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Research article

A novel salt-induced gene from sheepgrass, *LcSAIN2*, enhances salt tolerance in transgenic *Arabidopsis*

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ABSTRACT

Salt stress affects 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 throughout 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 *LcSAIN2* (*Leymus chinensis* salt-induced 2). Bioinformatics analysis predicted that 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 *LcSAIN2* gene. The expression analysis confirmed that *LcSAIN2* was induced by salinity, PEG, ABA, and cold stresses, especially by high salinity. Overexpression of *LcSAIN2* in *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 *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.

1. Introduction

Adverse environmental stresses affect plant growth and crop yields [1]. To cope with various environment stresses, plants have developed various adjustment mechanisms to defense and adapt to stress conditions [2,3]. Extensive stress-induced genes have been identified and characterized to elucidate the mechanisms by which plant respond to salinity stress, including transcription factors, enzymes, molecular chaperones, ion channels, receptor, signaling molecules, and the genes involved in producing compatible solutes (e.g., soluble sugar and proline), resulting in stress tolerance [4–8]. The molecular regulatory mechanisms in the expression of stress-responsible genes can be mainly divided into ABA-dependent and ABA-independent regulatory pathways [9–12].

Despite the above described salinity resistant genes in model plants and crops, wild species can adapt harsh environments and soil conditions and has rich stress resistance genes worth to explore. Sheepgrass (*Leymus chinensis* (Trin.) Tzvel) belongs to *Leymus* genus (Gramineae: Triticeae) and is an important forage species for livestock in the steppes of northern Eurasia and China [13,14]. It can grow and develop well in high salt (600 mM NaCl) and drought stress, and it also can withstand extreme low temperature stress. Although they have close phylogenetic relationship, sheepgrass has significantly higher stress tolerance than wheat or barley [15,16]. Intergeneric hybrids of wheat and *Leymus* demonstrated that the hybrid wheat exhibited higher stress tolerance than the control, suggesting the transfer of resistance genes from *Leymus* to wheat might support wheat improvement [17–19]. Therefore, sheepgrass can be an excellent gene pool for molecular breeding to enhance stress tolerance in wheat and other Triticeae crops.

In a previous study we found a large number of stress responsive genes in sheepgrass via a 454 high throughout sequencing technique. Some genes have been identified and found that they can improve drought and salt tolerance of transgenic *Arabidopsis thaliana* [20]. In order to validate more useful genes for crop improvement, we further isolate a novel stress inducible gene *LcSAIN2*, characterize its expression pattern under different stress

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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

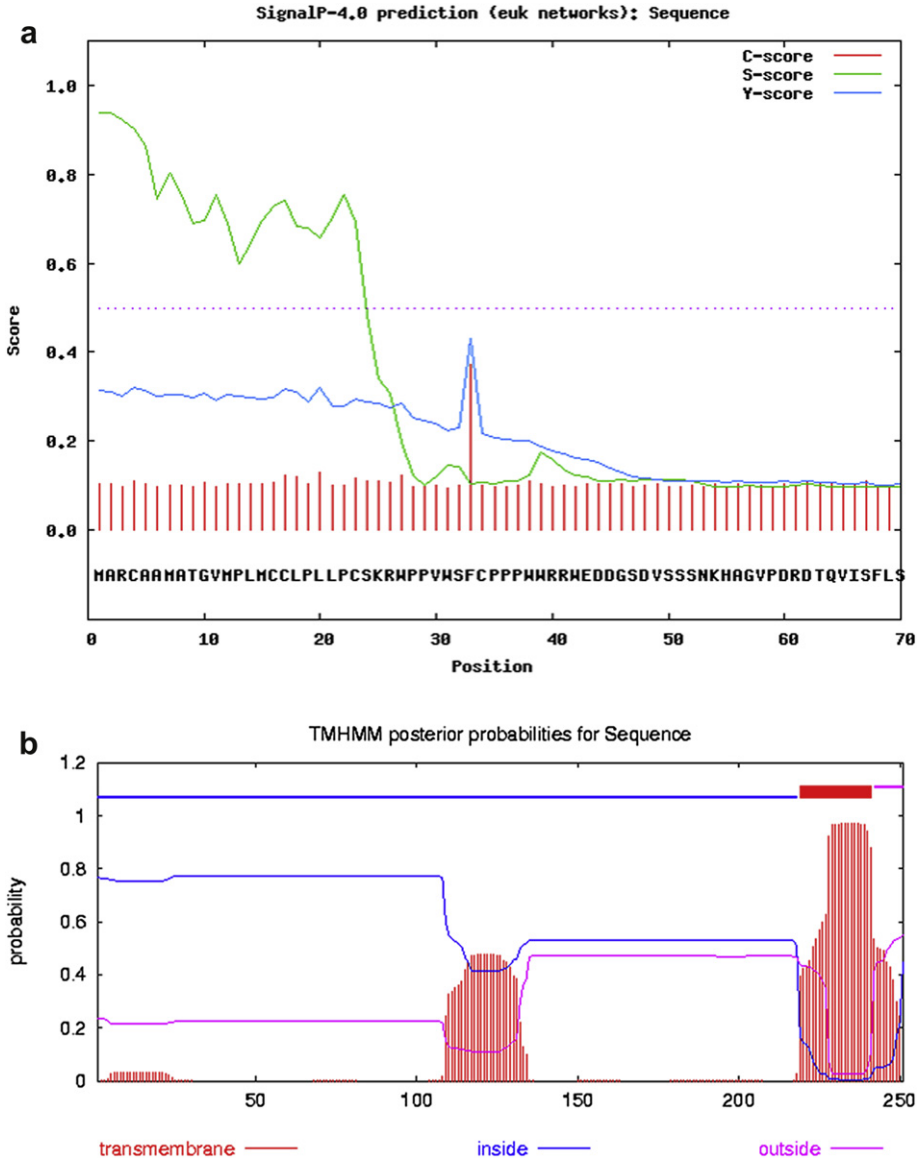

Fig. 1. Sequence analysis of *LcSAIN2*. a. SignalP 4.0 server predicts the presence of an N-terminal signal peptide. b. TMHMM 2.0 server predicted transmembrane helix.

Table 1
Primer sequences used for RT-PCR and qRT-PCR.

Name	Squence	Name	Sequence	Function
LcACTIN-S	GCACCCTGTGTTGCTCACT	*LcACTIN*-AS	TACCTTGATCTTCATGCTGCTC	RT-PCR
LcSAIN2-S	TATGCTGTCTGCCGCTGCTC	*LcSAIN2*-AS	TCCACCGCCGTCTATGCTC	RT-PCR
LcACTIN-S	GTGCTTTCCCTCTATGCAAGTGGT	*LcACTIN*-AS	CTGTTCTTGGCAGTCTCCAGCTC	qPCR
LcSAIN2-S	TCCTCTTTCGCTTCTCTCTCCTC	*LcSAIN2*-AS	TCAACGGCATAACGCCAGTC	qPCR
AtACTIN2-S	TGCTGACCGTATGAGCAAAG	*AtACTIN2*-AS	GATTGATCCTCCGATCCAGA	qPCR
AtMYB2-S	AACGTCTTCGAATTCTCCGGCTGA	*AtMYB2*-AS	ATCGTTGAACTCTCCGAAACCCGT	qPCR
AtRD26-S	GCACGAGTATCGCTTAATAGAACA	*AtRD26*-AS	CGACACAACACCCAATCATC	qPCR
AtRD29B-S	GCAAGCAGAAGAACCAATCA	*AtRD29B*-AS	CTTTGGATGCTCCCTTCTCA	qPCR
AtRAB18-S	CCACGAGAAGAAGGGGATGAT	*AtRAB18*-AS	CGAATGCGACTGCGTTACAA	qPCR

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 sheepgrass 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 sheepgrass. 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

Table 2
Prediction of stress response cis-acting element of *LcSAIN2*.

Factor or site name	Site	Signal sequence	Function
ABRELATERD1	417 (−) 599(−)	ACGTG	ABA responsive element
ACGTATERD1	418 (+) 560 (+) 418 (−) 560 (−)	ACGT	Early responsive to dehydration and dark-induced senescence
CE1	702 (−)	TGCCACCGG	Cis-acting element associated to ABRE, involved in ABA responsiveness
TC-rich repeats	42 (−)	GTTTTCTTAC	Cis-acting element involved in defense and stress responsiveness
MYB1AT	477 (+)	WAACCA	MYB recognition site found in the promoters of the stress-responsive genes
MYB2CONSENSUSAT	250 (−)	YAACKG	MYB recognition site found in the promoters of the stress-responsive genes
MYBCORE	250 (+)	CNGTTR	Binding site for regulation of genes that are responsive to water stress
MYCATERD1	606 (+) 101 (−)	CATGTG	MYC recognition sequence necessary for expression of erd1 in water-stress
MYCCONSENSUSAT	101 (+) 606 (+) 101 (−) 606 (−)	CANNTG	Regulates the transcription of CBF/DREB1 genes in the cold
WRKY71OS	84 (+) 43 (−) 413 (−) 485 (−)	TGAC	Binding site of rice WRKY71, a transcriptional repressor of the gibberellin signaling pathway
WBOXHVISO1	412 (−)	TGACT	Sugar-responsive elements of the iso1 promoter
WBOXNTERF3	412 (−) 484 (−)	TGACY	Found in the promoter region of a transcriptional repressor ERF3 gene
TCA-element	22 (+) 44 (+)	CAGAAAAGGA	Cis-acting element involved in salicylic acid responsiveness
TGACG-motif	41 (−) 83 (+)	TGACG	Cis-acting regulatory element involved in the MeJA-responsiveness
DOFCOREZM	27 (+) 531 (+) 536 (+) 383 (−) 398 (−) 634 (−) 658 (−)	AAAG	Core site required for binding of Dof proteins

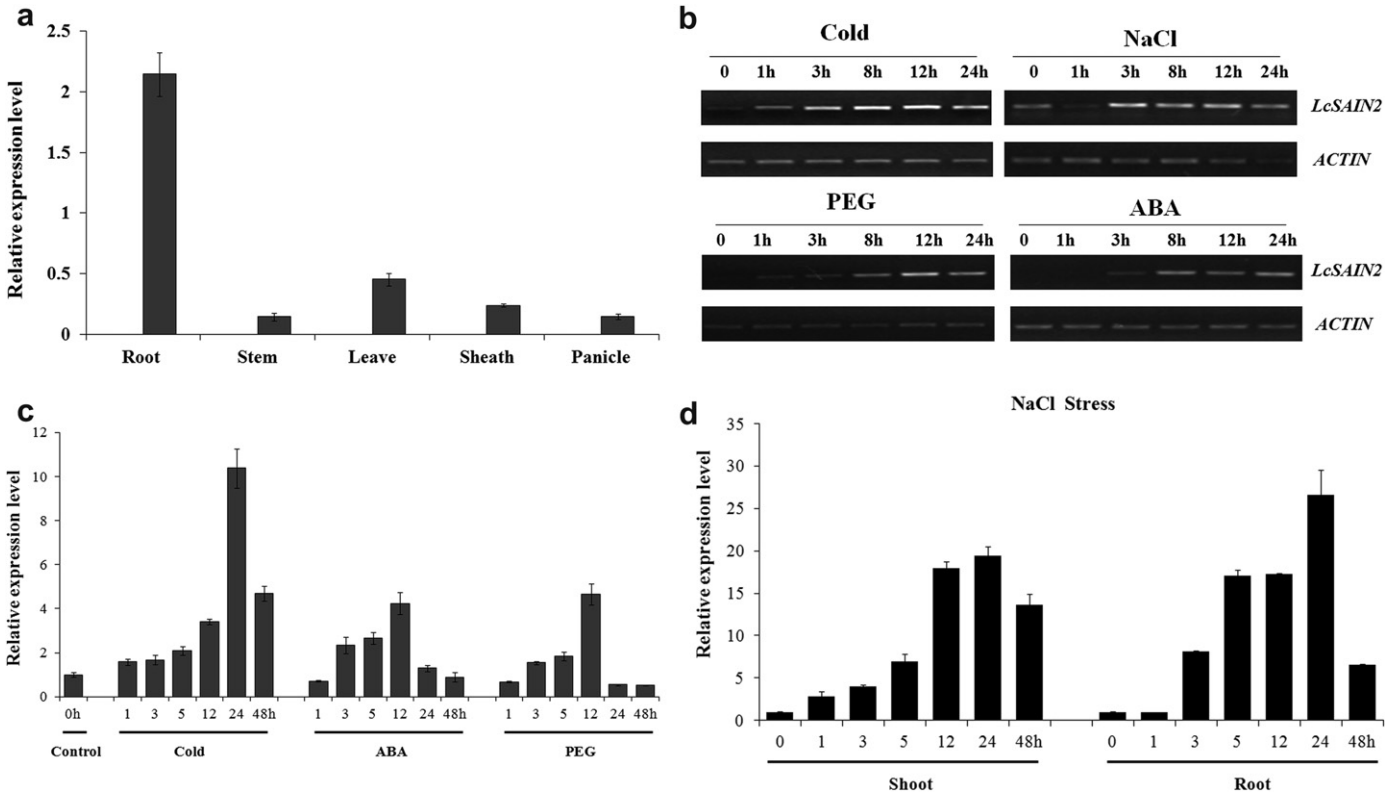

Fig. 2. Expression pattern of *LcSAIN2* in sheepgrass. a. Expression patterns of *LcSAIN2* in different tissues. b. Expression of *LcSAIN2* in 4 week seedlings under salinity, PEG, ABA, and cold treatment. c. Expression of *LcSAIN2* in 8 week seedlings under cold, ABA and PEG treatment. The transcript level of *LcSAIN2* at 0 h was used as control. d. Expression patterns of *LcSAIN2* in roots and shoots under 400 mM NaCl treatment.

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

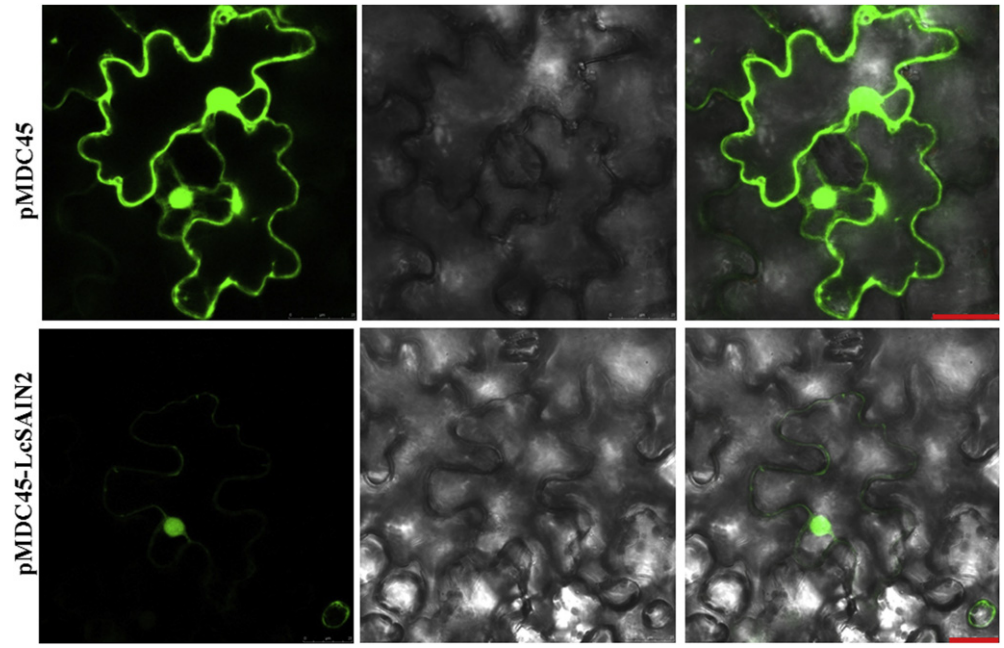

Fig. 3. Subcellular localization of the LcSAIN2 protein in tobacco epidermal cells. Transient expression of pMDC45-LcSAIN2 fusion and pMDC45 construct in tobacco epidermal cells. Green fluorescence was observed using a confocal microscope at 48 h after *A. tumefaciens* infiltration. Micrographs showing cells expressing GFP (control, upper lane) or LcSAIN2::GFP (bottom lane) fusion protein. From left to right, the pictures showed fluorophores, bright-field, and overlay of two illuminations. Bar = 25 μm.

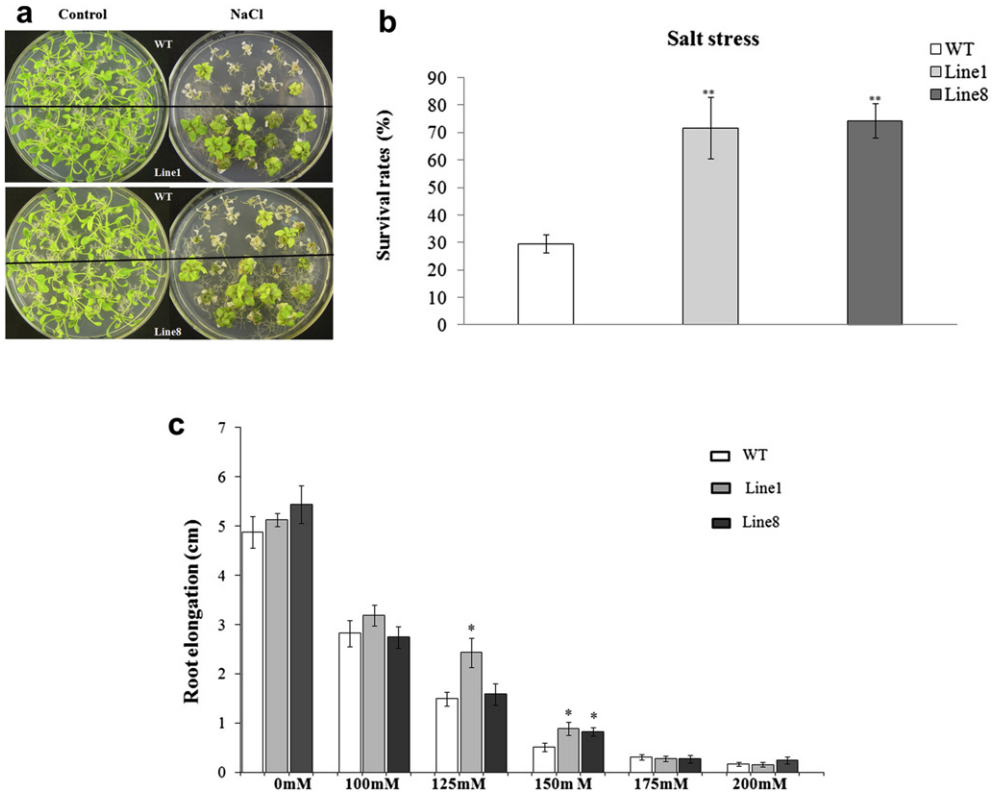

Fig. 4. Overexpression of *LcSAIN2* in transgenic plants enhanced resistance to salt stress. a. Growth of WT and transgenic lines treated with 150 mM NaCl. Seven-day-old seedlings were transferred to a medium containing 150 mM NaCl for three weeks before the images were taken. b. Survival rates of WT and transgenic seedlings were calculated after salt treatments. c. Measurements of root elongation in WT and transgenic plants. Four-day-old seedlings were shifted to agar medium containing various concentrations of NaCl for 7 d and the root elongating were recorded. For b and c, 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 $P < 0.05$, respectively.

transcripts from sheepgrass. In the present study, we cloned a novel salt stress induced gene of *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 *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 LcSAIN2 has one transmembrane helix and Plant-mPLoc program predicted that LcSAIN2 is localized in nucleus. Transient expression of LcSAIN2 in tobacco leaf cells also indicated that LcSAIN2 is mainly localized in the nucleus (Fig. 3). With a likely function in the nucleus [33,34], we speculate that *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 *LcSAIN2* in *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 *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 *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 *LcSAIN2* led to higher expression of all four stress-related genes (Fig. 6). It was well known that transcription factors *MYB2* and *RD26* involved in the ABA-dependent pathway and *RD29B* and *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 *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 *LcSAIN2* gene from sheepgrass. *LcSAIN2* was induced by various abiotic stresses, but especially by salt stress. It was mainly localized in the nucleus. Overexpression of *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 *Arabidopsis*. *LcSAIN2* may also provide a useful gene for molecular breeding of important crops to improve stress tolerance. At present, the work of transforming *LcSAIN2* into rice and introgressing wheat is in progress in our laboratory.

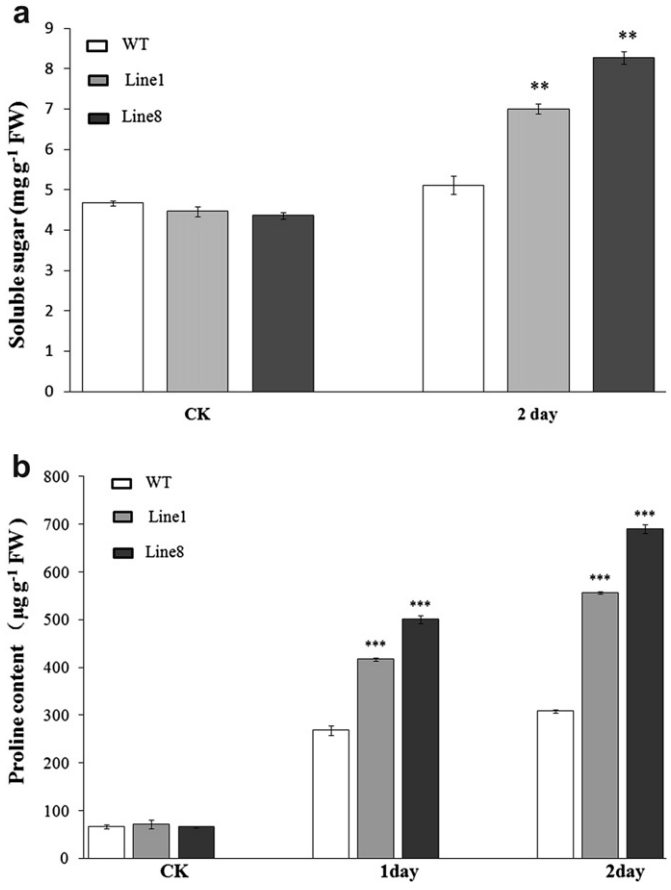

Fig. 5. Measurement of soluble sugars and proline content of transgenic and WT plants after 150 mM NaCl treatment. a. Soluble sugars content in transgenic and WT plants after 2 d treatment of 150 mM NaCl. b. Proline contents of transgenic and WT seedlings exposed to 150 mM NaCl for 1 d and 2 d. Each 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 the greenhouse at an 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.

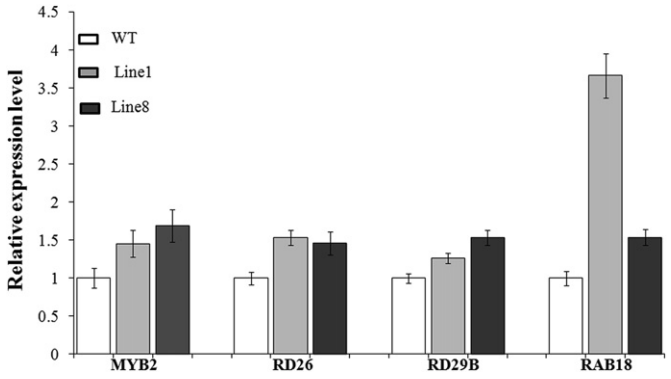

Fig. 6. Relative expression levels of four stress associated genes in response to salt treatment. Three-week old *Arabidopsis* seedlings grown on MS growth medium were used for real-time quantitative PCR.

A. thaliana plants (ecotype Columbia, Col-0) were grown in a soil mix of peat moss and vermiculite (2:1, v/v) at 23 °C under a 16-h photoperiod. Transformed *Arabidopsis* seeds were sterilized in 30% (v/v) bleach for 15 min, rinsed five times with sterile water, and were selected on Murashige and Skoog (MS) agar supplemented with 30 μg ml^{-1} hygromycin, transferred to the soil mix described above and then grown under the same conditions [21].

Tobacco (*N. tabacum*) seeds were grown in pots with peat moss and vermiculite (2:1, v/v) in greenhouse conditions at average temperature of 23 °C under a 16/8 h day/night cycle.

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′-CTCCAAATCCACCGCCGTCTATGC-3′ and 3GSP 5′-GATGTGCTGTCTGCCGCTGCTCC-3′. Genomic DNA was prepared from seedlings of sheepgrass using the Plant Genomic DNA Rapid Extraction kit (Bioteke Corporation, China). The genomic DNA sequence was amplified by PCR using 5′-TGGCTCTCATTTCTCC TCTTTCG-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′-TATGCTGACGAACCCCATCTGC-3′, SP2 5′-CAGAAAACGAAGCCACGCAGAG-3′ and SP3 5′-AGCGGCAGACAGCA-CATCAA-3′, and three arbitrary degenerate (AD) primers of AD1 5′-NTCGA(G/C)T(A/T)T(G/C)G(A/T)GTT-3′, AD2 5′-NGTCGA(G/C)(A/T) GANA(A/T)GAA-3′ and AD3 5′-(A/T)GTGNAG(A/T)ANCANAGA-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 sheepgrass seedlings and the cDNA was reverse transcribed using the above-mentioned protocols. *LcACTIN* and *AtACTIN2* (Table 1) were used as internal reference genes for assessing expression levels in sheepgrass 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 ($2^{-\Delta\Delta}$CT 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 pMDC45 (pCAMBIA1300) 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 pMDC45 and pMDC45-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*I and *Sac*I sites (forward, 5′-GGGGTAC-CATGGCTAGATGTGCGCCT-3′ *Kpn*I site underlined) and reverse, 5′-CGAGCTCTTAGATAGACATGAAAACAC-3′ *Sac*I site underlined) The recombinant plasmid was electroporated into *A tumefaciens* (EHA105) for *Arabidopsis* transformation using the floral dip method [29], 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% sulfosalicylic acid. Two microliters 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 spectrophotoelectrically 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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