A novel salt-induced gene from sheepgrass, \textit{LcSAIN2}, enhances salt tolerance in transgenic \textit{Arabidopsis}

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1. Introduction

Adverse environmental stresses affect plant growth and development, and limits the productivity of crops. Sheepgrass can grow well under various environmental and soil conditions and is a good wild resource in Triticeae. Using 454 high throughput sequencing technique, a large number of salt stress responsive genes have been picked out from sheepgrass. In this study, a novel salt-induced gene and its promoter were cloned and the gene was designated as \textit{LcSAIN2} (\textit{Leymus chinensis} salt-induced 2). Bioinformatics analysis predicted that \textit{LcSAIN2} has one transmembrane helix and is localized in nucleus. Experiments of subcellular localization in tobacco leaf cells also indicated that it was mainly localized in nucleus. Several stress responsive elements were found in the promoter region of the \textit{LcSAIN2} gene. The expression analysis confirmed that \textit{LcSAIN2} was induced by salinity, PEG, ABA, and cold stresses, especially by high salinity. Overexpression of \textit{LcSAIN2} in \textit{Arabidopsis} enhanced salt tolerance of transgenic plants by accumulating osmolytes, such as soluble sugars and free proline, and improving the expression levels of some stress-responsive transcription factors and key genes. Our results suggest that \textit{LcSAIN2} might play an important positive modulation role in salt stress tolerance and be a candidate gene utilized for enhancing stress tolerance in wheat and other crops.

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treatments, and speculate upon its structure and regulatory mechanism in salt stress tolerance.

2. Results

2.1. Isolation and characterization of LcSAIN2

A partial cDNA sequence of an unknown function stress-induced gene designated as LcSAIN2 was previously isolated via 454 high throughout sequencing, and the full length of gene was obtained by the RACE technique. LcSAIN2 (GenBank accession number: JX861860) had a full length of 1025 bp including 5’ untranslated region of 205 bp and 3’ untranslated region of 64 bp. It contained a 756 bp open reading frame encoding a protein with 251 amino acids, which showed higher homologies (67%) with a wheat cDNA clone: WT003_F22 (GenBank accession number: AK332187.1). Genomic sequence was amplified and analyzed, suggesting that the gene did not contain introns. The SignalP 4.0 server predicted the presence of an N-terminal signal peptide in the protein and its cleavage sites was between the 32 and 33 amino acids (Fig. 1a). The TMHMM 2.0 server predicted one transmembrane helix between residues 219 and 241 (Fig. 1b). To identify the putative cis-acting regulatory elements, about 1150 bp of sequence upstream from the start codon was isolated. Possible stress related ABA-responsive cis-elements ACGT, MYB and MYC recognition site elements were identified in sequence upstream of the LcSAIN2 gene (Table 2).

2.2. Expression pattern of LcSAIN2 in different tissues and response to abiotic stresses

The qRT-PCR was performed to determine the expression pattern of LcSAIN2 in different tissues. Transcripts of LcSAIN2 under control conditions were primarily observed in roots, with less in leaves, the sheath, panicle and stems (Fig. 2a).

To analyze the effect of stresses on the expression of LcSAIN2, the sheepgrass seedlings were exposed to diverse abiotic stresses. Semi-quantitative RT-PCR and qRT-PCR was performed using the total RNA extracted from 4-week and 8-week sheepgrass plants subjected to stress treatments at different time intervals. The time-dependent expression profiling revealed different transcript regulation.
patterns for LcSAIN2 in response to various abiotic stresses. Seedlings treated with cold showed a highest transcript levels at 12 h. Treatment with PEG and ABA also led to a significant increase in transcript levels after 3 h and the highest at 12 h (Fig. 2b, c).

For salt treatment, 4-weeks old sheegrass plants subjected to 400 mM NaCl, LcSAIN2 transcript levels showed an increase at 3 h, detected by semi-quantitative RT-PCR (Fig. 2b). In addition, the LcSAIN2 expression patterns of roots and shoots under salt treatment were also studied by qRT-PCR using 8-week old sheegrass. As shown in Fig. 2d, the transcription level of LcSAIN2 in the roots was nearly the same as in the shoots, and the transcription level increased after 1 h under salt stress. These results indicated that LcSAIN2 was responsive to salt stress and other abiotic stresses.

2.3. Subcellular localization of LcSAIN2

To determine the subcellular localization of LcSAIN2 in vivo, the open reading frame sequence was inserted into the vector pMDC45 fused with GFP reporter gene under the control of the CaMV 35S promoter, and transiently expressed in tobacco (Nicotiana tabacum) leaf cells. The vector pMDC45 was as a control. Fluorescent signals from pMDC45-LcSAIN2::GFP was mainly detected in the nucleus and weakly in the plasma membrane, while pMDC45::GFP alone were present throughout the whole cell (Fig. 3).

2.4. Over-expression of LcSAIN2 increases tolerance to salt stress

In order to analyze the function of LcSAIN2, salt tolerance of the WT and transgenic LcSAIN2 plants were tested. WT and transgenic seeds were germinated on MS medium plates for 7 days and then transferred to MS plates supplemented 150 mM NaCl treatment. After 3 weeks of treatment most of WT seedlings were albinos and wilting (Fig. 4a). Under normal growth conditions, no obvious differences were detected in either WT or transgenic seedlings. Furthermore, survival rates were also determined. As shown in Fig. 4b, the two transgenic lines showed significantly higher survival rates of 72% and 74%, respectively, as compared to the WT plants (~30%) (Fig. 4b).

To study the role of LcSAIN2 in enhancing the salinity stress on root growth, four-day-old seedlings were shifted to agar medium containing 100–200 mM NaCl. Seven days later, root elongation was measured. Root elongation was significantly higher in transgenic plants compared to WT plants at 150 mM NaCl (Fig. 4c).

2.5. Soluble sugars and proline content under salt tolerance in transgenic Arabidopsis

Previous studies indicated that plant adaptation to abiotic stress is related to the accumulation of soluble sugars and proline, and higher proline content under salt stress can facilitate plant tolerance to high salinity [32]. To explore the physiological and biochemical characteristics that may be responsible for improved tolerance of the transgenic plants to salt stress (150 mM NaCl) than WT plants, soluble sugars and proline content were determined. Our results suggested that the levels of soluble sugar were evaluated in both transgenic plants and control plants under salt stress conditions, but the increased levels of soluble sugar were significantly higher in transgenic plants compared to control plants (Fig. 5a). Further, the analysis of proline content revealed similar levels in transgenic plants and wild type plants without salt treatment. However, after 1 or 2 days, proline contents were significantly increased in both transgenic lines compared with WT (Fig. 5b).

2.6. Expression of stress-associated genes in transgenic Arabidopsis

The relative expression levels of four previously identified stress-associated genes were measured in these transgenic over-expression lines. Table 1

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Name</th>
<th>Sequence</th>
<th>Function</th>
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<td>GCACCTGTCTGTCTGCTACT</td>
<td>LcACTIN-AS</td>
<td>TACCTGTACGTCTGCTACT</td>
<td>RT-PCR</td>
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<tr>
<td>LcSAIN2-S</td>
<td>TATGCTGTCGCTGGGCTCTGTC</td>
<td>LcSAIN2-AS</td>
<td>TCACCCGCTGCTGCTACT</td>
<td>RT-PCR</td>
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<td>LcACTIN-S</td>
<td>GTGTCTATTCCCTATGAGCTG</td>
<td>LcACTIN-AS</td>
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<td>qPCR</td>
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<td>LcSAIN2-S</td>
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<td>LcSAIN2-AS</td>
<td>TCACCCGCTAAGGCCAGCTC</td>
<td>qPCR</td>
</tr>
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<td>LcACTIN-S</td>
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<td>qPCR</td>
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<td>LcMYB2-S</td>
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<td>ATCGTTGAACTCTCCGAAACCGCT</td>
<td>qPCR</td>
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<tr>
<td>ARID26-S</td>
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<td>CGACACAAACACCAATACATC</td>
<td>qPCR</td>
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<td>ARID29B-S</td>
<td>GAAAGAAGAAAGAAAACAACTCA</td>
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<td>qPCR</td>
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<tr>
<td>ARAB18-S</td>
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<td>ARAB18-AS</td>
<td>CGAAGTGGCTGCTTACAAAA</td>
<td>qPCR</td>
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Table 2

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<th>Signal sequence</th>
<th>Function</th>
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<tr>
<td>ABRELATERID1</td>
<td>417 (-) 599 (-)</td>
<td>AGCTG</td>
<td>ABA responsive element</td>
</tr>
<tr>
<td>AGCTERTERID1</td>
<td>418 (+) 560 (+) 418 (-) 560 (-)</td>
<td>AGCTG</td>
<td>Early responsive to dehydration and dark-induced senescence</td>
</tr>
<tr>
<td>CEI</td>
<td>702 (-)</td>
<td>TGCCACCAGG</td>
<td>Cis-acting element associated to ABRE, involved in ABA responsiveness</td>
</tr>
<tr>
<td>TC-rich repeats</td>
<td>42 (-)</td>
<td>GTTTTCTTAC</td>
<td>Cis-acting element involved in defense and stress responsiveness</td>
</tr>
<tr>
<td>MYB1AT</td>
<td>477 (+)</td>
<td>WAAACA</td>
<td>MYB recognition site found in the promoters of the stress-responsive genes</td>
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<tr>
<td>MYB2CONSENSUSAT</td>
<td>250 (-)</td>
<td>YAACKG</td>
<td>MYB recognition site found in the promoters of the stress-responsive genes</td>
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<tr>
<td>MYBCORE</td>
<td>250 (-)</td>
<td>CNGCTR</td>
<td>Binding site for regulation of genes that are responsive to water stress</td>
</tr>
<tr>
<td>MYCATERD1</td>
<td>606 (+) 101 (-)</td>
<td>CATGTC</td>
<td>MYC recognition sequence necessary for expression of erd1 in water-stress conditions</td>
</tr>
<tr>
<td>MYC CONSENSUSAT</td>
<td>101 (-) 606 (+) 101 (-) 606 (-)</td>
<td>CANNAG</td>
<td>Regulates the transcription of CBF/DREB1 genes in the cold</td>
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<tr>
<td>WRKY71OS</td>
<td>84 (+) 43 (-) 413 (-) 485 (-)</td>
<td>TGCAG</td>
<td>Binding site of rice WRKY71, a transcriptional repressor of the gibberellin signaling pathway</td>
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<tr>
<td>WBOXHVISOSI</td>
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<td>TACAGG</td>
<td>Sugar-responsive elements of the iso1 promoter</td>
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<tr>
<td>WBOXONTER3</td>
<td>412 (-) 484 (-)</td>
<td>TACAGG</td>
<td>Found in the promoter region of a transcriptional repressor ERF3 gene</td>
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<tr>
<td>TCA-element</td>
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<td>CAGARAAAGGA</td>
<td>Cis-acting element involved in salicylic acid responsiveness</td>
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<tr>
<td>TGAC-motif</td>
<td>41 (-) 83 (+)</td>
<td>TGACG</td>
<td>Cis-acting regulatory element involved in the MeJA-responsiveness</td>
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<tr>
<td>DOFCOREZM</td>
<td>27 (+) 531 (+) 536 (+)</td>
<td>AAAG</td>
<td>Core site required for binding of Dof proteins</td>
</tr>
<tr>
<td>DOFCOREZM</td>
<td>383 (-) 398 (-) 634 (-) 658 (-)</td>
<td>AAAG</td>
<td>Core site required for binding of Dof proteins</td>
</tr>
</tbody>
</table>

ABRELATERID1, MYB1AT, MYB2CONSENSUSAT, MYBCORE, MYCATERD1, MYC CONSENSUSAT, WRKY71OS, WBOXHVISOSI, WBOXONTER3, TCA-element, TGAC-motif, DOFCOREZM
lines using qRT-PCR. The expression of the ABA-induced gene RD29B and RAB18 was increased in LcSAIN2 transgenic plants compared with control plants. The transcription factors MYB2 and RD26 also exhibited increased expression levels in two transgenic lines (Fig. 6).

3. Discussion

Using 454 high throughout sequencing and molecular biology technique, we have found a great number of salt stress responsive
transcripts from sheepgrass. In the present study, we cloned a novel salt stress induced gene of \textit{LcSAIN2} and its promoter from these transcripts, which had relatively high homology (67\%) with an unknown EST sequence of wheat. Several stress responsive elements were found in the promoter region of \textit{LcSAIN2} and the gene was significantly induced by salinity, PEG, ABA, and cold stresses (Table 2, Fig. 2a). The TMHMM 2.0 server predicted that \textit{LcSAIN2} has one transmembrane helix and Plant-mPLoc program predicted that \textit{LcSAIN2} is localized in nucleus. Transient expression of \textit{LcSAIN2} in tobacco leaf cells also indicated that \textit{LcSAIN2} is mainly localized in the nucleus (Fig. 3). With a likely function in the nucleus [33,34], we speculate that \textit{LcSAIN2} is a stress responsive gene and may play roles in salt and other abiotic stresses response.

Previous studies showed that overexpression of several transcription factors improved salinity stress tolerance in transgenic plants [35–38]. In the present study, we found that overexpression of \textit{LcSAIN2} in \textit{Arabidopsis} also significantly improved salt tolerance. This salt tolerance was supported by phenotypic performance and physiological indicator changes, such as survival rates, root elongation. The results revealed that the survival rates and root elongation were significantly higher in transgenic lines than WT (Fig. 4). Plants may enhance stress tolerance by accumulating osmolytes, such as soluble sugars and free proline to adjust the osmotic potential and protect cell structures [39,40]. The increased soluble sugars and proline content under different environmental stresses significantly improved plant stress tolerance [32,39,41,42]. To investigate the potential mechanisms underlying the improved stress tolerance of \textit{LcSAIN2} in overexpressed plants, the levels of soluble sugars and free proline were measured under salinity stress conditions. The results showed that the contents of soluble sugars and free proline in the transgenic plants were significantly higher than those in WT plants under salt stress (Fig. 5). Therefore, we proposed that higher tolerance against salt stresses in transgenic \textit{Arabidopsis} might be achieved by osmolytes accumulation.

In order to dissect the enhanced salt tolerance at the molecular level, expression of four stress-responsive genes were monitored between the transgenic plants and control. Our results showed that overexpression of \textit{LcSAIN2} led to higher expression of all four stress-related genes (Fig. 6). It was well known that transcription factors \textit{MYB2} and \textit{RD26} involved in the ABA-dependent pathway and \textit{RD29B} and \textit{RAB18} could be induced by different stresses through an ABA-dependent pathway, and that higher expression of these genes improved the plant tolerance to multiple stresses [11,35,37,38]. We inferred that \textit{LcSAIN2} might act as an activator to increase the expression of the above stress responsive genes to enhance tolerance of the transgenic plants under salt stress.

In conclusion, this study identified and characterized a novel \textit{LcSAIN2} gene from sheepgrass. \textit{LcSAIN2} was induced by various abiotic stresses, but especially by salt stress. It was mainly localized in the nucleus. Overexpression of \textit{LcSAIN2} led to the up-regulated expression of several key stress responsive genes, greater accumulation of soluble sugars and free proline, and enhanced tolerance to salt stress in transgenic \textit{Arabidopsis}. \textit{LcSAIN2} may also provide a useful gene for molecular breeding of important crops to improve stress tolerance. At present, the work of transforming \textit{LcSAIN2} into rice and introgressing wheat is in progress in our laboratory.
**Fig. 5.** Measurement of soluble sugars and proline content of transgenic and WT plants after 150 mM NaCl treatment. a. Soluble sugars content in transgenic and WT plants after 2 day treatment of 150 mM NaCl. b. Proline contents of transgenic and WT seedlings exposed to 150 mM NaCl for 1 and 2 day. Each column represents an average of three replicates, and bars indicate SDs. **“** and *** indicate significant differences in comparison with the control at $P < 0.01$ and $P < 0.001$. 

4. Materials and methods

4.1. Plant growth conditions and stress treatment

Sheepgrass (variety Zhongke No 2) was grown in soil mix of peat moss and vermiculite (2:1, v/v) in greenhouse conditions at average temperature of 23 °C under long-day conditions (16 h light/8 h dark). For abiotic stress analyses, 4 or 8 week seedlings were treated with cold, NaCl, ABA and PEG. For cold stress treatment, plants were placed in a dark chamber at 4 °C. For NaCl, abscisic acid (ABA) and drought stress treatments, seedlings were irrigated with 400 mM NaCl, 100 μM ABA and 20% PEG6000, respectively. Total of 15 plants were sampled per treatment at various intervals (0, 1, 3, 5, 12, 24, and 48 h). To determine the gene expression level of the shoots and roots under salt stress, the above and below ground of 8 week seedlings were sampled separately and stored at −80 °C for RNA analysis. For expression analysis in different tissues, leaf, stem, sheath, panicle and root were collected from a 2-year-old sheepgrass grown in the greenhouse conditions as above described.

4.2. Cloning of the LcSAIN2 gene and sequence analysis

Based on the transcriptomic data of sheepgrass using 454 high throughout sequencing technique, a lot of candidate salinity induced transcripts were identified. Among these transcripts, one of the transcripts, designated as LcSAIN2, was an unknown function gene and significantly induced by salt stress treatment according to our previous experimental verification. Therefore, we are interested in this gene and want to explore its function.

To obtain the full-length cDNA of LcSAIN2, 2 weeks old sheepgrass (variety Zhongke No 2) seedlings were irrigated with 400 mM NaCl for 48 h were harvested. Total RNA was isolated using the Trizol Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The full-length cDNA sequence was amplified using a SMART™ RACE cDNA Amplification Kit (Clontech, Japan) and sequenced. The RACE primers used in the present study are: 5GSP 5′-TCTCAAATCCACCACCGTCTATGC-3′ and 3GSP 5′-GATGTGCTGTGCTGGCCGCTGCTCC-3′. Genomic DNA was prepared from seedlings of sheepgrass using the Plant Genomic DNA Rapid Extraction kit (Biotek Corporation, China). The genomic DNA sequence was amplified by PCR using 5′-TGGCTCTCATCTTCTCC TCTTTGC-3′ forward primer and 5′-CCAGAAGGACCCAGGCTTC-3′ reverse primer.

The obtained full-length cDNA and genomic DNA sequences of LcSAIN2 were further analyzed. Sequence assembling was performed using DNAMAN v5.0 (Lynnon Biosoft Inc., Vandreuil, Quebec, Canada), and SignalP4.0 [22] was used to predict the signal peptide. The transmembrane segments in protein were predicted using TMHMM 2.0 [23]. The subcellular localization of LcSAIN2 protein was predicted using Plant-mPLoc program [24].

To identify the putative cis-acting regulatory elements, the promoter sequence was isolated using TAIL-PCR [25] with the gene specific primers of SP1 5′-TATGCTGACGAAACCCCATCTG-3′, SP2 5′-CAGAAAACGAGGCAGCGGCACTGAG-3′ and SP3 5′-AGGGCAGAgACATCAATCCAT-3′, and three arbitrary degenerate (AD) primers of AD1 5′-NTCA(C/G)(T/A)/T/G(C/G)(A/T)/TGT-3′, AD2 5′-NCTCGA(G/C)(A/T)/TGA(A/T)/GAA-3′ and AD3 5′-(A/T)GTGCA(A/T)/T/A/ACANAGA-3′. PCR procedure was performed as described by Liu et al. [25]. Promoter cis-elements were identified using the algorithm developed by Higo et al. [26] which are available at www.dna.affrc.go.jp/PLACE/index.html.
4.3. Expression analysis

Total RNA was extracted from both Arabidopsis and sheegrass seedlings and the cDNA was reverse transcribed using the above-mentioned protocols. *LeACTIN* and *AtACTIN2* (Table 1) were used as internal reference genes for assessing expression levels in sheegrass and Arabidopsis, respectively. For semi-quantitative RT-PCR, the following programs were used, 5 min denaturation at 94 °C, followed by 28 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, completed by an extension step of 10 min 72 °C. SYBR® PrimeScript™ PCR Kit (TaKaRa, Japan) was used for quantitative RT-PCR (qRT-PCR). Each 20 μL qRT-PCR was subjected 40 cycles of 94 °C for 5 s and 60 °C for 20 s. Each qRT-PCR was run in triplicate. After the PCR program, data were quantified using the comparative CT method [2 - ΔΔCT method] [27]. All semi-quantitative RT-PCR and qRT-PCR experiments described in this section were reproduced at least three times using independent cDNA preparations. Primer sequences for semi-quantitative RT-PCR and qRT-PCR are listed in Table 1.

4.4. Tobacco transient expression and confocal microscopy

The *LcSAIN2* gene was combined with green fluorescent protein (GFP) to yield a fusion protein. The open reading frame (ORF) sequence of the gene was recombined into the vector pMD45 (pCAMBA1300) under the control of the Cauliflower mosaic virus (CaMV) 35S promoter were used to transform Agrobacterium tumefaciens (EHA105) cells. The intact leaves of 4-week-old wild type tobacco (*N. tabacum*) plants were injected with *A. tumefaciens* strain EHA105 harboring pMD45 and pMD45-LcSAIN2, respectively. Transgene-derived expression was monitored 2–3 d after infiltration by confocal microscopy on a Leica TCS SP5 microscope (Leica Microsystems, Wetzlar, Germany). Fluorophores were excited using an argon laser at 488 nm (GFP) and bright-field images were collected using the transmitted light detector.

4.5. Vector construction and genetic transformation

The ORF sequence was inserted into the vector pSN1301 vector [28] under the control of the Cauliflower mosaic virus (CaMV) 35S promoter via the *Kpn* and *Sac* sites (forward, 5′-GGGGTACCATGTTGCTAGATGTGCGCCT-3′ *Kpn* site underlined) and reverse, 5′-GGAGCTTCTAGATGACATGAAAACAC-3′ *Sac* site underlined) The recombinant plasmid was electroporated into *A. tumefaciens* (pCAMBIA1300) under the control of the Cauliflower mosaic virus (CaMV) 35S promoter via the *Kpn* and *Sac* sites [28], and T1 transgenic plants were confirmed by PCR analysis using the gene-specific primers of *LcSAIN2* as described above.

4.6. Salt stress analysis and osmolytes content measurement

For the salt treatment, two T3 generation (Line1 and Line 8) and wild-type (WT) seeds were surface-sterilized and sown on MS medium. Seven-day-old *Arabidopsis* seedlings were transferred to MS agar plates supplemented with 150 mM NaCl for another 3 weeks to assess their survivability and at least 40 plants of each transgenic line or WT were used for calculating the survival rates. To determine the effects of NaCl on root growth, 4-day-old plants grown on vertically standing MS agar plates were transferred to vertically standing plates containing 0–200 mM NaCl for 7 d and the root elongation was calculated. All assays were repeated at least three times and the error bars indicated the standard deviation (SD).

Three-week-old transgenic or WT plants were transferred to half-strength MS medium supplemented with 150 mM NaCl. After 0, 1, and 2 d salt treatment, the leaves of *Arabidopsis* wild-type and transgenic lines were harvested. Total soluble sugar content was measured as described previously [30], and proline content was determined according to a previously described method [31]. Briefly, leaves were harvested, weighted, and extracted in 3% sulfoisalicylic acid. Two microilers of supernatant of each extract was incubated with 3 mL of ninhydrin reagent (2.5% (w/v) ninhydrin, 60% (v/v) glacial acetic acid, 40% 6 M phosphoric acid) and 2 mL of glacial acetic acid at 100 °C for 40 min, and the reaction was terminated in an ice bath. Toluene (5 mL) was added and the absorbance was measured spectrophotometrically at the wavelength of 520 nm using a UVB 2450 UV spectrophotometer (SHIMADZU, Japan). Each date point had three replicates and the error bars indicated the standard deviation (SD).

4.7. Statistical analysis

The data of *Arabidopsis* seedling growth parameters, soluble sugar and proline content were subjected to ANOVA analysis using SPSS 15.0, and the results were edited in Excel.

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References


