

# Trehalose-6-phosphate phosphatase (TPP) participates in cold acclimatization of *Actinidia arguta* depending on the ethylene signal transduction pathway

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## Research Article

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# Abstract

*Actinidia arguta* (kiwiberry) is a perennial deciduous vine that has a very strong overwintering ability. We speculated that trehalose metabolism, which plays a pivotal role in the stress tolerance of plants, may be involved in the cold acclimatization of *A. arguta*. Transcriptome analysis showed that the expression of *AaTPPA*, encoding a trehalose-6-phosphate phosphatase (TPP), was upregulated in response to low temperature. *AaTPPA* expression levels were much higher in lateral buds, roots, and stem cambium than in leaves in autumn. In *AaTPPA*-overexpressing (OE) *Arabidopsis thaliana*, trehalose levels were 8 ~ 11 times higher than that of the wild type (WT) and showed different phenotypic characteristics from WT or overexpression lines of *OtsB*, the *E. coli* TPP gene. Surprisingly, *AaTPPA*-OE *A. thaliana* had significantly higher freezing tolerance than WT and *OtsB*-OE lines. Transient overexpression of *AaTPPA* in *A. arguta* leaves increased ROS scavenging capacity and the contents of soluble sugars and proline. *AaERF64*, an ethylene-responsive transcription factor, was induced by ethylene treatment, and bound to the GCC-box of the *AaTPPA* promoter to activate its expression. *AaTPPA* expression was also induced by abscisic acid (ABA). Overall, the temperature decrease in autumn induces *AaERF64* expression through the ethylene signal transduction pathway, which in turn upregulates *AaTPPA* expression, leading to the accumulation of osmotic protectants including soluble sugars and proline in the overwintering tissues of *A. arguta*.

## Introduction

Environmental and biological factors that are unfavorable or detrimental to the normal growth and development of plants are referred to as stresses. Drought, waterlogging, heat, cold, salinity, and invasion by pathogens are the six main stresses that plants face inevitably during their survival (VanWallerdael et al. 2019). Among them, cold stress is one of the major abiotic stresses that affect plant growth in early spring and late autumn, which is categorized into two terms: chilling stress (0–20°C) and freezing stress (< 0°C) (Guo et al. 2018). To cope with cold stress, most plants have evolved two strategies including cold tolerance and cold acclimatization. Cold tolerance is one of the strategies for plants to respond to temporary low temperatures, during which the contents of osmotic protectants and the activities of antioxidant enzymes increase, and stress-signaling phytohormones including gibberellin (GA), abscisic acid (ABA), ethylene, and jasmonic acid (JA) are also produced (Shinozaki and Yamaguchi-Shinozaki 2000). Cold acclimatization is another strategy for plants to cope with long seasonal weather changes, which is triggered upon prior exposure to non-freezing chilling stress. During this process, freezing-protective substances, such as soluble sugars and anti-freezing proteins, accumulate in overwintering tissues of plants to protect biological membranes and biomolecules from damage, and at the same time, morphological changes occur to minimize energy consumption (Ding et al. 2020; Kaplan et al. 2007).

Recently, it has been reported that trehalose ( $\alpha$ -D-glucopyranosyl-1,1- $\alpha$ -D-glucopyranoside) metabolism plays a distinct role in plant tolerance to stresses, such as drought, salt, cold, and so on (Hassan et al. 2022). Trehalose is a non-reducing disaccharide that exists in most organisms except vertebrates (Avonce et al. 2006). Generally, in plants, trehalose-6-phosphate (Tre6P) is first synthesized from UDP-glucose and glucose-6-phosphate by trehalose-6-phosphate synthase (TPS), which is in turn

dephosphorylated to produce trehalose by trehalose-6-phosphate phosphatase (TPP) (Cabib and Leloir 1958). Finally, it is hydrolyzed into two glucose molecules by trehalase (TRE) (Phan et al. 2022). In plant genomes, TPSs and TPPs are a large family of genes. For example, *Arabidopsis thaliana* alone has 11 TPS genes and 10 TPP genes (Lunn 2007). The plant TPS genes are further divided into two classes; the class I genes have TPS enzyme activity, and the class II genes have no enzymatic activity (Blazquez et al. 1998; Paul et al. 2008). Regrettably, the reason why plants have so many TPS and TPP genes has not been fully uncovered. Perhaps they might be related to the critical biological roles of the trehalose metabolites. Here, trehalose itself is an effective osmotic protectant, but its levels in most plants are too low to provide osmotic protection. Instead, trehalose and Tre6P act as signaling molecules that induce a set of factors related to stress tolerance and sugar metabolism in plants (Iturriaga et al. 2009). However, so far, only SUCROSE-NON-FERMENTING1-RELATED KINASE1 (SnRK1) has been identified as a target of Tre6P, and no other direct targets of Tre6P and trehalose have been found (Tsai and Gazzarrini 2014). Tre6P binds directly to SnRK1 and inhibits its activity, thereby controlling the expression of over 1,000 genes regulated by SnRK1 (Baena-Gonzalez et al. 2007).

Kiwiberry (*Actinidia arguta* (Sieb. et Zucc.) Planch ex Miq) is a perennial deciduous vine tree belonging to the genus *Actinidia* of the *Actinidiaceae* family. *A. arguta* is known for its fruit having uniquely delicious taste and rich nutrients and is also one of the valuable cold-tolerant fruit tree resources that do not freeze to death even under  $-34^{\circ}\text{C}$  in winter (Calugaru-Spataru et al. 2013; Latocha 2017). However, its sprouting buds are very sensitive to frost damage in spring. Recently, due to the "false vernalization" phenomenon caused by global warming, the production of kiwiberries has decreased significantly, causing great economic loss to fruit farming in China.

From the transcriptome data of *A. arguta* under chilling stress, we found that the expression level of a TPP gene (*AaTPPA*) increased in response to low temperature. *AaTPPA* overexpression in *A. thaliana* and transient overexpression in *A. arguta* leaves enhanced the cold tolerance of these plants by accumulating soluble sugars and proline and increasing the scavenging activity of reactive oxygen species (ROS). Furthermore, we found that *AaTPPA* was transcriptionally activated by *AaERF64* which was induced by ethylene treatment, and that their expression levels increased in overwintering tissues, such as lateral buds, stem cambium, and roots, in response to the temperature decrease in autumn.

## Materials and methods

### Plant materials and treatments

Two *A. arguta* species, *A. arguta* cv. Kuilv (KL) (cold resistant variety) and *A. arguta* cv. Longcheng No.2 (L2) (cold susceptible variety), were used in this study. *A. arguta* (KL) seedlings were supplied from the Horticulture Branch of Heilongjiang Province, and *A. arguta* (L2) seedlings were grown in pots from hardwood cuttings. *A. arguta* (KL) plants at different growth stages (cuttings, 2-month-old seedlings, 5-month-old plantlets) were treated under different low temperatures (4, 9,  $15^{\circ}\text{C}$ ) for different times (0, 2, 4, 6 h), respectively, and sprouting buds, young leaves, and mature leaves were collected for RNA extraction.

5-month-old *A. arguta* (L2) plantlets grown in pots were sprayed with ethephon (ethylene precursor) (10  $\mu\text{mol/L}$ ), abscisic acid (ABA) (100  $\mu\text{mol/L}$ ), or jasmonic acid methyl ester (MeJA) (10  $\mu\text{mol/L}$ ), respectively, once a day for 15 d. After spraying, each pot was covered with a transparent plastic bell to prevent leakage of the phytohormones. Mature leaves, lateral buds, stem cambium, and fine roots were collected for RNA extraction and biochemical analysis. All samples included three biological replicates.

### Transcriptome analysis

*A. arguta* (KL) plantlets grown in pots for 5 months were set outside at 4–10°C on 9 May 2020 (Harbin, China) for cold treatment. Leaves were collected after 0, 2, 4, and 6 h treatment, and were used for RNA extraction. Total plant RNA was extracted according to the instructions of RNAprep Pure Plant Kit (TIANGEN, Beijing, China). cDNA was synthesized according to the method of Clontech SMARTer PCR cDNA Synthesis Kit and sequenced on the Illumina HiSeq X sequencing platform. RNA-seq was set up with three biological replicates. Reads of RNA-seq data were mapped to the reference genome sequence of *Actinidia chinensis* cv. Hongyang using HISAT2v2.0.5. The values of gene fragments per kilo million bases were generated using Perl script. Differential expression analysis was performed using the DESeq R package (1.18.0). The resulting *P*-values were adjusted using Benjamini and Hochberg's approach (Love et al. 2014).

## Gene cloning and sequence analysis

The open reading frames (ORF) of *AaTPPA* and *AaERF64* were amplified from the cDNA of *A. arguta* (KL) leaves using 2 × Phanta Max Master Mix (Vazyme), *OtsB* were amplified from the genome DNA of *E. coli DH5a* (primers are listed in Table S3). PCR amplified products were purified using Gel Extraction kit (TIANGEN), ligated with a linear pMD18-T vector (Takara), and then transformed into *E. coli DH5a*. Transformants were confirmed by PCR test with M13-F/R primers, and sent for sequence analysis. The protein-coding sequences (CDS) of *AaTPPA*, *OtsB*, and *AaERF64* were cloned into the binary vector GV1300 between *Kpn* and *BamH* restriction sites, respectively, under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Constructed GV1300-*AaTPPA*, GV1300-*OtsB*, and GV1300-*AaERF64* vectors were used for subcellular localization assay and *A. thaliana* transformation. The CDSs of *AaTPPA* and *OtsB* were also cloned into the *E. coli* expression vector pET-14b for procaryotic expression and purification (Fig. S3).

## Quantitative real-time PCR (RT-qPCR) analysis

Samples were ground in liquid nitrogen, and then total RNA was extracted using RNAprep Pure Plant Kit (TIANGEN, Beijing, China). cDNA was synthesized according to the instruction of PrimeScript Reverse Transcriptase kit (Takara, Japan). 18S rRNA was used as the internal reference for *A. arguta*, and *AtActin* was used as the internal reference for *Arabidopsis thaliana*. qPCR was carried out using Premix Ex Taq™ (Takara) mix on Lightcycler480 system (Roche, USA). Three biological replicates were used for each sample. All data were analyzed by the relative quantification ( $2^{-\Delta\Delta\text{Ct}}$ ) method (Livak and Schmittgen 2001). The gene-specific primers are listed in Table S4.

# Bioinformatic analysis

Multiple alignments were performed using the ClustalW BLOSUM protocol of MEGA7. Phylogenetic trees were constructed using the Neighbor-Joining (NJ) method and enriched using iTOL (<https://itol.embl.de/tree/>). The nuclear localization signal peptides were predicted using the online website INSP (<http://www.csbio.sjtu.edu.cn/cgi-bin/INSP/INSP.py>). The protein 3D-structures were predicted using the online website Phyre<sup>2</sup> (<http://www.sbg.bio.ic.ac.uk/phyre2/>). Cis-acting elements of the promoter region were analyzed using the online website PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

## Recombinant protein expression and purification.

To determine TPP enzyme activity, CDSs of *AaTPPA* and *OtsB* were inserted into pET-14b *E. coli* expression vector at *NdeI* and *BamHI*, respectively. The recombinant vectors were transformed into *E. coli* Rosetta (DE3), and the positive colonies were verified by PCR test. The transformants were incubated at 37°C until OD<sub>600</sub> reached 0.6 ~ 1.0, and after the addition of IPTG to 0.5 mmol/L, were incubated at 28°C for the induction of the recombinant proteins (Fig. S2a, c). His6-tagged *AaTPPA* and *OtsB* were purified by the Ni-affinity chromatography according to the instruction of His-tag Protein Purification Kit (Beyotime Biotech Inc., China) (Fig. S2b, d).

## Subcellular localization

GV1300-*AaTPPA*, GV1300-*OtsB*, and GV1300-*AaERF64* plasmids were transferred into *Agrobacterium tumefaciens* strain GV3101. GV1300 empty vector was used as the control plasmid. Tobacco (*Nicotiana benthamiana*) leaves were infiltrated with the transformed *Agrobacterium* suspensions (OD<sub>600</sub> = 0.6-1.0) and were kept in a dark chamber for 1 d, then under normal conditions for 2 d, after which the transformed leaves were observed under a fluorescence microscope. These vectors were also transfected into onion epidermal cells. The onion epidermis was spread flat on Murashige and Skoog 1962 (MS) culture media comprising 8 g/L agar and 30 g/L sucrose (pH 5.8), and co-cultured with the transformed *Agrobacterium* for 24 h in the dark and 48 h in the light, and then observed under a fluorescence microscope. DAPI was used as a nuclear labeling dye.

## A. thaliana transformation

*Agrobacterium tumefaciens* strain GV3101 harboring the purpose plasmids were used to transform *A. thaliana* (Col-0) by the floral dip method (Davis et al. 2009). T<sub>0</sub> seeds were screened on MS media containing hygromycin (Hyg) (50 mg/L). Selected T<sub>1</sub> plants were verified by genotyping with primers used for cloning the corresponding genes, and then at T<sub>2</sub> generation, the positive transgenic lines were tested for the expression levels of the corresponding genes. Two T<sub>3</sub> transgenic lines with high expression levels of the transgenes were chosen for further gene functional analysis.

## Morphological analysis of the transgenic A. thaliana

T<sub>3</sub> seeds of *AaTPPA* and *OtsB*-OE lines were observed for morphology, and the seed size was calculated using ImageJ software. Seeds of *AaTPPA* and *OtsB*-OE lines were inoculated on MS media (each plate contained 76 seeds), and the germinated seeds were counted after 10 d cultivation. Seedlings of *AaTPPA* and *OtsB*-OE lines grown on MS media for 12 d were measured for size and primary root length. All seedlings grown on MS media for 12 d were transplanted into pots. *AaTPPA* and *OtsB*-OE plants grown for 28 d were observed and measured for leaf size and chlorophyll content. Vivid rosette leaves of the plants grown for 45 d were counted, and then the ratio of surviving versus total leaves was calculated to evaluate the degree of senescence. Adult plants were measured for branch numbers, height, and silique counts.

### **Cold tolerance verification of the transgenic *A. thaliana***

T<sub>3</sub> seeds of *AaTPPA* and *OtsB*-OE lines were inoculated on MS media without Hyg antibiotics. WT seeds were also inoculated on the same plate, and each plate contained 4 × 19 seeds. All plates were cultured in an incubator for 14 d, and then set in a refrigerator at -10°C for 0.5, 1, 2, and 4 h, respectively. After freezing treatments, 3 plates of each group were used for ion leakage assay, and others continued to culture under normal conditions for 2 d, and then the survival rate was measured. *AaTPPA* and *OtsB*-OE plants grown for 21 d were subjected to freezing stress at -10°C for 0, 1, 2, and 4 h, respectively, and then continued to grow under normal conditions for 2 d, after which the leaf wilting rate was calculated. After culturing 4 h-treated plants for 7 d, the reviving rate was calculated. For chlorophyll fluorescence, NBT, and DAB assay, 21-day-old plants were exposed to sunny outside of -2 ~ -4°C for 0, 1, and 2 h in winter.

## **Construction of the plant expression vector with DsRed marker**

GV1300 vector was digested by *EcoRI* and *PmeI* restriction enzymes and then ligated with chemically synthesized loxP fragment using Exnase II recombinase to construct the GVloxP vector. Next, the GVloxP vector was cut at *KpnI* and *SpeI* restriction sites within the loxP construct and then recombined with DsRed expression cassette cloned from MtPT4-p3 vector (purchased from Addgene (Timoneda et al. 2021)) to construct the GVloxP/*DsRed* vector (Fig. S5). The CDSs of *AaTPPA* and *AaERF64* were introduced into the GVloxP/*DsRed* vector at *SmaI* and *PspXI* restriction sites under enhanced CaMV 35S promoter, respectively, and these recombinant vectors (GVloxP/*DsRed*-*AaTPPA* and GVloxP/*DsRed*-*AaERF64*) were transfected into *A. arguta* leaves.

### **Transient transformation of *A. arguta* leaves**

GVloxP/*DsRed*-*AaTPPA* and GVloxP/*DsRed*-*AaERF64* plasmids were transformed into *A. tumefaciens* strain GV3101(pSoup). GVloxP/*DsRed* vector was used as the mock plasmid. Young leaves of *A. arguta* (L2) plantlets grown in pots for 2 months were infiltrated with the transformed *Agrobacterium* suspensions (OD<sub>600</sub> = 0.6-1.0) and then were kept in a dark chamber for 1 d. The treated plantlets continued to grow under normal conditions for 5 d, after which the infiltrated leaves at attached state were observed under a fluorescence stereo-microscope (Nikon SMZ25, Japan). The plantlets with red

fluorescent leaves were set in a refrigerator at -4°C for 2 h for cold stress treatment, and then the fluorescent leaves were collected for further analysis.

## Analysis of physiological characteristics

Conductivity was determined by DDS- A conductivity meter for  $E_0$  (water control),  $E_1$  (sample was soaked into water and shaken at RT for 1 h), and  $E_2$  (sample was boiled for 15 min). Ion leakage was calculated as the formula:  $REL (\%) = 100 \times (E_1 - E_0) / (E_2 - E_0)$  (Xu et al. 2021). For chlorophyll fluorescence assay, plants were placed in a dark room for 30 min, and then whole plants were imaged using a PHOTON SYSTEMS INSTRUMENTS (PSI Ltd.). The optimal/maximal rate of the quantum yield of photosystem II ( $F_v/F_m$ ) was measured with FluorCam7 software. The histochemical analyses of  $H_2O_2$  and  $(O_2^-)$  were visualized by staining with 3,3-diaminobenzidine (DAB) and nitro blue tetrazolium chloride (NBT), respectively (Daudi and O'Brien 2012; Kumar et al. 2014). The leaves of *A. thaliana* and *A. arguta* were immersed in NBT (0.1 g/mL NBT, 50 mmol/L  $KH_2PO_4$ , pH 6.4) and DAB (0.05 g/mL DAB, 50 mmol/L Tris-HCl, pH 5.6) staining solutions after cold stress, and incubated for 3 h at 37°C in dark, after which chlorophyll was discarded in Carnoy's solution (ethanol: lactic acid: glycerol = 3:1:1). The relative average intensity of NBT and DAB staining was analyzed using ImageJ software. Malondialdehyde (MDA), a product of membrane lipid peroxidation, was determined by the thiobarbituric acid method as described previously (Soleimanzadeh et al. 2010). 0.3 g of leaves were ground in liquid nitrogen, and placed in 4 mL of 50 mmol/L potassium phosphate buffer (pH 7.8) in a centrifuge tube. The supernatant was taken, and mixed with 2 mL of 0.5% thiobarbituric acid (TBA), and the mixture was heated in a boiling water bath for 15 min. The absorbance was read at 450 nm, 532 nm, and 600 nm, respectively.

## Metabolites and enzyme activities assay

To measure trehalose and sucrose contents, samples were prepared according to the method of (Wang et al. 2020). 21-day-old *A. thaliana* leaves (100 mg) were harvested and rapidly frozen in liquid nitrogen. Each sample was grounded, and evenly mixed with 500  $\mu$ L chloroform:acetonitrile (3:7, v/v) and incubated in an ice-bath with occasional shaking. The organic materials were extracted twice with 400  $\mu$ L water at 4°C. The supernatants were dried in a vacuum dryer. The dry samples were quantified by gas chromatography coupled with mass spectrometry (GC-MS), according to the method of (Garg and Singla 2016). For the determination of Tre6P, samples were prepared as above, and then analyzed using an ultra-performance liquid chromatograph tandem quadrupole mass spectrometer (UPLC-MS/MS) as described previously (Lin et al. 2023).

The content of soluble sugars was measured by the anthrone method. 0.5 g samples were grounded with 2 mL PBS, and 0.1 mL of the supernatant was mixed with 3.5 mL of anthrone, and placed in a water bath at 90°C for 10 min. After cooling, the  $OD_{620}$  was measured. The proline content was measured as follows: 0.5 g samples were ground with 2 mL PBS, and 1 mL of the supernatant was mixed with 1.5 mL of ninhydrin and 1 mL of glacial acetic acid, placed in a boiling water bath for 30 min, and then the  $OD_{520}$  was measured.

TPP (E.C.3.1.3.12) enzyme activity was assayed by monitoring phosphate ion (Pi) release from Tre6P (Garg and Pandey 2016). The reaction volume was set to 0.3 ml containing 25 mM Tris-HCl (pH 7.0), 10 mM MgCl<sub>2</sub>, and 1 mM Tre6P with 0.05 ml of enzyme solution. Pi concentration was measured by the zinc acetate method as follows: 0.3 mL reaction solution was mixed with 0.9 mL of a solution containing 100 mM zinc acetate and 15 mM ammonium molybdate (pH 5.0), and incubated in ice for 1 min. The OD<sub>350</sub> was immediately measured. Enzyme activities were expressed per mg of protein. SOD, POD, and CAT activities were assayed according to the method of (Huang et al. 2022a).

### **Cloning of the AaTPPA Promoter and its activity assay by GUS staining**

First two sections (1685 bp) upstream to *AaTPPA* CDS were cloned by conventional PCR amplification with forward primers designed based on the conserved sequences between the TPPA genes of four *Actinidia* species (*A. chinensis* cv. Hongyang, *A. chinensis* cv. Red5, *A. eriantha* cv. White, *A. rufa* cv. Fuchu) (Fig. S8a, b), the remaining five sections (2453 bp) were cloned by TAIL-PCR (thermal asymmetric interlaced PCR) according to the manual of Genome Walking Kit (Takara) (Fig. S8c-g). Primers used for cloning the promoter are listed in Table S5. For the promoter activity assay, the CaMV 35S promoter of the GUS expression cassette in pCambia1303 vector was substituted with four segments of the promoter region and 5'-UTR of *AaTPPA* gene at *NcoI* and *HindIII* restriction sites, respectively. These recombinant vectors were transformed into *A. thaliana* using *A. tumefaciens*-mediated transformation (refer to *A. thaliana* transformation method). GUS staining was conducted on AaTPPAproX::GUS transgenic *A. thaliana*. Seedlings and various organs of the plants were stained with X-Gluc solution in darkness at 37°C overnight. Subsequently, Carnoy's solution was used to remove chlorophyll before taking photographs.

## **cDNA library screen by Yeast-one-hybrid (Y1H) method**

The bait sequences were inserted into the pAbAi vector at *SalI* and *HindIII* restriction sites to construct pAbAi-bait plasmids. The pAbAi-bait plasmids were single-digested with *BbsI* to excise the *URA3* gene, and then 1 µg of the linearized plasmid was transformed into Y1HGold yeast competent cells, and determined for the minimum inhibition concentration (MIC) of aureobasidin A (AbA) required to inhibit the bait strain at background levels. The competent cells of the bait strain were co-transformed with *A. arguta* cDNA library inserted into pGADT7 vector, and screened on SD/-Ura/-Leu media containing the MIC of AbA. Normally growing yeast colonies were selected for PCR test and sequencing.

## **Yeast-two-hybrid (Y2H) assay for self-transcriptional activity**

The full CDS, N-terminal (1–69 aa), and C-terminal domain (70–169 aa) sequences of *AaERF64* were cloned into the pGBKT7 (BD) vector at *NdeI* and *EcoRI* restriction sites, and transformed into Y2HGold yeast competent cells, respectively. BD-Lam was used as a negative control, and BD-53 was used as a positive control. The transformed yeast cells were screened on SD/-Leu media, and the self-activation test was performed on SD/-Leu/-Trp/-His/-Ade and SD/-Leu/-Trp/-His/-Ade/+X-α-Gal media at different dilutions.

# Dual luciferase assay

Dual luciferase assay was performed as described previously (Duan et al. 2019). The CDS of *AaERF64* was cloned into the pGreenII-62-SK vector to prepare the effector vector, and the *AaTPPAproS3* sequence was cloned into the pGreenII-0800 vector to prepare the reporter vector (Fig. 7e). The constructed vectors were transformed into *A. tumefaciens* GV3101 (pSoup), respectively, and then co-infiltrated to tobacco (*N. benthamiana*) leaves. The leaves were treated with a solution containing 0.1 mmol/L potassium D-fluorescein and 0.01% TritonX-100 and then observed using a Tanon 5200 fully automated chemiluminescence image analysis system. Firefly luciferase (Fluc) and renal luciferase (Rluc) activities were determined using a GloMax® 20 light detector according to the instruction for Dual-Luciferase® Reporter Assay System (Promega, Beijing). 20 µL of cell lysates of infiltrated sections were added to a light tube containing 100 µL of LAR II to measure Fluc activity, and subsequently, 100 µL of Stop & Glo® Reagent was added to measure Rluc activity.

## Electrophoretic mobility shift assay (EMSA)

The CDS of *AaERF64* was cloned into pGEX-4T-1 expression vector and transformed into *E. coli* host cells to express the recombinant protein GST-AaERF64, which was purified by GST-tag affinity chromatography (Fig. S11). Biotin-labeled DNA probes were synthesized by AZENTA LIFE SCIENCES (Beijing, China). The biotin-tagged hot and mut-probes were mixed with the GST-AaERF64 protein in buffer (0.25 mol/L HEPES, 0.5 mol/L KCl, 20 mmol/L MgSO<sub>4</sub>, respectively, and 10 mmol/L DTT) and incubated at 25°C for 30 min. 6.6% PAGE was performed at 80 V for 30 min, and then the EMSA gel was scanned using the Typhoon FLA7000 system (USA).

## Statistical analysis

All data in this study were analyzed and plotted using GraphPad Prism 6.0 and IBM SPSS 25.0 (Chicago, IL), and statistical significance was tested using Student's *t*-test with differences considered significant at \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001. Duncan's multiple comparisons were used at significance levels of *P* < 0.05.

## Results

### Identification of the cold-responsive TPP gene of *A. arguta* and its subcellular localization

From the transcriptome data of *A. arguta* cv. Kuilv (KL), we found 39 trehalose-metabolism-related genes including 21 TPSs, 17 TPPs, and one TRE. Comparative analysis of their expression levels between normal (CK) and low temperature (LT) conditions showed that among these genes, TPP#3, #5, #8, and #9 expressed differentially in response to cold stress (Fig. 1a). Since *A. arguta* (KL) is a tetraploid plant (2n = 4x=116) (Liu et al. 2011), these cold-responsive TPPs might be alleles. Therefore, we cloned all cDNAs of TPP#3, #5, #8, and #9, and found more than 22 different sequences (Fig. S1a). Multiple alignment and phylogenetic tree of these sequences showed that the cloned TPP genes were largely divided into three

groups regardless of the primers used for PCR amplification (Fig. S1b). The identities between groups were over 84.6% (Fig. S1c), suggesting that these cold-responsive TPP genes of each group were alleles or duplicate genes, as expected. Among these genes, we selected the Group I TPP gene as the purpose cold-responsive TPP gene, which has the highest expression difference between CK and LT conditions (TPP#5; Appendix S1). This cold-responsive TPP gene of *A. arguta* had the highest homology with *TPPA* (trehalose-6-phosphate phosphatase A) of *Populus trichocarpa* and *Arabidopsis thaliana* (Fig. 1b). From this, we termed the cold-responsive *A. arguta* TPP gene as *AaTPPA*. The similarity of *AaTPPA* with *AtTPPA*, *PtrTPPA*, and *OtsB* (the *E. coli* TPP gene) was 66.8%, 73.3%, and 21.4%, respectively (Fig. S2).

The TPP gene family belongs to the HAD (L-2-haloacid dehalogenase) superfamily of phosphatases and other hydrolases, and the proteins of this superfamily are characterized by having three conserved motifs (HAD motif I, II, and III) that form the catalytic active site (Fieulaine et al. 2005). *AaTPPA* also had all the HAD motifs, considering that it could have the enzymatic activity of trehalose-6-phosphate phosphatase (Fig. S2). To determine its enzymatic activity, the His6-tag-linked *AaTPPA* protein was expressed in *E. coli*, and purified by Ni-affinity chromatography (Fig. S3). The His6-tag-linked *OtsB* protein was used as a positive control. The recombinant *AaTPPA* protein was mainly expressed in the form of an inclusion body in the *E. coli* host cells, while the recombinant *OtsB* protein was present in the cytoplasmic soluble fraction, suggesting that *AaTPPA* is largely different from *OtsB* in physiochemical properties. The enzymatic activity of *AaTPPA* was more than twice that of *OtsB* at 9–25°C and decreased largely above 35°C (Fig. 1d).

*AaTPPA* was predicted to have a monopartite nuclear localization signal (NLS) of RKKAK(73–77) (Fig. S2a). In addition, the predicted 3D structure of *AaTPPA* showed that it has a catalytic domain similar to *OtsB*, but *AaTPPA* has an additional N-terminal domain of an unknown function which is common in plant TPP genes (Fichtner and Lunn 2021) (Fig. 1c). The analysis of subcellular localization in onion epidermal cells and tobacco leaf epidermal cells showed that both *AaTPPA* and *OtsB* were present in the cell nucleus and the whole cytoplasm (Fig. 1e, f). This result is consistent with a previous report that *AtTPPA*, *AtTPPB*, *AtTPPC*, *AtTPPF*, and *AtTPPH* are present in the cytosol and nucleus (Krasensky et al. 2014).

### ***AaTPPA* expression is induced in response to mild low-temperature**

*A. arguta* acquires cold acclimatization as the weather temperature gradually decreases in autumn (Van Labeke et al. 2015). Thus, it was necessary to determine at what temperature the expression of the cold-responsive *AaTPPA* was induced. As a result, the expression level of *AaTPPA* in mature leaves gradually increased under the mild low-temperature of 15°C, and it was 3.5 times higher at 6 h than before the low-temperature treatment. *AaTPPA* expression decreased gradually over time at 4°C and 9°C (Fig. 2a).

*A. arguta* has a strong overwintering ability, but the developing young tissues are very susceptible to frost damage in spring (Marosz 2009). So, we investigated the low-temperature responsiveness of *AaTPPA* at different leaf growth stages. At the sprouting bud stage, *AaTPPA* did not respond to mild low temperature, and at the young leaf stage, its expression level increased about twice only after 6 h at 15°C (Fig. 2b). In

addition, *AaTPPA* expression was not induced at all in the sprouting buds and the young leaves under 4°C and 9°C treatments.

Based on the above results, we investigated changes in *AaTPPA* expression levels in mature leaves of *A. arguta* grown in a garden as the weather temperature dropped in autumn. Consistent with the indoor experiment, the expression levels of *AaTPPA* in mature leaves gradually increased until August 23, 2022 (Harbin, China), when the daily low-temperature elapsed 13°C, and then decreased as the temperature dropped below 10°C (Fig. 2d). The weather temperatures below 10°C suppressed the growth of shoot apical meristems, and accelerated leaf senescence (Fig. 2c). Leaves began to fall on September 25, the deadline for sampling. This result indicated that *AaTPPA* was transiently induced in mature leaves in response to temporary mild low temperature, and its expression was not maintained at the elevated level during the entire autumn, suggesting that temporary expression of *AaTPPA* in leaf tissues might not make a critical contribution to the acquisition of overwintering ability of *A. arguta*.

Therefore, to determine the main acting sites of *AaTPPA* for acquiring cold acclimatization of *A. arguta*, the plant's roots, stem cambium, lateral buds, and mature leaves were collected on September 25 in 2022, and *AaTPPA* expression levels were analyzed (Fig. 2e). The result showed that *AaTPPA* expression levels in lateral buds, roots, and stem cambium were much higher than that in leaves. Since lateral buds, roots, and stem cambium are dormant tissues in winter, the high expression levels of *AaTPPA* in these tissues implied that this gene might play an essential role in the cold acclimatization of *A. arguta*.

### **Developmental and morphological characteristics of *AaTPPA*-overexpressing *Arabidopsis***

Among the transgenic *A. thaliana* plants, #4 and #7 lines with high expression levels of *AaTPPA* were selected for *AaTPPA*-overexpressing (OE) lines (Fig. S4a). We also constructed *A. thaliana* transformed with *OtsB* which only has a catalytic domain, unlike the plant TPP enzymes, and *OtsB*-OE #6 and #7 lines were selected as positive controls (Fig. S4b). Seeds of the selected OE lines were all hygromycin-resistant at T<sub>3</sub> generation, confirming that these lines were homozygous.

First, we measured trehalose and Tre6P levels in *AaTPPA* and *OtsB*-OE *A. thaliana* lines. Surprisingly, trehalose levels in *AaTPPA*-OE lines were 8 ~ 11 times higher than that of the wild type (WT), whereas no significant changes were observed in *OtsB*-OE lines (Fig. 3a). T6P levels in *A. thaliana* were too low to distinguish significant differences between WT and the OE lines, but the result showed a tendency that T6P levels of *OtsB*-OE lines were lower than those of WT and *AaTPPA*-OE lines (Fig. S4c). To assay changes in Tre6P levels, we further investigated the expression levels of several SnRK1 marker genes (Fig. S4e). As a result, changes in the expression levels of SnRK1 marker genes in *OtsB*-OE lines were consistent with the previous report (Zhang et al. 2009), but in *AaTPPA*-OE lines, there was no significant difference from WT except for the expression of *AtDXP*. This suggested that Tre6P levels of *AaTPPA*-OE *A. thaliana* did not change significantly enough to affect the SnRK1 activity, unlike that of *OtsB*-OE lines.

Next, we compared the developmental and morphological characteristics between *AaTPPA* and *OtsB*-OE *A. thaliana*. The seeds of all the OE lines were smaller and misshapen compared to WT (Fig. 3b, c).

Especially, the seeds of *AaTPPA*-OE lines were smaller even than those of *OtsB*-OE lines. However, the small seed size did not affect the germination rate, suggesting that overexpression of the TPP genes only affected seed filling but not embryogenesis (Fig. S5a). Seedlings of the OE lines grown in MS media were also smaller in size and had short primary root lengths at first (Fig. S5b, c; Fig. 3d, e), but afterward, there was no significant difference in growth compared to WT (Fig. S5d), demonstrating that the initial growth disturbance was only due to a lack of nutrients in the seeds of the TPP-OE lines. *AaTPPA* overexpression rather seemed to enhance vegetative growth in the later developmental stages (Fig. 3f).

*AaTPPA*-OE lines had elongated rosette leaves like WT, but *OtsB*-OE lines had broad-shaped leaves (Fig. S5d; Fig. 3g). In addition, chlorophyll contents of rosette leaves were significantly lower in *OtsB*-OE lines, but there was no difference between *AaTPPA*-OE lines and WT (Fig. S5e). As in previous studies, bolting periods of all the OE lines were delayed than WT (Fig. 3f), and even at the time when all the rosette leaves of WT had wilted, the rosette leaves of the OE lines remained vivid (Fig. 3h). Interestingly, *AaTPPA*-OE lines had more branches and siliques than WT, but *OtsB*-OE lines had significantly fewer branches and siliques (Fig. 3i; Fig. S5g). No significant difference was observed in heights between the OE lines and WT (Fig. S5f).

Overall, *AaTPPA*-OE *A. thaliana* had much higher trehalose levels and showed distinct developmental and morphological characteristics. Like *OtsB*-OE lines, *AaTPPA*-OE lines had misshapen seeds and delayed reproductive growth and senescence. However, unlike *OtsB*, *AaTPPA* overexpression did not affect the leaf shape and chlorophyll content. Furthermore, *AaTPPA*-OE lines had more branches and siliques compared to WT and *OtsB*-OE lines.

### **Heterogeneous overexpression of *AaTPPA* enhances the freezing tolerance of *Arabidopsis***

First, we investigated the freezing tolerance of the OE *A. thaliana* lines at the seedling stage. The survival rate of *AaTPPA*-OE #4 line was significantly higher than that of WT after freezing treatment at -10°C for 0.5 and 1 h. On the contrary, *OtsB*-OE lines showed a relatively low survival rate compared to WT (Fig. S6a, b). Meanwhile, the ion leakages of both *AaTPPA*-OE and *OtsB*-OE seedlings were lower than that of WT after 1 h of freezing treatment, suggesting that the TPP overexpression had a protective effect on biological membranes (Fig. S6c).

Next, *A. thaliana* plants grown in pots were subjected to freezing stress at -10°C. The leaf wilting rates of *AaTPPA*-OE lines of 4 h treatment group were significantly lower than that of other lines (Fig. 4a, b). Surprisingly, after normal cultivation of this group for 7 d, the reviving rate of *AaTPPA*-OE #4 line was twice that of WT, while the reviving rates of *OtsB*-OE lines were significantly lower than that of WT (Fig. 4c).

Freezing stress-treated *A. thaliana* lines were stained with NBT to assay the levels of superoxide anion ( $O_2^-$ ) (Fig. S6d, e). Under normal conditions,  $O_2^-$  levels of all the OE lines were significantly lower than that of WT, suggesting that the TPP overexpression increased ROS scavenging capacity. Under freezing conditions,  $O_2^-$  levels of all the lines decreased as freezing time increased, and its levels were maintained

higher in all the OE lines than in WT. This result could be interpreted that photosystem I (PSI), the generator of  $O_2^-$  in chloroplasts, was destructed under extreme freezing conditions, but the TPP overexpression prevented PSI from freezing destruction. The fact that the plant photosystems were protected in the OE lines was also proved by chlorophyll fluorescence assay. After cold stress at  $-2^\circ\text{C}$  for 2h,  $F_v/F_m$  values remained the highest in *AaTPPA*-OE lines, and the values of *OtsB*-OE lines were also higher than that of WT (Fig. 4d, e).

We also evaluated the degree of hydrogen peroxide ( $H_2O_2$ ) accumulation using DAB staining (Fig. S6f, g). *AaTPPA*-OE lines tended to accumulate less  $H_2O_2$  than WT and *OtsB*-OE lines under normal and freezing stress, but no significant difference was recognized. Moreover, the activities of SOD, POD, and CAT increased more in *AaTPPA*-OE lines than in WT and *OtsB*-OE lines under normal and cold stress conditions (Fig. S6h, i, j), and the MDA contents also increased more in *AaTPPA*-OE lines after cold stress (Fig. 4f), indicating that *AaTPPA* overexpression plays a positive role in activating the plant antioxidant system.

The above results implied that *AaTPPA* overexpression could have a protective effect on biological membranes and biomolecules by accumulating osmotic protectants. For proof of concept, the contents of sucrose, soluble sugars, and proline, which are the main osmotic protectants of plants, were measured, showing that under normal and cold stress conditions more sucrose and soluble sugars were accumulated in both *AaTPPA* and *OtsB*-OE lines than those in WT, and proline contents were higher only in *AaTPPA*-OE lines (Fig. 4g, h, i).

In summary, *AaTPPA* overexpression enhanced the cold tolerance of *A. thaliana*, but *OtsB* overexpression did not affect the cold tolerance obviously and was rather negative in some aspects.

### **Transient overexpression of *AaTPPA* in *A. arguta* leaves improves cold tolerance indices**

Through the *Agrobacterium tumefaciens*-mediated transformation, target genes can be transiently overexpressed in plant leaf tissues. However, this method has low reproducibility, because the expression efficiency of the target gene is different each time depending on the growth state of the infiltrated leaves and the experimenter's technique. To improve the reproducibility of this transient transformation method, we created a new plant expression vector (GVloxP/DsRed) which contains a red fluorescence protein (DsRed) as a marker, allowing to monitor the degree of transfection in the infiltrated leaves by a non-invasive manner (Fig. S7). The overexpression vector, GVloxP/DsRed-*AaTPPA*, was prepared by replacing the CDS of the hygromycin resistance gene (*HygR*) of this vector with *AaTPPA* CDS (Fig. 5a).

Young leaves of *A. arguta* cv. Longcheng No.2 (L2) plantlets grown in pots were infiltrated with *Agrobacterium* containing GVloxP/DsRed-*AaTPPA* vector, and after 3 d, they were observed under a fluorescence stereomicroscope, and leaves with obvious red fluorescence were collected to determine the gene expression level (Fig. 5b, c). The result demonstrated that the *AaTPPA* expression level was incomparably higher in *AaTPPA*-OE group than in EV (the mock group only infiltrated with GVloxP/DsRed empty vector) and CK (the control group not transfected).

Next, *A. arguta* (L2) plantlets with red-fluorescent leaves that were transformed as described above were subjected to cold stress at -4°C for 2 h, and then several cold tolerance-related indices, such as ROS accumulation, ion leakage, MDA, soluble sugars, and proline contents, were analyzed. Interestingly, DAB staining showed that H<sub>2</sub>O<sub>2</sub> accumulation in EV group was particularly higher, explaining that the leaf tissues might be damaged by the transfection process, or that the overexpression of marker genes, such as *DsRed* and *HygR*, might be harmful to the physiology of the plant (Fig. 5d, e). Nevertheless, H<sub>2</sub>O<sub>2</sub> level of OE group was significantly lower than that of CK group after cold stress. In addition, after cold stress, the ion leakage and MDA contents of *AaTPPA*-OE leaves were significantly lower than those of CK and EV groups (Fig. 5f). Soluble sugars and proline contents were higher in *AaTPPA*-OE group even under normal conditions. Cold stress increased the contents of these osmotic protectants in all groups, but *AaTPPA*-OE group still maintained the highest level (Fig. 5f).

### **Cloning of the *AaTPPA* promoter and verification of its transcriptional activity**

Since the genome sequence of *A. arguta* has not been published yet, the promoter sequence of *AaTPPA* had to be cloned to seek upstream transcription factors that affect the *AaTPPA* expression in response to low temperature. According to the genome data of *A. chinensis* cv. Red5, the homologous TPPA gene had an exceptionally long 5'-UTR (3,162 bp) upstream of the ATG translational start codon (Fig. S8h). Therefore, to obtain the promoter sequence of *AaTPPA*, we had to clone a region of at least 4,000 bp upstream of the ATG codon. By combining the conventional PCR and TAIL-PCR (thermal asymmetric interlaced PCR), a total region of 4,138 bp upstream of the ATG codon was cloned and sequenced (Fig. S8, Appendix S2). Alignment with the TPPA gene sequence of *A. chinensis* cv. Red5 showed that among the whole cloned sequence, the promoter region was approximately 1185 bp, and the remaining section (2953 bp) belonged to 5'-UTR (Fig. S9a).

In the previous study on the promoters of TPP genes, ~2000 bp sequence upstream to CDS was considered to be the promoter region (Wang et al. 2022). So, to determine whether the long 5'-UTR of *AaTPPA* affects the gene expression, the CaMV 35S promoter of the GUS gene in pCAMBIA1303 vector was substituted by four sections of the cloned 5'-UTR plus promoter region to construct the promoter activity checking vector (Fig. S9a, b). The *AaTPPA*pro2 section contained a 1685 bp sequence from the ATG codon to the middle of *intron 1*, the *AaTPPA*pro3 section contained a 2688 bp sequence including the almost *intron 1*, and the *AaTPPA*pro9 included the whole 5'-UTR and promoter region of *AaTPPA* (4138 bp). The *AaTPPA*pro8 section contained only the predicted promoter region (Fig. S9a). The GUS expression cassettes driven by the above four different sections were transformed into *A. thaliana*, and the screened transgenic T<sub>1</sub> plants were verified by PCR test on the corresponding sections (Fig. S9c-j).

GUS gene driven by *AaTPPA*pro9 was expressed in almost all tissues including roots, vascular tissues, mesophyll, stems and branches, sepals and petals, siliques, and developing seeds (Fig. 6a). In comparison, GUS driven by *AaTPPA*pro8 had the same expression pattern as *AaTPPA*pro9, except for young leaves and developing seeds. On the contrary, *AaTPPA*pro2 and *AaTPPA*pro3-driven GUS were not expressed in roots and were mainly expressed in vascular tissues. *AaTPPA*pro3::GUS was also

specifically expressed in developing seeds. The above results showed that the AaTPPApro8 sequence had sufficient promoter activity in almost all tissues except for developing seeds and young leaves.

Next, to determine whether the AaTPPApro8 section has cold-stress responsive characteristics, we analyzed the expression levels of the AaTPPApro8-driven GUS gene under normal and low temperature (9°C) conditions (Fig. 6b, c). The result showed that GUS staining was more intense in the low-temperature treatment group than in the normal group, indicating that the AaTPPApro8 sequence has a low-temperature responsive cis-acting element.

Taken together, it can be concluded that *AaTPPA* has a long 5'-UTR of about 3000 bp and that the AaTPPApro8 sequence of 1274 bp has a low-temperature responsive characteristic as well as constitutive promoter activity.

### **AaERF64 binds to the AaTPPA promoter and activates its transcription**

We searched for cis-acting regulatory elements in the promoter region of 1185 bp upstream from the predicted transcriptional start site using the online software PlantCARE (Fig. 7a, Table S1). Based on the motif analysis, we adopted three sections which are clustered with functional motifs, and each fragment was inserted into pAbAi vector, respectively, to prepare bait vectors. Among the Y1HGold strains transformed with these bait vectors, only the AaTPPAproS3 introduced strain was completely suppressed on 300 ng/mL aureobasidin A (AbA) containing medium, and the others were insensitive to AbA because of their constitutive promoter activity. Therefore, proteins interacting with the AaTPPAproS3 section were screened from the *A. arguta* cDNA library using the yeast-one-hybridization (Y1H) method. As a result, three ethylene-responsive transcription factors (ERF) were identified as transcription factors. From the transcriptome data of *A. arguta*, the ERF gene (gene28680) which was responsive to low temperature was selected for further study (Table S2). Its interaction with the *AaTPPA* promoter was reconfirmed by using the Y1H assay (Fig. 7b). This ERF gene had the highest homology with ERF64 of *Actinidia deliciosa* (*AdERF64*) in NCBI's nucleotide database (Fig. S10a), so we called it *AaERF64* (Appendix S3).

In plants, ERF genes constitute a large family of transcription factors which is a part of the AP2/ERF (APETALA2/ETHYLENE RESPONSE FACTOR) superfamily (Riechmann et al. 2000). Generally, proteins of AP2/ERF superfamily have an AP2 DNA-binding domain of approximately 60–70 amino acids (Sakuma et al. 2002). AP2/ERF superfamily is divided into 12 phylogenetic groups in *A. thaliana*, and some of them are further divided into several subgroups (Nakano et al. 2006). The phylogenetic tree with 122 ERF family members of *A. thaliana* showed that *AaERF64* belongs to the V group of the ERF family, and has the closest relation to ERF#003 (At5g25190.1) of *A. thaliana* (Fig. S10a). This group is characterized to have one CMV-2 motif (Nakano et al. 2006). Bioinformatic analysis showed that both the AP2 DNA-binding domain and CMV-2 motif are well conserved in the *AaERF64* protein (Fig. S10c). In addition, the N-terminal contains NLS peptide, indicating that this protein could be localized in the cell nucleus. The predicted 3D structure showed that the *AaERF64* protein is composed of two domains including the AP2 DNA-binding domain (4–69 aa) and a poorly predicted C-terminal domain (70–169 aa). The C-terminal

domain was thought to be a transcriptional activation domain due to containing the CMV-2 motif (Fig. S10b).

Subcellular localization assay of AaERF64 in tobacco leaf epidermal cells showed that AaERF64 was localized in the cell nucleus, satisfying the first requirement for acting as a transcription factor (Fig. 7c). Its transcriptional activation ability was examined by the Y2H self-transactivation test (Fig. 7d). The result demonstrated that AaERF64 had a transcriptional activation function and that the C-terminal domain (70–169 aa) was a transcriptional activation domain, as expected. In addition, the dual luciferase assay also demonstrated that AaERF64 could interact with an element of the *AaTPPA* promoter to activate the transcription of the downstream gene in plant cells (Fig. 9e). Furthermore, we transiently transfected *A. arguta* leaves with the GVloxP/DsRed-AaERF64 vector and proved that AaERF64 overexpression upregulates the expression of the endogenous *AaTPPA* in *A. arguta* genome (Fig. 8a). These results revealed that AaERF64 acts as a transcriptional activator of *AaTPPA* in *A. arguta*.

Next, we searched for the AaERF64-binding cis-element within the *AaTPPA* promoter sequence. All the ERF family proteins have the conservative AP2 DNA-binding domain, which has been known to specifically bind to GCC-box (Ohme-Takagi and Shinshi 1995). We found three GCC-box motifs in the AaTPPAproS3 section, which was used as a bait sequence for the AaERF64 screen (Fig. 7f). Three DNA fragments (EM1 ~ 3) containing each GCC-box motif were introduced into the pAbAi vector, respectively, and their binding potential with AaERF64 was examined by employing the Y1H assay. The result revealed that the EM2 fragment (22 bp) interacted with AaERF64. Furthermore, electrophoretic mobility shift assay (EMSA) proved that AaERF64 directly binds to the GCC-box motif (GCCGAC) of the EM2 sequence (Fig. 7g).

### **AaERF64 expression is induced through the ethylene signal transduction pathway**

As shown above, *AaTPPA* expression levels were particularly high in overwintering tissues of *A. arguta* in autumn (Fig. 2e). This time, we investigated changes in the expression levels of *AaTPPA* and *AaERF64* in these tissues according to the temperature decrease in autumn (Fig. 8b). Based on the previous experimental data, we selected three sampling times; the first time was the day when the weather temperature was the highest in summer, 2023, the second time was the day when the daily low-temperature dropped below 15°C, and the last time was the day when the daily low-temperature dropped below 10°C. At the last time, the shoot apical buds began to degenerate.

In mature leaves, stem cambium, and lateral buds, the expression levels of *AaTPPA* were positively correlated to those of *AaERF64*, supporting the prior result that *AaERF64* overexpression induced upregulation of *AaTPPA* expression (Fig. 10a). Unexpectedly, in roots, no significant changes were observed in the expression levels of *AaERF64* depending on the period, but the expression levels of *AaTPPA* gradually increased as the temperature became colder. This suggested that *AaERF64* has no action in roots and that *AaTPPA* has a high expression level specifically in roots, and may be regulated by other pathways.

In mature leaves, consistent with the results in 2022 (Fig. 2d), the expression levels of *AaERF64* and *AaTPPA* were the highest at the mild low temperature (daily high-temperature 24°C, daily low-temperature 14°C), and afterward, the expression of both genes decreased. However, *AaERF64* expression in lateral buds and stem cambium continued to increase until the last day (September 15, 2023), and so did *AaTPPA* expression. This result suggested that under excessively low temperature conditions, leaves become withered, leading to the reduction of overall gene expression, whereas in lateral buds and stem cambium, expression of genes, especially the genes related to cold stress, continue to be activated even in late autumn.

Next, we conducted research to identify the signal transduction pathway that induces the expression of the *AaERF64* transcription factor. According to previous studies, plant TPP genes were induced by several phytohormones, such as abscisic acid (ABA), jasmonic acid methyl ester (MeJA), and gibberellin (GA) (Ge et al. 2008; Lin et al. 2020; Huang et al. 2022b), and the cis-acting element analysis of the *AaTPPA* promoter predicted that there exists MeJA and ABA-responsive motifs (Fig. 7a, Table S1). For *AaERF64* as an ethylene-responsive factor, we selected ethephon (the precursor of ethylene), MeJA, and ABA as the putative inducible phytohormones (Fig. S12c). In *A. arguta* leaves, *AaTPPA* was induced by ethylene and ABA, and *AaERF64* was induced only by ethylene treatment. Induction of *AaERF64* and *AaTPPA* expression by ethylene occurred at 1–2 d of treatment, whereas induction of *AaTPPA* expression by ABA peaked at 12 h and then decreased. Interestingly, ABA treatment rather suppressed *AaERF64* expression. This suggested that the induction of *AaTPPA* expression by ABA occurs through a pathway different from *AaERF64* and that the induction by ABA occurs transiently in leaves, which is reversible when the ABA signal disappears.

We further investigated changes in the expression levels of *AaTPPA* and *AaER64* in the overwintering tissues of *A. arguta* by ethylene treatment (Fig. 8c). In the lateral buds and stem cambium, the expression levels of *AaTPPA* and *AaER64* continued to increase until 15 d of treatment, reaching over 10-fold higher than before treatment. However, their expression levels in leaves did not increase further after 5 d of treatment. Soluble sugars and proline are the main osmotic protectants in plants (Ding et al. 2020). Undoubtedly, lateral buds, stem cambium, and roots of *A. arguta* also contained much soluble sugars and proline in late autumn (Fig. S12a, b). Ethylene treatment also increased the contents of soluble sugars and proline in the lateral buds and stem cambium more than in the leaves (Fig. 8c). However, in roots, *AaTPPA* and *AaER64* expression did not respond to ethylene treatment, and there were no significant changes in the contents of soluble sugars and proline. This suggested that the increase in *AaTPPA* expression and the accumulation of soluble sugars and proline in roots in autumn might occur depending on other signaling pathways, maybe by ABA (Fig. 8b).

## Discussion

***AaTPPA* overexpression dramatically increases trehalose levels of *Arabidopsis***

Schluepmann et al. reported that trehalose levels of *OtsB*-OE *Arabidopsis* plants were below the detection limit of 2 nmol/g FW, which were not significantly different from WT (Schluepmann et al. 2003). Meanwhile, trehalose levels in *AtTPPF*-OE *A. thaliana* seedlings were ~ 7 µg/g FW, not different from WT, but the levels in *AtTPPI*-OE seedlings were twice that of WT (Lin et al. 2019; Lin et al. 2023). In our experiments, *AaTPPA* had a substantially higher TPP enzymatic activity than *OtsB* (Fig. 1d). *OtsB* overexpression did not affect trehalose levels of *A. thaliana*, consistent with the previous study, but *AaTPPA* overexpression resulted in 8 ~ 11 times higher trehalose levels compared to WT (Fig. 3a). This suggests that TPP enzyme activities vary depending on plant species and types of TPP genes.

We also investigated the effect of *AaTPPA* overexpression on the levels of Tre6P, the substrate of the TPP enzyme reaction. The quantitative assay of Tre6P and changes in the expression levels of SnRK1 marker genes indicated that Tre6P levels were reduced in *OtsB*-OE lines, but there was no such trend in *AaTPPA*-OE lines (Fig. S4c, e). This result is similar to the previous reports that *OtsB* overexpression lowered Tre6P levels by more than 5-fold in *A. thaliana*, while *AtTPPI* overexpression only lowered them by about 20% compared to WT (Schluepmann et al. 2003; Lin et al. 2023).

The difference in the change of Tre6P levels was reflected in the developmental and morphological characteristics of *AaTPPA* and *OtsB*-OE *A. thaliana* lines. Schluepmann et al. reported that mature leaves of *OtsB*-OE *Arabidopsis* were lighter green and larger than those of WT, and these differences in leaf phenotypes were caused by the change in Tre6P levels, not by trehalose (Schluepmann et al. 2003). In our study, rosette leaves of *AaTPPA*-OE lines showed similar phenotypes to WT, unlike *OtsB*-OE lines (Fig. 3g, Fig. S4d, e). This result was also regarded as indirect evidence that Tre6P levels were not significantly changed in rosette leaves of *AaTPPA*-OE lines.

However, *AaTPPA*-OE lines showed *OtsB*-like phenotypes in other organs; both had small and misshapen seeds, late bolting, and delayed senescence (Fig. 3b, c, f, h). Previous studies reported that *OtsB*-OE *Arabidopsis* seeds often had an asymmetrical shape and lower oil content than WT (Zhai et al. 2021). When *OtsB* was expressed in peas under an embryo-specific promoter, Tre6P levels and starch accumulation were reduced, resulting in the formation of wrinkled seeds (Meitzel et al. 2020). Previous studies also indicated that *OtsB*-OE *Arabidopsis* bolted up to 3 weeks later than WT, and had a pronounced apical dominance and plenty of seed set (Schluepmann et al. 2003). *JcTPPJ*-OE *Arabidopsis* also bolted 2–3 d later and had more rosette leaves compared to WT (Zhao et al. 2019). The reason why *AaTPPA* overexpression did not affect the leaf morphology while affecting the reproductive growth and seed formation may be because the modes of trehalose metabolism are different between these tissues. In *Arabidopsis* leaves, *AtTPS1* participates in trehalose metabolism, but during embryogenesis in seeds, other types of TPSs, *AtTPS2* and *AtTPS4*, are expressed instead of *AtTPS1* (Fichtner and Lunn 2021). These different TPS genes may have different interactions with *AaTPPA*.

Although the bolting period was delayed, *AaTPPA*-OE lines had more branches and siliques, unlike WT and *OtsB*-OE lines (Fig. 4i, Fig. S5g). In plant leaf cells, Tre6P plays a role as an indicator of sucrose levels. Sucrose accumulation in mesophyll cells increases Tre6P levels which serve as a signal to activate

sucrose transport channels (SWEETs) to export more sucrose from the mesophyll to sink tissues (Fichtner and Lunn 2021). From this point of view, we can assume that, unlike *OtsB*, *AaTPPA* overexpression does not significantly reduce Tre6P levels in leaf cells, so excessive sucrose accumulation in mature leaves finally increases Tre6P levels to reach a threshold, triggering the activation of transporters to export the accumulated sucrose to phloem, which results in vigorous sprouting of branches.

Taking the above results together, we suppose that *AaTPPA* overexpression dramatically increases trehalose levels, but does not significantly reduce Tre6P levels at least in leaves. On the contrary, *OtsB* overexpression greatly reduces Tre6P levels but does not significantly change trehalose levels. Previous studies suggested that the reason why *OtsB* overexpression did not change trehalose levels was because the trehalase activity of plant cells was too high to degrade the synthesized trehalose immediately, preventing its accumulation (Van Houtte et al. 2013). Moreover, there was a report that trehalase activity was increased in yeast TPS and TPP fusion gene (*ScTPS1-ScTPS2*) transgenic plants than in non-transgenic plants (Romero-Reyes et al. 2023). In this regard, it might be thought that the rate of trehalose synthesis in *AaTPPA*-OE *Arabidopsis* leaves was high enough to exceed the capacity of trehalase.

More interestingly, despite the high enzymatic activity of *AaTPPA*, Tre6P levels in leaves of *AaTPPA*-OE lines seemed not to be decreased significantly, indicating that the TPS enzyme activity was also increased. To determine whether *AaTPPA* overexpression might have affected the expression of *AtTPS1*, the major TPS gene in *Arabidopsis*, we measured its transcription levels, but no significant difference was observed between WT and the OE lines (Fig. S5d). This suggested that TPS activity in *AaTPPA*-OE plant leaves was post-translationally regulated. In other words, *AaTPPA* might interact directly or indirectly with the *AtTPS1* protein to regulate its enzymatic activity. Therefore, we measured the TPS, TPP, and TRE enzyme activities, but no difference was found between WT and the OE lines. Perhaps the regulation of TPS and TRE activities under *in vitro* conditions would be different from the *in vivo* environment. Previous studies have suggested that genes of trehalose metabolism might interact with each other. Some class II TPS proteins perform regulatory functions by forming complexes with class I TPS in rice (Zang et al. 2011). In addition, it has been claimed that some TPP enzymes exhibit the “moonlight effect”, performing unknown regulatory functions rather than catalytic activity (Kretzschmar et al. 2015; Claeys et al. 2019). The fact that the biochemical and morphological characteristics of TPS-TPP fusion gene transgenic plants are consistent with *AaTPPA*-OE plants also supports the evidence of *AaTPPA* and *AtTPS1* interaction. In rice overexpressing fusion of *OtsA* and *OtsB*, the TPS and TPP genes of *E. coli*, trehalose levels increased to almost 0.1% of fresh weight without significant change in Tre6P levels, and there were no noticeable abnormal phenotypes, such as growth retardation and changes in root morphology, that were observed in rice overexpressing *OtsA* or *OtsB*, respectively (Jang et al. 2003). In addition, when the fusion of *ScTPS1* and *ScTPS2*, the yeast TPS and TPP genes, were overexpressed in *Arabidopsis*, trehalose levels increased more than 4 times compared to WT, and no differences in morphology and development were recognized, while only *ScTPS1*-OE lines showed abnormal phenotypes (Miranda et al. 2007). Considering the above data, it is believed that the accumulation of trehalose in *AaTPPA*-OE plants might be the result of the interaction of *AaTPPA* with *AtTPS1*, thus increasing Tre6P synthesis, the rate-limiting step of trehalose metabolism.

Protein 3D-structure prediction showed that OtsB has only a catalytic domain, whereas AaTPPA has an additional N-terminal domain of unknown function (Fig. 1c). From this, unlike OtsB, the regulatory action of AaTPPA is believed to be due to its N-terminal domain. In the future, it is necessary to verify the role of this N-terminal domain by investigating transgenic lines that are introduced with N-terminal truncated *AaTPPA*. In detail, much attention should be paid to elucidating the interaction between the N-terminal domain of TPPA and the N-terminal regulatory domain or C-terminal TPP-like domain of TPS1 whose functions are still unknown (Fichtner et al. 2020).

### **AaTPPA plays a role in enhancing the plant freezing tolerance**

There were several examples showing that the introduction of a plant TPP gene could increase the tolerance of plants to cold stress. *AtTPPI* overexpression in *Arabidopsis* enhanced cold tolerance, and *OsTPP1* overexpression in rice increased tolerance to salt and cold stress (Lin et al. 2023; Ge et al. 2008). However, there have been no published reports on the effect of *OtsB* on plant stress tolerance. Therefore, we studied the effect of *AaTPPA* on the freezing tolerance of *A. thaliana* in contrast with *OtsB*-OE lines. From the results, we concluded that *AaTPPA* overexpression significantly enhanced the freezing tolerance of *A. thaliana*, but *OtsB* did not contribute to the freezing tolerance (Fig. 4a, b, c). In addition, among *AaTPPA*-OE plants, the #4 line, which had the highest *AaTPPA* expression and trehalose levels, was superior in all physiological and biochemical indices related to cold tolerance, suggesting that trehalose levels are positively related to the cold tolerance of the plants. Previous studies also showed that there was a certain correlation between trehalose levels and the stress tolerance of plants. In *Arabidopsis*, rice, and wheat, overexpression of TPS-TPP fusion genes of *E. coli* or yeast origin increased trehalose levels as well as the tolerance to salt, drought, cold, and heat stresses (Garg et al. 2002; Jang et al. 2003; Miranda et al. 2007; Romero-Reyes et al. 2023). In addition, exogenous applications of trehalose to plants could increase the tolerance to various stresses (Luo et al. 2023; Islam et al. 2023; Zhang et al. 2022; Yuan et al. 2022; Yang et al. 2022). The above data suggest that trehalose plays a more decisive role than Tre6P in improving the stress tolerance of plants.

However, the impact of Tre6P on the stress tolerance of plants cannot be completely ignored. In plants, there exists the sucrose:Tre6P nexus model: sucrose accumulation increases Tre6P levels, which conversely inhibits the sucrose biosynthesis, thereby maintaining the constant of sucrose versus Tre6P ratio (generally 1000:1) in plant cells (Figueroa and Lunn 2016). According to this theory, in plant leaf cells, the decrease in Tre6P levels enhances the sucrose biosynthesis, and suppresses its export to phloem, resulting in the accumulation of sucrose. Sucrose is one of the osmotic protectants and directly contributes to the stress tolerance of plants. Indeed, our results showed that in both *AaTPPA* and *OtsB*-OE lines, several stress tolerance-related indices, such as ion leakage, ROS scavenging, *Fv/Fm* value, and the content of soluble sugars, were superior to WT, which could be explained in relation to the sucrose accumulation due to the reduction in Tre6P levels by TPP overexpression. Nevertheless, the overall freezing tolerance of *OtsB*-OE lines was inferior to WT. It might be because the excessive reduction of Tre6P levels caused by uncontrollable TPP enzyme activity had a negative impact on the growth and development of the plants.

*AaTPPA* overexpression can increase the tolerance of *A. arguta* to freezing stress. Because tissue culture-dependent plant transformation system has not been established yet for *A. arguta*, we could not conduct the freezing tolerance verification by using *AaTPPA*-OE *A. arguta* plants. Instead, we conducted assays for several cold tolerance-related indices of *AaTPPA* transiently overexpressing *A. arguta* leaves (Fig. 5). The results demonstrated that ROS scavenging activity and the content of osmotic protectants such as soluble sugars and proline were higher than that of control and mock groups. These results were consistent with previous studies showing that the introduction of TPP genes activated the expression of stress-related genes participating in the antioxidant system and the production of osmotic protectants in other plants (Lin et al. 2019; Huang et al. 2022b; Lin et al. 2023).

The results of the transient overexpression of *AaTPPA* in *A. arguta* leaves and the fact that its expression levels increased in the overwintering organs in response to low temperature promise that this gene will greatly contribute to the freezing tolerance of *A. arguta* as in *A. thaliana*. However, to increase the cold tolerance of *A. arguta* by introducing the TPP gene, a technology that can spatiotemporally control the gene expression must be employed, because the indiscriminate overexpression of TPP genes may have negative effects on plant growth and development in some aspects.

### ***AaTPPA* participates in the cold acclimatization of *A. arguta* in autumn**

There are dozens of TPP genes in a plant genome, and these genes are ascribed to have been formed and evolved through gene duplication. The *Arabidopsis* genome has 10 TPP genes, while the rice genome has 13 TPP genes, and every TPP gene is expressed differently depending on tissues, development stages, and environmental conditions (Vandesteene et al. 2012; Du et al. 2022). Through transcriptome analysis, we found that there were at least 17 TPP genes in the *A. arguta* genome, and among them, we selected *AaTPPA* as the research target, whose expression level increased in response to low temperature (Fig. 1a).

The expression level of *AaTPPA* increased in response to mild low temperatures around 15°C, and its induction by low temperature was more sensitive in mature tissues than in young tissues (Fig. 2a, b). In the fields, the period meeting these conditions is at the beginning of autumn when the weather temperature begins to decrease. Indeed, the expression analysis of *AaTPPA* from midsummer to late autumn showed that its expression began to increase after the daily low-temperature began to fall below 15°C. When the temperature decreased further, the expression levels of *AaTPPA* continued to increase in the overwintering tissues, such as lateral buds, roots, and stem cambium, even as the shoot apical meristems and young tissues withered and degenerated (Fig. 2c, d, e, 8b). These results strongly implied that *AaTPPA* might be involved in the overwintering preparation of *A. arguta* in autumn.

For plants to overcome cold winter safely, osmotic protectants such as inorganic and organic ions, soluble sugars, and soluble proteins that prevent cell damage from freezing must be sufficiently accumulated in overwintering tissues (Ding et al. 2020). Trehalose metabolism could be involved in the accumulation of these osmotic protectants. In *A. thaliana* plants and *A. arguta* leaves overexpressing *AaTPPA*, the osmotic protectants, such as soluble sugars and proline, were accumulated along with a

marked increase in trehalose levels. As a result, the cell membranes were protected from freezing, reducing ion leakage, and the antioxidant enzyme activities were preserved, increasing ROS scavenging capacity (Fig. 4, 5, Fig. S6). Changes in Tre6P levels might also have an impact on the accumulation of osmotic protectants in overwintering tissues. According to previous studies, a decrease in Tre6P levels in sink tissues serves as a sugar shortage signal, which activates sucrose transporters and promotes the decomposition of stored starch. As a result, the sink tissues import and produce more sucrose (Paul et al. 2018). According to this theory, upregulation of *AaTPPA* expression in the overwintering tissues would decrease Tre6P levels, leading to enhancing sucrose import and starch decomposition, which would supply more nutrients necessary to synthesize the osmotic protectants.

We identified the *AaERF64* transcription factor that binds to the *AaTPPA* promoter. *AaERF64* binds to the GCC-box of the *AaTPPA* promoter to activate its transcription and showed the same expression pattern as *AaTPPA* in response to the temperature decrease in autumn (Fig. 7, 8a, b). Furthermore, we demonstrated that *AaERF64* was induced by ethylene treatment (Fig. 8c, Fig. S12c). In plants, ethylene acts as a stress-induced signaling molecule, and its biosynthesis is generally enhanced under low temperature conditions (Murakami et al. 2014). The ethylene signal transduction pathway includes ethylene receptors, CTR1 (constitutive triple response 1), EIN2, EIN3 (ethylene insensitive 2 and 3), EIL (EIN3-like), ERF proteins, and ethylene-responsive genes (Huang et al. 2018). There were reports that ethylene biosynthesis was enhanced by low temperature in *Actinidia* plants and that the ripening of fruits was promoted by internally synthesized ethylene or its external application (Mworia et al. 2010; Wang et al. 2015). The above reports and our results support the idea that ethylene is the most upstream signaling molecule that increases *AaTPPA* expression in response to the temperature decrease in autumn. In addition, the expression level of *AaTPPA* was transiently increased by ABA treatment, and it is considered that *AaTPPA* expression is induced in response to temporary stress depending on the ABA signaling pathway, especially in roots (Fig. S12c). The induction of *AaTPPA* expression by ABA was reversible, whereas its induction by ethylene was irreversible because ethylene can feedback activate its endogenous biosynthesis (Mworia et al. 2010).

Combining the above results, we propose a model of the cold acclimatization mechanism of *A. arguta* (Fig. 9). The temperature decrease in autumn enhances ethylene biosynthesis in *A. arguta* plants. The synthesized ethylene induces the expression of the *AaERF64* transcription activator through its signal transduction pathway in overwintering tissues, such as lateral buds and stem cambium. *AaERF64* binds to the *AaTPPA* promoter, increasing its expression level. *AaTPPA* expression is also induced by ABA in roots. *AaTPPA* catalyzes the conversion reaction from Tre6P to trehalose, increasing trehalose levels and decreasing Tre6P levels in the corresponding tissue cells. Trehalose serves as a signaling molecule that activates the expression of stress-related genes involved in the production of osmotic protectants. Meanwhile, the reduction of Tre6P levels in overwintering tissues would enhance the sucrose import from source leaves, and accelerate the decomposition of stored starch, resulting in the production of more osmotic protectants, such as soluble sugars and proline. As a result, *A. arguta* plants acquire the overwintering ability to survive even under extreme freezing conditions.

## Declarations

## Author contribution

TE and PL conceived the research project and set up the experiments. JP, NE, and YX conducted metabolites and biochemical analysis. LM and JL performed the data analysis. TE and JP wrote the manuscript. JL and HK edited the manuscript. FM and PL organized and supervised the overall project. FM and GJ critically revised the manuscript for publication. All authors have read and agreed to the published version of the manuscript.

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## Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

All relevant data can be found within the manuscript and its supporting materials.

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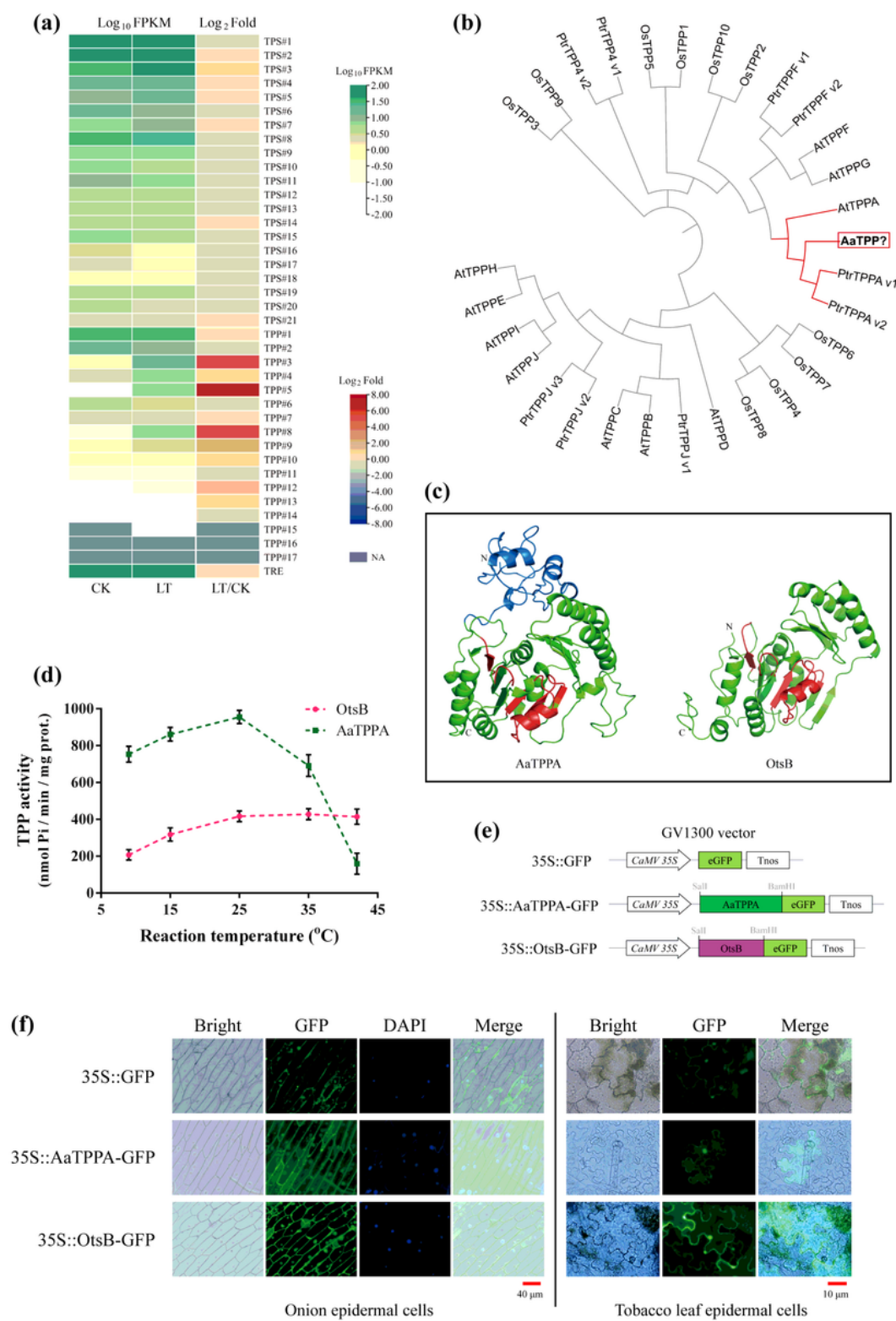
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## Figures



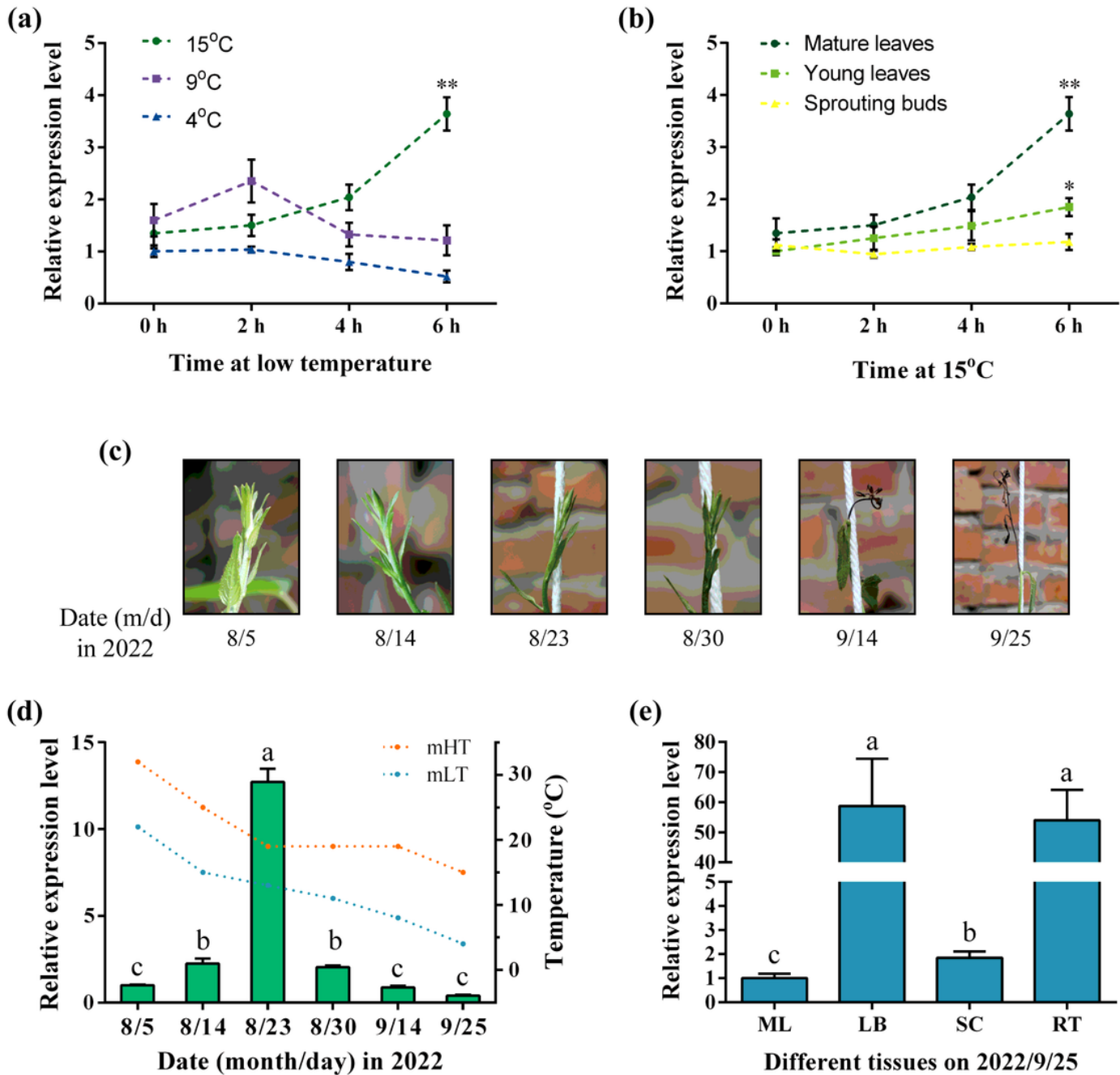
**Figure 1**

Identification and classification of the cold-responsive TPP gene of *A. arguta*, and verification of its enzymatic activity and subcellular localization.

(a) Comparative analysis of transcriptional levels of trehalose metabolism-related genes from the transcriptome data of *A. arguta* between normal (CK) and low temperature (LT: 4~10°C) conditions. (NA:

Data was not accessible)

- (b) Phylogenetic tree of the cold-responsive *AaTPPA* with TPP genes of *Populus trichocarpa*, *Arabidopsis thaliana*, and *Oryza sativa*.
- (c) 3D structure of *AaTPPA* and *OtsB* protein. (Blue region presents the N-terminal domain of *AaTPPA*. Red regions present HAD motifs.)
- (d) TPP enzyme activity of *AaTPPA* and *OtsB* depending on reaction temperatures. Data are presented as mean  $\pm$  SD (n = 3).
- (e) Schematic diagram of the expression cassettes of *AaTPPA* and *OtsB* in GV1300 plant expression vector. (CaMV 35S: the cauliflower mosaic virus (CaMV) 35S promoter, eGFP: enhanced green fluorescence protein, Tnos: NOS terminator)
- (f) Subcellular localization of *AaTPPA* and *OtsB* observed in epidermal cells of onion and tobacco leaf. (DAPI: fluorescent reagent staining the cell nucleus)



**Figure 2**

Expression pattern of *AaTPPA* in *A. arguta* under different low temperature conditions.

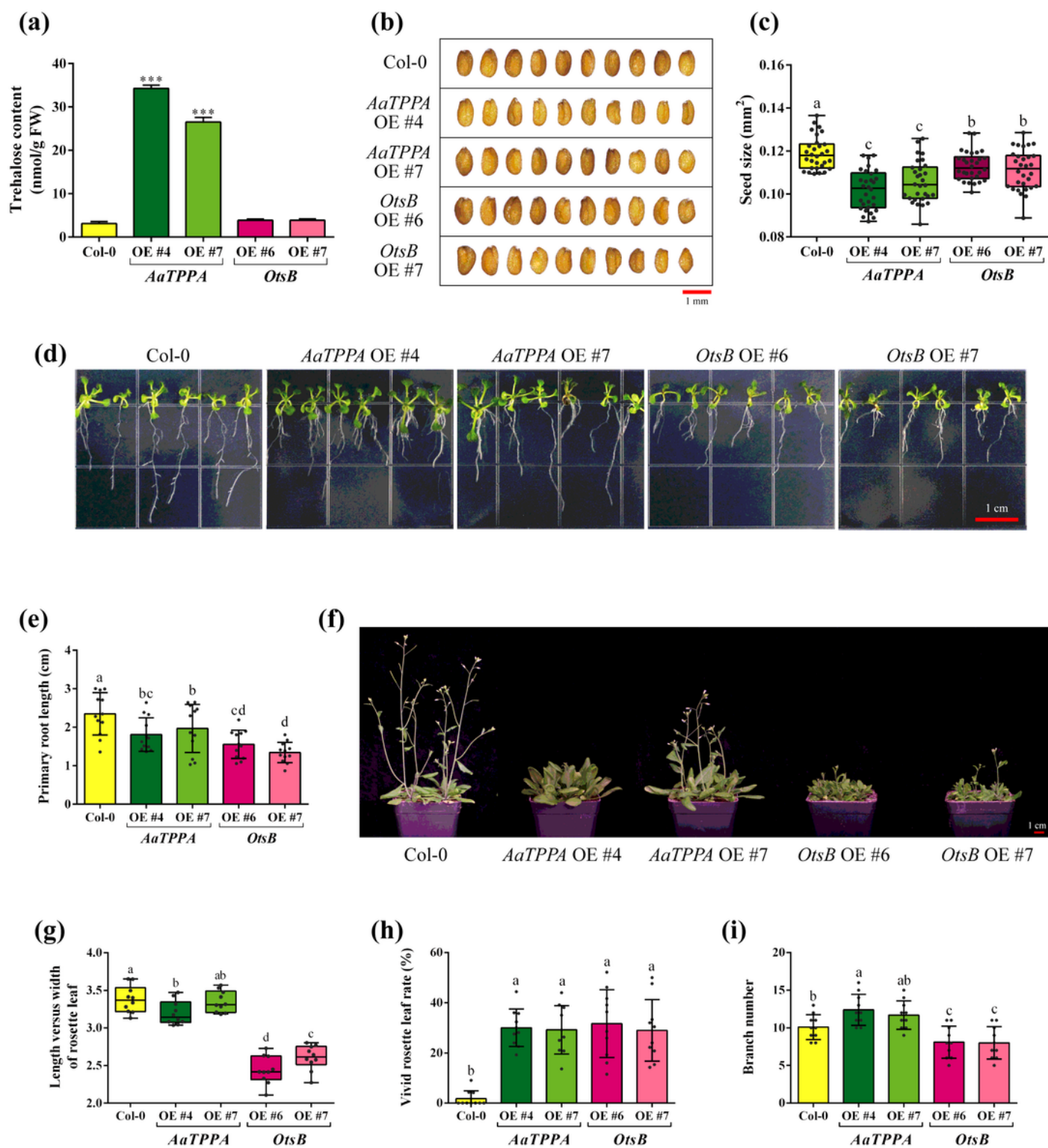
(a) Changes in *AaTPPA* expression levels in mature leaves when treated at different temperatures for different periods. Data are presented as mean  $\pm$  SD (n = 3). \*\* $P$  < 0.01 by Student's *t*-test.

(b) Changes in *AaTPPA* expression levels in sprouting buds, young leaves, and mature leaves according to treatment periods at mild low-temperature (15°C). Data are presented as mean  $\pm$  SD (n = 3). \* $P$  < 0.05; \*\* $P$  < 0.01 by Student's *t*-test.

(c) Growth status of *A. arguta* shoot apical meristems according to sample collection days.

(d) *AaTPPA* expression levels in mature leaves, and elapsed weather temperature according to sampling days. Data are shown as mean  $\pm$  SD (n=3), and Duncan's multiple comparison is employed at significance levels of  $P < 0.05$ . (mHT: the minimum value among the daily high-temperatures elapsed up to the sampling time point, mLT: the minimum value among the daily low-temperatures elapsed up to the sampling time point.)

(e) Relative expression levels of *AaTPPA* in mature leaves (ML), lateral buds (LB), stem cambium (SC), and roots (RT) on the last sampling day (2022/9/25). Data are shown as mean  $\pm$  SD (n=3), and Duncan's multiple comparison is employed at significance levels of  $P < 0.05$ .



**Figure 3**

Developmental and morphological characteristics of *AaTPPA* and *OtsB*-OE *A. thaliana* lines.

(a) Trehalose contents of *AaTPPA* or *OtsB*-OE lines. (n=3), \*\*\* $P < 0.001$  by Student's *t*-test.

(b, c) Seed morphology and size of *AaTPPA* and *OtsB*-OE lines. (n=30)

(d, e) Root morphology and primary root length of *AaTPPA* and *OtsB*-OE lines grown on MS media for 14 days. (n=12)

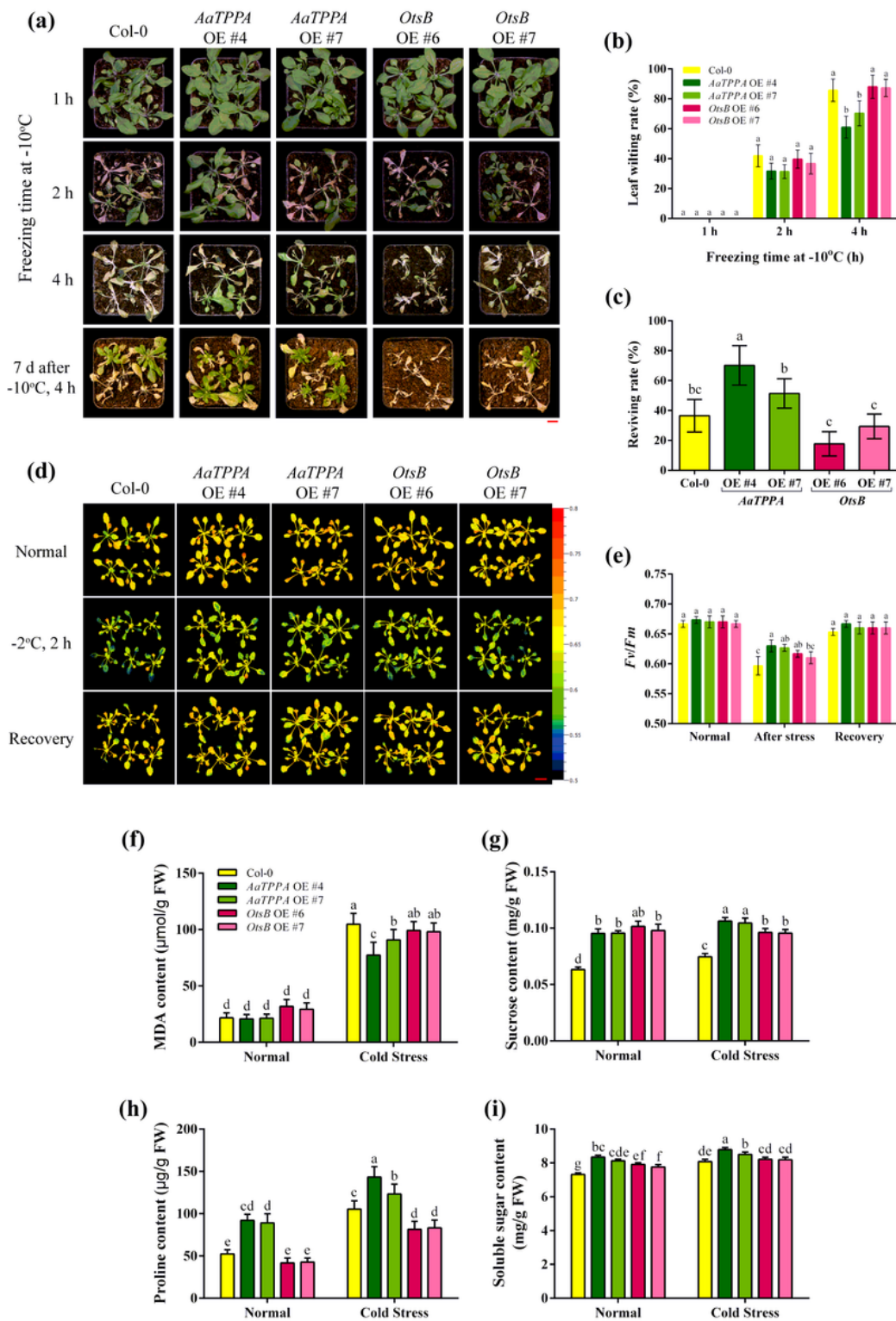
(f) Photos of *AaTPPA* and *OtsB*-OE lines grown for 32 days show different bolting periods.

(g) Length versus width ratio of the largest rosette leaves of *AaTPPA* and *OtsB*-OE lines grown for 21 d. (n=10)

(h) Percentage of vivid rosette leaves of *AaTPPA* and *OtsB*-OE lines grown for 45 d. (n=10)

(i) Branch numbers of adult *AaTPPA* and *OtsB*-OE lines. (n=10)

All data are shown as mean  $\pm$  SD, and Duncan's multiple comparison is employed at significance levels of  $P < 0.05$ .



**Figure 4**

Cold tolerance of *AaTPPA*-OE *A. thaliana* compared to *OtsB*-OE lines.

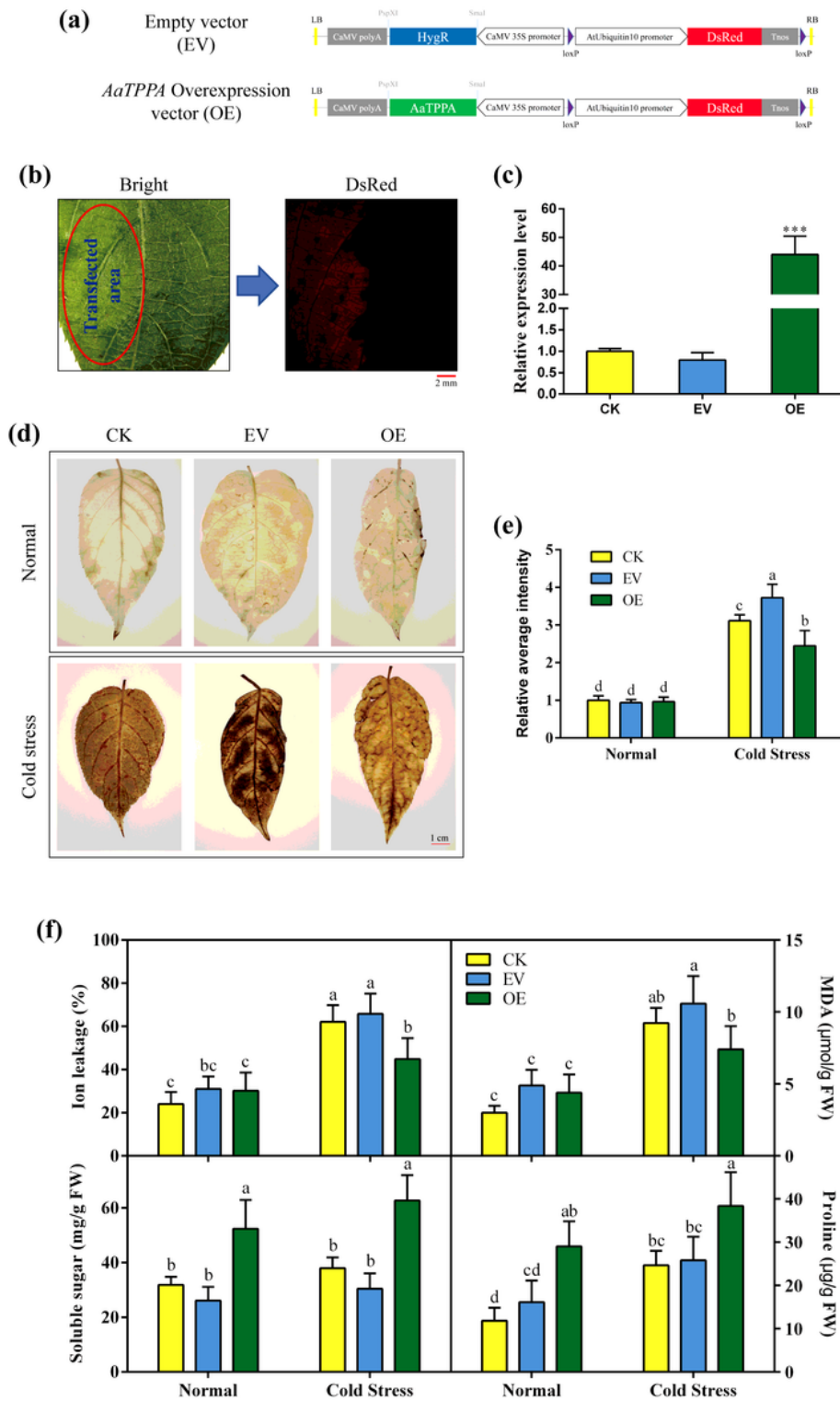
(a, b) Leaf wilting rate of *A. thaliana* lines after 2 d post freezing stress at -10°C.

(c) Survival rate of *A. thaliana* lines after 7 d post freezing treatment at -10°C for 4 h.

(d, e) Chlorophyll fluorescence images and maximal quantum yield of PSII ( $F_v/F_m$ ) of *A. thaliana* lines before and after cold stress at  $-2^{\circ}\text{C}$  for 2 h.

(f, g, h, i) Sucrose, soluble sugars, proline, and MDA contents of *A. thaliana* lines under normal conditions and after cold stress treatment at  $-2^{\circ}\text{C}$  for 2 h.

All data are shown as mean  $\pm$  SD ( $n=3$ ), and Duncan's multiple comparison is employed at significance levels of  $P < 0.05$ .



**Figure 5**

Cold tolerance of the transient *AaTPPA*-overexpressing *A. arguta* leaves

(a) Construct of *AaTPPA*-overexpressing vector (GVloxP/*DsRed-AaTPPA*) with the red fluorescent protein marker.

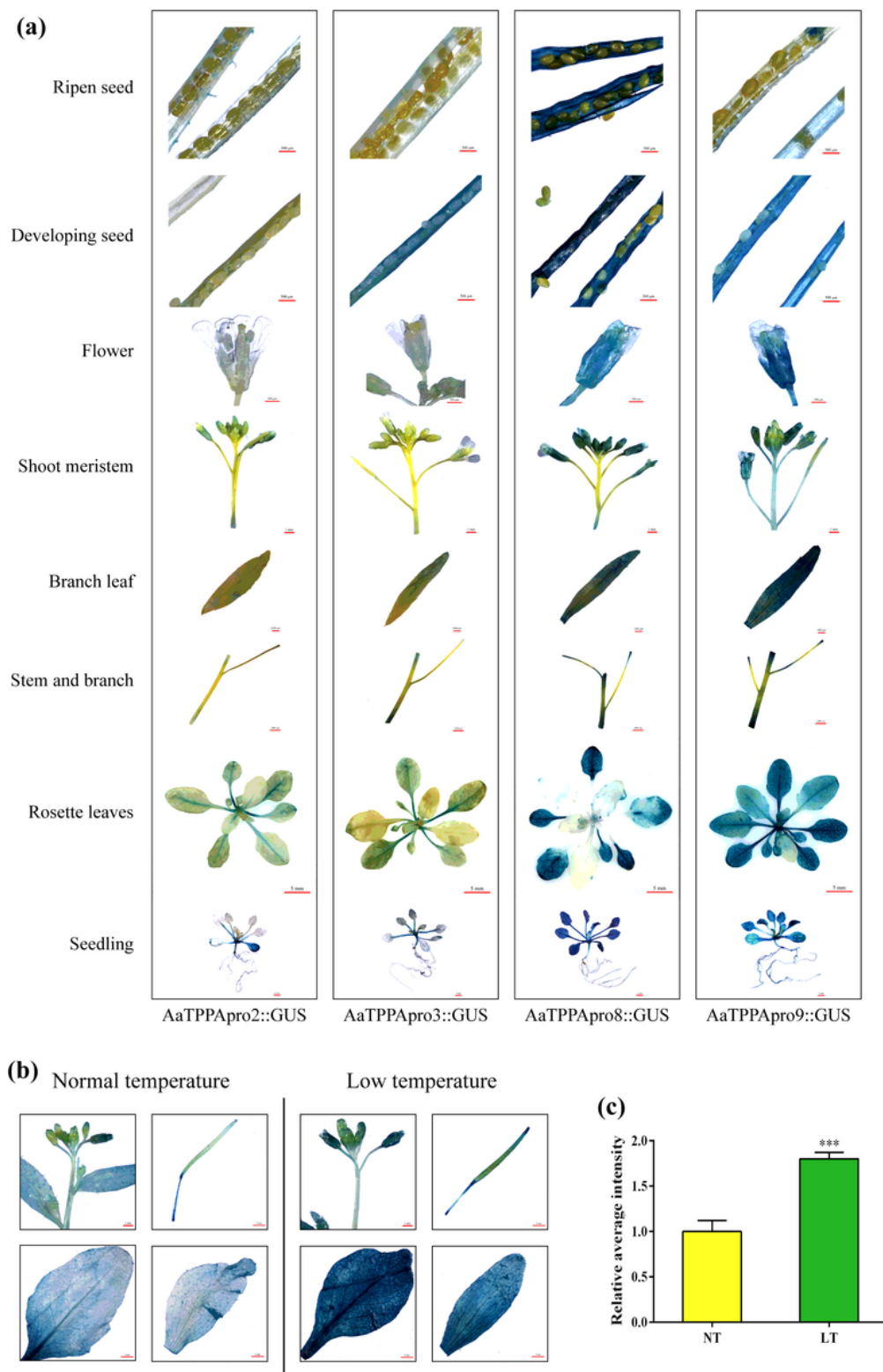
(b) Images showing the expression of the red fluorescent marker gene (*DsRed*) in *A. arguta* leaves infiltrated with *Agrobacterium* containing GVloxP/*DsRed-AaTPPA* vector.

(b, c) Expression levels of *AaTPPA* in *A. arguta* leaves infiltrated with *Agrobacterium* containing GVloxP/*DsRed-AaTPPA* vector.

(d, e) DAB staining of the transient *AaTPPA*-OE *A. arguta* leaves under normal conditions and after cold stress treatment at -4°C for 2 h.

(f) Assay of some cold tolerance indices (ion leakage, MDA, soluble sugars, proline) of the transient *AaTPPA*-OE *A. arguta* leaves under normal conditions and after cold stress treatment at -4°C for 2 h. (CK: not infiltrated, EV: infiltrated with *Agrobacterium* containing GVloxP/*DsRed* empty vector, OE: infiltrated with *Agrobacterium* containing GVloxP/*DsRed-AaTPPA* vector)

All data are shown as mean  $\pm$  SD (n=3), and Duncan's multiple comparison is employed at significance levels of  $P < 0.05$ .



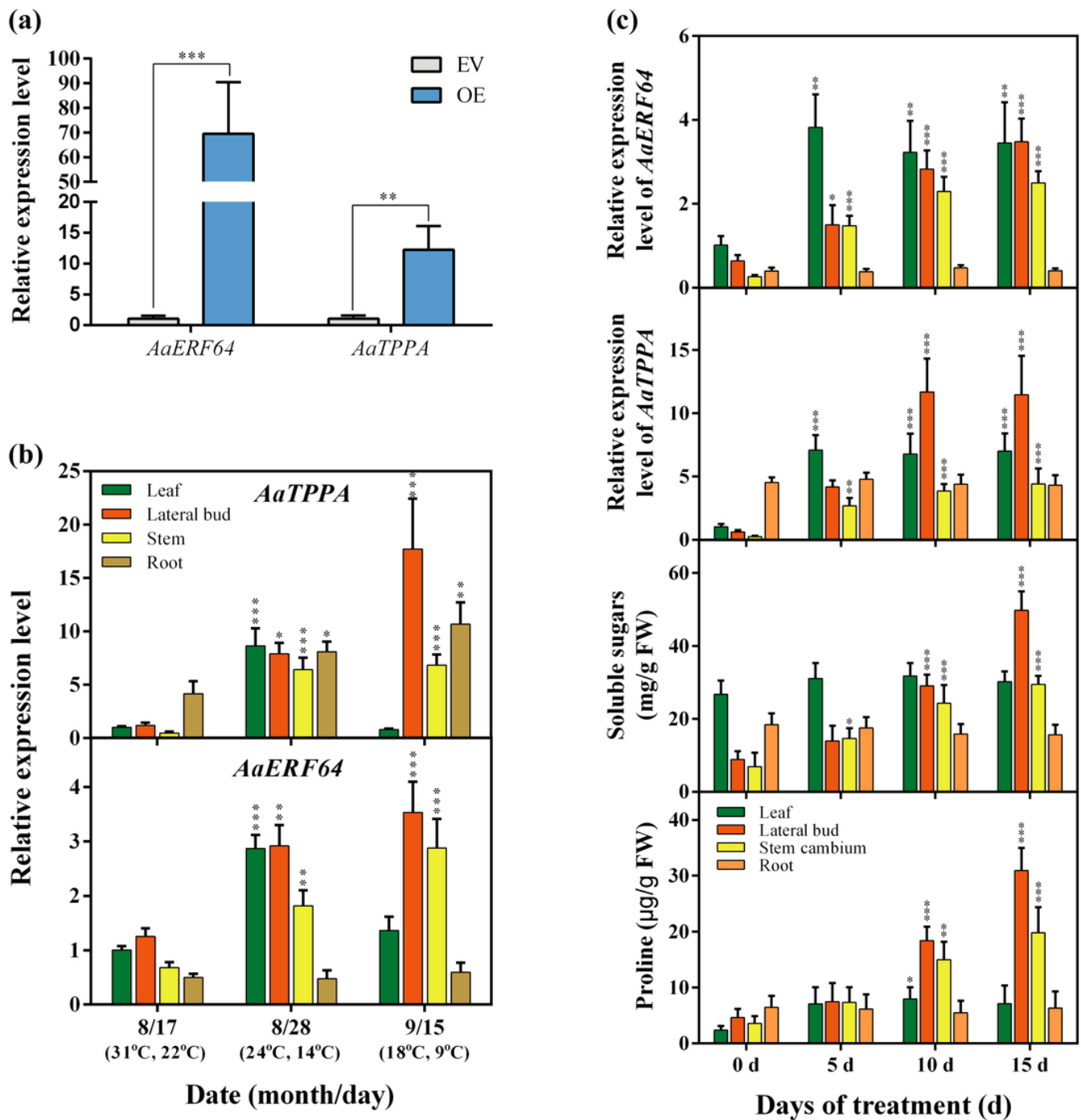
**Figure 6**

Verification of the *AaTPPA* promoter region.

(a) Expression patterns of *GUS* driven by different *AaTPPA* promoter sections in the transgenic *A. thaliana* T<sub>2</sub> plants at different growth stages and tissues.



- (a) Motif analysis of the *AaTPPA* promoter region.
- (b) Verifying the interaction of AaERF64 with the *AaTPPA* promoter S3 section by Y1H assay.
- (c) Subcellular localization of AaERF64 in tobacco leaf epidermal cells.
- (d) Self-transactivation activity of AaERF64 verified by Y2H assay.
- (e) Dual-Luciferase assay shows the interaction of AaERF64 with the *AaTPPA* promoter S3 section in tobacco leaf cells. Data are presented as mean  $\pm$  SD (n=3). \*\*\* $P < 0.001$  by Student's *t*-test.
- (f) Determination of the GCC-box in the *AaTPPA* promoter S3 section to which AaERF64 binds by Y1H assay.
- (g) Verification of the binding between AaERF64 and the GCC-box motif using EMSA.



**Figure 8**

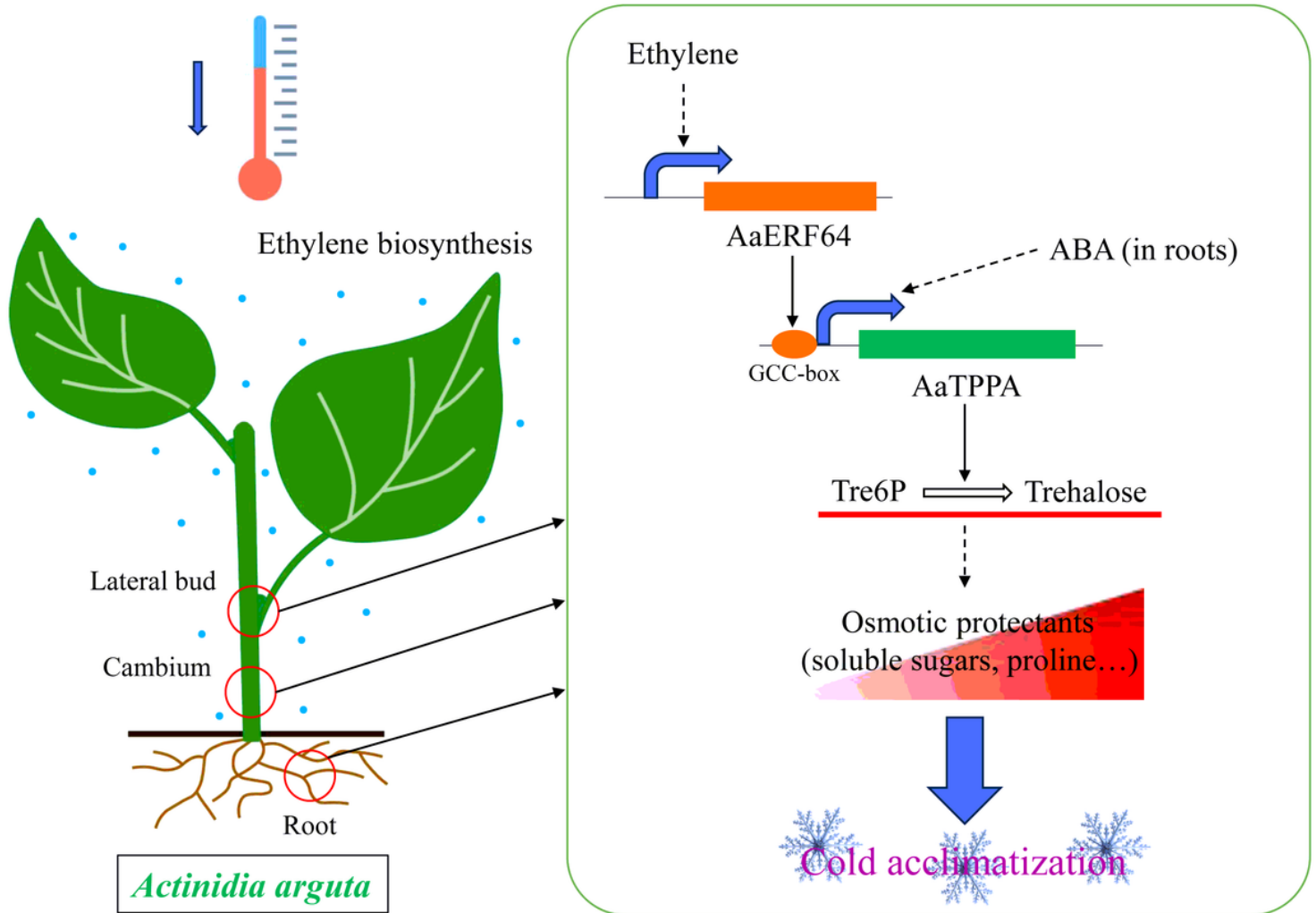
*AaTPPA* expression is upregulated by the *AaERF64* transcription factor depending on the ethylene signal transduction pathway in *A. arguta*.

(a) Upregulation of *AaTPPA* expression by the transient overexpression of *AaERF64* in *A. arguta* leaves.

(b) Expression levels of *AaERF64* and *AaTPPA* in different tissues of *A. arguta* according to the weather temperature decrease in autumn.

(c) Induction of *AaTPPA* and *AaERF64* expression and the accumulation of soluble sugars and proline in different tissues of *A. arguta* by ethylene treatment.

All data are presented as mean  $\pm$  SD (n=3). \* $P$  < 0.05, \*\* $P$  < 0.01, and \*\*\* $P$  < 0.001 by Student's  $t$ -test.



**Figure 9**

A schematic model illustrating the role of *AaTPPA* in the cold acclimatization of *A. arguta*.

The temperature decrease in autumn triggers the ethylene biosynthesis in *A. arguta*, which induces the expression of the *AaERF64* transcription factor in overwintering tissues, such as lateral buds and stem cambium. *AaERF64* directly binds to the GCC-box motif of the *AaTPPA* promoter and upregulates its transcription. ABA is also involved in the induction of *AaTPPA* expression in roots. Subsequently, *AaTPPA* catalyzes the reaction of trehalose synthesis. The decrease in Tre6P levels and the increase in trehalose levels cause the accumulation of osmotic protectants, such as soluble sugars and proline, conferring the plant to adapt to cold in winter. (The solid lines represent the direct regulation, and the dashed lines represent the indirect regulation.)

## Supplementary Files

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