected from >10 randomly selected tea plants.

The *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was used as wild-type (WT) controls and to generate the *CsbZIP6* overexpression (OE) lines. Transgenic arabidopsis plants were produced by *Agrobacterium tumefaciens*-mediated transformation (strain GV3101) (Clough and Bent, 1998). The *CsbZIP6*-OE lines in the Col-0 background constitutively express *CsbZIP6* with a C-terminal enhanced green fluorescent protein (eGFP) tag under the control of the 35S promoter. For the experiments, seeds from T_3 homozygote OE lines were used.

To construct the overexpression vector, the *CsbZIP6* open reading frame (ORF) was amplified using the following primer pair: 5′-CACCATGACGGCGGAGGAAGAAACG-3′ and 5′-TGCTTTCCGTTACACTGAGTC-3′. The amplified fragment was cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and sequenced ('pENTR-CsbZIP6'). The *CsbZIP6* ORF was then cloned from pENTR-CsbZIP6 into pH7FWG2 which contains a C-terminal eGFP fragment using LR clonase II enzyme mix (Invitrogen) (Takahashi *et al*., 2011). The resulting plasmid was designated as 'pH7FWG2-CsbZIP6' and used for *Agrobacterium*-mediated transformation of arabidopsis Col-0 plants.

Growth conditions

After 5 min surface sterilization in 10 % NaClO, seeds were rinsed five times with sterile water and stratified at 4 °C for 2–4 d before being planted on half-strength Murashige and Skoog (1/2 MS) medium containing 1·5 % sucrose. For the germination test, seeds were grown on 1/2 MS medium with or without 0·5 μM ABA for 10 d. For aseptic growth, seeds were grown on 1/2 MS medium for 7 d and then transferred to 1/2 MS medium containing 0·5 μM ABA for 14 d. Plants were grown in a growth chamber on a 10 h light/14 h dark regime at a light intensity of 100 μmol m^{-2} s^{-1} with daytime temperatures of 22 °C and night-time temperatures of 18 °C.

For freezing treatment, seedlings were grown on 1/2 MS medium for 10 d, and then transplanted to a soil mixture consisting of 3:2:1 peat moss:vermiculite:perlite for 20 d in the growth chamber on a 10 h light/14 h dark regime at a light intensity of 100 μmol m^{-2} s^{-1} with daytime temperatures of 22 °C and night-time temperatures of 18 °C. Then, plants were grown at 4 °C for 2 d under a normal photoperiod followed by 8 h at –6 °C, and the leaves were collected for measurement of electrolyte leakage (EL), malondialdehyde (MDA) and total soluble sugar (TSS) contents. For phenotype observation, the plants were grown under normal conditions (10 h 22 °C light/14 h 18 °C dark) for another 2 d after freezing treatment, and the photos were taken. For analysis of the percentage unstressed plants, the plants were grown under normal conditions (10 h 22 °C light/14 h 18 °C dark) for another 7 d after freezing treatment, and then the plants of which more than half of the leaves were withered as well as the dead plants were counted as stressed plants.

Promoter region amplification

The promoter region of *CsbZIP6* was amplified using the Genome Walking Kit (Takara) according to the manufacturer's protocol. Genomic DNA was isolated from the tea plant cultivar LJ43 and used as the PCR template. The primary reaction used the *CsbZIP6* gene-specific primer (GSP) 1 (5′-ACG GCTCTTGAGGAAGGCTTGATACTC-3′) and the AP1 primer provided in the kit. The secondary reaction used the *CsbZIP6* GSP 2 (5′-TGTCGTCCACTGAAAACACCCTATC CA-3′) and the AP2 primer provided in the kit. To analyse *cis*-acting regulatory elements, *CsbZIP6* promoter sequences in cultivars HL and ZN21 were amplified in reference to the sequence in LJ43 using the primers pair: 5′-GGCTGGTATCAA GTCAACTGAAAAT-3′ and 5′-TCCTCCGCCGTCATCGGT AAACGTA-3′.

Phylogenetic analysis of bZIP proteins

Alignment and phylogenetic tree assembly were conducted using default settings and the Neighbor–Joining algorithm of MEGA version 5 with 1000 bootstrap trials. Amino acid sequences of the bZIP proteins used for the analysis are listed in Supplementary Data Table S1.

Measurement of electrolyte leakage (EL)

For each condition, a total of six leaves were collected from three plants per line (two leaves per plant) and processed for EL measurements. Collected leaves were cut up and placed into tubes containing 4 mL of distilled water. The tubes were subjected to a vacuum three times at 5 min intervals to remove any air bubbles adherent to the surface of the leaves. Then, tubes were shaken at 200 rpm and 25 °C for 2 h. The conductivity of the solutions was measured at 25 °C (R1) using the conductivity meter Orion 5 Star (ThermoFisher Scientific). The solutions were then boiled at 100 °C for 20 min to lyse the plant cell walls completely. After cooling to 25 °C, the electrolyte conductivity of the boiled solutions was recorded (R2). The EL percentage was defined as follows: EL (%) = (R1/R2) × 100 %.

Measurement of malondialdehyde (MDA)

To measure MDA content, 0·2 g leaf samples were finely ground in liquid nitrogen and then homogenized in 5 mL of 10 % trichloroacetic acid. After centifugation, 3 mL of the supernatant was mixed with 3 mL of 0·67 % thiobarbituric acid. The mixture was boiled for 30 min and then rapidly cooled on ice. After centrifugation, the absorbance of the mixture was measured spectrophotometrically at 450, 532 and 600 nm, and the MDA content was calculated as previously described (Xu *et al*., 2008).

Measurement of total soluble sugar (TSS) content

To measure TSS content, 0·1 g leaf samples from WT and OE plants were lyophilized and homogenized. Soluble sugars were extracted with 1·5 mL of 80 % ethanol and shaken at 250 rpm at 60 °C for 30 min. After centrifuging at 11 000 *g* for 10 min, the supernatants were processed for TSS determination by the phenol–sulphuric acid method (DuBois *et al*., 1956).

Microarray analysis

Seedlings were germinated, grown on 1/2 MS medium for 2 weeks and then transferred to soil. Thirty-day-old plants (WT, 6-OE-1 and 6-OE-2) were grown at 4 °C ('cold') or normal conditions ('normal') for 4 d before the leaves were sampled, snap-frozen in liquid nitrogen and stored at –80 °C until RNA isolation. Each replicate was collected from one seedling. Three biological replicates were used for the analysis, except for the 'normal' 6-OE-2 samples which lack a replicate because of sub-standard RNA quality. The Agilent Arabidopsis Gene Expression microarray (4 × 44K, Design ID: 021169) was used in this experiment. Total RNA was extracted from leaf samples using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was quantified by spectrophotometry using the NanoDrop ND-2000 (Thermo Scientific), and RNA integrity was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies). The sample labelling, microarray hybridization and washing steps were performed according to the manufacturer's standard protocols. Briefly, total RNA was reverse-transcribed to double-stranded cDNA, then synthesized into cRNA and labelled with Cyanine-3-CTP. The labelled cRNAs were hybridized onto the microarray which was washed and then scanned by the Agilent Scanner G2505C (Agilent Technologies). Feature Extraction software (version 10.7.1.1, Agilent Technologies) was used to analyse array images to obtain raw data. The Genespring (version 13.1, Agilent Technologies) statistical tool was used for the basic analysis of the raw data. Raw data were first normalized with the quantile algorithm. Probes in any one out of all conditions that were flagged as 'Detected' were chosen for further analysis. Differentially expressed genes (DEGs) between the OE and WT plants were then identified through fold change (FC) analysis and assessed for statistical significance using the Student's *t*-test. The threshold for DEGs was an FC $\geq$ 2·0 and a *P*-value $\leq$0·05. Afterwards, Gene Ontology (GO) enrichment analysis was applied to assess the roles of these DEGs. The MapMan analysis was performed with MapMan version 3.5.1R2, and the DEGs were compared with public domain Ath_AGI_TAIR9_Jan2010 data sets (Thimm *et al*., 2004). All microarray data generated in this study have been deposited in the NCBI GEO database and are accessible through GEO Series accession number GSE84570 (release date: 1 July 2017).

Quantitative RT-PCR

Total RNA was extracted from leaf samples using Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA samples (1 μg) were then treated with RNase-free DNase I (Takara Biomedicals, Tokyo, Japan) to remove residual genomic DNA. First-strand cDNA was synthesized using a PrimeScript™ RT reagent Kit (Takara) according to the manufacturer's protocol. qRT-PCR was performed using the SYBR Premix Ex Taq™ II (Takara) on an ABI 7500 fast real-time

PCR system (Applied Biosystems). Triplicate quantitative assays were performed for each sample, and expression levels were normalized to the reference genes *CsPTB* and *AtEF* (AT5G19510) using the formula $2^{-\Delta Ct}$ (Yuan *et al*., 2008; Hao *et al*., 2014). All primer sequences used for qRT-PCR are listed in Supplementary Data Table S2.

Statistical analysis

Significance was determined by a one-way analysis of variance (ANOVA) for the treatment comparisons in Figs 4, 5 and 6, and by Fisher's least significant difference (LSD) test for differences between groups ($P < 0{\cdot}05$).

RESULTS

Effects of abiotic stress exposure on CsbZIP6 *transcription*

A previous study classified CsbZIP6 as a group C bZIP TF based on the phylogenetic tree of CsbZIPs and AtbZIPs and demonstrated that its transcript abundance was induced by ABA but not by 3 d cold stress treatment (Cao *et al*., 2015). The baseline expression level of *CsbZIP6* exceeded that of the reference gene *CsPTB* and was almost twice as high in leaves as in roots (Cao *et al*., 2015). Here, the *CsbZIP6* expression pattern was examined in the leaves of six different tea plant cultivars during cold acclimation. qRT-PCR analysis showed that the basal level of *CsbZIP6* expression was higher than that of *CsPTB* and *CsCBF1* (JX028828) in the leaves of non-acclimated tea plants, and the transcript abundance of *CsbZIP6* was highest in samples collected on 13 January, when the air temperature was lower than at any other collection time and tea plants were acclimatizing to cold conditions (Fig. 1). In contrast to *CsCBF1*, the induction of *CsbZIP6* was less in terms of magnitude in leaves (Fig. 1B). The level of *CsbZIP6* induction in response to cold acclimation differed between the different tea cultivars. For example, *CsbZIP6* expression was nearly five times higher on 13 January than on November 6 in HL but only 1·3 times higher in ZN21 (Fig. 1). Therefore, *CsbZIP6* transcription appears to be induced in response to cold temperatures.

Using genome walking PCR, a 2201 bp region upstream of the *CsbZIP6* start codon (ATG) was successfully amplified from total genomic DNA of the LJ43 cultivar and sequenced (Supplementary Dataset S1). *CsbZIP6* promoter sequences were also amplified from HL and ZN21 cultivars as these two cultivars showed the biggest difference in *CsbZIP6* induction (Supplementary Dataset S1). Analysis of the *CsbZIP6* promoter region by PLACE (a database of plant *cis*-acting regulatory DNA elements) revealed the presence of several *cis*-acting elements associated with stress responses, including an ABA-responsive *cis*-element (ABRE; ACGTG), a cold-responsive *cis*-element (CBF HV; RYCGAC), a low temperature response element (LTRE), and MYB and MYC recognition sites (Table 1) (Higo *et al*., 1998). Although some bases in *CsbZIP6* promoters were different among the three cultivars, there was no difference in the ABRE, CBF HV and LTRE elements, indicating that the alteration in cold induction was not caused by the promoters.

Phylogenetic analysis of CsbZIP6

There are 75, 89, 131 and 126 predicted members of the bZIP family of transcription factors in arabidopsis, rice, soybean and maize, respectively (Jakoby *et al*., 2002; Liao *et al*., 2008; Nijhawan *et al*., 2008; Wei *et al*., 2012). To investigate the evolutionary and structural relationship between CsbZIP6 and previously characterized bZIPs involved in the abiotic stress response in arabidopsis, rice, soybean and maize, phylogenetic analysis using amino acid sequences was performed. Most of the bZIP proteins branched into two main clades (Fig. 2). While the ABFs clustered into Clade I, CsbZIP6 fell into the oppositely branched Clade II. CsbZIP6 was most closely related to AtbZIP63, which has previously been reported to function as a regulator of ABA-mediated abiotic stress responses, followed by AtbZIP1, OsbZIP52 and GmbZIP62 (Matiolli *et al*., 2011).

Generation of CsbZIP6 *overexpression and reporter lines in arabidopsis*

To examine the function of CsbZIP6, transgenic lines that constitutively overexpress *CsbZIP6* were developed in *A. thaliana* ecotype Col-0. Two independent OE lines, 6-OE-1 and 6-OE-2, were confirmed by qRT-PCR (Fig. 3). As expected, the *CsbZIP6* transcript levels were significantly higher in both OE lines than in their WT counterparts where *CsbZIP6* expression was undetectable (Fig. 3A).

To determine its subcellular localization, the full-length *CsbZIP6* cDNA was cloned in-frame to the 5′ end of eGFP. Targeting ability was tested in arabidopsis roots, and results showed that the CsbZIP6::eGFP fusion proteins were targeted to arabidopsis nuclei (Fig. 3).

Effects of CsbZIP6 *overexpression on ABA sensitivity*

Since *CsbZIP6* is induced by ABA, we further tested if CsbZIP6 is involved in ABA sensitivity and the ABA-dependent regulation pathway by germination tests using the OE and WT plants lines. There were no differences in the germination rates of OE and WT plants in 1/2 MS medium (Fig. 4A, B). However, the addition of 0·5 μM ABA to the 1/2 MS medium led to lower germination rates in the OE lines than in WT plants (Fig. 4A, B). The effects of ABA on seedling development were also investigated, and no significant differences between the shoot or root growth of OE and WT plants were found in 1/2 MS medium. However, the roots of OE plants grown in the presence of 0·5 μM ABA were significantly shorter than the roots of WT plants raised under the same conditions (Fig. 4C, D). These results indicate that *CsbZIP6*-OE plants are more sensitive to ABA, and we propose that CsbZIP6 is involved in ABA signalling.

Effects of CsbZIP6 *overexpression on freezing tolerance*

Because the expression of *CsbZIP6* is induced during cold acclimation in the tea plant, we next investigated whether CsbZIP6 is involved in the adaptation to cold stress. While no striking phenotypic differences between homozygous OE lines

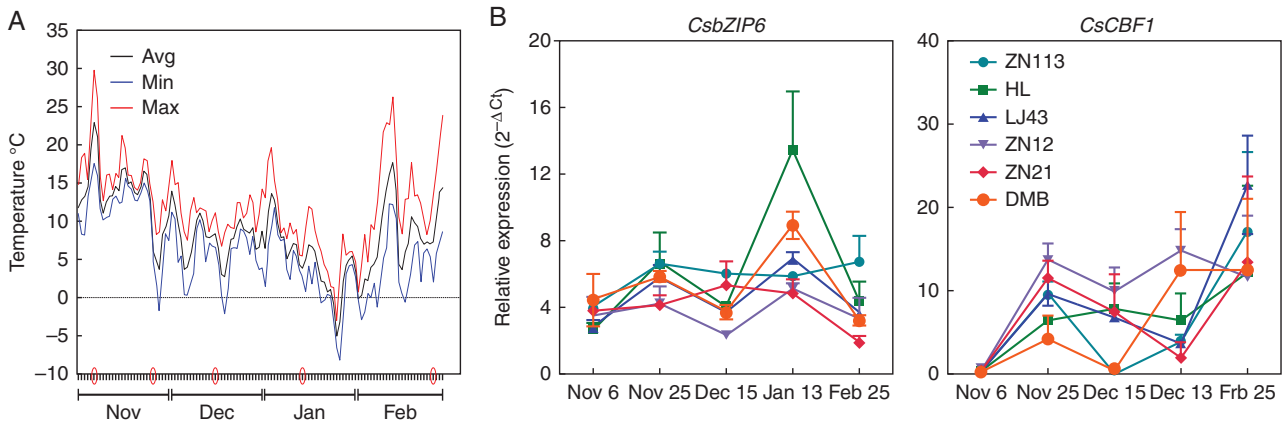

FIG. 1. Changes in air temperature and *CsbZIP6* expression levels in the leaves of six tea plant cultivars during cold acclimation. (A) Changes in the air temperature from November 2015 to February 2016. The maximum (Max), minimum (Min) and average (Avg) daily temperatures recorded are shown. Leaf sampling time points are indicated with red circles on the *x*-axis. The Avg temperatures on sampling days were 22·9 °C (6 November), 6·1 °C (25 November), 8·4 °C (15 December), 3·9 °C (13 January) and 7·3 °C (25 February). (B) Expression analysis of *CsbZIP6* and *CsCBF1* transcript abundance in the leaves of six tea plant cultivars during cold acclimation. Transcript abundance was determined by qRT-PCR. Data are shown as the mean ± s.e.m. ($n = 3$). All values are expressed relative to the *CsPTB* expression level.

TABLE 1. *Putative* cis*-acting regulatory elements in the promoter region (Supplementary Dataset S1) upstream of the* CsbZIP6 *start codon in LJ43, ZN21 and HL cultivars*

Motif name	Sequence	No. of motifs in two strands of DNA sequences			Description
		LJ43	ZN21	HL	
ABRELATERD1	ACGTG	1	1	1	ABRE-like sequence
ACGTATERD1	ACGT	8	8	8	Dehydration-responsive element
DOFCOREZM	AAAG	21	19	20	Stress-responsive element
GATABOX	GATA	18	18	18	Light-responsive element
GT1GMSCAM4	GAAAAA	4	4	4	Salinity-responsive element
LTRECOREATCOR15	CCGAC	1	1	1	Low temperature- and ABA-responsive element
MYB1AT	WAACCA	4	4	4	MYB recognition site; dehydration, ABA-responsive element
MYBCORE	CNGTTR	2	2	3	
MYB2CONSENSUSAT	YAACKG	1	1	2	
MYBPLANT	MACCWAMC	2	2	2	
MYCCONSENSUSAT	CANNTG	8	8	8	MYC recognition site; dehydration, ABA-responsive element
MYCATERD1	CATGTG	1	1	1	
MYCATRD22	CACATG	1	1	1	
CBF HV	RYCGAC	2	2	2	Dehydration-responsive element (DRE)-binding proteins
C-repeat/DRE	TGGCCGAC	1	1	1	Regulatory element involved in cold and dehydration responsiveness
DPBF CORE	ACACNNG	1	1	1	bZIP-binding core sequence

The PLACE database (http://www.dna.affrc.go.jp/PLACE/) was used to perform the analysis.
Abiotic stress-responsive motifs are listed in alphabetical order.

and WT plants raised under normal growth conditions were apparent (Fig. 5A), the OE lines displayed more severe freezing damage and a lower ratio of unstressed plants than WT plants following an 8 h exposure to –6 °C (Fig. 5B, C).

The control of membrane integrity and membrane-associated functions is crucial for cold tolerance, and cold stress-induced membrane damage can result in EL which is an indicator for the amount of damaged cells (Verslues *et al.*, 2006). Under normal conditions, both the OE lines and WT plants had comparable levels of relative EL (Fig. 6). However, under freezing conditions, the leaves of the OE lines released 75·6 and 66·0 % of their total electrolytes, respectively, whereas WT leaves released 46·5 %, indicating that *CsbZIP6*-OE plants were more vulnerable to membrane damage when exposed to freezing temperatures than WT plants.

When plants are exposed to cold temperatures, accumulated soluble sugars can act as osmoprotectants to protect the cell structure by binding water molecules during the dehydration induced by extracellular freezing (Ingram and Bartels, 1996). We therefore compared the total soluble sugar content in OE and WT plants exposed to normal or freezing conditions. In the two OE lines, the TSS content was either marginally ($P = 0·07$) or significantly ($P < 0·05$) less than in WT plants (Fig. 6).

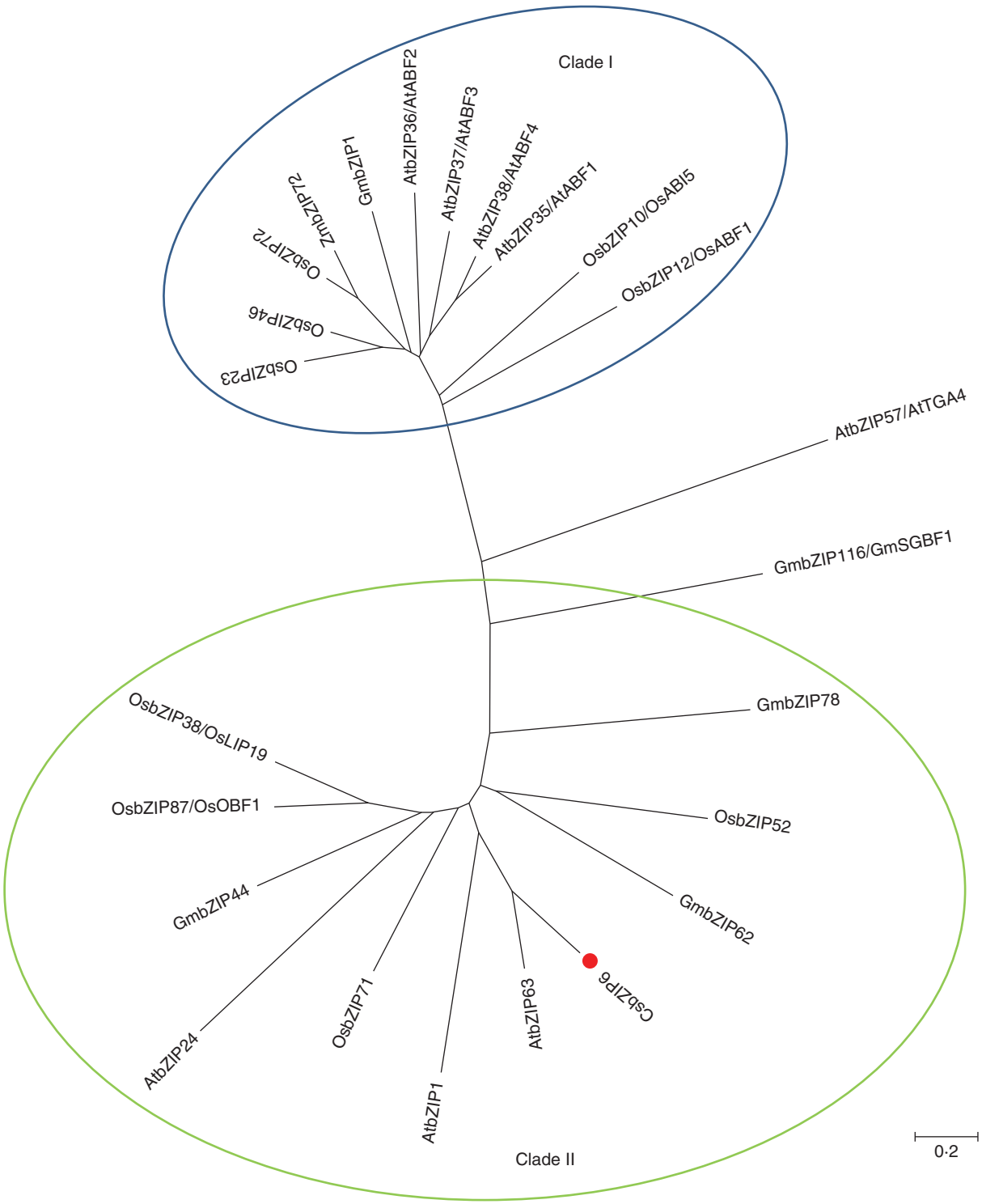

FIG. 2. Phylogenetic tree of CsbZIP6 and known bZIP transcription factors that function in the abiotic stress response. bZIP transcription factors from arabidopsis, rice, soybean and maize were subjected to the Neighbor–Joining algorithm phylogenetic tree construction using MEGA software version 5 with default settings. The amino acid sequences used to generate this phylogenetic analysis are listed in Table S1.

We also measured MDA which is a breakdown product of peroxidized polyunsaturated fatty acids in plant membranes and can indicate lipid peroxidation levels (Weber *et al*., 2004). In freezing conditions, the MDA content in one of the two OE lines was slightly higher than in WT plants, indicating a higher degree of lipid peroxidation in *CsbZIP6*-OE plants (Fig. 6). Altogether, these observations suggest that the constitutive overexpression of *CsbZIP6* in arabidopsis lowers the plant's tolerance to cold stresses.

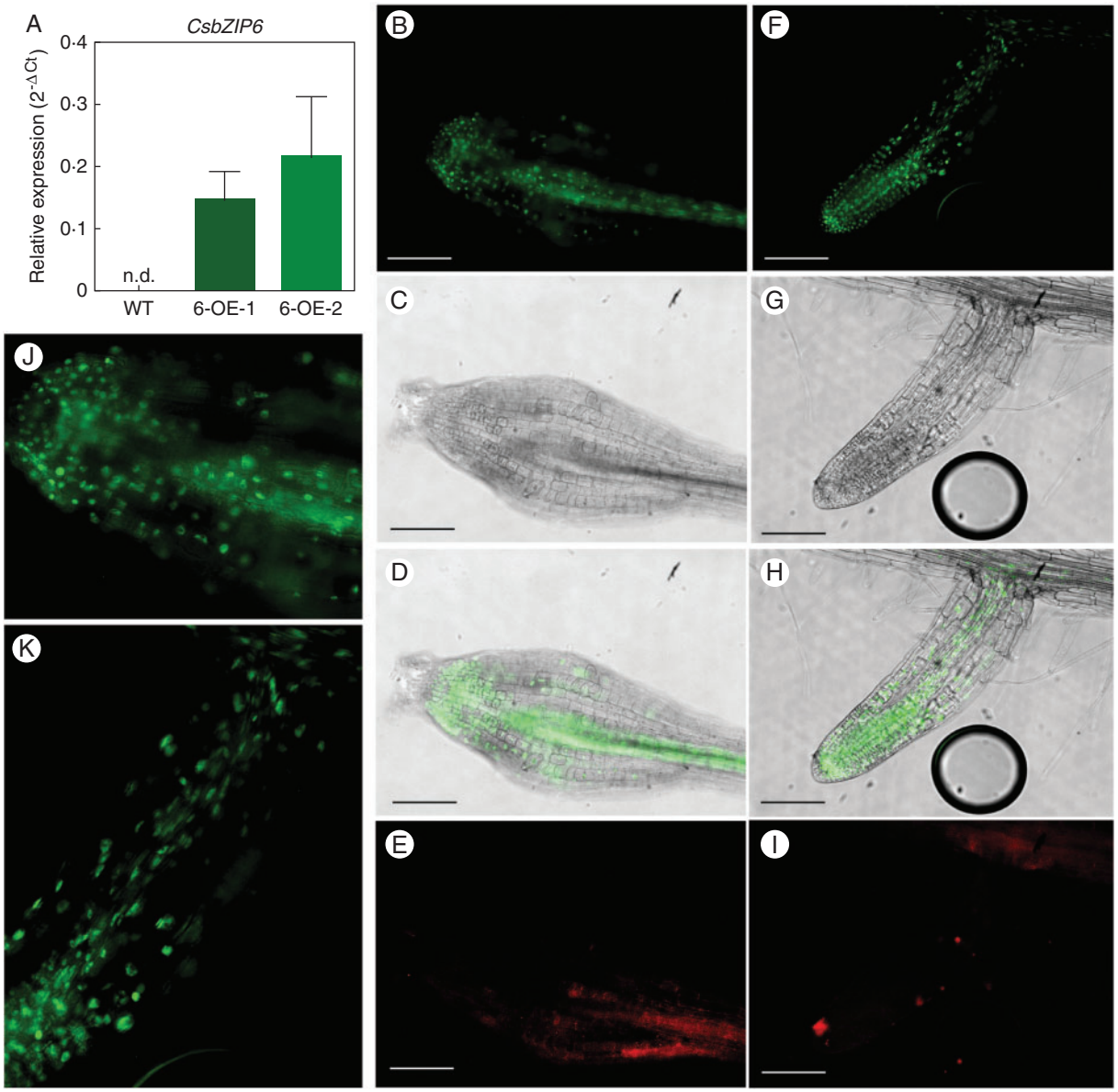

FIG. 3. *CsbZIP6* expression in the leaves of **a**rabidopsis overexpression (OE) plants and protein subcellular localization. (A) Expression of *CsbZIP6* in the leaves of arabidopsis Col-0 and two *CsbZIP6*-OE lines, respectively. Data are shown as the mean ± s.d. ($n = 3$). (B–K) Subcellular localization of CsbZIP6 in arabidopsis roots. Localization of C-terminal GFP tag of CsbZIP6::eGFP proteins in arabidopsis roots by fluorescence microscopy. Representative images were taken under fluorescence (B, E, F, I, J, K), under transmitted light (C, G) or as an overlay of both channels (D, H). The green signals indicate GFP and the red signals indicate non-specific background. (J, K) Magnification of (B) and (F), respectively. Scale bar = 50 μm.

Effect of CsbZIP6 *overexpression on the expression of cold-responsive genes in arabidopsis*

To obtain further insights into the molecular mechanisms by which CsbZIP6 mediates sensitivity to cold stress in arabidopsis plants, we compared the gene expression profiles in the leaves of both *CsbZIP6*-OE lines and WT plants. DEGs were selected based on fold change (FC > 2·0) and *P*-value ($P < 0{\cdot}05$). Under normal conditions, 418 genes were differentially expressed in WT and OE plants, while more than three times as many genes (1263) were differentially expressed in WT and OE plants under cold stress conditions, thereby supporting a specific role for CsbZIP6 in the cold stress response in leaves (Fig. 7A). Since the OE lines exhibit an increased sensitivity to freezing stress, we also compared the DEGs in OE and WT leaves exposed to cold stress with the complete cold-altered transcriptome of WT leaves. In summary, 344 of the genes that were differentially expressed in cold-treated OE and WT plants overlapped with cold stress-responsive genes in WT plants (Fig. 7B). Of these 344 DEGs, 124 DEGs showed the same and 220 DEGs the inverse regulation in the two comparisons (Fig. 7B).

We further characterized the network of DEGs by GO enrichment analysis with a significance cut-off at $P < 0{\cdot}05$. Closer examination of the DEGs between OE and WT plants

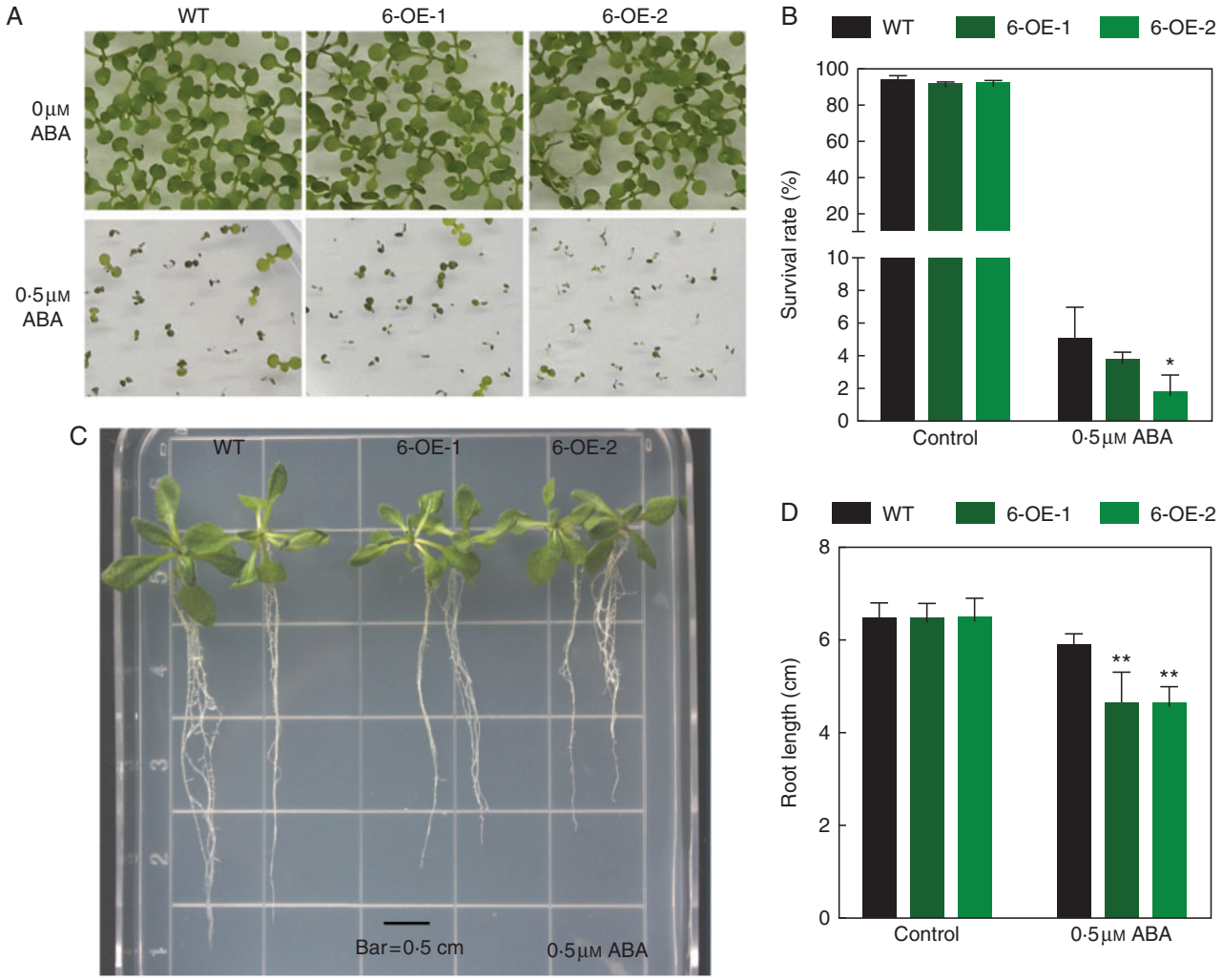

FIG. 4. ABA sensitivity of *CsbZIP6*-OE plants. (A) Homozygous seeds from WT plants or two OE lines were germinated on 1/2 MS medium with or without 0·5 μM ABA for 10 d. (B) The survival rate of the seeds grown on 1/2 MS medium with or without 0·5 μM ABA after 10 d. Experiments were performed in triplicate ($n = 50$ each). Data are shown as the mean ± s.d. (C, D) ABA response of root growth. Seeds were germinated for 7 d on ABA-free medium, and the seedlings were then transferred to medium with or without 0·5 μM ABA. Root length was measured 14 d after the transfer. Data are shown as the mean ± s.d. Significance is indicated by one ($P < 0·05$) or two ($P < 0·01$) asterisks.

under normal and cold conditions revealed 75 and 81 enriched GO terms belonging to 'biological processes', respectively (Supplementary Data Table S3 and S4). Notably, under normal conditions, 21 enriched GO terms had >10 genes mapped, including plant hormone-related pathways (containing 'ABA-activated signalling pathway' and 'response to ABA') and abiotic stress-related pathways (containing 'response to cold', 'response to oxidative stress' and 'response to water deprivation') (Fig. 7C). Under cold conditions, 29 enriched GO terms had >10 genes mapped, including sugar-related pathways and abiotic stress pathways (containing 'response to cold' and 'response to water deprivation') (Fig. 7C). We speculate that these pathways are important determinants of the phenotypic differences of the *CsbZIP6*-OE lines exposed to cold and ABA stress conditions.

The fundamentally different abiotic stresses drought, salinity and freezing all share the commonality that they decrease the availability of water to plant cells. The genes differentially expressed under cold conditions that mapped to 'response to cold' and 'response to water deprivation' GO terms are listed in Table 2. Interestingly, most cold-inducible genes were downregulated while the cold-repressible genes *AtMYB15*, *AtDIN10* and *AtBT5* were upregulated in the OE lines.

To analyse further the transcriptomic changes of the OE lines in response to cold treatment, we next compared gene expression changes via microarray using the MapMan software (Thimm *et al*., 2004). Starch metabolism was downregulated in the OE lines under cold conditions, revealing that it might play an important role in CsbZIP6 regulating plant response to cold stress (Fig. 7D). In conclusion, we hypothesize that reduced starch degradation might decrease the soluble sugar content and thus make plants more vulnerable to osmotic cell wall damage. The microarray data were further confirmed by qRT-PCR, showing six and three genes that were down- or upregulated, respectively, in the leaves of the *CsbZIP6*-OE lines (Supplementary Data Fig. S1).

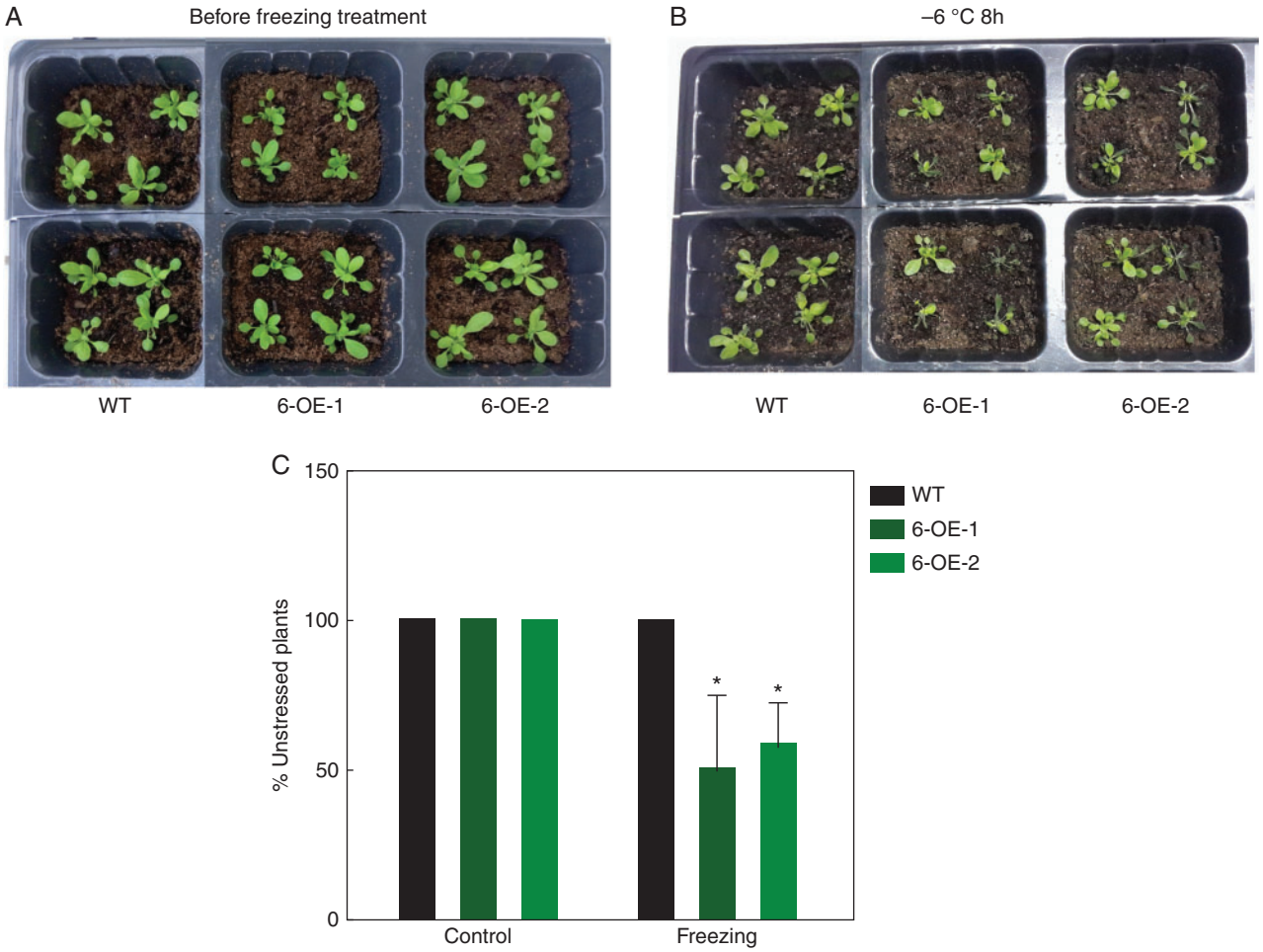

FIG. 5. Freezing tolerance of *CsbZIP6*-OE arabidopsis. (A, B) Growth performance of WT and two *CsbZIP6*-OE transgenic arabidopsis lines under normal conditions (before freezing) and after freezing treatment (–6 °C for 8 h). One-month-old plants were grown at 4 °C for 2 d, transferred to –6 °C for 8 h and then grown under normal conditions (10 h 22 °C light/14 h 18 °C dark) for an additional 2 d. (C) Percentage unstressed plants of OE lines and WT plants after freezing treatment. The plants were grown under normal conditions (10 h 22 °C light/14 h 18 °C dark) for another 7 d after freezing treatment, and then the plants of which more than half the leaves were withered as well as the dead plants were counted as stressed plants. Data are shown as the mean ± s.d. ($n = 12$). Significant ($P < 0{\cdot}05$) differences in OE lines compared with the WT is indicated by an asterisk.

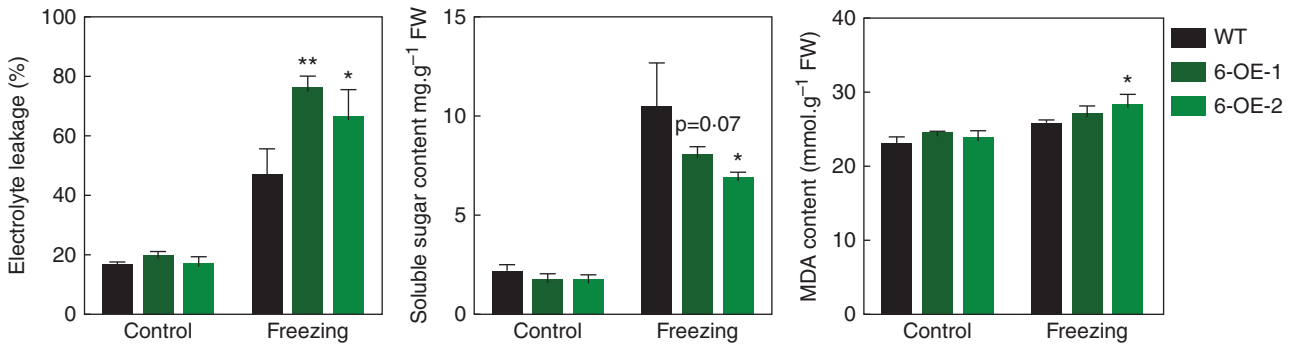

FIG. 6. Analysis of electrolyte leakage, soluble sugar content and MDA content in OE lines and WT plants. One-month-old plants were grown at 4 °C for 2 d, transferred to –6 °C for 8 h, and the leaves were collected and measured. Data are shown as the mean ± s.d. ($n = 3$). Significance of differences is indicated by one ($P < 0{\cdot}05$) or two ($P < 0{\cdot}01$) asterisks.

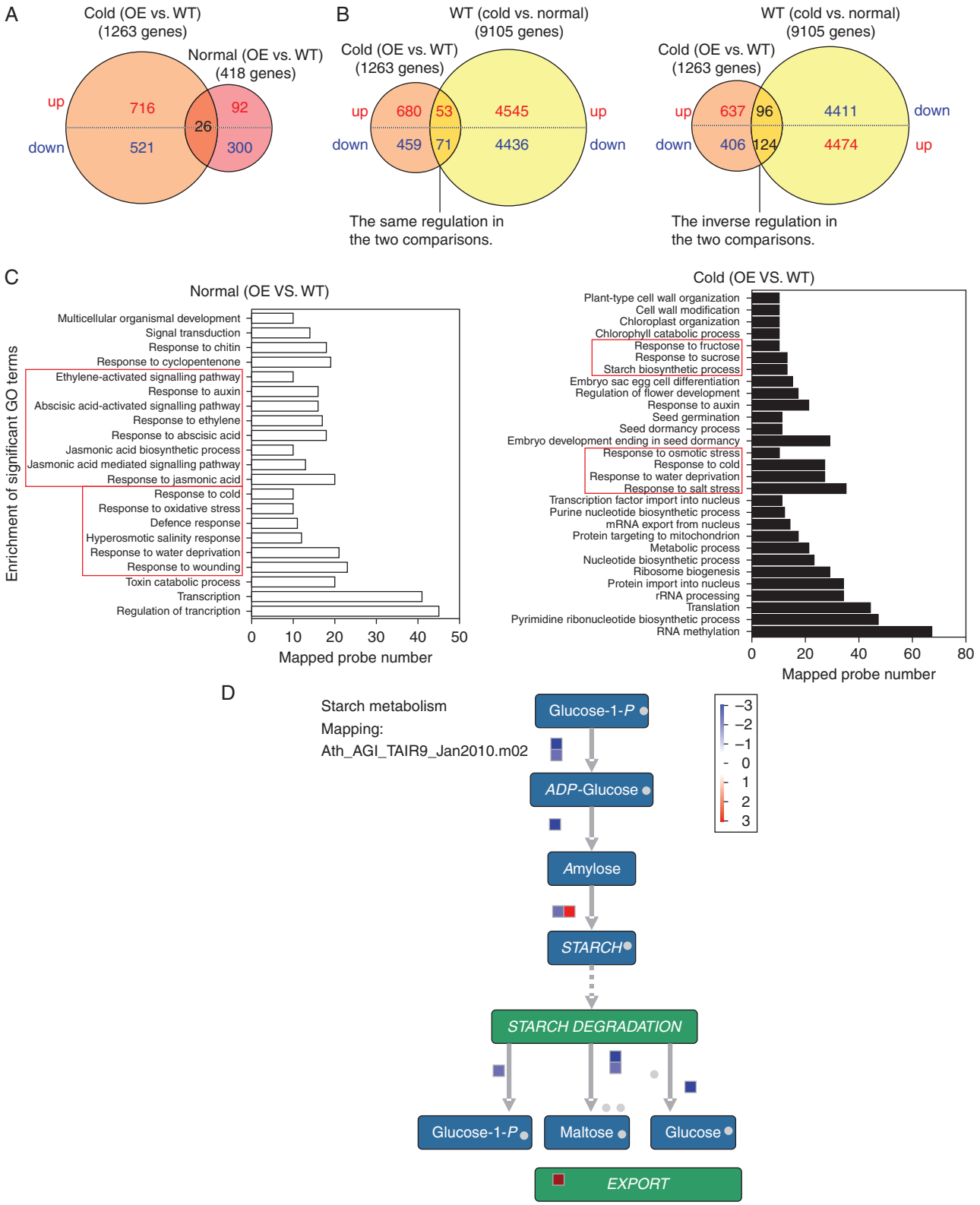

FIG. 7. Differential expression analyses in WT and *CsbZIP6*-OE plants. (A) Venn diagram showing the number of significantly differentially expressed genes ($P < 0{\cdot}05$, 2-fold cut-off) in the OE lines compared with WT plants under cold and normal conditions. (B) Venn diagram showing the number of significantly differentially expressed genes ($P < 0{\cdot}05$, 2-fold cut-off) in OE lines compared with WT plants and the significantly differentially expressed genes ($P < 0{\cdot}05$, 2-fold cut-off) in response to cold in the WT. The overlapping area indicates the number of DEGs showing the same and the inverse regulation in the two comparisons, respectively. (C) Significantly ($P < 0{\cdot}05$, 10-mapped-gene cut-off) enriched GO terms among the 418 and 1263 differentially expressed genes between OE and WT plants under normal and cold conditions, respectively. (D) MapMan starch metabolism map showing differences in transcript levels between OE and WT plants under cold conditions.

TABLE 2. *Microarray analysis of WT and* CsbZIP6-*OE plants*

Probe name	TAIR identifiers	Gene name	GO term	Description	Fold change		
					(OE-1 + OE-2) vs. WT		Cold vs. normal WT
					Cold	Normal	
Cold-inducible genes							
A_84_P16574	AT3G57260	*BGL2*	Response to cold	β-1,3-Glucanase 2	–3·5	X	7·32
A_84_P17366	AT1G01250	*ERF023*	Response to water deprivation; response to cold	DREB subfamily	–3·13	X	6·96
A_84_P18510	AT4G04490	*CRK36*	Response to cold	Cysteine-rich RLK 36	–2·85	X	12·66
A_84_P16776	AT5G06760	*LEA4-5*	Response to water deprivation; response to cold	Late embryogenesis abundant 4–5, protects enzyme activities from freeze–thaw cycles	–2·7	X	8·04
A_84_P15628	AT3G57240	*BG3*	Response to cold	Glycosyl hydrolase	–2·67	X	21·82
A_84_P14611	AT3G29320	*PHS1*	Response to water deprivation; response to cold	α-Glucan phosphorylase 1	–2·44	X	3·53
A_84_P16114	AT1G09350	*GolS3*	Response to cold	Galactinol synthase 3	–2·31	X	729·9
A_84_P291304	AT1G29395	*COR413IM1*	Response to water deprivation; response to cold	Cold regulated 413 inner membrane 1	–2·31	X	6·43
A_84_P598557	AT5G57380	*VIN3*	Response to cold	Vernalization insensitive 3	–2·19	–2·0	6·81
A_84_P167173	AT4G25480	*DREB1A*/*CBF3*	Response to water deprivation; response to cold	C-repeat binding factor 3	–2·18	–1·79	21·39
A_84_P21841	AT1G02930	*GSTF6*	Response to water deprivation	Glutathione *S*-transferase F6	–2·13	X	6·65
A_84_P234853	AT5G15960	*KIN1*	Response to water deprivation; response to cold	Cold and ABA inducible protein KIN1, anti-freeze protein	–2·07	X	268·86
A_84_P15651	AT3G63010	*GID1B*	Response to water deprivation; response to cold	GA insensitive dwarf 1B	–2·04	–1·61	2·87
A_84_P855617	AT5G20630	*GER3*	Response to cold	Germin like protein 3	2·97	X	2·62
Cold-repressive genes							
A_84_P820158	AT2G18050	*HIS1-3*	Response to water deprivation	Histone H1-3	–3·28	–1·73	–18·52
A_84_P860582	AT4G34000	*ABF3*/*BZIP37*	Response to water deprivation	Abscisic acid responsive elements-binding factor 3	–2·40	–1·26	–3·84
A_84_P13518	AT1G19640	*JMT*	Response to water deprivation	Jasmonic acid carboxyl methyltransferase	–2·17	X	–6·21
A_84_P21458	AT4G37610	*BT5*	Response to cold	BTB and TAZ domain protein 5	12·36	X	–35·4
A_84_P14131	AT5G20250	*DIN10*	Response to cold	Dark inducible 10, raffinose synthase 6	10·14	–2·21	–23·19
A_84_P870349	AT3G23250	*MYB15*	Response to water deprivation	MYB domain protein 15	2·39	X	–4·72
Genes not regulated by cold in the present study							
A_84_P12977	AT5G01600	*FER1*	Response to cold	Ferritin 1	–3·38	X	X
A_84_P799845	AT1G49480	*RTV1*	Response to cold	Related to vernalization 1	–2·69	X	X
A_84_P17929	AT1G56600	*GolS2*	Response to water deprivation; response to cold	Galactinol synthase 2	–2·5	X	X
A_84_P17859	AT5G57560	*TCH4*	Response to cold	Cell wall modifying enzyme	4·32	X	X
A_84_P857901	AT3G22310	*PMH1*	Response to water deprivation; response to cold	Putative mitochondrial RNA helicase 1	3·16	X	X
A_84_P12714	AT3G23830	*GRP4*	Response to water deprivation; response to cold	Glycine-rich RNA binding protein 4	2·78	1·38	X
A_84_P10108	AT4G36020	*CSDP1*	Response to water deprivation; Response to cold	Cold shock domain protein 1	2·65	X	X
A_84_P807865	AT2G36530	*LOS2*	Response to cold	Low expression of osmotically responsive genes 2	2·53	X	X
A_84_P11113	AT5G08620	*STRS2*	Response to water deprivation; response to cold	Stress response suppressor 2	2·48	1·17	X
A_84_P811094	AT1G56070	*LOS1*	Response to cold	Low expression of osmotically responsive genes 1	2·4	X	X
A_84_P849175	AT1G55490	*CPN60B*	Response to cold	Chaperonin 60β	2·04	X	X
A_84_P813301	AT5G08280	*HEMC*	Response to cold	Hydroxymethylbilane synthase	2·02	X	X
A_84_P24062	AT3G23920	*BAM1*	Response to water deprivation	β-Amylase	–2·51	–1·45	X

(continued)

TABLE 2. *Continued*

Probe name	TAIR identifiers	Gene name	GO term	Description	Fold change		
					(OE-1 + OE-2) vs. WT		Cold vs. normal WT
					Cold	Normal	
A_84_P20156	AT2G41430	*ERD15*	Response to water deprivation	Early responsive to dehydration 15	–2·19	–1·51	X
A_84_P18627	AT4G39090	*RD19*	Response to water deprivation	Responsive to dehydration 19	–2·29	–1·75	X
A_84_P851466	AT1G55740	*SIP1*	Response to water deprivation	Seed imbibition 1	2·29	X	X
A_84_P50830830	AT1G47128	*RD21*	Response to water deprivation	Dehydration stress-responsive gene	–2·05	–1·20	X
A_84_P251265	AT1G63720	*HRGP*	Response to water deprivation	Hydroxyproline-rich glycoprotein family protein	–2·08	X	X
A_84_P116602	AT2G01830	*AHK4*	Response to water deprivation	Histidine kinase 4	2·07	X	X
A_84_P22337	AT4G21440	*MYB102*	Response to water deprivation	R2R3 family MYB transcription factor	–2·96	X	X
A_84_P815408	AT2G04030	*CR88*	Response to water deprivation	Chloroplast heat shock protein 90	2·26	X	X
A_84_P854478	AT1G54160	*NF-YA5*	Rsponse to water deprivation	Nuclear transcription factor Y subunit A-5	–2·0	X	X

Differential expression of genes ($P < 0{\cdot}05$, 2-fold cut-off) between OE and WT plants under cold conditions mapped in the GO terms 'response to cold and response to water deprivation' ($P < 0{\cdot}05$, false discovery rate $< 0{\cdot}05$), their expression in OE compared to WT plants under normal conditions, and their response to cold stress in WT plants.

Fold change is shown for each comparison; 'X' indicates no significant differential expression.

Significance was determined by a P-value $< 0{\cdot}05$. A caret (^) indicates marginal significance ($0{\cdot}05 < P < 0{\cdot}07$).

DISCUSSION

In this study, we showed that *CsbZIP6* is induced during cold acclimation. Constitutive overexpression of *CsbZIP6* decreases the cold tolerance in transgenic arabidopsis plants by negatively regulating cold-related genes.

CsbZIP6 gene expression was regulated by both ABA and cold exposure (Fig. 1B) (Cao *et al*., 2015). Sequence analysis identified several types of stress-responsive elements in the *CsbZIP6* promoter region including ABRE, DRE, MYB and MYC recognition sites which are recognized by bZIP, CBF, MYB and MYC TFs, respectively (Table 1). Since these elements play crucial roles in the plant response to ABA and abiotic stress exposures such as cold, CsbZIP6 may also be involved in ABA- and abiotic stress-mediated signalling.

Even though *CsbZIP6* expression was not affected by a 3 d cold stress (4 °C) treatment (Cao *et al*., 2015), it was induced during tea plant cold acclimation in the present study (Fig. 1B). Actually, there was no significant alteration in the EL of leaves of LJ43 between normal conditions (15 °C) and cold stress conditions (2 °C treatment for 1–7 d), and leaves released 29–33 % of their total electrolytes in that process (Huang *et al*., 2015). Notably, the leaves released 40–46 % of their total electrolytes on 13 January in this study (Supplementary Data Fig. S2). This suggests that the different state of the leaves between short-term cold stress and natural cold acclimation may influence the expression of *CsbZIP6*, and the cold-responsive induction of *CsbZIP6* expression might require a prolonged exposure to cold temperatures or a certain strength of low temperature. Alternatively, *CsbZIP6* could be seasonally regulated by the circadian clock which has been shown to be strongly linked with cellular responses to cold temperatures. Circadian rhythms in plants are crucial for both the photoperiodic measurement of the seasons and for essential adaptations for survival in the cold (Eriksson and Webb, 2011). Furthermore, the expression of *CBF* genes as well as some of their downstream targets is also under circadian control (Fowler *et al*., 2005).

Protein sequence alignment revealed that OsbZIP52 and CsbZIP6 share 52 % amino acid identity and are classified as members of group C (Supplementary Dataset S2). OsbZIP52 is a negative regulator involved in drought and cold stress responses in rice (Liu *et al*., 2012). In this study, we showed that the function of CsbZIP6 in response to cold stress was similar to that of OsbZIP52. AtbZIP63, which shares 42 % amino acid identity with CsbZIP6, has been proposed to play a role in energy metabolism, seed maturation and germination under osmotic stress (Supplementary Dataset S2) (Veerabagu *et al*., 2014). In addition, AtbZIP63 has been reported to represent an important node in the glucose–ABA interaction network and may participate in the fine-tuning of the ABA-mediated abiotic stress response depending on sugar availability (Matiolli *et al*., 2011). Recently, Mair and colleagues (2015) showed that *AtbZIP63* is regulated by sucrose non-fermenting-related kinase 1 (SnRK1)-dependent phosphorylation and functions in the energy starvation response and metabolic regulation. Particularly under stress conditions, the use of available energy resources needs to balance the growth and defence needs of plants (Mair *et al*., 2015). The data derived from Genevestigator showed that similarly to the *CsbZIP6* cold response, *AtbZIP63* was significantly upregulated by cold stress in several studies (Zimmermann *et al*., 2004). Since CsbZIP6 participates in the cold response, AtbZIP63 might also be involved in cold response regulatory pathways, and its role in the plant cold response warrants further investigation. Three tea plant CsSnRKs have been isolated in our laboratory and shown to be

transcriptionally regulated by cold acclimation (Yue *et al*., 2015). Our microarray data identified three sugar metabolism-related pathways that were over-represented in the DEGs between OE and WT plants under cold conditions, indicating a functional CsSnRK–bZIP6 pathway in the tea plant which should be explored further in future studies.

Abscisic acid is important in seed dormancy and seed germination processes (Agarwal and Jha, 2010). It was reported that many *bZIP* genes mediate plant responses to ABA and abiotic stress tolerance, such as *AtABF* genes, *OsbZIP23*, *OsbZIP71*, etc, (Kang *et al*., 2002; Kim *et al*., 2004; Xiang *et al*., 2008; Liu *et al*., 2014). For example, *OsbZIP71*-RNAi (RNA interference) lines were more sensitive to ABA and osmotic stresses, and the inducible lines were insensitive to ABA and more tolerant to osmotic stress (Liu *et al*., 2014). *CsbZIP6*-OE plants were hypersensitive to ABA at both seed germination and the seedling growth stage (Fig. 4). Under cold conditons, the DEGs could be mapped to 'seed germination' and 'seed dormancy' processes, indicating that CsbZIP6 could also affect these processes, and the induction of the ABA level in response to cold may be involved in this (Lang *et al*., 1994) (Fig. 7C). We also found that *CsbZIP6*-OE plants had a lower unstressed plants ratio than WT plants in response to freezing temperatures (Fig. 5). Indirect evidence implied that altered phenotypic characteristics such as EL percentage, MDA content and TSS content might contribute to the reduced cold tolerance of *CsbZIP6*-OE plants. A reasonable explanation is that CsbZIP6 mediates ABA sensitivity which is involved in cold stress tolerance.

To date, a number of *COR* genes have been characterized. Even though *COR15A*, *COR47* and *COR78* had previously been reported to be downstream genes of CBFs, their relative expression levels did not change in the *CsbZIP6*-OE lines exposed to cold conditions, indicating that these *COR* genes were not regulated by *CsbZIP6* (Supplementary Data Table S5). Jia *et al*. (2016) reported that CBFs only regulate 7 % of *COR* genes, and the *cbf* triple mutants do not totally abolish cold acclimation, supporting that other cod response pathways different from CBF exist. Transcript profiling indicated that *CsbZIP6* overexpression only moderately affects the transcriptome of arabidopsis plants growing under normal conditions. However, there were three times as many DEGs under cold conditions as there were under normal conditions, indicating that CsbZIP6 plays an important role in the response to cold stress (Fig. 7A). Sugar plays an essential role in tea plant cold acclimation; the contents of starch, TSS and individual sugars were changed in tea plant leaves during cold acclimation (Yue *et al*., 2015). Since starch metabolism was down-regulated in the OE lines under cold conditions (Fig. 7D), CsbZIP6 may be involved in cold stress adaptation by regulating the sugar level.

The expression pattern of several genes was altered in *CsbZIP6*-OE plants, with enrichment of plant hormone-related and cold, salinity, drought and oxidative stress response pathways under normal conditions and enrichment of sugar-related and cold, salinity, drought and oxidative stress response pathways under cold conditions (Fig. 7C). These genes represent a list of potential targets which could be used to study how cold stress response could be controlled. The cross-talk of different stress responses allows plants to adapt/acclimate to a range of different stresses (Pastori and Foyer, 2002). In addition to drought and salinity, chilling and freezing temperatures can also cause osmotic stress as well as their direct effect on metabolism, and freeze-induced membrane damage results primarily from the severe dehydration associated with freezing (Thomashow, 1999; Chinnusamy *et al*., 2004). Oxidative stress signalling has been postulated to play a role in freezing tolerance, and this could explain the oxidative stress-related GO terms that were significantly changed in the DEGs between OE lines and WT plants. It is also similar to the report that CBF-regulated genes are not only involved in the cold stress response, but are also implicated in the drought and salt stress response (Jia *et al*., 2016). Thirteen of the 14 cold-induced genes listed in Table 2 were down-regulated in the OE lines under cold conditions, indicating that *CsbZIP6* overexpression results in hypersensitivity to cold stress due to the suppressed induction of cold-related genes, suggesting that it functions as a negative regulator in the plant cold response. Notably, the expression of *AtCBF3*, a cold-inducible positive regulator involved in the cold response, was down-regulated in the *CsbZIP6*-OE lines compared with WT plants under cold conditions (Table 2) (Liu *et al*., 1998). However, AtMYB15, which can negatively regulate CBFs, was upregulated in *CsbZIP6*-OE plants (Table 2) (Agarwal *et al*., 2006). These results indicate that the AtMYB15–AtCBF3 cold-responsive signalling pathway may play an important role in CsbZIP6 regulating plant response to cold stress in arabidopsis. CsbZIP6 has CBF HV promoter elements; we propose that there is a feedback regulation loop between the CBF3-mediated ABA-independent pathway and the CsbZIP6-mediated ABA-dependent pathway.

The potential role of CsbZIP6 as a negative regulator in the cold stress response, combined with the specific induction of *CsbZIP6* expression during cold acclimation, sheds new light on the cold stress response of plants at a physiological level. In arabidopsis, certain bZIP TFs have been shown to be involved in abiotic stress and ABA signalling but in the tea plant, the function of most *bZIP* genes in terms of the abiotic stress response is less well characterized. The knowledge gained in this study not only reveals an important regulatory function for CsbZIP6 in cold stress tolerance but also provides a foundation for the future investigation of cold-induced signalling pathways in which CsbZIP6/AtbZIP63 participate.

In conclusion, we determined the cold sensitivity of *CsbZIP6*-overexpressing transgenic arabidopsis plants. Our data revealed that CsbZIP6 functions as a negative regulator in the arabidopsis response to cold stress on both physiological and transcriptional levels. Nevertheless, the results described in this study highlight the need to dissect further the pathways through which CsbZIP6 regulates the tea plant's tolerance of cold stress conditions.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Dataset S1: the promoter sequence of *CsbZIP6* (upstream of the ATG codon) in LJ43, HL and ZN21 cultivars. Dataset S2: alignment of OsbZIP52 and AtbZIP63 with CsbZIP6 by NCBI Blastp. Table S1: the amino acid sequences used to generate the phylogenetic tree. Table S2: primer sequences for quantitative RT-PCR. Table S3: 75 significant GO terms ($P < 0{\cdot}05$) belonging

to 'biological process' were mapped by DEGs between OE and WT plants under normal conditions. Table S4: 81 significant GO terms ($P < 0{\cdot}05$) belonging to 'biological process' were mapped by DEGs between OE and WT plants under cold conditions. Table S5: expression of *COR* genes between OE and WT plants under normal and cold conditions, and their response to cold stress in WT plants. Fig. S1: quantitative RT-PCR validation. Fig. S2: analysis of electrolyte leakage in the six tea plant cultivars on November 6 and January 13.

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