The Wheat Aquaporin Gene *TaAQP7* Confers Tolerance to Cold Stress in Transgenic Tobacco

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Aquaporin proteins (AQPs) have been shown to be involved in abiotic stress responses. However, the precise role of AQPs, especially in response to cold stress, is not understood in wheat (*Triticum aestivum*). In the present study, quantitative real time polymerase chain reaction (qRT-PCR) analysis revealed that *TaAQP7* expression increased in leaves, but decreased in roots after cold treatment. Expression of *TaAQP7* in tobacco plants resulted in increased root elongation and better growth compared with wild-type (WT) plants under cold stress. Moreover, after cold treatment, the transgenic tobacco lines exhibited higher chlorophyll contents, lower levels of malondialdehyde (MDA), and less ion leakage (IL) than WT plants. Thus, expression of *TaAQP7* enhanced cold stress tolerance in transgenic tobacco. Taken together, our results suggest that *TaAQP7* confers cold stress tolerance by relieving membrane damage in the transgenic plants.

Key words: TaAQP7, Wheat, Cold Stress

Introduction

Low temperature inhibits water uptake by roots. Aquaporin proteins (AQPs) are known to transport water and other small molecules through biomembranes. In rice, the decrease in root hydraulic conductivity under cold stress is related to the function of aquaporins (Ahamed *et al*., 2012). In maize and cucumber, the decrease in root hydraulic conductivity caused by cold stress may be the result of aquaporin dysfunction caused by oxidation or intercellular accumulation of hydrogen peroxide (Lee *et al*., 2004, 2005; Aroca *et al*., 2005). Plant AQPs can be classified into five subfamilies: plasma membrane intrinsic proteins (PIPs); tonoplast membrane intrinsic proteins (TIPs); nodulin 26-like intrinsic proteins (NIPs); X (for unrecognized) intrinsic proteins (XIPs); and small basic intrinsic proteins (SIPs) (Weaver *et al*., 1991; Kammerloher *et al*., 1994; Chaumont *et al*., 2001; Johanson *et al*., 2001; Johanson and Gustavsson, 2002; Danielson and Johanson, 2008). PIPs are further divided into the subfamilies PIP1 and PIP2 (Schäffner, 1998; Chaumont *et al*., 2000). Many *AQP* genes have been identified in a number of plant species (Sade *et al*., 2010) including 35 in *Arabidopsis* (Johanson *et al*., 2001), 36 in maize (Chaumont *et al*., 2001), and 33 in rice (Sakurai *et al*., 2005).

Activities of AQPs can be directly regulated by phosphorylation, which may be induced in response to a number of stimuli, including abiotic stresses (Johansson *et al*., 2000; North and Nobel, 2000; Horie *et al*., 2011), plant hormones (Bienert *et al*., 2006), and light (Chaumont *et al*., 2005; Kaldenhoff and Fischer, 2006). Cold stress affects the expression of *AQP genes*. *AtPIP1;1*, *AtPIP1;2*, *AtPIP1;5*, *AtPIP2;2*, *AtPIP2;3*, *AtPIP2;4*, and *AtPIP2;7* were found to be downregulated, while *AtPIP2;5* and *AtPIP2;6* were upregulated in cold-stressed roots and aerial parts of *Arabidopsis thaliana* (Jang *et al*., 2004). In addition, *OsPIP2;7* was generally upregulated in roots but downregulated in shoots of rice at the early stage of chilling stress

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(Li *et al*., 2008). These results indicate that different members of the AQP family respond differentially to cold stress. Thus, mediation of cold stress responses by AQPs appears to be complex.

As a major crop of world-wide importance, wheat (*Triticum aestivum*) production is severely constrained by drought, salinity, extreme temperature, and other environmental stress factors. A better understanding of the mechanisms employed by wheat plants to tolerate abiotic stresses will be helpful for wheat genetic improvement. To date, more than 35 *AQP* genes have been identified in the wheat genome. Although some common wheat and durum wheat *AQP genes* such as *TaNIP*, *TdPIP1;1*, *TdPIP2;1*, and *TaAQP8* have been found to be involved in drought or salt stress tolerance (Forrest and Bhave, 2008; Gao *et al*., 2010; Ayadi *et al*., 2011; Hu *et al*., 2012), their role in cold tolerance has not been studied. Recently, we have isolated the cDNA of 1019 bp corresponding to the wheat gene *TaAQP7* (GenBank HQ650109) that encodes a novel PIP2 protein of 286 amino acids, and have characterized the function of the protein in transgenic tobacco during drought stress (Zhou *et al*., 2012). In the present study, we found that expression of *TaAQP7* confers cold stress tolerance to tobacco plants by protecting the membrane integrity in transgenic tobacco.

Materials and Methods

Plant materials and treatment

The seeds of wheat (*Triticum aestivum* L. cv. Chinese Spring) were surface-disinfected and germinated as described previously (Zhou *et al*., 2012). For cold treatment, the 10-d-old seedlings were transferred into Petri dishes and maintained at 4 °C for different time periods (0, 1, 2, 6, 12, 24 h). Leaf and root samples from both treated and control plants were subsequently frozen in liquid nitrogen and stored at −80 °C for extraction of total RNA.

Quantitative real time polymerase chain reaction (qRT-PCR) analysis

The expression of *TaAQP7* in wheat seedlings after cold treatment was examined by qRT-PCR in a detection system (MJ Research Opticon 2; BioRad, Foster City, CA, USA) according to the methods previously described (Zhou *et al.*, 2012). In all qRT-PCR experiments, a relative quantification method was employed to assess relative expression of the tested genes with three replicates of each condition (Livak and Schmittgen, 2001).

Low-temperature stress tolerance assays of the transgenic and wild-type (WT) plants

The recombinant plasmid pCAMBIA1304-*TaAQP7-GFP* under the control of the CaMV 35S promoter was transformed into tobacco, and the plants of the T_2 generations of three independent transgenic tobacco lines (OE6, OE9, and OE13) expressing *TaAQP7* were obtained, as we described previously (Zhou *et al*., 2012). Among the transgenic lines, OE6 and OE9 had higher *TaAQP7* expression levels. The transgenic lines and WT plants were cultured in Murashige and Skoog (MS) medium under a 16-h light/8-h dark cycle at 25 °C for one week. Then the seedlings were transferred to growth chambers of 4 °C for 2 d followed by recovery at 25 °C for one week, and then the whole seedlings were sampled to measure the root length. Furthermore, transgenic lines and WT plants were cultured in MS medium under a 16-h light/8-h dark cycle at 25 °C for one week and then transplanted to containers filled with a mixture of soil and sand (3:1) where they were regularly watered. Six-week-old tobacco plants similar in growth status were exposed to −20 °C for 1.5 h, then returned to room temperature for 10 d of recovery, after which photographs were taken of them. After 2 d of recovery from the −20-°C treatment, leaves were sampled for analysis of the chlorophyll and malondialdehyde (MDA) contents, as well as of the ion leakage (IL). The same measurements were taken on seedlings exposed to 4 °C for two weeks.

Measurement of chlorophyll and MDA contents, and IL

Chlorophyll content was extracted using 95% ethanol and analysed by UV spectrophotometry as described in Yang *et al*. (2009). MDA content was measured according to Heath and Packer (1968). IL was determined as described by Jiang and Zhang (2001).

Results

Cold treatment differentially influences TaAQP7 expression in leaves and roots of wheat seedlings

To investigate the response of *TaAQP7* to cold stress, wheat seedlings were incubated in a growth chamber at 4 °C or 25 °C, and qRT-PCR was performed with leaf and root samples. A no-treatment

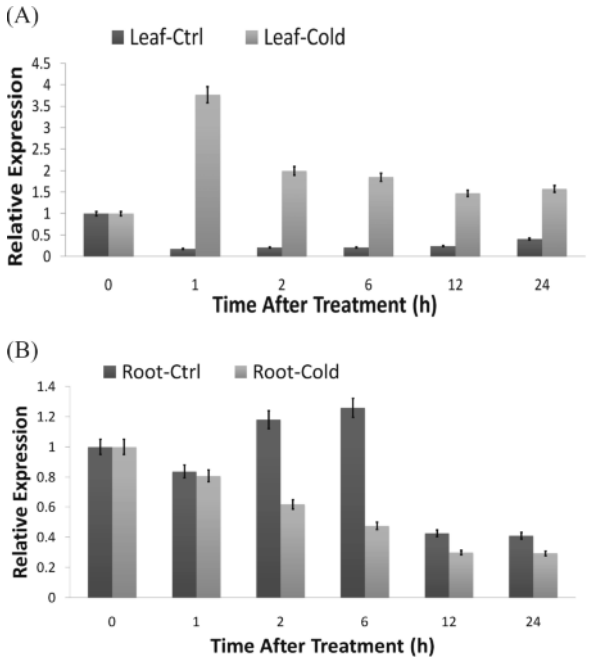

Fig. 1. *TaAQP7* transcript levels in wheat seedlings under cold stress. (A) Expression of *TaAQP7* in leaves of wheat seedlings under cold stress. Ten-day-old wheat seedlings were subjected to 24 h of cold (4 °C) treatment, and leaves were sampled for qRT-PCR analysis of *TaAQP7* transcripts. (B) Expression of *TaAQP7* in roots of wheat seedlings under cold stress. Wheat seedlings were treated as in (A), and root *TaAQP7* transcript levels were determined. Untreated controls were included for each time point. Data are means ± SD of four replicates.

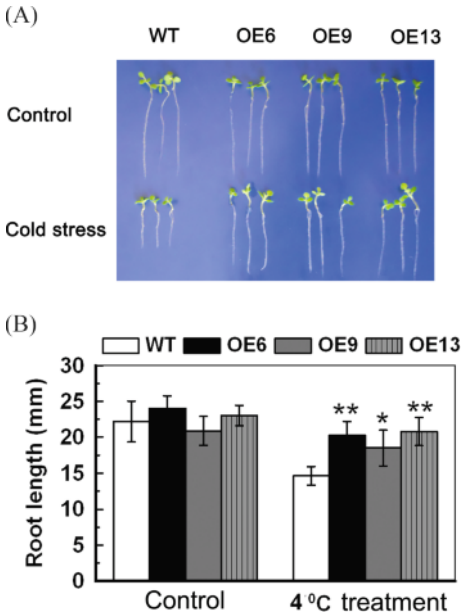

Fig. 2. Effect of cold treatment on *TaAQP7*-expressing tobacco plants during early seedling development. One-week-old transgenic tobacco seedlings were subjected to low temperature (4 °C) for 2 d, followed by recovery at 25 °C for one week. Plants growing at 25 °C were used as control. (A) Photographs of seedlings; (B) root length. Data are means ± SD of four replicates. Similar results were observed in three independent transgenic plants, compared to the respective controls, with $^{*}p < 0.05$ and $^{**}p < 0.01$.

control was always included. *TaAQP7* expression increased in leaves (Fig. 1A), but decreased in roots in response to cold treatment (Fig. 1B) compared with the control plants at the same time points. Previously, *OsPIP2;7* had been reported to be generally upregulated in roots, but downregulated in shoots of rice plants at the early stage of chilling stress (Li *et al.*, 2008). These results imply that the AQPs-mediated cold stress response may be a complex process.

Expression of TaAQP7 improves tolerance of transgenic tobacco plants to cold stress

T_2 generations of three independent transgenic tobacco lines (OE6, OE9, and OE13) expressing *TaAQP7* were obtained in our previous study (Zhou *et al.*, 2012). Among the transgenic lines, OE6 and OE9 had higher *TaAQP7* expression levels than OE13. One-week-old tobacco seedlings were transferred to a growth chamber of 4 °C for 2 d. After recovery for one week at 25 °C, root length was measured. Statistical analysis revealed that, under cold stress, root growth of the transgenic lines was suppressed to a lesser extent than that of WT plants (Figs. 2A, B), while no obvious difference was observed between the transgenic plants and the WT plants in MS medium.

Six-week-old transgenic lines and WT plants were exposed to −20 °C for 1.5 h, then the plants were allowed to recover at 25 °C for 10 d, and their phenotypes were observed. After this extreme cold stress, the WT plants died, while the transgenic plants survived despite having some wilted leaves (Fig. 3). These results suggest that expression of *TaAQP7* could improve the tobacco plants' tolerance to cold stress.

Expression of TaAQP7 in transgenic tobacco plants improves chlorophyll content and decreases MDA content and IL under cold stress

Enhanced cold tolerance in the transgenic lines compared with WT plants led us to look for differences

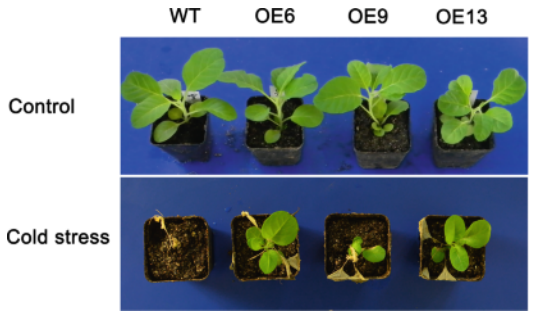

Fig. 3. Response of the transgenic lines OE6, OE9, and OE13 and WT plants to extreme cold shock. Six-week-old tobacco plants were exposed to −20 °C for 1.5 h, then returned to 25 °C for 10 d, and photographs were taken at this time.

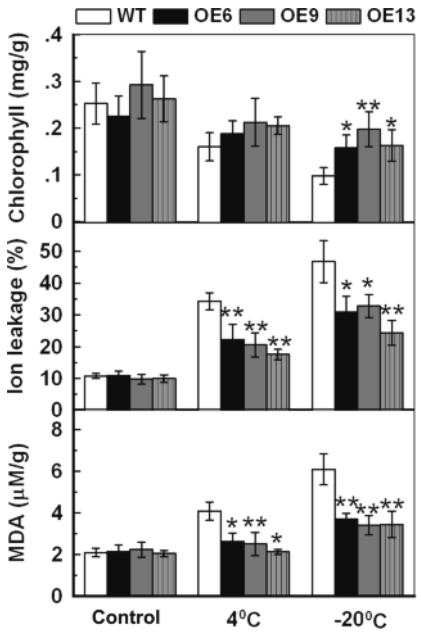

Fig. 4. Leaf contents of chlorophyll and MDA, as well as IL in the transgenic lines (OE6, OE9, OE13) and WT plants under cold stress. Six-week-old tobacco plants were exposed to either −20 °C for 1.5 h followed by 2 d recovery at 25 °C, or 4 °C for two weeks. Plants growing at 25 °C were used as controls. Data are means ± SD of four replicates, compared to respective controls, with $^{*}p < 0.05$ and $^{**}p < 0.01$ (g corresponds to fresh weight).

in physiological parameters known to be affected by cold stress. The transgenic lines had a higher chlorophyll content than WT plants after the −20-°C treatment, but no difference was seen after the 4-°C treatment (Fig. 4). IL, an important indicator of membrane injury, was higher in WT plants than in the transgenic plants after both the 4-°C and −20-°C treatment, suggesting that the transgenic plants suffered less membrane damage than WT plants (Fig. 4). MDA is the product of lipid peroxidation caused by reactive oxygen species (ROS), and is in general used to evaluate ROS-mediated injuries in plants (Moore and Roberts, 1998). MDA contents displayed a pattern similar to those of IL and were lower in the transgenic lines relative to WT plants after cold treatment (Fig. 4). These physiological parameters confirm that the transgenic lines are more tolerant to cold stress.

Discussion

Cold stress damages plants in many ways. For instance, extracellular freezing and thawing cause cell shrinkage and expansion, leading to plant tissue injury (Peng *et al.*, 2008). In addition, cold stress can impact plant-water relations by directly/indirectly inducing desiccation in plant cells (including chilling-induced inhibition of root hydraulic conductivity and extracellular freezing-induced cellular dehydration) (Sanders and Markhart, 2001; Peng *et al.*, 2008). AQPs have been shown to respond to various environmental stresses, including cold stress (Aroca *et al.*, 2005; Guo *et al.*, 2006; Yu *et al.*, 2006; Cui *et al.*, 2008; Mahdieh *et al.*, 2008; Peng *et al.*, 2008; Gao *et al.*, 2010; Sade *et al.*, 2010), and this may be directly related to their function in the transport of water across membranes.

AQPs have been widely reported to be either negatively or positively affected by cold stress. Overexpression of *PIP1;4* and *PIP2;5* led to the enhancement of water uptake upon cold stress in *A. thaliana* (Jang *et al.*, 2007). Overexpression of *OsPIP2;7* improved the transpiration rate and tolerance to low temperature in rice (Li *et al.*, 2008). Expression of *RcPIP2s* and *Panax ginseng PIP1* in *A. thaliana* enhanced the freezing tolerance and cold acclimation of the transgenic plants, which was presumably due to their increased capacity to resist freeze desiccation (Peng *et al.*, 2007, 2008). However, downregulation of *PIP* transcripts in *Arabidopsis* and rice during cold acclimation was beneficial in preventing cellular dehydration and thereby increasing freezing tolerance (Jang *et al.*, 2004; Yu *et al.*, 2006; Heinen *et al.*, 2009). Thus, the differential performance of AQPs under cold stress might be related to different cold response mechanisms. Notably, although transcript levels of some *PIPs* were found to increase significantly in wheat leaves after cold treatment (Herman *et al.*, 2006), no function of wheat AQPs in cold stress tolerance has been reported.

Here, we report that *TaAQP7*, a wheat aquaporin gene, is a positive regulator in cold tolerance. Changes in the expression of *TaAQP7* in response to low temperature suggested that *TaAQP7* was involved in the cold stress response. The functional investigation of *TaAQP7* under chilling (4 °C) and freezing (−20 °C) stress was carried out with transgenic tobacco. The transgenic lines exhibited longer roots under chilling stress, a better growth status after freezing treatment, as well as a higher chlorophyll content, a lower MDA content, and reduced IL, as compared to WT plants. IL is an important indicator of membrane injury. MDA is the product of lipid peroxidation caused by ROS and is generally used to assess ROS-mediated injuries in plants (Moore and Roberts, 1998). The lower MDA content and reduced IL suggest that the transgenic lines suffered less membrane damage after chilling and freezing treatments, indicating that expression of *TaAQP7* could help plants to preserve membrane integrity under cold stress. These results are consistent with previous reports that *OsPIP2;7*-expressing rice plants exhibited increased cold stress tolerance by reducing membrane injury (Li *et al*., 2008).

In conclusion, *TaAQP7*, a wheat aquaporin gene, was characterized as a positive regulator of cold tolerance. Expression of *TaAQP7* in tobacco conferred tolerance to cold stress through relieving membrane damage. Future work will put emphasis on the detailed regulation mechanism of *TaAQP7* involved in cold stress.

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