biologia plantarum
an international journal for experimental botany

BIOLOGIA PLANTARUM (2021) 65: 47-59 DOI: 10.32615/bp.2020.155

Molecular cloning and functional analysis of the thioredoxin gene *SikTrxh* from *Saussurea involucrata*

L. ZHANG[1,+], C. LIU[2,+], F.F. CHENG[3,+], X.Y. GUO[3], Y.X. LI[3], A.Y. WANG[3], and J.B. ZHU[3,*]

[1] *Shihezi University of Medicine, Shihezi 832003, P.R. China*
[2] *Agriculture College of Xinjiang Agriculture University, Urumqi, 830052, P.R. China*
[3] *College of Life Science, Shihezi University, Shihezi 832003, P.R. China*

[*]Corresponding author: E-mail: jianboz9@sina.com

Abstract

Thioredoxins are oxidoreductases that help to maintain redox homeostasis in plants under abiotic stress. In this study, a new thioredoxin gene, *SikTrxh*, was cloned from *Saussurea involucrata* (Kar. & Kir.), a perennial herb that grows in the high alpine mountains of Central Asia. Bioinformatics analysis shows that the full-length cDNA of *SikTrxh* consisted of 565 bp with a 354-bp open reading frame and encoded a 117 amino acid protein. Using quantitative reverse transcription (RT) PCR, we found that the expression of the *SikTrxh* gene was induced by salt, cold, and drought stresses, suggesting that this protein played a significant role in plant defense. Subcellular localization confirmed that the protein was localized to the mitochondria. A vector carrying *SikTrxh* was inserted into tobacco, and successfully modified plants were identified by RT-PCR. Physiological indicators and antioxidant enzyme activities were measured under low temperature, and salt and drought stresses. Our results show that malondialdehyde content and relative electrolyte leakage increased in both wild-type and *SikTrxh*-overexpressing transgenic plants; however, these increases were significantly higher in the wild-type plants than in the transgenic plants. We also found that photosystem II photoinhibition was lower in the transgenic plants than in the wild-type plants, and that activities of reactive oxygen species-scavenging enzymes were higher in the transgenic plants than in the wild-type plants. We conclude that *SikTrxh* can reduce toxic effects of reactive oxygen species to protect the plasma membrane, thereby increasing plant resistance to abiotic stresses.

Keywords: abiotic stresses, reactive oxygen species, thioredoxins, tobacco.

Introduction

Adverse environmental conditions, such as extreme temperatures, saline-alkaline conditions, and drought, can inhibit the growth, development, and productivity of plants. Plants have evolved versatile redox signaling and regulation mechanisms, some of which are controlled by thioredoxins (TRXs), to maintain redox homeostasis in harsh environmental conditions. TRX is a generic term for the large family of small, versatile, redox proteins found in all organisms (Meyer *et al*. 2005, 2009, Park *et al*. 2009, Rivas *et al*. 2004). Trxs are small (roughly 10 - 12 kDa) oxidoreductases that contain a highly conserved Trp-Cys-(Gly/Pro)-Pro-Cys (WC(G/P)PC) active site motif (Holmgren 1989). The redox activity of these proteins relies on the two Cys residues that, when reduced, induce

Received 13 February 2020, *last revision* 21 October 2020, *accepted* 29 October 2020.
Abbreviations: APX - ascorbate peroxidase; CAT - catalase; cDNA - complementary DNA; F_0 - minimal fluorescence; F_m - maximal fluorescence; F_v - variable fluorescence; F_v/F_m - maximal photochemical efficiency of PS II; MDA - malondialdehyde; $O_2^{\bullet-}$ - superoxide radical anion; MS - Murashige and Skoog; PEG - polyethylene glycol 6000; PS II - photosystem II; REL - relative electrolyte leakage; ROS - reactive oxygen species; RT-PCR - reverse transcription PCR; SOD - superoxide dismutase; Trxh - thioredoxin-h.
Acknowledgements: We would like to thank the Key Areas of Corps Science and Technology Reform Project (2020AB009), the National GMO Major Project (2016ZX080005004-009), the Key Scientific and Technological Projects for the Cultivation of New Varieties of Genetically Modified Organisms (2018ZX0800501B-005), and the National Natural Science Foundation of China (31360053) for their support. [+]These authors contributed equally.
Conflict of interest: The authors declare that they have no conflict of interest.

reductive cleavage of a disulfide bond in target proteins *via* two nucleophilic substitution reactions (Serrato *et al.* 2003). These reactions allow Trxs to activate or deactivate the target protein and, consequently, the associated cellular redox pathway (Gromer *et al.* 2004). Trxs receive electrons from either one of two compartment-specific systems: the ferredoxin-TRX system, which reduces Trxs with electrons coming from ferredoxin through the action of ferredoxin-TRX reductase, or the NADPH-TRX system, in which NADPH-TRX reductase furnishes electrons from NADPH to Trxs (Gromer *et al.* 1998).

TRX proteins occur widely in plants, animals, and microorganisms. Large quantities of TRX have been identified in plants (Buchanan *et al.* 2005). Plant TRX proteins are divided into 6 types, according to their amino acid sequences and cellular distribution: f, m, x, y, h, and o (Nuruzzaman *et al.* 2008). It is thought that members of different families regulate various redox-dependent cellular processes for a variety of proteins (Reichheld *et al.* 2002, Gelhaye *et al.* 2005, Hall *et al.* 2010, Meyer *et al.* 2012). The TRX type h proteins (TRXh) form the largest TRX family. TRXh has the most extensive cellular distribution, and different Trxh proteins have been located in subcellular structures such as cytoplasm (Montrichard *et al.* 2009), nucleus (Gelhaye *et al.* 2004), endoplasmic reticulum (Serrato *et al.* 2002), plasma membrane (Marcus *et al.* 1991), and mitochondria (Meng *et al.* 2010). They are the most structurally complex TRX proteins and have the greatest number of biochemical properties. The TRXh is considered to be the most versatile type in terms of redox pathways and other cellular processes involved in redox (Gelhaye *et al.* 2004, 2005). There are many Trxh proteins in plants, 11 *Trxh* genes have been identified in *Arabidopsis thaliana* (Meyer *et al.* 2005), and there are at least 10 *Trxh* genes in rice (Xie *et al.* 2009). However, only a few functions have been elucidated for the h-type Trxs, which represent the largest group of these proteins identified by sequencing (Park *et al.* 2009).

Trxh5 in *A. thaliana* is important in resisting virus and microbial infection (Sweat *et al.* 2007, Park *et al.* 2017). Trxh1 and Trxh4 in rice are involved separately in the fine regulation of the stress response to high salt (Zhang *et al.* 2011) and low phosphorus (Ying *et al.* 2017). Trxh5 in barley is involved in the regulation of grain germination (Wong *et al.* 2002). Further research showed that during seed germination, Trxh regulates disulfide bonds to increase the activity of starch hydrolase and increase the solubility of seed storage protein, thus promoting seed germination (Hägglund *et al.* 2013). Trxh is an electron donor that affects the activity of several oxidative stress response enzymes, including methionine sulfoxide reductase, peroxidase, and glutathione reductase (Rouhier *et al.* 2001, Fomenko *et al.* 2002). It is also thought to participate in other plant developmental pathways, including self-incompatibility (Cabrillac *et al.* 2001), carbon metabolism, and nitrogen metabolism (Baumann *et al.* 2002). The cited studies indicate that the *Trxh* gene is important in regulating plant growth and development as well as being involved in the stress response. In addition the development of proteomics tools has permitted the identification of potential targets of many TRXh proteins as reviewed in Montrichard *et al.* (2009).

In the present study, we investigated the expression of a new *Trxh* gene isolated from *Saussurea involucrata* (Kar. & Kir.), referred to as *SikTrxh*. *S. involucrata* is a perennial herb that grows in the high mountains of central Asia, at altitudes of 2400 - 4100 m, where the climate is often below freezing and snowy. *S. involucrata* has evolved unique physiological and biochemical mechanisms that allow it to survive under these conditions, making it a good subject for the study of abiotic stress tolerance in plants. Therefore, we introduced the *SikTrxh* gene into tobacco plants, and investigated the tolerance of the transformed tobacco plants to cold, drought, and salt. We also studied the expression of genes and activities of antioxidant enzymes that alleviate oxidative damage, including superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT), together with the accumulation of the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2) and superoxide radical anions ($O_2^{\bullet-}$).

Materials and methods

Plants and growth conditions: *Saussurea involucrata* (Kar. & Kir.) seedlings were cultured in Murashige and Skoog (MS) solid medium in a growth chamber with a 70 - 80 % relative humidity, a 16-h photoperiod, a photon flux density of 200 µmol m^{-2} s^{-1}, and day/night temperatures of 21/19 °C. Seeds of wild-type tobacco (*Nicotiana tabacum* L. cv. NC 89) and transgenic tobacco plant lines were germinated in MS medium for two weeks and then transplanted to containers filled with a 3:1 soil:sand mixture. The seedlings were grown for eight weeks either in a controlled environmental chamber or a greenhouse. The chamber was set to 25/20 °C day/night temperatures, a 16-h photoperiod, and a photon flux density of 500 - 600 µmol m^{-2} s^{-1}. The greenhouse was lit by natural light supplemented with high-pressure sodium lights; photon flux density was from 600 µmol m^{-2} s^{-1} at the pot level to 1600 µmol m^{-2} s^{-1} at the top of the plant. The positions of the plants were changed daily, and the plants were watered with a nutrient medium.

Cloning and sequence analysis of *SikTrxh* gene: Total RNA was isolated from leaves of *S. involucrata* using the TRIzol reagent (*Takara Biotechnology*, Shiga, Japan), and DNase I according to the manufacturer's instructions. A full-length cDNA library of *S. involucrata* was constructed using the Creator SMART cDNA library construction kit (*Clontech*, Mountain View, CA, USA) following the procedure described by Zhu *et al.* (2006). Ninety-six single clones were randomly selected and sequenced. One cDNA sequence showed homology to a gene encoding a thioredoxin-like protein. This cDNA sequence was named *SikTrxh*. The 5' end, the CDS region, and the 3' end of the *SikTrxh* gene were identified using open reading frame finder and aligned with known sequence databases. The obtained *SikTrxh* sequence was subjected to sequence alignment using DNAMAN (v. 8.0). Phylogenetic tree

was reconstructed by MEGA 4.1 software (http://www.megasoftware.net/) using the Neighbor-Joining method and 1 000 bootstrap replicates, bootstrap scores of < 50 % were deleted.

Construction of *SikTrxh* gene expression vector in plants: *SikTrxh* cDNA was amplified using the forward primer 5'-GGATCCAAAATGGCGGAAGAAGGA-3' (the BamHI site is underlined) and the reverse primer 5′-GAGCTCGGAAACACATAAGTTGCTG-3′ (the SacI site is underlined) to construct the pBI121-SikTrxh recombinant plasmid. PCR products were digested with BamHI and SacI enzymes and ligated with the pBI121 vector, which contained the CaMV 35S promoter. After double digestion with the same enzymes, the β-*glucuronidase* (*GUS*) gene was replaced with the *SikTrxh* gene. The identity of the insert was confirmed by sequencing.

Subcellular localization of *SikTrxh*: The full-length open reading frame of *SikTrxh* without a stop codon was amplified by PCR with the primer SikTrxh-Sub, containing BamHI and XbaI restriction sites (Table 1 Suppl.). The fragment was then fused to the pCAMBIA2300-GFP expression vector to generate pCAMBIA2300- SikTrxh-GFP, which is driven by the CaMV 35S promoter. The p35S-SikTrxh-GFP plasmid and MT-rk mitochondria marker plasmid (Nelson *et al.* 2007) were transformed into *Agrobacterium tumefaciens* strain GV3101. These transformed constructs were infiltrated into tobacco abaxial leaf tissue using a syringe. The infiltrated leaves were incubated for 2 d in an irradiated cultivation chamber before being peeled. The subcellular localization of the GFP fusion protein was then imaged using a Leica SP8 confocal laser scanning microscope (*Leica Microsystems*, Heidelberg, Germany) with the excitation wavelengths set at 488, 561, and 633 nm.

Plant transformation and transgenic tobacco identification: To create the transgenic tobacco plants, the pBI121-SikTrxh recombinant plasmid was introduced into *A. tumefaciens* strain GV3101 and then transformed into the tobacco using cotyledons as explants. Selected shoots were regenerated in MS medium containing 50 mg dm^{-3} kanamycin. When they had rooted, the kanamycin-resistant primary transformants (the T_0 generation) were transferred to soil and allowed to growth till maturation. Kanamycin-resistant plants were tested for the presence of the *SikTrxh* gene using PCR. The seeds from T_0 plants were sown, and two fully independent transgenic T_1 lines (OE-2 and OE-3) with comparatively high content of *SikTrxh* were used for further analysis.

Stress tolerance: Six-week-old seedlings of T_2 wild-type and transgenic lines (OE-2 and OE-3) were randomly divided for cold stress tolerance tests. The seedlings were placed for 2 h in a cold chamber at 4, -1, or -2 ºC, control seedlings were kept at 25 °C. Photographs of the seedlings were taken after they were exposed to the cold environment, the phenotypic changes in the whole plants were observed and analyzed. After a further 2 d of 4 °C cold treatment, the second and third fully expanded leaves from the tops of all the plants were harvested for determining the activity of antioxidant enzymes and gene expressions (see below). After a disinfectant rinse, wild-type and transgenic seeds were separated and placed in a half MS medium. The seeds were stored at normal (20 ºC) for 7 d or at low (4 ºC) temperature for 14 d. Survival rate was recorded.

The drought tolerance tests were carried out in a greenhouse maintained under natural irradiance at temperatures 22 - 25 ºC. The six-week-old seedlings of both wild-type and transgenic T_2 plants were watered, and each type of them were subsequently divided into two groups, *i.e.*, a drought treatment group and a control group. Water was withheld from seedlings in the drought treatment for 12 d. Seedlings in the control group were watered every 2 d. After 12 d, the first and second fully expanded leaves from the tops of all the plants were harvested to determine the activity of antioxidant enzymes and genes expressions. Photographs of the plants were taken after 0, 3, 6, 9, and 12 d, the corresponding phenotypic changes were observed. After disinfection, seeds were separated and sown in the half MS mediums with different polyethylene glycol (PEG)-6000 concentrations (0, 5, 10, and 15 %, m/v). Survival rate was recorded.

Six-week-old wild-type and transgenic T_2 plants were watered using a 150 mM NaCl solution for 12 d for the salinity tolerance tests. The control group was grown at 25 °C with non-saline water. After 12 d, the first and second fully expanded leaves from the tops of all plants were harvested to determine the activity of antioxidant enzymes and gene expressions. Photographs of the plants were taken after 0, 3, 6, 9 and 12 d, the phenotypic changes were observed. Disinfected seeds of both wild-type and transgenic plants were separated and seeded in the half MS medium containing a range of NaCl concentrations (0, 100, 150, and 200 mM). Survival rates were recorded.

All tests were repeated three times to produce representative results, and each sample was prepared in triplicate.

Physiological measurements: Malondialdehyde (MDA) content was determined by the modified thiobarbituric acid reaction using a spectrophotometer (*UV-160A, Shimadzu Scientific Instruments*, Kyoto, Japan) as described by Du *et al.* (1992). Leaves excised from the tobacco plants were washed in deionized water, and leaf discs were punched out. Membrane damage was quantified by the MDA concentration.

Relative electrolyte leakage (REL) was determined using an *EC215* conductivity meter (*Markson Science*, Del Mar, CA, USA), following the method described by Lutts and Kinet (1996). Relative conductance was calculated by the equation REL = (EC1 - CW)/(EC2 - CW) × 100, where EC1 is electrical conductivity during the first measurement, EC2 is electrical conductivity after boiling, and CW is conductivity of deionized water.

Maximal photochemical efficiency of PS II (F_v/F_m) was measured from the tobacco leaves using a portable fluorescence analyzer (*DUAL-PAM-100*, *Walz*, Effeltrich,

Germany). Leaves were dark-adapted for 30 min and then exposed to a saturating light pulse for 1 s. The minimal fluorescence (F_0) in the dark-adapted state (fluorescence intensity when all PS II reaction centers are open) and the maximal fluorescence (F_m) during the application of the saturating pulse (fluorescence intensity when all PS II reaction centers are closed) were measured. Variable fluorescence ($F_v = F_m - F_0$) was then calculated (Van Kooten *et al*. 1990).

Chlorophyll content was measured using the technique described by Zhao *et al*. (2016). Leaves were frozen in liquid nitrogen before being placed in ethanol and heated in a water bath. Total chlorophyll content was quantified at absorbance A_{654} on an *Infinite M200 Pro* microplate reader (*Tecan Group*, Männedorf, Switzerland) using the equation: chlorophyll content = A_{654} /(39.8 × 200 mg) × 100, where A_{654} is the absorbance reading and 200 mg is the fresh mass of the initial leaf sample (Tetley *et al*. 1974). These experiments were repeated three times.

After the cold, drought, and salt treatments, samples of wild-type and transgenic T_2 tobacco plant leaves were collected. The leaves were cut into pieces and homogenized in an ice bath with 4 cm^3 of 50 mM sodium phosphate buffer (pH 7.8) containing 1 % (m/v) polyvinylpyrrolidone and 10 mM β-mercaptoethanol. The homogenate was transferred to a tube and centrifuged at 1 509 *g* and 4 °C for 15 min. The supernatant was then used to determine enzyme activity. We assessed the activity of superoxide dismutase (SOD) using the methods described by Beauchamp and Fridovich (1971), with the radiation absorption value read at 560 nm. Ascorbate peroxidase (APX) activity was determined as the decrease in absorbance of ascorbate at 290 nm, using the method described by Nakano and Asada (1981). The activity of catalase (CAT) was determined using the method of Cakmak and Marschner (1992). Absorbance was recorded by an *Infinite M200 Pro* microplate reader (*Tecan Group*).

Hydrogen peroxide and $O_2^{\bullet-}$ content were determined from a standard curve, as described by Benikhlef *et al*. (2013). Absorption values were recorded at 415 and 530 nm using a spectrophotometer (*UV-160A*, *Shimadzu Scientific Instruments*, Kyoto, Japan).

Gene expression values: We evaluated the expression of *SikTrxh* gene in roots, stems, and leaves of *S. involucrata* growing under cold, drought, or salt stress. The *S. involucrata* seedlings with the same size and phenotype were selected for stress treatments after six weeks of growth. For the cold stress, the *S. involucrata* seedlings were treated at 4 °C for 24 h. For the drought stress, the *S. involucrata* seedlings were then taken out of the medium and submerged in 20 % (m/v) PEG 6000 for 24 h. For the salt stress, the *S. involucrata* seedlings were taken out of the medium, and their roots were submerged in 200 mmol NaCl for 24 h. Untreated plants were used as the controls. All plants were cultured in a 21 °C (daily) and 19 °C (overnight) cycle incubator in parallel. The leaves at the same position were harvested at 0, 1, 3, 6, 12, and 24 h during the process of the cold, drought, or salt treatments to measure the expression patterns of *SikTrxh* gene.

The leaves of wild-type and transgenic T_2 tobacco plants treated with the above mentined stress conditions were also used for the ROS-related stress-responsive genes superoxide dismutase (*NtSOD*), ascorbate peroxidase (*NtAPX*), catalase (*NtCAT*), late embryogenesis-abundant protein (*NtLEA5*), and Δ1-pyrroline-5- carboxylate synthetase (*NtP5CS*). Regularly watered plants grown in a greenhouse at 25 ºC were used as control samples.

Three replicates were collected and stored in liquid nitrogen at -80 °C for RNA isolation. Total RNA was extracted from the samples using *TRIzol* reagent (*Takara Biotechnology*), following the manufacturer's protocol. First-strand cDNA was synthesized using Oligo (dT) primer and *PrimeScript RTase* (*Takara Biotechnology*). Transcripts were then quantified by PCR analysis using *SYBR Premix Ex Taq* (*Takara Biotechnology*). Each RT-PCR reaction was performed in 25 mm^3 of final volume on *iQ5 Multicolor* real time PCR detection system (*Bio-Rad*, Hercules, USA). The sequences of primers used for gene expression analysis in this study are listed in Table 1 Suppl.

Statistical analysis: *GraphPad Prism 7.0* and *SigmaPlot 12* (SYSTAT software) were used for statistical analysis of the data. Relative expression of *SikTrxh* under abiotic stresses are reported as means ± standard deviation of three replicate samples, leaves from three individual seedlings were considered as a replicate sample. Significant differences between the wild-type plants and each transgenic plant line were determined using Duncan's multiple range test.

Results

The full-length cDNA of *SikTrxh* consisted of 565 bp with a 354-bp open reading frame that encodes a 117 amino acid protein. To better understand the features of SikTrxh, Fig. 1*A* shows the sequence analysis of Trxh proteins isolated from *S. involucrata* and other plant species. It can be seen that SikTrxh contained the typical conserved dithiol/disulfide active site sequence WCGPC. A phylogenetic tree was constructed with rice and *Arabidopsis* h-type Trxs. The results showed that *SikTrxh1*, together with *OsTRXh1*, *OsTRXh2*, *OsTRXh3*, *OsTRXh6*, *AtTRXh1*, *AtTRXh3*, *AtTRXh4* and *AtTRXh5*, belongs to the TRXh subgroup I (Fig. 1*B*).

We used GFP as a fusion protein marker to identify the localization of the protein. In epidermal cells of tobacco leaves transiently expressing SikTrxh-GFP, GFP signals were localized to the mitochondria (Fig. 2*A*). The mitochondrial localization of SikTrxh-GFP was confirmed by co-localization with the mitochondrial marker MT-rk (Fig. 2*B-D*).

To investigate *SikTrxh* expression in different organs of *S. involucrata*, we used real time quantitative RT-PCR to detect *SikTrxh* in the plant roots, stems, and leaves. *SikTrxh* was expressed in all the organs, but the highest expressions were recorded in the roots, followed by the leaves and the stems (Fig. 3*A*).

The relative expression of *SikTrxh* under different

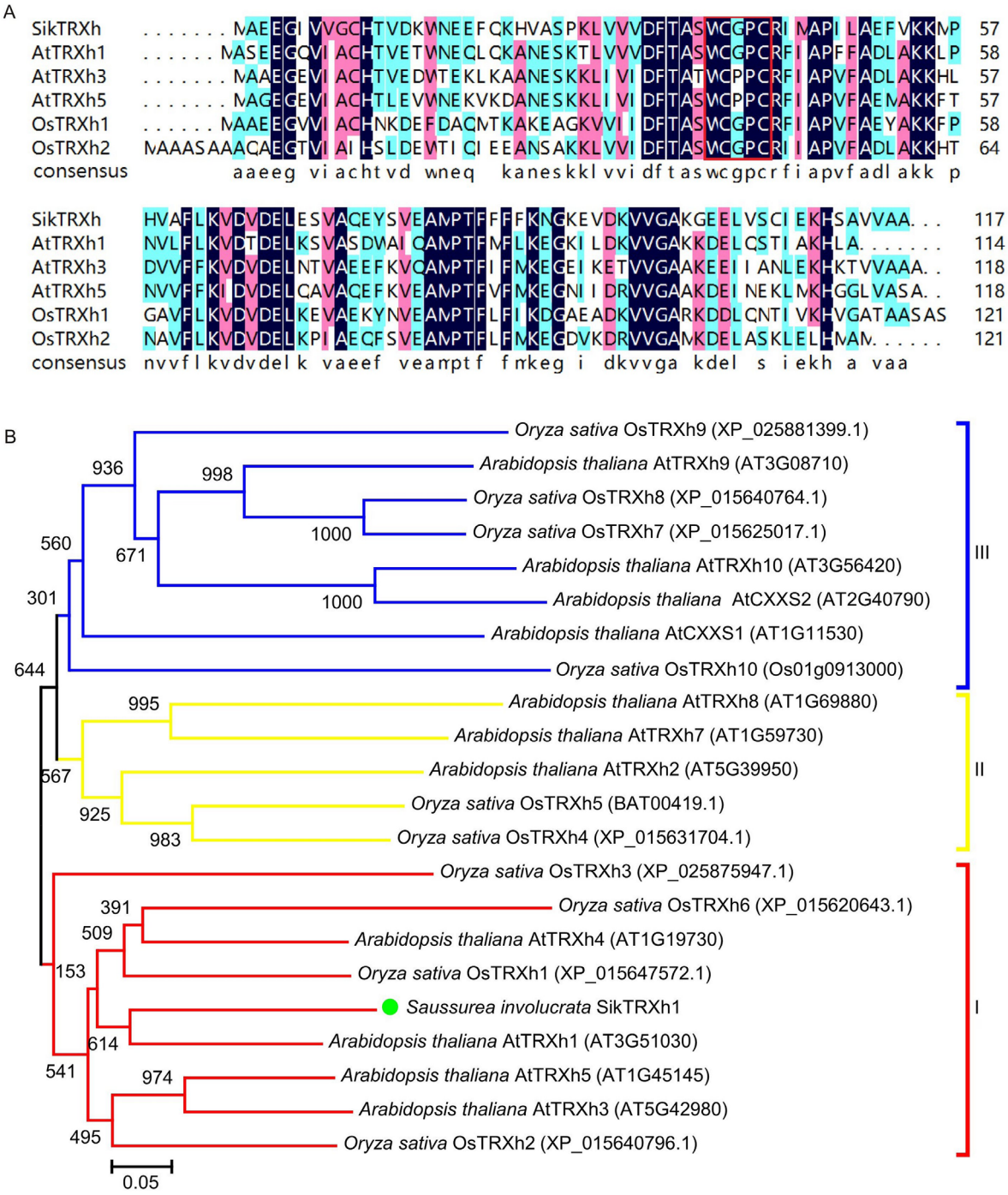

Fig. 1. Sequence analysis of *Saussurea involucrata thioredoxin-h* (*SikTrxh*). *A* - sequence comparison between the SikTrxh protein and homologous proteins from other species. *B* - phylogenetic relationships of plant *TRXh* genes; *Oryza sativa japonica*: *OsTRXh1* (XP_015647572.1), *OsTRXh2* (XP_015640796.1), *OsTRXh3* (XP_025875947.1), *OsTRXh4* (XP_015631704.1), *OsTRXh5* (BAT00419.1), *OsTRXh6* (XP_015620643.1), *OsTRXh7* (XP_015625017.1), *OsTRXh8* (XP_015640764.1), *OsTRXh9* (XP_025881399.1), and *OsTRXh10* (Os01g0913000). *Arabidopsis thaliana*: *AtTRXh1* (AT3G51030), *AtTRXh2* (AT5G39950), *AtTRXh3* (AT5G42980), *AtTRXh4* (AT1G19730), *AtTRXh5* (AT1G45145), *AtTRXh7* (AT1G59730), *AtTRXh8* (AT1G69880), *AtTRXh9* (AT3G08710), *AtTRXh10* (AT3G56420), *ATCXXS1* (AT1G11530), and *ATCXXS2* (AT2G40790).

abiotic stresses was detected by quantitative RT-PCR. To test response to cold temperature, expression of *SikTrxh* transcripts in *S. involucrata* was initially induced with exposure to cold, a temperature of 4 ºC (Fig. 3*B*). The gene reached its highest expression at 1 h. Expression decreased slightly after that, then gradually decreased to levels close to the control expression at 24 h. To test for exposure to drought, expression of *SikTrxh* was induced

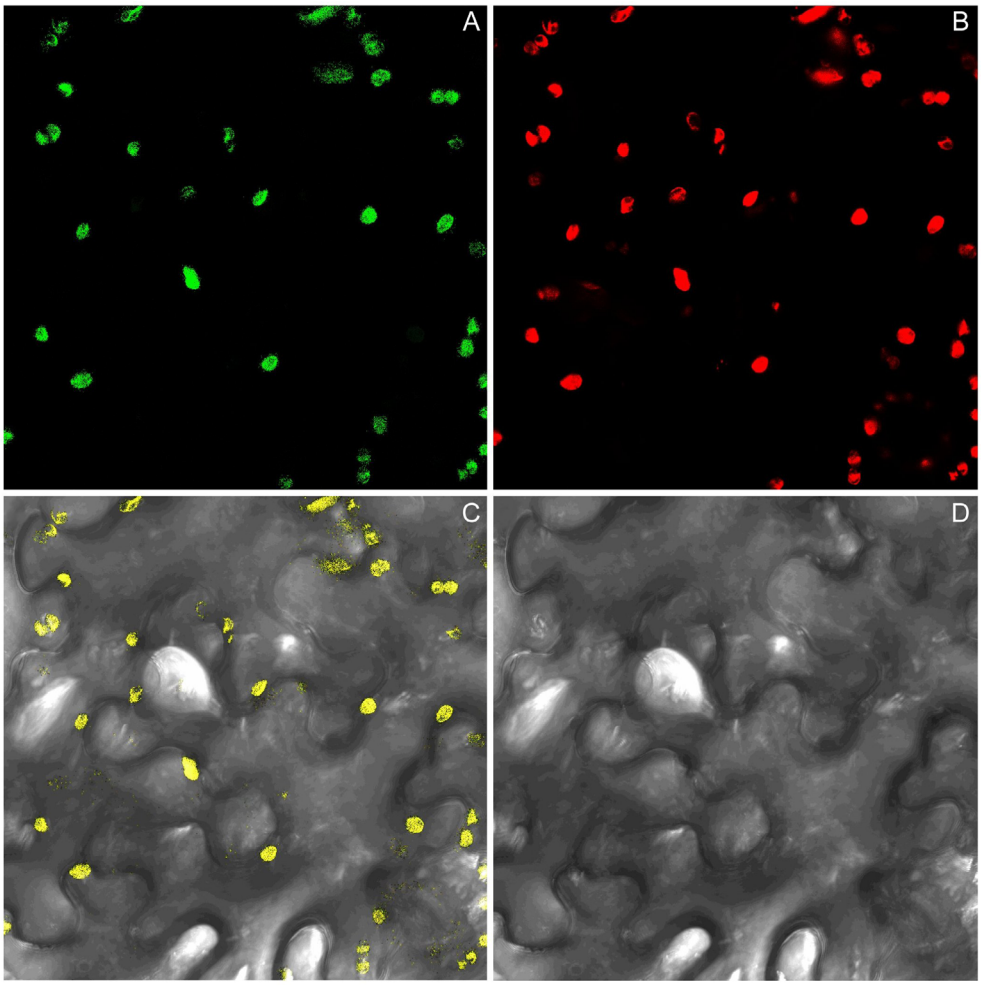

Fig. 2. Subcellular localization of *Saussurea involucrata* thioredoxin-h (SikTrxh). Confocal images of tobacco leaf epidermal cells transiently expressing SikTrxh-green fluorescent protein (GFP) and a mitochondria marker (MT-rk). *A* - fluorescence image of tobacco leaf epidermal cells expressing the SikTrxh-GFP fusion protein. *B* - fluorescence image of mitochondria in tobacco leaf epidermal cells with MT-rk. *C* - merged fluorescence image of tobacco leaf epidermal cells expressing the SikTrxh-GFP fusion protein and MT-rk marker. *D* - image of tobacco leaf epidermal cells under bright light. *A*,*B* - dark field and *C*,*D* - bright field.

when *S. involucrata* was exposed to 10 % (m/v) PEG-6000, reaching peak expression after 3 h (Fig. 3*C*). Expression gradually decreased, although expressions at 6 h were still three times higher than of the pre-treatment control. After 12 h, expression was one third that of the control, and after 24 h, only one fifth that of the control. In the test of exposure to salt, after 1 h in 150 mM NaCl, *SikTrxh* expression increased rapidly, reaching expression nearly five times that of the control (Fig. 3*D*). Expression then gradually decreased, after 3 h, expression was just less than four times that of the control, after 6 h, 2.5 times that of the control, and after 12 h, less than two times that of the control. After 24 h, expression slightly increased to over three times that of the control. These results indicate that *SikTrxh* expression was induced by low temperature, drought, and salt stress. We hypothesize that this gene regulates stress responses in *S. involucrata*.

We generated five independent kanamycin-resistant primary transgenic tobacco plants (T_0 generation). PCR analysis confirmed that the *SikTrxh* gene was successfully introduced into the plants. Three of the transgenic tobacco plants were confirmed by PCR amplification using a CaMV35S forward and gene-specific reverse primer pair. We selected two independent transgenic tobacco lines for further study (OE-2 and OE-3). Semiquantitative RT-PCR and quantitative RT-PCR showed that both tobacco lines showed *SikTrxh* expression, and *SikTrxh* expression was slightly higher in the OE-2 line than in the OE-3 line (Fig. 1 Suppl.).

Both the wild-type and the transgenic plants performed well under normal conditions, and there were no significant differences in their growth (Fig. 2 Suppl.). After the 4 ºC treatment, wilting was observed in wild-type plants, but the growth of the overexpressing *SikTrxh* plants was not affected, they had sturdy and erect stems with green ovate-lanceolate leaves. At -1 °C, the stems of the wild-type plants became flaccid and the leaves displayed obvious wilting, whereas the stem and leaves of the two transgenic plants sagged slightly. When subjected to -2 °C for 2 h, the leaves of the wild-type plants became severely wilted, and the

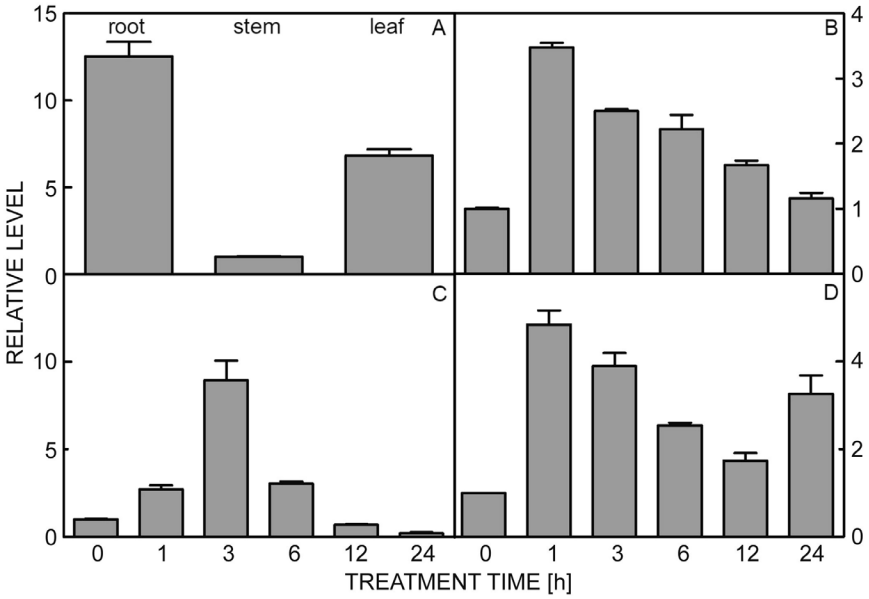

Fig. 3. Expression pattern analyses of *Saussurea involucrata thioredoxin-h* (*SikTrxh*). *A* - tissue-specific expressions of *SikTrxh* in leaf, root, and stem of 6-week-old *S. involucrata*. *B* - expression of *SikTrxh* in *S. involucrata* under low temperature treatment (4 °C) for 0, 1, 3, 6, 12, and 24 h. *C* - expression of *SikTrxh* in *S. involucrata* under drought treatment caused by 10 % (m/v) PEG 6000 for 0, 1, 3, 6, 12, and 24 h. *D* - expression of *SikTrxh* in *S. involucrata* under 150 mM NaCl treatment for 0, 1, 3, 6, 12, and 24 h.

plants were no longer viable. In contrast, the leaves of the OE-2 line showed moderate wilting and those of the OE-3 line showed slight wilting. All transgenic plants remained viable after the cold treatment. We tested the survival rates of the plants by seedling analysis. The tobacco seedlings showed no differences under normal conditions, but after 8-h exposure to -2 ºC, all seedlings wilted. They quickly recovered after 2-d exposure to 25 ºC. The survival rate of the control group was 20 %, but the survival rates of the transgenic strains were 60 and 40 % (Fig. 2 Suppl.).

The transgenic lines showed increased resistance to drought stress. The transgenic plants began to wilt after 9 d of drought stress, while the wild-type plants began to wilt after 6 d. After 9 d of drought treatment, the wild-type plants were thoroughly wilted (Fig. 3 Suppl.). Similarly, after 12 d of drought treatment, the control seedlings turned yellow. After a 2 d recovery period, the control group survival rate was 27.5 %, and the survival rates of the two transgenic strains were 55 and 45 % (Fig. 3 Suppl.). These results suggest that *SikTrxh* is important in increasing drought tolerance in tobacco plants.

Salt stress causes water shortages in tissues. This results in cellular osmotic stress and the accumulation of reactive oxygen species. A 150 mM NaCl solution was used to test the salt tolerance of six-week-old wild-type and transgenic T_2 plants. After 6 d of treatment with the NaCl solution, the leaves of the wild-type tobacco plants yellowed but the leaves of the transgenic plants did not change color. After 9 d, the leaves of the wild-type plants were severely dehydrated and the leaves of transgenic plants began to turn yellow. Most of the leaves of both types of plant, had thickened. After 12 d, most of the wild-type plants was withered and the leaves of the transgenic strains were yellowed (Fig. 4 Suppl.). When two-week-old wild-type and transgenic plants were treated with salt stress for 12 d, the transgenic plants were in a better conditions than the wild-type plants. The survival rates of the transgenic plants were 60 and 46 % and the survival rate of the wild-type plants was 22 % (Fig. 4 Suppl.).

We found that malondialdehyde (MDA) content increased and relative electrolyte leakage (REL) became elevated when the wild-type and transgenic plants were exposed to cold, drought, and salt stresses (Fig. 4*A-B*). Both MDA and REL were considerably higher in the wild-type plants than in the transgenic lines. The differences were significant ($P < 0.01$). Proline accumulation also increased in all plants after stress but more in the transgenic plants than in the wild-type plants (Fig. 4*C*). Proline content in the wild-type plants was significantly lower than in the transgenic plants ($P < 0.01$). The OE-2 line consistently had the highest proline content. Before the stress treatments, the maximum photochemical efficiency of photosystem II (F_v/F_m) was almost equal for wild-type and transgenic plants (Fig. 4*D*). The F_v/F_m decreased continuously in all plants under salt, cold, and drought stresses. However, the decrease was more pronounced in the wild-type plants, and the differences between wild-type and transgenic plants were significant ($P < 0.01$). Chlorophyll content was significantly higher in the transgenic plants than in the wild-type plants (Fig. 4*E*).

Superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) activities were measured to investigate effects of *SikTrxh* expression on reactive oxygen species scavenging under salt, cold, heat, and drought stresses. The reactive oxygen species hydrogen peroxide (H_2O_2) and the superoxide anion ($O_2^{\bullet-}$) were also measured. We found that SOD, APX, and CAT activities varied in response to salt, cold, and drought stresses

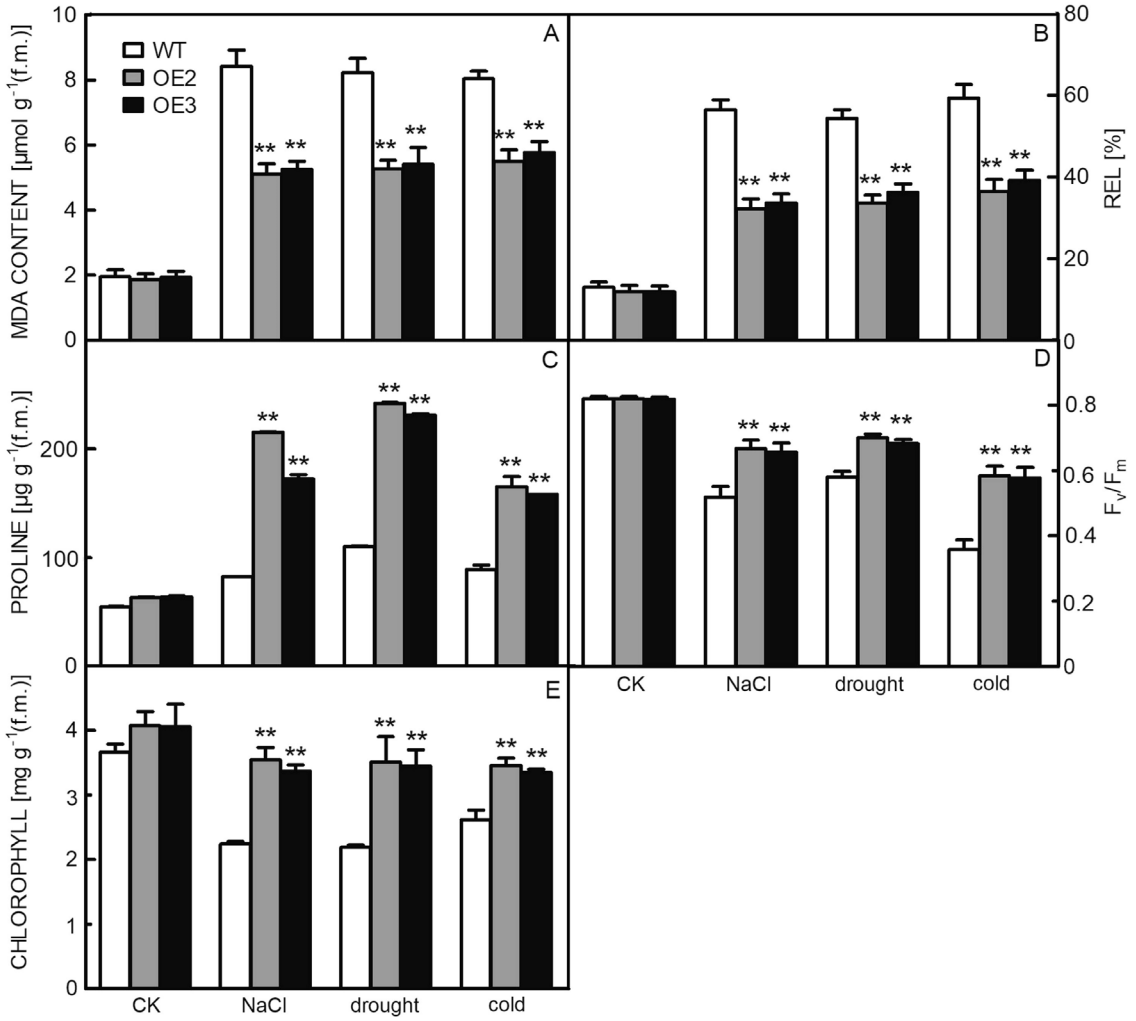

Fig. 4. Physiological analysis of wild-type and transgenic *Saussurea involucrata thioredoxin-h* tobacco plants after salt (NaCl), drought, and cold stresses. Malondialdehyde (MDA) content (*A*), relative electrolyte leakage (REL) (*B*), proline content (*C*), maximal photochemical efficiency of photosystem II (F_v/F_m) (*D*), and chlorophyll content (*E*). CK - 25 °C under normal conditions. Data for the wild-type plants are means ± SEs of three replicates, and data for the transgenic plants are means ± SEs of three different lines; ** -significant differences ($P < 0.01$).

(Fig. 5*A-C*). Transgenic lines routinely showed higher enzyme activities than the wild-type plants. The differences between wild-type and transgenic plants in each treatment were significant. The *SikTrxh*-overexpressing line OE-2 regularly showed the highest enzyme activity.

Before the salt, cold, and drought stress tests, H_2O_2 and $O_2^{\bullet-}$ content was low and almost identical between the wild-type and transgenic plants (Fig. 5*D-E*). Following exposure to salt, cold, and drought stresses, ROS content increased, considerably more in the wild-type plants than in the transgenic plants. Differences between wild-type and transgenic plants were significant for both the cold and drought stresses.

Gene expression patterns of the tobacco genes for stress response (*NtSOD*, *NtAPX*, and *NtCAT*) and membrane protection (*NtLEA5* and *NtP5CS*) were determined for wild-type and transgenic plants. Transgenic lines showed significantly higher gene expressions than wild-type plants for each treatment (Fig. 6).

Discussion

Abiotic stresses such as salt, drought, and cold caused persistent damage and seriously affected crop growth and yield. Major abiotic stresses can be expected to damage more than 50 % of a crop. The impact on a crop may be even more severe due to instability caused by environmental degradation (Mittler *et al.* 2006). Plants are more susceptible to environmental factors than animals because they are fixed in the soil and lack mobility. Plants have evolved complex mechanisms to improve their tolerance to abiotic and biotic stresses (Nakashima *et al.* 2006). Plants growing in extreme environments have been naturally selected to form stable genetically controlled structures and functions that result in physiological and biochemical mechanisms that adapt the plants to these conditions. *S. involucrata*, a medicinal plant found in Xinjiang, is one such plant. *S. involucrata* is a typical alpine perennial plant that has strong antioxidant and free

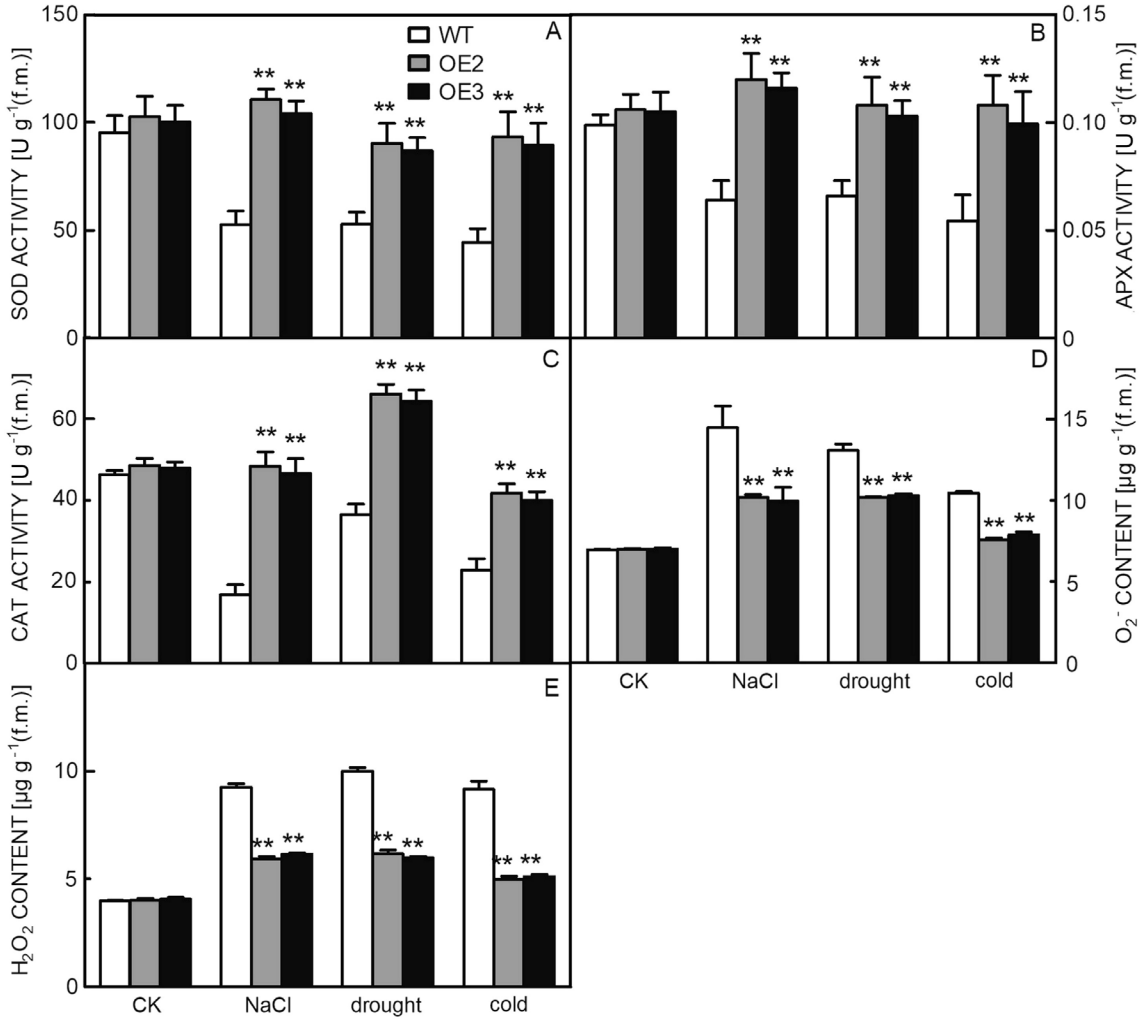

Fig. 5. Antioxidant enzyme activities and accumulation of reactive oxygen species in wild-type and transgenic *Saussurea involucrata thioredoxin-h* plant lines (OE-2 and OE-3) under salt (NaCl), drought, and cold stresses. SOD superoxide dismutase, APX - ascorbate peroxidase, CAT - catalase, $O_2^{\bullet-}$ - superoxide radical anions. CK - 25 °C under normal conditions. Data for the wild-type and transgenic plants are means ± SEs of three replicates; ** - significant differences between wild-type and transgenic plants at $P < 0.01$.

radical scavenging abilities that adapt it to the extreme environment.

In this study, a new thioredoxin gene *SikTrxh* was cloned using the full-length cDNA library RT-PCR. Analysis of the SikTrxh amino acid sequence shows that the gene belongs to the first subgroup of the plant thioredoxin gene family, *TRXh*, and it is located in the mitochondria. Plant mitochondria are the main sites of ROS production (Venditti *et al*. 2014). Abiotic stressors, such as drought, cold, and salt, increase ROS production and so lead to ROS-related damage (Giraud *et al*. 2008).

Verification of gene expression by quantitative RT-PCR provided basic information on probable gene functions. Many new genes have been identified by functional analysis or gene expression analysis from organisms subjected to different stresses (Bruzzone *et al*. 2008, Jozefczuk and Adjaye 2011). Studies have shown that *Trxh* is expressed in response to abiotic stress. For example, the expression profile of plant thioredoxin varies in different tissues (Reichheld *et al*. 2002). The thioredoxin gene *ScTRXh1* was isolated from sugar cane and classified into the 1 subgroup of thioredoxin genes. Studies have also shown that the relative abundance of *ScTRXh1* expressed in sugar cane roots, stems, and buds was higher by a factor of 12 - 33 than in the leaves (Guo *et al*. 2012). However, the *SikTrxh* used in this study had different expression characteristics. The *SikTrxh* was principally expressed in the roots, followed by the leaves, and little in the stems. This result suggests that *SikTrxh* has different biological functions in *S. involucrata*. Zhang *et al*. (2008) cloned the thioredoxin gene *LbTRXm2* of *Limonium bicolor* and found that the gene was inhibited when the plant was subjected to low temperature, salt (NaCl), or PEG stress. Wang *et al*. (2011) showed that after salt stress, *GmTRXh1* expression in salt-tolerant soybean cultivars is significantly higher than in salt-sensitive cultivars. Yan *et al*. (2006) showed that the expression of thioredoxin in rice is induced by low temperature stress. Serrato *et al*. (2003) showed that under oxidative stress, thioredoxin is enriched in the nucleus of wheat seeds. However, we found that *SikTrxh* showed

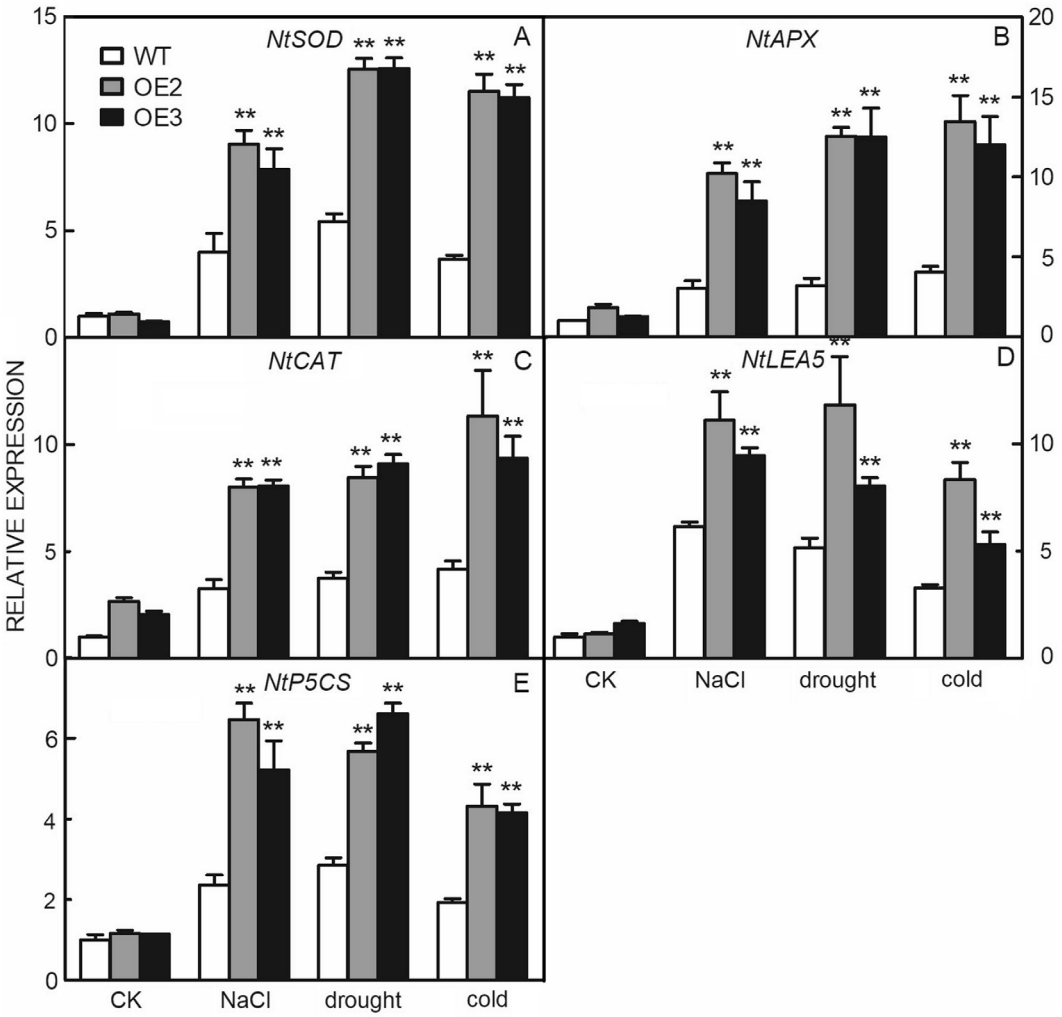

Fig. 6. Relative expressions of *Nicotiana tabacum superoxide dismutase* (*NtSOD*) (*A*), *Nicotiana tabacum ascorbate peroxidase* (*NtAPX*) (*B*), *Nicotiana tabacum catalase* (*NtCAT*) (*C*), *Nicotiana tabacum late embryogenesis-abundant protein* (*NtLEA5*) (*D*), and *Nicotiana tabacum Δ1-pyrroline-5- carboxylate synthetase* (*NtP5CS*) (*E*) in wild-type and transgenic *Saussurea involucrata thioredoxin-h* plants (lines OE-2 and OE-3) under salt (NaCl), drought, and cold stresses. CK - 25 °C under normal conditions. Data are means ± SEs of three replicates; ** - significant differences between wild-type and transgenic plants at $P < 0.01$.

significant up-regulated and down-regulated changes. Some characteristics of thioredoxin gene expression in *S. involucrata* suggest that *TRX* gene functionality differs depending on its source. The results of our study not only enriched the study on thioredoxin gene of *S. involucrata*, but also form the foundation of a deeper investigation into the function of *SikTrxh* and its activity in resistance to abiotic stress.

The phenomenon of abiotic stress causing excessive production of reactive oxygen species in plants and leading to oxidative damage of biological macromolecules has been confirmed in soybean, pea, barley, wheat, *Arabidopsis thaliana* and other plants (Holmgren *et al*. 1989, Meyer et al. 2005, Delisle *et al*. 2001, Yamamoto *et al*. 2001, Darkó *et al*. 2004, Tamás *et al*. 2004). When a plant is under stress, its defense system enzymes will be activated to resist any damage caused by the stress (Gelhaye *et al*. 2004). Trxh is an important redox agent. The expression of *Trxh* genes is often associated with an increase in oxygen anions. Plant antioxidant responses can be involved in a variety of pathways. Li *et al*. (2010) found *TRX* gene overexpression in barley together with decreased H_2O_2 and MDA content. Hence, the study shows that *TRX* genes can effectively relieve H_2O_2 oxidation damage of barley seedling leaves. Also the ability of seedlings to resist aluminum stress was enhanced, and the tolerance to high temperature, drought, and strong irradiance at the later stage of grain filling was improved. Prabu *et al*. (2011) showed that the expression of the sugarcane *ScNAC23* transcription factor gene highly interacted with the *thioredoxin h1* gene, which may be due to regulation by the formation of disulfide bonds between *ScNAC23* cysteine residues and sugarcane thioredoxin, when induced by low temperature (4 ºC) and drought stress. Zhang *et al*. (2011) found that *OSTRXh1*, which can be secreted into the ectoplasmic body, was involved in the regulation of the ectoplasmic reactive oxygen species, and thus affected the growth, development, and stress response of rice. These studies provide important theoretical basis

for the importance of *TRX* genes in plant resistance.

The transgenic *SikTrxh* tobacco strains were more resistant to low temperature, drought, and high salinity than the wild-type plants. *SikTrxh* gene overexpression reduces the damage caused by various plant stressors, SikTrxh activates the anti-stress signaling pathway of tobacco, thereby increasing stress resistance in the transgenic plants.

Many studies have shown that abiotic stress leads to ROS accumulation, mainly in the form of H_2O_2 and $O_2^{\bullet-}$. These important signal transduction molecules increase intracellular free Ca^{2+} and act as secondary signal messengers (Misra *et al*. 2009). However, excessive amounts of H_2O_2 and $O_2^{\bullet-}$ during stress can lead to malondialdehyde accumulation and membrane damage. Membrane damage and lipid peroxidation have been used to indicate plant tolerance to abiotic stress (Wang *et al*. 2012). We used change in MDA content and relative membrane conductivity to analyze membrane lipid peroxidation and membrane damage under different stresses. Our results showed that different stresses caused severe membrane lipid peroxidation and cell membrane damage in tobacco, which was consistent with the results of previous studies. Transgenic tobacco was significantly less susceptible to membrane lipid peroxidation and membrane damage than wild-type tobacco (the control group). Free proline is also important in plant self-protection and cellular proline content is directly related to stress resistance (Sleator *et al*. 2001). Our results showed that under different stress treatments, proline content in the leaves of transgenic tobacco plants was significantly higher than in wild-type plants, thus confirming the stress resistance of transgenic tobacco plants.

Membrane damage and elevated content of ROS can lead to chlorophyll degradation (Stepien *et al*. 2009). We found that chlorophyll content was higher in transgenic *SikTrxh*-overexpressed tobacco than in wild-type plants, likely due to high antioxidant enzyme activities in the transgenic plants. SOD, CAT, and APX activities were significantly higher in the transgenic plants than in the wild-type plants. These three enzymes are important components of the antioxidant enzyme system: CAT directly catalyzes hydrogen peroxide (H_2O_2) to produce water and oxygen, APX takes ascorbic acid as the substrate to decompose H_2O_2, and SOD is a key enzyme in the ROS scavenging system that rapidly transforms $O_2^{\bullet-}$ into O_2 or H_2O_2. The increased activity of these antioxidant enzymes removes reactive oxygen species produced by stress and thus protects macromolecules from oxidative damage.

Corresponding to enzyme activity, the expression of encoding genes (*NtSOD*, *NtCAT*, and *NtAPX*) were significantly higher in transgenic strains OE-2 and OE-3 than in the wild-type plants. It is generally thought that the redox state of proteins is controlled by the oxidation-reducing agents of cells, Trx is an important oxidation-reducing agent. Some enzymes belonging to antioxidants contain conserved cysteine residues. Catalase, glutathione peroxidase, peroxidase, SOD (Cu-Zn), APX, and 2-cys peroxiredoxins contain 5, 1, 8, 2, 2, and 1 conserved cysteine residues, respectively, which may be targets of Trxh (Wong *et al*. 2004). It is possible that the reduced Trxh can transfer electrons to these enzymes, thus providing a reduction equivalent for the regulation of enzyme activity, or it may be that Trxh is involved in the regulation of signal cascades dependent on the redox state; this area needs further investigation.

Conclusions

The *SikTrxh* gene was isolated from the *S. involucrata*. Overexpression of the *SikTrxh* gene increased the survival rate of transgenic tobacco plants subjected to low temperature, drought, or salt. The transgenic tobacco plants showed increased activity of CAT, SOD, APX and other antioxidant enzymes which could remove ROS produced in response to low temperature, drought, and salt stress more effectively than in the wild type plants. The enhanced enzyme activities also reduced oxidative damage to membrane lipids and proteins, thus increasing the resistance of transgenic tobacco plants to low temperature, drought, and salt stresses. To better counter abiotic stresses, it is necessary to breed new stress-resistant plant cultivars. Our experiments with transgenic tobacco plants showed that a *TRXh* gene can increase plant resistance to low temperature, drought, and high salt stresses by increasing antioxidant enzyme activities and therefore provide a theoretical foundation for the cultivation of resistant crop cultivars.

References

Baumann, U., Juttner, J.: Plant thioredoxins: the multiplicity conundrum. - Cell. mol Life Sci. **59**: 1042-1057, 2002.

Beauchamp, C., Fridovich, I.: Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. - Anal. Biochem. **44**: 276-287, 1971.

Benikhlef, L., L'Haridon, F., Abou-Mansour, E., Serrano, M., Binda, M., Costa, A., Lehmann, S., Métraux, J.P.: Perception of soft mechanical stress in *Arabidopsis* leaves activates disease resistance. - BMC Plant Biol. **13**: 1-12, 2013.

Bruzzone, C.M., Belcher, J.D., Schuld, N.J., Newman, K.A., Vineyard, J., Nguyen, J., Chen, C., Beckman, J.D., Steer, C.J., Vercellotti, G.M.: Quantitative real-time polymerase chain reaction (qRT-PCR) restriction fragment length polymorphism (RFLP) method for monitoring highly conserved transgene expression during gene therapy. - Translational Res. **152**: 290-297, 2008.

Buchanan, B.B., Balmer, Y.: Redox regulation: A broadening horizon. - Annu. Rev. Plant Biol. **56**: 187-220, 2005.

Cabrillac, D., Cock, J.M., Dumas, C., Gaude, T.: The S-locus receptor kinase is inhibited by thioredoxins and activated by pollen coat proteins. - Nature **410**: 220-223, 2001.

Cakmak, I., Marschner, H.: Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. - Plant Physiol. **98**: 1222-1227, 1992.

Delisle, G., Champoux, M., Houde, M.: Characterization of oxalate oxidase and cell death in Al-sensitive and tolerant wheat roots. - Plant Cell Physiol. **42**: 324-333, 2001.

Du, Z., Bramlage, W.J.: Modified thiobarbituric acid assay for measuring lipid oxidation in sugar-rich plant tissue extracts. -

J. Agr. Food Chem. **40**: 1566-1570, 1992.
Darkó, É., Ambrus, H., Stefanovits-Bányai, É., Fodor, J., Bakos, F., Barnabás, B.: Aluminium toxicity, Al tolerance and oxidative stress in an Al-sensitive wheat genotype and in Al-tolerant lines developed by *in vitro* microspore selection. - Plant Sci. **166**: 583-591, 2004.
Fomenko, D.E., Gladyshev, V.N.: CxxS: fold-independent redox motif revealed by genome-wide searches for thiol/disulfide oxidoreductase function. - Protein Sci. **11**: 2285-2296, 2002.
Gelhaye, E., Rouhier, N., Gerard, J., Jolivet, Y., Gualberto, J., Navrot, N., Ohlsson, P.I., Wingsle, G., Hirasawa, M., Knaff, D.B., Wang, H., Dizengremel, P., Meyer, Y., Jacquot, J.P.: A specific form of thioredoxin h occurs in plant mitochondria and regulates the alternative oxidase. - Proc. nat. Acad. Sci. USA **101**: 14545-14550, 2004.
Gelhaye, E., Rouhier, N., Jacquot, J.P.: The thioredoxin *h* system of higher plants. - Plant Physiol. Biochem. **42**: 265-271, 2004.
Gelhaye, E., Rouhier, N., Navrot, N., Jacquot, J.P.: The plant thioredoxin system. - Cell. mol. Life Sci. **62**: 24-35, 2005.
Giraud, E., Ho, L.H.M., Clifton, R., Carroll, A., Estavillo, G., Tan, Y.F., Howell, K.A., Ivanova, A., Pogson, B.J., Millar, A.H., Whelan, J.: The absence of alternative oxidase 1a in *Arabidopsis* results in acute sensitivity to combined light and drought stress. - Plant Physiol. **147**: 595-610, 2008.
Gromer, S., Urig, S., Becker, K.: The thioredoxin system–from science to clinic. - Med. Res. Rev. **24**: 40-89, 2004.
Gromer, S., Wissing, J., Behne, D., Ashman, K., Schirmer, R.H., Flohé, L., Becker, K.: A hypothesis on the catalytic mechanism of the selenoenzyme thioredoxin reductase. Biochem J. **332**: 591-592, 1998.
Guo, J.L., Zheng, R., Ling, H., Fu, H.Y., Su, Y.C., Wang, H.B.: Cloning and expression analysis of a thioredoxin gene *ScTRXh1* from sugarcane (*Saccharum complex*). - Genomics appl. Biol. **6**: 574-581, 2012.
Hall, M., Mata-Cabana, A., Akerlund, H.E., Florencio, F.J., Schröder, W.P., Lindahl, M., Kieselbach, T.: Thioredoxin targets of the plant chloroplast lumen and their implications for plastid function. - Proteomics **10**: 987-1001, 2010.
Hägglund, P., Björnberg, O., Navrot, N., Jensen, J.M., Maeda, K., Kirkensgaard, K., Shahpiri, A., Sultan, A., Bunkenborg, J., Gubler, F., Barrero, J.M., Henriksen, A., Finnie, C., Svensson, B.: The barley grain thioredoxin system - an update. - Front. Plant Sci. **4**: 151, 2013.
Holmgren, A.: Thioredoxin and glutaredoxin systems. - J. Biol. Chem. **264**: 13963-13966, 1989.
Jozefczuk, J., Adjaye, J.: Quantitative real-time PCR-based analysis of gene expression. - Methods Enzymol. **500**: 99-109, 2011.
Li, Q.Y., Niu, H.B., Ma, Y., Ren, J.P., Li, Y.C., Yin, J.: Effects of overexpressing *Trxs* on antioxidant enzymes activities in transgenic barley seedling leaves under ultraviolet B stress. - J. Henan agr. Univ. **4**: 365-369, 2010.
Lutts, S., Kinet, J.M., Bouharmont, J.: NaCl-induced senescence in leaves of rice (*Oryza sativa* L.) cultivars differing in salinity resistance. - Ann. Bot. **78**: 389-398, 1996.
Marcus, F., Chamberlain, S.H., Chu, C., Masiarz, F.R., Shin, S., Yee, B.C., Buchanan, B.B.: Plant thioredoxin h: an animal-like thioredoxin occurring in multiple cell compartments. - Arch. Biochem. Biophys. **287**: 195-198, 1991.
Meng, L., Wong, J.H., Feldman, L.J., Lemaux, P.G., Buchanan, B.B.: A membrane-associated thioredoxin required for plant growth moves from cell to cell, suggestive of a role in intercellular communication. - Proc. nat. Acad. Sci. USA **107**: 3900-3905, 2010.
Meyer, Y., Belin, C., Delorme-Hinoux, V., Reichheld, J.P., Riondet, C.: Thioredoxin and glutaredoxin systems in plants: molecular mechanisms, crosstalks, and functional significance. - Antioxid. Redox Signal. **17**: 1124-1160, 2012.
Meyer, Y., Buchanan, B.B., Vignols, F., Reichheld, J.P.: Thioredoxins and glutaredoxins: unifying elements in redox biology. - Annu. Rev. Genet. **43**: 335-367, 2009.
Meyer, Y., Reichheld, J.P., Vignols, F.: Thioredoxins in *Arabidopsis* and other plants. - Photosynth. Res. **86**: 419-433, 2005.
Misra, M.K., Sarwat, M., Bhakuni, P., Tuteja, R., Tuteja, N.: Oxidative stress and ischemic myocardial syndromes. - Med. Sci. Monitor: internat. med. J. exp. clin. Res. 15: 209-219, 2009.
Mittler, R., Kim, Y., Song, L., Coutu, J., Coutu, A., Ciftci-Yilmaz, S., Lee, H., Stevenson, B., Zhu, J.K.: Gain- and loss-of-function mutations in *Zat10* enhance the tolerance of plants to abiotic stress. - FEBS Lett. **11**: 6537-6542, 2006.
Montrichard, F., Alkhalfioui, F., Yano, H., Vensel, W.H., Hurkman, W.J., Buchanan, B.B.: Thioredoxin targets in plants: the first 30 years. - J. Proteomics **72**: 452-474, 2009.
Nakano, Y., Asada, K.: Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplast. - Plant Cell Physiol. **22**: 867-880, 1981.
Nakashima, K., Fujita, Y., Katsura, K., Maruyama, K., Narusaka, Y., Seki, M., Shinozaki, K., Shinozaki, K.Y.: Transcriptional regulation of ABI3- and ABA-responsive genes including *RD29B* and *RD29A* in seeds, germinating embryos, and seedlings of *Arabidopsis*. - Plant mol. Biol. **60**: 51-68, 2006.
Nelson, B.K., Cai, X., Nebenführ, A.: A multicolored set of *in vivo* organelle markers for co-localization studies in *Arabidopsis* and other plants. - Plant J. **51**: 1126-1136, 2007.
Nuruzzaman, M., Gupta, M., Zhang, C., Wang, L., Xie, W., Xiong, L., Zhang, Q., Lian, X.: Sequence and expression analysis of the thioredoxin protein gene family in rice. - Mol. Genet. Genomics **280**: 139-151, 2008.
Park, S.C., Jung, Y.J., Kim, I.R., Lee, Y., Kim, Y.M., Jang, M.K., Lee, J.R.: Functional characterization of thioredoxin h type 5 with antimicrobial activity from *Arabidopsis thaliana*. – Biotechnol. Bioprocess Eng. **22**: 129-135, 2017.
Park, S.K., Jung, Y.J., Lee, J.R., Lee, Y.M., Jang, H.H., Lee, S.S., Park, J.H., Kim, S.Y., Moon, J.C., Lee, S.Y., Chae, H.B., Shin, M.R., Jung, J.H., Kim, M.G., Kim, W.Y., Yun, D.J., Lee, K.O., Lee, S.Y.: Heat-shock and redox-dependent functional switching of an h-type *Arabidopsis* thioredoxin from a disulfide reductase to a molecular chaperone. - Plant Physiol. **150**: 552-561, 2009.
Prabu, G., Kawar, P.G., Pagariya, M.C., Prasad, D.T.: Identification of water deficit stress upregulated genes in sugarcane. - Plant mol. Biol. Rep. **29**: 291-304, 2011.
Reichheld, J.P., Mestres-Ortega, D., Laloi, C., Meyer, Y.: The multigenic family of thioredixin h in *Arabidopsis thaliana*: specific expression and stress response. - Plant Physiol. Biochem. **40**: 685-690, 2002.
Rivas, S., Rougon-Cardoso, A., Smoker, M., Schauser, L., Yoshioka, H., Jones, J.D.: CITRX thioredoxin interacts with the tomato Cf-9 resistance protein and negatively regulates defence. - Eur. mol. Biol. Organ. J. **23**: 2156-2165, 2004.
Rouhier, N., Gelhaye, E., Sautiere, P.E., Brun, A., Laurent, P., Tagu, D., Gerard, J., De Fa, E., Meyer, Y., Jacquot, J.P.: Isolation and characterization of a new peroxiredoxin from poplar sieve tubes that uses either glutaredoxin or thioredoxin as a proton donor. - Plant Physiol. **127**: 1299-1309, 2001.
Serrato, A.J., Cejudo, F.J.: Type-h thioredoxins accumulate in the nucleus of developing wheat seed tissues suffering oxidative stress. - Planta **217**: 392-399, 2003.
Serrato, A.J., Pérez-Ruiz, J.M., Cejudo, F.J.: Cloning of thioredoxin h reductase and characterization of the thioredoxin

reductase-thioredoxin h system from wheat. - Biochem. J. **367**: 491-497, 2002.

Sleator, R.D., Gahan, C.G., Hill, C.: Identification and disruption of the proBA locus in *Listeria monocytogenes*: role of proline biosynthesis in salt tolerance and murine infection. - Appl. environ. Microbiol. **67**: 2571-2577, 2001.

Stepien, P., Johnson, G.N.: Contrasting responses of photosynthesis to salt stress in the glycophyte *Arabidopsis* and the halophyte *Thellungiella*: role of the plastid terminal oxidase as an alternative electron sink. - Plant Physiol. 149: 1154-1165, 2009.

Sweat, T.A., Wolpert, T.J.: Thioredoxin h5 is required for victor insensitivity mediated by a *CC-NBS-LRR* gene in *Arabidopsis*. - Plant Cell **19**: 673-687, 2007.

Tamás, L., Šimonovičová, M., Huttová, J., Mistrík, I.: Aluminium stimulated hydrogen peroxide production of germinating barley seeds. - Environ. exp. Bot. **51**: 281-288, 2004.

Tetley, R.M., Thimann, K.V.: The metabolism of oat leaves during senescence: I. respiration, carbohydrate metabolism, and the action of cytokinins. - Plant Physiol. **54**: 294-303, 1974.

Van Kooten, O., Snel, J.F.H.: The use of chlorophyll fluorescence nomenclature in plant stress physiology. - Photosynth. Res. **25**: 147-150, 1990.

Venditti, P., Napolitano, G., Di Meo, S.: Role of enzymatic and non-enzymatic processes in H_2O_2 removal by rat liver and heart mitochondria. - J. Bioenerg. Biomembr. **46**: 83-91, 2014.

Wang, S., Liang, D., Li, C., Hao, Y., Ma, F., Shu, H.: Influence of drought stress on the cellular ultrastructure and antioxidant system in leaves of drought-tolerant and drought-sensitive apple rootstocks. - Plant Physiol. Biochem. **51**: 81-89, 2012.

Wang, W.Q., Hou W.S.: Clone and analysis of thioredoxin gene from soybean (*Glycine max* L.). - Soybean Sci. **3**: 351-355, 2011.

Wong, J.H., Cai, N., Balmer, Y., Tanaka, C.K., Vensel, W.H., Hurkman, W.J., Buchanan, B.B.: Thioredoxin targets of developing wheat seeds identified by complementary proteomic approaches. - Phytochemistry **65**: 1629-1640, 2004.

Wong, J.H., Kim, Y.B., Ren, P.H., Cai, N., Cho, M.J., Hedden, P., Lemaux, P.G., Buchanan, B.B.: Transgenic barley grain overexpressing thioredoxin shows evidence that the starchy endosperm communicates with the embryo and the aleurone. - Proc. nat Acad. Sci, USA **99**: 16325-16330, 2002.

Xie, G., Kato, H., Sasaki, K., Imai, R.: A cold-induced thioredoxin h of rice, ostrx23, negatively regulates kinase activities of osmpk3 and osmpk6 *in vitro*. - FEBS Lett. **583**: 2734-2738, 2009.

Yamamoto, Y., Kobayashi, Y., Matsumoto, H.: Lipid peroxidation is an early symptom triggered by aluminum, but not the primary cause of elongation inhibition in pea roots. - Plant Physiol. **125**: 199-208, 2001.

Yan, S.P., Zhang, Q.Y., Tang, Z.C., Su, W.A., Sun, W.N.: Comparative proteomic analysis provides new insights into chilling stress responses in rice. - Mol. Cell. Proteomics **5**: 484-496, 2006.

Ying, Y., Yue, W., Wang, S., Li, S., Wang, M., Zhao, Y., Wang, C., Mao, C., Whelan, J., Shou, H.: Two h-type thioredoxins interact with the E2 ubiquitin conjugase PHO_2 to fine-tune phosphate homeostasis in rice. - Plant Physiol. **173**: 812, 2017.

Zhang, C.J., Zhao, B.C., Ge, W.N., Zhang, Y.F., Song, Y., Sun, D.Y., Guo, Y.: An apoplastic h-type thioredoxin is involved in the stress response through regulation of the apoplastic reactive oxygen species in rice. - Plant Physiol. **157**: 1884-1899, 2011.

Zhang, D.W., Yang, C.P., Wang, Y.C., Ban, Q.Y., Ma, H.: Cloning and expression analysis of thioredoxin gene (*Trx*) from *Limonium bicolor*. - Plant Physiol. Commun. **44**: 201-205, 2008.

Zhao, F., Ma, J., Li, L., Fan, S., Guo, Y., Song, M., Wei, H., Pang, C., Yu, S.: *GhNAC12*, a neutral candidate gene, leads to early aging in cotton (*Gossypium hirsutum* L). - Gene **576**: 268-274, 2016.

Zhu, J.B., Liu, H.L., Wang, Z., Zhou, P.: Construction of full-length cDNA library from the *Saussurea involucrate* Kar. *et* Kir laminae. - Acta agr. boreali-occident. sin. **6**: 170-173, 2006.