Expression of a *Medicago falcata* small GTPase gene, *MfARL1* enhanced tolerance to salt stress in *Arabidopsis thaliana*

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**Article Info**

**Abstract**

To understand the role of small GTPases in response to abiotic stress, we isolated a gene encoding a small GTPase, designated *MfARL1*, from a subtracted cDNA library in *Medicago falcata*, a native legume species in semi-arid grassland in northern China. The function of *MfARL1* in response to salt stress was studied by expressing *MfARL1* in *Arabidopsis*. Wild-type (WT) and transgenic plants constitutively expressing *MfARL1* showed comparable phenotype when grown under control conditions. Germination of seeds expressing *MfARL1* was less suppressed by salt stress than that of WT seeds. Transgenic seedlings had higher survival rate than WT seedlings under salt stress, suggesting that expression of *MfARL1* confers tolerance to salt stress. The physiological and molecular mechanisms underlying these phenomena were elucidated. Salt stress led to a significant decrease in chlorophyll contents in WT plants, but not in transgenic plants. Transgenic plants accumulated less amounts of H$_2$O$_2$ and malondialdehyde than their WT counterparts under salt stress, which can be accounted for by the higher catalase activities, lower activities of superoxide dismutase, and peroxidase in transgenic plants than in WT plants. Transgenic plants displayed lower Na$^+$/$K^+$ ratio due to less accumulation of Na$^+$ than wild-type under salt stress conditions. The lower Na$^+$/$K^+$ ratio may result from less accumulation of Na$^+$ due to reduced expression of *AtHKT1* that encodes Na$^+$ transporter in transgenic plants under salt stress. These findings demonstrate that *MfARL1* encodes a novel stress-responsive small GTPase that is involved in tolerance to salt stress.

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**1. Introduction**

Being sessile organism, plants are frequently exposed to changing environment that results in holdback of growth and development. Plants have evolved various mechanisms to avoid and/or alleviate the adverse effects of abiotic stress. Upon exposure of plants to a stressed environment, numerous molecular and physiological processes are altered [1]. Environmental signals are often sensed by plants and transduced to activate downstream targets through intricate signaling network, in which transcription factors and signal-transducing GTPases play key roles. Small GTPase proteins are monomeric G proteins that are related to the subunit of heterotrimeric G proteins with molecular masses of 20–40 kDa. Similar to heterotrimeric G proteins, small GTP-binding proteins can cycle between GDP and GTP-bound states [2]. Once stimulated by upstream signals, the GDP-bound inactive form can be converted into the GTP-bound active form by guanine nucleotide exchange factors (GEF), thus activating downstream targets. Conversely, GTPase-activating proteins (GAP) can catalyze the GTP form to the GDP form [3].

Members of small GTPase share several common structural features, including four guanine nucleotide-exchange factors and an effector-binding domain [2]. However, small GTPases also exhibit a remarkable diversity in both structure and function. They are further divided into four main subfamilies in plants: Rab, Rop, Ran and Arf [4]. A diverse function of plant small GTPases has been reported in the literature. For instance, the Rab and Arf GTPase families have been suggested to regulate distinct steps of membrane trafficking [5,6]. Ran GTPases are involved in mediation of transport of proteins and RNA across the nuclear envelope [7].
Rop GTPases regulate several physiological processes, ranging from pollen growth and root hair development to abscisic acid (ABA) response [8]. These findings highlight that small GTPases may act as important molecular switches associated with plant signaling.

In recent years, the involvements of small GTPases in response of plants to abiotic stresses have been investigated. Several genes in the Rab GTPase family have been shown to be responsive to abiotic stress, including expression of SsRab2 to water stress [9], OsRab7 to cold stress [10] and AtrabG3e to salt/osmotic stress [11]. Among them, OsRab7 is differentially regulated by several environmental stimuli including cold, salt, dehydration and phytohormone ABA [10]. In addition, OsRab7 is localized to the vacuolar membrane, suggesting that OsRab7 may be implicated in a vesicular transport to the vacuole in plant cells [10]. In Arabidopsis thaliana, transgenic plants of overexpressing AtrabG3e exhibited accelerated endocytosis in roots, leaves and protoplasts. In addition, the transgenic plants also showed increased tolerance to salt stress due to reduced accumulation of reactive oxygen species [11]. These results imply that vesicle trafficking may play an important role in plant adaptation to stress. Furthermore, expression of AtRop2 renders seed germination less sensitive to ABA [8], suggesting that Rop GTPases negatively regulate ABA signaling. A recent study demonstrated that overexpression of OsRAN2 in rice confers tolerance to cold stress by regulating cell cycle [12]. In contrast to cold stress, overexpression OsRAN2 in rice renders transgenic plants hypersensitive to salinity and osmotic stress [13].

ADP-ribosylation factor (Arf) is an important regulator of membrane-trafficking pathways, and was initially identified due to their ability to stimulate the ADP-ribosyltransferase activity of cholera toxin A [14]. The production of three types of vesicle coat proteins (COPI, COPII and clathrin) is related to Arf GTPases [15]. The production of three types of vesicle coat proteins (COPI, COPII and clathrin) is related to Arf GTPases [15]. Similar domains have been found in many reported small GTP-binding proteins to membrane [2,3].

2. Results

2.1. Isolation and sequence analysis of MfARL1 cDNA

On the basis of the segment sequence of MfARL1, RACE was performed to obtain the full-length cDNA. The assembled results showed that MfARL1 had a 618 bp open reading frame encoding a protein of 205 amino acid residues, with a calculated molecular mass of about 23 kDa. Sequence comparison revealed that the putative protein was highly homologous to the small GTP-binding proteins (Fig. 1, Supplemental Fig. S1), and accordingly was designated MfARL1. The sequence data have been deposited in GenBank (accession No. JX292786).

MfARL1 protein contains 6 conserved functional domains. Similar domains have been found in many reported small GTP-binding proteins (Supplemental Fig. S1). Domains I–IV are involved in GTP-binding, and domain E is an effector region which can be recognized by GTPase-activation proteins (GAPs), and is essential for regulation of GTPases. Domain P, the C-terminal motif, is a prenylation site and important for attachment of small GTP-binding proteins to membrane [2,3].

Phylogenetic trees based on the full-length amino acid sequences of MfARL1 proteins were constructed using the MEGA 5 software (Fig. 1). The resulting trees contained four families, Rab, Ran, Rop and Arf. According to the phylogenetic trees, MfARL1 protein had the highest similarity with AtARLB1, a small GTP-binding protein of Arf family in A. thaliana with unknown function.

2.2. Expression pattern and subcellular localization of MfARL1

The MfARL1 was isolated from the salt stress suppression subtractive hybridization library. To validate the response of MfARL1 to various abiotic stresses, RT-qPCR was performed. As shown in Fig. 2A, transcripts of MfARL1 were found to be accumulated after 2 h of exposure to salt stress, and peaked after 5 h of salt stress. Expression of MfARL1 was also up-regulated by treatments with low temperature (4 °C) and osmotic stress (20% PEG6000) (Fig. 2B). Transcripts of MfARL1 were detected in roots, stems, leaves, flowers and pods under non-stressed conditions, with the expression being greatest in leaves, followed by roots and stems, lowest in flowers (Fig. 2C).

To examine the subcellular localization of MfARL1, an open reading frame of MfARL1 was fused to the C-terminus of the GFP reporter gene of pEGAD [22]. The recombinant constructs of the MfARL1-GFP fusion gene and GFP alone were introduced into tobacco leaf epidermal cells by Agrobacterium injection. The results showed that the MfARL1–GFP fusion protein was specifically localized in the cell membrane, whereas GFP alone showed ubiquitous distribution in the whole cell (Fig. 3).
2.3. Expression of MfARL1 enhanced tolerance to salt stress

To study the function of MfARL1, an overexpressing construct, under the control of a Cauliflower mosaic virus (CaMV) 35S promoter, was transformed into A. thaliana (Col-0) and five transgenic lines were obtained. The transgenic lines were confirmed by hygromycin selection, β-Glucuronidase (GUS) staining, and RT-qPCR. Compared with the untransformed WT Arabidopsis, the abundance of MfARL1 transcript was much higher in the over-expressing lines (Supplemental Fig. S2). Three independent transgenic lines (Line 1, 2 and 3) were used for further physiological studies throughout this paper. Given that the expression of MfARL1 was induced by salt stress in M. falcata, we investigated the role of MfARL1 played in response to salt stress by comparing the performance of transgenic plants expressing MfARL1 and wild-type seedlings under salt stress.

The effect of salt stress on germination of WT and transgenic seeds expressing MfARL1 was investigated. Exposure of both WT and transgenic seeds to NaCl reduced their germination rate, and no difference in seed germination between WT and the transgenic seeds in the absence of NaCl in the incubation medium was observed. However, germination of MfARL1-expressing transgenic seeds was less suppressed by NaCl than that of WT seeds (Fig. 4A). For instance, seed germination rate of the three MfARL1-expressing lines was 92.2%, 67.8 and 75.6% when incubated in 1/2 MS medium supplemented with 100 mM NaCl, while germination rate for wild-type seeds was 50.0% under the same conditions, suggesting that expressing MfARL1 confers transgenic seeds more tolerant to salt stress during seed germination.

In addition to seed germination, the effect of NaCl on seedling growth was also examined by exposure of four-week-old seedlings to 1/2 MS medium supplemented with and without 200 mM NaCl for 7 days. The MfARL1-expressing plants and WT plants exhibited comparable phenotype in the absence of NaCl, while the transgenic plants appeared healthier than WT in the presence of 200 mM NaCl (Fig. 4B).

Moreover, the MfARL1-expressing seedlings exhibited higher survival rate under salt stress when grown in the agar plate. For example, the survival rate of the three MfARL1-expressing lines was 30.8%, 21.8 and 39.2% after treatment with 150 mM NaCl for 14 days, respectively, while the survival rate for WT seedlings was only 10.0% when challenged by the same salt stress (Fig. 5).

2.4. Expression of MfARL1 conferred transgenic plants higher chlorophyll content, less H$_2$O$_2$ and MDA under salt stress

A decrease in chlorophyll content is often observed when plants suffer from salt stress [23]. There were significant decreases in contents of both chlorophyll $a$ and chlorophyll $b$ of WT plants when challenged with NaCl (Fig. 6A and B). In contrast, no significant
changes in both chlorophyll $a$ and chlorophyll $b$ contents were observed for the three transgenic lines expressing *MfARL1* when treated with the same NaCl concentration (Fig. 6A and B).

To investigate whether the transgenic plants differ from WT in their sensitivity to oxidative stress associated with salt stress, H$_2$O$_2$ and MDA contents in WT and transgenic plants grown in the absence and presence of NaCl were measured. As shown in Fig. 7, there were significant increases in H$_2$O$_2$ contents in both WT and transgenic plants upon exposure to solution containing NaCl. However, the salt stress-induced accumulation of H$_2$O$_2$ in the three transgenic lines was significantly less than in WT plants. A similar differential increase in MDA content in WT and the transgenic plants was also observed when the seedlings were treated with NaCl. For instance, MDA content in WT and the transgenic plants was comparable in the absence of NaCl in the incubation solution, and MDA content was increased by 319%, 181%, 66% and 73% in WT and the three transgenic lines after exposure to solution containing NaCl, respectively (Fig. 7B).

We further explored the physiological mechanisms by which the transgenic lines accumulate less H$_2$O$_2$ and MDA than WT plants under salt stress by measuring the activities of antioxidant enzymes. The CAT activity of transgenic plants showed little change in response to NaCl treatment, while wild-type plants exhibited significant decreases in CAT activity by the same treatment (Fig. 8A). In contrast to CAT activity, SOD activity in WT and the transgenic plants were increased by salt stress with the increase being significantly higher in WT than in the transgenic plants (Fig. 8B). A marked increase in activity of POD was also observed in both WT and the transgenic plants with the increase in WT being approx. 2.3 times greater than in the transgenic Line 3 (Fig. 8C).

### 2.5. Na$^+$ content of transgenic plants under salt stress

Na$^+$ is toxic to plant cells when accumulated in excess amounts under salt stress, leading to damage to plants. To further elucidate the physiological mechanisms underlying the enhanced tolerance of the *MfARL1*-expressing plants, the effects of salt stress on Na$^+$ and K$^+$ concentrations in shoots of WT and the transgenic plants were investigated. No differences in both Na$^+$ and K$^+$ concentrations in shoots of WT and the transgenic plants were found when they were grown in the control medium without NaCl (Fig. 9A and B). There were marked increases in Na$^+$ concentrations in both WT and the transgenic plants when they were exposed to solution containing 200 mM NaCl. However, Na$^+$ concentration in the three transgenic lines was significantly lower than that in WT (Fig. 9A). In contrast to Na$^+$, K$^+$ concentrations in both WT and the transgenic plants were equally reduced when NaCl was present in the growth medium (Fig. 9B). Accordingly, an increase in Na$^+$/K$^+$ ratio in both WT and the transgenic lines was observed under salt stress. For instance, Na$^+$/K$^+$ ratio in WT plants was increased from 0.06 to 2.39, while Na$^+$/K$^+$ ratio in the transgenic Line 3 was increased to 1.45 when these plants were treated with NaCl (Fig. 9C). Furthermore, the effects of salt stress on the expression of *AtHKT1* gene that encodes a Na$^+$ influx transporter in both WT and the transgenic lines were examined at transcriptional levels by real-time quantitative. As shown in Fig. 9D, transcripts of *AtHKT1* in the transgenic plants were significantly less than in WT under
conditions of salt stress. This result suggests that MfARL1 may down-regulate the expression of AtHKT1, thus contributing to enhanced tolerance of MfARL1-expressing lines due to less accumulation of Na⁺ in shoots of the transgenic plants.

3. Discussion

There is increasing evidence demonstrating that small GTPase proteins are involved in mediation of numerous physiological processes, ranging from pollen growth and root hair development to response to abiotic stress [8,12]. Signal transduction pathway mediated by GTP-binding protein-coupled receptor is involved in many physiological processes in plants [24]. Small GTP-binding protein, acting as molecular switches, can be “activated” by GTP and “inactivated” by the hydrolysis of GTP to GDP [4]. Among the GTPases, a few studies have demonstrated that Arf GTPases are involved in the regulation of membrane trafficking [5,6]. In recent years, several small GTPases have been reported to be involved in response of plants to abiotic stresses. For example, overexpression of AtRabG3e in A. thaliana leads to less sensitive to salt stress than WT plants. Further studies revealed that Na⁺ was accumulated into the vacuolar in the transgenic plants, thus minimizing toxic effect of Na⁺ in the cytosol and facilitating water uptake due to effective osmoregulation [11]. In rice, it has been shown that OsRAN2 plays different roles when plants exposed to cold, salinity and osmotic stress [12]. The expression of OsRAN2 is up-regulated by cold stress, and overexpression of OsRAN2 in rice confers tolerance to cold stress [12]. However, there has been no report to evaluate the role of Arf GTPases in response and adaptation of plants to abiotic stresses. In the present study, we isolated a novel Arl gene, designed MfARL1, that encodes a small GTPase protein from a legume pasture plant native to the semi-arid grassland in northern China, from a SSH cDNA library associated with salt stress in M. falcata. MfARL1 have high similarity to AtARLB1 in terms of their sequences, but the function of AtARLB1 remains to be characterized. Our results showed that expression of MfARL1 in Arabidopsis seedlings conferred the transgenic plants more tolerant to salt stress. To the best knowledge of authors, this is the first report showing the involvement of a plant Arl GTPase protein in response to salt stress. We further demonstrated that the enhanced tolerance of transgenic plant expressing MfARL1 to salt stress could be accounted for by reduced accumulation of Na⁺, and H₂O₂ as well as MDA under salt stress.

Photosynthetic activity is suppressed by salt stress, and the reduction in photosynthetic activity can be accounted for by the decline in chlorophyll content [23]. In the present study, we found that WT plants and MfARL1-expressing plants differed in their chlorophyll contents in response to salt stress. For example, no changes in both chlorophyll a and chlorophyll b contents of MfARL1-expressing transgenic plants were observed when they were exposed to solution containing NaCl (Fig. 6). In contrast, a significant decrease in photosynthetic activity and chlorophyll contents was found in WT plants when challenged by the same salt stress (Fig. 6). The higher chlorophyll content in the MfARL1-expressing transgenic plants would allow plants to maintain photosynthetic rate, thus facilitating normal growth of plants under salt stress.

Plants suffering from salt stress often display symptoms of oxidative damage as indicated by marked accumulation of reactive oxygen species such as H₂O₂ [25]. MDA has been widely recognized...
The less accumulation of H2O2 under salt stress may result in a similar less accumulation of H2O2 in the transgenic plants [11]. A similar less accumulation of H2O2 in the transgenic plants was also observed [11]. The less accumulation of H2O2 and MDA in the transgenic plants were significantly less than in WT under conditions of salt stress (Fig. 9A). In contrast to Na+, there was no difference in K+ concentrations in both WT and transgenic plants in the absence or presence of NaCl in the growth medium (Fig. 9B). Consequently, the transgenic plants exhibited a lower Na+/K+ ratio than WT under salt stress (Fig. 9C). The lower Na+/K+ ratio is beneficial for plants to maintain physiological processes under salt stress, thus contributing to the enhanced tolerance of the transgenic plants to salt stress. In plants, influx of Na+ into the cytosol and efflux of Na+ from the cytosol to apoplast and vacuoles are mediated by several transporters, including SOS1 [27], HKT1 [28], and NHX1 [29]. Mutations in the AtHKT1 gene suppressed sos3 mutant phenotypes, and analysis of ion contents in the sos3hkt1 mutant demonstrated that AtHKT1 is involved in mediation of Na+ influx into plant cells [28]. In addition, mutations that disrupted AtHKT1 function could also suppress sensitivity of sos1 and sos2 to salt stress [30]. These results indicate that suppressing the expression of AtHKT1 can enhance the ability of salt tolerance in Arabidopsis. In the present study, we found that the expression levels of AtHKT1 in the transgenic plants were significantly less than in WT under conditions of salt stress (Fig. 9D). The less expression of AtHKT1 in MfARL1-expressing may prevent Na+ influx into plant cells, thus conferring plants more tolerance to salt stress by alleviating toxic effect of Na+ on plant cells. The reduced accumulation of Na+ in the transgenic plants under salt stress is in contrast to the transgenic Arabidopsis plants overexpressing AtRabG3e in which Na+ is transported into the vacuoles, thus the overall Na+ content in the transgenic plants is not reduced under salt stress [11].

In summary, expression of MfARL1 conferred the enhanced tolerance of the transgenic Arabidopsis seedlings to salt stress by maintaining relatively higher chlorophyll contents, suppressed accumulation of H2O2 and MDA, and reduced Na+ influx into plant cells via down-regulation of AtHKT1 expression. Therefore, our findings highlight that MfARL1, encoding a small GTPase protein, may act as an important regulator involved in response to salt stress.

4. Materials and methods

4.1. Plant material and treatments

Seeds of M. falcata L. cv Humeng were soaked in concentrated sulfuric acid for approximately 8 min, and then thoroughly rinsed with tap water. After chilled at 4 °C for 2 d, the seeds were grown in a pot (diameter 10 cm) filled with vermiculite: peat soil (2:1) under controlled conditions (26 °C day/20 °C night, 14-h photoperiod, and 50% relative humidity) as described by Wang et al. [31].

Four-week-old seedlings were treated with varying abiotic stresses. Salt and osmotic stresses were achieved by exposing 4-week-old seedlings to 1/2 MS medium supplemented with 200 mM NaCl and 20% PEG6000 for different periods, respectively. For cold treatments, the seedlings were exposed to 4 °C for 5 h and 10 h. Shoots were sampled for isolation of RNA and quantitative PCR.
irrigated with and without 200 mM NaCl for 5 days were used in the experiment. Data are means ± SE with three replicates. Asterisks represent statistically significant differences between WT and transgenic lines. *P ≤ 0.05, **P ≤ 0.01.

4.2. Cloning the full-length sequence of MfARL1 cDNA

To identify gene fragments in response to abiotic stresses, suppression subtractive hybridization (SSH) was used to construct a cDNA library. SSH was carried out using a PCR-Select cDNA Subtraction Kit (Clontech) according to the manufacturer’s instruction and the methods of Diatchenko et al. [32]. Briefly, shoots of M. falcata treated with solution containing 200 mM NaCl and 0 mM NaCl (control) for 5 h were collected for RNA isolation, and then RNA was reverse-transcribed to cDNA. After digestion, two rounds of hybridization were performed to separate salt responsive cDNA. After enriched by PCR, these cDNA was ligated to pGEM-T Easy vector (Promega), and then transformed into Escherichia coli. The selected positive clones were sequenced using ABI 3730xl sequencer.

A positive clone was identified from the subtracted cDNA library of M. falcata. On the basis of the sequence of the clone, rapid-amplification of cDNA end (RACE) was performed to get the full-length cDNA using GeneRacer™ RLM-RACE Kit (Invitrogen). To obtain 5’ end, amplify the first-strand cDNA using a reverse gene-specific primer (5’-CCC TCC AGT CCA AGC ACT TGA TGG-3’) and GeneRacer™ 5’ primer (homologous to the GeneRacer™ RNA Oligo). Each reaction contained 4.5 µL of 10 µM GeneRacer™5’ primer, 1.5 µL of 10 µM reverse gene-specific primer, 1 µL RT template, 5 µL of 10× Pfx amplification buffer, 1.5 µL of dNTP (10 mM each), 0.5 µL Platinum Pfx DNA polymerase (2.5 U/µL) and 1 µL MgSO4 (50 mM) in a final volume of 50 µL. The thermal cycle used was 94 °C for 2 min, 5 cycles of 94 °C for 30 s, 72 °C for 2 min, 5 cycles of 94 °C for 30 s, 70 °C for 2 min, 22 cycles of 94 °C for 30 s, 65 °C for 30 s, 68 °C for 2 min, and 68 °C for 10 min. A forward gene-specific primer (5’-ACT GGA GGG TTT TCT GCT TCT GCC-3’) and GeneRacer™ 3’ primer were used to obtain 3’ end of this gene by the same method. The complete full-length sequence was then assembled by results of 5’-RACE and 3’-RACE using the DNAMAN software.

4.3. RNA isolation and real-time quantitative PCR

Total RNA was isolated using RNAiso Plus reagent (TaKaRa) and treated with RNase-free DNase I (Promega). The total RNA was reverse-transcribed into first-strand cDNA with PrimeScript® RT reagent Kit (TaKaRa).

Real-time quantitative PCR (RT-qPCR) was performed using ABI StepOne Plus instrument. Gene-specific primers used for RT-qPCR were designed using software Primer Premier 5, and were as follows: for MfARL1 (5’-AAC TGC TTG GAC TGG AGG GTT T-3’ and 5’-CAC GTA AGC ACT GGA CTC TTT C-3’), for AtHKT1 (5’-CAT GTG CCT CCT AAT CCC T-3’ and 5’-ACC ATA CTC GCT ACG CCT TTT-3’), MtActin gene (accession No. BT141409) and AtActin11 gene (accession No. NM_112046) were used as internal control with primers (5’-ACC AGG GTT TCA GAT G-3’ and 5’-ACC TCC GAT CCA GAC A-3’) and (5’-TCT TCT TTC CCT CTA CGC T-3’ and 5’-CAT TAC GAT TTT ACG CTC TTT-3’). The primer sequence of MtActin has been used previously [18,33], and AtActin11 was always used as a reference gene to normalize the expression [34,35]. Each reaction contained 10.0 µL of SYBR Green Master Mix reagent (TOYOBIO), 0.8 µL cDNA samples, and 1.2 µL of 10 µM gene-specific primers in a final volume of 20 µL. The thermal cycle used was 95 °C for 2 min, 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The relative expression level was analyzed by the comparative Ct method using the Microsoft Excel 2010 as described by Livak and Schmittgen [36].

4.4. Subcellular localization of MfARL1

The open reading frame (ORF) of MfARL1 was ligated to the C-terminal of green fluorescent protein (GFP) in pEGAD [22]. This construct was transformed to Agrobacterium tumefaciens GV3101 by electroporation. The Agrobacterium was infiltrated to leaves of Nicotiana tabacum as described by Sparkes et al. [37]. After 48 h of culture, GFP fluorescence in transformed tobacco epidermal cells was observed under a Zeiss LSM 510 confocal microscope.
4.5. Transformation and regeneration of Arabidopsis

The ORF of MfARL1 was amplified with the primers 5'-GGC GGA TCC ATG TTT TCG TTA TTT TAT G-3' (BamHI site underlined) and 5'-CTT GAG CTC CTA GGC AGG ACC TGG ACC C-3' (SalI site underlined). The EcoRI/BamHI — digested product was inserted the downstream of cauliflower mosaic 35S (CaMV 35S) promoter of pSN1301 [38]. After pSN1301: MfARL1 was transformed to A. tumefaciens EHA105 by electroporation, transformation of A. thaliana (Col-0) was performed using the A. tumefaciens — mediated floral dip method as described by Zhang et al. [39]. Several independent lines of the T3 generation were randomly chosen for further physiological studies.

4.6. Determination of tolerance to salt stress

To determine seed germination rate in the absence and presence of NaCl in the medium, sterile seeds were pointed to 1/2 MS plate (0.8% agar) supplemented with and without 100 mM NaCl at 25 °C. There were 40 seeds in each plate and the seeds were considered to be germinated at the emergence of the plumule and scored. Seed germination was recorded after 48 h of incubation.

The effect of NaCl on seedling growth was also examined by irrigation of four-week-old seedlings using 1/2 MS medium supplemented with and without 200 mM NaCl for 7 days.

To determine the survival rate of wild-type and transgenic plants, 40 two-day-old seedlings each replicate were transfer to 1/2 MS agar plate supplemented with and without 150 mM NaCl for 14 days. Seedlings that survived the salt treatments can be distinguished from the dead plants, and the survival rate was determined by counting the survived seedlings.

4.7. Determination of chlorophyll, H$_2$O$_2$ and malondialdehyde (MDA)

Four-week-old seedlings that were irrigated with or without 200 mM NaCl for 5 days harvested for determination of chlorophyll, H$_2$O$_2$ and MDA contents. Chlorophyll was extracted and determined following protocols used by Arnon [40]. Hydrogen peroxide was measured as described by Alexieva et al. [41]. MDA content in leaves was determined following the protocol described by Kramer et al. [42].

4.8. Determination of antioxidant enzyme activity

Seedlings irrigated with 200 mM NaCl for 5 days were used for determination of antioxidant enzymes. Leaves were sampled for determination of antioxidant enzyme activity. Catalase (CAT) activity was assayed using the method by Aebi [43]. Superoxide dismutase (SOD) activity was measured spectrophotometrically based on inhibition in the photochemical reduction of nitroblue tetrazolium described by Giannopotitis and Ries [44]. Peroxidase (POD) was determined through measuring the oxidation of guaiacol [45].

4.9. Determination of Na$^+$ and K$^+$ concentration

Four-week-old seedlings of transgenic and WT plants were irrigated with 1/2 MS solution containing 200 mM NaCl for 5 days, washed with ultrapure water for five times, fixed at 105 °C for 10 min and baked at 80 °C for 24 h to constant weight. As much as 50 mg of dry material was weighed and placed in a digestion tube, and 5 mL of nitric acid and 1 mL of hydrogen peroxide were added for digestion. The digested fluid volume was finalized to 100 mL and ion content was measured by ICP-AES (Thermo).

4.10. Statistical analyses

All data were analyzed by analysis of variance using SPSS17.0 statistics program. Statistical differences are referred to as significant when $P < 0.05$.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2012.12.004.

References


