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Overexpression of genes encoding enzymes involved in trehalose synthesis from *Caragana korshinskii* enhances drought tolerance of transgenic plants

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Abstract

Trehalose, which plays important roles in resistance to abiotic stresses and preservation of biological activity in plants, is synthesized by two key enzymes, trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP). Therefore, the expressions of the *TPS* and *TPP* genes directly affect trehalose synthesis and stress resistance of plants. In this study, *CkTPS* and *CkTPP* from *Caragana korshinskii* were identified, and the role of trehalose synthesis in the adaptation of this desert plant to adverse conditions was investigated. Higher *CkTPS* and *CkTPP* expressions were observed in the roots, whereas expressions were much lower in leaves and stems, and their expressions were upregulated under drought stress. Histochemical analyses showed that *β-glucuronidase* expression driven by the *CkTPS* and *CkTPP* promoters was strongly induced by abiotic stresses and phytohormones, such as abscisic acid, gibberellin, methyl jasmonate, and mannitol, which suggests that trehalose synthesis may be regulated by various signaling pathways. To determine the functional mechanism underlying the role of trehalose synthesis in regulating drought response in plants, *CkTPS* and *CkTPP* were introduced into *Arabidopsis*. Compared to wild-type (WT) plants, these transgenic plants showed higher germination rate, survival, less damage, better shoot growth, and longer roots under drought stress. Moreover, transgenic plants had a significantly higher content of proline, chlorophyll, trehalose, and activities of antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), and lower malondialdehyde (MDA) content than WT controls. Double-transgenic plants carrying *CkTPS* and *CkTPP* showed better growth and stronger drought tolerance than either single transgenic plant line. These results provide a theoretical and experimental basis for further understanding the function and regulatory mechanism of *CkTPS* and *CkTPP*, as well as the possibility of their application for improving drought tolerance in crops through genetic engineering.

Keywords: *Caragana korshinskii*, drought tolerance, transgenic *Arabidopsis*, trehalose-6-phosphate phosphatase, trehalose-6-phosphate synthase.

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Abbreviations: ABA - abscisic acid; CAT - catalase; GUS - β-glucuronidase; MDA - malondialdehyde; POD - peroxidase; ROS - reactive oxygen species; RWC - relative water content; SOD - superoxide dismutase; TPP - trehalose-6-phosphate phosphatase; TPS - trehalose-6-phosphate synthase; WT - wild type.

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Introduction

The growth and development of plants in natural ecosystems are strongly influenced by biotic and abiotic factors. Drought is a major abiotic stress that severely restricts the sustainable development of agriculture and animal husbandry. It causes the growth retardation, yield reduction, and quality decline of crops by affecting the morphological, physiological, and biochemical processes (Mcdowell *et al*. 2010, Kaya *et al*. 2020, Raja *et al*. 2020). Drought causes a decrease of soil water potential, makes it difficult for plants to absorb water and reduces the relative water content (RWC) of plants. Water deficiency disturbs the electron transport in chloroplast and mitochondria and causes the excessive accumulation of reactive oxygen species (ROS), which leads to lipid peroxidation and protein and DNA damage (Møller *et al*. 2007, Farooq *et al*. 2020). Lipid peroxidation is a series of reactions in biological membranes, which leads to the accumulation of highly toxic malondialdehyde (MDA). Therefore, the content of MDA is used to evaluate the degree of biological membrane damage (Cui *et al*. 2017, Sohag *et al*. 2020). Plants have evolved a series of antioxidant defence mechanisms to effectively counteract the drought-induced ROS, including antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). However, under severe drought stress, the antioxidant activity is disturbed, and the ability of scavenging ROS decreases, leading to intensification of oxidative damage. In addition, water deficiency induces the accumulation of organic compounds in cells, including proline, sugars, and glycine betaine, which act as compatible solutes or osmolytes to protect plants from the injuries caused by drought stress (Ahammed *et al*. 2020).

Trehalose is a non-reducing disaccharide that acts as a storage substance and stress protector, which is widely distributed in many organisms (Avonce *et al*. 2006). Exogenous trehalose can alleviate the inhibition of plant growth by various abiotic stresses by regulating stomata opening and maintaining membrane stability. The plants treated with trehalose show increased relative water content (RWC) and antioxidant enzyme activities, as well as lower MDA content (Kosar *et al*. 2021). Trehalose biosynthesis and signaling have been investigated extensively in bacteria, yeast, fungi, insects, plants, and animals (Elbein *et al*. 2003, Lunn *et al*. 2014). The biosynthesis of trehalose in plants is catalyzed by two consecutive enzymatic reactions, *i.e*., trehalose-6-phosphate synthase (TPS) catalyzes the transfer of glucose groups from uridine diphosphate glucose (UDP-Glc) to glucose-6-phosphate (Glc-6-P) to produce trehalose-6-phosphate (T6P), which is then dephosphorylated by trehalose-6-phosphate phosphatase (TPP) to produce trehalose (Cabib and Leloir 1958). In plants, the components of the trehalose biosynthesis pathway not only influence growth and development but are also involved in responses to both biotic and abiotic stresses (Fernandez *et al*. 2010, Delorge *et al*. 2014).

Plant *TPS* and *TPP* genes were first identified in *Arabidopsis* (Vogel *et al*. 1998, Leyman *et al*. 2001), and then were detected in various other plants and have been found to form a large gene family (Yang *et al*. 2012). Eight putative *TPS* genes, referred to as *StTPSs*, and their promoter sequences have been identified in potato (*Solanum tuberosum*); the transcriptions of *StTPSs* are upregulated by salt, osmosis (mannitol), and heat stress, and their expressions in flowers (sepals and petals) and roots are significantly higher than those in leaves (Xu *et al*. 2017). *Cis*-acting element analysis of 13 *PtTPS* and 10 *PtTPP* genes in the *Populus tomentosa* genome revealed the involvement of these genes in responses to plant hormones and environmental stresses. Expression profiles analysis further confirmed the difference in expression of the *PtTPSs* and *PtTPPs* under salt, cold, and methyl jasmonate treatments (Gao *et al*. 2021). In cassava, 12 *MeTPS* and 10 *MeTPP* genes have been identified, of which *MeTPS1* is constitutively expressed, while the others are regulated by various factors (Han *et al*. 2016). Nine putative *ScTPS* genes have been identified in sugarcane hybrids and shown to exhibit divergent expression in response to simulated drought, salinity, and ABA (Hu *et al*. 2020). *TPS* and *TPP* genes have also been identified in other plants, including *Prunus mume* (Yang *et al*. 2020), *Moringa oleifera* (Lin *et al*. 2018), and *Dendrobium officinale* (Yu *et al*. 2020), and shown to exhibit various specific expression patterns.

Previous studies have shown that *TPS* and *TPP* are important for genetically improving plant resistance. Overexpression of *OsTPS1* improves the tolerance of *Oryza sativa* seedlings to low-temperature, high-salinity, and drought stress without significantly affecting the plant phenotype (Li *et al*. 2011). Similar observations have been reported in rice plants overexpressing the *OsTPP1* gene, which enhances tolerance to salt, osmotic, and cold stress (Ge *et al*. 2008). Transgenic *Nicotiana benthamiana* overexpressing *MeTPS1* (from *Manihot esculenta*) accumulates significant amounts of trehalose in leaves, stem, and roots, and shows stronger tolerance to natural drought and attenuated symptoms of dehydration (Fernandez *et al*. 2010). Overexpression of *ThTPS* in *Tamarix hispida* (Wang *et al*. 2022) and *AtTPPF* in *Arabidopsis thaliana* have also been found to increase the drought stress tolerance of transgenic plants (Lin *et al*. 2019).

The overexpression of the *TPS* and *TPP* genes from yeast and *Escherichia coli* in higher plants also improves the tolerance of transgenic plants to abiotic stresses but often leads to growth distortion, which may be due to the excessive accumulation of trehalose-6-phosphate (T-6-P) during plant development (Romero *et al*. 1997, Pilon-Smits *et al*. 1998). To reduce the accumulation of the intermediate product T-6-P in plant cells, a chimeric translational fusion of *TPS* and *TPP* from yeast, referred to as *TPS-TPP* driven by the CaMV 35S /stress-regulated rd29A promoter, has been introduced into *A. thaliana* and the transgenic plants display a significantly improved tolerance to drought, freezing, salt, and heat (Miranda *et al*. 2007). A gene encoding a biofunctional fusion of *EcTPS* and *EcTPP* from *E. coli*, referred to as *TPSP* driven by the CaMV35S promoter has also been introduced into tomato, and transgenic plants show normal growth with an

accumulation of a higher amount of trehalose in leaves and increased tolerance to drought and salt stress (Lyu *et al*. 2013). These results indicate that overexpression of the *TPSP* fusion gene can enhance resistance to abiotic stress in plants without causing growth distortion, which has also been confirmed in *Nicotiana tabacum* (Karim *et al*. 2007).

Caragana korshinskii Kom., a deciduous legume shrub widely distributed in the arid and semi-arid grasslands and deserts of northwest China, shows strong resistance to wind erosion and sand burial. This desert plant has well-developed roots with a variety of symbiotic microorganisms that can effectively improve soil structure and reduce water loss. Therefore, it is a pioneer plant for soil erosion and desertification control in arid and semi-arid areas. Due to its strong adaptation to drought stress and high content of biomolecules, such as coumarin, flavonoids, amino acids, and alkaloids, this legume shrub has been used as high-quality animal feed after fermentation, which makes it an ideal forage for livestock in deserts and desert grasslands (Jia *et al*. 2010). *C. korshinskii* has potential for application in agricultural production, vegetation restoration, and ecological protection, and it is, therefore, important to study its mechanism of drought resistance and to identify the key regulatory genes involved.

Materials and methods

Plants and growth conditions: *Caragana korshinskii* Kom. seeds were collected from Alxa, Inner Mongolia, China. The seeds were germinated on damp filter paper at a temperature of 23 °C, a 16-h photoperiod, an irradiance of 200 μmol m^{-2} s^{-1}, and relative humidity of 20 % for 7 d, and then placed in quartz sand and irrigated with Hoagland nutrient solution. Seedlings were harvested for the isolation of RNA and DNA after 3 weeks.

Arabidopsis thaliana L. (ecotype Columbia) were sterilized with 75 % (v/v) ethanol for 5 min, resuspended in anhydrous ethanol, followed by washing with distilled water, and then were grown on 1/2 Murashige and Skoog (MS) medium at a temperature of 23 °C, a 16-h photoperiod, and irradiance of 45 μmol m^{-2} s^{-1}. Seedlings with four true leaves were transplanted into pots with peat soil and cultured under the same conditions.

Cloning and bioinformatics analyses of *CkTPS* and *CkTPP*: Total RNA was extracted from 3-week-old *C. korshinskii* seedlings using a *MiniBEST* Plant RNA extraction kit (*TaKaRa*, Dalian, China). First-strand cDNA was synthesized using a *TaKaRa* RNA PCR kit (*AMV ver.3.0*), and it was used as the template for PCR to amplify the cDNA of *CkTPS* and *CkTPP*. Nested PCR was performed to obtain the *CkTPS* fragment utilizing the degenerate primers TPSZJ-F1/F2/R (Table 1 Suppl.) designed based on the conserved amino acid sequence of plant TPS genes. The 5′-terminal sequence of *CkTPS* was isolated using a *SMARTer RACE* cDNA amplification kit (*Clontech*, Palo Alto, CA, USA), and the reverse primers TPS-J-R1/R2 (Table1 Suppl.) designed based on the isolated *CkTPS* fragment. The 3′-terminal sequence of *CkTPS* was isolated using a *TaKaRa* RNA PCR kit (*AMV ver.3.0*) and the forward primers TPS-J-F1/F2 designed based on the isolated *CkTPS* fragment. The cDNA sequence of *CkTPP* was cloned using the same method as described above with the primers TPPZJ-F1/F2/R, TPP-J-R1/R2, and TPP-J-F1/F2 (Table 1 Suppl.). The full-length cDNA sequences of *CkTPS* and *CkTPP* were amplified using the primer pairs CkTPS-full-F/R and CkTPP-full-F/R, respectively (Table 1 Suppl.)

PEG-simulated drought stress and expression analyses of *CkTPS* and *CkTPP*: *C. korshinskii* seedlings were irrigated with Hoagland nutrient solution, with or without 5 % polyethylene glycol (PEG6000) for 3 or 7 d, and the roots, stems, and leaves were collected for total RNA extraction using a *MiniBEST* plant RNA extraction kit (*TaKaRa*). The cDNA was synthesized using *EasyScript® All-in-One* first-strand cDNA synthesis super mix for qPCR (*One-Step gDNA Removal*, *TransGen Biotech*, Beijing, China), and the transcription profiles of *CkTPS* and *CkTPP* were examined by real-time quantitative PCR. The *C.korshinskii* actin gene, *CkActin*, amplified using primer pair CkActin-F and CkActin-R, was used as an internal reference (Table 1 Suppl.). Real-time qPCR was performed with a *480II* real-time PCR cycler (*Roche*, Hilden, Germany) using *TransStart Tip Green qPCR SuperMix* (*TransGen Biotech*). The relative expression differences were calculated by the $2^{-\Delta\Delta CT}$ method.

Cloning and sequence analyses of the *CkTPS* and *CkTPP* promoters: Genomic DNA was extracted from *C. korshinskii* seedlings using a *TaKaRa MiniBEST* plant genomic DNA extraction kit. The *CkTPS* and *CkTPP* promoters were cloned by genome walking using a *TaKaRa* genome walking kit with primers designed according to the manufacturer's instructions; TPS-SP1–1/2/3 and TPS-SP2–1/2/3 (Table 1 Suppl.) were used for the *CkTPS* promoter and TPP-SP1–1/2/3, TPP-SP2–1/2/3, and TPP-SP3–1/2/3 (Table 1 Suppl.) were used for the *CkTPP* promoter. The sequence of *Pro.CkTPS* was amplified using the primer pair, pCkTPS-F and pCkTPS-R (Table 1 Suppl.), under the following conditions: initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min, followed by extension at 72 °C for 5 min. The sequence of *Pro.CkTPP* was verified using the primer pair pCkTPP-F and pCkTPP-R (Table 1 Suppl.) under the following conditions: initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min, followed by extension at 72 °C for 5 min. The transcriptional initiation site and regulatory elements were analyzed using *BDGP* (http://www.fruitfly.org/seq_tools/promoter.html) and *PlantCARE* (http://bioinformatics.psb.ugent.be/ webtools/plantcare/html/), respectively.

Histochemical detection of GUS expression driven by *Pro.CkTPS* and *Pro.CkTPP*: The sequence of *Pro.CkTPS* was amplified with the primer pair, pCkTPS-F2 and pCkTPS-R2 (Table 1 Suppl.), and inserted between

the *Bam*HI and *Sac*I sites of the binary vector pORE R1 (provided by Prof. Qi Zhi) to generate the plasmid, pORE R1-*Pro.CkTPS::GUS*. The sequence of *Pro.CkTPP* was amplified with the primer pair, pCkTPP-F2 and pCkTPP-R2 (Table 1 Suppl.), and inserted between the *Sal*I and *Sac*I sites of the binary vector, pORE R1, to generate pORE R1-*Pro.CkTPP::GUS*. The recombinant plasmids were transformed into competent *Agrobacterium tumefaciens* strain GV3101 cells (*Biomed*, Beijing, China), and transferred into *Arabidopsis* using the floral dipping method (Clough and Bent 1998). The transgenic lines were obtained by screening on a 1/2 MS medium containing kanamycin (40 mg dm^{-3}) and identified by genomic PCR with the primer pair pORE-F and pORE-R (Table 1 Suppl.), using a *TransDirect* plant tissue PCR kit (*TransGen Biotech*). *GUS* gene expression was detected by reverse transcription PCR (RT-PCR) using the primer pair, GusF and GusR (Table 1 Suppl.), under the following conditions: initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 35 s, followed by extension at 72 °C for 5 min. *Actin2* of *Arabidopsis* (*AtActin2*) was used as an internal reference with the primer pair, Actin2-F and Actin2-R (Table 1 Suppl.), under the following conditions: initial denaturation at 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 20 s, followed by extension at 72 °C for 5 min.

The seeds of transgenic *Arabidopsis* were sown in 1/2 MS medium containing kanamycin (40 mg dm^{-3}), and 4-, 8-, 12-, and 16-d-old transgenic seedlings were collected for GUS histochemical staining. To investigate the effects of abiotic stress and plant hormones, 2-, 6-, 10- and 14-d-old transgenic seedlings were transplanted onto a 1/2 MS medium containing methyl jasmonate (MeJA; 100 μM), gibberellins (GA; 160 μM), abscisic acid (ABA; 50 μM), mannitol (100 mM), and NaCl (100 mM), and cultured at 24 °C for 2 d, after which they were stained for GUS. The 28- and 38-d-old transgenic *Arabidopsis* plants cultured on peat soil were irrigated with Hoagland nutrient solution containing the above additives every 12 h, and GUS staining was performed after 2 d. Histochemical staining for GUS was performed using a GUS staining kit (*COMIN*, Jiangsu, China) according to the manufacturer's instructions, and *Pro.CkTPS::GUS* and *Pro.CkTPP::GUS* expressions were examined with a stereo microscope (*SMZ18*; *Nikon*, Tokyo, Japan).

Genetic transformation of *Arabidopsis*: The coding sequence of *CkTPS* was amplified and inserted between *Xba*I and *Bam*HI sites of the pBI101 vector to generate pBI101-CkTPS. The coding sequence of *CkTPP* was amplified and inserted between two *Bam*HI sites of the pBI101 vector to generate pBI101-CkTPP. The recombinant plasmids, pBI101-CkTPS and pBI101-CkTPP, were introduced into *A. tumefaciens* GV3101 and the transgenic *Arabidopsis* lines were screened on 1/2 MS medium containing kanamycin (40 mg dm^{-3}). Moreover, to establish *CkTPS* and *CkTPP* double transformant lines, the coding sequence of *CkTPP* was amplified and inserted between the *Xba*I and *Sac*I sites of the pBI101 vector to generate pBI101-NPTII-CkTPP-HPT, and then introduced into transgenic *Arabidopsis* homozygous for *CkTPS*, and the transgenic lines were screened on 1/2 MS medium containing kanamycin (40 mg dm^{-3}) and hygromycin (25 mg dm^{-3}). *CkTPS* and *CkTPP* expression in transgenic *Arabidopsis* lines were investigated by reverse transcription (RT)-PCR using the primer pairs, CkTPS-full-R/F and CkTPP-full-F/R (Table 1 Suppl.). *AtActin2* was used as an internal reference. Three transgenic lines homozygous for each transgene were selected for further analyses.

Stress tolerance analyses of transgenic *Arabidopsis*: Seeds of WT and transgenic *Arabidopsis* were sown in 1/2 MS medium containing 0, 5, 10, 15, or 20 % PEG6000, and the germination rate was measured after 14 d. The 7 d-old WT and transgenic seedlings were transplanted into a 1/2 MS medium containing 0, 5, 10, 15, or 20 % PEG6000 for 7 d to observe their growth and measure the primary root length.

In addition, 14-d-old WT and transgenic *Arabidopsis* plants cultured in peat soil were irrigated with Hoagland nutrient solution with or without 5 % PEG6000 for 7 d. The leaves were collected at the same position of the plants to measure the fresh mass (f.m.), and the water-saturated mass (w.s.m.) was determined after floating in distilled water for 24 h in the dark. Then the dry mass (d.m.) was measured after drying at 80 °C for 48 h. The relative water content (RWC) was calculated as follows: RWC [%] = [(f.m. - d.m.)/(w.s.m. - d.m.)] ×100. The chlorophyll content was measured using a *SPAD-502* portable chlorophyll meter (*Minolta*, Tokyo, Japan). The activities of SOD, POD, and CAT, and the content of trehalose, proline, and MDA were measured using corresponding assay kits (*COMIN*) according to the manufacturer's instructions.

Statistical analyses: The data were obtained from three independent biological experiments, and each experiment was repeated at least three times. Data are expressed as means ± standard deviations (SDs). The statistical significance of differences was assessed by one-way analysis of variance (*ANOVA*) using Fisher's least significant differences (LSD) test. In all analyses, $P < 0.05$ was taken to indicate statistical significance. All analyses were performed using *GraphPad Prism 6.0* software (San Diego, CA, USA) and *SPSS* (SPSS Inc., Chicago, IL, USA).

Results

The 3117 bp *CkTPS* cDNA fragment was amplified by PCR and found to contain an open reading frame (ORF) of 2 655 bp encoding a protein of 884 amino acids with a predicted molecular mass of 99 kDa and a theoretical isoelectric point of 7.64 (Fig. 1 Suppl.). Phylogenetic analyses showed that *CkTPS* was closely related to those of *Cicer arietinum* (XP_004515603.1), *Medicago truncatula* (XP_013443169.1), *Petunia* × *hybrida* (ADO16162.1), *Nicotiana tabacum* (BAI99252.1), and *Arabidopsis thaliana* (CAA69879.1) (Fig. 2*A* Suppl.). The 1 100 bp

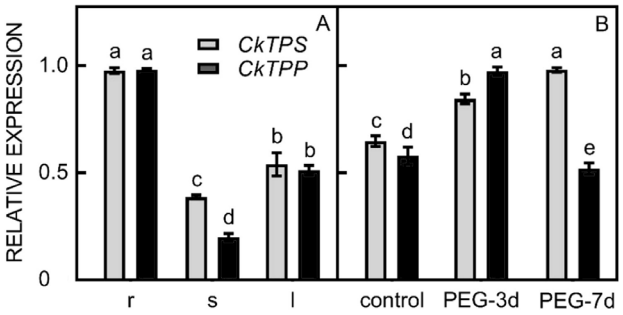

Fig. 1. The expression patterns of *CkTPS* and *CkTPP* in different tissues of *C. korshinskii* (*A*). Expressions of *CkTPS* and *CkTPP* under drought stress induced by PEG6000 (*B*). Values are means ± SDs of three independent biological replicates. Letters indicate significant differences ($P < 0.05$). r - root; s - stem; l - leaf.

CkTPP cDNA fragment was amplified by PCR and found to contain an ORF of 855 bp encoding a protein of 284 amino acids with a predicted molecular mass of 32.3 kDa and a theoretical isoelectric point of 9.2 (Fig. 3 Suppl.). Phylogenetic analyses showed that *CkTPP* was closely related to those from *C. arietinum* (XP_004500616.1) and *M. truncatula* (KEH34903.1), but more closely related to those from *Glycine max* (XP_003523824.2) and *Glycine soja* (KHN19743.1) (Fig. 2*B* Suppl.).

Real-time qPCR analyses showed that the *CkTPS* and *CkTPP* genes were stably expressed in roots, stems, and leaves of *C. korshinskii* with a higher relative expression in roots and lower relative expression in stems (Fig. 1*A*). Under conditions of drought stress, the expressions of these genes gradually increased with time, peaking at 3 d and 7 d, respectively, and then the expression of *CkTPP* decreased to below that of controls (Fig. 1*B*). These results indicate that the expressions of both genes were induced by drought stress and that *CkTPS* responds to drought stress slowly but continuously while *CkTPP* shows a rapid but limited response.

The 1 101 bp *Pro.CkTPS* sequence (Fig. 1 Suppl.) and 1 912 bp *Pro.CkTPP* sequence (Fig. 3 Suppl.) were cloned by genome walking, and analyses of transcriptional regulatory elements were performed using the *PlantCARE* database. The results showed that *Pro.CkTPS* and *Pro. CkTPP* sequences contained not only core promoter elements, such as the TATA-box and CAAT-box, but also *cis*-acting regulatory elements related to abiotic stress and plant hormones, including elements responsive to radiation (G-box, GA motif, and GT1motif), MeJA (CGTCA motif), GA (P-box and GARE motif), and ABA (ABRE). In addition, *cis*-acting elements involved in defense and stress responses (TC-rich repeats), heat stress response (HSE), and ethylene response (ERE) were also found in the *Pro.CkTPP* region.

Arabidopsis was transformed with the pORE R1-*Pro. CkTPS::GUS* and pORE R1-*Pro.CkTPP::GUS* plasmid constructs and transgenic lines were identified by genomic PCR with the primer pair pORE-F and pORE-R. Three *Pro.CkTPS* and *Pro.CkTPP* lines were selected for reverse transcription (RT)-PCR detection with the primer pair, GUS-F and GUS-R (Table 1 Suppl., Fig. 4 Suppl.). *Actin2* gene was used as an internal reference. The results showed that the *GUS* genes driven by *Pro.CkTPS* and *Pro. CkTPP* were stably expressed in the transgenic lines. Then transgenic plants were subjected to histochemical staining for GUS at different developmental stages under different conditions of abiotic stress and hormone induction to investigate the expression of *CkTPS* and *CkTPP*.

As shown in Fig. 2*A*, under normal conditions, no *Pro.CkTPS*::*GUS* expression was detected in the early developmental stages, and only weak expression was detected in the mature leaves of 16-d-old transgenic *Arabidopsis*. However, different GUS expression patterns were observed when plants were treated with ABA, GA, MeJA, and mannitol. GUS signals were detected in leaves treated with GA and mannitol; stronger signals were detected in the leaves treated with MeJA; a weak signal was detected at the leaf edge when treated with ABA; while no GUS expression was observed after NaCl induction. In the reproductive stage, little GUS activity was detected in pods when treated with mannitol, and no signal was detected in flowers. These results indicate that *CkTPS* was transcribed at a low level under normal conditions, while its expression can be induced by mannitol-simulated drought stress and plant hormones, such as GA, ABA, and MeJA.

As shown in Fig. 2*B*, under normal conditions, *Pro. CkTPP::GUS* expression was first observed in cotyledons of 4-d-old transgenic seedlings, and then it was detected in roots and leaves. GUS signals were detected in leaves and tops when treated with GA; in leaves of seedlings and in mature plants when treated with MeJA and NaCl, and also in leaves of mature plants following ABA and mannitol treatment. GUS expression was also detected in reproductive tissues, *i.e.*, flowers, under MeJA and mannitol treatment, and pods under mannitol treatment. These results indicate that *CkTPP* is transcribed at a low level under normal conditions, and its expression can be induced by abiotic stress and plant hormones.

The plasmids pBI101-*CkTPS*, pBI101-*CkTPP*, and pBI101-NPTII-*CkTPP*-HPT were transferred into *Arabidopsis*. Three homozygous lines for *CkTPS* (S1, S2, S3), *CkTPP* (P1, P2, P3), and double transformants of *CkTPS* and *CkTPP* (SP1, SP2, SP3) were identified by RT-PCR (Fig. 3), and drought tolerance of the transgenic plants was determined by examining the morphology and physiology under conditions of drought stress.

Wild-type (WT) and transgenic *Arabidopsis* seeds were sown in 1/2 MS medium containing 0, 5, 10, 15, or 20 % PEG6000 for 14 d, and the germination rates were recorded (Fig. 4*A*). There were no significant differences in germination rate among the transgenic lines under normal conditions, while the germination rates of S1, P1, and SP3 lines were markedly higher than WT, and those of the other transgenic lines were slightly higher than WT under conditions of 5 % PEG treatment. With increasing PEG concentration, almost all transgenic lines showed higher germination rate than WT; transgenic lines P2, P3, SP2, and SP3 showed significantly higher germination

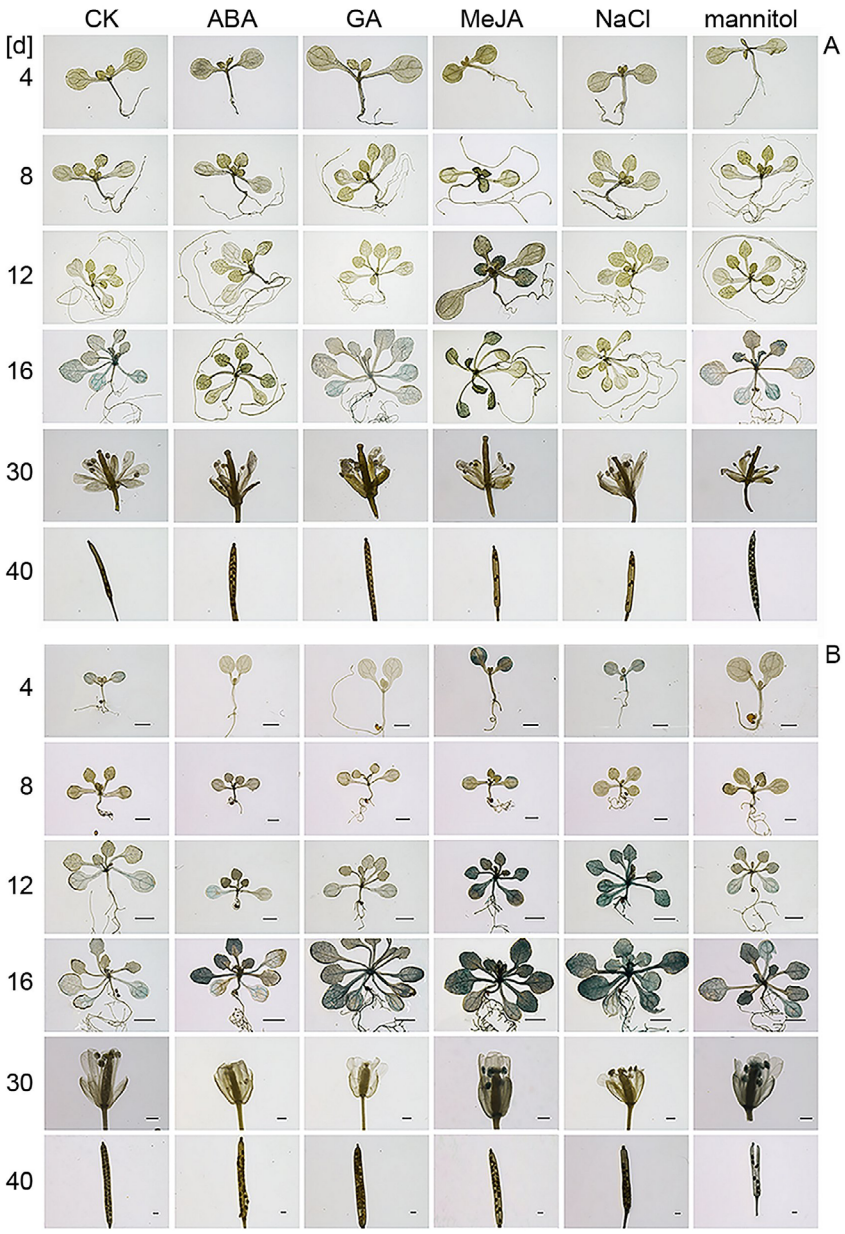

Fig. 2. GUS staining of transgenic *Arabidopsis* expressing the *GUS* reporter gene driven by *Pro.CkTPS* and *Pro.CkTPP*. Expression of *Pro.CkTPS::GUS* in *Arabidopsis* at different growth stages under different induction conditions (*scale bars* 2 mm) (*A*). Expression of *Pro.CkTPP::GUS* in *Arabidopsis* at different growth stages under different induction conditions (*scale bars*: 2 mm) (*B*). CK: transgenic seedlings grow on 1/2 MS medium; ABA: transgenic seedlings grown on 1/2 MS medium containing ABA (50 μM); GA: transgenic seedlings grown on 1/2 MS medium containing GA(160 μM); MeJA: transgenic seedlings grown on 1/2 MS medium containing MeJA (100 μM); NaCl: transgenic seedlings grown on 1/2 MS medium containing NaCl (100 mM); mannitol: transgenic seedlings grown on 1/2 MS medium containing mannitol (100 mM).

rates (68.9 - 72.2 %) than WT (55.6 %) under 10 % PEG treatment; P3 (75.6 %) and SP3 (68.9 %) showed significantly higher germination rates than WT (52.2 %) under 15 % PEG treatment; and SP1 (38.9 %) showed significantly higher germination rate than WT (5.6 %) under 20 % PEG treatment.

Next, 7-d-old WT and transgenic seedlings were transplanted into a 1/2 MS medium containing 0, 5, 10, 15, or 20 % PEG6000 for 7 d to observe shoot growth and primary root length. As shown in Fig. 5*A*, there were no significant differences in the growth of WT and transgenic lines under normal conditions. However, with increasing PEG concentration, WT seedlings showed more severe growth inhibition than transgenic seedlings. The leaves of transgenic seedlings were slightly larger than WT with 5 % PEG treatment (Fig. 5*B*), while under conditions of 10, 15, and 20 % PEG treatment, the leaves of transgenic seedlings were significantly larger than those of WT. SP1 showed superior growth compared to the other lines under conditions of PEG treatment (Fig. 5*C-E*). The root lengths

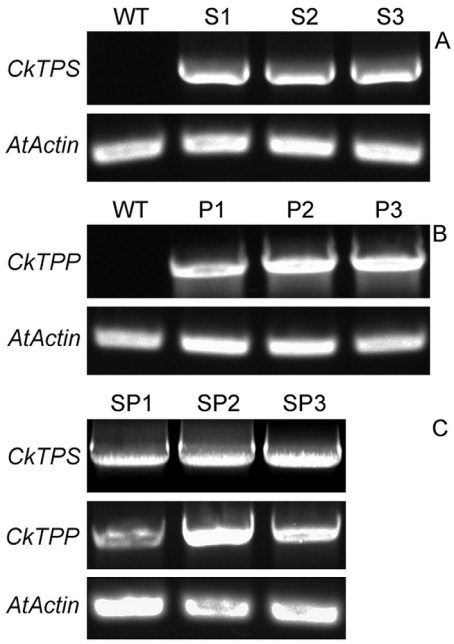

Fig. 3. RT-PCR identification of transgenic *Arabidopsis*. Expression of *CkTPS* in transgenic *Arabidopsis* (*A*). Expression of *CkTPP* in transgenic *Arabidopsis* (*B*). Expression of *CkTPS* and *CkTPP* in double transformants (*C*).

of WT and transgenic seedlings were also measured. There were no significant differences in root length between WT and transgenic lines under normal conditions. However, the roots of all transgenic lines were markedly longer than WT under 5, 10, and 15 % PEG treatments (Fig. 4*B*, Fig. 5*G-I*). The roots of transgenic line SP1-3 were significantly longer than WT roots and roots of other transgenic lines under conditions of 20 % PEG treatment were 50.5 - 50.7 % longer than those of WT. These results indicate that transgenic lines overexpressing *CkTPS* or *CkTPP* had a higher germination rate, longer roots and better growth than WT under conditions of drought stress. Moreover, the double transgenic lines overexpressing *CkTPS* and *CkTPP* simultaneously showed superior performance.

WT and transgenic *Arabidopsis* were transplanted into peat soil for further analyses. As shown in Fig. 6, after irrigating with Hoagland nutrient solution containing 5 % PEG for 1 week, the leaves of transgenic plants were slightly shrunken and yellowed, while the growth of WT plants was obviously suppressed with shrivelled and yellow leaves. The chlorophyll content and RWC of transgenic lines were higher than those of WT plants under conditions of drought stress (Fig. 7*A*,*B*). The accumulation of proline in transgenic lines was higher, particularly in SP1 - SP3, while the MDA content of transgenic lines was lower than that of WT (Fig. 7*C*,*D*). The activities of SOD in S2, S3, and SP2, POD in S2, P1, and SP1, and CAT in P1 - P3 and SP1 - SP3, were significantly higher than those

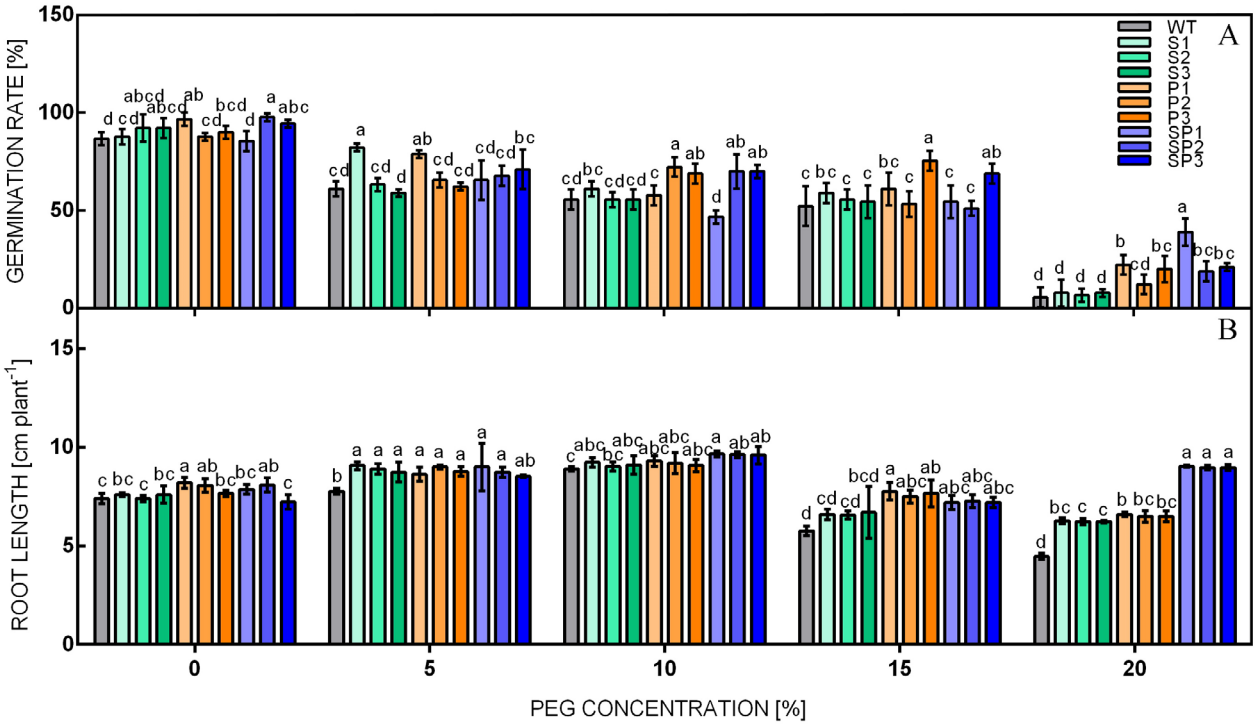

Fig. 4. Detection of drought tolerance of transgenic *Arabidopsis*. Germination rates of WT and transgenic lines cultured in 1/2 MS medium containing different concentrations of PEG for 2 weeks (*A*). Root length of WT and transgenic lines cultured in 1/2 MS medium containing different concentrations of PEG for 2 weeks (*B*). WT - wild-type; S1 to S3 - transgenic lines overexpressing *CkTPS*; P1 to P3 - transgenic lines overexpressing *CkTPP*; SP1 to SP3 - double transformants overexpressing *CkTPS* and *CkTPP*. Means ± SDs of three independent biological replicates. Different letters indicate significant differences ($P < 0.05$).

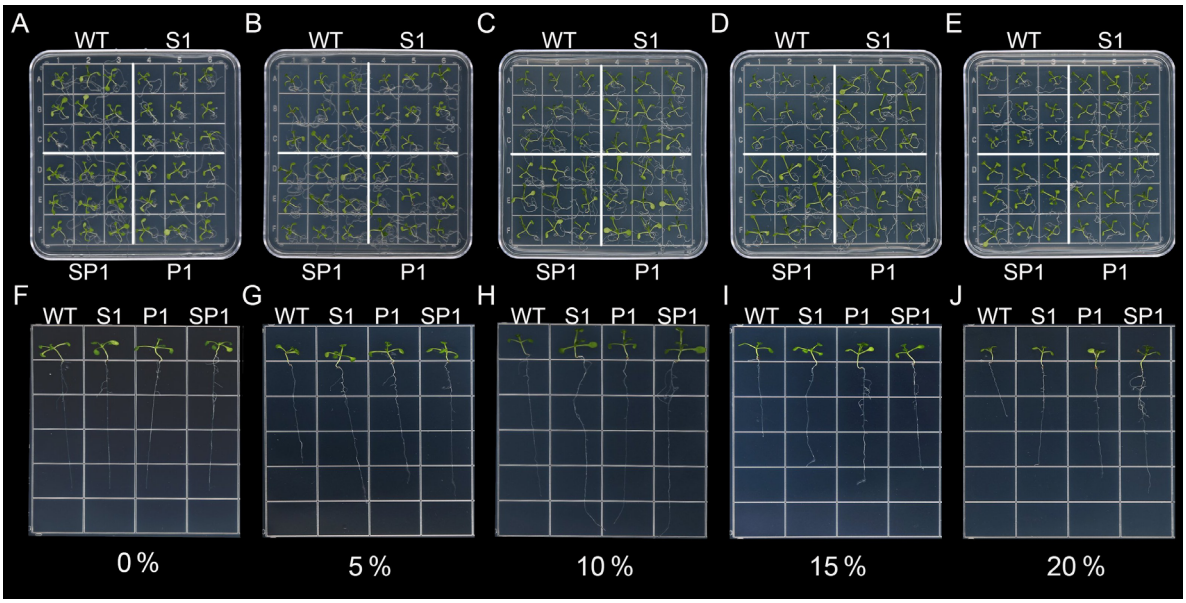

Fig. 5. Morphological traits and root length of WT and transgenic *Arabidopsis* under conditions of drought stress. Morphological traits of WT and transgenic *Arabidopsis* cultured in 1/2 MS medium containing 0, 5, 10, 15 or 20 % PEG *(A-E)*. Root length of WT and transgenic *Arabidopsis* cultured in 1/2 MS medium containing 0, 5, 10, 15, or 20 % PEG *(F-J)*. WT - wild-type; S1 - transgenic lines overexpressing *CkTPS*; P1 - transgenic lines overexpressing *CkTPP*; SP1 - double transformants overexpressing *CkTPS* and *CkTPP*.

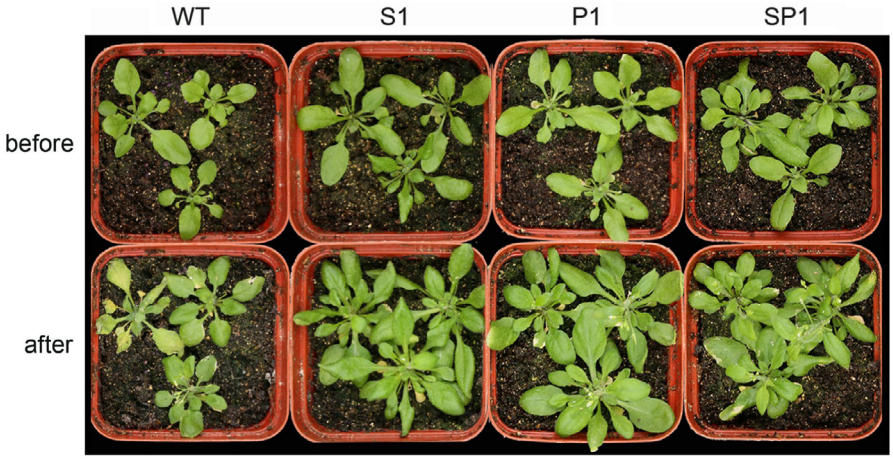

Fig. 6. Phenotypic differences in WT and transgenic lines before and after 5% PEG stress for 1 week. WT - wild-type; S1 - transgenic lines overexpressing *CkTPS*; P1 - transgenic lines overexpressing *CkTPP*; SP1 - double transformants overexpressing *CkTPS* and *CkTPP*.

in WT (Fig. 7*E,F,G*). Trehalose content was increased in all lines under drought stress, and the effect was particularly marked in P2 and SP2 **(**Fig. 7*H*). Moreover, the double-transgenic lines SP1 and SP2 showed higher proline and trehalose content and SOD activity compared to transgenic lines individually overexpressing *CkTPS* or *CkTPP* under drought stress.

Discussion

The *TPS* and *TPP* genes have been isolated from various plants and some have been used for genetic improvement of crops (Han *et al*. 2016, Acosta-Pérez *et al*. 2020). However, there are few reports on *TPS* and *TPP* genes from xerophytic shrubs. Xerophytic mesquite, including *Caragana*, play important roles in their ecosystems and have great economic value. Therefore, it is essential to understand the expression features and functions of *TPS* and *TPP* genes of these plants. This study revealed the expression features of *CkTPS* and *CkTPP* and their functions in improving the drought tolerance of transgenic *Arabidopsis*.

The expression patterns of *TPS* and *TPP* genes are varied in different plants, and therefore further analyses of the expression and regulatory mechanisms of *CkTPS* and *CkTPP* are important to understand their functions and potential applications for the genetic improvement of crops.

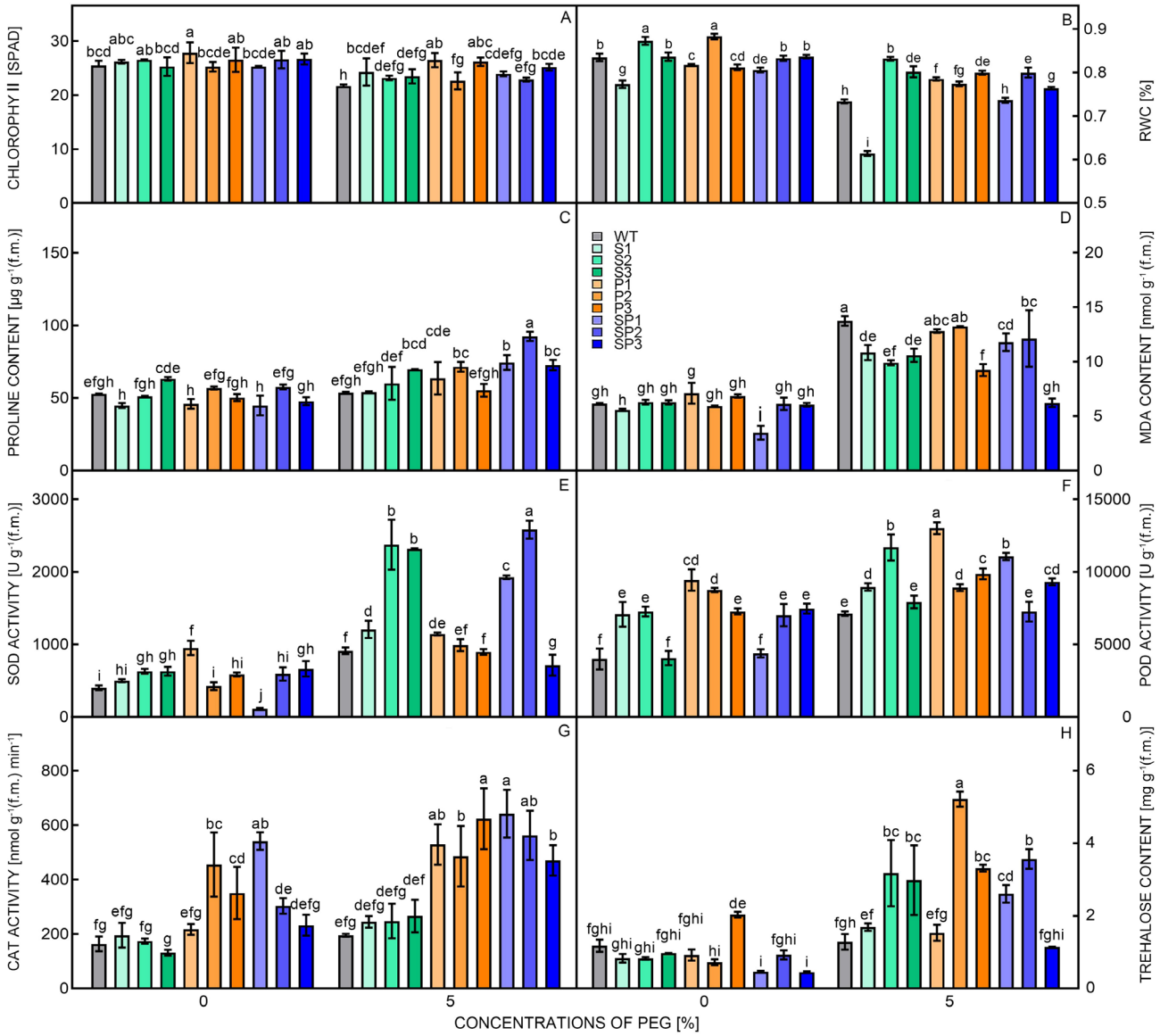

Fig. 7. Detection of physiological indexes of transgenic *Arabidopsis* under normal and drought stress conditions. Chlorophyll content (SPAD) (*A*), RWC (*B*), proline content (*C*), MDA content (*D*), SOD activity (*E*), POD activity *(F)*, CAT activity *(G)*, trehalose content *(H)*. WT - wild-type; S1 to S3 - transgenic lines overexpressing *CkTPS*; P1 - P3 - transgenic lines overexpressing *CkTPP*; SP1 - SP3 - double transformants overexpressing *CkTPS* and *CkTPP*. Means ± SDs of three independent biological replicates. Different letters indicate significant differences ($P < 0.05$).

The real-time PCR results showed that *CkTPS* and *CkTPP* of *C. korshinskii* were stably expressed in various tissues with higher expression in roots than in stems and leaves, and they were induced by drought stress (Fig. 1). The expression pattern was consistent with the *CsTPS* genes in cucumber, where all of seven *CsTPS* genes are expressed in the roots, stems, and leaves, and their expressions were higher in the roots than in the other tissues. The *CsTPS* genes showed different expression under PEG-simulated water stress (Dan *et al*. 2021), which indicates that they are involved in drought response in different ways. The expression characteristics of *TPSs* and *TPPs* in the other plant species have also been reported. The potato *StTPS* genes showed higher expression in flowers and roots than in leaves, and *StTPS1* and *StTPS7* were frequently regulated by various factors, which suggests that they may be the major *TPSs* involved in the response to environmental stresses (Xu *et al*. 2017). Similarly, the four *MtTPS* genes from *M. truncatula* were induced by drought, salt, and cold stress (Song *et al*. 2021). These results indicate that *TPS* and *TPP* genes are differentially expressed in various tissues and play different functions in resistance to various stress conditions. The expression of *CkTPS* and *CkTPP* under conditions of drought stress suggested that the trehalose biosynthesis pathway may play a key role in the adaptation of desert plants to the dry environment.

The promoters of *CkTPS* and *CkTPP* contain some *cis*-acting elements that respond to abiotic stresses, such

as irradiance and phytohormones, including GA, MeJA, ethylene, and ABA. These observations suggested that the trehalose biosynthesis pathway may be involved in the responses to biotic and abiotic stresses, and regulate growth and development *via* various phytohormone signaling pathways in *C. korshinskii*. The expression of *Pro.CkTPS::GUS* was stronger in the leaves of transgenic *Arabidopsis* after treatment with various phytohormones, while the expression of *Pro.CkTPP::GUS* was stronger in the roots and leaves, which showed different expression patterns from those in *C. korshinskii* (Fig. 2). These differences in expression patterns may be due to differences in characteristics in growth and development and environmental adaptation between herbaceous plants and trees. GUS expression driven by the promoters of *CkTPS* and *CkTPP* was also detected in reproductive organs, including pods and anthers, of transgenic *Arabidopsis* under stress conditions, which suggests that the trehalose biosynthesis pathway may be involved in the regulation of growth, development, and protection of reproductive organs. Some phytohormone-responsive *cis*-acting elements were also detected in the *TPS* and *TPP* promoters of potato (Xu *et al*. 2017), which suggests that the *TPS* and *TPP* genes may be involved in growth and development *via* various phytohormone signaling pathways in potato. Similarly, the *PtTPS* and *PtTPP* genes of *Populus tomentosa* were confirmed to be involved in plant hormone and environmental stress responses, which showed different expression patterns under salt, cold, and MeJA treatments (Gao *et al*. 2021). Previous studies have shown that MeJA can regulate plant growth and development, induce the expression of defense genes, synthesize defense substances, and form defense organs; GA, as a phytohormone essential for plant growth and development, can regulate several processes of plant growth and development; ABA can increase the activity of protective enzymes and decrease membrane lipid peroxidation (Tiwari *et al*. 2017). These results suggest that *CkTPS* and *CkTPP* may affect plant growth, development, and stress resistance through the above-mentioned signal transduction pathways and protect plants from damage due to adverse environmental conditions.

Studies on *TPS* and *TPP* genes in many plants have confirmed that their expression or overexpression can improve the growth and development of transgenic plants under conditions of drought stress (Lyu *et al*. 2013, Xu *et al*. 2017, Lin *et al*. 2019). In this study, we examined the drought tolerance of transgenic *Arabidopsis* lines overexpressing *CkTPS* and *CkTPP* alone or in combination. Compared to the WT plants, all transgenic lines showed higher germination rates, less damage, better growth, and longer roots under conditions of drought stress, with the double transformants showing a significant advantage over the other lines (Figs. 4, 5, 6). Transgenic plants have deep roots, which facilitate strong water and nutrient absorption, thus promoting growth under drought conditions. Similar results have been reported in other studies in which rice seedlings overexpressing *OsTPS1* showed a higher survival rate and *Arabidopsis* seedlings overexpressing *AtTPPI* had longer roots than WT plants under drought conditions (Li *et al*. 2011, Lin *et al*. 2020). Under conditions of drought stress, plant growth differed between different transgenic lines, which may be due to the different insertion sites and means of regulation of transgenes. Our results indicate that overexpression of *CkTPS* and *CkTPP* enhanced drought tolerance of transgenic *Arabidopsis*, and the synergistic overexpression of *CkTPS* and *CkTPP* showed a greater effect than either transgene alone.

To understand the mechanisms by which *CkTPS* and *CkTPP* enhanced drought tolerance of transgenic plants, some physiological indexes of transgenic *Arabidopsis* cultured in the greenhouse were investigated. Compared to WT, the transgenic plants had a higher content of chlorophyll, proline, and trehalose, in addition to higher RWC (Fig. 7). The double transformant lines showed a significant advantage over the other lines, particularly with regard to the content of proline and trehalose. Drought causes a decrease of soil water potential, and some organic compounds including proline, sugars, glycine betaine, and sugar alcohols accumulate in cells and act as osmolytes to reduce the osmotic potential of cells, and to protect plants from the injuries of drought stress (Akram and Irfan 2016, Hussain *et al*. 2019, Parveen *et al*. 2019). Overexpression of *CkTPS* and *CkTPP* leads to an increase in trehalose content, which is considered an important osmoticum (Kosar *et al*. 2019). On the other hand, water deficiency causes damage of photosystems and reduction of the chlorophyll content (Baker *et al*. 2007, Farouk and Qados 2011, Talebi 2014, Dąbrowski *et al*. 2019, Kosar *et al*. 2019, Parveen *et al*. 2019). The enhanced chlorophyll content in transgenic plants overexpressing *CkTPS* and *CkTPP* may be due to the protective effect of trehalose, which prevents oxidative degradation of chlorophylls, or the trehalose stabilizes key enzymes in the chlorophyll biosynthesis pathway. The result is consistent with previous studies, where the trehalose-treated fenugreek plants had a higher content of photosynthetic pigments (Farouk and Qados 2011), and the seeds pretreated with trehalose caused a significant increase in chlorophyll content in radish plants (Yang *et al*. 2022). Moreover, the transgenic lines had higher activities of antioxidant enzymes, including SOD, POD, and CAT, and lower MDA content compared to WT *Arabidopsis*. These results are consistent with previous studies, where transgenic rice overexpressing *OsTPS1* showed higher content of trehalose and proline than WT (Li *et al*. 2011). Previous studies have shown that overexpression of *AtTPPF* leads to an increase in trehalose content, which may play a role in the scavenging of ROS (Lin *et al*. 2019). The protective effect of trehalose on proteins under drought stress may also be the reason for the enhancement of antioxidant enzyme activities in transgenic plants. The strong drought tolerance may also be due to the higher chlorophyll content, resulting in improved photosynthesis in transgenic plants under conditions of drought stress. Meanwhile, the higher trehalose content of transgenic *Arabidopsis* may also play an important role in osmotic protection, stabilizing proteins and membranes and preserving viability.

Previous studies have shown that overexpression of

TPS caused growth defects in transgenic plants, possibly due to the accumulation of trehalose-6-phosphate (T-6-P; Li *et al*. 2011, Yu *et al*. 2020). Therefore, the expression of TPS and TPP fusion constructs was used to alleviate this effect. A bifunctional yeast TPS-TPP fusion construct, 35S::TPS1-TPS2, was introduced into *Arabidopsis*, and the transgenic plants showed greater tolerance to multiple abiotic stresses, with advantages in germination rate, survival rate, and trehalose accumulation (Dan *et al*. 2021). However, the *TPS* and *TPP* genes of plants are longer than those of bacteria, and fusion expression may affect protein folding and thus enzyme activity. The method in which the two exogenous genes are expressed separately in the same plant is safer. Our experimental findings confirm that the simultaneous overexpression of *CkTPS* and *CkTPP* results in greater drought tolerance in transgenic plants.

In conclusion, *CkTPS* and *CkTPP* were highly expressed in roots and their expression was regulated by abiotic stresses in *C. korshinskii* suggesting that the trehalose biosynthesis pathway plays a key role in adaptation to the desert environment in this plant. Overexpression of *CkTPS* and *CkTPP* enhanced drought tolerance in transgenic *Arabidopsis*, and the synergistic overexpression of *CkTPS* and *CkTPP* had a better effect. The improved tolerance was achieved by the stronger ability to absorb water and nutrients, improved photosynthesis, and greater osmotic and antioxidant adjustments in transgenic plants. These results provide a theoretical and experimental basis for further understanding the function and regulatory mechanism of *CkTPS* and *CkTPP*, as well as their application for improving drought tolerance in crops through genetic engineering.

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