Overexpressing a Putative Aquaporin Gene from Wheat, TaNIP, Enhances Salt Tolerance in Transgenic Arabidopsis

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(Received August 12, 2009; Accepted March 24, 2010)

High soil salinity is a major abiotic stress in plant agriculture worldwide. Here, we report the characterization of a novel aquaporin gene TaNIP (Triticum aestivum L. nodulin 26-like intrinsic protein), which was involved in salt tolerance pathways in plants. TaNIP was identified and cloned through the gene chip expression analysis of a salt-tolerant wheat mutant RH8706-49 under salt stress. Quantitative reverse transcription–PCR (Q-RT–PCR) was used to detect TaNIP expression under salt, drought, cold and ABA treatment. The overexpression of TaNIP in transgenic Arabidopsis produced higher salt tolerance than wild-type plants. Localization analysis showed that TaNIP proteins tagged with green fluorescent protein (GFP) were localized to the cell plasma membrane. Under salt stress treatment, TaNIP-overexpressing Arabidopsis accumulated higher K\(^{+}\), Ca\(^{2+}\) and proline contents and lower Na\(^{+}\) level than the wild-type plants. The overexpression of TaNIP in transgenic Arabidopsis also up-regulated the expression of a number of stress-associated genes. Our results suggest that TaNIP plays an important role in salt tolerance in Arabidopsis and can also enhance plants’ tolerance to other abiotic stresses.

Keywords: Aquaporin • Salt tolerance • TaNIP • Wheat.

Abbreviations: AA, atomic absorption; AQP, aquaporin; GFP, green fluorescence protein; MS, Murashige and Skoog; PEG, polyethylene glycol; Q-RT-PCR, quantitative real-time reverse transcription–PCR; TaNIP, Triticum aestivum L. nodulin 26-like intrinsic protein.

The nucleotide sequence of TaNIP has been submitted to GenBank under the accession number DQ530420.

Introduction

High soil salinity severely affects agricultural productivity (Boyer 1982). It has been estimated that 20% of cultivated land worldwide is impaired by salinity (Flowers et al. 1995). Under high salt stress, plants are affected throughout the growth process from germination, to seedling growth and development, to vegetative growth and to flowering and fruiting (Sairam et al. 2004). Identifying salt tolerance genes and understanding their functions have become the most urgent tasks in agricultural research today.

Water-selective channel proteins, also known as aquaporins (AQPs), mediate and regulate rapid transmembrane water flow during growth and development processes such as seed germination, cell elongation, stomatal movement, phloem loading and unloading, reproductive growth and stress responses (Eisenbarth et al. 2005). The first plant AQP γ-TIP was isolated from Arabidopsis and is a vacuole intrinsic membrane protein (Maurel et al. 1993). So far, 35 AQP genes have been isolated from Arabidopsis (Quigley et al. 2001), 31 from Zea mays (Chaumont et al. 2001) and at least 14 from Mesembryanthemum crystallinum (Tyerman et al. 1999).

The plant AQP family is divided into four families according to amino acid sequence homology and protein subcellular localization (Johanson et al. 2001): plasma membrane intrinsic proteins (PIPs) (Kammerloher et al. 1994); tonoplast membrane intrinsic proteins (TIPs) (Karlsson et al. 2000); nodulin 26-like intrinsic proteins (NIPs) (Weaver et al. 1991); and small basic intrinsic protein (SIp) (Chaumont et al. 2001). Twenty-four PIP and 11 TIP genes were recently identified in wheat (Forrest and Bhave, 2008); however, to date, there have been no identified NIP genes reported in wheat.

The transcription of AQPs can be induced by environmental stresses. Heterologous overexpression of rice OsPIP-1 and OsPIP2-2 in Arabidopsis improved tolerance to salinity and dehydration, and prevented growth inhibition in the main roots (Guo et al. 2006). When treated with 100 µM NaCl, maize rapidly adapted to salt stress by accumulating ABA, thus regaining water potential within 2 h. In addition, the expression of multiple PIP-type genes was induced (Zhu et al. 2005).

To explore the expression pattern of AQP genes in wheat, quantitative reverse transcription–PCR (Q-RT–PCR) was performed with a salt-tolerant wheat mutant RH8706-49 under 135 mM NaCl salt stress. In the current study, TaNIP, an AQP gene, was identified and its expression was found to be up-regulated by salt stress. TaNIP was cloned and overexpressed in Arabidopsis and its effects on the stress tolerance of Arabidopsis to various abiotic stresses were evaluated.
Results

Cloning of the TaNIP gene

Using the differential expression gene chip result from a salt-tolerant wheat mutant RH8706049 under salt stress, we obtained 81 clusters in total in our gene chip analysis. Fig. 1 shows one of the clusters (the 64th cluster). There are 56 cDNA sequences included in this cluster. Dozens of novel genes have been identified in our laboratory. One of these novel genes was TaNIP. The sequence (Supplementary Fig. 1) was obtained by clustering analysis using Cluster and Tree View software (Eisen et al. 1998), NCBI BLAST search and DNAStar programs. Its expression patterns and involvement in stress tolerance in Arabidopsis were analyzed in this study.

A 930 bp band was obtained by RT–PCR amplification (Supplementary Fig. 2), which matched the band size with predicted sequence. This gene shares the same conserved structural domains as the AQPs and is a member of the AQP family. It contains two Asn–Pro–Ala (NPA) basic sequences (Supplementary Fig. 3) and thus belongs to the NIPs. This gene was named TaNIP (Triticum aestivum L. nodulin 26-like intrinsic protein) and submitted to GenBank (accession No. DQ530420).

Expression of the TaNIP gene in salt-tolerant and salt-sensitive wheat mutants

To validate the gene chip results, TaNIP expression was examined in the salt-tolerant wheat mutant RH8706-49 and salt-sensitive wheat mutant H8706-34 under salt stress (Fig. 2). The results indicate that at 72 h after salt stress, TaNIP was up-regulated in both wheat lines, but its expression was much higher in the salt-tolerant mutant than in the salt-sensitive mutant (Student’s t-test, significant at P < 0.01).

Expression pattern of the TaNIP gene under various abiotic stresses

To explore the response of TaNIP gene to various abiotic stresses, Q-RT–PCR was used to analyze the TaNIP gene expression pattern in roots and leaves of a salt-tolerant wheat mutant RH8706-49 under NaCl, ABA, polyethylene glycol (PEG) and cold treatments (Fig. 3). As shown in Fig. 3A, in root, TaNIP gene expression increased under NaCl stress and showed the highest level at 72 h after stress. Under ABA treatment, TaNIP gene expression increased and showed the highest level at 12 h. Under cold treatment, TaNIP gene expression was inhibited slightly but later increased gradually. PEG treatment inhibited the expression of the TaNIP gene.

The expression patterns of TaNIP in the roots and in the leaves were not identical. In the leaves (Fig. 3B), TaNIP gene expression was up-regulated after stress treatments and the highest level was 3- to 5-fold (Student’s t-test, significant at P < 0.05) more than that of those not subjected to stress treatments. The TaNIP expression patterns also varied with various stress treatments. TaNIP expression was up-regulated and reached the highest level at 12 h after NaCl treatment. Under ABA and cold treatments, TaNIP expression decreased slightly at first but later increased and eventually reached the highest level 12 h after treatment. Under PEG treatment, the TaNIP expression decreased at 1 h and then increased at 6, 12 and 72 h after treatment.

Subcellular localization of TaNIP protein

The seeds of transgenic Arabidopsis plants harboring pCAMBIA1300-TaNIP–GFP (green fluorescent protein) or the
control vector pCAMBIA1300–GFP were germinated under normal condition for 6 d. Then their root cells were examined by confocal microscopy (Fig. 4). In the root cells of pCAMBIA1300–TaNIP–GFP-transformed plants (Fig. 4A, B, C), GFP-tagged TaNIP protein was mainly localized at the cell plasma membranes while no fluorescence was observed in the nuclei. In contrast, fluorescence was found in cell nuclei, cytoplasm and plasma membrane in the root cells of GFP-expressing plants (Fig. 4D).

The TaNIP gene markedly improved salt tolerance of the transgenic Arabidopsis plants

Total RNA was extracted from both wild-type plants and homozygous transgenic Arabidopsis plants, and cDNA was synthesized by reverse transcription. PCR was carried out with TaNIP-specific primers. The PCR band of the TaNIP gene was visible from the homozygous transgenic plants, while no visible band was obtained from the wild-type plants (Supplementary Fig. 4).

To understand the relationship between TaNIP expression and salt tolerance in plants, 4 d cultured seedlings of different homozygous transgenic lines of Arabidopsis of the T3 generation and the seedlings of wild-type Col-0 were transferred to 1/2 Murashige and Skoog (MS) medium containing 100 mM NaCl for vertical culture. The phenotype was examined and root growth was measured after 5 d of culture (Fig. 5). The results indicated that the roots of the transgenic plants were much longer (∼1.7- to 2-fold) (Student’s t-test, significant at P < 0.05) than the roots of the wild-type plants.

Ion content determination using an atomic absorption (AA) spectrometer

The ion content in plants was analyzed with an AA spectrometer and the results are shown in Fig. 6. In the absence of salt treatment, Na+, K+ and Ca2+ levels were higher in TaNIP transgenic plants than in the wild-type plants. After salt treatment, the Na+ content (Fig. 6B) increased in both transgenic and wild-type plants, but the amount of Na+ increase in the transgenic plants was less than that in the wild-type plants. Salt treatment only resulted in a moderate increase in K+ content (Fig. 6C). In contrast, Ca2+ content increased to a similar extent in both transgenic and wild-type plants (Fig. 6A). Further analysis indicated that the K+/Na+ ratio in transgenic Arabidopsis plants was three times higher than in the wild-type plants after salt treatment (Fig. 6D).
Proline content in transgenic Arabidopsis under salt treatment

Proline accumulation facilitates cell tolerance to high salinity. The analysis of proline content revealed a higher proline level in transgenic plants than in the wild type in the absence of salt treatment (Student’s t-test, significant at P < 0.05). After salt treatment, the proline content increased in both transgenic and wild-type plants (Fig. 7).
Expression of stress-associated genes in transgenic Arabidopsis

To evaluate the implications of TaNIP gene in stress response pathways, the expression of nine genes involved in certain stress signaling pathways in Arabidopsis plants was analyzed by Q-RT–PCR. The results indicated that ADH1 (de Bruxelles et al. 1996) expression in plants overexpressing TaNIP increased to approximately 1.5-fold higher than in the wild-type plants (Fig. 8). The expression of the ABA-induced gene RD29B (Yamaguchi-Shinozaki et al. 1994) had the most significant increase in TaNIP transgenic plants, being approximately 11-fold higher than in the wild-type plants (Student’s t-test, significant at $P < 0.01$). In contrast, the expression of a cold-regulated gene, COR15a (Lin et al. 1992), was severely inhibited, and the expression of the P5CS1 gene, a rate-limiting enzyme in proline synthesis (Yoshida et al. 1999), remained approximately the same.

Discussions

In this study, the TaNIP gene was identified using previous expression gene chip results of a salt-tolerant wheat mutant RH8706-49. The biochemical properties and structures of the TaNIP protein were predicted using online database resources. TaNIP belongs to the AQP family and shares high sequence homology with NIP from rice, maize, sorghum, zucchini and Populus (Gardiner et al. 2004, Tuskan et al. 2006, Yamaji et al. 2008).

Plants often face abiotic stresses such as salinity, osmosis, drought and cold. The expression of the TaNIP gene in the root and leaves of wheat was determined by Q-RT–PCR. PCR results revealed up-regulation of TaNIP expression under salt treatment. The results indicated that TaNIP expression was induced by salts and thus the gene could be associated with the salt tolerance pathway in wheat (Fig. 3). TaNIP gene expression was also up-regulated after ABA treatment, suggesting its association with the ABA tolerance pathway as well. TaNIP displayed the opposite expression pattern after PEG treatment.

Subcellular localization experiments indicated that the TaNIP protein was mainly localized on the plasma membrane, which conforms to the characteristics of membrane intrinsic proteins (Fig. 4). Therefore, TaNIP may be involved in regulation of transmembrane transport, which regulates the cellular ion concentration to some extent.

TaNIP transgenic Arabidopsis plants showed higher salt tolerance, and under salt treatment their roots grew significantly longer (~1.7- to 2-fold) than those of the wild-type plants (Fig. 5).
The vacuole is the largest organelle of mature plant cells; it stores nutrients and metabolites, which protect cells from potential injury due to toxic substances. When ions are transported into a cell, salt ions are transported through the Na+/H+ countertransporter protein, on one hand to the apoplast and thus the extracellular place, and on the other hand to the vacuole so as to reduce the injurious effect of salt ions on cytosolic enzymes and keep metabolic reactions running normally (Barkla et al. 1996). The K+ and Na+ levels are important for the health of cells, and the K+∕Na+ ratio is an important indicator of plant salt tolerance (Cuin et al. 2008).

In this study, TaNIP-overexpressing Arabidopsis plants accumulated less Na+ and more K+ than the wild-type plants under salt stress (Fig. 6B, C). Potassium uptake and sodium extrusion is an important approach to increase salt tolerance in wheat (Munns et al. 2006). In transgenic plants, more Na+ was extruded, so a low Na+ content was detected, which indicated that TaNIP enhances salt tolerance in transgenic plants. Because AQPs mediate and regulate rapid transmembrane water flow during growth and development processes, transgenic plants can absorb more water quickly and dilute the Na+ content when TaNIP is overexpressed. The K+∕Na+ ratio of the transgenic plants was 3-fold higher than that of the wild-type plants (Fig. 6D), which suggested a theoretical basis for high salt tolerance in transgenic Arabidopsis. Meanwhile, a higher Ca2+ content was detected in transgenic Arabidopsis. Ca2+ can enhance the activity of AQPs (Cabanero et al. 2005) and also enhance the activity of AQPs in transgenic Arabidopsis (Fig. 7), which regulated downstream SAD1 and RD29B expression. Consequently, the K+/Na+ ratio and the Ca2+ concentration are increased mainly by the Ca2+-dependent SOS pathway (Zhu 2002). However, the detailed mechanisms are still unknown and need to be studied further.

Materials and Methods

Plant and growth conditions

The salt-tolerant wheat mutant RH8706-49 and the salt-sensitive mutant H8706-34 were single-seed descent lines obtained by hybridization of Punong 3665/Bainong 3039 F1, another culture, ethylmethane sulfonate (EMS) mutation and repeated salt stress selection. They have been stably cultured for 15 generations. Wheat seeds were soaked in water till rootling and germination, and then transferred to an aquaculture tank (23°C, light 16 h/dark 8 h). At the two leaves and a bud stage, the plants are ready for stress treatment (Ge et al. 2007). Arabidopsis (Col-0) plants were germinated and cultured on MS medium. All plant tissues were immediately frozen in liquid nitrogen after harvesting and stored at −80°C for further analysis.

Microarray analysis

Plants of the salt-tolerant wheat line RH8706-49 were treated with 135 mM NaCl for 0, 1, 6, 12 and 72 h. The roots were then taken for preparation of total RNA using the TRIzol (Invitrogen, Shanghai, PR China) reagent. Total RNA was purified with the RNeasy Mini kit (Qiagen, Beijing PR China). Double-stranded cDNA was synthesized with the one-cycle cDNA Synthesis Kit (Affymetrix, Shanghai, PR China), and then purified with the GeneChip Sample Cleanup Module (Affymetrix). The purified cDNA was used to prepare biotin-labeled cRNA using a GeneChip IVT Labeling Kit, according to the manufacturer’s instructions. The biotin-labeled cRNA was fragmented at 94°C for 35 min, which yielded the targets used for hybridization. The targets were hybridized with the Affymetrix Wheat Genome Array P/N520254, and washing and scanning were carried out according to the assay procedure. The hybridization image was analyzed with Affymetrix Microarray Suite 5.0 software and the data were normalized (Zhao et al. 2007). Clustering analysis was carried out with the Cluster and Tree View software (Eisen et al. 1998).

Cloning of the TaNIP gene

Total RNA was extracted from leaves of the salt-tolerant wheat mutant RH8706-49 at the two leaves and a bud stage. RNA extraction and cDNA synthesis were performed as reported previously (Ge et al. 2007). The full-length cDNA sequence was
obtained and primers were designed using the Primer Premier 5.0 software: upstream 5′-AAGCAACCCCAACCGAAG-3′ and downstream 5′-GGTATTCCTTCTGGG-3′. The cDNA was used as the template for PCR amplification.

**Analysis of the TaNIP gene expression pattern**

The salt-tolerant mutant RH8706-49 at the two leaves and a bud stage was treated separately with either 170 mM NaCl, 16.7% PEG or 4°C cold. Leaves and roots were harvested at 0, 1, 6, 12 and 72 h after each individual treatment. Subsequently cDNA was synthesized via reverse transcription as reported previously (Ge et al. 2007). Q-RT–PCR was performed on a Rotor-Gene 3000 quantitative PCR machine (Gene Company Ltd.). The results were analyzed with the RG3000 6.0 software (Gene Company Ltd.). The wheat β-actin gene (GenBank accession No. AB181991) was used as an internal reference control. Quantitative analysis was performed with the ΔΔCt comparative method (Livak et al. 2001). The primers for Q-RT–PCR were: TaNIP upstream 5′-GAGGATCAGACGTGGTCGAACT TGCTATCCTTCGTTTGG ACCTT-3′, downstream 5′-AGCGGTGTTTGTGGAGGAG-3′; and β-actin upstream 5′-TGCTATCCTTCGTTTGG ACCTT-3′, downstream 5′-AGCGGTGTTTGTGGAGGAG-3′.

**Vector construction and transformation to Arabidopsis**

The TaNIP gene was subcloned into the dual expression vector pCAMBIA1300. For the TaNIP overexpression construct, two primers were used (upstream: 5′-TCTAGAAGCAACCCCAAC CGGA-3′, downstream: 5′-GGATCCAGACGTGGTCGAACT TGCTATCCTTCGTTTGG ACCTT-3′; the XbaI and SacI sites, respectively, are underlined). For the TaNIP subcellular localization construct, two primers were used (upstream: 5′-TCTAGAAGCAACCCCAAC CGGA-3′, downstream: 5′-GGATCCAGACGTGGTCGAACT TGCTATCCTTCGTTTGG ACCTT-3′; the XbaI and BamHI sites, respectively, are underlined). These two constructs were transformed into Agrobacterium tumefaciens GV3101 using the freeze–thaw method and then transformed into Arabidopsis as reported previously (Clough et al. 1998). Transgenic Arabidopsis was selected using hygromycin and transplanted into vermiculite soil and cultured at 22°C. Two weeks later, the plants were treated with either water or 100 mM NaCl for 10 d. The parts of the plant that were above ground were harvested. For ion content determination, the plant materials were washed with ultrapure water, treated at 105°C for 10 min and baked at 80°C for 48 h. Then 50 mg of the dry material was weighed, placed into a quartz beaker and dissolved with 3 ml of nitric acid. The volume of dissolved solution was brought to an accurate 50 ml, which was diluted 25-fold and used for ion content determination on a VARIAN atomic absorption spectrometer (AA240FS+AA240Z). Proline content determination was performed as reported previously (Troll et al. 1955).

**Expression of stress-related genes in transgenic Arabidopsis**

The expression of nine genes involved in specific stress signaling pathways in TaNIP transgenic Arabidopsis plants was analyzed by Q-RT–PCR to study the mechanism of TaNIP-mediated salt tolerance and the gene’s role in the salt tolerance pathway. The primers are listed in Table 1.

**Supplementary data**

Supplementary data are available at PCP online.

<p>| Table 1 Primers used in Q-RT–PCR |</p>
<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
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<tbody>
<tr>
<td>RD298</td>
<td>5′-GTGAAAGATGCTACTCTCGGGTTCGTC-3′</td>
</tr>
<tr>
<td>KIN2</td>
<td>5′-TCTAGAAGCAACCCCAAC CGGA-3′</td>
</tr>
<tr>
<td>COR15a</td>
<td>5′-TCTGACGAGACCATCAGATGCTCTGG-3′</td>
</tr>
<tr>
<td>PSC5</td>
<td>5′-TTCTGACGAGACCATCAGATGCTCTGG-3′</td>
</tr>
<tr>
<td>ADH</td>
<td>5′-CTCTGGTGTGCTGGTGGTGAGG-3′</td>
</tr>
<tr>
<td>FRY1</td>
<td>5′-CTCTGGTGTGCTGGTGGTGAGG-3′</td>
</tr>
<tr>
<td>SAD1</td>
<td>5′-GGCAACAAATCCTTACGAG-3′</td>
</tr>
<tr>
<td>SOS2</td>
<td>5′-GGATCGAACGAATCCGAG-3′</td>
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<td>Actin</td>
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Funding

This work was supported by the National Natural Science Fund [30971766]; the Hebei Provincial Natural Science Fund [No. C2009000278].

References


