Heterologous Expression of the Wheat Aquaporin Gene TaTIP2;2 Compromises the Abiotic Stress Tolerance of Arabidopsis thaliana

Chunhui Xu*, Meng Wang*, Li Zhou*, Ta Yong Quan, Guangmin Xia*

The Key Laboratory of Plant Cell Engineering and Germplasm Innovation, Ministry of Education, School of Life Sciences, Shandong University, Jinan, Shandong, China

Abstract
Aquaporins are channel proteins which transport water across cell membranes. We show that the bread wheat aquaporin gene TaTIP2;2 maps to the long arm of chromosome 7b and that its product localizes to the endomembrane system. The gene is expressed constitutively in both the root and the leaf, and is down-regulated by salinity and drought stress. Salinity stress induced an increased level of C-methylation within the CNG trinucleotides in the TaTIP2;2 promoter region. The heterologous expression of TaTIP2;2 in Arabidopsis thaliana compromised its drought and salinity tolerance, suggesting that TaTIP2;2 may be a negative regulator of abiotic stress. The proline content of transgenic A. thaliana plants fell, consistent with the down-regulation of P5CS1, while the expression of SOS1, SOS2, SOS3, CBF3 and DREB2A, which are all stress tolerance-related genes acting in an ABA-independent fashion, was also down-regulated. The supply of exogenous ABA had little effect either on TaTIP2;2 expression in wheat or on the phenotype of transgenic A. thaliana. The expression level of the ABA signalling genes ABI1, ABI2 and ABF3 remained unaltered in the transgenic A. thaliana plants. Thus TaTIP2;2 probably regulates the response to stress via an ABA-independent pathway(s).

Introduction
A range of abiotic stresses, including soil salinity, drought and extreme temperature, can compromise crop yield and quality. Improving tolerance to these stresses is thus a major priority in many crop breeding programmes. The effectiveness of water transporters, such as the aquaporins, is an important component of the plant response to stress [1]. The aquaporins belong to a highly conserved major intrinsic protein family, and combine with the cell membrane system to control the flow of water between and within the cell. Their structure is characterized by the formation of six transmembrane domains connected by five loops [2–4]. Based on their sub-cellular localization and sequence, plant aquaporins have been classified into four sub-families, namely the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the nodulin 26-like intrinsic proteins (NIPs) and the small basic intrinsic proteins [5]. There are also three new subclasses of aquaporins in moss Physcomitrella patens, named GlpF-like intrinsic protein (GIP), hybrid intrinsic protein (HIP) and X intrinsic protein (XIP) [6].

The Panax ginseng aquaporin gene PgTIP1 [7], tomato (Solanum lycopersicum) SlTIP2;2 [8] and cotton (Gossypium hirsutum) GhPIP2;7 [9] have been shown to positively regulate salinity and drought tolerance, but negative effects on stress tolerance of certain aquaporins have also been documented [10]. The stress inducibility of aquaporin genes is variable, with examples including both their up- [11–15] and down-regulation [13,14,16,17] as well as their insensitivity [13–15]. Among the wheat aquaporins, it is known that certain TIPs show a marked affinity with ammonia [18], while the activity of particular NIP [19] and PIP genes [20–22] have been associated with an improved response to abiotic stress. Of a set of 35 wheat aquaporin genes, 24 were shown to be PIPs and 11 were TIPs that have diverse sequence characteristics [23]. However, as
yet there is an insufficient understanding of the role of the aquaporins in the abiotic stress response of wheat. The bread wheat cultivar Shanrong No. 3 (SR3) is a derivative of an asymmetric somatic hybrid between cv. Jinan 177 (JN177) and an accession of tall wheatgrass (Thinopyrum ponticum) [24]. SR3 has proven to show an enhanced level of both salinity and drought tolerance over JN177 [25].

A microarray-based gene expression study has shown that in A. thaliana ABA-independent manner. Gene is involved in the down-regulation of proline synthesis and

Table 1. Sequences of PCR primers used.

| Name        | Forward sequence (5'→3')                          | Reverse sequence (5'→3')                          |
|-------------|----------------------------------------------------|---------------------------------------------------|
| TaTIP2      | GCTCTAGATGGCCGGCTCCATCGCCTTCG                      | CGAGCTTCTAGATCGTCCGCCGGACGGGA                     |
| TaTIP2:2    | CTCTCATCTCCAGTCTTCGGTTC                           | CACGTACCGGATACGGACGC                               |
| Actin       | GTCCAATTATGAGGGATACGCC                             | GACACTTCCATGAGAAACAAACCTAC                       |
| GC island1  | TTGTTGTTATATAATTGGGATTG                           | CAAAAGTTTTTACAAATACTACATAC                       |
| GC island2  | TTGTTAGGGGATGGTTACATTAAA                         | AAAATACGTACTACAAACACAC                          |
| TaTIP2:2-GFP| GCTCTAGATGGCCGGCTCCATCGCCTTCG                      | GCGGATCCGATGCATTGCCTGGGCAGACGGGA                 |

Table 1. Sequences of PCR primers used.

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Materials and Methods

Plant materials and growing conditions

SR3 seedlings were raised in half-strength Hoagland's liquid medium [27] at 22°C under a 16h photoperiod with the light intensity of 3,000 lux. At the three leaf stage, a portion of the seedlings was exposed to abiotic stress by the addition to the medium of either 150mM NaCl, 18% w/v PEG or 100 μM ABA. After 0h, 0.5h or 48h of this treatment, RNA was extracted from both the leaf and the root using an RNAiso plus kit (Takara, Dalian, China), following the protocol recommended by the manufacturer. A. thaliana plants were cultured on either half-strength MS medium or soil under the same environmental conditions as the wheat seedlings.

Isolation of TaTIP2;2

The TaTIP2;2 cDNA sequence (GenBank accession number AY525640) was used to design a primer pair (TaTIP2;2 sequences given in Table 1) able to amplify the gene's open reading frame from a template of cDNA prepared from salinity stressed SR3 seedlings. For cloning purposes, an XbaI restriction site was included in the forward primer and a SacI site in the reverse primer. The PCR comprised an initial denaturation of 95°C/5min, followed by 25-30 cycles of 94°C/30s, 55°C/30s, 72°C/60s, ending with a final extension of 72°C/10min. The resulting amplicons were gel-purified, digested with XbaI and SacI and ligated with XbaI/Sacl digested pSTART [28]. The resulting construct was transferred into Agrobacterium tumefaciens strain EHA105 for the agroinfection of A. thaliana Col-0 via the floral dip method [29]. Homozygous transgenic segregants in the T3 generation were used for phenotypic and gene expression analysis. The same primer pair was used to recover the SR3 TaTIP2;2 genomic sequence, and the resulting amplicon was gel-purified and ligated with the pMD18-T vector (Takara, Dalian, China) to allow for its sequencing.

A transmembrane domain prediction of the predicted TaTIP2;2 protein was obtained using the TMHMM tool provided at http://www.cbs.dtu.dk/services/TMHMM. The software package DNAMAN v6.0 (http://www.lynnon.com/) was employed to obtain the intron/exon structure of the genomic sequence. The phylogeny of TaTIP2;2 was investigated by comparing its protein sequence with those of TIP sequences from A. thaliana, rice, maize, barley and wheat represented in the GenBank database.

Chromosomal location of TaTIP2;2

The genomic DNA of a full set of wheat cv. Chinese Spring nulli-tetrasomic lines [30] and a partial set of ditelocentric lines [31] was used as a template for PCRs primed with TaTIP2. The PCR comprised an initial denaturation of 95°C/5min, followed by 25 cycles of 94°C/30s, 55°C/30s, 72°C/60s, ending with a final extension of 72°C/10min. The resulting amplicons were separated by agarose electrophoresis.

Analysis of TaTIP2;2 expression

The cDNA first strand was synthesized using a Tianscript RT kit (Tiangen, Beijing, China), and this was used as the template for a semi-quantitative RT-PCR (sqRT-PCR), primed by TaTIP2 (primer sequences given in Table 1). The wheat Actin gene (GenBank accession AB181991) was used as a reference (primer sequences given in Table 1). The PCR comprised an initial denaturation of 95°C/5min, followed by 35 cycles of 94°C/30s, 55°C/30s, 72°C/60s, ending with a final extension of 72°C/10min.
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Sub-cellular localization of TaTIP2;2 in wheat protoplasts

The TaTIP2;2 gene without its stop codon was cloned into the XbaI and BamHI sites of the pUC-GFP vector to form a TaTIP2;2::GFP gene fusion. The sequence was amplified using the primer pair TaTIP2;2::GFP (primer sequences given in Table 1), and transferred into wheat cv. Yangmai158 protoplasts isolated from embryogenic calli to perform a purpose, three week old seedlings cultured on MS medium which they were transferred onto half strength MS medium containing either 300mM mannitol (drought treatment) or 60°C/30s, 72°C/30s, ending with a final extension of 72°C/10min. The amplified fragments were gel-purified, ligated with the pEASY T1 vector (Transgene) and transformed into E. coli. A random selection of at least 15 clones per amplicon was sequenced. BiQ Analyzer software (http://biq-analyzer.bioinf.mpi-inf.mpg.de/) was used to calculate the the ratios of cytosine methylation at each CG/CNG/CNN site.

Phenotyping of transgenic A. thaliana

Seed of both wild type and transgenic A. thaliana was surface-sterilized (0.1% w/v HgCl₂, 15min), rinsed five times in water, and held at 4°C on half strength MS medium for 36h. To investigate the response to drought and salinity stress, a portion of the seeds was then held at 22°C for 3d, following which they were transferred onto half strength MS medium containing either 300mM mannitol (drought treatment) or 150mM NaCl (salinity treatment). The plates were orientated vertically and held for 10d at 22°C. A second portion of the seed was used to measure seedling proline content. For this purpose, three week old seedlings cultured on MS medium were processed as described by Troll and Lindsley [34].

The expression of stress-related genes in transgenic A. thaliana

RNA of four week old A. thaliana plants on half strength MS was extracted with the same method for SR3 and used as a template for quantitative RT-PCRs targeting the stress-related genes ABI1, ABI2, ABI3, SOS1, SOS2, SOS3, CBF3, DREB2A, DREB2B, MYB2, MYC2, RAB18, RD29B and PSCS1. Relevant primer sequences and the analytical method adopted were as reported elsewhere [35,36].

Results

The sequence characteristics of TaTIP2;2

The SR3 TaTIP2;2 ORF comprises a 747bp sequence, predicted to encode a 248 residue polypeptide. Its sequence is the same with the previously reported TaTIP2;2 (GenBank accession number AY525640) [18]. Alignment of its opening reading frame with the TaTIP2;2 genomic sequence showed that the genomic copy consists of three exons (130bp, 249bp and 367bp) and two introns (83bp and 92bp) (Figure 1A). TMHMM analysis indicated that the protein contains six transmembrane domains (Figure 1B), typical of the aquaporins. An alignment of TaTIP2;2 protein with other plant TIP proteins confirmed the conventional classification of the aquaporin gene family, the three wheat TIP2 proteins clustered closely with one another, and their sequence was ~89% homologous with that of the rice homologue OsTIP2;2 (Figure S1).

When DNA of the Chinese Spring nulli-tetrasomic lines was amplified with the TaTIP2 primer pair, only lines deficient for chromosome 7B (7BA and 7BD) failed to amplify the expected product. The PCR profile of the ditelocentric line carrying the long arm but not the short arm of 7B (7BL) was the same as that of the euploid, while that of the line carrying the short arm but not the long arm (7BS) was the same as that of the lines deficient for chromosome 7B (Figure 1C). Thus the gene must be located on the long arm of chromosome 7B.

Expression of TaTIP2;2 in response to abiotic stress and exogenous ABA

The outcome of the sqRT-PCR experiments showed that TaTIP2;2 was expressed in both the leaf and root of SR3 (Figure 1D). In the root, the gene was down-regulated following a 0.5h exposure to either salinity or drought stress. In the leaf, it was also down-regulated following a 0.5h exposure to salinity, but its down-regulation in response to PEG treatment was delayed. There was no apparent effect of exogenous ABA on its expression (Figure 1D).

Methylation of the TaTIP2;2 promoter under stress treatment

The 2.5kbp region upstream of the SR3 TaTIP2;2 start codon contained two major CG islands, one located at positions -425 to -616, and the other at -1982 to -2,272. Bisulphate PCR analysis showed that almost all the cytosines present at CG dinucleotide sites were hyper-methylated irrespective of the presence of salinity stress (Figure 2A), while the methylation intensity at the CNG trinucleotide sites was increased by the imposition of salinity stress (Figure 2B).

Sub-cellular distribution of TaTIP2;2 protein

Both GFP on its own and the TaTIP2;2::GFP fusion protein were transiently expressed in wheat protoplasts. In the case of the construct containing only GFP, confocal microscopy identified signal throughout the cytoplasm and nucleus (Figure 3A-C). However, in the case of the TaTIP2;2::GFP construct, there was no fluorescence on the cytoplasm membrane; rather the signal was concentrated in the tonoplast of the central
vacuole and throughout the endomembrane system (Figure 3D-F).

**Figure 1. Characteristics of TaTIP2;2.** (A) The genomic copy consists of three exons (indicated by filled bars) and two introns (lines). (B) Domain prediction of the gene product. Internal residues indicated by full lines, external ones by dotted lines. The transmembrane domains are shaded grey. (C) Chromosomal location of TaTIP2;2 based on aneuploid stocks of cv. Chinese Spring. 7BA, 7BD are deficient for chromosome 7B, 7BS for chromosome arm 7BL and 7BL for chromosome arm 7BS. (D) sqRT-PCR analysis shows that TaTIP2;2 was expressed in both the root and leaf, and was down-regulated by drought (PEG) and salinity, but not by ABA.

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**Figure 3. Sub-cellular localization of TaTIP2;2 protein in wheat protoplasts.** (A-C) A transgene encoding GFP alone generates signal throughout the protoplast. (A) Fluorescent image, (B) bright field image, (C) merger of A and B. (D-F) The TaTIP2;2-GFP fusion is deposited in the endomembrane system. (D) Fluorescent image, (E) bright field image, (F) merger of D and E. Bars, 10μM.

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Heterologous expression of TaTIP2;2 in stressed *A. thaliana*

Two homozygous transgenic $T_3$ *A. thaliana* selections (OE1 and OE2) carried TaTIP2;2 driven by the CaMV 35S promoter, and successfully expressed the gene (Figure 4A). The behaviour of OE1 and OE2 differed from that of the wild type...
control in response to exposure to ten days of salinity or osmotic (mannitol) stress. In the presence of NaCl, the growth of the transgenic plants ceased before the cotyledons had fully opened, and most of the seedlings were bleached; in contrast, the cotyledons of the wild type plants opened normally, and bleaching was less frequent (Figure 4B, C). The effect of mannitol stress was to shorten the length of the transgenics’ primary roots compared to those of the wild type (Figure 4D, E). Thus the constitutive expression of TaTIP2;2 in A. thaliana compromised the level of drought and salinity. There was no differential response to the supply of 5μM ABA (Figure 4F). The proline content in OE1 and OE2 was lower than in the wild type (Figure 4G).

Effect of TaTIP2;2 heterologous expression on the expression of abiotic stress-related genes

As revealed by quantitative RT-PCR, the expression level of the ABA signalling genes ABI1, ABI2 and ABF3 was not obviously altered by the presence of the TaTIP2;2 transgene (Figure 5A), similar results were also produced from DREB2B, MYB2, MYC2, RAB18 and RD29B (data not shown), but that of SOS1, SOS2, SOS3, CBF3, DREB2A and P5CS1 was markedly lowered (Figure 5B,C).

Discussion

The participation of TaTIP2;2 in the determination of stress tolerance

Response of plant to drought and osmotic stress is intimately related with water transport, in which the aquaporins are involved [8]. Although a number of attempts have been made to characterize the expression profile of various aquaporin genes [3,37], their in vivo function has been much less well researched [7,10,19,38]. Only few examples have been presented which suggest a role for them in stress response. In wheat, Gao et al. [19] showed that TaNIP activity can promote stress tolerance by increasing the K+/Na+ ratio via the regulation of the SOS pathway; wheat PIP aquaporins genes TaAQP7 [21] and TaAQP8 [20], durum wheat PIP genes TdPIP1;1 and TdPIP2;1 [22], cotton PIP gene GhPIP2;7 [9] were also found to be beneficial to stress tolerance. However, many instances of variation in TIP aquaporin expression induced by abiotic stress have been documented. Thus, the down-regulation of both ZmTIP2;2 and TaTIP2;2 was induced by salinity [15,26,39] and that of OsTaTIP2;2 by low temperature [17]. Here, we have demonstrated that the response of TaTIP2;2 to the imposition of stress suggests that it acts as a negative regulator of salinity and drought stress. The observation that this response is independent of ABA is consistent with previous indications that TIP genes generally are not subject to hormonal regulation [14]. Gene expression can be regulated in a number of ways, but a prominent mechanism is represented by promoter methylation. We have shown that the TaTIP2;2 upstream sequence experienced an increase in C-methylation at CNG sites as a result of the imposition of salinity stress, and that TaTIP2;2 expression was reduced by this treatment. With respect to the regulation of gene expression, C-methylation at CNG sites is thought to be more important than at CG sites [40]. As a result, it is possible that these two events are causally related.

It has been suggested that the TIPs are deposited in the tonoplast. With the A. thaliana TIPs, AtTIP1;1 and AtTIP2;2 localized to the tonoplast of the root central vacuole and vacuolar bulbs [41]. Through an analysis of signal peptides or anchors, the site of TaTIP2;2 deposition has been bioinformatically predicted to lie within the plasma membrane [23], a conclusion inconsistent with the present experimental results, which showed that the protein was present not only in wheat protoplast tonoplasts, but also distributed throughout the endomembrane system (Figure 3D). The P. ginseng protein PgTIP1 appears to enhance the level of stress tolerance, but its sub-cellular location has not been determined [7]. A tomato TIP protein SITIP2;2 was found localized to the tonoplast, and overexpression of it has increased stress tolerance [8]. Contradictory to this, TaTIP2;2 has a negative effect on stress response. Potentially the localization of a given TIP may be related to its effect on the stress response, but as yet there are insufficient relevant data available to reach any sensible conclusion on this issue.
characterized as hydrogen peroxide channels, suggesting their role in the signalling of stress induced by reactive oxygen species [42]. Salinity stressed A. thaliana lines heterologously expressing PgTIP1;1 can accumulate more Na⁺ and are more drought tolerant than the wild type [7]. TaTIP2;2 maintains the osmotic water permeability of tonoplast and extends the capacity of vacuole for osmotic buffering under stress [8]. Evidence for the involvement of TIPs in stress response signalling remains scanty.

Proline is frequently used by plants as an osmolyte, and its accumulation is a common response to a wide range of abiotic stresses [43,44]. Glutamate semialdehyde, the precursor of proline, is formed by the reduction of glutamate catalysed by pyrroline-5-carboxylate synthetase (P5CS) [43]. However, although the expression of TaTIP1 was found to be responsible for increasing the proline content of transgenic A. thaliana, it had no effect on the transcription of P5CS1 [17]. In the present experiments, in contrast, the heterologous expression of TaTIP2;2 did reduce P5CS1 expression (Figure 5C), which may explain the lower proline content of the transgenic lines (Figure 4G). The implication is that TaTIP2;2 expression reduced the osmotic tolerance of transgenic A. thaliana partially via the suppression of proline synthesis.

The SOS pathway genes SOS1, SOS2 and SOS3 are all positive regulators of salinity tolerance in A. thaliana [45]. SOS3 [46] is a calcium sensor, which activates the kinase activity of SOS2 [47]. The resulting complex up-regulates activity of SOS1 [48–50], a Na⁺/H⁺ antiporter located on the cell membrane that exports Na⁺ to the extracellular space and reduces the severity of the salinity stress [48,50]. Heterologous expression of TaTIP has been shown to reduce the level of SOS2 expression, although surprisingly, the phenotypic effect of this reduction was to enhance abiotic stress resistance [19]. All three SOS genes were down-regulated by the TaTIP2;2 transgene (Figure 5B), leading to our hypothesis that the negative impact of TaTIP2;2 expression on the salinity tolerance of transgenic A. thaliana operates via its regulation of the SOS pathway.

Both CBF3 [51] and DREB2A [52] are positive stress regulators acting independently of ABA. Their up-regulation has been shown to enhance the level of drought and salinity stress tolerance. Here, the heterologous expression of TaTIP2;2 down-regulated both genes (Figure 5C), the effect of which would be expected to inhibit plant growth in the presence of abiotic stress. ABA plays a central role in determining stress tolerance [53]. The products of ABA1 [54] and ABI2 [55] are both negative regulators of ABA signalling, while that of ABA3 [56] is a positive regulator. None of these three genes was transcriptionally affected by the heterologous expression of TaTIP2;2 (Figure 5A). Expression of other tested ABA responsive stress regulators MYB2, MYC2 [57,58], RAB18 [59,60] and RD29B [58] was also not changed by the overexpression of TaTIP2;2 (data not shown). These results suggest that the altered stress tolerance of the transgenic A. thaliana plants was not induced through an ABA-dependent pathway. Together with the response of TaTIP2;2 in ABA treated wheat is not obvious (Figure 1D), it can be concluded...
Figure 5. The effect of the heterologous expression of TaTIP2;2 on the expression of abiotic stress-related genes in A. thaliana. (A) ABI1, ABI2 and ABF3 expression was not affected. (B) SOS1, SOS2 and SOS3 were down-regulated. (C) CBF3, DREB2A and P5CS1 were down-regulated. Col: wild type Col-0 ecotype, OE1 and OE2: T3 selections expressing TaTIP2;2. Standard deviation was calculated with STDEVP function of Microsoft Excel 2010. Asterisks indicate significant differences between means (Student's t-test, P<0.05 or 0.01).

that TaTIP2;2 is ABA-independent. In the study of A. thaliana [13], rice [61], Brassica napus [12], Craterostigma plantagineum [62] and radish [14] aquaporin genes, ABA-dependent and independent members were both found, suggests that ABA-independent mechanism is commonly involved in the function of aquaporins.

As a channel protein gene, TaTIP2;2 is behooved to lie at the most downstream of the signalling pathways. How its overexpression regulated several upstream genes of these signalling pathways still needs to be revealed. Our results show that TaTIP2;2 localizes in the endomembrane system (Figure 3). Given that tonoplast aquaporins are important for maintaining the osmotic equilibrium in the cytoplasm [63], it could be suggested that overexpression of TaTIP2;2 change the water balance inside the cells, and therefore lead to the change of the osmotic pressure in some compartments. This could be detected by osmosensors [57, 58, 64], which then triggers the signalling pathways and change the expression of stress responsive genes. On the other hand, aquaporins could transport solutes together with water, like H2O2, ammonia, glycerol, and so on [42, 65–67]. Some of the solutes (for example, H2O2) are secondary messengers for stress response[68], which could also activate stress signalling and change the expression of the related genes.

In summary, it appears that TaTIP2;2 acts as a negative regulator of the abiotic stress response. It is down-regulated by salinity-induced C-methylation in its promoter region. A possible scenario for improving the stress tolerance of wheat and other crops could be to decrease its expression using a genetic engineering approach.

Supporting Information

Figure S1. Phylogeny of plant TIP proteins. AAtIPs from A. thaliana, HvTIPs from barley, OsTIPs from rice, TaTIPs from wheat, ZmTIPs from maize. TaTIP2;2 shown in bold type. (TIF)

Author Contributions

Conceived and designed the experiments: CX GX. Performed the experiments: MW LZ. Analyzed the data: CX MW. Contributed reagents/materials/analysis tools: CX LZ MW TQ. Wrote the manuscript: CX GX.

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