PeSTZ1, a C2H2-type zinc finger transcription factor from Populus euphratica, enhances freezing tolerance through modulation of ROS scavenging by directly regulating PeAPX2

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Summary
In the present study, PeSTZ1, a cysteine-2/histidine-2-type zinc finger transcription factor, was isolated from the desert poplar, Populus euphratica, which serves as a model stress adaptation system for trees. PeSTZ1 was preferentially expressed in the young stems and was significantly up-regulated during chilling and freezing treatments. PeSTZ1 was localized to the nucleus and bound specifically to the PeAPX2 promoter. To examine the potential functions of PeSTZ1, we overexpressed it in poplar 84K hybrids (Populus alba × Populus glandulosa), which are known to be stress-sensitive. Upon exposure to freezing stress, transgenic poplars maintained higher photosynthetic activity and dissipated more excess light energy (in the form of heat) than wild-type poplars. Thus, PeSTZ1 functions as a transcription activator to enhance freezing tolerance without sacrificing growth. Under freezing stress, PeSTZ1 acts upstream of ASCORBATE PEROXIDASE2 (PeAPX2) and directly regulates its expression by binding to its promoter. Activated PeAPX2 promotes cytosolic APX that scavenges reactive oxygen species (ROS) under cold stress. PeSTZ1 may operate in parallel with C-REPEAT-BINDING FACTORS to regulate SCOF-1 expression. Overall, PeSTZ1 up-regulation reduces malondialdehyde and ROS accumulation by activating the antioxidant system. Taken together, these results suggested that overexpressing PeSTZ1 in 84K poplar enhances freezing tolerance through the modulation of ROS scavenging via the direct regulation of PeAPX2 expression.

Keywords: freezing stress, poplar, PeSTZ1, PeAPX2, reactive oxygen species, transgenic.

Introduction
Cold, heat, salinity and drought are the most common abiotic stresses. They limit the geographic distribution of plant species, affect their growth and development, and reduce crop yield (Shi et al., 2018; Zhu, 2016). Chilling stress (0–15 °C) can alter cell membrane fluidity, disrupt protein complexes and inhibit photosynthesis (Yadav, 2010). Freezing stress (<0 °C) can cause even more serious injuries to plants (Shi et al., 2018; Xie et al., 2018). Thus, plants have evolved molecular mechanisms to alleviate cold stress, which are mediated by C-REPEAT-BINDING FACTOR (CBF)-dependent or CBF-independent signalling pathways (Ma et al., 2015; Park et al., 2015; Shi et al., 2018).

Several COLD-REGULATED (COR) genes have been identified in plants including LOW-TEMPERATURE INDUCED (LTI), RESPONSIVE TO DESICCATION (RD) and EARLY DEHYDRATION-INDUCIBLE (ERD) genes (Huang et al., 2013; Jaglo-Ottosen et al., 1998; Xie et al., 2018). Earlier studies revealed that transcription factors (TFs) and pivotal protein kinases participate in the CBF-dependent pathway by activating downstream target COR genes (Gilmour et al., 1998; Jaglo-Ottosen et al., 1998; Liu et al., 1998). In contrast, CBF-independent TFs such as RAV1, C2F1, ZAT12, ZF, ZAT10, HSFC1 and HY5 modulate COR expression (Catala et al., 2011; Park et al., 2015; Vogel et al., 2005).

Transcription factors ZAT12, ZF and ZAT10 belong to the cysteine-2/histidine-2 (C2H2)-type zinc finger protein family found in Arabidopsis (Catala et al., 2011; Park et al., 2015; Vogel et al., 2005). The C2H2-type zinc finger, also named the TRANSCRIPTION FACTOR II-A (TFII-A)-type finger, was first identified in Xenopus sp. and represents an important class of eukaryotic TFs (Nguyen et al., 2016). The C2H2-type zinc finger domain includes the ‘CX2-4CX3FX5LX2HX3-5H’ motif, which is unique to plant zinc finger proteins, and consists of about two pairs of conserved cysteine and histidine tetrahedrally bound to a zinc ion and 30 amino acids (Pabo et al., 2001; Takatsui, 1999).

In response to abiotic stress induction, certain C2H2-type zinc finger protein genes were authenticated from petunia, soybean, Arabidopsis, rice and potato (Kim et al., 2011; Mittler et al., 2006; Sakamoto, 2004; Xu et al., 2008). The SOYBEAN COLD-INDUCIBLE ZINC FINGER-1 (SCOF-1), a TFII-A-type zinc finger TF from soybean, enhanced ABSCISIC ACID (ABA)-RESPONSIVE ELEMENT (ABRE)-dependent gene expression mediated by SOYBEAN G-BOX 1 (SGBF-1) (Kim et al., 2011). Overexpression of SCOF-1 enhances cold tolerance in soybean, sweet potato and potato (Kim et al., 2001, 2011, 2016). The petunia zinc finger protein gene ZPT2-3 is up-regulated by drought and cold stresses (Sugano et al., 2003). Overexpressing ZPT2-3 in petunia enhanced its tolerance to drought stress (Sugano et al., 2003). The drought- and salt stress-responsive C2H2-type zinc finger protein ZFP252 in rice strengthens salt and drought stresses tolerance by maintaining the homeostasis of intracellular osmotic pressure (Xu et al., 2008). Overexpressing the SALT TOLERANCE
ZINC FINGER (STZ/ZAT10) gene may enhance tolerance to drought, salt, osmotic, cold, photoinhibitory light and oxidation (Kim et al., 2001, 2011, 2016; Mittler et al., 2006; Nguyen et al., 2016; Rossel et al., 2007; Sakamoto, 2004; Shi and Chan, 2014; Xie et al., 2012; Xu et al., 2008). The transcription inhibitor protein STZ/ZAT10 negatively regulates RD29A expression in Arabidopsis and can enhance stress tolerance after slow growth (Lee et al., 2002; Sakamoto, 2004; Sakamoto et al., 2000; Zhang et al., 2004). As a positive TF in Arabidopsis, however, STZ/ZAT10 may also enhance osmotic stress tolerance by interacting with MITOGEN-ACTIVATED PROTEIN KINASE3/6 (MPK3/6) (Nguyen et al., 2012, 2016). Both gain- and loss-of-function mutations in ZAT10 reinforced stress tolerance (Mittler et al., 2006). However, it is uncertain whether STZ/ZAT10 functions as a transcription repressor or activator when it enhances stress tolerance in transgenic plants (Mittler et al., 2006).

Common plant responses to cold stress include the accumulation of cryoprotectant molecules such as soluble sugars, sugar alcohols and low-molecular-weight nitrogenous compounds (glycinebetaine and proline; Xing et al., 2018; Zhuo et al., 2017), and activated antioxidative defence systems which include ascorbate peroxidase (APX), peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT; Xie et al., 2018; Zhuo et al., 2017). These enzymes prevent the accumulation of reactive oxygen species (ROS) in plant cells (Wu et al., 2018). Both SOD and the ascorbate–glutathione (AsA-GSH) cycle play extremely important roles in scavenging ROS (Shi et al., 2013, 2014). While SOD is the first line of plant ROS defence, catalysing the conversion of oxygen ions (\( \text{O}_2^- \)) to oxygen (O\(_2\)) and hydrogen peroxide (H\(_2\)O\(_2\)) (Huang et al., 2016), APX uses AsA to reduce H\(_2\)O\(_2\) to water in the AsA-GSH cycle (Xing et al., 2018).

Several subtypes of APX have been found in various organelles and in the cytosol, and their adjustment modes differ under abiotic stresses (Xing et al., 2018). Overexpression of STZ/ZAT10 enhanced tolerance to exogenous H\(_2\)O\(_2\), improved ROS detoxification and reinforced plant APX2 expression under both normal and stress conditions (Mittler et al., 2006; Rossel et al., 2007). Therefore, we proposed that STZ might regulate plant APX2 expression.

Whether in early spring, summer or autumn, plants are severely damaged and their yield is reduced due to sudden freezing injury, which is of great importance to understand the stress resistance mechanism of plants under such extreme conditions (Liu et al., 2019). In 2007, widespread freezes during the spring months in the eastern and midwestern portions of the United States (US) resulted in over $2 billion in crop damage (Gu et al., 2008). An increase in devastating spring frosts is expected as a direct result of global climate change, despite the overall increases in average temperature (Ding et al., 2019; Gu et al., 2008). Plants can vary greatly in their ability to cold acclimatize and withstand freezing temperatures (Shabalina, 2017). The poplar BAK hybrid (Populus alba × Populus glandulosa) is a common perennial deciduous plant in cold temperate zones, and it can naturally resist local winters (Feng et al., 2015; He et al., 2018). Asexual reproduction is frequent in poplar (Fang et al., 2006), but the sudden low temperature will make a large number of seedlings too late to undergo cold acclimation and death. Thus, understanding poplar seedlings' cold resistance for tree genetic breeding is crucial.

The main functions of C2H2-type zinc finger proteins have been widely studied in herbaceous plants. However, their specific roles in woody plants (including trees), especially in the enhancement of freezing stress tolerance, remain unknown. The transcriptome data set of PeSTZ1 was selected for bioinformatics and functional analyses (Chen et al., 2014; Park et al., 2015). This gene is up-regulated in Populus euphratica in response to chilling and freezing stress (Chen et al., 2014; Ma et al., 2013). In the present study, a C2H2-type zinc finger protein gene, PeSTZ1, was identified and transferred into the poplar BAK hybrid (P. alba × P. glandulosa) and its function was verified by means of molecular biology and plant physiological indexes (He et al., 2018; Su et al., 2018b). The aims of these experiments were to identify the genetic factors responsible for conferring cold and freezing tolerance in woody plants and their modes of action. We demonstrated that PeSTZ1 enhances cold tolerance in poplar by regulating COR gene expression.

**Results**

**PeSTZ1 involvement in abiotic stress**

To elucidate the potential function of zinc finger proteins in the cold stress response of ligneous plants, the transcript levels of 33 genes involved in chilling and freezing responses were displayed as a heat map. A bioinformatics analysis of the transcriptome data set showed that the expression levels of 33 genes encoding zinc finger proteins were up-regulated or down-regulated by at least twofold when *P. euphratica* was subjected to 4 and −4 °C under weak lighting (Chen et al., 2014). Hierarchical clustering (average linkage) revealed that 15 of these genes were up-regulated at low temperatures and nine of these 15 were up-regulated by more than twofold in response to chilling and frostbite damage under our experimental conditions (Figure 1a). Unigene9835, also known as PeSTZ1, was up-regulated during chilling and freezing. PeSTZ1 was dramatically more up-regulated during freezing than chilling (Figure 1a). To confirm whether PeSTZ1 was induced by cold stress, a quantitative real-time polymerase chain reaction (qRT-PCR) was run to analyse its expression patterns in different tissues and under various treatments.

*Populus euphratica* plants were subjected to heat, chilling, freezing and dehydration stresses and exogenous ABA, and their PeSTZ1 expression patterns were assessed. PeSTZ1 transcription decreased with heat treatment duration, and after 36 h of treatment, it was only 0.108× that of the control (Figure 1b). Foliar PeSTZ1 transcription instantaneously increased in response to chilling stress (4 °C), and it was 17× higher than that of the control after 3 h of treatment. After 6 and 12 h of treatment, however, it had decreased to 5× that of the control (Figure 1c). Foliar PeSTZ1 expression transiently increased under freezing stress (−4 °C), and it was 26.38× higher than that of the control after 1 h of treatment, but declined to ~20× higher than that of the control after 3 and 6 h of treatment, and it was only 1.4× greater than that of the control after 9 h of treatment (Figure 1d). Foliar PeSTZ1 transcription instantaneously increased in response to dehydration stress. It was 15.9× higher than that of the control after 2 h of treatment but only 2.2× higher than that of the control after 12 h (Figure 1e). Foliar PeSTZ1 transcription transiently increased in response to ABA treatment and was 14.8× greater than that of the control after 3 h, ~9.6× higher than that of the control after 6 h and only 6.7× greater than that of the control after 12 h (Figure 1f). PeSTZ1 was mainly expressed in the stem rather than in the other tissues (leaf, root; Figure 1g). In addition, the PeSTZ1 promoter contained ABRE and MBS elements that are involved in the response to abiotic stress (Figure S1). These results indicated that PeSTZ1 is induced by low temperature, water stress and ABA treatment.
Molecular characterization of PeSTZ1

According to a previous study, PeSTZ1, a C2H2-type zinc finger TF in P. euphratica, was up-regulated during chilling and freezing. The PeSTZ1 coding sequencing (CDS) is 759 bp long, encodes 252 amino acids and has a molecular mass of ~29 kD and an isoelectric point of 6.92. The domain architecture analysis of PeSTZ1 suggested that it has a two zinc finger (C2H2) domain located in the amino acid sequences 93–117 and 146–171 (Figure 2a). The multiple amino acid sequence alignment indicated that the sequences of PeSTZ1, PtrSTZ1, OsZFP252 and AtSTZ all contained the same conserved domains (Figure 2b), which was consistent with the aforementioned results. To identify proteins homologous to PeSTZ1, an evolutionary tree was constructed for poplar, Arabidopsis, rice and maize STZ family members according to the results of the amino acid sequence alignment (Figure 2c). The nucleic acid sequence of PeSTZ1 was roughly similar to that of ATSTZ1 (At1g27730) from Arabidopsis. Thus, PeSTZ1 and ATSTZ1 may have similar biological functions.

Previous studies showed that ZAT10/STZ, a C2H2-type zinc finger TF, reinforced plant tolerance to abiotic stresses (Mittler et al., 2006; Nguyen et al., 2012, 2016; Xie et al., 2012; Xu et al., 2008). Arabidopsis STZ1, a functional homolog of PeSTZ1, might be localized to the same cellular structures as PeSTZ1. However, the specific subcellular localization of STZ is unknown. To determine the subcellular localization of PeSTZ1, a 35S:PeSTZ1-green fluorescent protein (GFP) fusion protein was transfected into tobacco leaves and Arabidopsis leaf protoplasts. A concurrent 4,6-diamidino-2-phenylindole (DAPI) staining disclosed that the 35S:PeSTZ1-GFP fusion protein was localized to the nucleus (Figure 2d).

To elucidate the potential biological functions of PeSTZ1, transgenic poplar B4K (P. alba × P. glandulosa) hybrids overexpressing PeSTZ1 were generated. Each transgenic line was verified by PCR, qRT-PCR and histochemical staining with β-glucuronidase (GUS) (Figure S2b, c, d). The aforementioned three methods are commonly used to verify transgenic plants (He et al., 2018).

PeSTZ1 overexpression improves freezing stress tolerance in transgenic poplars

Because chilling and freezing stresses up-regulate PeSTZ1 (Figure 1a, c and d), we deduced that PeSTZ1 plays a pivotal role in regulating freezing stress tolerance. Eight-week-old wild-type (WT) and transgenic poplars were raised in the same environment and then subjected to freezing treatment. After exposure to −7 °C for 12 h, the WT leaves presented severe freezing injury whereas the 35S:PeSTZ1 poplar leaves showed only minor freezing damage (Figure 3a).

The relative electrical conductance (REC), malondialdehyde (MDA) content and soluble sugar content reflect the degree of chilling injury (Niu et al., 2012; Shi et al., 2013). Therefore, REC,
MDA and soluble sugar were measured in 35S:PeSTZ1 and WT poplars before and after freezing. The REC increased in both transgenic and WT poplars after freezing treatment. After 12 h of freezing, the 35S:PeSTZ1 poplar leaves had moderate membrane damage (~38.9%–53.7% ion leakage) whereas the WT poplar leaves showed severe membrane damage (~73.8% ion leakage; Figure 3b). Changes in foliar MDA content in response to freezing stress were also compared between the WT and 35S:PeSTZ1 poplars. The WT poplar leaves had relatively higher MDA levels than those of the 35S:PeSTZ1 poplars after freezing exposure (Figure 3c). The 35S:PeSTZ1 poplar leaves had higher soluble sugar content than those of the WT plants in response to freezing stress (Figure 3d). In general, 35S:PeSTZ1 poplars had better tolerance to freezing stress than WT poplars.

Photosynthetic parameters during freezing stress

To determine the physiological effects of PeSTZ1 up-regulation in response to freezing stress, we plotted a slow dynamic fluorescence induction curve with a pulse-amplitude modulation chlorophyll fluorometer (PAM100). As shown in Figure 3e and f, both $F_{m}/F_{0}$ and $Y_{II}$ decreased. The chlorophyll fluorescence characteristics of the WT poplars decreased more than those of the 35S:PeSTZ1 poplars under short-term freezing conditions. Fluorescence quenching by photosynthesis reflects photosynthetic activity (Su et al., 2018a). Non-photochemical quenching (NPQ) is a mechanism employed by plants to protect themselves from the adverse effects of high light intensity, and helps to regulate and protect photosynthesis in environments where light energy absorption exceeds the capacity for light utilization in photosynthesis (Horton and Ruban, 2005; Muller et al., 2001). Thus, NPQ reflects the ability of the plant to dissipate excess light energy in the form of heat and to protect itself from light exposure (Suzuki et al., 2011). The levels of qP and NPQ were higher in 35S:PeSTZ1 plants than in WT plants under short-term freezing conditions (Figure 3g and h). Therefore, 35S:PeSTZ1 poplars had a better response to freezing stress than WT poplars.

Figure 2  Amino acid multiple sequence alignment, phylogenetic tree of different PeSTZ1 protein family members and subcellular localization of PeSTZ1. (a) Domain architecture analysis of PeSTZ1 proteins. (b) Multiple alignment of the amino acid sequences of PeSTZ1 proteins from Populus, rice and Arabidopsis. (c) Phylogenetic analysis of the PeSTZ1 homologs from Arabidopsis, rice, maize and Populus. (d) Subcellular localization of 35S: GFP and 35S: PeSTZ1-GFP in transiently expressed tobacco leaves and Arabidopsis leaf protoplasts. DAPI (blue) was applied to mark the nucleus. Tobacco: Bars = 10 μm; Arabidopsis: Bars = 5 μm.
PeSTZ1 enhances freezing tolerance via PeAPX2 regulation

Figure 3  The 35S:PeSTZ1 poplar lines showed tolerance to freezing stress during short-term freezing treatment. (a) Phenotypic differences in short-term freezing treatment. Quantitative measurement of REC (relative electrical conductance) (b), MDA content analysis (c), soluble sugar (d) in the leaves of WT and 35S:PeSTZ1 plants under normal and freezing stress conditions. The parameters of the slow dynamic fluorescence induction curve were measured in 35S: PeSTZ1 and WT plants during short-term freezing treatment and control. (e) Maximal PSII quantum yield ($F_{v}/F_{m}$). (f) Quantum yield of photochemical energy conversion in PSII ($Y_{II}$). (g) Photochemical quenching parameter ($qP$). (h) Non-photochemical quenching parameter (NPQ). Values are means ± SE ($n = 20$). All asterisks denote significant differences: **$P \leq 0.01$. 
**PeSTZ1** promotes ROS scavenging in response to freezing stress

The levels of H$_2$O$_2$ and O$_2^-$ were measured by histochemical staining with 3, 3′-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT), respectively (Shi et al., 2013; Xing et al., 2018). Under normal conditions, there was no obvious difference between the transgenic and WT plants. Under the freezing treatment, however, the blue and brown spots indicating H$_2$O$_2$ and O$_2^-$, respectively, were darker in the WT plants than in the 3SS:PeSTZ1 plants (Figure 4a). H2DCFDA and PI were applied to cold-stressed 3SS:PeSTZ1 and WT poplar roots to reveal dead cells and H$_2$O$_2$, respectively. After 3 h of freezing stress, the root tips of the transgenic poplars showed less green fluorescence (H2DCFDA) and red fluorescence (PI) than those of the WT plants (Figure 4b). Consistent with previous results, the H$_2$O$_2$ content in the 3SS:PeSTZ1 poplars was significantly lower than that in the WT plants after freezing treatment. Under normal conditions, no obvious difference was detected between the transgenic line and the WT plants (Figure 4c).

Ascorbate peroxidase, POD, SOD and CAT are major ROS scavengers and maintain cellular ROS homeostasis (He et al., 2018; Xing et al., 2018). To elucidate the correlations between the levels of antioxidant enzymes and ROS accumulation, POD, SOD and APX activities were measured in 3SS:PeSTZ1 and WT poplars under freezing stress. Under normal conditions, overexpression plants had slightly higher APX content than WT plants whereas their POD and SOD contents did not differ (Figure 4d–f). Under cold stress, however, 3SS:PeSTZ1 showed distinctly higher POD, SOD and APX activities than WT plants (Figure 4d–f). Therefore, PeSTZ1 may regulate APX gene expression and indirectly enhance cold tolerance by activating the antioxidant system and maintaining ROS homeostasis.

**PeSTZ1 regulates PeAPX2 expression**

Previous reports indicated that ZAT10/STZ1 proteins may be involved in the antioxidant response by regulating APX2 expression (Mittler et al., 2006; Rossel et al., 2007). Predictive bioinformatics with the JASPAR database and PlantPAN v.3.0 demonstrated that putative STZ1 proteins may combine with the PeAPX2 promoter (Chow et al., 2018; O’Malley et al., 2016). The highest number of binding sites was determined for the promoter fragment of PeAPX2 containing the CATTAAACACTG motifs. The specific binding sites are shown in Table S1. A yeast one-hybrid (Y1H) assay was run to verify whether PeSTZ1 binds to the PeAPX2 promoter. The AD-BeSTZ1 and Phis2-PeAPX2 vectors were constructed for the Y1H assay which was then used to demonstrate the interaction between the PeAPX2 promoter and PeSTZ1 (Figure 5a). Forty millimolar of 3-amino-1, 2, 4-triazole (3-A) was added to prevent a false positive in the Y1H assay. The results suggested that the co-transformed AD-BeSTZ1 and Phis2-PeAPX2Pro yeast strains could grow on the synthetic-defined (SD) medium without tryptophan, leucine and histidine (/-Trp/-Leu/-His) containing 40 µM 3-AT (Sun et al., 2017). In contrast, no yeast spot was found in the control medium (Figure 5b). These results indicate that PeSTZ1 binds to the PeAPX2 promoter.

To confirm whether PeSTZ1 binds to the PeAPX2 promoter, an electrophoretic mobility shift assay (EMSA) was conducted. The 28-bp PeAPX2 promoter fragments and mutant fragments were labelled with biotin (Figure 5c), and the purified HIS-BeSTZ1 fusion proteins were used in the subsequent experiments. The EMSA disclosed dark stripes of the DNA–protein complex after the purified His–PeSTZ1 fusion proteins, and the labelled WT probes were co-incubated (Figure 5d). In the presence of unlabelled competitor probe with the same sequence, these complexes formed at a very low rate (Figure 5d). Moreover, the dark stripes completely disappeared when the PeAPX2 promoter fragments were mutated in the probe (Figure 5d). The Y1H and EMSA assays established that PeSTZ1 directly binds to the PeAPX2 promoters.

To verify whether TFs activate downstream gene promoters, a transient expression assay was run in Nicotiana benthamiana leaves. The PeAPX2 promoter fragment containing the CATTAAACACTG motif was inserted into the pGreenII 0800-LUC vector to generate a reporter construct. The PeSTZ1 CDS was fused to a pGreenII 62-5K vector to generate an effector plasmid (Figure 5e). The dual-fusion assay revealed that PeSTZ1 induced PeAPX2 expression under both normal and freezing conditions, and that cold stress enhanced this induction (Figure 5f). Therefore, PeSTZ1 activated PeAPX2 expression. In addition, an analysis of the promoter region of PeAPX2 indicated that the PeAPX2 promoter contains several elements and binding sites of other TFs (binding sites of ATBP-1, MYBHv1 and MYB), which may be involved in stress responses (Figure S4).

**Analysis of the cold-responsive gene transcript levels in the WT and the transgenic lines**

PeSTZ1 increases freezing stress tolerance through directing gene expression in poplars. To understand this molecular mechanism, the patterns of cold-responsive gene expression were evaluated in WT and overexpressing plants. The genes involved in cold stress response in Arabidopsis include APX1, APX2, ZAT12, COR47, LEA, CSP3, LT165, MPK3, MPK6, CBFI, CBF2 and CBF3 (Ding et al., 2015; Jaglo-Ottosen et al., 1998; Shi et al., 2018; Vogel et al., 2005; Xie et al., 2018; Xing et al., 2018). Homologous cold response genes in poplars may have similar functions. The transcriptional levels of several cold-responsive genes in addition to PeMPK3 and PeMPK6 were significantly up-regulated in overexpressing plants in response to freezing stress (Figure 6a–l). The relative expression of PeMPK3 in WT was down-regulated after 3 h of freezing treatment, but the relative expression of PeMPK6 was not changed (Figure 6h and i). The relative expression of PeCBF1/1/2/3 was notably increased after the freezing treatment, but there was no significant difference between WT and overexpressed poplars (Figure 6j–l). Therefore, PeSTZ1 may either directly or indirectly regulate these genes to enhance freezing tolerance in poplars.

**Discussion**

Transcriptome data showed that gene regulation and signalling pathways participated in the cold stress responses of *P. euphratica* (Chen et al., 2014). To illustrate the function of zinc finger proteins under low-temperature stress in ligneous plants, the transcript levels of 33 zinc finger proteins involved in chilling and freezing were displayed as a heat map (Figure 1a). The bioinformatics analysis of the transcriptome data set and qRT-PCR verified that PeSTZ1 was up-regulated in response to low-temperature stress and its levels were substantially higher during freezing than chilling (Figure 1a, c and d). The qRT-PCR suggested that PeSTZ1 was induced by abiotic stresses including heat, chilling, freezing, drought and exogenous ABA (Figure 1). Members of the STZ family were identified in Arabidopsis and were regulated by a variety of adverse circumstances (Kim et al., 2001; Lee et al.,...
PeSTZ1 may increase cold resistance in Arabidopsis, potato, sweet potato, soybean and other plants (Kim et al., 2001, 2011, 2016; Park et al., 2015). To evaluate the function of PeSTZ1 in ligneous plants, the gene was cloned from *P. euphratica*. The multiple amino acid sequence alignment showed that PeSTZ1, PtrSTZ1, OsZFP252 and AtSTZ contain two C2H2-type zinc finger motifs (Figure 2b) and may, therefore, share a crucial conserved function. PeSTZ1 is a TFIIIA-type zinc finger TF structurally similar to STZ/ZAT10, and it may play a vital role in the nucleus (Figure 2d). It is an extensively studied zinc finger protein in Arabidopsis (Sakamoto, 2004; Xu et al., 2008). The conserved ‘CX2-4CX3FX5LX2HX3-5H’ motif lies in the C2H2-type zinc finger domain unique to plant zinc finger proteins that consists of ~30 amino acids and two pairs of conserved cysteine and histidine tetrahedrally bound to a zinc ion (Pabo et al., 2001; Takatsuji, 1999). The phylogenetic analysis suggested that the sequences of PeSTZ1, PtrSTZ1 and At1g27730 were highly homologous (Figure 2c) which corroborated the aforementioned results.

The ZAT gene family may increase tolerance to drought, salt, osmosis, cold, photoinhibitory light and oxidative stresses when overexpressed in transgenic plants (Kim et al., 2001, 2011, 2016; Mittler et al., 2006; Nguyen et al., 2016; Rossel et al., 2007; Sakamoto, 2004; Shi and Chan, 2014; Xie et al., 2012; Xu et al., 2008). The Arabidopsis ZAT10 protein negatively regulates...
RD29A expression (Lee et al., 2002; Sakamoto, 2004; Sakamoto et al., 2000; Zhang et al., 2004). The Arabidopsis STZ/ZAT10 is a transcription repressor that enhances stress tolerance after growth delay (Park et al., 2015; Sakamoto, 2004), but it is also a positive TF in osmotic stress tolerance and may be regulated by MAP kinases in Arabidopsis (Nguyen et al., 2012, 2016). In the present study, transgenic 84K poplar lines overexpressing PeSTZ1 were generated (Figure S2a, b, c, d). The OxPeSTZ1 plants had higher cold stress tolerance than the WT plants (Figure 3a). There were no significant differences in morphology among the nine transgenic lines and the WT (Figure S2e). In addition, we found that overexpressing PeSTZ1 in poplars did not affect plant growth during normal conditions (Figure S3a, b, c, d), similar to that observed for rice overexpressing ZFP252 (Sakamoto, 2004; Xu et al., 2008). Plant growth is regulated by various internal factors in different plants (Zhu, 2016). The present study indicated that PeSTZ1 could be of great practical benefit in the molecular breeding of trees with enhanced cold tolerance.

Figure 5 PeSTZ1 distinguishes the promoter of PeAPX2 and regulates PeAPX2 expression. (a) The AD-PeSTZ1 and Phis2-Promoter vectors used for yeast one-hybrid assay. Phis2, AD, AD-PeSTZ1 and Promoter-Phis2 represent the p53His2 vector and the pGADT7-Rec2 vector, the PeSTZ1 gene was fused to the pGAD vector, and the promoter of PeAPX2 was fused to the Phis2 vector, respectively. (b)’1H assay displaying direct binding of PeSTZ1 transcription factor to the promoter of PeAPX2. The AD and the Promoter-Phis2, the AD-PeSTZ1 and Phis2, and AD and Phis2 to represent negative control, respectively. Yeast clones were grown in SD/Leu-Trp-His plate containing 0 mM 3-AT (as control) or 40 mM 3-AT. (c) Diagram of the wild-type and mutated probes used for electrophoretic mobility shift assay (EMSA). The wild probe is a putative PeSTZ1 transcription factor binding site (CATTAACACTG) on the PeAPX2 promoter. In the mutant probe, the putative binding site sequences CATTACACTG was replaced with CACCGCTGGTG. (d) EMSA assays were applied to identify the interactions between HIS-PeAPX2 protein and the PeAPX2 promoter. (e) The schematic diagrams of the effector and reporter constructs used for a dual-luciferase assay. (f) Dual-luciferase assay. Transient expression assay of relative luciferase activity, shown as a ratio of LUC to REN in Nicotiana benthamiana leaves. Pro35S: REN (pGreenII 62-SK) was applied to an internal control. Left histograms in (f), leaves of N. benthamiana were instantaneously co-transformed and existed in normal conditions for 60 h. Right histograms in (f), leaves of N. benthamiana were co-transformed and grown under normal conditions for 57 h and then −7 °C growth chamber for additional 3 h before harvest. Values are means ± SE (n = 20). All asterisks denote significant differences: **P ≤ 0.01. Data in Figure 5 were derived from experiments that were performed at least three times with similar results, and representative data from one repetition were shown.
Relative electrical conductance and MDA content are valid indicators of membrane damage (He et al., 2018; Shi et al., 2013). In response to freezing stress, both REC and MDA were markedly lower in OxPeSTZ1 plants than in WT plants (Figure 3b and c). The PeSTZ1 protein may improve cell membrane integrity under cold stress. Concurrently, osmoprotectants such as soluble sugars may accumulate in plants in response to abiotic stresses (Shi et al., 2013; Zhuo et al., 2017). Our study showed that freezing stress-induced increases in soluble sugar content were higher in OxPeSTZ1 poplar lines than in WT plants (Figure 3d). The homologs STZ/ZAT10 and SCOF-1 reinforced cold stress tolerance in potato by preserving cell membrane integrity (Kim et al., 2011, 2016). The $F_v/F_m$ ratio corresponds to the maximum photosystem II (PSII) quantum yield that reflects potential maximum light energy conversion efficiency in plants, while $Y (II)$ represents the quantum yield of photochemical energy conversion and reflects the actual light energy conversion efficiency of plants in PSII (Su et al., 2018a; Suzuki et al., 2011). Fluorescence quenching caused by photosynthesis reflects the photosynthetic activity (Suzuki et al., 2011), and NPQ reflects the ability of the plant to dissipate excess light energy in the form of heat and the ability of the plant to protect itself from light exposure (Su et al., 2018a). Under environmental stress, PSII efficiency declines in plants, and freezing stress damages membrane systems and alters PSII electron transport. Nevertheless, the OxPeSTZ1 poplar lines had higher $F_v/F_m$ and $Y (II)$ than the WT plants (Figure 3e and f). The former presented only minor damage under extremely cold conditions (Figure 3a). Plants must maintain a balance between photochemical reactions (temperature insensitive) and metabolic/developmental (temperature sensitive) processes in order to avoid photoinhibition (Horton and Ruban, 2005). Photosynthesis can be maintained by NPQ mechanisms, which are used by the plant to dissipate the excess energy, not used in photosynthesis, as heat (Muller et al., 2001). At the same time, this protects the PSII reaction centre from damage that can occur upon exposure to freezing temperatures (Oquist and Huner, 2003; Oquist et al., 1993). In the present study, OxPeSTZ1 poplar lines dissipated more excess light energy as heat to prevent light damage than WT plants (Figure 3h). The photosynthetic system of OxPeSTZ1 poplar was less damaged under freezing stress than that of WT plants. Furthermore, PSII was inhibited under freezing stress. This effect may be the result of MDA and ROS accumulation (Figures 3b and 4a). Therefore, PeSTZ1 overexpression in plants enhances their freezing tolerance.

Both $O_2^−$ and $H_2O_2$ are generated by various stresses and are markers of ROS production (Cui et al., 2015; Xing et al., 2018). Low levels of ROS production are considered stress signals, but massive ROS generation destroys cell membrane integrity, causes

Figure 6 Quantitative real-time PCR analysis of the transcript levels of cold-responsive genes in wild-type and transgenic lines before and after freezing stress. (a) PeAPX1, (b) PeAPX2, (c) PeZAT12, (d) PeCOR47, (e) PeLEA, (f) PeCSP3, (g) PeLtI65, (h) PeMPK3, (i) PeMPK6, (j) PeCBF1, (k) PeCBF2 and (l) PeCBF3 expression levels. Error bars are means ± SE ($n$ = 20). Asterisks indicate significant differences: *$P \leq 0.05$; **$P \leq 0.01$.
toxic substances to accumulate and results in cell death (Huang et al., 2009; Suzuki et al., 2012). In the present study, we found that under freezing stress, ROS generation was significantly lower in the leaves of OxPeSTZ1 poplar lines than in the leaves of WT plants (Figure 4a). In response to freezing stress, the number of dead cells in the root tips of WT plants was significantly greater than in the root tips of OxPeSTZ1 lines (Figure 4b). Under extreme conditions, however, ROS accumulation may actually activate the antioxidant system to enable the plant to contend with the stress (de la Garma et al., 2015; Mori and Schroeder, 2004). Ascorbate peroxidase, POD, SOD and CAT are major ROS scavengers that maintain the homeostasis of cellular ROS (He et al., 2018; Xing et al., 2018). In the present study, we revealed that the OxPeSTZ1 poplar lines had higher antioxidant enzyme (APX, SOD and POD) activities and less cellular injury (lower ROS and MDA levels and cell death rates) than WT plants under freezing stress (Figures 3 and 4). The qRT-PCR analysis indicated that the COR gene was up-regulated in OxPeSTZ1 poplar lines relative to WT plants under cold stress (Figure 6). Therefore, these genes participate in ROS scavenging and enhance cold tolerance under freezing stress.

ASCORBATE PEROXIDASE2 encodes the cytosolic APX2 to scavenge H₂O₂ in plant cells (Wu et al., 2018). Overexpressing STZ/ZAT10 in Arabidopsis showed enhanced tolerance to exogenous H₂O₂ and improved ROS detoxification (Mittler et al., 2006; Rossel et al., 2007). Moreover, ZAT10 overexpression up-regulates APX2 in plants under both controlled and stressful conditions (Rossel et al., 2007). In the present study, the Y1H assay verified that PeSTZ1 binds to the PeAPX2 promoter and may regulate PeAPX2 expression (Figure 5b). The C2H2-type zinc finger TF from soybean SCOF-1 enhanced ABRE-dependent gene expression mediated by SGBF1, which, in turn, enhanced cold tolerance in transgenic plants (Kim et al., 2001). Arabidopsis STZ/ ZAT10 is a transcription repressor that enhances stress tolerance after growth delay (Sakamoto, 2004). The bioinformatics prediction using JASPAR database disclosed that the putative STZ1 promoters might bind with the PeAPX2 promoter (O’Malley et al., 2016). Our sequence analysis showed that the binding sites were the PeAPX2 promoter fragments containing the CATTAA- CACTG motifs (Figure 5c). The EMSA suggested that the PeAPX2 promoters might bind with the PeSTZ1 protein (Figure 5d), and PeAPX2 expression might then be regulated by PeSTZ1. In addition, PeSTZ1 can induce PeAPX2 expression under both normal and freezing conditions, and cold stress enhanced this induction (Figure 5f). Other TFs (MYB TFs) with similar functions to PeSTZ1 may also regulate the expression of PeAPX2 under freezing conditions (Figure S4). The present study indicated that PeSTZ overexpression in plants may up-regulate PeAPX2 so that it synthesizes more cytosolic APX2 to remove the ROS generated by intracellular stress.

The mpk3 and mpk6 mutants showed enhanced expression of CBF genes and increased freezing tolerance, demonstrating that the MKK4/5-MPK3/6 cascade negatively regulates cold stress response (Zhao et al., 2017). Interestingly, the MEKK1-MKK2-MPK4 pathway constitutively suppressed MPK3 and MPK6 activities and had a positive role in the cold response (Shi et al., 2017). In both poplar and Arabidopsis, MPK3 showed down-regulation after freezing treatment (Figure 6h). However, PeMPK6 did not exhibit the similar expression trend to PeMPK3 after 3 h of freezing stress, which might indicate the different role of PeMPK6 in freezing-treated poplars (Figure 6i). However, previous studies showed that MPK3 and MPK6 expression trends were different in poplar under adverse conditions (Su et al., 2018b).

The CBFs in poplar are also involved in cold stress, and their expressions were significantly up-regulated under cold treatment (Li et al., 2016; Tian et al., 2016). Under freezing stress, inhibition of MPK3/6 activity in Arabidopsis up-regulated the expression of CBFs and enhanced freezing resistance (Shi et al., 2018; Zhao et al., 2017). Similar results were obtained in poplar, where PeMPK2 expression was inhibited, but PeCBF1/2/3 expression was activated after cold treatment (Figure 6). ZAT10/AtSTZ1 are cold-induced TFs that induce the expression of COR genes under cold stress by the CBF-independent pathway (Ding et al., 2019; Park et al., 2015). There was no significant difference in PeCBF1/2/3 expression between WT and overexpressed poplar lines (Figure 6j-l), which indicated that PeSTZ1 might not rely on CBFs to regulate COR genes.

The reliable data derived from this study were used to build a model explaining the function of PeSTZ1 in response to freezing in poplar (Figure 7). A C2H2-type zinc finger TF, PeSTZ1, had a positive effect on cold resistance. It was preferentially expressed in young stems and dramatically up-regulated by chilling and freezing. Under cold stress, by activating the calcium signalling pathway in plant cells and stimulating downstream cold signal transduction (Ma et al., 2015), the up-regulated PeSTZ1 acts upstream of PeAPX2 and directly regulates its expression by binding to the CATTAAACACTG motif of its promoter. The activated PeAPX2 then promotes cytosolic APX2 accumulation to scavenge ROS under freezing stress. The CBF-independent ZAT10 TFs modulate COR expression (Park et al., 2015; Shi et al., 2018). PeSTZ1 and CBFs may collaborate to regulate COR gene expression and PeSTZ1 up-regulation reduced MDA and ROS accumulation by activating the antioxidant system. Overexpressing PeSTZ1 in 84K poplar enhanced freezing tolerance by modulating ROS scavenging via the direct regulation of PeAPX2 expression. Thus, we obtained a novel line of poplar with increased freezing tolerance.

Experimental procedures

Plant materials and stress treatment

One-year-old P. euphratica seedlings were grown on a seed plot [15.0 h light (06:00–21:00); 22–25 °C] at Haidian, Beijing, China (40°000N, 116°200E, 49 m above sea level; He et al., 2018). The plants were watered with 1 L Hoagland nutrient solution every 2 weeks for 2 months before treatment (He et al., 2018; Wang et al., 2016).

To examine PeSTZ1 expression patterns in different tissues and treatments, 60-day P. euphratica seedlings were subjected to various abiotic stresses. For heat stress, similarly grown P. euphratica seedlings were subjected to 45 °C in a growth chamber for 0, 5, 10, 20, 40 min, 1, 3, 6, 9, 12 and 36 h. For chilling stress, similarly grown P. euphratica seedlings were subjected to 4 °C in a growth chamber for 0, 1, 3, 6, 9 and 12 h. For freezing stress, similarly grown P. euphratica seedlings were subjected to −7 °C in a growth chamber for 0, 30 min, 1, 3, 6, 9 and 12 h. For water stress, similarly grown P. euphratica seedlings were dehydrated by being removed from the soil and exposed to air at 70% relative humidity and 23 °C under dim light for 12 h (He et al., 2018). For ABA stress and organizational expression pattern, the treatment was performed as described previously (He et al., 2018). At least 40 seedlings were used per test. At the aforementioned time intervals, leaves were excised.
PeSTZ1 enhances freezing tolerance via PeAPX2 regulation

Figure 7 A working model for PeSTZ1 response to freezing in poplar. Under cold stress, activating the Ca2+ signalling pathway in plant cells and stimulating downstream cold signal transduction, the upregulated PeSTZ1 acts upstream of PeAPX2 and directly regulates its expression by binding to the CATTAA CACTG motif of its promoter. The activated PeAPX2 then promotes cytosolic ascorbate peroxidase accumulation to scavenge ROS under freezing stress. The CBF-independent ZAT10 transcription factors may modulate COR expression. PeSTZ1 and CBFs may collaborate to regulate COR gene expression. Overexpressing PeSTZ1 in 84K poplar enhances freezing tolerance by modulating ROS scavenging via the direct regulation of PeAPX2 expression.

from the plants, immediately frozen in liquid nitrogen and stored at −80 °C until further use.

RNA extraction and qRT-PCR analysis

Total RNA was isolated from the collected materials with a plant total RNA extraction kit (Aidlab, Beijing, China) according to the manufacturer’s protocol and treated with DNase I (Aidlab, Beijing, China). A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, West Palm Beach, FL) was used to measure RNA quality and quantity. Approximately 2 μg total RNA was used for reverse transcription with a Tiangen Fast Quant RT Kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer’s instructions. The qRT-PCR was conducted as previously described (Bustin et al., 2009; He et al., 2018). Primer Premier v.6.6 (Sigma-Aldrich Corp., St. Louis, MO) was used to develop the primers listed in Table S2. At least 20 replicates (five biological replicates × four technical replicates) were performed per experiment.

Cloning and bioinformatics analysis of PeSTZ1

The cDNA of PeSTZ1 was cloned by PCR with a PrimeStar® high-fidelity thermostable DNA polymerase reagent kit (Takara Biotechnology Co. Ltd., Dalian, Liaoning, China) according to the manufacturer’s instructions. The primers used are displayed in Table S2. Homologous amino acid sequences were acquired from http://popgenie.org/. Multiple alignment of the amino acid sequences was run with ClustalW (http://align.genome.jp/). The phylogenetic tree for PeSTZ1 was constructed in MEGA7. The theoretical isoelectric point and the molecular weight were determined with ExPaSY (http://www.expasy.org). Hierarchical and k-means clustering analyses were performed on log2-normalized data in CLUSTER. The resulting clusters were visualized in TREEVIEW (http://rana.lbl.gov/EisenSoftware.htm). Bioinformatics analysis of the transcriptome data set generated a heat map in R mapping software (R Core Team, Vienna, Austria).

Genetic transformation of poplar and molecular verification of transgenic plants

The 759 bp PeSTZ1 CDS was inserted into the pCAMBIA-1301 vector at the Smal and Sac sites under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The construct was introduced into Agrobacterium tumefaciens strain EHA105 for poplar transformation and the corresponding vector was transformed into poplar 84K (P. alba × P. glandulosa) as described elsewhere (He et al., 2018).

Total DNA was extracted from hygromycin-resistant seedlings with a plant total DNA extraction kit (Aidlab, Beijing, China) according to the manufacturer’s instructions. Transformation was confirmed by PCR using 20 bp of CAMV 35S promoter as a forward primer and 20 bp of PeSTZ1 as a reverse primer. Then, a qRT-PCR was run to verify the PeSTZ1 expression levels in the transgenic lines. Total RNA was obtained from the leaves of WT and transgenic plants with a plant total RNA extraction kit (Aidlab, Beijing, China). A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, West Palm Beach, FL) was used to determine RNA quality and quantity. Approximately 2 μg total RNA was used for reverse transcription with a Tiangen Fast Quant RT Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer’s instruction. The qRT-PCR was conducted as described previously (He et al., 2018). The internal controls were the previously screened reference genes PeActin and PeUBQ (He et al., 2018; Wang et al., 2014). The primers used are listed in Table S2.

GUS staining

Histochemical staining was performed as previously described to validate the expression of GUS co-transformed with PeSTZ1 (Jin et al., 2017). Briefly, leaves from different transgenic lines were incubated at 37 °C in a solution consisting of 0.1 M sodium phosphate buffer (pH 7.0), 2 mM 5-bromo-4-chloro-3-indolyl-β-d-glucuronide (X-Gluc), 10.0 mM EDTA (pH 7.0), 0.10% Triton X-100, 0.50 mM potassium ferricyanide and 0.50 mM potassium ferrocyanide for 12 h. The isolated leaves were cleared of chlorophyll with 75% ethanol for 12 h and photographed.

Subcellular localization

To confirm the subcellular localization of PeSTZ1, the 35S:PeSTZ1-GFP fusion proteins were instantaneously transfected into tobacco leaves according to a previously published protocol (Cui...
et al., 2012; He et al., 2018). The constructed vector 35S:PeSTZ1-GFP was also instantaneously transfected into Arabidopsis leaf protoplasts via polyethylene glycol (PEG) treatment (Yoo et al., 2007). The nuclear dye DAPI (10 mg/mL; Sigma-Aldrich Corp., St. Louis, MO) was used to stain the nuclei (Duan et al., 2017; He et al., 2018; Xie et al., 2018). Laser confocal fluorescence microscopy (Leica TCS SP8; Leica, Wetzlar, Germany) was used to observe the instantaneously transformed tobacco leaves and Arabidopsis protoplasts. The LAS-AF software (Leica, Wetzlar, Germany) recorded the images. The following argon ion laser lines were used: 488 nm for GFP, 380 nm for DAPI and 488 nm for chlorophyll. Fluorescence was detected at 495–515 nm for GFP, 430–450 nm for DAPI and 650 nm for chlorophyll.

**Stress tolerance assay and physiological measurements**

One-month-old clonally propagated seedlings were transplanted to small pots (10 cm length × 10 cm width × 10 cm height) with the same soil (potting soil, turfy soil, and vermiculite 1:1:1) and then grown for 2 months in a greenhouse (light cycle: 16.0 h of light, 8.0 h of dark; temperature: 20 °C). Eight-week-old WT 84K poplar plants and two overexpressing 84K poplar lines (PeSTZ1-5 and PeSTZ1-8) were directly exposed to −7 °C for 12 h without cold acclimation under weak light conditions (Chen et al., 2014). The parameters were determined from previous optimization experiments. The control plants were maintained in the same conditions except for temperature, which was 20 °C. Electrolyte leakage, MDA and soluble sugar were measured in the WT 84K poplar plants and two overexpressing lines (PeSTZ1-5 and PeSTZ1-8) at 6 and 12 h after freezing treatment and under control conditions. The parameters of the slow dynamic fluorescence induction curves were plotted for 35S: PeSTZ1 and WT plants during the 12-h freezing treatment and under control conditions. The relative electrical conductance and soluble sugar were measured as described previously (Shi et al., 2013, 2014). The MDA was monitored according to a previously published protocol (He et al., 2018). The parameters of the slow dynamic fluorescence curves, including Fm/Fo, Y (II), qP and NPQ, were measured using a previously published method (He et al., 2018). At least 20 replicates (five biological replicates × four technical replicates) were performed per experiment.

**Histochemical staining, cell viability and antioxidant enzyme activity**

Eight-week-old WT 84K poplar plants and two overexpressing 84K poplar lines (PeSTZ1-5 and PeSTZ1-8) were directly exposed to −7 °C for 3 h without cold acclimation and under weak light conditions. The control plants were maintained in the same conditions except for temperature, which was 20 °C. Leaves at the same positions on the stems/branches were subjected to histochemical staining and H2O2 and antioxidant enzyme activity measurements. The root tips of 84K poplar lines were then examined to assess root cell viability.

In situ O2− and H2O2 accumulation levels were examined by histochemical staining with NBT and DAB (Mei5 Biotech, MF073-01, Beijing), respectively (Huang et al., 2013; Xing et al., 2018). Root cell viability was determined with H2DCF-DA (D6883 HZB1212; Sigma-Aldrich Corp., St. Louis, MO) and PI (Sigma-Aldrich Corp., St. Louis, MO; Cruz-Ramirez et al., 2004; Deng et al., 2015). The H2O2 content and the APX, POD and SOD activity levels were measured with customized kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. At least 20 replicates (five biological replicates × four technical replicates) were performed per experiment.

**Yeast one-hybrid assays**

The Y1H assays were conducted to verify the physical interactions between the promoters and the TFs (An et al., 2018). The promoter fragment of PeAPX2 was amplified from *P. euphratica* and cloned into the phi52 vector. The primers used are displayed in Table S2. The CDSS of PeSTZ1 were inserted into the pGADT7 vector to generate recombinant GAD-PeSTZ1 constructs. The Y1H assay was conducted according to the manufacturer’s instructions (Matchmaker Gold Y1H Library Screening System; Clontech Laboratories, Mountain View, CA).

The pGAD-Rec2-53 and the Phis2-Promoter (phi52S.1), pGAD-PeSTZ1 and phi52S.1, and pGAD-Rec2-53 and phi52S.1 were used to represent negative controls. The pGAD-Rec2-53 and p53His2S.2 were provided in the kit as a positive control. The plasmids were co-transformed into yeast Y187 strains which were then plated on the SD-Trp/-Leu/-His medium containing either 0 mM 3-AT (control) or 40 mM 3-AT (3-amino-1, 2, 4-triazole) as described previously (An et al., 2018).

**Electrophoretic mobility shift assay**

The HIS-PeSTZ1 fusion proteins were obtained from in vitro prokaryotic expression. The cDNAs encoding full-length PeSTZ1 were cloned into PET28a to generate His-fusion recombinant vectors, which were then expressed in *Escherichia coli* BL21. Then, 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was applied to induce the protein. His-fusion proteins were purified with a His-Tagged Protein Purification kit (Beyotime, GS008 and GS009, China) according to the manufacturer’s instructions.

The PeAPX2 promoter fragment containing CATTAACACTG motifs was synthesized by Sangon (Beijing, China). The EMSA Probe Biotin Labeling kit and a Chemiluminescent EMSA kit (GS008 and GS009; Beyotime, Biotechnology, Shanghai, China) were used for the subsequent EMSA assays, which were performed according to the manufacturer’s protocols. Briefly, biotin-labelled probes and fusion proteins were mixed in a binding buffer for 30 min at 22 °C. The unlabelled probes were used for probe competition, and the HIS protein was used as a negative control.

**Dual-luciferase reporter (DLR) assay**

The PeAPX2prom fragment containing the CATTAACACTG motifs was inserted into pGreenII 0800-LUC vectors to generate the reporter construct. The 35Spro:PeSTZ1 effectors were generated by recombining the PeSTZ1 genes into the pGreenII 62-SK vector. Transformation and dual-luciferase (LUC) activity determination were conducted as described elsewhere (Zhang et al., 2018). In brief, the recombinant plasmids were introduced into *A. tumefaiciens* GV3101 that were then cultured until an optical density at 600 nm of 0.15. The reporter and effector were combined in equal volumes, maintained at 20 °C without shaking for 3 h, instantaneously transfected into *N. benthamiana* leaves and incubated for another 60 h. The LUC and Renilla luciferase (REN) activity levels were determined in a Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The transactivation was expressed as the ratio of LUC: REN. At least 20 replicates (five biological replicates × four technical replicates) were performed per experiment.
Statistical analyses

Microsoft Excel 2010 (Microsoft Corp., Redmond, WA) and Statistical Product and Service Solutions v. 17.0 (SPSS, Chicago, IL) were used to analyse the experimental data. Both one-way analysis of variance and two-way analysis of variance were used to determine the significance of the differences among treatments. Student’s t-test was run to calculate P-values (*P < 0.05; **P < 0.01). The data were normalized, and all samples were normally distributed in terms of homogeneity of variance.

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Accession numbers

The sequence data reported here have been submitted to the GenBank database under the accession numbers displayed in Table S3.

References

An, J.P., Wang, X.F., Li, Y.Y., Song, L.Q., Zhao, L.L., You, C.X. and Hao, Y.J. (2018) EIN3-LIKE1, MYB1, and ETHYLENE RESPONSE FACTOR3 act in a regulatory loop that synergistically modulates ethylene biosynthesis and anthocyanin accumulation. Plant Physiol. 178, 808–823.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R. et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622.

Catala, R., Medina, J. and Salinas, J. (2011) Integration of low temperature and light signaling during cold acclimation response in Arabidopsis. Proc. Natl. Acad. Sci. USA, 108, 16475–16480.

Chen, J., Tian, Q., Pang, T., Jiang, L., Wu, R., Xia, X. and Yin, W. (2014) Deep-Sequencing transcriptome analysis of low temperature perception in a desert tree, Populus euphratica. BMC Genom. 15, 326.

Chow, C.N., Lee, T.Y., Hung, Y.C., Li, G.Z., Tseng, K.C., Liu, Y.H., Kuo, P.L. et al. (2018) PlantPan3.0: a new and updated resource for reconstructing transcriptional regulatory networks from ChIP-seq experiments in plants. Nucl. Acids Res. 47, D1155–D1163.

Cruz-Ramirez, A., Lopez-Bucio, J., Ramirez-Pimentel, G., Zurita-Silva, A., Sanchez-Calderon, L., Ramirez-Chavez, E., Gonzalez-Ortega, E. et al. (2004) The xipotl mutant of Arabidopsis reveals a critical role for light signaling during cold acclimation response in Arabidopsis. Plant Physiol. 135, 1321–1332.

Ding, Y., Shi, Y. and Yang, S. (2019) Advances and challenges in uncovering cold tolerance regulatory mechanisms in plants. New Phytol. https://doi.org/10.1111/nph.15696.

Duan, M., Zhang, R., Zhu, F., Zhang, Z., Gou, L., Wen, J., Dong, J. et al. (2017) A lipid-anchored NAC transcription factor is translocated into the nucleus and activates glyoxalase I expression during drought stress. Plant Cell, 29, 1748–1772.

Fang, S., Tian, Y. and Yuan, F. (2006) Effects of cutting density on growth, yield and quality of poplar clone seedlings. Front. For. China, 1, 64–69.

Feng, F., Ding, F. and Tyree, M.T. (2018) Investigations concerning cavitation and frost fatigue in clonal B&K poplar using high-resolution cavitation measurements. Plant Physiol. 168, 144–155.

de la Garma, J.G., Fernandez-Garcia, N., Bardis, E., Pallol, B., Rubio-ASENSIO, J.S., Bru, R. and Olmos, E. (2015) New insights into plant salt acclimation: the roles of vesicle trafficking and reactive oxygen species signalling in mitochondria and the endomembrane system. New Phytol. 205, 216–239.

Gilmour, S.J., Zarka, D.G., Stockinger, E.J., Salazar, M.P., Houghton, J.M. and Thomashow, M.F. (1998) Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. Plant J. 16, 433–442.

Gu, H., Hansson, P.J., Mac Post, V., Kaiser, D.G., Yang, B., Nernani, R., Pallardi, S.G. et al. (2008) The 2007 eastern US spring freezes: increased cold damage in a warming world? Bioscience, 58, 253–262.

Huang, X.S., Wang, W., Zhang, Q. and Liu, J.H. (2013) A basic helix-loop-helix transcription factor, TmrbHLH, of Triticum aestivum confers cold tolerance and drought tolerance in rice via stomatal aperture control. New Phytol. 197, 912–922.

Huang, S.B., Van Aken, O., Schwarzlander, M., Belt, K. and Millar, A.H. (2016) ZmGSTZ1 enhances freezing tolerance via AP2 transcriptional reprogramming. Plant Physiol. 172, 361–371.

Huang, X.Y., Chao, D.Y., Gao, J.P., Zhu, M.Z., Shi, M. and Lin, H.X. (2009) A functional NAC transcription factor, PtrbHLH, of Poncirus trifoliata confers cold tolerance and drought tolerance in Populus trichocarpa. New Phytol. 185, 785–793.

Jin, Y.-L., Tang, R.J., Wang, H.H., Jiang, C.M., Bao, Y., Yang, Y., Liang, M.X. et al. (2017) Overexpression of Populus trichocarpa CBF1 confers cold tolerance via stomatal aperture control. Proc. Natl. Acad. Sci. USA, 114, 2913–2918.

Kim, Y.-H., Kim, M.D., Park, S.C., Yang, K.S., Jeong, J.C., Lee, H.S. and Kwak, S.-S. (2015) LOS2, a functional enolase. Sci. Rep., 5, 12181.

Kortenkamp, C., Department of Plant Sciences, University of California, Davis, CA, USA, and Shinozaki, K. (1998) Two transcription factors, DREB1 and DREB2, with functions specifically in an integrated cold regulatory network. Plant J., 15, 213–227.

Lee, H., Guo, Y., Ciftci, M., Xiong, L., Stevenson, B. and Zhu, J.K. (2002) LOS2, a functional enolase. Sci. Rep., 5, 12181.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) Two transcription factors, DREB1 and DREB2, with an ERF/EBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell, 10, 1391–1406.

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Liu, C., Schiappi, M.R., Mao, B., Wang, W., Wang, A. and Chu, C. (2019) The bZIP73 transcription factor controls rice cold tolerance at the reproductive stage. *Plant Biotechnol. J.* https://doi.org/10.1111/pbi.13104.

Ma, T., Wang, J.Y., Zhou, G.K., Yue, Z., Hu, Q.J., Chen, Y., Liu, B.B. et al. (2013) Genomic insights into salt adaptation in a desert poplar. *Nat. Commun.* 4, 2797.

Ma, Y., Dai, X., Xu, Y., Luo, W., Zheng, X., Zeng, D., Pan, Y. et al. (2015) COLD1 confers chilling tolerance in rice. *Cell.* 160, 1209–1221.

Müller, K., Kim, Y., Song, L., Coutou, J., Coutou, A., Ciftci-Yilmaz, S., Lee, H. et al. (2006) Gain- and loss-of-function mutations in Zat10 enhance the tolerance of plants to abiotic stress. *FEBS Lett.* 580, 6537–6542.

Mori, I.C. and Schroeder, J.I. (2004) Reactive oxygen species activation of plant Ca2+ channels. A signaling mechanism in polar growth, hormone transduction, stress signaling, and hypothetically mechanotransduction. *Plant Physiol.* 135, 702–708.

Muller, P., Li, X.P. and Nyogi, K.K. (2001) Non-photosynthetic chemquenching. A response to excess light energy. *Plant Physiol.* 125, 1558–1566.

Nguyen, X.C., Kim, S.H., Lee, K., Kim, K.E., Liu, X.M., Han, H.J., My, H.T.H. et al. (2012) Identification of a C2H2-type zinc finger transcription factor (ZAT10) from Arabidopsis as a substrate of MAP kinase. *Plant Cell Rep.* 31, 737–745.

Nguyen, X.C., Kim, S.H., Hussein, S., An, J., Yoo, Y., Han, H.J., Yoo, I.S. et al. (2016) A positive transcription factor in osmotic stress tolerance, ZAT10, is regulated by MAP kinases in Arabidopsis. *J. Plant Biol.* 59, 55–61.

Niu, C.F., Wei, W., Zhou, Q.Y., Tian, A.G., Hao, Y.J., Zhang, W.K., Ma, B. et al. (2012) Wheat WRKY genes TaWRKY2 and TaWRKY19 regulate abiotic stress tolerance in transgenic Arabidopsis plants. *Plant Cell Environ.* 35, 1156–1170.

O’Malley, R.C., Huang, S.S.C., Song, L., Lewsey, M.G., Bartlett, A., Nery, J.R., Galli, M. et al. (2016) Cistrome and epistrome features shape the regulatory DNA landscape (vol 165, pg 1280, 2016). *Cell.* 166, 1588–1598.

Oquist, G. and Huner, N.P. (2003) Photosynthesis of overwintering evergreen plants. *Annu. Rev. Plant Biol.* 54, 329–355.

Oquist, G., Hurry, V.M. and Huner, N. (1993) Low-temperature effects on photosynthesis and correlation with freezing tolerance in spring and winter cultivars of wheat and rye. *Plant Physiol.* 101, 245–250.

Pabo, C.O., Pesach, E. and Grant, R.A. (2001) Design and selection of novel Cys2His2 zinc finger proteins. *Annu. Rev. Biochem.* 70, 313–340.

Park, S., Lee, C.M., Doehy, C.J., Gilmour, S.J., Kim, Y. and Thomashow, M.F. (2015) Regulation of the Arabidopsis CBF regulon by a complex low-temperature regulatory network. *Plant J.* 82, 193–207.

Rosell, J.B., Wilson, P.B., Hussain, D., Woo, N.S., Gordon, M.J., Mewett, O.P., Howell, K.A. et al. (2007) Systemic and intracellular responses to photooxidative stress in Arabidopsis. *Plant Cell.* 19, 4091–4110.

Sakamoto, H. (2004) Arabidopsis Cys2/His2-Type zinc-finger proteins function as transcription regulators under drought, cold, and high-salinity stress conditions. *Plant Physiol.* 136, 2734–2746.

Sakamoto, H., Ariaki, T., Meshi, T. and Inawabuchi, M. (2000) Expression of a Hairy Leaf 6, an AP2/ERF transcription factor, interacts with OsWOX3B and regulates trichome formation in rice. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 51, 135–166.

Shibata, S. (2017) Plant Stress Physiology. Wallingford, Oxfordshire, UK; Boston, MA, USA: CABl.

Shi, H. and Chan, Z. (2014) The cysteine2/histidine2-type transcription factor ZINC FINGER OF ARABIDOPSIS THALIANA 6-activated C-REPEAT-BINDING FACTOR pathway is essential for melatonin-mediated freezing stress resistance in Arabidopsis. *J. Pineal Res.* 57, 185–191.

Shi, H., Ye, T., Chen, F., Cheng, Z., Wang, Y., Yang, P., Zhang, Y. et al. (2013) Manipulation of arginase expression modulates abiotic stress tolerance in Arabidopsis: effect on arginine metabolism and ROS accumulation. *J. Exp. Bot.* 64, 1567–1579.

Shi, H., Ye, T., Zhu, J.K. and Chan, Z. (2014) Constitutive production of nitric oxide leads to enhanced drought stress resistance and extensive transcriptional reprogramming in Arabidopsis. *J. Exp. Bot.* 65, 4119–4131.

Shi, Y., Ding, Y. and Yang, S. (2018) Molecular regulation of CBF signaling in cold acclimation. *Trends Plant Sci.* 23, 623–637.

Su, J., Yang, L., Zhuo, Q., Wu, H., He, Y., Liu, Y., Xu, J. et al. (2018a) Active photosynthetic inhibition mediated by MPK3/MPK6 is critical to effector-triggered immunity. *PLoS Biol.* 16, e2004122.
up-regulation of polyamine turnover, antioxidant protection, and proline accumulation. Plant, Cell Environ. 41, 2021–2032.

Supporting information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Analysis of the Cis elements of the PeSTZ1 gene.
Figure S2 Analysis of the transgenic poplar plants overexpressing PeSTZ1.

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Figure S3 Compared with the wild type, the growth of 35S PeSTZ1 poplars was not affected under normal conditions.
Figure S4 Analysis of the Cis elements of the PeAPX2 gene.
Table S1 Specific binding sites for bioinformatics prediction using the JASPAR database.
Table S2 Primer sequences used for cloning PeSTZ1 cDNA and for qRT-PCR.
Table S3 Accession numbers.