

# The *Thellungiella salsuginea* Tonoplast Aquaporin TsTIP1;2 Functions in Protection Against Multiple Abiotic Stresses

Li-Li Wang<sup>1,2</sup>, An-Ping Chen<sup>3</sup>, Nai-Qin Zhong<sup>1,2</sup>, Ning Liu<sup>1,2</sup>, Xiao-Min Wu<sup>1,2</sup>, Fang Wang<sup>1,2</sup>, Chun-Lin Yang<sup>1,2</sup>, Michael F. Romero<sup>3</sup> and Gui-Xian Xia<sup>1,2,\*</sup>

<sup>1</sup>Institute of Microbiology, University of Chinese Academy of Sciences, Beijing, 100101 China

<sup>2</sup>State Key Laboratory of Plant Genomics, Beijing, 100101 China

<sup>3</sup>Physiology and Biomedical Engineering, Mayo Clinic College of Medicine, Rochester, MN 55905 USA

\*Corresponding author: E-mail, xiagx@im.ac.cn; Fax: +86 10 64845674

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**Examination of aquaporin (AQP) membrane channels in extremophile plants may increase our understanding of plant tolerance to high salt, drought or other conditions. Here, we cloned a tonoplast AQP gene (*TsTIP1;2*) from the halophyte *Thellungiella salsuginea* and characterized its biological functions. *TsTIP1;2* transcripts accumulate to high levels in several organs, increasing in response to multiple external stimuli. Ectopic overexpression of *TsTIP1;2* in *Arabidopsis* significantly increased plant tolerance to drought, salt and oxidative stresses. *TsTIP1;2* had water channel activity when expressed in *Xenopus* oocytes. *TsTIP1;2* was also able to conduct H<sub>2</sub>O<sub>2</sub> molecules into yeast cells in response to oxidative stress. *TsTIP1;2* was not permeable to Na<sup>+</sup> in *Xenopus* oocytes, but it could facilitate the entry of Na<sup>+</sup> ions into plant cell vacuoles by an indirect process under high-salinity conditions. Collectively, these data showed that *TsTIP1;2* could mediate the conduction of both H<sub>2</sub>O and H<sub>2</sub>O<sub>2</sub> across membranes, and may act as a multifunctional contributor to survival of *T. salsuginea* in highly stressful habitats.**

**Keywords:** Aquaporin • Channeling activity • Stress tolerance • *Thellungiella salsuginea*.

**Abbreviations:** AQP, aquaporin; DAB, 3,3-diaminobenzidine; GFP, green fluorescent protein; MDA, malondialdehyde; MS, Murashige and Skoog; MV, methyl viologen; ORF, open reading frame; ROS, reactive oxygen species; RT-PCR, reverse transcription-PCR; RWC, relative water content; TIP, tonoplast intrinsic protein; TW, turgid weight.

## Introduction

Aquaporins (AQPs), a large family of channel proteins, are present in all living organisms from bacteria to higher plants and mammals (Maurel et al. 1993, Maurel et al. 2008, Danielson and Johanson 2010). However, plants contain a greater number of AQP genes than other organisms. Based on their subcellular

localizations and protein sequence similarity, plant AQPs were previously divided into four subfamilies, i.e. the plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin 26-like intrinsic proteins (NIPs) and small basic intrinsic proteins (SIPs). More recent studies in moss and other plants revealed three additional subfamilies of plant AQPs, the uncharacterized intrinsic proteins (XIPs), GlpF-like intrinsic proteins (GIPs) and hybrid intrinsic proteins (HIPs), thus expanding the plant AQP protein family into seven subclasses (Gustavsson and Gerhardsson 2005, Danielson and Johanson 2008, Bienert et al. 2011, Soto et al. 2012).

In addition to H<sub>2</sub>O, plant AQPs are permeable to a wide range of small neutral molecules; these molecules include glycerol, urea, boric acid, silicon, CO<sub>2</sub>, NH<sub>3</sub>/NH<sub>4</sub><sup>+</sup> and H<sub>2</sub>O<sub>2</sub> (Biela et al. 1999, Gerbeau et al. 1999, Uehlein et al. 2003, Loque et al. 2005, Ma et al. 2006, Bienert et al. 2007, Schnurbusch et al. 2010). The reported substrate selectivity of plant AQPs is diverse. Many AQPs can conduct water or another small molecule, and a number of them are permeable to more than one substrate. For example, tobacco NtAQP1 can conduct water and CO<sub>2</sub> (Uehlein et al. 2003), rice OsNIP2;1 can conduct selenite and silicon (Zhao et al. 2010), tobacco NtTIPa can conduct water, glycerol and urea (Gerbeau et al. 1999), and rice OsLsi1 and *Arabidopsis* AtNIP5;1 can conduct silicon, boron and arsenic (Mitani-Ueno et al. 2011). Although such capacities of plant AQPs were mainly detected in heterologous systems such as *Xenopus* oocytes and yeast, these results are in good agreement with findings showing multiple functions of some plant AQPs (Maurel et al. 2008, Maurel et al. 2009). Several factors including subcellular localization, and heteromerization of the AQP proteins were shown to be involved in the regulation of divergent channeling activities of plant AQPs (Uehlein et al. 2008, Otto et al. 2010).

Plant AQPs perform important functions in the responses to environmental stimuli (Yu et al. 2006, Maurel et al. 2008, Aroca et al. 2012). AQP expression responds to diverse abiotic stresses such as drought, salinity and low temperature (Smart et al. 2001, Alexandersson et al. 2005, Sakurai et al. 2005, Guo et al.

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2006). However, there are various expression profiles of AQPs. Different AQPs may be either up-regulated or down-regulated by the same stress and those in the same subgroup may have distinct expression features under different stress conditions (Alexandersson et al. 2005, Guo et al. 2006). Similar to these expression characteristics, the physiological relevance of AQPs to abiotic stress responses is multifaceted. Several studies showed that overexpression of AQPs could either increase or decrease the stress tolerance of transgenic plants. For instance, expression of wheat or rice AQPs conferred improved salt or drought tolerance to transgenic plants (Lian et al. 2004, Gao et al. 2010, Hu et al. 2012), and tomato SlTIP2;2 and ginseng PgTIP1 increased tolerance to both salt and drought stresses (Peng et al. 2007, Sade et al. 2009). In contrast, overexpression of some AQPs decreased tolerance to salt, dehydration or cold stress in transgenic plants (Aharon et al. 2003, Katsuhara et al. 2003, Wang et al. 2011). Also, overexpression of an AQP could cause sensitivity to one kind of stress, but tolerance to another stress (Jang et al. 2007a, Jang et al. 2007b). Based on these data, it has been proposed that AQPs are components of an important tuning mechanism by which the plant is able to adapt to unfavorable conditions (Maurel et al. 2008, Aroca et al. 2012). Therefore, accurately selecting the right candidate genes for transgenic manipulation and exploring the mode of their functions in different circumstances are vital for understanding the physiological roles of this large family of proteins.

Extremophile plants are tolerant to highly stressful growth conditions. To date, few studies have examined the AQP proteins in extremophiles. In the present study, we identified and functionally characterized a tonoplast aquaporin gene (*TsTIP1;2*) from *Thellungiella salsuginea* (salt cress), which is a close relative of *Arabidopsis thaliana* and is considered a model halophyte plant (Inan et al. 2004, Volkov and Amtmann 2006, Taji et al. 2008, Amtmann 2009, Wu et al. 2012). We show that the expression of *TsTIP1;2* increased in response to various stresses in *T. salsuginea*, and ectopic overexpression of *TsTIP1;2* enhanced tolerance to drought, salt and oxidative stresses in transgenic *Arabidopsis*. The molecular mechanisms by which *TsTIP1;2* exerts the multiple functions were analyzed and discussed.

## Results

### Cloning of the *TsTIP1;2* gene and phylogenetic analysis

A salinity response gene (GenBank accession No. EE683447) in *T. salsuginea* was isolated through functional overexpression of plant cDNAs in fission yeast cells as previously described (Liu et al. 2007). The cDNA encodes a deduced protein of 254 amino acids with a calculated mol. wt of 25.7 kDa. A blast search indicates that the protein shares high homology with the plant tonoplast aquaporins (TIPs). Domain analysis shows that the protein contains two Asn-Pro-Ala (NPA) motifs that are conserved in aquaporin proteins (**Supplementary Fig. S1A**).

By BLAST search of the reported genome and cDNA sequences of *T. salsuginea* (Taji et al. 2004, Taji et al. 2008, Wu et al. 2012), we identified a total of 11 *TIP* genes (**Supplementary Table S1**) according to the sequence similarity of their derived proteins with the tonoplast aquaporins of *A. thaliana* (Johanson et al. 2001). Phylogenetic analysis of TIPs from *T. salsuginea* and *A. thaliana* was conducted and the *T. salsuginea* TIPs were named based on their phylogenetic relationships with *Arabidopsis* TIPs. The sequence of the cDNA we identified using a yeast system is identical to that of BY822233, and the gene was designated as *TsTIP1;2* (**Supplementary Fig. S1B**).

### Subcellular localization of *TsTIP1;2*

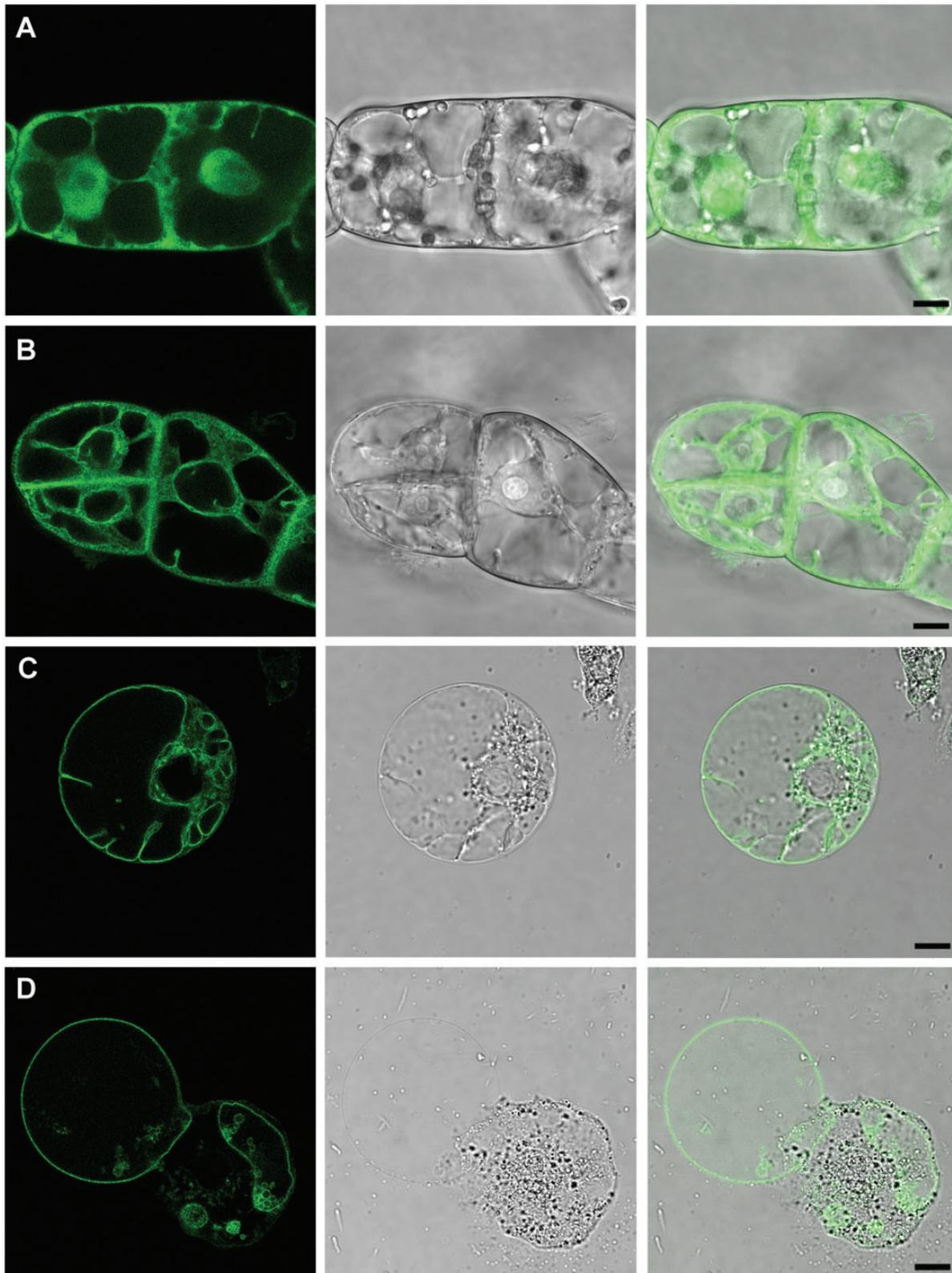
To verify the sequence-based prediction of the tonoplast location of *TsTIP1;2*, the subcellular localization of the protein was determined by examination of a *TsTIP1;2*-green fluorescent protein (GFP) fusion protein. The plasmid construct encoding *TsTIP1;2*-GFP under the control of the 35S promoter was transformed into BY-2 cells by *Agrobacterium*-mediated transformation. As shown in **Fig. 1**, the GFP fluorescence appeared mainly on the vacuolar membranes, indicating that *TsTIP1;2* protein was indeed located on tonoplasts.

### Expression of *TsTIP1;2* in different organs and in response to abiotic stresses

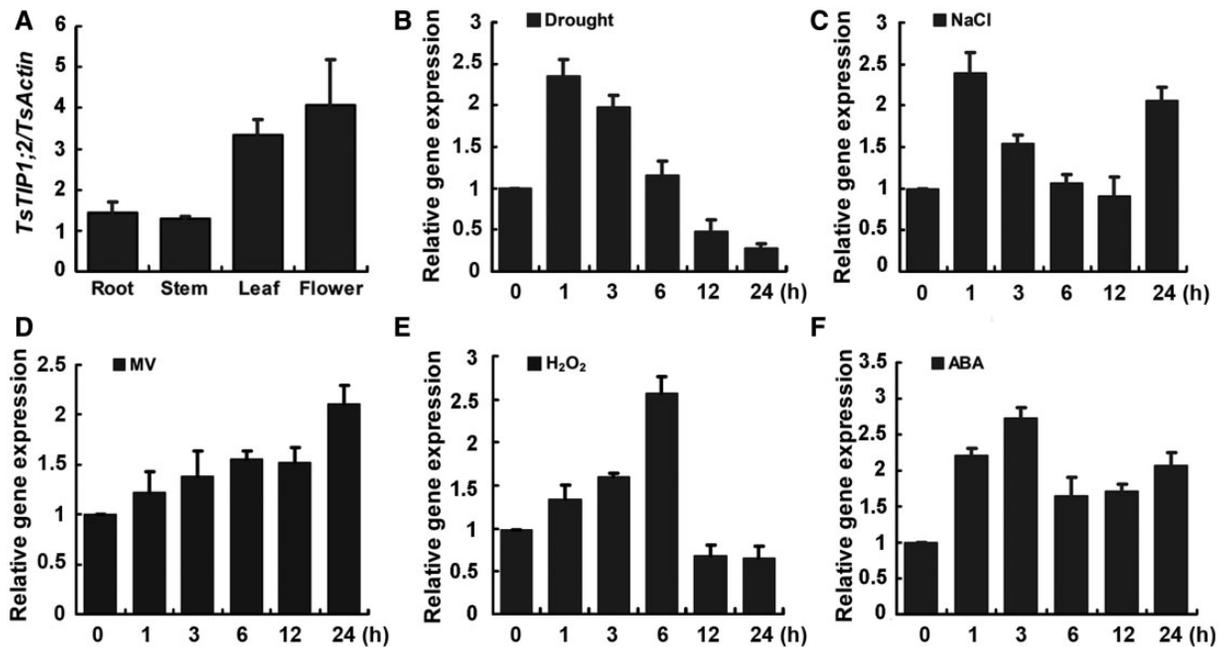
The relative expression levels of *TsTIP1;2* mRNA in flower, leaf, root and stem were analyzed by real-time PCR. As shown in **Fig. 2A**, *TsTIP1;2* is expressed ubiquitously in all the tested organs. *TsTIP1;2* expression levels appear to be lower in the roots and stem, but the transcripts accumulated to a fairly high level in the leaves and flowers.

We next measured the relative expression of *TsTIP1;2* in response to several abiotic and hormonal stresses. *TsTIP1;2* transcripts increased in response to all the tested treatments, including salt, drought, methyl viologen (MV), H<sub>2</sub>O<sub>2</sub> and ABA (**Fig. 2B–F**). Under conditions of drought and ABA application, *TsTIP1;2* expression was induced, with the highest levels occurring at 1–3 h. Upon salt stress treatment, *TsTIP1;2* expression was stimulated to a peak value at 1 h and then decreased gradually until it recovered to its original level. The transcript abundance increased again 24 h after NaCl treatment, which may be a secondary response to the stress. After MV treatment, *TsTIP1;2* expression was gradually elevated. After H<sub>2</sub>O<sub>2</sub> treatment, *TsTIP1;2* expression showed the highest level at 6 h. These results indicated that *TsTIP1;2* expression is responsive to multiple stressors, suggesting that its function may be associated with the physiological processes that protect the plant against these environmental dangers.

The stress responses of another 20 *TsTIP* genes expressed in the seedlings of *T. salsuginea* were also analyzed and compared with that of *TsTIP1;2*. The expression levels of *TsTIP3;1*, *TsTIP3;2* and *TsTIP5;1* were too low to be detected. Among the other seven genes, the transcript abundance of *TsTIP1;1*, *TsTIP1;2* and



**Fig. 1** Subcellular localization of TsTIP1;2-GFP fusion proteins in BY2 cells. (A) GFP fluorescence in control BY2 cells. (B) GFP fluorescence in *TsTIP1;2-GFP* transgenic BY2 cells. (C) Protoplast derived from a *TsTIP1;2-GFP* transgenic BY2 cell. (D) Vacuole released from the protoplast derived from the *TsTIP1;2-GFP* transgenic BY2 cell after short mechanical pressure. The GFP was visualized under a laser scanning confocal microscope at an excitation wavelength of 488 nm. Scale bars = 10  $\mu$ m.



**Fig. 2** Expression patterns of *TsTIP1;2*. (A) Expression of *TsTIP1;2* in various organs of *T. salsauginea*. (B–F) Time course analysis of *TsTIP1;2* expression in response to abiotic stresses. Total RNA was isolated from 2-week-old plants treated with drought stress (B), 300 mM NaCl (C), 25  $\mu$ M MV (D), 10 mM H<sub>2</sub>O<sub>2</sub> (E) or 50  $\mu$ M ABA (F), for 0, 1, 3, 6, 12 and 24 h, respectively. The levels of *TsTIP1;2* and *TsActin2* were measured by SYBR Green I real-time PCR. The 0 h value was used as reference. Each column represents means  $\pm$  SE from three biological replicates.

*TsTIP1;3* in the *TsTIP1* subclass was higher than those of the other five *TIP* genes under normal growth conditions, but most of these genes were clearly responsive to the environmental stresses (Supplementary Fig. S2).

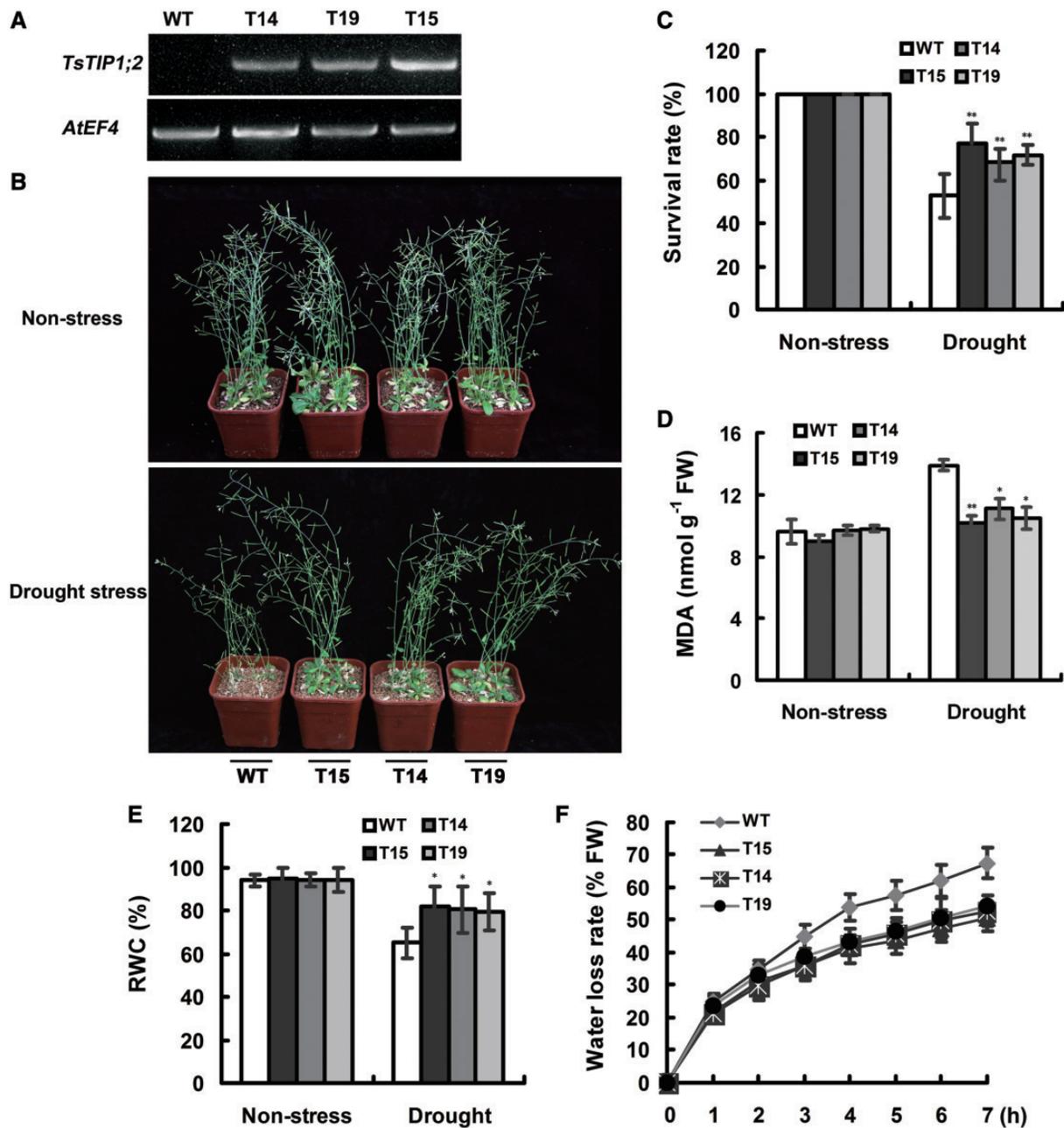
### Ectopic expression of *TsTIP1;2* in Arabidopsis enhanced tolerance to drought and salt stresses

A gain-of-function approach was employed to assess the functions of *TsTIP1;2* in abiotic stress responses. As Arabidopsis has been used frequently in functional characterization of genes from extremophytes, we employed this model glycophyte plant as our experimental system.

A number of studies reported that overexpression of plant AQPs affected plant tolerance to drought stresses (Aharon et al. 2003, Lian et al. 2004, Sade et al. 2009), but either positive or negative effects were observed. To see which is the case for *TsTIP1;2*, three independent transgenic Arabidopsis lines with high transgene expression (Fig. 3A) were tested for their tolerance to salt and drought stress. First, 2-week-old wild-type and transgenic plants were subjected to drought stress by stopping irrigation for 4 weeks. As shown in Fig. 3B, the transgenic plants grew bigger with greener leaves after the treatment as compared with the wild-type control. The survival rates of the transgenic plants were also higher, >68%. However, only 52% of wild-type plants survived after drought stress (Fig. 3C). The improved drought tolerance of the

transgenic plants was further examined by measurement of malondialdehyde (MDA) accumulation, which is an indicator of damage caused by abiotic stresses (Taulavuori et al. 2001, Dong et al. 2006). Indeed, the transgenic plants accumulated much less MDA than the wild-type plants after drought treatment (Fig. 3D). To see if increased drought tolerance is associated with water status, the relative water content (RWC) and water loss rates of leaves from wild-type and transgenic plants were measured and compared. The water content of the transgenic leaves was higher and the water loss rates were lower than in the wild-type leaves (Fig. 3E, F). These results indicate that overexpression of *TsTIP1;2* could confer improved drought tolerance to the transgenic plants by decreasing the water loss rate, which alleviated or prevented damage caused by the drought stress.

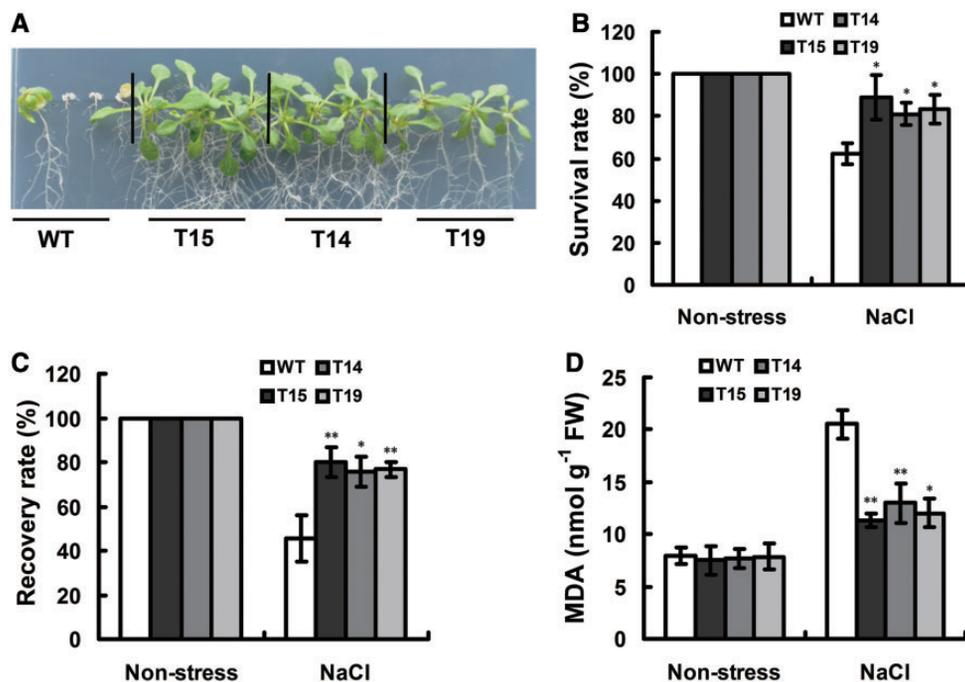
The performance of the transgenic plants under salt stress conditions was then investigated. Five-day-old WT and transgenic seedlings were transferred to half-strength Murashige and Skoog (1/2 MS) medium containing 0 or 150 mM NaCl and grown for 1 week and then recovered on 1/2 MS solid medium for 10 d. Under normal growth conditions, the performance of wild-type and transgenic plants was similar; however, the growth of wild-type seedlings was seriously inhibited under the stress treatment and many did not recover after 10 d (Fig. 4A). In contrast, the transgenic seedlings showed significantly improved tolerance of the salt stress. The survival rate of the wild-type plants was only around 60%, whereas



**Fig. 3** Effects of drought stress on wild-type (WT) and *TsTIP1;2* transgenic Arabidopsis plants. (A) Expression of *TsTIP1;2* in WT and transgenic plants (lines T14, T19 and T15). (B) Phenotypic comparison of the WT and transgenic plants before and after drought stress treatment. Upper panel: plants grown under normal conditions. Lower panel: plants subjected to water withholding for 4 weeks. (C) Survival rate of plants under drought stress. Each value is the average of three biological experiments ( $n=30$  for each experiment) and bars indicate the SE. (D) Malondialdehyde (MDA) contents in Arabidopsis plants growing under normal conditions or after drought treatment for 1 week. Each data point represents the mean  $\pm$  SE. Three biological repeats were performed. Asterisks indicate significant differences compared with wild-type plants ( $*P < 0.05$ ,  $**P < 0.01$ , Student's  $t$ -test). (E) Relative water contents (RWCs) in aerial parts of the pot-grown plants before or after withholding water for 1 week. (F) Water loss rate in aerial parts of the pot-grown plants. For E and F, error bars indicate the SE and each value is the average of four experiments. Asterisks indicate significant differences compared with the WT control ( $*P < 0.05$ , Student's  $t$ -test).

the transgenic plants had a survival rate of  $>80\%$  under these conditions (Fig. 4B). The recovery rate was reduced to 43% for the wild-type plants, but this rate was  $>75\%$  for all the transgenic lines (Fig. 4C). Likewise, the transgenic plants had

lower MDA levels than the wild-type control in salt-stressed conditions (Fig. 4D). Thus, *TsTIP1;2* expression could also provide the transgenic plants with enhanced tolerance to salt stress.



**Fig. 4** Effects of salt stress on wild-type (WT) and *TsTIP1;2* transgenic Arabidopsis plants. (A) Phenotype of WT and *TsTIP1;2* transgenic plants (lines T15, T14 and T19) recovered from salt stress treatment. Five-day-old WT and transgenic seedlings were transferred to 1/2 MS solid medium supplemented with 150 mM NaCl and grown for 1 week and then recovered on 1/2 MS solid medium for 10 d. (B) Survival rate of the salt-treated plants. (C) Recovery rate of the salt-treated plants after recovery. For B and C, each value is the average of three biological experiments ( $n = 30$  for each experiment) and bars indicate the SE. (D) MDA levels in plants. Each data point represents the mean  $\pm$  SE from three biological repeats. Asterisks indicate a significant difference compared with WT plants (\* $P < 0.05$ , \*\* $P < 0.01$ , Student's *t*-test).

### Overexpression of *TsTIP1;2* improved tolerance to oxidative stress

The induced expression of *TsTIP1;2* in response to treatment with  $H_2O_2$  or MV prompted us to investigate the possible involvement of *TsTIP1;2* in the development of oxidative stress tolerance. As  $H_2O_2$  is unstable, we chose MV, a strong and steady oxidative stressor that causes production of reactive oxygen species (ROS; mainly  $H_2O_2$ ) in the chloroplasts (Dodge 1971, Kraus and Fletcher 1994). Growth of wild-type and transgenic plants was examined after recovering from MV treatment. As shown in Fig. 5A, the growth of all plants was reduced by MV stress, but the transgenic plants showed better growth. The FW of the wild-type plants was  $<30\%$  of the value recorded for untreated plants and the Chl content of stressed plants was no more than 30%. In contrast, transgenic lines had higher FW ( $>60\%$ ) and greater Chl content (about 60%) (Fig. 5C, D). Also, the transgenic plants had lower MDA and ROS levels (Fig. 5B; Supplementary Fig. S3). These experimental data revealed that besides drought and salinity tolerance, *TsTIP1;2* overexpression could produce increased oxidative stress tolerance in the transgenic plants.

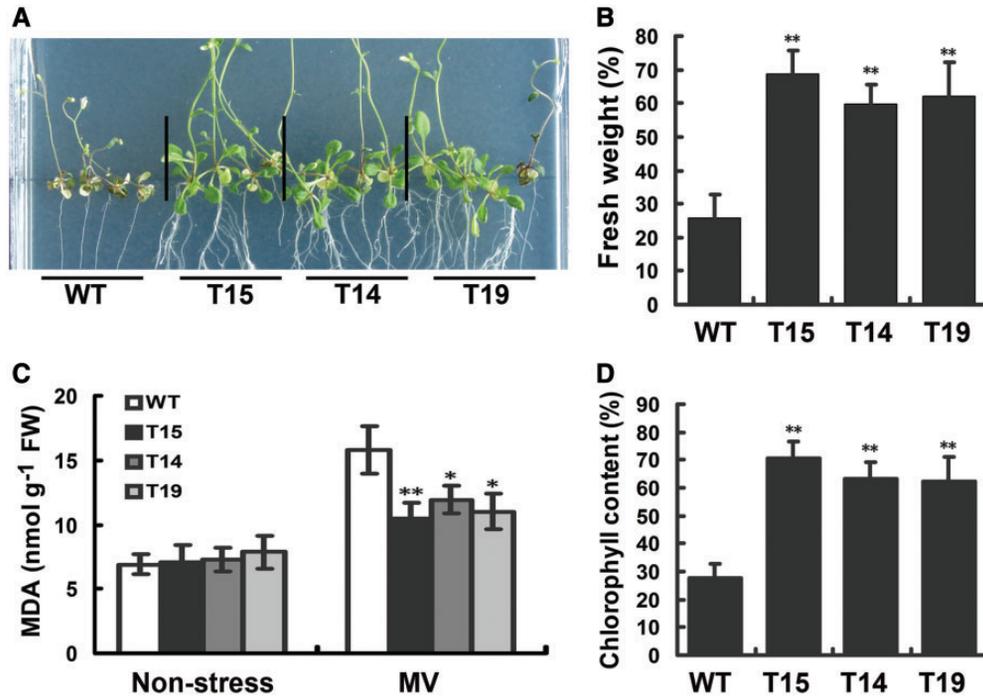
### *TsTIP1;2* has water transport capacity in *Xenopus* oocytes

To understand the mechanism by which the *TsTIP1;2* transgenic plants acquired increased tolerance to abiotic stresses, we

assessed the substrate-conducting activities of *TsTIP1;2*. The water transport capacity of *TsTIP1;2* was tested in the *Xenopus* oocyte system. *TsTIP1;2* complementary RNA (cRNA) was injected into *X. laevis* oocytes to express *TsTIP1;2* protein. Water-injected oocytes were used as negative controls, and the previously characterized Arabidopsis AtTIP1;1 was used as a positive control (Maurel et al. 1993). After incubation for 3 d, the rates of change in oocyte volume were measured in the hypo-osmotic solution and then the water permeability coefficient (Pf) values were calculated and statistically analyzed. As shown in Fig. 6A, expression of AtTIP1;1 conferred a rapid osmotically driven increase in relative volume, while expression of *TsTIP1;2* enabled an increase in relative volume at a slower rate. Water-injected oocytes showed no obvious increase in relative volume during several minutes under the same conditions. The Pf values of *TsTIP1;2*, AtTIP1;1 and water-injected oocytes were 32, 66 and  $7.7 \mu\text{m s}^{-1}$ , respectively (Fig. 6B). Compared with water-injected control, the oocytes injected with *TsTIP1;2* cRNA showed a 4.2-fold increase in Pf, indicating that *TsTIP1;2* possesses water transport activity. In comparison with AtTIP1;1, the apparent water channel activity of *TsTIP1;2* is weaker (Fig. 6B).

### *TsTIP1;2* mediates $H_2O_2$ diffusion across the membrane

The enhanced tolerance of *TsTIP1;2* transgenic plants to oxidative stress suggested that *TsTIP1;2* may also be permeable to



**Fig. 5** Effects of oxidative stress on wild-type (WT) and *TsTIP1;2* transgenic *Arabidopsis* plants. (A) Phenotype of WT and *TsTIP1;2* transgenic plants (lines T15, T14 and T19) recovered from oxidative stress treatment. Five-day-old WT and transgenic seedlings were transferred to 1/2 MS solid medium supplemented with 1  $\mu$ M MV and grown for 1 week and then recovered on 1/2 MS solid medium for 10 d. (B) MDA contents in plants. Each data point represents the mean  $\pm$  SE from three biological repeats. (C) Relative fresh weight of plants under MV stress, as a percentage of weight of unstressed plants. (D) Relative Chl contents in plants under MV stress, as a percentage of Chl content in unstressed plants. For C and D, each data point is the average of three experiments ( $n = 15$  for each experiment) and bars indicate the SE (\* $P < 0.05$ , \*\* $P < 0.01$ , Student's *t*-test).

H<sub>2</sub>O<sub>2</sub>. To see if this holds true, we employed the yeast system to test H<sub>2</sub>O<sub>2</sub> permeability of *TsTIP1;2*. *TsTIP1;2*-transgenic yeast cells were grown on solid medium containing various concentration of H<sub>2</sub>O<sub>2</sub> at 30°C for 6 d. As seen in Fig. 7A, expression of *TsTIP1;2* markedly affected cell growth and the effect increased with increasing concentrations of H<sub>2</sub>O<sub>2</sub> in the medium. We monitored the H<sub>2</sub>O<sub>2</sub> levels in yeast cells using the intracellular ROS-sensitive fluorescent dye CM-H<sub>2</sub>DCFDA. Upon addition of H<sub>2</sub>O<sub>2</sub> to the medium, *TsTIP1;2*-expressing yeast cells showed a dramatic fluorescence increase and this change was not so obvious in the control cells (Fig. 7B; Supplementary Fig S4), indicating that *TsTIP1;2* had the capability to take up H<sub>2</sub>O<sub>2</sub>. To confirm such an activity of *TsTIP1;2*, transgenic yeast cells were treated with AgNO<sub>3</sub>, a known blocker of AQP channels (Hooijmaijers et al. 2012), and the cellular H<sub>2</sub>O<sub>2</sub> content was measured. As shown in Fig. 7C, AgNO<sub>3</sub> blocked the increase of the fluorescent signal in *TsTIP1;2* transgenic yeast cells after H<sub>2</sub>O<sub>2</sub> stress treatment. These data suggested that the inhibited growth of the *TsTIP1;2* transgenic yeast cells was a result of H<sub>2</sub>O<sub>2</sub> influx.

### Overexpression of *TsTIP1;2* led to influx of Na<sup>+</sup> into the vacuoles

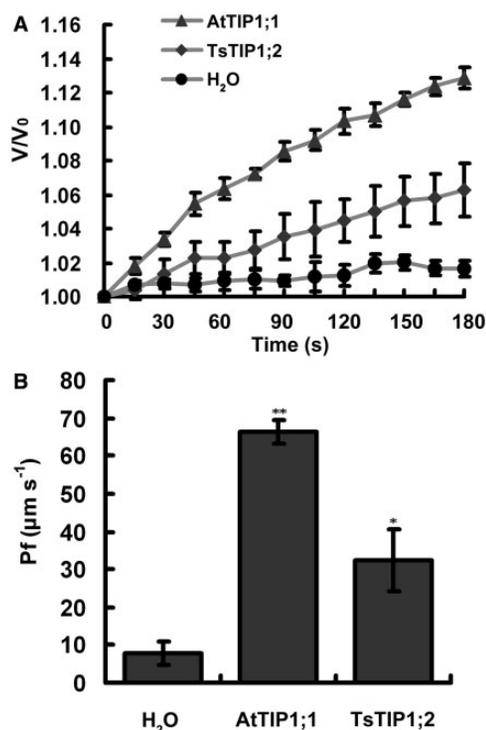
It has been reported that some animal AQPs transport Na<sup>+</sup> across membranes (Yool and Campbell 2012). Thus we assessed the possible ion-conducting ability of *TsTIP1;2* proteins using

the *Xenopus* oocyte system with the method of Romero et al. (1998a). However, we were unable to detect Na<sup>+</sup> channeling activity of *TsTIP1;2* (data not shown).

Then, to see if the improved salt tolerance of the *TsTIP1;2*-overexpressing plants resulted from a change in cellular Na<sup>+</sup> levels, the Na<sup>+</sup> contents of wild-type and transgenic seedlings were examined using the fluorescent dye Sodium Green. As Fig. 8A shows, treatment with 300 mM NaCl for 30 min caused a clear reduction of the tonoplasts and an increase of the fluorescent signals in both wild-type and transgenic cells. However, the amounts of intravacuolar Na<sup>+</sup> were obviously higher in the transgenic cells as compared with the wild-type cells, implying that *TsTIP1;2* expression resulted in Na<sup>+</sup> influx into the vacuoles. To quantify the change of the ion contents, the Na<sup>+</sup> levels of the wild-type and transgenic plants were measured following the method described by Herbig et al. (2002). In the absence of NaCl treatment, Na<sup>+</sup> content was similar between the two types of plants. When treated with 100 mM NaCl for 10 d; however, the Na<sup>+</sup> content was apparently higher in the transgenic plants as compared with the wild-type control (Fig. 8B).

### Discussion

Along with emerging findings of multiple substrates of individual AQPs, recent research indicates that AQP proteins have



**Fig. 6** Water channel activity assessment of *TsTIP1;2*. (A) The swelling kinetics of *Xenopus* oocytes injected with H<sub>2</sub>O, or cRNA coding for AtTIP1;1 and *TsTIP1;2*, respectively. The water-injected oocytes were used as a negative control. AtTIP1;1-injected oocytes were used as a positive control.  $V/V_0$  represents the volume changes upon immersion in hypo-osmotic solution.  $V_0$  represents the volume at the initial time. Three biological replicates were performed. Bars indicate the SE. (B) Water permeability coefficient (Pf) of H<sub>2</sub>O, *TsTIP1;2* and AtTIP1;1. The Pf values were calculated according to the equation described by Zhang and Verkman. (1991). Asterisks indicate significant differences in comparison with oocytes injected with water (\* $P < 0.05$ , \*\* $P < 0.01$ , Student's *t*-test).

multiple functions involved in plant growth and development and in responses to environmental stresses (Maurel et al. 2008, Maurel et al. 2009). Previously, it was reported that an AQP could confer both drought and salt tolerance to transgenic plants (Peng et al. 2007, Sade et al. 2009). In this study, we observed that ectopic overexpression of an extremophyte AQP in *Arabidopsis* plants could improve the tolerance to at least three types of abiotic stresses, thus adding a solid piece of evidence to show the importance of plant AQPs in stress tolerance.

### **TsTIP1;2 is capable of conducting H<sub>2</sub>O**

Plant AQPs play fundamental roles in H<sub>2</sub>O homeostasis, which is crucial for biological processes such as seed germination, cell elongation and osmoregulation (Lin et al. 2007, Peng et al. 2007, Ma et al. 2008, Sade et al. 2009, Lee et al. 2012). Similar to many other AQP genes, expression of *TsTIP1;2* was up-regulated in response to drought stress. *TsTIP1;2* transgenic *Arabidopsis*

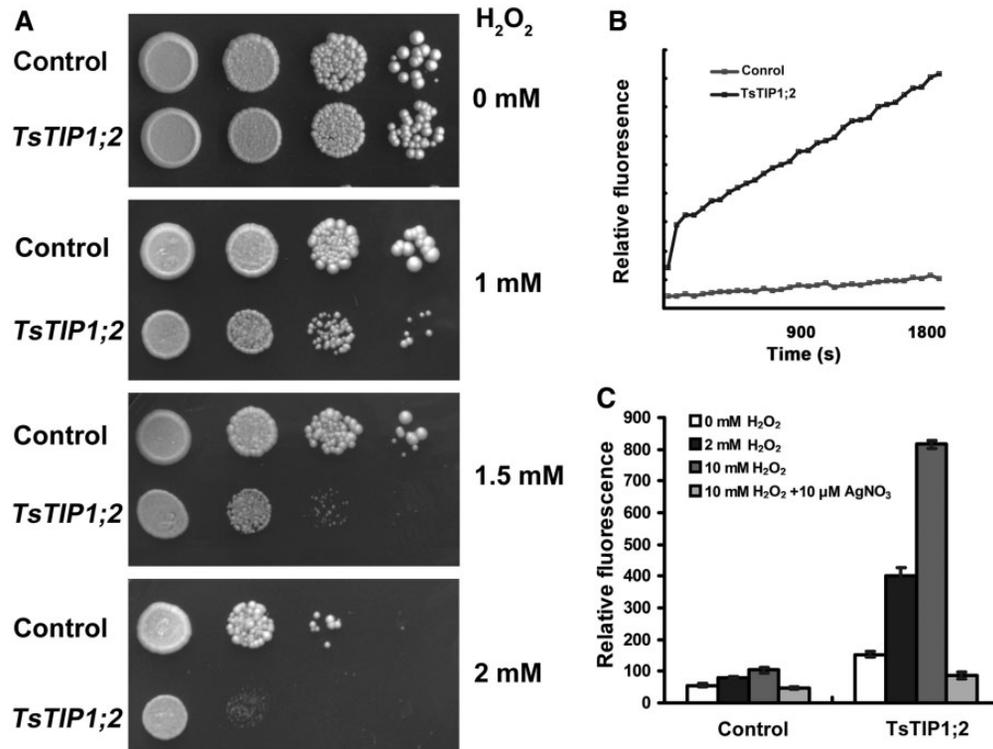
plants exhibited a substantially increased tolerance to drought stress, and the water content increased while the water loss rate decreased in transgenic plants as compared with the wild-type control under water-deficit conditions (Fig. 3). These data implied that overexpression of *TsTIP1;2* provided the transgenic plants with the ability to decrease rapid loss of water under drought conditions. Previously, Jang et al. (2007b) reported that ectopic overexpression of the cucumber AQP gene *CsPIP1;1* and the fiddlehead gourd AQP gene *CfPIP2;1* in *Arabidopsis* altered the transcript levels of endogenous PIP genes. To see if this was the case for *TsTIP1;2* transgenic plants, we analyzed the expression patterns of the endogenous AQP genes in *TsTIP1;2*-overexpressing plants. Indeed, expression of several *Arabidopsis* AQP genes decreased under drought condition in the transgenic plants, but far fewer genes were affected under salinity conditions (Supplementary Fig. S5). These results showed that *TsTIP1;2* overexpression perturbed the expression of the endogenous AQPs, particularly under drought stress. Thus, the increased drought tolerance of the *TsTIP1;2*-transgenic plants might be a consequence of integrated effects of *TsTIP1;2* and the endogenous AQPs.

### **TsTIP1;2 is able to facilitate H<sub>2</sub>O<sub>2</sub> transport**

Several AQPs are able to facilitate H<sub>2</sub>O<sub>2</sub> entry into yeast cells (Bienert et al. 2007, Dynowski et al. 2008, Hooijmaijers et al. 2012). Although these studies provided important new insights into the substrate selectivity of AQPs, the physiological relevance was unexplored. In our study, we detected that *TsTIP1;2* mediated influx of H<sub>2</sub>O<sub>2</sub> into the yeast cells and enhanced fluorescence in the cytoplasm after H<sub>2</sub>O<sub>2</sub> treatment (Supplementary Fig. S4), thus resulting in inhibited growth of the transgenic cells. In plants, overexpression of *TsTIP1;2* increased oxidative stress tolerance of the transgenic plants after MV treatment, accompanied by a decrease of H<sub>2</sub>O<sub>2</sub> level in the leaf cells (Fig. 5; Supplementary Fig. S3). It is known that MV treatment causes H<sub>2</sub>O<sub>2</sub> production in the chloroplasts and results in an increase in the amount of ROS in the cytosol (Dodge 1971, Kraus and Fletcher 1994). Overexpression of *TsTIP1;2* may help the influx of excess H<sub>2</sub>O<sub>2</sub> into the vacuoles that contain peroxidases, CuZn-superoxide dismutases (SODs) and ascorbate/phenolic/peroxidase systems (Ogawa et al. 1996, Andrews et al. 2002, Takahama 2004, Zipor and Oren-Shamir 2013), thus leading to reduction of cytosolic ROS and alleviation of the injury caused by MV treatment. In agreement with our assumption, Takahama (1992) demonstrated that vacuoles of mesophyll cells of *Vicia faba* participated in H<sub>2</sub>O<sub>2</sub> scavenging by vacuolar peroxidase.

### **TsTIP1;2 indirectly mediates Na<sup>+</sup> influx into vacuoles**

It has been shown that *Arabidopsis* plants overexpressing the wheat *TaNIP* gene accumulated higher K<sup>+</sup> and Ca<sup>2+</sup>, and lower Na<sup>+</sup> than the wild-type plants under salt stress (Gao et al. 2010). However, *Arabidopsis* plants overexpressing wheat *TaAQP8*



**Fig. 7** H<sub>2</sub>O<sub>2</sub> permeability of yeast cells expressing *TsTIP1;2*. (A) *Saccharomyces cerevisiae aqy-null* strain cells transformed with the empty vector pYES2 alone or pYES2-*TsTIP1;2* were spotted in 10-fold dilutions on medium without or with 1, 1.5 or 2 mM H<sub>2</sub>O<sub>2</sub>, respectively. The *S. cerevisiae aqy-null* strain transformed with the empty vector pYES2 was used as the control. (B) *TsTIP1;2*-mediated H<sub>2</sub>O<sub>2</sub> diffusion across yeast membranes. The fluorescence of CM-H<sub>2</sub>DCFDA-loaded *S. cerevisiae aqy-null* cells transformed with *TsTIP1;2* or empty vector pYES2 was recorded over time. The assay was performed for 30 min after addition of 10 mM H<sub>2</sub>O<sub>2</sub>. (C) Bar diagram showing the average increase in fluorescence. Cells were treated with 2 or 10 mM H<sub>2</sub>O<sub>2</sub> for 30 min before fluorescence measurement. White bars, control; black bars, treatment with 2 mM H<sub>2</sub>O<sub>2</sub>; dark gray bars, treatment with 10 mM H<sub>2</sub>O<sub>2</sub>; light gray bars, pre-incubation with 10 μM AgNO<sub>3</sub> prior to 10 mM H<sub>2</sub>O<sub>2</sub> treatment. The experiment was repeated more than three times.

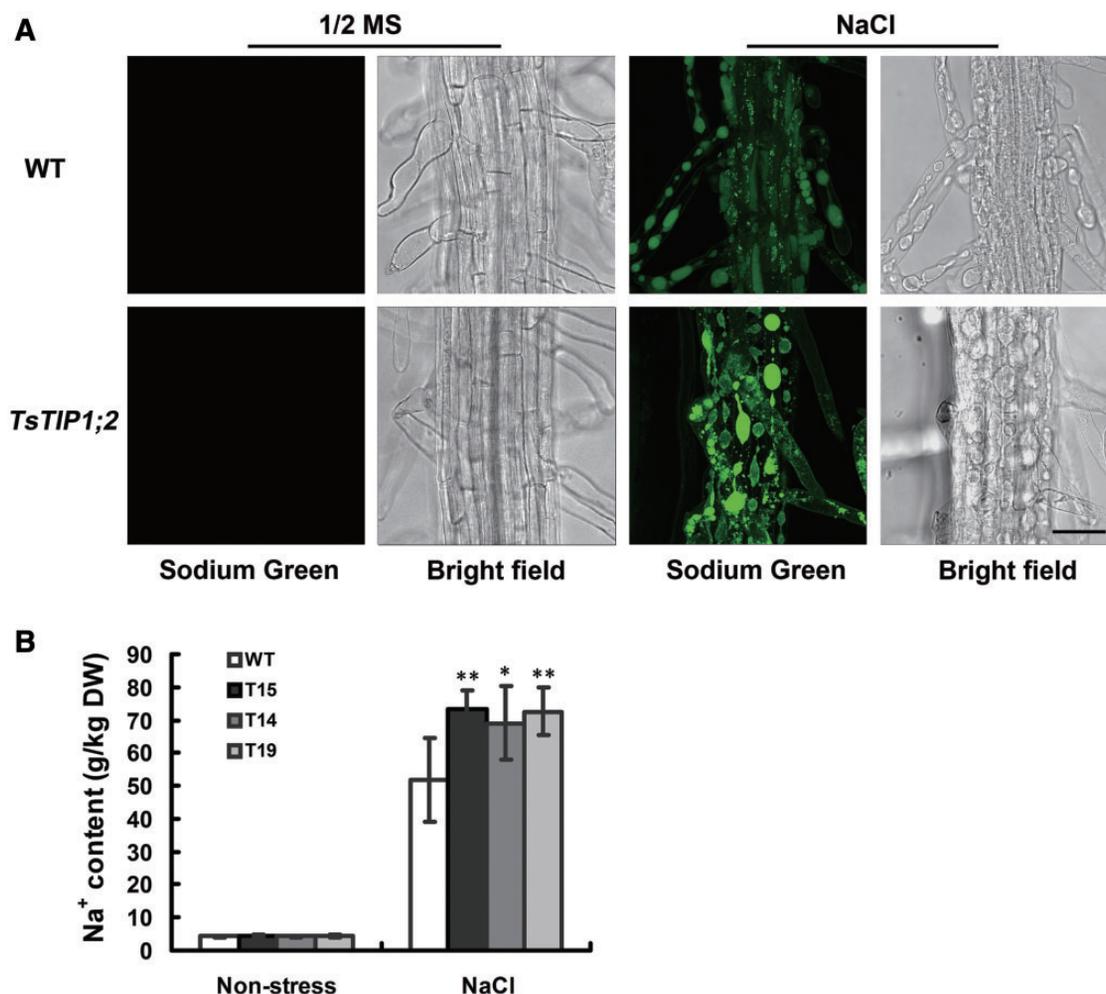
accumulated higher Na<sup>+</sup> and K<sup>+</sup> than wild-type plants in roots under salt stress (Hu et al. 2012). Peng et al. (2007) reported that overexpression of the ginseng *PgTIP1* in *Arabidopsis* plants resulted in an increase in the Na<sup>+</sup> level, but no change in the K<sup>+</sup> level under salt stress. Although these studies indicated that AQPs affected ion contents in plant cells under salt stress, the mechanistic details remain unclear.

In our study, we found that *TsTIP1;2* overexpression elevated the cellular Na<sup>+</sup> levels in transgenic plants, but without significantly affecting K<sup>+</sup> levels under salt stress (Fig. 8B; Supplementary Fig. S6). Moreover, our results showed higher Na<sup>+</sup> accumulation in the vacuoles of transgenic plant cells in comparison with wild-type cells (Fig. 8A), indicating that *TsTIP1;2* proteins are involved in sequestration of Na<sup>+</sup> ions into the vacuoles under high-salinity conditions. It is known that sequestration and accumulation of Na<sup>+</sup> ions in the vacuoles is an effective mechanism for increasing plant salt stress tolerance (Zhu 2003, Yamaguchi and Blumwald 2005, Ruiz-Lozano et al. 2012). The correlation between increased Na<sup>+</sup> amounts in the vacuoles and the enhanced salinity tolerance of the *TsTIP1;2* transgenic plants also supported this idea. Previously, several

animal AQPs were shown to have Na<sup>+</sup>-conducting activity (Yool and Campbell 2012). We were unable, however, to detect any Na<sup>+</sup>-transporting ability for *TsTIP1;2* using the *Xenopus* oocyte system. Hence, we speculate that the function of *TsTIP1;2* may be coupled with other ion-transporting machinery such as an ion exchanger, antiporter or other transporters in plant cells. Our results provide an intriguing clue for exploring the mechanisms by which plant AQPs function in the development of salt stress tolerance.

### AQPs may play crucial roles in the protection against abiotic stresses in *T. salsuginea*

Although the important roles of AQPs in plant water balance are well documented in glycophytes, the contribution of these proteins to hypertolerance to abiotic stresses in extremophytes remains unclear. *Thellungiella salsuginea* is a halophytic plant highly tolerant to cold, drought, oxidative and salt stresses (Taji et al. 2004, Wu et al. 2012). In our study, we report on several features of *TsTIP1;2* and some other homologs in *T. salsuginea*. We tested the expression patterns of eight *TsTIP*



**Fig. 8** Assessment of  $\text{Na}^+$  content in wild-type (WT) and *TsTIP1;2* transgenic plants (lines T15, T14 and T19). (A)  $\text{Na}^+$  content in plant cells was measured using the  $\text{Na}^+$  fluorescent dye indicator Sodium Green before and after treatment with 300 mM NaCl. Scale bar = 50  $\mu\text{m}$ . (B) Quantitative analysis of  $\text{Na}^+$  levels in plants. Error bars indicate the SE of more than four biological repeats. Asterisks indicate a significant difference in comparison with the wild-type plants (\* $P < 0.05$ , \*\* $P < 0.01$ , Student's *t*-test).

genes, and observed that although the basal expression levels of these genes are divergent, most of them were apparently responsive to the abiotic stresses. Also, we compared the water-conducting activity of *TsTIP1;2* with that of its Arabidopsis homolog AtTIP1; the results indicated that *TsTIP1;2* possessed a weaker water-channeling capacity than AtTIP1 in *Xenopus* oocytes. Different water-channeling activities of AQPs in *T. salsuginea* may be associated with water use efficiency of this extremophyte plant. More importantly, overexpression of *TsTIP1;2* was linked with development of at least three types of stress tolerance in the transgenic Arabidopsis plants. We speculate that such a functional diversity of *TsTIP1;2* may reflect a common feature of some other aquaporin proteins in *T. salsuginea*. Taken together, our results implied that AQP proteins may have crucial roles in the adjustment of the water relations in *T. salsuginea* for the plant to adapt to the harsh environment. Factors such as regulated gene expression and varied substrate-conducting and -selectivity activities of AQPs

may be responsible for the functional spectrum of this family of stress-related proteins.

## Materials and Methods

### Plant growth and stress treatment

Seeds of *T. salsuginea* were surface sterilized with 70% ethanol for 30 s and 10% NaClO for 10 min, and washed five times with sterile water. The surface sterile seeds were plated on 1/2 MS medium containing 1% sucrose. Plates were placed in darkness for 3 weeks at 4°C and then transferred to the greenhouse. Seedlings were grown at 22°C under a 16 h light/8 h dark photoperiod (100  $\mu\text{m m}^{-2} \text{s}^{-1}$ ). Seedlings grown for the indicated times were subjected to the following treatments: drought, 300 mM NaCl, 10 mM  $\text{H}_2\text{O}_2$ , 25  $\mu\text{M}$  MV and 50  $\mu\text{M}$  ABA. For drought stress treatment, seedlings were allowed to air-dry on filter paper for the indicated times. For NaCl,  $\text{H}_2\text{O}_2$ , MV and

ABA treatments, seedlings were placed on filter paper moistened with 300 mM NaCl, 10 mM H<sub>2</sub>O<sub>2</sub>, 25 μM MV and 50 μM ABA, respectively.

Seeds of *Arabidopsis thaliana* (ecotype Columbia) were surface sterilized by the above methods. The seeds were plated on 1/2 MS medium containing 1% sucrose. Plates were placed in darkness for 3 d at 4°C and then transferred to the greenhouse. Seedlings were grown at 22°C under a 16 h light/8 h dark photoperiod (100 μm m<sup>-2</sup> s<sup>-1</sup>).

### Vector construction and plant transformation

The cDNA fragment, containing the *TsTIP1;2* open reading frame (ORF), was amplified using specific primers with *Bam*HI and *Sac*I sites (forward, 5'-AAA GGA TCC GCC ATT GGT TTC ATC GTC-3'; reverse, 5'-AAA GAG CTC TCA ATA ATC AGT GGT GGG-3', restriction sites are underlined) and inserted into the pPZP111 expression vector under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter (Hajdukiewicz et al. 1994). The recombinant construct was named 35S:*TsTIP1;2*-PZP111. The recombinant plasmid was introduced into the *Agrobacterium tumefaciens* strain EHA105. Transformation of *Arabidopsis* plants was performed by the *Agrobacterium*-mediated floral dip method (Clough and Bent 1998).

### Subcellular localization analysis

To construct pPZP-*TsTIP1;2*-GFP constructs, the *TsTIP1;2* ORF was amplified using *Bam*HI and *Sac*II sites (forward, 5'-AAA GGA TCC GCC ATT GGT TTC ATC GTC-3'; reverse, 5'-AAA CCG CGG CCA ACC GTT GAA TCA GTG AAG-3', restriction sites are underlined). The transgenic tobacco (*Nicotiana tabacum* L.) cv BY-2 cells were generated by the method of Nagata et al. (1992).

Images of transgenic BY-2 cells were viewed under a laser scanning confocal microscope (Leica TCS SP5, Leica Microsystems). The GFP was detected at an excitation wavelength of 488 nm.

### Phylogenetic analysis

The protein sequence alignment were performed using the ClustalW program, and a phylogenetic tree was generated by the Neighbor-Joining method using the MEGA program version 4 (Tamura et al. 2007).

### RNA extraction, semi-quantitative RT-PCR and real-time PCR analysis

Total RNAs were extracted from *T. salsuginea* and *A. thaliana* using an RNeasy Kit (Qiagen) and the TRIzol reagent (Invitrogen). Contaminating genomic DNA was digested by RNase-free DNase I, and first-strand cDNA was then reverse transcribed with SuperScript III RNase H<sup>-</sup> Reverse Transcriptase (Invitrogen) from 2 μg of total RNA. Real-time PCR analysis was carried out with the SYBR green PCR kit (TOYOBO). Specific primers used in semi-quantitative reverse

transcription-PCR (RT-PCR) and real-time PCR are listed in **Supplementary Table S2**.

### Measurement of malondialdehyde content

For MDA measurement under NaCl and MV stress conditions, 5-day-old seedlings were transplanted onto 1/2 MS medium or 1/2 MS medium supplemented with NaCl or MV for 1 week, respectively. The whole plants were harvested for measurements of MDA content using the method reported by Cui and Wang (2006). For MDA measurement under drought stress, 2-week-old seedlings were grown in pots without watering for 1 week. The aerial parts of plants were harvested for measurement of MDA content.

### Measurements of relative water content and water loss rate

For measurement of RWC, the FW of whole aerial parts of watered or drought-treated plants was determined. Then these plants were saturated in distilled water at 4°C for 8 h and their turgid weights (TWs) were measured. Plants were dried at 70°C overnight and the DW was recorded. The RWC was calculated by the equation  $RWC = (FW - DW) / (TW - DW) \times 100$  (Peng et al. 2007). For measurement of water loss rate, aerial parts from watered 2-week-old wild-type and *TsTIP1;2* transgenic plants were weighed every 1 h until signs of wilting appeared.

### Xenopus oocyte expression and osmotic water permeability analysis

The coding region of *TsTIP1,2* was subcloned into *Xenopus* expression vector pGEMHE using *Eco*RI and *Hind*III restriction sites. After digestion and linearization of the plasmid, the cRNAs were synthesized in vitro using the T7 mMessage mMachine kit (Ambion). Female *X. laevis* were purchased from Xenopus Express, oocytes were removed and collagenase was dissociated. The procedure was approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC). The oocytes were injected with 50 nl of water or RNA solution (10 ng of RNA). Equal injection of RNA was confirmed by UV absorbance ( $A_{260}$ ) and ethidium bromide staining. Oocytes were incubated at 16°C in OR3 medium (~200 mOsm) (Romero et al. 1998b) and were studied at least 3 d after injection.

Osmotic swelling was analyzed from volume changes photographed by a Zeiss Axiocam microscope. At time zero, oocytes were quickly transferred from ND96 solution (~200 mOsm) (Chen et al. 2012) into 2-fold diluted ND96 (~100 mOsm) to induce swelling. Images were captured every 15 s for 3 min and analyzed using Adobe Photoshop Element Image software. The osmotic water permeability coefficient (Pf) of oocytes, determined as described by Zhang and Verkman (1991), is defined as:  $Pf = V_0 \times [d(V/V_0)/dt] / [S \times V_{water} \times (Osm_{in} - Osm_{out})]$ . The oocyte diameter (*R*) was measured as the maximal cross-sectional area of the oocytes and it was used to calculate the

oocyte volume ( $V_0$ ) and oocyte surface area ( $S$ ).  $d(V/V_0)/dt$  is the relative volume increase per unit time.  $V_{\text{water}}$  is the molar volume of water ( $18 \text{ cm}^3 \text{ mol}^{-1}$ ),  $\text{Osm}_{\text{in}}$  is 200 mOsm and  $\text{Osm}_{\text{ou}}$  is 100 mOsm. Pf values ( $\mu\text{m s}^{-1}$ ) are given as mean  $\pm$  SE.

### Fluorescence staining of $\text{Na}^+$

For evaluation of the  $\text{Na}^+$  content in the vacuoles of transgenic and wild-type plants, roots of 10-day-old seedlings were stained with  $10 \mu\text{M}$  Sodium Green Indicator (Molecular Probes) for 2 h, washed three times with double-distilled water, treated with double-distilled water or 300 mM NaCl, and visualized with a laser scanning confocal microscope (Leica, Germany).

### Determination of $\text{Na}^+$ or $\text{K}^+$ ion contents in plants

For ion content determination, 5-day-old seedlings of transgenic and wild-type control plants were separately transplanted on 1/2 MS medium plates containing 1/2 MS medium or NaCl (100 mM) for 10 d. The whole plants were harvested, washed four times with double-distilled water and then baked at  $60^\circ\text{C}$  for 48 h. Measurement of  $\text{Na}^+$  content was performed by the method described previously (Herbik et al. 2002). A 50 mg aliquot of dry material was dissolved in a microwave oven with 13 ml of  $\text{HNO}_3$  (14.4 M) and 2 ml of  $\text{H}_2\text{O}_2$  (8.8 M). Metal content was analyzed by ICP-OES (inductively coupled plasma optical emission spectrometry; Optima-2000 DV, Perkin Elmer).

### $\text{H}_2\text{O}_2$ fluorescence assays in yeast

For fluorescence assays in yeast, cells of the *Saccharomyces cerevisiae aqy-null* strain (*MAT $\alpha$  leu2::hisG trp1::hisG his3::hisG ura3-52 aqy1::KanMX4 aqy2::HIS3*) (Leitao et al. 2012) transformed with pYES2 or pYES2 containing *TsTIP1;2* were grown to logarithmic phase, incubated with  $25 \mu\text{M}$  5- (and 6-) chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate acetylesther (CM- $\text{H}_2\text{DCFDA}$ , Molecular Probes) for 40 min, washed more than four times with HEPES buffer (pH 7.0), and then resuspended to the same  $\text{OD}_{600}$ . Fluorescence before and after  $\text{H}_2\text{O}_2$  stress treatment was measured with multi-mode microplate readers Synergy<sup>TM</sup> H4 (BioTek), excited at a wavelength of 492 nm and emission was measured at a wavelength of 527 nm at room temperature ( $25^\circ\text{C}$ ) (Bienert et al. 2007, Dynowski et al. 2008).  $\text{AgNO}_3$  inhibition assay was conducted according to Bienert et al. (2007). Yeast cells were treated with  $10 \mu\text{M}$   $\text{AgNO}_3$  for 45 min and washed three times with HEPES buffer (pH 7.0) before  $\text{H}_2\text{O}_2$  treatment, and the fluorescence signal was measured.

### $\text{H}_2\text{O}_2$ detection in plants

Detection of  $\text{H}_2\text{O}_2$  in plants was carried out using the method of 3,3-diaminobenzidine (DAB) staining (Wang et al. 2008) with subtle modifications. Arabidopsis seedlings were incubated with  $0.1 \text{ mg ml}^{-1}$  DAB for 20 h in the dark, and Chl was removed with 100% ethanol.

### Supplementary data

Supplementary data are available at PCP online.

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### Disclosures

The authors have no conflicts of interest to declare.

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