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Overexpression of the stress-associated protein (SAP) SAP_{MR219} in *Oryza sativa* (var. MR219) increases salt tolerance in transformed *Arabidopsis thaliana* plants

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Abstract

The stress-associated protein (SAP) family provides salinity stress tolerance in plants. We performed functional studies of *SAP* $_{MR219}$, which is a member of the SAP family that is induced after salinity stresses. Computational analysis of the *SAP* $_{MR219}$ cDNA clone that was isolated from rice root and shoot revealed significant homology with the SAP gene families from rice (89% to 36%) and *Arabidopsis* (44% to 32%). This clone has a 516-bp coding region encoding a 171 amino acid protein with a predicted molecular mass of 18.31 kDa. *In silico* analysis demonstrated that the *SAP* $_{MR219}$ gene product encoded a cytoplasmic zinc finger protein that might perform its functions *via* protein–protein interactions aided by its AN1 and A20 zinc finger domains. The *SAP* $_{MR219}$ gene was isolated, cloned and introduced into *Arabidopsis thaliana* under the control of the CaMV35S promoter. Five transgenic *Arabidopsis* lines were obtained by the floral-dip transformation method using *Agrobacterium tumefaciens* strain GV3101. The survivability of the transgenic lines under salinity stress was evaluated at 100, 150, 200 and 250 mM NaCl. At 250 mM NaCl, the germination rates of transgenic lines were approximately 50%, whereas the wild-type plants did not grow. Our results indicate that *SAP* $_{MR219}$ may play a significant role in the response to salt stress tolerance in plants.

Keywords: SAP $_{MR219}$, *Arabidopsis*, SAP, stress tolerance, transgenic plants.
Abbreviations: SAP, stress-associated protein; cDNA, DNA complementary to RNA; bp, base pair; CaMV35S, cauliflower mosaic virus.

Introduction

Salinity is one of the major environmental factors affecting plant growth and productivity and is generally defined as the presence of excessive amounts of soluble salt that hinders the normal functions that are required for plant growth and development. The presence of high levels of sodium chloride (NaCl) in soil, which may occur naturally or from the irrigation or the hydraulic lifting of salty underground water is the primary cause of salinity. This phenomenon significantly affects plant development and damages plant physiological functions (Martínez-Atienza et al., 2007). The current transgenic plant strategies offer new opportunities to improve tolerance to abiotic stresses. Current engineering approaches rely on the transfer of one or several genes that participate in regulatory pathways and signalling; encode enzymes that are present in pathways leading to the synthesis of functional and structural protectants, such as antioxidants and osmolytes; or encode stress-tolerance-conferring proteins (Kathuria et al., 2007). One way of achieving stress-tolerance is to overexpress transcription factor genes that control multiple genes from various pathways or to overexpress genes that are involved in abiotic signal perception and transduction (Kanneganti and Gupta, 2008). In recent years, the stress-associated protein (SAP) family has emerged as an important gene family that is involved in multiple abiotic stress responses in plants. In total, 18 and 14 indicated members of the SAP gene family have been identified in rice and *Arabidopsis*, respectively (Vij and Tyagi, 2006). SAP gene members have characteristic A20/AN1 zinc-finger domains and play an essential function in regulating the immune response (Huang et al., 2004). Plant SAPs contain A20/AN1, A20 or AN1, zinc-finger domains at the N- or C-termini. Some SAP members also contain extra Cys2-His2 RING motifs at the C-terminus (Jin et al., 2007). The overexpression of *ZFP177*, which is another rice zinc-finger A20/AN1 gene, in tobacco plants increased tolerance to both H_2O_2 and low- and high-temperature stresses but caused an oversensitivity to salt and dehydration stresses (Huang et al., 2008). Similarly, the overexpression of *AlSAP*, which is a stress-associated protein from the halophyte grass *Aeluropus littoralis*, in tobacco confers an increased tolerance to salt, drought, cold, and heat stresses (Ben Saad et al., 2010). *AtSAP12*, which is another member of the SAP family in *Arabidopsis*, undergoes a strong upregulation of its transcript levels after 6 h of salt treatment and cold (Ströher et al., 2009). The underlying biochemical and molecular mechanisms by which these SAP genes provide strong

tolerance to various abiotic stresses remain unknown. Recently, *AtSAP5*, which is a member of the *A. thaliana* SAP gene family, has been shown to act as an E3 ubiquitin ligase through its AN1 domain and to confer tolerance to dehydration stress (Kang et al., 2011). In addition to the abovementioned studies, little is known regarding members of the SAP family in plants. The overexpression of *AtSAP10*, which is a member of the *Arabidopsis* SAP family that contains an A20 and AN1 zinc finger domain at the N- and C-termini, confers strong tolerance to high-temperature stress and to several toxic metals. In the present study, we describe the isolation and functional characterisation of *SAP* $_{MR219}$, which is a member of SAP gene family, from the rice cultivar MR219. An *in silico* analysis of the *SAP* $_{MR219}$ protein indicated that this protein is likely to be localised in the cytoplasm and in chloroplasts. We also present direct evidence for the salt tolerance of the *SAP* $_{MR219}$ gene in *A. thaliana*. Our results indicate that *SAP* $_{MR219}$ may play an important role in the response of salinity stress to transgenic *Arabidopsis* plants

Results

Characterisation of SAP $_{MR219}$ ***using a bioinformatic approach***

The identity values of *SAP* $_{MR219}$ at the amino acid level ranged *SAP* $_{MR219}$ from 89% to 36% with *Oryza sativa* and 44% to 32% with that of *Arabidopsis* stress-associated proteins. Computational analysis of the cDNA clone that was isolated from the rice root and shoot library indicated that its 516-bp coding region encodes a 171 amino acid protein with a predicted molecular mass of 18.31 kDa. The maximum amino acid content was Ala (11.1%). Based on the theory of protein stabilisation, the unstable index of the stable protein is lower than 40 (Zhang et al., 2006). *SAP* $_{MR219}$ cDNA encodes a putative stable protein. Homology searches that were run with the full-length amino acid sequences of the cDNA clone revealed significant similarity to SAPs from rice and *Arabidopsis* (Xiao et al., 2011).

The AN1 domain of *SAP* $_{MR219}$ showed 88, 86, 86, 86 and 84% identity to *Saccharum officinarum* (SAP1), *Hordeum vulgare* (F2CT42), *Triticum aestivum* (SAP2), *Aegilops tauschii* (F775_32779) and *Zea mays* (ZEAMMB73_059434), respectively. In contrast, the A20 domain showed 100, 100, 97, 94, 91% identity to its homologues from *Zea mays* (ZEAMMB73_059434), *Saccharum officinarum* (SAP1), *Festuca arundinacea* (ZNF1), *Triticum aestivum* (SAP2) and *Gossypium arboretum* (D2I9L5), respectively. Hydropathy (Kyte and Doolittle, 1982) indicated that SAP $_{MR219}$ proteins are highly hydrophilic, with four clear hydrophilic stretches and a region of lower hydrophilicity between them. Based on the theory that when the amino acid score is lower, the hydrophilicity is stronger, we expect that when the amino acid score is higher, the hydrophobicity is stronger (Xiao et al., 2011). The prediction results of the secondary structure of the SAP $_{MR219}$ protein by PSIPRED software showed that the secondary structure consisted of 1 helix and 1 strand (Fig. 1). A model of *SAP* $_{MR219}$ that was predicted using PHYRE2 software is depicted in Fig. 2 (Kelley and Sternberg, 2009). Eighty-four percent of the residues were modelled at >90% confidence. The two conserved domains (the N-terminal domain (in blue) and the C-terminal domain (in red)) were clearly separated according to the predicted protein model. PHYRE2 software uses the alignment of hidden Markov models and a folding simulation system called Poing to model regions of a protein of interest to a three-dimensional structure. The prediction of the subcellular localisation of *SAP* $_{MR219}$ with PSORT II software showed that this protein is likely to be localised in the cytoplasm and in chloroplasts. The deduced amino acid sequence of *SAP* $_{MR219}$ showed no signal peptide, as predicted in SignalP 4.1 software. SignalP 4.1 software was used to predict the presence and the location of signal peptide cleavage sites in amino acid sequences (Petersen et al., 2011). The rice *SAP* $_{MR219}$ protein showed no transmembrane domain using the TMHMM Server version 2.0. The plot was obtained by calculating the total probability that a residue sits in a helix, inside, or outside summed over all of the possible paths through the model.

Selection and analysis of transformants

The transgenic seedlings produced well-established roots and green leaves within the selection medium at approximately one month after planting (Fig. 3). The transformants were grown to maturity by transplantation (preferably after the development of 5 to 7 adult leaves) into heavily moistened potting soil.

Characterisation of transgenic plants

Only 5 lines of the T_1 plant survived and produced 9 T_2 seeds (Fig. 4). The genomic DNA that was isolated from the transgenic *Arabidopsis* lines was used as a template to amplify the target gene by PCR to confirm that the putative transgenic *Arabidopsis* plants were indeed transformed with the rice *SAP* $_{MR219}$ gene. The leaf tissues were sampled from all five transformed *Arabidopsis* lines along with wild-type *Arabidopsis* plants (as controls) to isolate the genomic DNA from plant tissues. Then, a PCR analysis was performed to detect the presence of transgenes using two sets of primers that amplify either *SAP* $_{MR219}$ cDNA or the CaMV35S promoter region. The PCR amplification of the putative overexpression transgenic plants using the *SAP* $_{MR219}$ ov-F and *SAP* $_{MR219}$ ov-R primers resulted in a 516-bp fragment. This experiment, which used *SAP* $_{MR219}$-specific primers, was conducted to ensure that the isolated genomic DNA was of good quality and could be used in the next step. Thus, all of the samples that were tested gave positive results as expected. Another primer from the promoter region was used to detect the transgenic plants. As shown in Fig. 5, a 700-bp fragment was detected in transgenic *Arabidopsis* plants using the forward primer 35S and the reverse primer *SAP* $_{MR219}$ ov-R. No band was detected from wild-type plants.

Analysis of the T_2 ***generation of transgenic Arabidopsis plants***

In this study, the inheritance of *hpt*II was followed by the segregation of resistant or sensitive T_2 *Arabidopsis* seedlings on MS media containing 25 mg ml^{-1} hygromycin. Of the tested transgenic plants, the progeny populations from 3 lines (5, 4, and 1) showed conventional Mendelian inheritance (3:1), suggesting that these lines have only one copy of the transgene in their genome (Christou and klee, 2004). However, other lines showed various inheritance behaviours (Table 1). The leaves were removed from selected T_2 plants and from the control (wild-type) plants for genomic DNA extraction to confirm the presence of transgenes. The transgenes were amplified by PCR using promoter primers. All of the samples run on an agarose gel showed an amplified 700-bp fragment that represents a fragment of *SAP* $_{MR219}$ and

Table 1. Progeny segregation of survived lines. The inheritance of the transgene was tested by estimating the percentage of the seedlings that grew on hygromycin.

Line	Total seedlings tested	Resistance to Hygromycin	Sensitive to Hygromycin	Ratio
1	140	96	32	3:1
4	160	124	36	3:1
5	180	132	48	3:1
2	80	44	36	1:1
3	180	152	28	5:1

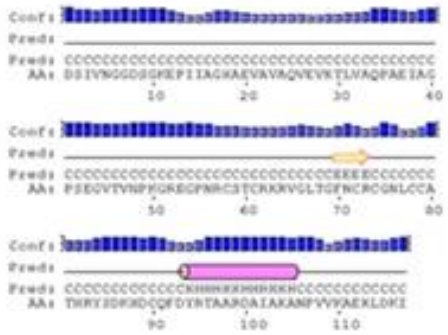


Fig 1. Prediction of secondary structure for SAP $_{MR218}$ protein. The PSIPHRED protein sequence analysis workbench was used (Jones, 1999). :helix; :coil : :strand; :confidence of prediction; Pred; Predicted secondary structure; AA; target sequence.

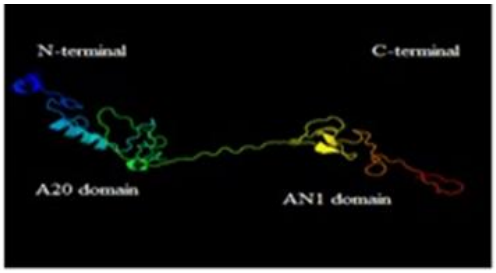


Fig 2. Phyre2 server predicted tertiary structure of SAP $_{MR219}$, with 99.9% confidence.The Protein folds ar shown in the colors of the rainbow from the N-terminus (blue) to the C-terminus (red).

a portion of the promoter. No amplified DNA fragment was found in the samples from wild-type plants (Fig. 6).

Expression of the SAP$_{MR219}$ transgene in transgenic Arabidopsis plants
RT-PCR

A RT-PCR reaction was performed (15-d-old lines 1, 2 and 5) with the wild-type plants (Fig. 7) using *SAP $_{MR219}$*-specific primers. The *Actin* gene was used as the internal control in this experiment. These results confirmed that *SAP $_{MR219}$* had been integrated into the *Arabidopsis* genome and transcribed in transgenic plants (Fig. 8).

SAP $_{MR219}$ overexpression improves the seed germination of Arabidopsis under salt stress

Several important points can be noted. No significant difference in germination was observed among the genotypes under control conditions (Fig. 9A). At 100 mM NaCl, no significant difference in germination was observed among the transgenic lines; however, the germination of the wild-type plants decreased significantly compared to control conditions (Fig. 9B). The germination of the wild-type plants decreased progressively with increasing NaCl concentrations from 100 mM to 200 mM NaCl (Fig. 9C-9D), and the complete inhibition of seed germination occurred at 250 mM NaCl (Fig. 9E). The germination of the transgenic lines decreased to 70% at 200 mM NaCl and to 40% at 250 mM NaCl. The percentage germination of the transgenic lines was significantly ($P<0.05$) higher compared to that of the control (wild-type) plants under 100, 150 and 200 mM NaCl during 7 d of germination. Based on seed germination, the transgenic plants carrying the transgene were tolerant to salt.

Discussion

In the present study, we reported the characterisation of SAP $_{MR219}$. This gene belongs to the SAP gene family. SAP $_{MR219}$ contains both A20 and AN1 domains at the N-terminal and C-terminal regions of the protein, respectively. The exact role of SAP $_{MR219}$ in stress tolerance is unclear. Members of the SAP family in rice could be categorised as regulatory proteins that are induced during the plant stress response because these proteins have a zinc-finger-containing domain. Generally, many of the zinc finger domain proteins act as

Fig 3. Putative transgenic *Arabidopsis* plants (ecotype Columbia-0) with pCMB-MS. Constructs identified as hygromycin-resistant seedlings with well established roots and green leaves within the hygromycin MS selection medium.

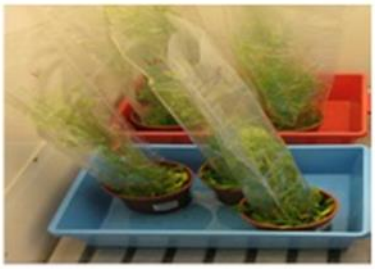


Fig 4. Putative mature transgenic *Arabidopsis* plants. Five transgenic *Arabidopsis* plants survived to produce T2 seeds.

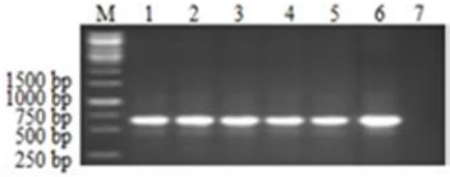


Fig 5. Agarose gel electrophoresis of PCR analysis of putative transgenic *Arabidopsis* plants using promoter primer.~ 700bp fragment was detected using 35S-F and SAP_{MR219}OV-R primers. Lane M, 1kb marker; Lane 1-5 putative transgenic plants; Lane 6, positive control (plasmid pCMB-SAP MR_{219}); Lane 8, negative control (wild-type plants).

transacting factors. Previous studies have demonstrated that SAPs can regulate the expression of downstream genes, particularly those genes that are involved in stress acclimation. However, whether the observed transcriptome changes are the result of the activation of some transcription factors by SAPs or whether SAPs bind to DNA and regulate transcription directly remains unknown. Recently, a cotton SAP (GaZnF) was reported to bind to an MYB-box element that is present in the *GUSP1* (cotton stress-responsive gene) promoter (Zahur et al., 2012), indicating a role as a transcriptional regulator of a SAP. However, OsiSAP1 may not act as transcriptional factor because this protein lacks a nuclear localisation signal and a DNA binding domain. OsiSAP1 was also hypothesised to perform its function *via* protein-protein interactions (Mukhopadhyay et al., 2004). An *in silico* analysis of the SAP $_{MR219}$ gene product did not predict any DNA binding domain or potential nuclear localisation signal. Hydropathy and pSORT analyses indicated that this protein is a hydrophilic soluble cytoplasmic and chloroplastic protein. The SAP $_{MR219}$ gene product encoding a zinc finger protein might perform its functions *via* protein–protein interactions aided by its AN1 and A20 zinc finger domains.

Materials and Methods

Bioinformatics analyses

A comparative sequence analysis of *SAP* $_{MR219}$ was performed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) against the protein database (http://blast.ncbi.nlm.nih.gov/). The physico-chemical properties of the SAP $_{MR219}$ protein were analysed online using the tool ProtParam (http://web.expasy.org/protparam/) in the toolkit of the ExPASy server. Protein domain prediction was performed using the tool InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/). The hydrophilicity and hydrophobicity of the *SAP* $_{MR219}$ protein were predicted using the tool ProtScale (http://web.expasy.org/protscale/).

The subcellular localisation of this protein was investigated using the PSORT II prediction program (http://psort.hgc.jp/form2-html). The putative domains were identified using the InterProScan search (http://www.ebi.ac.uk/interproscan/) and the Conserved Domain Database (CDD) in NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The

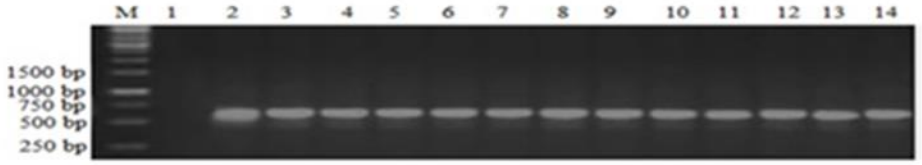


Fig 6. Agarose gel electrophoresis of PCR products of transgenic *Arabidopsis* plants. Amplification of genomic DNA from overexpression transgenic Arabidopsis plants using promoter primer.~ 700bp fragment was detected using 35S-F and *SAP* $_{MR219}$-R primers in transgenic plants. Lane M, 1kb marker; Lane 3-14 putative transgenic plants; Lane 2, positive control (plasmid pCMB-SAP MR_{219}); Lane 1, negative control (wild-type plants).

Fig 7. Salt stress sensitivity of wild-type and transgenic *Arabidopsis* seedlings. Photographs was taken after two weeks of growth on media containing 150 mM NaCl.

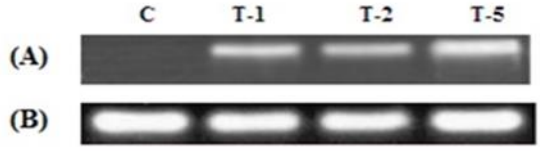


Fig 8. (A) Overexpression of *SAP* $_{MR219}$ in transgenic plants was confirmed by RT-PCR. Lanes T-1, T-2, T-5 transgenic plants; Lane C wild-type plant. (B) Actin RNA as an internal control.

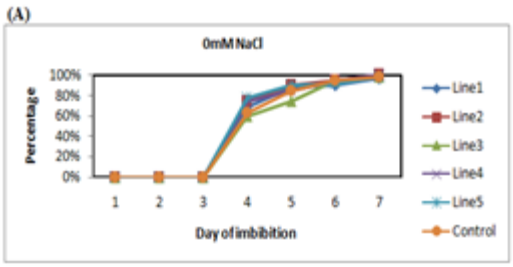
PSIPHRED protein sequence analysis workbench was used to determine the secondary structure of the SAP $_{MR219}$ protein, and the degree of amino acid sequence identity was determined using Washington University Basic Local Alignment Search Tool (Wu-Blast) from the European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/blast2). A multiple sequence alignment was performed using ClustalX software (www.clustal.org/). The membrane topology of the SAP $_{MR219}$ protein was investigated using TMHMM V2.0 software (http://cbs.dtu.dk/services/TMHMM). PHYRE2 software (www.sbg.bio.ic.ac.uk/phyre2/) was used to predict the 3D structure of this protein. The presence of a signal peptide was predicted using SignalP 4.1 software (http://ww.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011).

Plant materials and treatments

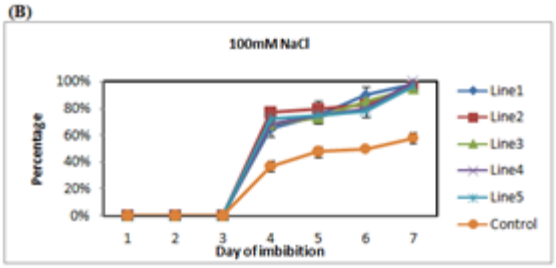
Rice seeds (var. MR219) were washed with distilled water. The seeds were surface-sterilised by the following treatments: 70% ethanol for 45 s and 80% Clorox for 20 min. The seeds were subsequently rinsed completely with distilled water 3 times and sown on soil. Two-week-old rice seedlings were transferred to 150 Mm NaCl for 2 h.

Construction of pCMB-MS

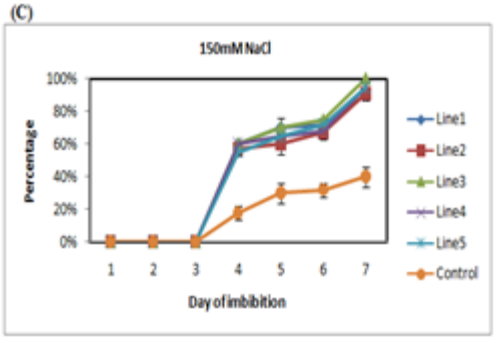
RT-PCR was performed using total RNA that was obtained from two-week-old rice seedlings to amplify the full-length *SAP* $_{MR219}$ cDNA. Total RNA was isolated using a TRI reagent kit (Sigma-Aldrich, Bangalore, India) according to the manufacturer's protocol. The reactions were performed using the following PCR primers: *SAP* $_{MR219}$-F:5'-CCCCCCAGATCTTATGGAGCACAAGGAGACT-3', carrying the *BstEII* site (underline); *SAP* $_{MR219}$-R: 5'-CCCCCCGGTNACCCTAAATTTTGTCAAGTTTCTC-3', carrying the *BglII* site (underline). Then, the obtained DNA fragment was digested by the restriction enzymes *BstEII* and *BglII*, and the released fragment was ligated into a transformation vector. The transformation vector pCAMBIA1301 was used for the overexpression vector construction. The *gus* gene was cut and replaced with *SAP* $_{MR219}$ cDNA. The double digestion of the pCAMBIA1301 vector was performed using the restriction enzymes *BstEII* and *BglII*. The digested product was used for ligation into the pCAMBIA1301 transformation vector. The *E. coli* strain TOP10 was used as the competent cell, with kanamycin (100 μg ml^{-1}) as the selective antibiotic. The presence of *SAP* $_{MR219}$



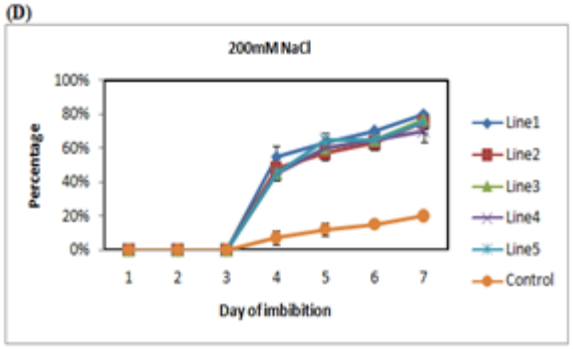
Germination of transgenic lines and wild-type plants during 7 d on MS medium with 0 mM NaCl



Germination of transgenic lines and wild-type plants during 7 d on MS medium with 0 mM NaCl



Germination of transgenic lines and wild-type plants during 7 d on MS medium with 150 mM NaCl



Germination of transgenic lines and wild-type plants during 7 d on MS medium with 200 mM NaCl

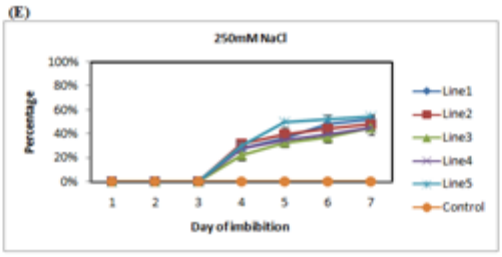


Fig 9 Germination of transgenic lines and wild-type plants during 7 d on MS medium with 250 mM NaCl

cDNA in the recombinant plasmid was confirmed by sequencing double digestion using an appropriate restrictionenzyme (underlined in a primer sequence) and PCR using the *SAP* $_{MR219}$ primers.

Sterilisation and treatment of Arabidopsis seeds

Seeds of *A. thaliana* (ecotype Col-0), which were provided by MPOB (Malaysian Palm Oil Board), were washed with distilled water. The seeds were surface-sterilised by the following treatments: The seeds were incubated in 70% ethanol for 2 min by inverting tubes several times to keep the seeds in solution. Then, the ethanol was removed before adding a solution of 50% Clorox containing a few drops of Tween 20. The samples were incubated for 15 min by inverting the tubes several times. The solution was removed as much as possible. Then, the samples were washed five times with a large volume of double-distilled H_2O.

Genetic transformation of Arabidopsis

Arabidopsis plants were grown on commercial potting soil (Agroniche, Malaysia) in a controlled growth chamber environment (Conviron ATC26; Controlled Environments, Winnipeg) under a 16-h photoperiod at a light intensity of 150 µE m^{-2} s^{-1}. The temperature was kept at 22°C day/20°C night, and the humidity was maintained at 40%. The plants were watered regularly (once every 2 to 3 d) to prevent desiccation. When the plants flowered, the first bolts were clipped to encourage the proliferation of secondary bolts. The plants were ready for inoculation at approximately 4 to 6 d after clipping. Before transformation, *Agrobacterium tumefaciens* GV3101 carrying genes of interest was prepared by culturing overnight at 28°C in an Erlenmeyer flask containing 50 ml of Luria-Bertani (LB) broth that was supplemented with 50 mg L^{-1} kanamycin and 20 mg L^{-1} rifampicin. The *A. tumefaciens* cells were pelleted by centrifugation at 3,000×g for 10 min at room temperature and resuspended in 5% sucrose solution to OD_{600}=0.4-0.7. Silwet 408 (500 µl L^{-1}) (Crompton Corporation, USA) was added to the bacterial suspension. Plant transformation was performed by dipping the above ground parts of the plant into the *A. tumefaciens* suspension for a few seconds with gentle agitation. The inoculated plants were wrapped to maintain a high humidity for 16 to 24 h in the dark at 4°C and then transferred to a growth chamber, where the plants were allowed to set seeds. The loose bolts were tied with tape or by other means. When seeds were mature (as determined by the silique colour that turned from green to brown), the seeds were harvested, kept at 37°C overnight and then transferred to 4°C until screening.

Analysis of putative transgenic Arabidopsis plants

A molecular analysis of the putative transgenic plants was performed using a Direct PCR Kit (Yeastern Biotech. Co., Ltd., Taiwan). The leaf tissue was cut into small pieces (0.5 to 1 mm²) using the Harris Uni-Core puncher provided in the kit and added to the 25 µl PCR reaction mixture. The amplification reaction mixture contained 12.5 µl of 2× PCR Premix, 1 µl of YB Direct DNA polymerase and 1 µl each of 1 µM forward and reverse primers. To confirm overexpression in transgenic plants, the 35S primer that was designed from the sequence of the CaMV35S promoter was used as a forward primer, while the *SAP* $_{MR219}$ov-R primer was used as the reverse primer. The primer sequences were as follows: 35S-F: 5'-TACAGTCTCAGAAGACAAAGG-GC-3'; *SAP* $_{MR219}$ov-R: 5'-CCCCCC GGTNACCCTAAA-TTTTGTCAAGTTTCTC-3'. The following primers were used to amplify the genomic DNA: *SAP*$_{MR219}$ov-F:5'-CCCCCCAGATCTTATGGAGCACAAGGAGACT-3'; *SAP* $_{MR219}$ov-R: 5'-CCCCCCGGTNACCCTAAATTTTGTCAA-GTTTCTC-3'. One microlitre of DNA was added to each amplification reaction mixture and amplified. As a positive control, the DNA sample was replaced with the pCMB-MS plasmid, while the genomic DNA of the non-transformed plants was used as a negative control. The reaction products were resolved by electrophoresis in a 1% agarose gel with 1× TAE running buffer.

Analysis of the T_2 generation of transgenic Arabidopsis

Transgenic *Arabidopsis* (T_1) plants were grown to maturity to produce T_2 seeds. The seeds of transgenic *Arabidopsis* plants were grown on ½ MS with 25 mg L^{-1} hygromycin. Antibiotic resistance T_2 seedlings were transferred to potting soil. PCR was performed using the 35S-F and SAP $_{MR219}$ov-R primers to confirm the presence of the transgene. The PCR product was analysed using 1% agarose gel electrophoresis.

Salt treatments and germination tests

For germination analysis, wild-type and transgenic surface-sterilised *Arabidopsis* seeds were sown on MS medium (Murashige and Skoog,1962) that was supplemented with 3% sucrose, after which the Petri dishes were placed at 8°C for 3 d in the dark and then transferred to normal growth conditions. The medium was supplemented with 0, 100, 150, 200 and 250 mM NaCl to determine the effect of salinity stress on seed germination. A seed was considered germinated when the radicle protruded through the seed coat. Germination was scored for three replicates each of 40 seeds after 3 d of dark imbibition at 23°C.

Expression analysis of the SAP$_{MR219}$ transgene in transgenic Arabidopsis

Extraction of the total RNA

The total RNA was extracted from the leaves of 15-d-old non-transgenic and transgenic plants (lines 1, 2 and 5) that were exposed to 150 mM stress treatments using RNzol-Total RNA Extraction Reagent (BioTeke Corporation, China) according to the established protocol.

Reverse transcriptase (RT)-PCR

The total RNA was first treated with RNase-free DNase I (Fermentas) at 37°C for 30 min, and then the DNase was inactivated by heating at 95°C for 5 min. The RNA was precipitated overnight in the presence of 0.2 M sodium acetate and 66% (v/v) ethanol, collected by centrifugation at 13,000 rpm for 10 min, washed with 70% ethanol and finally dissolved in RNase-free-water. The first-strand cDNA was synthesised using a RevertAid^{TM} First-Strand cDNA Synthesis Kit (Fermentas). The reaction mixture (20 µl) contained 3 µg of total RNA, each of the primer pairs (SAP $_{MR219}$ov), 5 µl of 5× reaction buffer, RiboLock^{TM} Ribonuclease Inhibitor (20 U/µl, 1 µl), 10 mM dNTP mix (2 µl), RevertAid^{TM} M-MLV Reverse Transcriptase (200 U/µl, 1 µl) and DEPC-treated water. The mixture was incubated at 42°C for 60 min, and the reaction was stopped by heating at 70°C for 10 min and then chilling on ice. PCR amplification was performed in a 25-µl PCR mixture containing *Taq* DNA polymerase (0.5 U, Fermentas), $MgCl_2$ (1.5 mM), 1× reaction buffer, dNTP mix (1 mM), 1 µM each primer (SAP $_{MR219}$ov) and 1 µl of the First-Strand cDNA reaction. PCR cycling was conducted in a programmed Mastercycler (Eppendorf) using 0.2-ml tubes. First, the samples were heated to 94°C for 3 min and then shifted to the following cycling profile: 30 s at 94°C for denaturation, 50 s 55°C for annealing and 60 s at 72°C for extension. After 30 cycles, the samples were incubated at 72°C for an additional 10 min. The PCR product was analysed by electrophoresis in a 1.2% agarose gel.

Statistical Analysis

All of the data are presented as the mean±SD of three replications, and the means were compared by an analysis of variance (ANOVA) using Statistical Analysis System software (SAS 9.0, SAS Institute, Cary, CA, USA).

Conclusion

In conclusion, *SAP* $_{MR219}$, which is a member of SAP gene family, was isolated from rice. This gene encodes a cytoplasmic and chloroplastic protein zinc finger protein that might act in the signal transduction of salinity stress responses. The overexpression of this gene in a heterologous (*Arabidopsis*) system led to an increase in salinity tolerance at the seed germination stage. These findings indicate that all five lines of the transformed plants had an enhanced ability to grow under salt-stress conditions. Thus, *SAP* $_{MR219}$ may play an important role in the plant salt-stress response during early growth. Further studies are required to determine the possible role of *SAP* $_{MR219}$ in the plant response to other abiotic stresses.

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