

# The *SbASR-1* Gene Cloned from an Extreme Halophyte *Salicornia brachiata* Enhances Salt Tolerance in Transgenic Tobacco

Bhavanath Jha · Sanjay Lal · Vivekanand Tiwari · Sweta Kumari Yadav · Pradeep K. Agarwal

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**Abstract** Salinity severely affects plant growth and development. Plants evolved various mechanisms to cope up stress both at molecular and cellular levels. Halophytes have developed better mechanism to alleviate the salt stress than glycophytes, and therefore, it is advantageous to study the role of different genes from halophytes. *Salicornia brachiata* is an extreme halophyte, which grows luxuriantly in the salty marshes in the coastal areas. Earlier, we have isolated *SbASR-1* (abscisic acid stress ripening-1) gene from *S. brachiata* using cDNA subtractive hybridisation library. *ASR-1* genes are abscisic acid (ABA) responsive, whose expression level increases under abiotic stresses, injury, during fruit ripening and in pollen grains. The *SbASR-1* transcript showed up-regulation under salt stress conditions. The *SbASR-1* protein contains 202 amino acids of 21.01-kDa molecular mass and has 79 amino acid long signatures of ABA/WDS gene family. It has a maximum identity (73 %) with *Solanum chilense* ASR-1 protein. The *SbASR-1* has a large number of disorder-promoting amino acids, which make it an intrinsically disordered protein. The *SbASR-1* gene was over-expressed under CaMV 35S promoter in tobacco plant to study its physiological functions under salt stress. T<sub>0</sub> transgenic tobacco seeds showed better germination and seedling growth as compared to wild type (Wt) in a salt stress condition. In the leaf tissues of transgenic lines, Na<sup>+</sup> and proline contents were

significantly lower, as compared to Wt plant, under salt treatment, suggesting that transgenic plants are better adapted to salt stress.

**Keywords** Salinity · *Salicornia brachiata* · Halophyte · *SbASR-1* · Transgenic plant

## Introduction

Among the different abiotic stresses, salinity is the major cause, reducing agricultural yield in salt-affected areas. Salinity is defined as the presence of a high concentration of dissolved salts in soil. NaCl constitutes a major portion of these salts and is highly toxic to plants. Soil salinity is caused by various factors like amount of evaporation, precipitation, weathering of rocks and uneven irrigation (Mahajan and Tuteja 2005). It leads to ionic (Na<sup>+</sup>, Cl<sup>-</sup> and SO<sub>4</sub><sup>2-</sup>), osmotic and oxidative stress conditions in glycophytes (Zhu 2002), whereas halophytes grow naturally in salt-affected areas and have developed efficient protective mechanism against salinity.

*Salicornia brachiata* Roxb (Amaranthaceae) is a leafless succulent annual halophyte and commonly grows in coastal marshes in India. *Salicornia* can grow in a wide range of salt concentrations (0.1–2.0 M) and can accumulate quantities of salt as high as 40 % of its dry weight. This unique characteristic provides an advantage to study the salt tolerance mechanisms. We have earlier developed an expressed sequence tag (EST) database of *S. brachiata* using cDNA subtractive hybridisation (Jha et al. 2009). The detailed functional analysis is being carried out for some important stress-related genes from EST database and others like *MAPK* (Agarwal et al. 2010a), *NHX-1* (Jha et al.

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B. Jha (✉) · S. Lal · V. Tiwari · S. K. Yadav · P. K. Agarwal (✉)  
Discipline of Marine Biotechnology and Ecology, CSIR- Central  
Salt and Marine Chemicals Research Institute,  
G.B. Road,  
Bhavnagar 364 002, Gujarat, India  
e-mail: bjha@csmcri.org  
e-mail: pagarwal@csmcri.org

2011a), *GSTU* (Jha et al. 2011b), *SOS-1*, *pAPX* and transcription factors like DREB (Gupta et al. 2010).

Abscisic acid stress ripening-1 (*ASR-1*) is a stress- and developmentally regulated gene found only in plant genome. This gene was first reported from tomato (Iusem et al. 1993). Since then, multiple homologues of *ASR-1* gene from many plant species (Carrari et al. 2004) except *Arabidopsis* (Yang et al. 2005) were reported. The *ASR* gene expression was reported to be induced by abscisic acid (ABA), salt, drought, cold and injury, during fruit ripening and also by biotic factors (Amitai-Zeigerson et al. 1995; Liu et al. 2010). The *ASR* proteins are hydrophilic in nature possessing a high content of charged residue such as His, Lys and Glu (Iusem et al. 1993; Amitai-Zeigerson et al. 1995) and do not have any N-terminal hydrophilic repeat sequence as specific in late embryogenesis abundant (LEA) proteins (Maskin et al. 2001). Maskin et al. (2007) showed that tomato *ASR-1* accumulates as LEA proteins at late stage of seed development. C-terminal end of LLA23 (a lily *ASR* protein) contains Lys (K)-rich motifs, characteristic of nuclear localising signal (NLS) and is conserved in other plant *ASR* proteins (Wang et al. 2005). *ASR* family protein works as a transcription factor and regulates hexose transporters and ABA-responsive genes (Frankel et al. 2007). Konrad and Bar-Zvi (2008) have shown that tomato *ASR-1* is found in unstructured form in the cytosol and protects cytosolic proteins under abiotic stress conditions. Tomato *ASR-1* protein gets dimerised to structured form in the presence of Zn, and this form is abundant in the nucleus (Goldgur et al. 2007). Several transgenic plants over-expressing *ASR* genes have been developed. Over-expression of lily *ASR* (*LLA23*) in *Arabidopsis* (Yang et al. 2005) and tomato *ASR-1* in tobacco (Kalifa et al. 2004) conferred enhanced salt and drought tolerance in transgenic lines. The over-expression of *MpASR* gene in *Arabidopsis* exhibited enhanced osmotic tolerance (Dai et al. 2011).

There are several reports on isolation and characterisation of *ASR-1* gene from different glycophytes; however, this gene has not been characterized from any halophytes so far. Halophytes have a unique genetic make-up with well-developed adaptation mechanism to survive in a saline condition. With this aim, we have isolated and characterised the *SbASR-1* gene from an extreme halophyte *S. brachiata*. T<sub>1</sub> transgenic tobacco lines were screened for functional validation of this gene under salt stress condition.

## Material and Methods

### In Silico Analysis

The *ASR-1* gene and protein sequences of different plants were obtained from National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>).

The phylogenetic tree of the amino acid sequences was constructed using MEGA5 software (Tamura et al. 2011) with the maximum likelihood method and 1,000 replicates. Indication of conserved domains of *SbASR-1* was carried out by query against InterPro (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>). Secondary structure prediction was carried out by ExPASy tools (<http://www.expasy.ch/tools/>). Folding characteristic of *SbASR-1* protein was predicted by FoldIndex program (Prilusky et al. 2005, <http://bioportal.weizmann.ac.il/fldbin/findex>). Primary *SbASR-1* protein sequence and other glycophtic *ASR-1* sequences were analysed for N-glycosylation sites, protein kinase sites and myristoylation sites by PROSCAN.BASE (<http://npsa-pbil.ibcp.fr>), whereas total phosphorylation site search was performed by Net Phos2.0 server (<http://www.cbs.dtu.dk/services/NetPhos/>). Physicochemical parameters of *SbASR-1* protein were studied by ProtParam (ExPASy tools).

### Construction of Plant Transformation Vector and Tobacco Transformation

The *SbASR-1* cDNA was PCR amplified using forward (*ASR1F*: 5'-GGCTCGAGATGGAAACAGGAGGC TATGGTTCAACCG-3') and reverse (*ASR1R*: 5'-GGGAATCTTAGAAGAAATGGTGCTTCTTTCC-3') primers containing *XhoI* and *EcoRI* sites, respectively. The amplicon was digested with *XhoI/EcoRI* and cloned into pRT 101 vector (Töpfer et al. 1987). Thereafter, the entire cassette containing CaMV 35S-*SbASR-1* gene and terminator was digested with *PstI* and cloned into the pCAMBIA 1301 vector at the *PstI* site. The resulting vector was mobilised into *Agrobacterium tumefaciens* (LBA 4404) and further transformed to tobacco (*Nicotiana tabacum* cv. *xanthii*) plants according to the standard protocol (Horsch et al. 1985).

### Confirmation of Transgene in Tobacco Plants

Genomic DNA of tobacco transgenic lines was extracted from leaf tissues using modified cetyltrimethylammonium bromide (CTAB) method as described by Bubner et al. (2004). Leaf tissues (2 g) were powdered with mortar and pestle in liquid nitrogen and homogenised in 4 ml hot (65 °C) extraction buffer (2 % CTAB w/v, 100 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 1 % PVP *M<sub>r</sub>* 40,000, 1 % β-mercaptoethanol). Further, 5 ml of chloroform/isoamyl alcohol (CI 24:1) was added, mixed and centrifuged; the supernatant was again mixed with 1 ml of 5 % CTAB (w/v in 0.35 M NaCl) buffer and extracted with 5 ml CI and centrifuged. The nucleic acid was precipitated from the supernatant by 2/3 volume of ice-cold isopropanol. RNA was removed by rehydrating pellets in 500 μl high salt TE buffer including RNase (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 1 M NaCl, 100 ng/μl RNase) at 37 °C for 15 min. Further, the

DNA was purified by CI extraction and precipitated by using 2× ice-cold absolute ethanol. The DNA pellet was washed with 80 % ethanol, dissolved in sterile double-distilled water and stored at −20 °C. Genomic DNA concentration was determined by NanoDrop Spectrophotometer (ND1000, Wilmington, USA) and diluted to 1, 10 and 100 ng/μl concentration. Real-time quantitative (RTQ) PCR condition was optimised for *GUS* gene primers (GQF: 5′-GTGAAGGGCCAACAGTTCC-3′, GQR: 5′-GGTAATGCGAGGTACGGTA-3′) and *NRA* gene (NCBI accession no. X06134) primers (NRAF: 5′-AATGCTGGCACTGATTGCAC-3′, NRAR: 5′-TCCTCTGCGCTGGAACAAG-3′) (*NRA* as an internal control, Guo et al. 2010). The PCR reactions were carried out using 3.75 ng *GUS* primer or 7.5 ng *NRA* primers in 20-μl reaction using QuantiFast SYBR Green PCR reaction kit (Qiagen, USA). The following PCR conditions were maintained for RTQ: 95 °C for 5 min, 1 cycle and 95 °C for 1 min, 55 °C for 30 s, and 72 °C for 30 s, 45 cycles. At the end of the PCR cycles, the products were put through a melt curve analysis. The amplified product was run on a 1.5 % agarose gel to confirm expected size. The experiments were repeated twice independently. Reactions were run in Real-Time iQ5 Cyclor (Bio-Rad, USA), and standard curves were plotted using threshold cycle (Ct) value to determine reaction efficiencies. The efficiency values were put in the following formula given by Shepherd et al. (2009) to determine the copy number ratio of *GUS* to *NRA*:

$$\text{Ratio} = \left\{ 1 + (E)^{Ct} \right\}_{\text{gene of interest}} / \left\{ 1 + (E)^{Ct} \right\}_{\text{control}}$$

#### Transcript Expression of *SbASR-1* Gene in Transgenic Lines

Total RNA was isolated from leaf tissues using the GITC method (Chomczynski and Sacchi 1987). The cDNA from both wild-type (Wt) and transgenic plants was prepared according to manufacturer's protocol (Promega, USA). The reverse transcriptase PCR was carried out using 500 ng cDNA, 150 ng *ASR-1* primers (ASRQF3 5′-CCGAGACCAAGAGGTATGG-3′, ASRQR3 5′-GAGGCAACAGCACCAAGA-3′) or 150 ng *actin* primers (QACTF 5′-CGTTTTGGATCTTGCTGGTCGT-3′, QACTR 5′-CAGCAATGCCAGGGAACATAG-3′), 200 μM dNTPs and 2.5 U *Taq* DNA polymerase in a 50-μl reaction with the following conditions: 95 °C for 5 min, 1 cycle; 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, 35 cycles; 72 °C for 7 min, 1 cycle. The PCR products were analysed via agarose gel electrophoresis.

#### Germination Assay and Growth Parameter Analysis

To test the stability of the transgene, the seeds from T<sub>0</sub> transgenic plants were germinated on Murashige and Skoog (MS) medium supplemented with hygromycin (20 mg/l). For analysis of salt stress tolerance of *SbASR-1* over-expressing

tobacco plants, seeds of two transgenic lines and Wt were germinated on MS medium (Murashige and Skoog 1962) supplemented with 0, 100, 150, 200 and 300 mM NaCl. Percent seed germination, root length, fresh seedlings' weight and leaf areas were scored after 30 days of growth. To study the growth for longer duration, the seeds were first germinated on the MS basal medium, and after 1 week of germination, the T<sub>1</sub> seedlings were transferred in jars on the MS basal medium or supplemented with 200 mM NaCl. In parallel, 6-week-old plants after germination were transferred to sterile soil in small plastic cups. After 2 weeks of acclimatisation, plants were treated with 10 ml of 400 mM NaCl every alternate day for 2 weeks.

#### Ion Contents

For analysis of ion content, 0.2 g of tissues from second leaf from the top of a 4-week-old plant grown in hydroponic medium (1/2 MS with or without 100 mM NaCl) was digested with 4 ml perchloric acid and nitric acid solution (3:1). The solution was dried on the hot plate and further diluted to 25 ml with deionised water and filtered through 0.2-μm filter. Ion content was measured by inductively coupled plasma optical emission spectrometer (Optima 2000DV, PerkinElmer, Germany).

#### Electrolyte Leakage and Membrane Stability Index

Electrolyte leakage was measured according to Lutts et al. (1996). Leaf discs of same size were collected from three plants for each treatment and washed thoroughly with deionised water to remove surface-adhered electrolytes. The samples were placed in closed vials containing 10 ml of deionised water and were then incubated at 25 °C on a rotary shaker for 24 h. Subsequently, the electrical conductivity (EC) of the solution ( $L_t$ ) was determined using Seven Easy conductivity meter (Mettler Toledo AG 8603, Switzerland). The samples were then autoclaved at 120 °C for 20 min and cooled at 25 °C before determining the final electrical conductivity ( $L_0$ ). The electrolyte leakage was defined as follows:

$$\text{Electrolyte leakage (\%)} = (L_t/L_0) \times 100.$$

Membrane stability index (MSI) of Wt and transgenic lines was determined as described by Sairam (1994); 200 mg young leaves of stress-treated plants was taken in 10 ml double-distilled water in duplicate. One set was incubated in a water bath at 40 °C for 30 min, and EC ( $L_1$ ) was recorded by a conductivity meter. The second set was boiled on the water bath for 10 min at 100 °C, and again, EC ( $L_2$ ) was recorded after cooling. Finally, the MSI was calculated using the formula below:

$$\text{MSI} = [1 - (L_1/L_2)] \times 100.$$

### Water Content

Total percent water content (PWC) in the leaves of stress-treated and untreated plants was estimated as described by Zhang and Blumwald (2001). Fresh weight (FW) of leaf discs of 6 cm<sup>2</sup> was recorded, wrapped in aluminium foil and dried at 70 °C for 48 h. The dry weight (DW) of discs was recorded, and PWC was calculated according to the following formula:

$$\text{PWC} = [(FW - DW)/FW] \times 100.$$

### Chlorophyll Content

Leaf chlorophyll content of the 4-week-old transgenic and Wt plants, grown under different salt concentration, was estimated according to Arnon (1949). Leaf tissues were homogenised in 80 % acetone and were then incubated in the dark for 6 h, and absorbance of the supernatant was recorded at 645 and 663 nm in T80+ UV-Vis Spectrophotometer (PG Instrument Ltd., UK) to calculate total chlorophyll amount.

### Proline Content

Free proline content in the leaves was determined using ninhydrin (Bates et al. 1973) with minor modifications. Leaf tissues (100 mg) were homogenised in 1.2 ml of 3 % aqueous sulphosalicylic acid and centrifuged at 13,000 rpm for 10 min. After centrifugation, 500 µl of supernatant was diluted to 1 ml with distilled water, reacted with 1 ml of 2 % ninhydrin and incubated at 90 °C for 1 h. The samples were cooled on ice, and 2 ml of toluene was added, vortexed and allowed to settle for 2 min. Upper phase was aliquoted to read the absorbance at 520 nm in T80+ UV-Vis Spectrophotometer (PG Instrument Ltd., UK). The proline content was calculated against a standard curve drawn from known concentration of L-proline (Sigma-Aldrich, USA) and expressed as microgrammes per gram of fresh weight.

### Statistical Analysis

Each experiment was carried out in three replicates. The two-factor ANOVA with replication on Microsoft Excel (Microsoft Corp., Redmond, WA, USA) was carried out. The critical difference values (C.D.) were calculated at the  $p=0.05$  level to find out the significant differences between the means of Wt and transgenic lines with or without salt treatment by the following formula:

$$\text{C.D.} = t \text{ value at } 5 \% \text{ level} \times \text{S.E.}$$

$$\times 1/\sqrt{\text{number of mean values to be compared.}}$$

Significantly different mean values are indicated by different letters on the graphs.

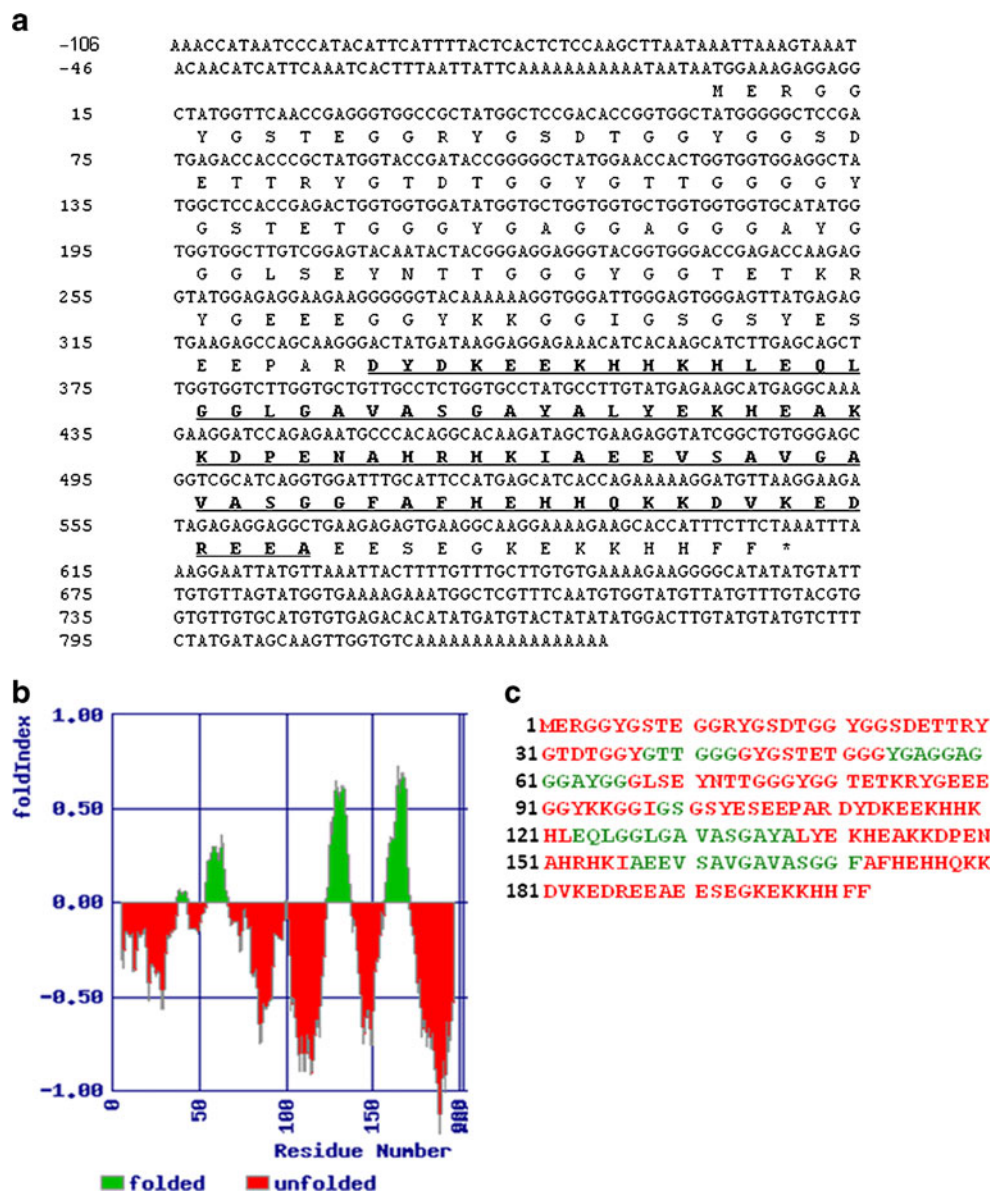
## Result and Discussion

### In Silico Analysis

The *SbASR-1* (accession number EU746399) was 938 bp long-containing 106 bp 5' UTR, 609 bp open reading frame and 223 bp 3' UTR regions. The *SbASR-1* gene encoded a polypeptide of 202 amino acid residues (Fig. 1a) with an estimated molecular mass of 21.01 kDa having an isoelectric point of 5.30. Analysis of SbASR-1 protein sequence by query against InterPro revealed the presence of a characteristic ABA/WDS signature sequence of 79 amino acid residues (Fig. 1a). The SbASR-1 amino acid sequence showed maximum identity (73 %) with *Solanum chilense* ASR-1. To study the evolutionary relationship, phylogenetic tree of amino acid sequences of SbASR-1 and other plant ASR-1 proteins was constructed by the maximum likelihood method (Supplementary Fig. 1). The phylogenetic tree revealed that SbASR-1 is closer to other Solanaceae members. Total amino acids' alignment of SbASR-1 with other 22 ASR-1 proteins from glycophytic plants was carried out by ClustalW program. Comparative analysis of different motifs (Virlovet et al. 2011) and sites of SbASR-1 and other glycophytic ASR-1 was carried out using an online server (Supplementary Table 1). The N-terminal His-rich region (motif A) was found in all glycophytic ASR-1, whereas in SbASR-1, it is dominated by glycine residues (Supplementary Fig. 2). The C-terminal region revealed three conserved domains, viz. Zn<sup>2+</sup>-dependent DNA-binding domain (motif B), hydrophobic residue-rich DNA-binding hindering domain (motif C) and NLS (motifs D and E, Supplementary Fig. 2). Most of the ASR proteins have K- or R-rich bipartite NLS on the C-terminal end (Wang et al. 2005). Similarly, C-terminal end of the SbASR-1 protein also contains conserved bipartite K-rich NLS (Supplementary Fig. 2). The LLA23 protein has K-rich NLS, and it was found to be distributed in the nucleus and cytosol of the vegetative cells of mature pollen grain (Wang et al. 2005). VvMSA (Grape ASR protein) was localised in the nucleus and interacted with the *VvHTI* promoter (Cakir et al. 2003). However, it was observed that ASR-1 proteins from Solanaceae family have monopartite NLS (Supplementary Fig. 2). The secondary structure of SbASR-1 was predicted using the PSIPRED protein structure prediction server (ExPASy tools) and revealed the presence of five coils and four alpha-helices (Supplementary Fig. 3). Approximately, 30 % of all eukaryotic proteins are either completely or partially disordered (Fink 2005). The intrinsically disordered proteins (IDPs) protect other cellular proteins under stress conditions and help in transcriptional regulation of other



**Fig. 1** **a** Nucleotide sequence and derived amino acid of *SbASR-1*. Amino acid sequence is denoted in *single-letter format* just below the cDNA nucleotide sequence. The *underlined* amino acid sequence shows the conserved domain of ABA/WDS protein super family. **b** *SbASR-1* folding prediction via FoldIndex program using the values predicted in the window=10 and step=1. *Positive and negative numbers* represent ordered and non-ordered protein, respectively. **c** The primary amino acid sequence of *SbASR-1*. Amino acids as shown in **b** have ordered and disordered regions which are represented by *green* and *red bold characters*, respectively



genes. It can undergo extensive post-translational modifications, such as phosphorylation, acetylation, ubiquitination, sumoylation, etc., allowing easier protein–protein interaction for modulation of biological functions because of their structural flexibility (Uversky and Dunker 2010). Folding prediction of *SbASR-1* protein using FoldIndex program revealed 66.3 % disorder-promoting amino acid residues (Fig. 1b, c). Similarly, in tomato, the *ASR-1* protein contained higher percentage of disorder-promoting amino acid residues (Goldgur et al. 2007). Uversky et al. (2000) reported that the natively unfolded protein or IDPs had generally low  $M_r$ s with less than 150 amino acid residues and showed either acidic or basic isoelectric pH. The *SbASR-1* protein exhibited an acidic isoelectric pH (5.3) compared to almost neutral isoelectric pH of tomato *ASR* protein confirming that *SbASR-1* is also an IDP.

The PROSCAN.BASE search revealed 29 N-myristoylation sites, 3 tyrosine kinase phosphorylation sites, 2 protein kinase C phosphorylation sites and 1 N-glycosylation site in *SbASR-1* protein (Supplementary Tables 1 and 2), whereas in other glycophytic plant *ASR-1* proteins, few N-myristoylation sites were found. The myristoylation occurs by covalent attachment of myristate group at the N-terminal glycine residue of eukaryotes and viruses (Farazi et al. 2001). Myristoylation of protein helps in protein–protein interaction and in regulating signalling pathways during apoptosis and salt stress adaptation (de Jonge et al. 2000). Ishitani et al. (2000) reported that mutation in myristoylation sites makes plants hypersensitive to salt stress. Therefore, it is possible that the presence of glycine-rich region in the *SbASR-1* may help in salt adaptation. The overall phosphorylation site search analysis by NetPhos 2.0 server exhibited seven, two and five serine, threonine and tyrosine

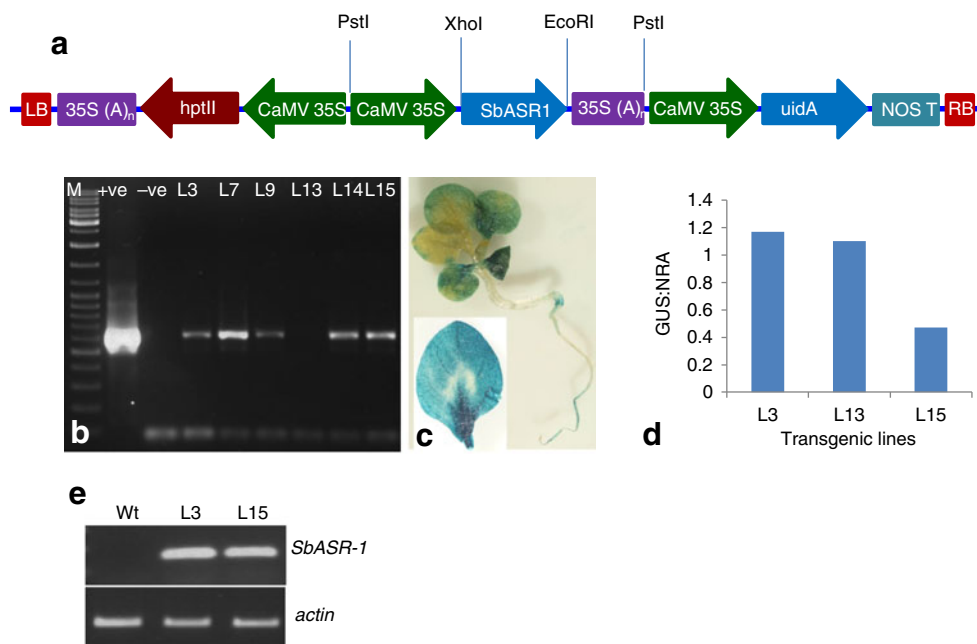
phosphorylation sites, respectively. It has one N-glycosylation site, which is found only in few glycophytic species. In *Arabidopsis*, it was reported that one isoform of glycosylating enzyme STT3A is involved in salt/osmotic stress-adapted mechanism (Koiwa et al. 2003)

### Analysis of Transgenic Lines

ASR protein developmentally regulates fruit ripening and is also expressed in salt and drought conditions. Earlier, we have reported that *SbASR-1* was constitutively expressed and reached to a maximum level at 250 mM NaCl. Time course analysis with 250 mM NaCl revealed that *SbASR-1* transcript peaks at 12 h (Jha et al. 2009). In the present study, *SbASR-1* gene was cloned into plant expression vector pCAMBIA 1301 (Fig. 2a), and transgenic tobacco was developed for functional validation. Transgenic plants were confirmed by PCR (Fig. 2b) and GUS staining (Fig. 2c). RTQ-PCR was done to determine the transgene integration and the copy number using *NRA* gene as the internal control (Guo et al. 2010). RTQ analysis revealed that L3 and L13 have GUS to NRA ratios close to 1, and therefore, it is highly likely that these lines have double insertion. The transgenic line, L15 with a ratio close to 0.5 probably showed a single-copy insertion (Fig. 2d). Transgenic lines

showed high expression of the *SbASR-1* gene by RT-PCR, whereas it was absent in the Wt plant (Fig. 2e)

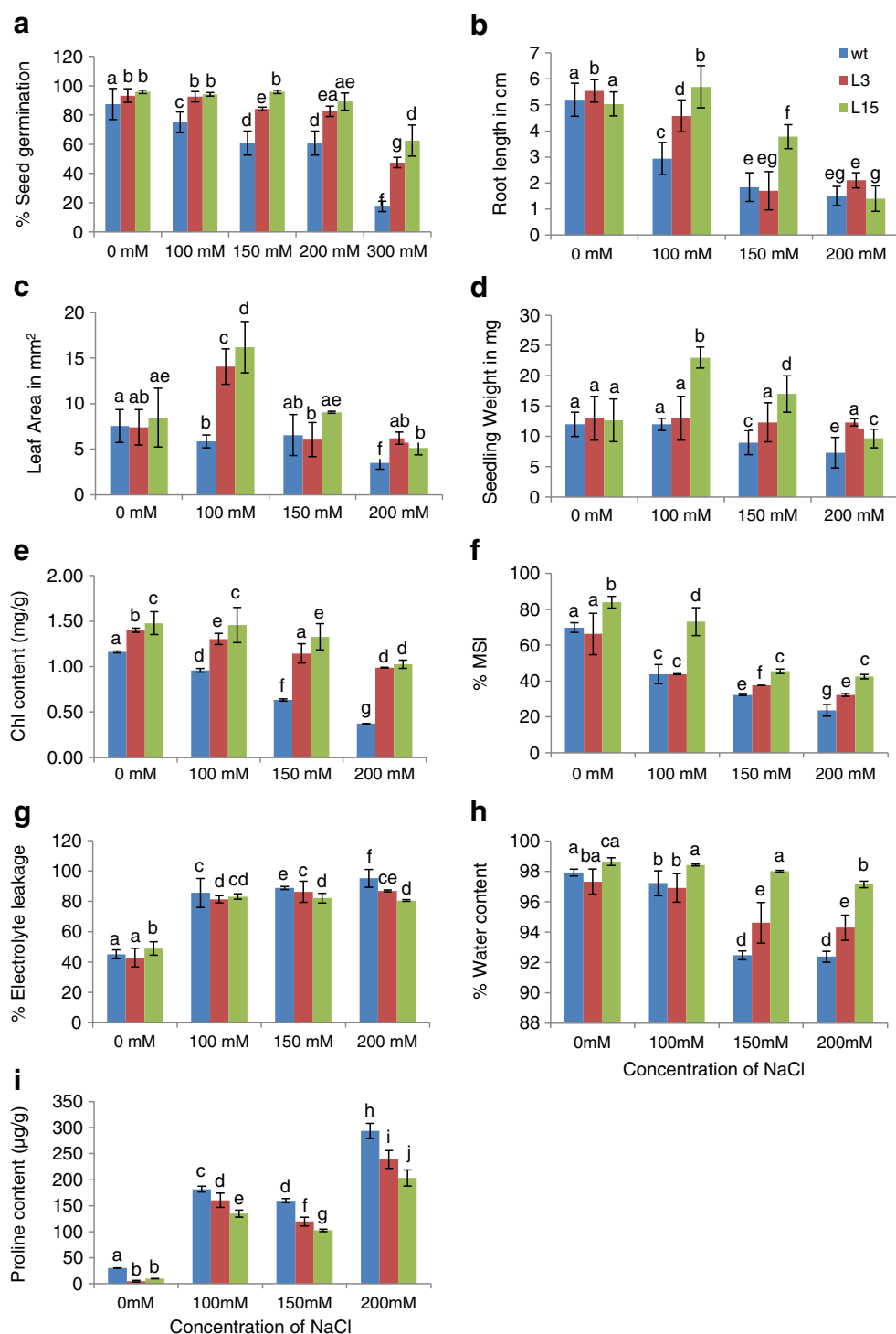
The seeds from T<sub>0</sub> transgenic lines L3 and L15 were germinated on the MS basal medium supplemented with 0, 100, 150, 200 and 300 mM NaCl. The percentage of seed germination decreased with increasing NaCl concentration in both Wt and transgenic lines; however, the germination was affected severely in Wt plants (Fig. 3a). Over-expression of peroxidase *TPX2* in tobacco increased the germination rate of transgenic lines suffering from osmotic stress (Amaya et al. 1999). The peroxidase gene is reported from cDNA library prepared through suppressive subtractive hybridisation experiment of Wt and transgenic tobacco line over-expressing tomato *ASR-1*. It was also observed that expression of seed imbibition protein homologue is higher in tobacco transgenic line compared to Wt (Kalifa et al. 2004). Therefore, it is expected that the relative increase in germination rate of the transgenic lines under salt stress in our experiments may be due to increased expression of these proteins. At 0 mM NaCl, the Wt and transgenic lines showed similar morphological features (Figs. 3a–d and 4a); however, at 100 mM and higher concentration of NaCl, the seedling growth was better in transgenic lines. The root length was significantly reduced at 100 to 200 mM NaCl (Figs. 3b and 4b–d). The seeds were germinated on the MS



**Fig. 2** **a** Diagrammatic representation of the pCAMBIA1301-*SbASR-1* construct used for tobacco transformation. *SbASR-1* was cloned into pCAMBIA 1301 vector downstream to CaMV 35S promoter and terminated with 35S poly A tail. **b** Confirmation of transgenic T<sub>0</sub> lines by using *SbASR-1*-specific primers. *M* denotes standard marker, *-ve* and *+ve* are the negative and positive controls, respectively, and *L3–L15* are transgenic lines. **c** Histochemical GUS assay of transgenic seedling and individual leaf (*inset*). Blue coloration of leaf and seedling

depicts expression of the *GUS* gene in transgenic lines. **d** Real-time quantitative PCR analysis to determine the ratio of *GUS* to *NRA* gene in transgenic lines. Three lines *L3*, *L13* and *L15* have a ratio of 1.17, 1.10 and 0.47, respectively. **e** Reverse transcriptase PCR of *SbASR-1* transgenic lines. Amplification was observed in PCR reactions with *SbASR-1*-specific primers using cDNA of transgenic lines, while there was no amplification from Wt cDNA; *actin* gene was used as an internal control

**Fig. 3** Graphs show growth parameter and physiological and biochemical analysis of Wt and transgenic lines under different NaCl concentration: **a** percent seed germination assay, **b** root length, **c** leaf area, **d** seedling fresh weight, **e** chlorophyll content, **f** percent membrane stability index (MSI), **g** percent electrolyte leakage, **h** percent water content, and **i** proline content in leaf tissue

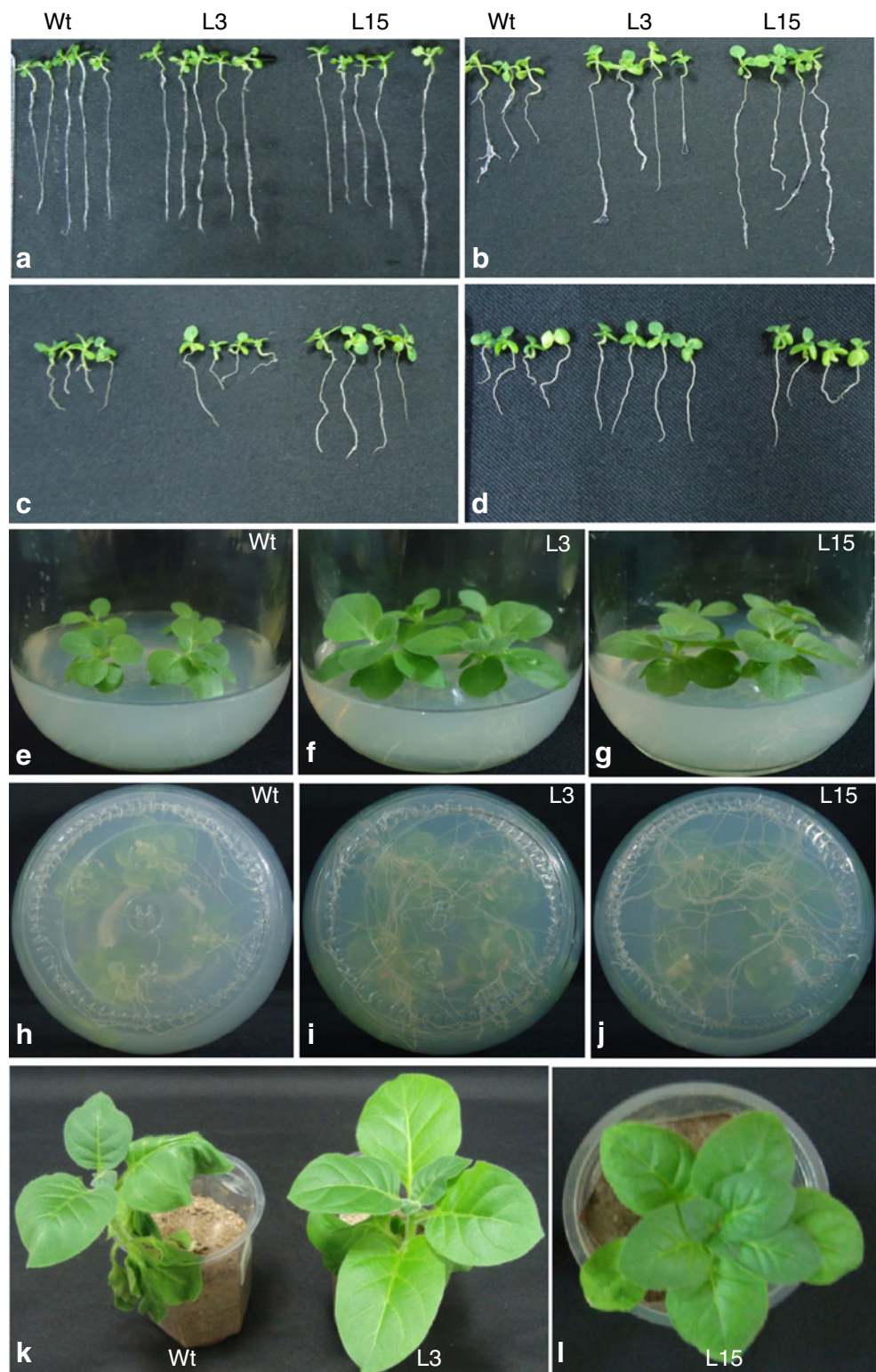


basal medium, and the seedlings were transferred on the MS basal medium with or without 200 mM NaCl. The transgenic lines showed better shoot and root growth after 4 weeks in the presence of salt (Fig. 4e–g). Wt plants showed very poor root growth in 200 mM NaCl, whereas transgenic lines exhibited denser and healthier roots (Fig. 4h–j). The

transgenic plants, grown in small plastic pots, treated with 400 mM NaCl every alternate day for 2 weeks showed less wilting compared to Wt plants (Fig. 4k, l). Similarly, the leaf area and seedling weight were found significantly higher in transgenic lines at higher NaCl concentration compared to Wt plants (Fig. 3c, d). The increase in root length of the



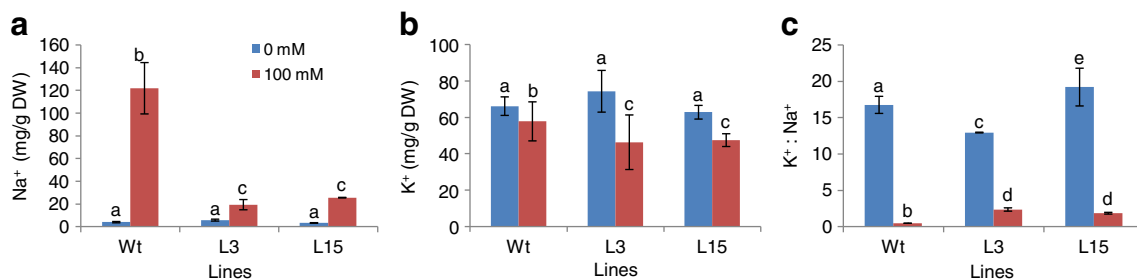
**Fig. 4** Analysis of **a–d** 4-week-old transgenic plants was observed at 0, 100, 150 and 200 mM NaCl; **e–g** morphological difference in shoot growth and **h–j** root growth of 4-week-old Wt, L3 and L15 plants, respectively, at 200 mM NaCl concentration supplemented to MS basal media. **k, l** Wt plant shows more wilting compared to L3 and L15 transgenic lines treated with 10 ml of 400 mM NaCl solution on each alternative day for 2 weeks



transgenic lines compared to Wt may be due to increased water uptake to overcome the secondary osmotic stress condition induced by salt (Agarwal et al. 2010b). Chlorophyll is considered as one of the markers of cellular stress, and the decrease in chlorophyll is considered as a common symptom

under salt stress. Chlorophyll content was significantly reduced in Wt as compared to transgenic plants at different NaCl concentrations (Fig. 3e). The reduced chlorophyll accumulation during salt treatment may be due to the interference of different chlorophyll biosynthesis stages in Wt plants.





**Fig. 5** Ion content analysis of Wt and transgenic lines at 0 and 100 mM of NaCl. **a** There is no significant difference in Na<sup>+</sup> concentration of Wt and transgenic lines at 0 mM NaCl treatment, whereas Wt plants accumulate significantly higher concentration of Na<sup>+</sup> compared

to transgenic lines at 100 mM of NaCl treatment. **b** Accumulation of K<sup>+</sup> ion in transgenic lines decreased significantly compared to Wt plants at 100 mM of NaCl stress. **c** K<sup>+</sup>/Na<sup>+</sup> is higher in transgenic lines compared to Wt plants at 100 mM of NaCl stress

Cell membrane stability is affected in response to abiotic stress in sensitive plants leading to increased electrolyte leakage. Electrolyte leakage is used to measure the membrane permeability of the cell. Salt stress causes damage to plant cell membrane, thereby increasing the electrolyte permeability (Shukla et al. 2011). Therefore, maintenance of membrane stability and integrity is quite important in preventing the harsh effect of stress (Levitt 1980). In the present study, the MSI was significantly higher in the transgenic lines as compared to Wt plants (Fig. 3f). The L15 line showed better membrane stability compared to L3. The electrolyte leakage was approximately 40 % in Wt and transgenic lines at 0 mM NaCl and significantly increased by increasing NaCl concentration (Fig. 3g). However, the electrolyte leakage was higher in Wt plants. Transgenic *Arabidopsis* over-expressing *MpAsr* showed less leakage and peroxidation under salt and osmotic stress conditions (Dai et al. 2011). MSI of 35 *S-TaABC1* transgenic plants under osmotic stress was found to be higher than that of Wt and vector control plants (Wang et al. 2011). The MSI was found higher when transgenic 35 *S::NtOsmotin* was compared with non-transgenic, whereas electrolyte leakage assay showed a reverse result (Das et al. 2011). Better membrane stability was also observed in cotton and strawberry plants transformed with *Osmotin* gene (Husaini and Abdin 2008; Parkhi et al. 2009). Higher membrane stability and lower electrolyte leakage had been reported in transgenic plants in mulberry using the *HVA1* gene (Lal et al. 2008) under simulated drought and salt conditions. In *SbASR-1* transgenic lines, PWC was higher in the L15 line compared to Wt and L3 at different NaCl concentrations. The L15 line also showed higher MSI, which is well correlated with its high PWC (Fig. 3f, h).

Proline and total soluble sugars are very important biochemical indicators of salinity tolerance in plants (Ashraf and Harris 2004). When plants face salt stress, proline accumulates in the cytosol and helps substantially in cytoplasmic osmotic adjustment (Leigh et al. 1981). It helps the plant cell by stabilising sub-cellular structures such as

membranes and proteins, scavenging free radicals and buffering cellular redox potential under salt stress (Ashraf and Foolad 2007). In the present study, transgenic lines showed lower proline content up to 200 mM NaCl (Fig. 3i). This suggests that transgenic plants do not face much salt stress; therefore, the proline remains low in these plants as compared to Wt. Similarly, the tomato *ASR-1* gene in transgenic tobacco showed less proline accumulation as compared to Wt (Kalifa et al. 2004).

The regulation of ions is an indispensable component of growth and adaptation. In plants, the balance of Na<sup>+</sup> and K<sup>+</sup> is important for salt tolerance. Since the leaves serve as a sink for the toxic ions under salt stress, ion (Na<sup>+</sup> and K<sup>+</sup>) content analysis was carried out in the leaves of Wt and transgenic plants. The *SbASR-1* transgenic lines showed significantly less Na<sup>+</sup> accumulation in L3 and L15 lines than Wt plants (Fig. 5a). Salinity also impedes the ratio of K<sup>+</sup> and Na<sup>+</sup> in the cell. However, an increase in K<sup>+</sup> concentration can alleviate the deleterious effect of salinity on growth and yield (Giri et al. 2007). In the present study, we observed that K<sup>+</sup> accumulation was decreased in L3 and L15 lines, compared to Wt plants (Fig. 5b). However, the ratio of K<sup>+</sup>/Na<sup>+</sup> was higher in the transgenic lines under salt stress (Fig. 5c). Similarly, Kalifa et al. (2004) reported less accumulation of Na<sup>+</sup> and K<sup>+</sup> in the *SLASR-1* over-expressed tobacco transgenic line.

In conclusion, it can be explained that halophytes have efficient mechanisms to cope with high salt stress by regulating different transcription factor and salt-tolerant genes. The *SbASR-1* amino acid showed N-terminal glycine-rich region, maximum number of myristoylation sites and higher ratio of disorder promoting amino acids as compared to glycohytic ASR-1 proteins. The *SbASR-1* gene showed significant salt tolerance in transgenic tobacco. The transgenic plants exhibited better growth, low proline content and lower amount of Na<sup>+</sup> and K<sup>+</sup> content under salt stress. This study reveals that *SbASR-1* gene is a potential candidate for imparting abiotic stress tolerance in crop plants.

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