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Overexpression of the wheat salt tolerance-related gene TaSC enhances salt tolerance in Arabidopsis

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Abstract

A novel gene named TaSC was cloned from salt-tolerant wheat. Northern blot showed that the expression of TaSC in salt-tolerant wheat was up-regulated after salt stress. Real-time quantitative PCR analyses showed that TaSC expression was induced by salt and ABA in wheat. Localization analysis showed that TaSC proteins were localized to the plasma membrane in transgenic Arabidopsis thaliana. The overexpression of TaSC in Col-0 and atsc (SALK_072220) Arabidopsis strains resulted in increased salt tolerance of the transgenic plants. TaSC overexpression in Col-0 and atsc significantly up-regulated the expression of AtFRY1, AtSAD1, and AtCDPK2. AtCDPK2 overexpression in atsc rescued the salt-sensitive phenotype of atsc. The TaSC gene may improve plant salt tolerance by acting via the CDPK pathway.

Key words: Arabidopsis thaliana, QRT-PCR, salt tolerance, signalling pathway, TaSC gene, wheat.

Introduction

High-salt stress may affect the development and production of individual plants (Pitman et al., 2002). Many studies have indicated that plants under salt stress can activate and synthesize a large number of functional proteins, including transcription factors, enzymes, molecular chaperones, ion channels, and transporters, which lead to a variety of physiological and metabolic responses against salt stress (Tran et al., 2004; OuYang et al., 2007; Ligaba et al., 2011). The wheat transcription factors TaPIMP1 (Liu et al., 2011), TaSRG (He et al., 2011) and TaMYBsd1u1 (Rahaie et al., 2010), as well as the rice transcription factor OsWRKY45 (Tao et al., 2011), can improve salt and drought tolerance in transgenic plants. CBL-interacting protein kinase (ZmCIPK) genes in corn were up-regulated under salt, drought, heat, and cold stresses (Chen et al., 2011). Under salt stress, plants accumulate proline to resist osmotic stress and promote the expression of salt-responsive genes whose promoter regions contain proline-responsive elements (PRE) (Satoh et al., 2002; Oono et al., 2003); this response improves salt tolerance. Δ1-Pyrroline-5-carboxylic acid synthetase (P5CS) plays an important role in the proline synthesis pathways and is regulated by abscisic acid (ABA) (Xiong et al., 2001a). P5CS overexpression in tobacco increases the free proline levels in transgenic plants (Kishor et al., 1995).

The signalling transduction networks activated in plants under stresses such as low temperature, drought, and salt include the plant mitogen-activated protein kinase (MAPK), Calcium-dependent protein kinase(CDPK) and the salt overly sensitive (SOS) pathways (Li and Nam, 2002). MAPK cascades play an important role in mediating biotic and abiotic stress responses (Liu et al., 2010). MAPK cascade pathways are composed of three serine/threonine protein kinases: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK (Takada et al., 1998). First, MAPKKK is phosphorylated and phosphorylated MAPKKK subsequently phosphorylates the downstream MAPKK which, in turn, phosphorylates MAPK (Gustin et al., 1998). Phosphorylated MAPK then activates a set of genes via the regulation of appropriate transcription factors. In Arabidopsis protoplasts, MKK2 is specifically activated by...
stress-induced MAPK kinase MEKK1 under cold and salt stresses (Teige et al., 2004). CDPK has been implicated in signalling pathways in response to different stresses (Chinnusamy et al., 2006). CDPK activates the stress-induced promoter to enhance the expression of stress proteins under drought and salt stresses (Sheen, 1996). The transcription of AtCDPK1 and AtCDPK2 genes increases in Arabidopsis after 1 h of exposure to salt or drought stresses (Urano et al., 1994). FRY1 and SAD1 are genes downstream of CDPK. Genetic analysis of the FRY locus of Arabidopsis suggests the involvement of IP1 in ABA, salt, and cold stress signalling (Xiong et al., 2001b). SAD1, a Sm-like protein in Arabidopsis, may directly or indirectly regulate the upstream signalling events that control stress-relative gene transcription (Xiong et al., 2001c). The SOS pathway is critical for plant salt-stress tolerance, and it plays a key role in regulating ion transport under salt stress (Batelli et al., 2007).

In this pathway, an increase in cellular Ca2+ concentration can trigger the interaction between SOS3 and SOS2, resulting in the formation of a protein kinase complex that further activates SOS2. Activated SOS2 can further phosphorylate SOS1, a Na+/H+ antiporter gene, at the plasma membrane. In response to NaCl treatment, transgenic plants overexpressing SOS1 had higher levels of Na+ in the xylem transpiration stream and in the shoot (Zhu, 2002; Shi et al., 2003).

Wheat is an important staple crop in many regions worldwide, and the study of its salt-tolerance mechanisms is of great significance for food security. Plant transcriptome analysis measures gene expression differences under different abiotic stresses in a high-throughput manner (Seki et al., 2002). Genome-wide expression analysis can be conducted via cDNA-AFLP, an AFLP-based transcript profiling method used to identify differentially expressed genes in any species without the need for prior sequence knowledge. This method has found widespread use in temporal quantitative gene expression analysis and in gene discovery studies due to its fragment detection ability (Vuylsteke et al., 2007). Using cDNA-AFLP technology, differentially expressed genes were analysed between the salt-tolerant wheat mutant RH8706-49 (abbreviated as 49) and the salt-sensitive mutant H8706-34 (abbreviated as 34) under salt stress, and many EST fragments were obtained. G09-94 was found to only be up-regulated in salt-tolerant wheat, its cDNA sequence was used as the initial sequence and the full-length cDNA of the gene was cloned. The gene was named TaSC (Triticum aestivum L. Salt-tolerant Correlative) and registered in GenBank (accession number: AY956330). The expression level of TaSC was measured, and its subcellular localization and salt-tolerance properties were also investigated.

cDNA-AFLP analysis and RT-PCR

Overall, seeds from the 49 and 34 lines were incubated at room temperature for 24 h, transferred to petri dishes, cultured for 10 d, and the one-leaf stage at 25 °C with light (16/8 h light/dark) and with hydroponics and then transferred to 1/2 strength Hoagland’s nutrient solution. Each line was treated with the following two conditions: one group was the non-treated control, and the other group was treated with 170 mM NaCl for 72 h. The leaves were then collected for RNA extraction using the RNeasy Plant Mini Kit (Qiagen), and the cDNA-AFLP experiments were performed according to Bachem’s method (Bachem et al., 1996). According to the cDNA-AFLP results, the expressed fragments induced by salt stress were sequenced, and a full-length cDNA sequence was obtained by BLAST in NCBI (http://www.ncbi.nlm.nih.gov/). RT-PCR was performed, and the PCR product was ligated into the T-vector (Takara Co. Ltd.). The following RT-PCR primer pairs were used: 5'-TCTAGAGAGGCAGGGTTTGTGCAGG-3' (forward) and 5'-GAGCTCTCCATTTCCAGGAGCGGTC-3' (reverse).

Northern blot analysis

Wheat mutants 49 and 34 cultured to the stage of two leaves and one shoot with hydroponics were treated with 170 mM NaCl for 72 h, and total RNA was extracted from their leaves. RNA from the non-treated wheat leaves was used as the control. α-32P-dCTP (5000 Ci mmol-1) (Furui Biological Engineering, Beijing, China) was integrated into the TaSC sequence as a PCR amplification probe. The Northern blot hybridization process was performed according to Sambrook et al. (2001). After washing the membrane, the hybridization results were detected using a CS-930 dual-wavelength chromatogram scanner (Shimadzu, Japan).

Expression vector construction and Arabidopsis transformation

A binary overexpression vector, pCAMBIA1300-CaMV35S-TaSC, was constructed and transformed into wild-type and atsc mutant Arabidopsis strains using the floral-dipping method. The resulting TaSC-1OE, TaSC-2OE, and TaSC-3OE transgenic homozygotes were used in the salt-tolerance experiment. The following primer pairs were used to construct the overexpression vector: 5'-TCTAGAGAGGCAGGGTTTGTGCAGG-3' (forward) and 5'-GAGCTCTCCATTTCCAGGAGCGGTC-3' (reverse).

Fusion expression vector construction and Arabidopsis transformation

The fusion gene expression vector pCAMBIA1300-CaMV35S-TaSC-GFP was constructed and transformed into wild-type Arabidopsis. The transgenic plants were cultured in tissue culture for 5 d and used for subcellular localization studies. The primer pairs used to construct the subcellular localization vector were the following: 5'-TCTAGAGAGGCAGGGTTTGTGCAGG-3' (forward) and 5'-GAGCTCTCCATTTCCAGGAGCGGTC-3' (reverse).

TaSC overexpression vector transformation of the Arabidopsis atsc mutant

The AtSC (AT1G36980) gene is a homologous gene to TaSC and shares 71% protein homology to TaSC. In this work, the atsc mutant (SALK-072220) strain that was obtained by T-DNA insertion into the third exon of the AtSC gene (see Supplementary Fig. S2 at JXB online) was purchased from the SALK database. It was found that AtSC gene expression was absent in the mutant (see Supplementary Fig. S3 at JXB online); therefore, it is an AtSC-knockout mutant. A binary overexpression vector, pCAMBIA1300-CaMV35S-TaSC, was transfected into the atsc mutant to acquire a 35S::TaSC (atsc) line.

Materials and methods

Experimental materials

The wheat salt-tolerant mutant RH8706-49 (abbreviated as 49) and the salt-sensitive mutant H8706-34 (abbreviated as 34) are near-isogenic lines with similar genetic backgrounds, but their salt-tolerance levels are different (Ge et al., 2007). The Arabidopsis thaliana ecotype Columbia (Col-0) and an Arabidopsis thaliana mutant with AtSC-knockout, which was obtained by T-DNA insertion (SALK-072220) (purchased from the Salk Institute Genomic Analysis Laboratory) were also used.
Salt tolerance of transgenic Arabidopsis and the determination of the ion, chlorophyll, and proline contents

The germination rate and seedling root length under salt stress of the wild type and three transgenic homozygotes, TaSC-1OE, TaSC-2OE, TaSC-3OE, were determined. For determination of the germination rate, the sterilized seeds were sown in MS medium containing 120 mM NaCl. The germination rates were then calculated after a 5 d culture in an illuminated incubator. To measure the seedling root length, the sterilized seeds were placed in MS medium and cultured vertically for 5 d, transferred into MS medium containing 100 mM NaCl and vertically cultured for another 4 d at 22 °C before measurement of root length under salt stress. The wild-type, atsc mutant, TaSC-2OE and 35S::TaSC (atsc) plants were grown in the salt-tolerant experiments, and some physiological indexes were detected. The plants were cultured for 2 weeks in the greenhouse and then irrigated with 170 mM NaCl solution for 10 d. Phenotypic changes were observed, and physiological parameters were measured after NaCl treatment. To determine the chlorophyll content (Arnon, 1949), the absorbance at 663 nm and 645 nm was measured using the UV spectrophotometer (PGENERAL UV-1800S). Atomic absorption was used to determine the Na⁺, K⁺, and Ca²⁺ content, as conducted by Sanui and Pace (1966). The proline content determination used the protocol of Gao et al. (2010). In all experiments, the untreated Arabidopsis lines were used as controls.

Quantitative real-time PCR

RH8706-49 and H8706-34 plants were cultured by hydroponics to the stage of two leaves and one shoot and subjected to a treatment of 170 mM NaCl or 50 µM ABA for different periods of time. Total RNA from the root was extracted at different time points (0, 1, 6, 12, 24, and 72 h). Reverse transcription was performed according to the protocol of the Takara RNA PCR Kit (AMV) (purchased from Takara Co. Ltd.). Real-time quantitative RT-PCR was performed using SYBR Premix ExTaq (purchased from Takara Co. Ltd.) in a Rotor-Gene 3000 quantitative PCR instrument (Corbett Research, Mortlake, NSW, Australia). The results were analysed using RG3000 6.0 software (Gene Company Ltd., Hong Kong). The β-actin gene from wheat (GenBank accession No. AB181991) was used as an internal reference. Using the ΔΔCt comparative quantitative method to analyse the samples, each sample was analysed three times. The quantitative primer pairs were the following: 5'-CGTCCAGGTCTCCTTCATCC-3' (forward) and 5'-TGGACATCGAGCTTCTATCC-3' (reverse) for TaSC; 5'-TGAGATCTCTTACCTGAGG-3' (forward) and 5'-AGGGGTGTTTGGAGAGGAT-3' (reverse) for β-actin.

Examination of stress-related gene expression and analysis of signalling pathways in transgenic Arabidopsis

Wild-type, atsc mutant, TaSC-2OE, and 35S::TaSC (atsc) Arabidopsis lines were cultured on soil for 15 d. Total RNA of the seedlings was extracted for reverse transcription to generate cDNA. Using real-time PCR, the expression of salt-tolerant genes such as AtSOS2, AtSOS3, AtFRY1, AtADH, AtCOR15a, AtKIN2, AtP5CS1, AtRD29B, AtCDPK1, and AtCDPK2 was detected. Actin was used as the internal reference in testing the expression of AtSOS2, AtSOS3, AtFRY1, and AtADH, which are the downstream genes of the SOS and the CDPK pathways. The sequences of the quantitative PCR primers are listed in Table 1.

Overexpression vector construction of AtCDPK2 and Arabidopsis atsc mutant transformation

Using Arabidopsis cDNA as a template, the AtCDPK2 (ATIG35670) gene was amplified by PCR. The binary vector pCAMBIA1300-CaM35S::AtCDPK2 was constructed and transformed into the atsc mutant, and the homozygous transgenic lines, 35S::AtCDPK2 (atsc), were subjected to the salt tolerance test. The primer pairs used to construct the vector were the following: 5'-atctctagagagacagagctgttggaacc-3' (forward) and 5'-agcccgagatctcctggagc-3' (reverse).

Table 1. The primer pairs and genes for quantitative PCR

<table>
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<th>Gene names</th>
<th>Primer sequences</th>
<th>Gene names</th>
<th>Primer sequences</th>
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<tr>
<td>AtSOS2</td>
<td>5'-ATTAGGCGCTGGATAGCACC-3' (forward)</td>
<td>Atkin2</td>
<td>5'-GTCTAGGAGCAACCCACAGAATGTC-3' (forward)</td>
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<td>5'-TGAGAGCTTGGTTGTTGGGTC-3' (reverse)</td>
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<td>AtSOS3</td>
<td>5'-GGAGGATCTCTCCCTGCT-3' (forward)</td>
<td>AtP5CS1</td>
<td>5'-TTCTGGATAGTTTCTGAGAGATT-3' (forward)</td>
</tr>
<tr>
<td></td>
<td>5'-CAGGAAAGGCTTACCCACTC-3' (reverse)</td>
<td></td>
<td>5'-TGAGATCTCTGATGTTCTGTTG-3' (reverse)</td>
</tr>
<tr>
<td>AtFRY1</td>
<td>5'-GCCGGTACAGCACTAGGATTTG-3' (forward)</td>
<td>AtRD29B</td>
<td>5'-GTGAAGATGACTATCTCAGGCTG-3' (forward)</td>
</tr>
<tr>
<td></td>
<td>5'-TGACACCGAGTTTTATTG-3' (reverse)</td>
<td></td>
<td>5'-TACCAGAGACTCAAGACTCCTGTTG-3' (forward)</td>
</tr>
<tr>
<td>AtSAD1</td>
<td>5'-GGGACACATCTTCATCAGAG-3' (forward)</td>
<td>AtCDPK1</td>
<td>5'-CGTCTGGTTGGCCAGTGAGAATCTGAG-3' (forward)</td>
</tr>
<tr>
<td></td>
<td>5'-CTTCGGGAGAACCACCT-3' (reverse)</td>
<td></td>
<td>5'-CCATGGGATGATCACAATCAGTGATG-3' (reverse)</td>
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<tr>
<td>AtADH</td>
<td>5'-CTTGTCGCTGGCTGGTTTAAGG-3' (forward)</td>
<td>AtCDPK2</td>
<td>5'-GTCCTGCTTACCTTCCCGGATAT-3' (forward)</td>
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<td></td>
<td>5'-CAAGAATTACCCTGCCATATGTTGCT-3' (reverse)</td>
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<tr>
<td>ArCOR15a</td>
<td>5'-GGGACACATCTTCATCAGAG-3' (forward)</td>
<td>Actinin</td>
<td>5'-AGGCACCTTAAACCCATAAACCG-3' (forward)</td>
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<td>5'-CTTGGGAGAACCACCT-3' (reverse)</td>
<td></td>
<td>5'-GGGACACGAGAATCTCCTGACG-3' (reverse)</td>
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</tbody>
</table>
Results

Cloning of the TaSC gene

In the cDNA-AFLP experiments, many differentially expressed fragments were observed. For example, the G09-94 EST fragment had weaker expression in the 34 and 49 lines before stress induction. Its expression increased in 49 after 170 mM NaCl stress for 72 h, but no significant increase was observed in 34 (Fig. 1). The length of the G09-94 EST fragment was 254 bp, and the full-length cDNA sequences in 34 and 49 were cloned via in silico cloning and RT-PCR. The two sequences were found to be identical using sequence alignment (see Supplementary Fig. S1A at JXB online) and shared 95% homology with the Hordeum vulgare gene (BAK02180.1) as well as 89% homology with the rice gene (NM_001069875.1). However, the function of this gene has not been reported. Consequently, it is a novel gene with unknown function and was named TaSC (Triticum aestivum L. Salt-tolerant Correlative) in GenBank, with the accession number of AY956330. Bioinformatic analysis showed that this gene encodes a protein with a signal peptide (software Anthe4_3c) (see Supplementary Fig. S1B at JXB online) and four transmembrane domains (http://www.sbc.su.se/-erikw/toppred2) (see Supplementary Fig. S1C2 at JXB online) and it was predicted to be a transmembrane protein.

Northern blot of the TaSC gene

Northern blot hybridization results showed that TaSC expression was similar in the non-treated 34 and 49 lines. After 72 h of NaCl treatment, no significant increase was found in 34; however, a significant increase was observed in 49 (Fig. 2). Quantitative results showed that TaSC gene expression in 49 was approximately twice the level in the controls. This result confirms that TaSC gene expression in 49 was enhanced by salt-induction, which was consistent with the cDNA-AFLP results.

Effects of salt stress on transgenic plants

The salt-tolerance test on the TaSC-OE Arabidopsis thaliana lines showed that, without salt treatment, the germination rate (Fig. 3A) and seedling root length (Fig. 3D) of the transgenic Arabidopsis lines were not significantly different compared to Col-0, the wild-type control. On the MS medium containing 120 mM NaCl, the germination rates of the three transgenic lines were 95%, 93%, and 89%, respectively (Fig. 3B, 3C), compared with a germination rate of 78% in the wild-type control. Furthermore, the controls grew more slowly than the transgenic lines after germination. The germination rate of the transgenic seeds showed a statistically significant difference compared with the controls (t test, P < 0.05). After germination, the 5-d-old seedlings were transferred onto MS medium containing 100 mM NaCl to culture for another 4 d. It was found that the average root length of the transgenic plants was significantly longer than that of the wild-type control (t test, P < 0.05) (Fig. 3E, 3F). The wild-type, atsc mutant, TaSC-2OE, and 35S::TaSC (atsc) lines were cultured for 14 d under normal conditions and then irrigated with 170 mM NaCl for 10 d. Consequently, the stems and leaves of the wild-type plants turned yellow and the atsc mutant became entirely yellow and stopped growing. Furthermore, the TaSC-2OE line grew well, and the 35S::TaSC (atsc) line grew better than the wild-type line but worse than the TaSC-2OE line (Fig. 3H, 3I). After 24 d of normal culture, no significant difference in phenotype was observed in all of the non-treated controls (Fig. 3G).

TaSC gene expression induced by NaCl and ABA

Real-time PCR was conducted to investigate TaSC gene expression induced by NaCl and ABA in wheat mutants 34 and 49. TaSC gene expression did not change when the salt-sensitive mutant 34 was treated with 170 mM NaCl; however, in the salt-tolerant mutant 49, the TaSC gene was down-regulated at 3 h and then up-regulated at 12 h after salt treatment. Ultimately, TaSC gene expression eventually increased 2-fold compared with non-treated 49 plants at 72 h (t test, P < 0.01) (Fig. 4). TaSC expression gradually decreased in 34 in wheat seedlings treated with 50 µM ABA. TaSC expression gradually increased in 49 and reached 2.3-fold the level in the control after 12 h of salt treatment (P < 0.01) and it eventually decreased to a level that was slightly higher than the level in the non-treated control after 72 h (Fig. 4).

TaSC protein subcellular localization

The TaSC protein has a signal peptide and four transmembrane domains, which suggests that the TaSC protein is located in the plasma membrane. Arabidopsis root cells transfected with the TaSC-GFP fusion gene or an empty vector were observed using confocal microscopy. The results showed that the fluorescence of the control plants with the empty vector was distributed at the nucleus and plasma membrane; however, the fluorescence of the
TaSC gene overexpression enhances salt tolerance of transgenic Arabidopsis

Chlorophyll, proline, and ion content

The TaSC-2OE, wild-type, atsc mutant, and 35S::TaSC (atsc) lines of Arabidopsis were treated with 170 mM NaCl for 10 d and the chlorophyll, proline, Na⁺, Ca²⁺, and K⁺ contents were determined, using non-treated plants of each line as a corresponding control. No significant differences among the non-treated plants of different lines were observed. After salt-stress treatment, the chlorophyll contents of all lines decreased. The TaSC-2OE line underwent the smallest decrease, from originally 1.6 mg chlorophyll g⁻¹ of leaf to 1.35 mg g⁻¹ of leaf, followed by the wild-type and 35S::TaSC (atsc) lines. The largest decrease in chlorophyll content was found in the atsc mutant line, which dropped from 1.53 mg g⁻¹ of leaf to 0.8 mg g⁻¹ of leaf (Fig. 6A). After exposure to salt stress, the proline content in the four Arabidopsis lines increased. The TaSC-2OE line underwent the largest elevation in proline content, which increased from 70 µg g⁻¹ of leaf to 155 µg g⁻¹ of leaf, followed by the 35S::TaSC (atsc) and wild-type lines. The atsc mutant line showed the smallest increase in proline content (Fig. 6B). The atomic absorption test showed that salt stress increased the Na⁺ concentration and decreased the K⁺ concentration in the four Arabidopsis lines. The TaSC-2OE line of Arabidopsis underwent the smallest increase in Na⁺ concentration and the smallest decrease in K⁺ concentration; therefore, it had a higher K⁺/Na⁺ ratio of 4.5 than the other lines (Fig. 6C, 6E). The K⁺/Na⁺ ratio in the 35S::TaSC (atsc) line was also higher than the ratio of the wild-type line, and the ratio in the atsc mutant line was the lowest at 1.1 (Fig. 6F). After salt stress, the Ca²⁺ concentrations in all the lines declined with the smallest decrease found in the TaSC-2OE line, and the largest decrease observed in the atsc mutant line (Fig. 6D).

The effect of TaSC overexpression on the expression of known salt-tolerant genes

The real-time PCR assay results of the downstream genes in the signalling pathway are illustrated in Fig. 7A. In the transgenic plants that overexpressed TaSC, the expression of AtFRY1 and AtSADI was 2.46- and 2.5-fold the levels in the controls, respectively. However, the expression of AtSOS2 and AtSOS3 did not change markedly. In the 35S::TaSC (atsc) lines, overexpression of TaSC improved the expression of AtFRY1 and AtSADI (Fig. 7A).

In the TaSC-2OE transgenic plants, real-time PCR results showed that the expression levels of the salt-tolerant genes AtADH, AtCOR15a, AtP5CS1, AtRD29B, and AtCDPK2 were significantly higher than the levels in the wild-type. They were 3.1-, 3.05-, 4.83-, 2.08-, and 2.25-fold greater than the control level, respectively, and the expression of AtKIN2 changed relatively little. In the atsc mutant line, AtKIN2 expression significantly increased and was 2.89-fold the level in the control, and AtRD29B expression significantly decreased to 0.31-fold of the control level. AtP5CS1 expression was slightly reduced to 0.75-fold of the control level, and AtCDPK1 expression decreased significantly to 0.23-fold of the level in the control.
The expression of the other tested genes did not change substantially. In the 35S::TaSC (atsc) line, overexpression of TaSC led to the enhanced expression of AtADH, AtCOR15a, AtP5CS1, AtRD29B, AtCDPK1, and AtCDPK2 (Fig. 7B).

**Phenotype of the atsc mutant and 35S::AtCDPK2 (atsc) lines under salt stress**

The atsc mutant and three 35S::AtCDPK2 (atsc) lines were treated with 170 mM NaCl, and their morphological properties were studied. The results showed that all non-treated plants grew similarly regardless of their genetic disparity (Fig. 8A). After treatment with 170 mM NaCl, the leaves and stems of the atsc mutant line were yellow and stopped growing; however, the 35S::AtCDPK2 (atsc) lines grew continuously (Fig. 8B, 8C). These results show that the AtCDPK2 gene can increase the salt tolerance of the atsc mutant, and the TaSC gene is probably upstream of the CDPK2 gene.

**Discussion**

Wheat is hexaploid, and its genome sequence is large and unknown. Furthermore, it is difficult to analyse and study the...
TaSC gene overexpression enhances salt tolerance of transgenic Arabidopsis

mechanism of tolerance in wheat genetically. Arabidopsis is the model plant organism; therefore, heterogeneous transformation was performed to study the function of wheat genes. A novel gene, TaSC, was cloned from wheat, transformed the gene into Arabidopsis, and then used the transgenic Arabidopsis plants to study the gene function.

TaSC was cloned using cDNA-AFLP and RT-PCR in this work. The gene showed significantly different expression levels in the salt-tolerant wheat mutant 49 compared with the salt-sensitive mutant 34 under salt stress (Figs 1, 2, 4). Furthermore, TaSC protein is located in the plasma membrane (Fig. 5). TaSC gene expression was up-regulated by salt stress, which increased to 2-fold of the original level in 49 after salt treatment for 72 h (Fig. 2).

To determine the function of the TaSC gene under salt stress, the salt tolerance of Arabidopsis that overexpressed TaSC was
analysed. Morphological analysis showed that, in the transgenic plants overexpressing TaSC, the germination rate (Fig. 3B), seedling root length (Fig. 3E), and salt-tolerance (Fig. 3H, 3I) were significantly higher in the controls after salt stress treatment. Mutant atsc (Salk-072220) plants were identified as salt-sensitive, and the overexpression of TaSC in the atsc mutant improved their salt tolerance (Fig. 3H, 3I). This finding indicates that TaSC overexpression improves the salt tolerance of Arabidopsis; therefore, TaSC is a salt-tolerance related gene.

Salt stress often leads to Na⁺ excess and K⁺ decrease, which causes an ion-toxic effect in cells, physiological drought, and lack of nutrients (Zhu et al., 2001). Therefore, the maintenance of a low intracellular Na⁺ concentration and a low cytosolic Na⁺/K⁺ ratio is essential for salt tolerance in plants (Lynch and Läuchli, 1984; Maathuis and Amtmann, 1999). Studies have shown that, among glycophytes such as wheat, Na⁺ efflux and the high K⁺/Na⁺ ratio are the key mechanisms involved in salt tolerance (Rus et al., 2001; Munns et al., 2006; Rodriguez-Navarro and Rubio, 2006). Ren et al. (2005) found that the rice gene OsHKT8 in durum wheat prevents the uptake of Na⁺ from the xylem and leaf sheath under salt stress to maintain a low Na⁺ and high K⁺ concentration as a Na⁺-selective transporter. The HKT gene can unload Na⁺ from xylem vessels into xylem parenchyma cells. To explore the mechanism by which TaSC overexpression improves plant salt tolerance further, the Na⁺ and K⁺ contents in transgenic and control plants were examined under normal growing conditions and salt-stress conditions. It was found that the TaSC-2OE transgenic line had a high K⁺/Na⁺ ratio. By contrast, the atsc mutant had a higher level of intracellular Na⁺, which resulted in a lower K⁺/Na⁺ ratio (Fig. 6F). Garg et al. (2002) observed that TPSP transgenic rice has a lower Na⁺/K⁺ ratio than non-transgenic rice, which may be the basis for the reduced Na⁺-toxicity under salt stress. Therefore, we speculate that TaSC overexpression ensures a good performance of physiological activities and imparts higher salt tolerance by increasing the K⁺/Na⁺ ratio when a plant is under salt stress.

Salt-tolerant related genes often function via signalling pathways. The experimental results show that the AtSOS1, AtSOS2, AtSOS3, and AtCDPK2 genes in the CDPK pathway were up-regulated in the TaSC-2OE plants and down-regulated in the atsc mutant; however, AtSOS2 and AtSOS3 gene expression levels did not undergo considerable changes (Fig. 7). Furthermore, the AtCDPK2 genes were transfected into the atsc mutant and subsequently increased the salt tolerance of the mutant (Fig. 8). The results show that TaSC may involve the CDPK pathway and is perhaps upstream relative to the AtCDPK2 genes. Protein kinase CDPK in the CDPK signalling pathway is Ca²⁺-dependent. Ca²⁺ activates CDPKs and regulates the expression of downstream transcription factors (Knight and Knight, 2001; Ludwig et al., 2004), which enhances the expression of the known salt-tolerant genes such as AtCOR15a, AtRD29B, AtP5CS1, and AtADH (Xiong and Zhu, 2002). It was found that the expression of these genes was significantly enhanced (Fig. 7B), and transgenic plants accumulated higher concentrations of Ca²⁺ (Fig. 6D). In TaSC-2OE plants, the Ca²⁺ concentration was higher, and the expression levels of salt-tolerance related genes were up-regulated, indicating that TaSC may exert a salt-tolerance effect through the CDPK pathway.

It has been shown that proline accumulation can modulate osmotic pressure and protect the cell structure from destruction (Boyer, 1982), which is positively correlated with plant stress tolerance (Kishor et al., 1995). In higher plants, P5CS is the rate-limiting enzyme in proline synthesis (Hu et al., 1992). Enhanced expression of this enzyme can improve proline synthesis, which results in proline accumulation and improves plant salt tolerance (Armengaud et al., 2004). Overexpression of the P5CS gene can enhance stress tolerance in transgenic tobacco (Kishor et al., 1995) and rice (Zhu et al., 1998), with a significant increase in the proline level as a common feature. The study found that TaSC overexpression in transgenic plants increased the free proline level (Fig. 6B) and the gene expression level of AtP5CSI (Fig. 7B). We speculate that increased

Fig. 7. Expression of salt-tolerance related genes in Arabidopsis. Two-week-old Arabidopsis seedlings grown on MS growth medium were used for a total RNA extraction. Real-time quantitative PCR was performed to analyse the salt tolerance-related genes after reverse transcription. (A) Quantitative analysis of the expression of selected genes in four different Arabidopsis lines; (B) the expression level detection of the known salt-tolerance genes. TaSC-2OE: TaSC-OE line; Col-0: wild type; atsc: AtSC mutant; 35S::TaSC (atsc): atsc mutant with TaSC overexpression. Values are the mean ±SD (with three biological replicates). The asterisks indicate a statistically significant difference when compared with the control samples (t test, P <0.01).
K+/Na+ ratio increased and chloroplast function was enhanced
pathway. Subsequently, proline accumulated, the intracellular
messenger Ca2+, ultimately, leading to the activation the CDPK
gene expression and enhanced the concentration of the second
when the cells were under salt stress, which up-regulated
salt-tolerant wheat strain 49 sensed the ABA accumulation signal
esis that
et al., 2002), which is consistent with the hypoth-
that the TaSC may be involved in the CDPK pathway.
In summary, we speculate that the TaSC gene promoter of the
salt-tolerant wheat strain 49 sensed the ABA accumulation signal
when the cells were under salt stress, which up-regulated TaSC
gene expression and enhanced the concentration of the second
message Ca2+, ultimately, leading to the activation the CDPK
pathway. Subsequently, proline accumulated, the intracellular
K'/Na+ ratio increased and chloroplast function was enhanced 
through the expression of downstream genes in the pathway.
These responses are conducive to plant growth and improving
salt tolerance in RH 8706-49.

TaSC gene overexpression enhances salt tolerance of transgenic Arabidopsis

Fig. 8. After treatment with 170 mM NaCl for 10 d, phenotype of
atsc mutant and 3S::AtCDPK2 (atsc) lines. (A) The whole-plant
phenotype of atsc mutant and 3S::AtCDPK2 (atsc) lines grown in
soil without NaCl for 24 d; (B) lateral view of atsc mutant and
3S::AtCDPK2 (atsc) lines with 170 mM NaCl irrigating for 10
d after grown in soil for 14 d; (C) polar view of atsc mutant and
3S::AtCDPK2 (atsc) lines with 170 mM NaCl irrigating for 10 d
after grown in soil for 14 d. atsc: AtSC mutant; 3S::AtCDPK2
(atsc) lines: atsc mutant with AtCDPK2 overexpression; L1, L2,
and L3 are different 3S::AtCDPK2 (atsc) lines.

AtP5CSI1 gene expression promotes high levels of free proline
synthesis in cells (Fig. 6B). This enhanced synthesis reduces the
cell osmotic potential and aids in water absorption as well as in
the restoration of the original cell ion concentrations (Xiong and
Zhu, 2002; Farooq et al., 2009), which resist damage caused by
salt stress and enhance salt tolerance in wheat 49 and transgenic
Arabidopsis.

Real-time PCR showed that the TaSC is up-regulated by ABA
and NaCl under stress (Fig. 4), which suggests that the promoter
responds to ABA and NaCl. Sanders and colleagues found that
the CDPK pathway was activated by ABA and other stress fac-
tors (Sanders et al., 2002), which is consistent with the hypothe-
sis that TaSC may be involved in the CDPK pathway.

In summary, we speculate that the TaSC gene promoter of the
salt-tolerant wheat strain 49 sensed the ABA accumulation signal
when the cells were under salt stress, which up-regulated TaSC
gene expression and enhanced the concentration of the second
message Ca2+, ultimately, leading to the activation the CDPK
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These responses are conducive to plant growth and improving
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