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RESEARCH PAPER

A MYB transcription factor from the grey mangrove is induced by stress and confers NaCl tolerance in tobacco

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Abstract

MYB transcription factor genes play important roles in developmental and various other processes in plants. In this study, functional characterization of AmMYB1, a single-repeat MYB transcription factor isolated from the salt-tolerant mangrove tree *Avicennia marina* is reported. *AmMYB1* cDNA was 1046 bp in length with an open reading frame of 783 bp, encoding 260 amino acids. The corresponding gene had two introns and three exons and was present as a single copy in *A. marina*. The deduced amino acid sequence showed similarities to MYB proteins reported in other plants, including the conserved MYB binding domain. RNA gel blot analysis showed that the *AmMYB1* transcript expression was more pronounced in green photosynthetic tissue and was strongly induced by stresses such as salt (500 mM), light (500 µE m^{-2} s^{-1}), and the exogenous application of ABA (100 µM). An analysis of the upstream sequence of *AmMYB1* gene revealed the presence of regulatory elements identical to those present in the promoters of stress inducible genes. The promoter was responsive to NaCl and could enhance reporter gene expression *in planta*. An *in vitro* DNA binding assay using the promoter region (TGGTTAG) of the *AtRD22* gene and a transactivation assay in yeast cells suggest the possibility of AmMYB1 protein regulating the expression of other genes during salt stress. Transgenic tobacco plants constitutively expressing the AmMYB1 transcription factor showed better tolerance to NaCl stress.

Key words: Avicennia marina, halophyte, MYB transcription factor, single-repeat MYB protein, salt tolerance.

Introduction

Plants have evolved different mechanisms to cope up with environmental stresses such as drought, salinity, and low temperature, which limit their growth and productivity. Regulation of gene expression is one such mechanism that is facilitated by stress-responsive transcription factors. In *Arabidopsis thaliana,* over 1600 transcription factors have been identified (Jung *et al*., 2008*a*), out of which 163 represent MYB (Myeloblastosis) family transcription factors (Yanhui *et al*., 2006). MYB is a large family of transcription factors, which play diverse physiological and developmental roles in plants and animals. MYB proteins are induced or repressed under different stress conditions and participate in the regulation of abiotic stress-responsive genes in plants (Chen *et al*., 2005). MYB transcription factors are DNA binding proteins with highly conserved MYB domains formed by single, double or triple imperfect repeats, with each repeat containing 50–53 amino acids. In plants, MYB domain consists of two repeats (R2 and R3) or a single repeat (R1 or R2). It is possible that single MYB-domain proteins are plant specific, bind DNA in a different way compared with the two-repeat or three-repeat MYB proteins, and therefore are likely to have different functions (Jin *et al*., 1999). In plants, the majority of MYB-like proteins possess a DNA binding domain with R2–R3 repeats. These proteins have been reported to play an important role in trichome initiation of *Arabidopsis thaliana* (Kirik *et al*., 2005) and also the regulation of phenylpropanoid pathway in *Vitis vinifera* (Deluc *et al*., 2006). Such developmental and regulatory roles played by MYB proteins may also lead to specialized structural adaptations to stress. MYB domain proteins

(CCA1and LHY) with a single repeat in *Arabidopsis* are known to be involved in circadian rhythm, leaf movement, photosynthetic gene expression, and hypocotyl growth (Jin *et al*., 1999).

The function of several other transcription regulators, such as the AP2 domain transcription factor and the homeobox-leucine rich protein has been extensively studied with reference to stress tolerance in plants like *Arabidopsis thaliana* and *Oryza sativa* (Uno *et al*., 2000; Oh *et al*., 2009), whereas little is known about the role of MYB family transcription factors in the abiotic stress tolerance of plants. Recent studies indicate the involvement of two repeat MYB-related genes such as *AtMYB2* of *Arabidopsis*, *OsMYB3R-2* of rice, *BcMYB1* of hamley, and *GmMYB76* of soybean in stress tolerance (Dai *et al*., 2007; Jung *et al*., 2008*a*; Liao *et al*., 2008). A 'SHAQKYF' class of the MYB family transcription factor belonging to the KANADY family has been shown to increase the photosynthetic capacity in rice (Zhang *et al*., 2009). In addition, previous studies have shown that ectopic expression of MYB genes resulted in increased tolerance to freezing, drought, and salt stress in non-halophytic plants (Dai *et al*., 2007; Pasquali *et al*., 2008). Although expressed sequence tags (ESTs) related to MYB proteins were reported in the model halophyte *Thellungiella halophila* (Wang *et al*., 2004), their roles in salt stress tolerance in halophytic plants have not been studied thus far. In the present study, the functional characterization of a single-repeat MYB transcription factor, designated as *AmMYB1* and isolated from *Avicennia marina*, a salt-tolerant (Tomlinson, 1986) mangrove plant, is reported.

Materials and methods

Growth of Avicennia marina *seedlings and stress treatments*

Avicennia marina seeds collected from their natural habitat in Pichavaram, Tamil Nadu, India were germinated in sand-filled trays under a 12/12 h (light/dark cycle) at 35±2 °C and the seedlings were watered daily. One-month-old *A. marina* seedlings were acclimatized for 3 d in a growth chamber maintained at 25 °C and 70% relative humidity (RH). A light/dark cycle of 16/8 h was followed with 100 µE m^{-2} s^{-1} photosynthetic photon flux density (PPFD). Seedlings were grown hydroponically in half-strength MS (Murashige and Skoog, 1962) basal medium. For ABA- and salt stress-treatments, one-month old seedlings were treated hydroponically with 100 µM ABA or with 250 or 500 mM NaCl for different time periods (Ganesan *et al*., 2008). A high light treatment (500 µE m^{-2} s^{-1}) was continuously provided up to 24 h after *A. marina* seedlings were exposed to continuous darkness for 7 d.

Isolation of MYB transcription factor cDNA from A. marina

EST clones were obtained from a cDNA library prepared using leaves of *A. marina* seedlings exposed to salt (500 mM NaCl) for 48 h (Mehta *et al*., 2005)**.** The plasmids representing ESTs (Am901243, Am901783, Am900384, and Am900882) and showing homology to different MYB transcription factor cDNAs were restriction digested using *Not*I and *Sal*I to determine the insert size. Plasmid DNA was extracted from clones having an insert size above 600 bp and sequenced using M13 forward and M13 reverse primers (Big-dye chain termination method with ABI Prism 310 DNA sequencer, Applied Biosystems).

AmMYB1 *sequence analysis*

Sequence analysis of *AmMYB1* was performed using BLAST (NCBI). Corresponding protein sequences were aligned using Clustal W. Phylogenetic analysis was done using the Neighbor–Joining (NJ) algorithm of MEGA 4.0 (Tamura *et al*., 2007). Further analysis for the presence of conserved MYB domain was done using SMART (Simple Modular Architecture Research Tool) (Letunic *et al*., 2006) and the deduced amino acid sequence was analysed using ExPaSy (Expert Protein Analysis System) tools. http://www.expasy.ch/tools/dna.html

Northern analysis

Total RNA was isolated from *A. marina* leaf samples using the LiCl method (Alemzadeha *et al*., 2005) after the seedlings were treated with NaCl, ABA, and high light for different time intervals. About 20 µg of total RNA was electrophoresed on 1.2% agarose with 1× MOPS buffer and transferred to a nylon membrane (Amersham, Pharmacia Biotech). Hybridization was performed with an α ^{32}P dCTP-labelled gene-specific 3' UTR cDNA probe. The membrane was washed with 2× SSC, 0.1% SDS at 65 °C for 15 min and the signal was detected using autoradiography after 2 d.

Isolation of the AmMYB1 *gene and its upstream region*

The upstream regulatory region of the *AmMYB1* gene was isolated from *A. marina* genomic DNA using the TAIL-PCR (Thermal Asymmetric Interlaced PCR) method (Liu *et al*., 1995). The specific PCR products from the tertiary TAIL-PCR reaction were purified and cloned in to T/A vector (MBI Fermentas). Sequence analysis was performed using PLACE (Plant *cis*-acting Regulatory DNA Elements) Signal Scan Program. The genomic clone of *AmMYB1* was obtained by PCR using gene-specific primers designed using 5' and 3' UTRs of *AmMYB1* cDNA. Exon–intron junctions were predicted using Genevestigator (https://www.genevestigator.com/gv/).

Southern analysis of AmMYB1 *in* A. marina

DNA gel-blot analysis was performed as described by Dai *et al.* (2007). Genomic DNA isolated (Michiels *et al*., 2003) from 1-month-old *A. marina* seedlings were digested with *Bam*HI, *Sac*I, and *Kpn*I, fractionated electrophoretically on a 0.8% agarose gel and blotted on to a nylon membrane (Amersham, Pharmacia Biotech). The membrane was prehybridized at 42 ºC for 3 h and hybridized with an α ^{32}P-dCTP labelled 3' UTR *AmMYB1* probe overnight. After hybridization, the membrane was washed twice with 2× SSC plus 0.1% SDS at 42 ºC for 15 min, and exposed to X-ray film for 2 d at –80 °C.

AmMYB1 expression in E. coli

The *AmMYB1* coding region was cloned using the *Bam*HI and *Sac*I restriction sites of pET-32a (+) (Novagen) to generate the pET-32a-*AmMYB1* expression vector. Expression and purification of AmMYB1 recombinant proteins were performed using the *E. coli* strain BL21 (DE3). The pET-32a (+) vector without the insert was used as a control. Cells containing the pET32a-*AmMYB1* plasmid were grown to an OD of 0.5 at 600 nm and then incubated with 1 mM IPTG for an additional 3 h. Cells were then washed and re-suspended in a binding buffer containing 20 mM TRIS, pH 8.0, 500 mM NaCl, 5 mM imidazole, and 1 mM PMSF. The resultant cell suspension was incubated on ice for 1 h. AmMYB1 protein was then purified from the cells using Ni^{+} agarose affinity columns according to the manufacturer's instructions (Novagen, USA). Purified AmMYB1 protein was used for the preparation of anti-AmMYB1 polyclonal antibodies in rabbit (Chromous Biotech, India).

Electrophoretic Mobility Shift Assay (EMSA)

The EMSA was performed as described previously by Diaz *et al*. (2002). One-hundred picomoles of the α ^{32}P end-labelled probe of the *AtRD22* promoter element (30 mer) was incubated with 500 ng of purified histidine tagged thioredoxin-*AmMYB1* fusion protein in 20 µl binding buffer at 25 °C for 30 min. The resulting DNA–protein complexes were loaded on to a 0.5× TRIS–borate–EDTA, 5% polyacrylamide gel. After electrophoresis at 10 V cm^{-1}, the gel was subsequently dried and visualized by autoradiography. For competitive binding assays, 5- or

10-fold excess of the unlabelled *AtRD22* promoter element (specific) and salmon sperm DNA (non-specific) competitors were incubated with the fusion protein at room temperature for 30 min prior to the addition of labelled probes.

Transactivation activity of AmMYB1 in yeast cells

Complete and partial regions of *AmMYB1* encoding sequence were amplified and cloned in the pGBKT7 vector (Clontech), fused with the GAL4 DNA Binding Domain (DBD) coding region under the control of the ADH1 promoter. The yeast strain AH109 harbouring the *HIS3* and *LacZ* reporter genes were transformed independently with the recombinant plasmids. Transformants were selected on synthetic dropout (SD) media lacking tryptophan (SD-Trp). HIS3 activity was assessed by conducting a viability test on a histidine-lacking medium. *LacZ* activity was tested by performing the galactosidase filter lift assay according to the manufacturer's instructions (Clontech).

Transformation of tobacco plants

AmMYB1 cDNA was amplified with specific primers containing *SacI* and *KpnI* restriction sites. The amplified fragment was digested and ligated to the binary vector pCAMBIA 1301 containing the 35S CaMV promoter which was already cloned in the multiple cloning site using *Hin*dIII and *Xba*I. The construct was used to transform tobacco through *Agrobacterium tumefaciens* LBA4404. Control plants were generated by transforming tobacco with pCAMBIA 1301 without *AmMYB1*. Leaf disc transformation and regeneration of transgenic plants were performed as previously described (Horsch *et al*., 1985). Transformed tobacco plants were identified using hygromycin (25 mg l^{-1}) as the selection marker. Shoots from T_0 transgenic plants were excised and transferred to the rooting medium [MS medium (pH 5.8) containing 2% sucrose and 0.8%, bacto-agar, containing 250 mg l^{-1} cefotaxime and 25 mg l^{-1} hygromycin] for the first subculture and propagated in the same medium without cefotaxime, using the shoot tip as the explant for subsequent cultures. Rooted T_0 plants were acclimatized in half-strength MS liquid medium for 10 d before they were used in salt-tolerance assays. T_0 plants were transplanted to the soil and grown under greenhouse conditions for the generation of T_1 and T_2 seeds.

Molecular analysis of AmMYB1 *transgenic tobacco plants*

PCR was carried out using the genomic DNA isolated from hygromycin-tolerant tobacco plants. DNA from the control plant was used as a negative control and plasmid DNA of pCAMBIA1301-35S *AmMYB1* was used as a positive control. PCR analysis of plasmid DNA (10 ng) and genomic DNA (50 ng) were performed using *AmMYB1* cDNA UTR-specific primers using the following reaction conditions: initial denaturation (94 °C) for 5 min; 30 cycles of denaturation, annealing, and extension at 94 °C for 45 s, 65 °C for 45 s, 72 °C for 1 min, and a final elongation at 72 °C for 10 min. Amplified PCR products were analysed by gel electrophoresis on a 1.0% agarose gel. Stable integration and copy number in the transformed plants were further confirmed by Southern blot analysis. Total genomic DNA from the transformants and control plants were digested with *Bam*H1 and transferred to a nylon membrane. An *AmMYB1*-specific 3' UTR fragment was used as a probe.

Protein extraction and Western blot hybridization

Leaves from control and transgenic tobacco plants were homogenized in liquid N_2 and suspended in 1.5 ml of 100 mM TRIS-HCl, pH 7.5 containing 50 mM EDTA and 3 mM dithiothreitol. The resultant homogenates were then centrifuged for 10 min at 7000 g. Supernatants were transferred to fresh tubes and protein concentrations were determined as described by Bradford (1976). Western blot analysis was carried out as described by Jung *et al.* (2008*b*). Ten microgram total protein was electrophoresed in a 12% SDS polyacrylamide gel, and transferred to a nitrocellulose membrane by electroblotting. The membrane was washed three times with TBS-T buffer (20 mM TRIS pH 7. 5, 150 mM NaCl, and 0.1% Tween 20), blocked for 4 h at 25 °C in the same solution with 5% non-fat milk and then probed with rabbit anti-AmMYB1 polyclonal antibody (1:1000 dilution; immunoglobulin G fraction) (Chromous Biotech, India). The membrane was washed three times in TBS-T buffer prior to incubation with alkaline phosphatase (AP) conjugated secondary antibody (1:2500 dilution; Pierce, USA) for 1 h. NBT/BCIP-based (Bangalore Genei, India) signal detection was performed following the manufacturer's instruction.

Salt-tolerance assays in tobacco plants

T_0 tobacco single copy AmMYB1 transgenic lines (1, 4, 26) and control transgenic plants were used for the chlorophyll leaching assay, chlorophyll estimation, and the whole-plant salt-tolerance assays. For chlorophyll leaching experiment, leaf discs of 1.5 cm diameter were prepared from healthy, fully expanded tobacco leaves from *AmMYB1*-expressing and control plants. The discs were incubated for 72 h in NaCl (50 mM, 100 mM, and 200 mM) or water. Chlorophyll content in leaf discs was then measured spectrophotometrically after extraction using 80% cold acetone (Lichtenthaler, 1987). Treatments were carried out under continuous white light at 25±2 °C. For the whole-plant salt-tolerance assay, plants were kept in hydroponic half-strength MS basal medium with 50 mM NaCl. The salt concentration was increased gradually up to 200 mM over 10 d. Control and *AmMYB1* transgenic lines were monitored for signs of wilting. Experiments were done in three experimental replicates each. Wherever applicable, Student's *t* test was carried out to calculate significant differences.

Germination assay

T_2 seeds of the control and *AmMYB1* transgenic tobacco lines were surface-sterilized with 70% ethanol for 1 min and washed three times with sterile distilled water. Surface-sterilized seeds were incubated in half-strength MS medium with 100 mM, or 200 mM NaCl, or without NaCl , or with 0, 1, or 2 μM ABA. The germination percentage was scored after 10 d. Seeds were considered germinated after the emergence of cotyledons.

Subcellular localization analysis of AmMYB1 in transgenic tobacco

The open reading frame (ORF) of *AmMYB1* was amplified using the *A. marina* cDNA clone as the template. Then the ORF was fused to the GFP in the pBS-SK GFP vector. From this plasmid, the *AmMYB1-GFP* fusion was restriction digested and cloned in the pCAMBIA binary vector at *Xba*I and *Bam*HI sites under the transcriptional control of the 2× 35S cauliflower mosaic virus promoter. Transgenic tobacco was obtained using the construct (*p35S: AmMYB1-GFP*) through *Agrobacterium*-mediated transformation. Leaf epidermal peels were prepared from *AmMYB1*:GFP positive T_0 tobacco plants and examined under a Confocal Laser Scanning Microscope (CLSM). Fluorescence was measured at 500–570 nm for GFP and 630–700 nm to detect chlorophyll autofluorescence. Leaf epidermal peels from untransformed tobacco plants were used for comparison.

Cloning of AmMYB1 *promoter and histochemical analysis for GUS expression in NaCl-treated seedlings*

The region upstream of the *AmMYB1* gene was amplified using primers listed in Supplementary Table S1 at *JXB* online and cloned upstream of the reporter gene (GUS) in pCAMBIA 1391Z vector at the *Hin*dIII/*Bam*HI sites. The construct was transformed into tobacco by *Agrobacterium*-mediated transformation as described by Horsch *et al*. (1985) and plants were selected using hygromycin as the resistance marker. Control plants were generated by transforming tobacco with pCAMBIA 1301 with 35S CaMV promoter driving GUS expression. Tobacco plants constitutively expressing GUS were treated as controls to verify NaCl induction of the *AmMYB1* promoter. Transgenic tobacco plants were identified by PCR and positive plants were selected to study the activity of the *AmMYB1* promoter. T_2 seeds were collected from

control and transgenic tobacco plants and allowed to germinate on two separate sterile filter papers soaked in half-strength MS medium containing hygromycin (25 mg l^{-1}) as the selection marker. Ten-day-old transgenic seedlings were then treated with NaCl (50 mM or 100 mM NaCl). Histochemical analysis for GUS expression was performed after 24 h of NaCl stress treatment as described by Jefferson *et al*. (1987).

Results

AmMYB1 *is a single-repeat MYB transcription factor in* A. marina

Sequence analysis showed that the *AmMYB1* cDNA (GenBank Accession number: EU091320) was 1046 bp in length, including a complete open reading frame of 783 bp with 5' and 3' UTRs of 86 bp and 173 bp, respectively. *AmMYB1* cDNA encodes 260 amino acids with a calculated mass of 28 kDa. The deduced amino acid sequence of the AmMYB1 protein revealed the presence of a single MYB repeat. AmMYB1 protein contained a putative nuclear localization signal and highly conserved tryptophan residues separated by 18–19 amino acids (Fig. 1A). The AmMYB1 amino acid sequence showed maximum identities of 54% and 46% to MYB proteins from *Glycine max* and *Bruguiera gymnorhiza* respectively. Phylogenetic tree construction using 14 other full-length MYB proteins resulted in AmMYB1 clustering with *Zea mays* MYB family proteins (Fig. 1B).

AmMYB1 *transcript level increases after NaCl and ABA treatments in* Avicennia marina *leaves*

Northern analysis revealed a higher accumulation of the *AmMYB1* transcript after 12, 24, and 48 h when the plants were grown in the stressful (500 mM) concentration of NaCl (Fig. 2A) in the medium, dropping thereafter from 24 h after recovery from the NaCl treatment. Interestingly, the *AmMYB1* transcript level remained high even after 10 d at the tolerable (250 mM) concentration of NaCl (Fig. 2A). In the case of abscisic acid-treated (100 μM) plants, *AmMYB1* transcript expression was enhanced at 12 h, dropping thereafter from 24 h and again increasing from 48 h for 10 d (Fig. 2A). *AmMYB1* mRNA accumulation was pronounced at 6, 12, and 24 h after exposure of plants to continuous light. However, *AmMYB1* was not detectable after 7 d of exposure to darkness (Fig. 2A). The level of *AmMYB1* mRNA was high in 14-d-old leaves, stems, and 30-d-old leaves. It was less detectable in 30-d-old stems and 14- and 30-d-old roots (Fig. 2B).

AmMYB1 *is a single copy gene in* A. marina *having stress responsive* cis*-acting elements*

The *AmMYB1* gene sequence revealed the presence of two introns and three exons. The size of the introns varied between 102–396 bp while the size of the exons varied between 155–384 bp. For all the exons, the intron–exon splice junctions conformed to the consensus sequence GT at the donor site and AG at the acceptor site (see Supplementary Fig. S1 at *JXB* online). The sequence upstream of the *AmMYB1* gene revealed potential binding sites for several stress-associated transcription factors such as *AtERD1, AtMYB1, AtMYC,* and *WRKY7*. Southern blot analysis of *A. marina* genomic DNA after digestion with restriction enzymes revealed a single band for all the three enzymes tested, suggesting the single copy nature of the gene in *A. marina* (see Supplementary Fig. S2 at *JXB* online).

AmMYB1 protein binds to AtRD22 cis*-acting elements in vitro*

To assess whether AmMYB1 is a transcriptional regulator of stress-responsive genes, the possibility of its binding *in vitro* to the TGGTTAG motif in the promoter region of the *AtRD22* gene (Abe *et al*.,1997, 2003) was tested by the electrophoretic mobility shift assay (EMSA). The binding efficiency of the purified AmMYB1 protein (Fig. 3A) to *AtRD22* only decreased on the addition of an excess of an unlabelled specific *AtRD22* competitor and not to the addition of a non-specific competitor (Fig. 3B).

AmMYB1 protein has transactivation activity

The transactivation ability of AmMYB1 was analysed in a yeast assay system. GAL4 DNA binding domain(BD)-AmMYB1 fusion plasmids were transformed into yeast cells (AH109) and assayed for their ability to activate transcription of the marker *HIS3* gene controlled by the GAL4 upstream activation sequence and their ability to promote yeast growth in the absence of histidine. Yeast transformants expressing full AmMYB1 protein could survive on histidine-lacking medium whereas the region containing only the N-terminal sequence (containing 160 amino acids) and the empty vector could not. β-galactosidase activity was also examined for the marker *lacZ* gene expression. The LacZ activity of each yeast transformant was similar to the *HIS3* activity (Fig. 4). These results indicate that *AmMYB1*, as reported in the case of several other transcription factors, exhibits transactivation activity and its activation domain is located in the C-terminal region rich in both proline and acidic amino acids (Asp and Glu).

Expression of AmMYB1 in transgenic tobacco

Three transgenic lines with a single copy insertion (L1, L4, and L26) were identified (see Supplementary Fig. S3A at *JXB* online) and used for protein expression study. Western blot analysis of transgenic plants indicated that the presence of a band with a molecular mass of about 28 kDa, corresponded with the predicted size of the AmMYB1 protein and no equivalent band was observed in control plants (see Supplementary Fig. S3B at *JXB* online).

AmMYB1 expression confers better salt tolerance in transgenic tobacco plants

In the presence of salt stress, chlorophyll loss was significantly reduced in *AmMYB1* transgenic lines L1 and L4 compared with the control and line 26 (Fig. 5A). Chlorophyll content of the leaf discs was also determined to assess the extent of salt stress-induced cellular damage. The chlorophyll content in transgenic lines L1 and L4 was significantly higher than the control and line 26 leaf discs at 200 mM NaCl (Fig. 5B). Line 4 (L4) had a higher

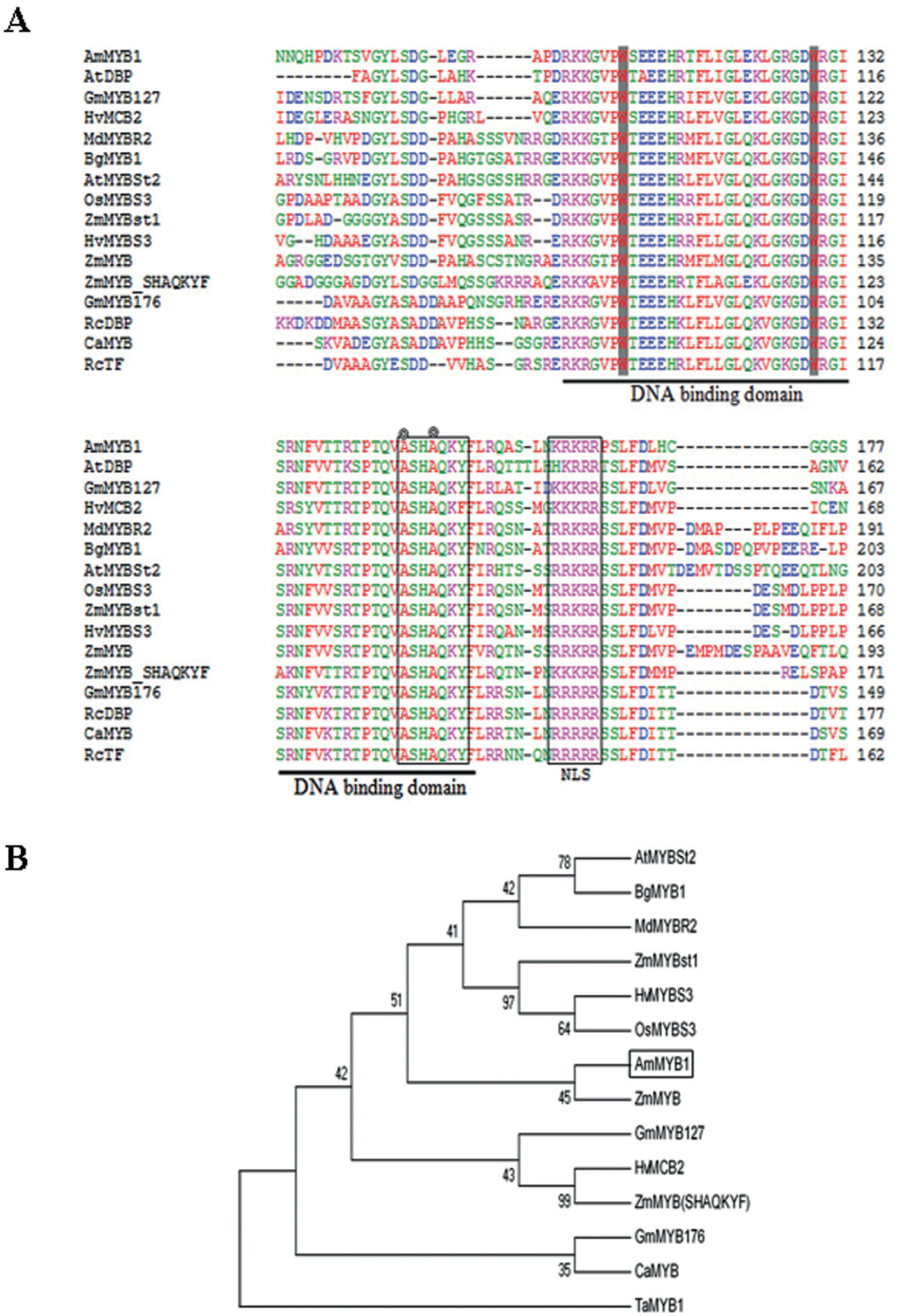

Fig. 1. (A) Clustal W alignment to compare the amino acid sequence of AmMYB1 with other plant MYB proteins. Highly conserved tryptophan residues are shaded grey. The dark line indicates the DNA binding domain. The NLS (Putative Nuclear Localization signal) and the SHAQKY motif are indicated by a box. Two alanine (A) residues that can potentially occupy a third tryptophan (W) residue are indicated by asterisks (*)**.** (B) Neighbour–Joining tree of predicted MYB1 proteins. Bootstrap values (1000 replicates) are indicated in the branches. GenBank accession numbers for MYB proteins are given in the Supplementary material at *JXB* online. (This figure is available in colour at *JXB* online.)

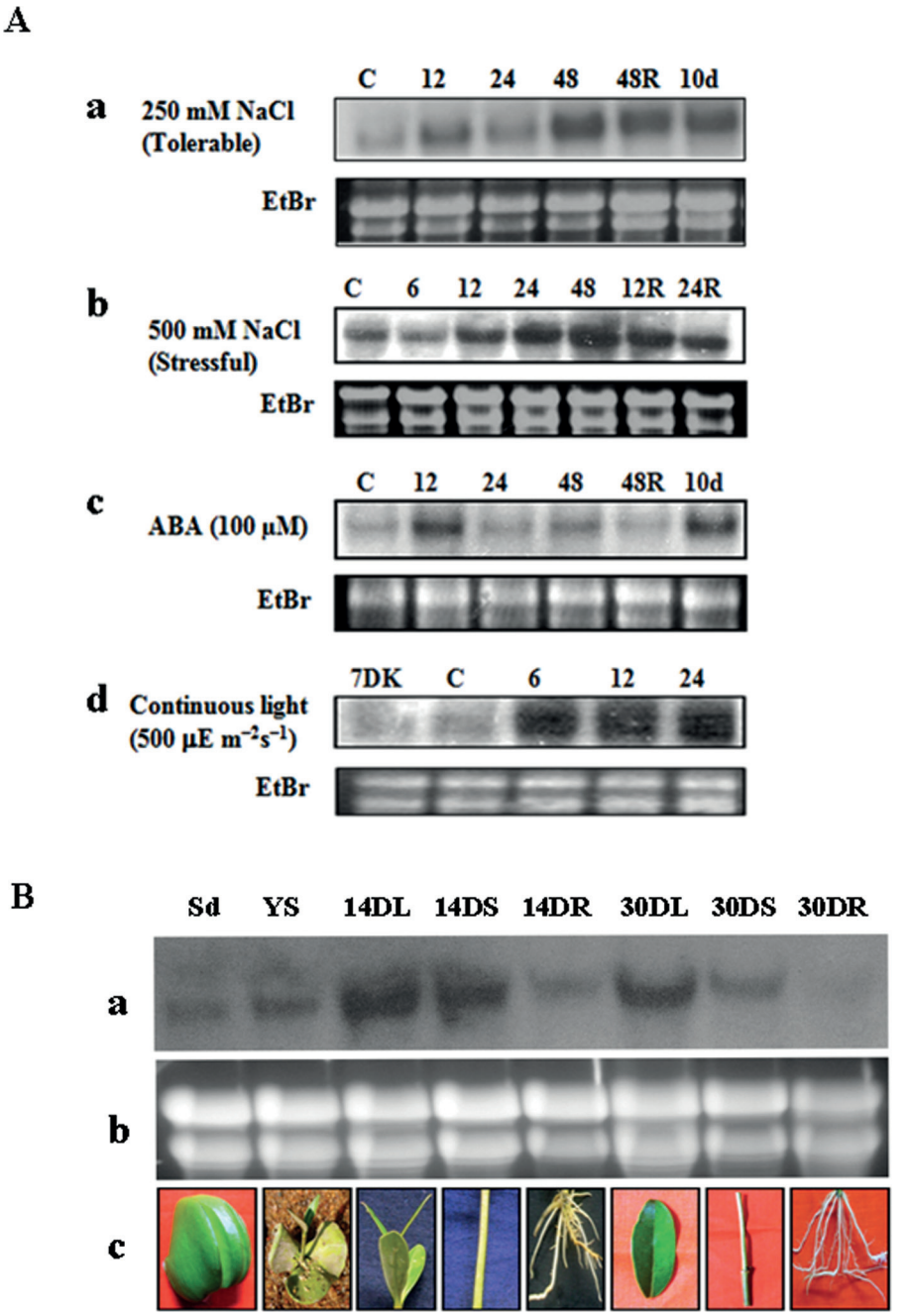

Fig. 2. (A) Northern analysis for *AmMYB1* expression in *A. marina* under various treatments. (a) RNA isolated from *A. marina* seedlings grown at a tolerable concentration (250 mM) of NaCl. (b) RNA isolated from *A. marina* seedlings grown at a stressful concentration (500 mM) of NaCl. (c) RNA isolated from *A. marina* seedlings treated with 100 µM ABA. (d) RNA isolated from *A. marina* seedlings treated with high light. 7Dk (7 d dark) and 6, 12, 24 h of continuous light. Lanes: 0, 6, 12, 24, 48 (h after NaCl, ABA, and light treatment) and 10D (days after NaCl and ABA treatment); 12R, 24R, and 48R (hours after recovery from NaCl or ABA treatment); EtBr indicates ethidium bromide-stained RNA. (B) *AmMYB1* mRNA accumulation during different development stages of *A. marina*. (a) RNA blot analysis of *AmMYB1.* (b) Ethidium bromide-stained gel. (c) Tissues of *A. marina* at different developmental stages. Lanes: Sd (seed), YS (young seedling), 14DL and 30DL (14- and 30-d-old leaves), 14DS and 30DS (14- and 30-d-old stems), 14DR and 30DR (14- and 30-d-old roots). (This figure is available in colour at *JXB* online.)

chlorophyll content in all the salt treatments compared with the control and the other two transgenic lines (L1 and L26).

To examine further the effect of constitutive *AmMYB1* expression on conferring salt tolerance to tobacco at the whole plant level, *AmMYB1* transgenic and control plants were kept in half-strength MS nutrient medium supplemented with different concentrations of NaCl. Symptoms of salt stress such as wilting and leaf rolling were only observed in control plants after 2 d in the 100 mM and 200 mM NaCl treatments (Fig. 6). Even though the leaves of control plants did not droop at 50 mM NaCl in the growth medium, only *AmMYB1* tobacco plants had healthy leaves. Amongst the transgenic lines, L1 and L4 showed better tolerance to salt stress

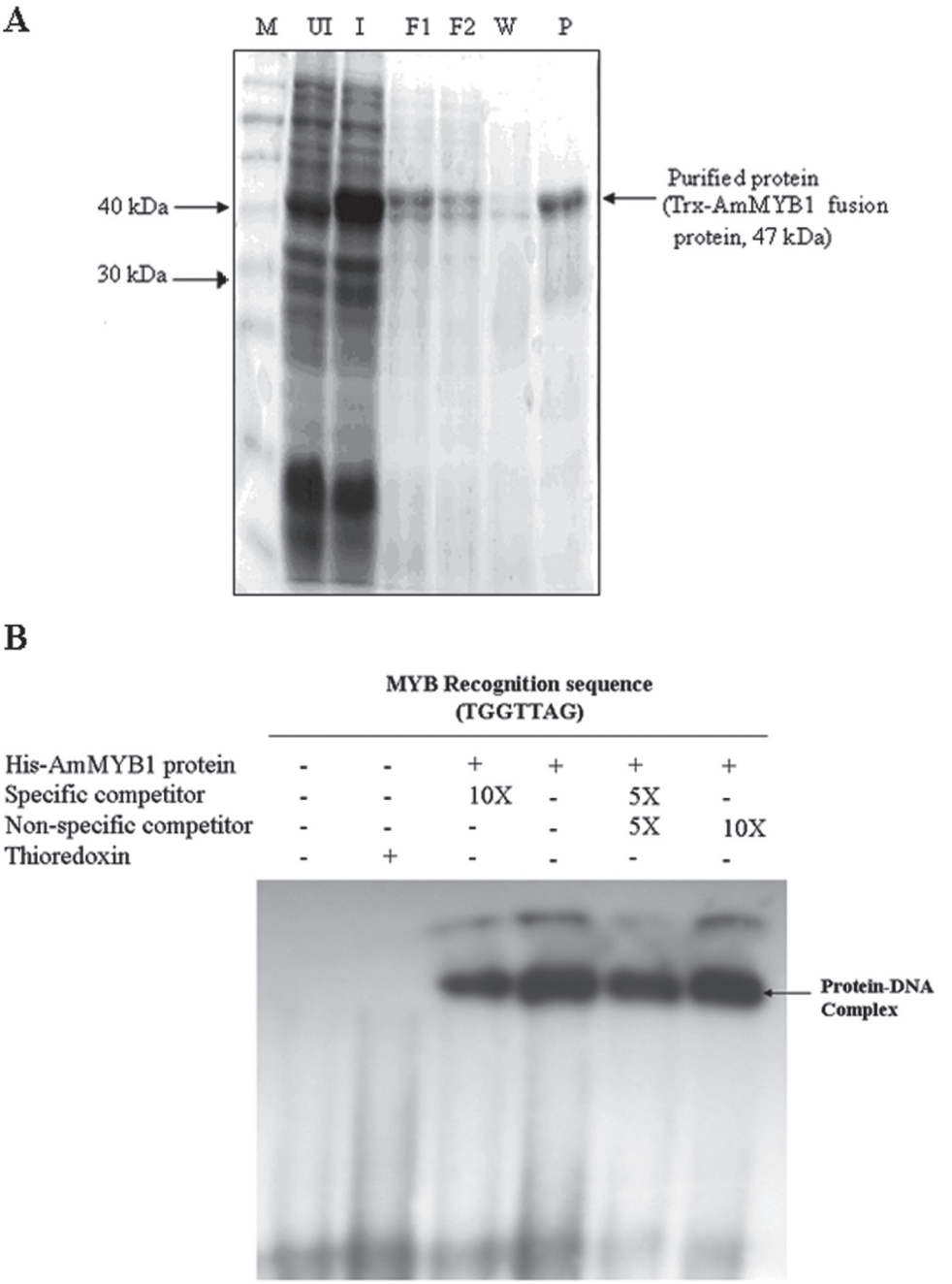

Fig. 3. Electrophoretic gel mobility shift assay. (A) Recombinant AmMYB1 protein purified from *E. coli.* (B) *In vitro* DNA binding assay. Unlabelled *AtRD22* ds DNA (30 mer) was used as the specific competitor and salmon sperm DNA was used as the non-specific competitor.

up to 100 mM NaCl compared with L26 at the whole plant level (Fig. 6).

To compare the relative stress tolerance between control and *AmMYB1* transgenic tobacco plants, germination rate and seedling development were assessed using T_2 transgenic seeds. Germination efficiency was calculated on the tenth day after planting seeds. Under normal conditions (–NaCl), both control and T_2 transgenic seeds germinated at a similar rate, and no phenotypic variations were observed. At 100 mM NaCl treatment, the percentage germination of the *AmMYB1* transgenic lines was higher than that of the control seeds (Fig. 7A; see Supplementary Fig. S4 at *JXB* online). Control seeds showed poor germination efficiency at 100 mM and 200 mM NaCl treatment. Seeds from the transgenic line L4 germinated well in both the concentrations of NaCl and was better than lines L1 and L26. In the presence of ABA, germination of control seeds was severely inhibited, while transgenic seeds were able to germinate comparatively better. For example, at 2 μM ABA treatment, approximately 57% of *AmMYB1* over-expressed seeds germinated compared with 19% of seeds from control plants (Fig. 7B; see Supplementary Fig. S5 at *JXB* online).

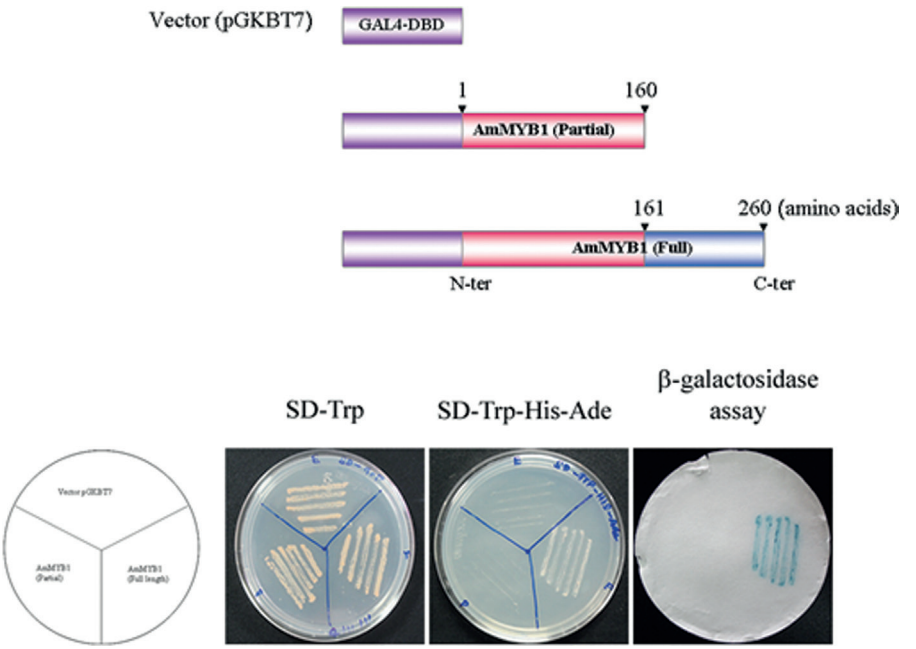

Fig. 4. Transactivation assay of AmMYB1 protein: Full and partial constructs of *AmMYB1* were fused with the GAL4 DNA-binding domains and expressed in the yeast strain AH109. The transformed yeasts were grown in a medium with (+HIS) or without (–HIS) histidine. LacZ activity was assessed by the β-galactosidase filter lift assay (GAL4-DBD: GAL4 DNA-binding domain). (This figure is available in colour at *JXB* online.)

AmMYB1 protein is localized in the nucleus

Sequence analysis of AmMYB1 amino acid showed the presence of a putative nuclear localization signal (from position 161 to 165), implying that the AmMYB1 protein may be localized in the nucleus. In order to verify this, the subcellular localization of AmMYB1 was examined by monitoring the GFP fluorescence in the AmMYB1-GFP transgenic tobacco leaf epidermal cells. Confocal imaging of the epidermal leaf peels revealed a nuclear localization of the AmMYB1 protein in *AmMYB1:GFP*-transformed transgenic tobacco (Fig. 8). No fluorescence was observed in the nucleus of cells from untransformed plants.

AmMYB1 *promoter is responsive to NaCl treatment*

Transgenic (T_2) tobacco seedlings expressing GUS under the control of the *AmMYB1* promoter were subjected to NaCl treatment to study the effect of NaCl on promoter activity. Results show that the leaf tissue was more responsive to NaCl treatment than shoot and root tissue (Fig. 9C, D). A basal level of GUS expression was also observed in unstressed transgenic seedlings (Fig. 9B). Transgenic tobacco seedlings expressing GUS under the control of a CaMV promoter showed activity in all the tissues (Fig. 9A).

Discussion

Transcription factors play important roles in the regulation of gene expression when plants encounter abiotic stress. MYB transcription factors are a large family of proteins, with each one regulating the expression of a cascade of genes specific to certain developmental processes or environmental cues. *A. marina MYB1* sequence information (Fig. 1A; see Supplementary Fig. S1 at *JXB* online) reveals the absence of a typical plant MYB domain similar to known plant homologues with two or three MYB repeats in the N-terminal region. The *AmMYB1* gene encodes a single MYB domain repeat consisting of 59 amino acids. Highly conserved amino acid residues present within the AmMYB1 single repeat is in agreement with similar MYB proteins reported from other plants (Baranowskij *et al*., 1994; Kirik *et al*., 1996; Mercy *et al*., 2003), and suggest their importance in maintaining the MYB repeat structure. Other interesting features associated with the AmMYB1 protein include the presence of acidic amino acid residues and a proline-rich domain located at the C-terminal region that are essential for transcriptional activation, as demonstrated by the ability of the AmMYB1 protein to activate transcription in yeast cells. The subcelluar localization study revealed that AmMYB1 is a nuclear localized protein with its proposed function as a transcription factor (Fig. 8).

In plants such as *Arabidopsis*, maize, and petunia, MYB proteins containing a single repeat have been reported to function in various plant-specific processes, but little is known about their function in stress tolerance (Wang *et al*., 2008; Zhu *et al*., 2009). A majority of transcription factors are induced during the early phase of response to salt, drought, and cold stresses and attain maximal induction after several hours (Thomashow, 2001). There was a need to investigate the stress- responsive nature of *AmMYB1* in *A. marina* both after short and prolonged exposure to NaCl. An abundance of *AmMYB1* mRNA after 48 h (Fig 2A), both in stressful and tolerable concentrations of salt for *A. marina* seedlings (Ganesan *et al*., 2008) suggests its early salt-responsive nature. Sustained accumulation of *AmMYB1*

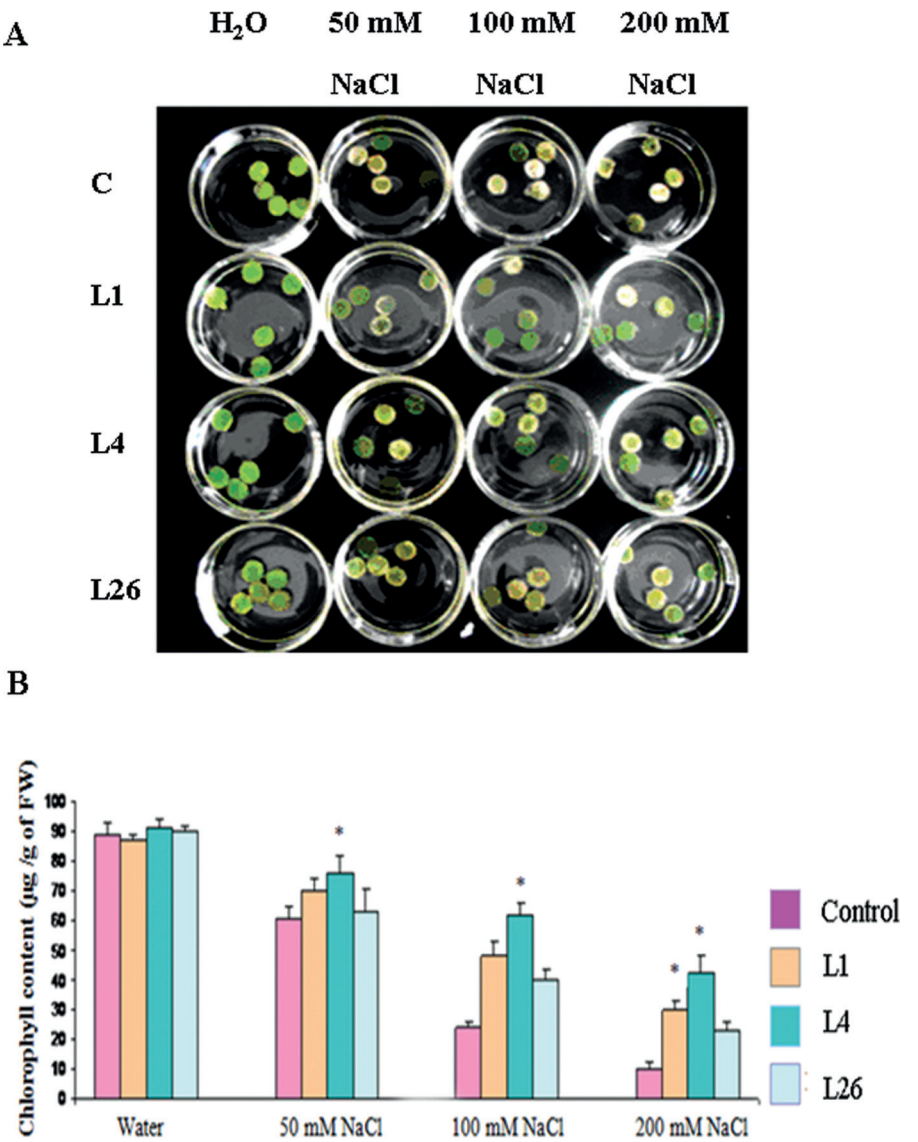

Fig. 5. (A) Leaf disc assay for chlorophyll bleaching. Leaf discs of control and AmMYB1 over-expressing transgenic tobacco lines, after incubation in water, 50 mM, 100 mM, and 200 mM NaCl solutions for 72 h under continuous white light at 25±2 °C. Results are representative of three independent experiments. (B) Chlorophyll content determined by the leaf disc senescence assay for salinity tolerance of control and transgenic tobacco lines over-expressing AmMYB1. Data represent the mean ±SD of three biological repeats. Asterisks indicate a *P* value <0.05 from Student's *t* test. (This figure is available in colour at *JXB* online.)

transcript for prolonged durations up to 10 d (Fig. 2A) indicates the possibility of its role in providing tolerance to NaCl stress in *A. marina*. Transcript expression for *AmMYB1* is similar to the observation made for MYBS3 (Su *et al*., 2010), a single repeat MYB protein identified from rice (Lu *et al*., 2002).

Enhanced salt tolerance accompanies the production of a high level of ABA during stress conditions (Zhang *et al*., 2006). MYB transcription factors have been found to operate either through an ABA-dependent or ABA-independent pathway. For example, AtMYB2 and HOS10 are induced by ABA and also induced by salt and drought stress (Abe *et al*., 2003, Zhu *et al*., 2005), but OsMYB4 and AtMYB60 are not induced by ABA in spite of their high expression levels upon salt and desiccation stress (Pasquali *et al*., 2008; Park *et al*., 2008). Similar expression patterns for *AmMYB1* transcript under salt and ABA (Fig. 2A), and the presence of an ABA responsive element in the promoter of the *AmMYB1* gene suggest the possibility of an ABA-dependent response. Regulation of gene expression related to photosynthesis is also mediated by MYB proteins (Saibo *et al*., 2009) and environmental signals like light and salt stress can trigger such a response (Churin *et al*., 2003). A light-induced increase in *AmMYB1* expression under continuous light after dark adaptation and higher levels of expression in photosynthetic tissues together indicate its involvement in the regulation of photosynthesis-related genes (Fig. 2A, B).

Based on our observations of *AmMYB1* transcript expression in the presence of salt stress and ABA, a DNA binding assay was performed for the AmMYB1 protein. The *cis*-acting element (TGGTTAG) of *AtRD22* has been described as the motif recognized by AtMYB2. Since *AtRD22* was reported to be responsive

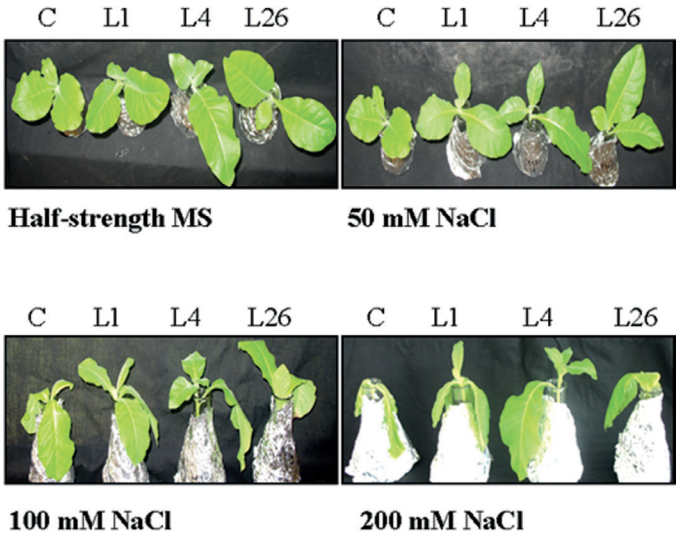

Fig. 6. Whole-plant salt tolerance assay. Effect of salt stress on tobacco plants from control (C) and transgenic lines (L1, L4, L26) was assessed by growing rooted plants hydroponically in half-strength MS medium in the presence of 50 mM, 100 mM, and 200 mM NaCl. Representative of three independent experiments are shown. (This figure is available in colour at *JXB* online.)

to ABA (Abe *et al*., 2003) like *AmMYB1*, the *cis*-acting element for *AtRD22* was chosen to study the DNA binding specificity of AmMYB1. The electrophoretic mobility shift assay indicated the formation of specific complexes between AmMYB1 protein and the *AtRD22 cis*-acting element. The ability of the Trx-AmMYB1 fusion protein to bind to the *AtRD22 cis*-acting element was significantly reduced by the addition of a 10-fold molar excess of unlabelled specific competitor DNA, while the addition of a 10-fold molar excess of non-specific competitor DNA had no discernible effect on the pattern typically observed (Fig. 3B), thereby indicating the possibility of AmMYB1 being a transcriptional regulator of stress-responsive genes.

The promoter region of *AmMYB1* was isolated to identify potential stress- and ABA responsive elements. In addition to the presence of stress-inducible *cis*-acting elements and ABRE, the presence of a MYB recognition sequence (MRS) that contained an *AtRD22*-like *cis*-acting element was interesting. It suggests the possibility of AmMYB1 regulating its own expression through binding to its *cis*-acting element during salt stress. Alternatively, a different MYB protein in the same plant may also bind to the MRS and regulate the expression of AmMYB1. Furthermore, the presence of a MYC binding site in the promoter region of *AmMYB1* is similar to that for *AtRD22*

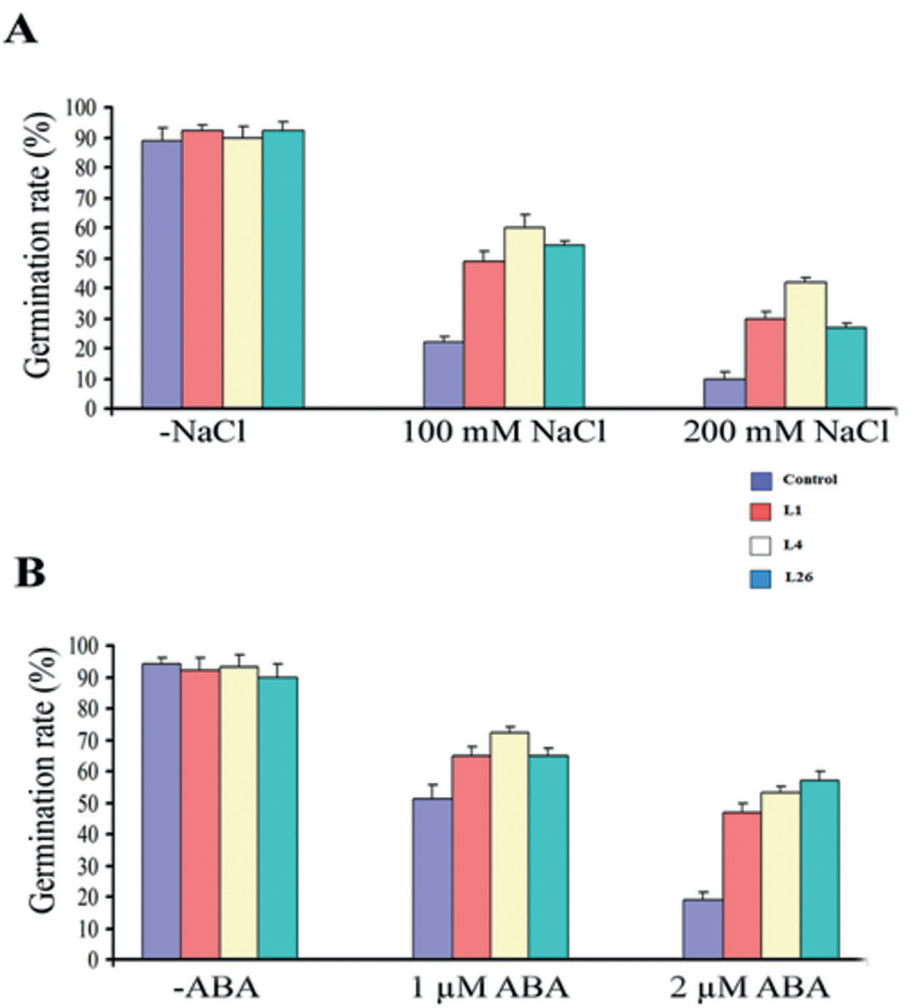

Fig. 7. Germination ability of *AmMYB1* transgenic and control seeds in half-strength MS medium containing (A) NaCl or (B) ABA. Data are presented as ±SD of three independent assays. (This figure is available in colour at *JXB* online.)

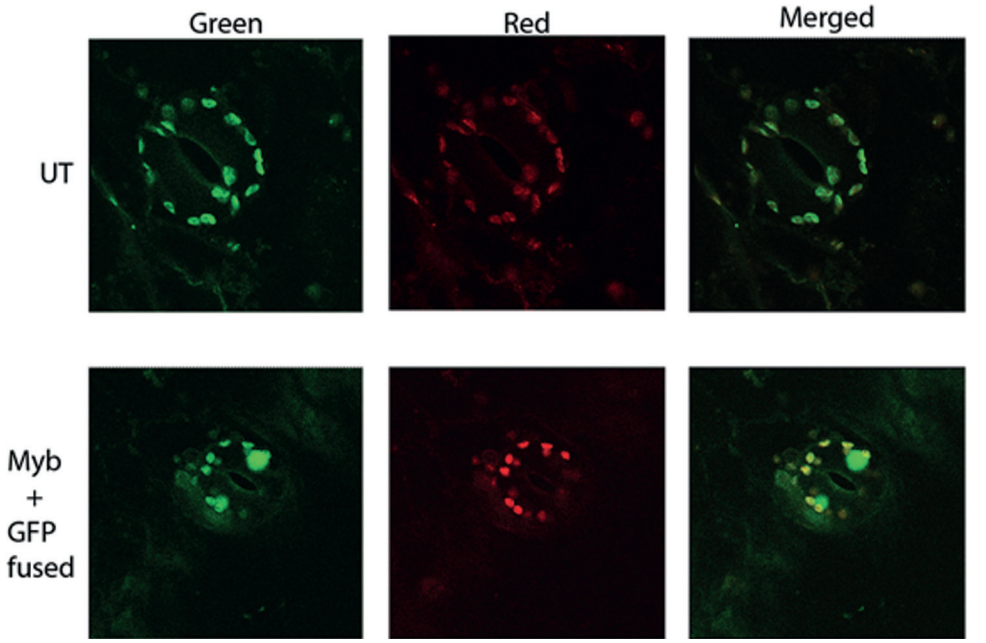

Fig. 8. Intracellular localization of AmMYB1:GFP fusion protein. Fluorescence microscopic images of guard cells from a tobacco plant transformed with 35S:AmMYB1:GFP fusion gene and an untransformed plant (UT). (This figure is available in colour at *JXB* online.)

where both MRS and MYC were reported to be responsible for triggering stress responses (Abe *et al*., 2003). The response of the *AmMYB1* promoter to NaCl treatment as verified through GUS expression, together with results from *AmMYB1* transcript expression indicate a role for AmMYB1 protein in the regulation of photosynthesis-related genes during salt stress (Figs 2B, 9).

Over expression of certain stress-induced genes can impart stress tolerance (Dai *et al*., 2007) and hence could be used to transform salt-sensitive crops. Over-expression of MYB transcription

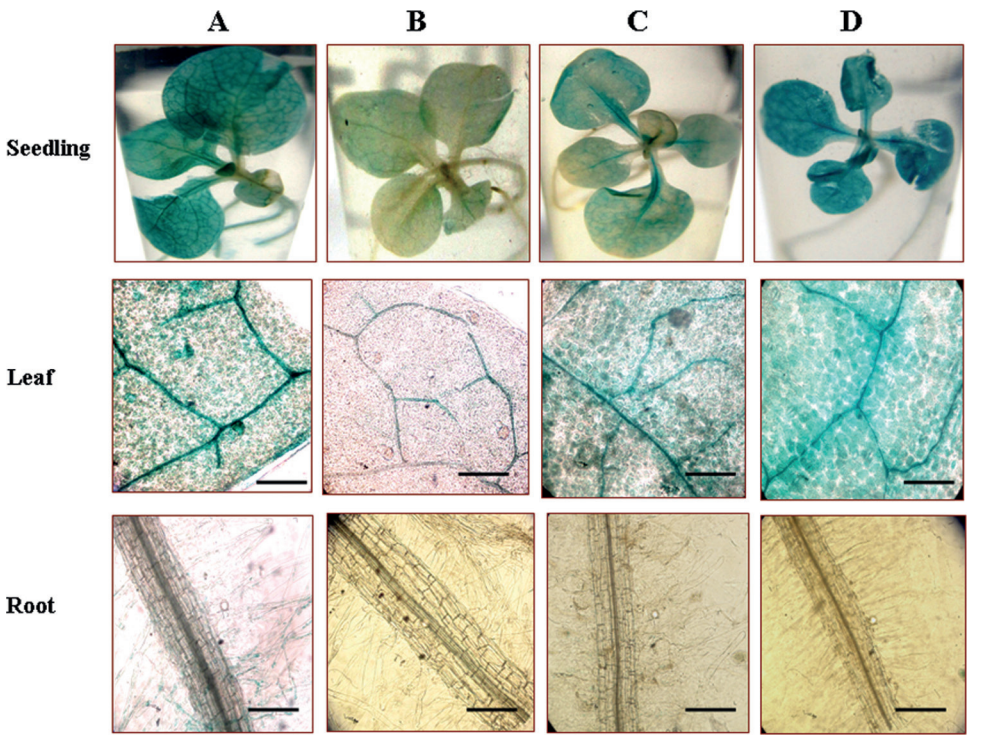

Fig. 9. Histochemical staining of GUS expression. Seedlings transformed with CaMV promoter:GUS were grown in half-strength MS medium (A). Seedlings transformed with *AmMYB1* promoter::GUS were grown on half-strength MS medium (B), treated with 50 mM NaCl (C), with 100 mM NaCl (D). Bars=2 mm. (This figure is available in colour at *JXB* online.)

factor genes has been shown to confer abiotic stress tolerance in transgenic plants (Vannini *et al*., 2004; Zhu *et al*., 2005; Jung *et al*., 2008*a*; Pasquali *et al*., 2008). It was investigated whether constitutively expressing AmMYB1 would confer salt tolerance in a heterologous system, by transforming tobacco and later assessing the relative tolerance using isolated leaf discs, whole plants and seeds. *AmMYB1* tobacco plants were able to tolerate salt stress as indicated by reduced chlorosis in leaf discs and significant reduction in symptoms of salt stress like wilting and yellowing in whole plants (Figs 5A, 6).

Common environmental stresses such as salt, drought, and osmotic stresses adversely affect seed germination and plant growth (Bailly, 2004). In nature, seeds encounter a combination of these environmental stresses, which affect their germination potential. T_2 transgenic tobacco seeds collected from line 4 showed a significant increase in germination potential (Fig. 7A; see Supplementary Fig. S4 at *JXB* online) which is similar to our observations in whole plant tolerance and the chlorophyll leaching assay in the presence of NaCl concentrations up to 200 mM in the medium. Reduced sensitivity of *AmMYB1* transgenic tobacco seeds to ABA during germination is similar to the observation made by Dai *et al.* (2007) in OsMYB3R-2, transgenic *Arabidposis*, suggesting that AmMYB1 can function as a regulator of ABA signalling (Fig. 7B; see Supplementary Fig. S5 at *JXB* online).

In conclusion, AmMYB1 is a salt-responsive single-repeat MYB protein that can potentially regulate the expression of other genes in *A. marina* during salt stress. In addition, the better salt tolerance of *AmMYB1* transgenic tobacco observed in the present study, suggests the possibility of transforming salt-sensitive crop plants with *AmMYB1* and testing for their tolerance.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1. Sequence of primers used in this study.

Supplementary Fig. S1. Promoter, gene, and deduced amino acid sequences of AmMYB1 from A. marina.

Supplementary Fig. S2. Southern analysis of AmMYB1 in the A. marina genome.

Supplementary Fig. S3. (A) Southern analysis of AmMYB1 integration in transgenic tobacco lines; (B) Western blot analysis of AmMYB1 transgenic tobacco lines.

Supplementary Fig. S4. Germination assay under NaCl treatment.

Supplementary Fig. S5. Germination assay under ABA treatment.

Supplementary material. Accession numbers for MYB proteins.

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