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Molecular cloning and expression analysis of the *MaASR1* gene in banana and functional characterization under salt stress

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ABSTRACT

Background: Abscisic acid (ABA)-, stress- and ripening-induced protein (ASR) is plant-specific hydrophilic transcriptional regulators involved in sucrose stress and wounding in banana. However, it is not known whether banana *ASR* genes confer salt stress tolerance. The contexts of the study was to analysis the sequence characterization of banana *ASR1*, and identify its expression patterns and function under salt stress using quantitative real-time PCR (qPCR) and overexpression in *Arabidopsis*. The purpose was to evaluate the role of banana *ASR1* to salt stress tolerance employed by plants.
Results: A full-length cDNA isolated from banana fruit was named *MaASR1*, and it had a 432 bp open reading frame (ORF) encoding 143 amino acids. *MaASR1* was preferential expression in roots and leaves compared to low expression in fruits, rhizomes and flowers. Under salt stress, the expression of *MaASR1* quickly increased and highest expression level was detected in roots and leaves at 4 h, and then gradually decreased. These results suggested that *MaASR1* expression was induced under salt stress. MaASR1 protein was localized in the nucleus and plasma membrane. *MaASR1* was transformed to *Arabidopsis* and verified by southern and northern analysis, transgenic lines L14 and L38 integrated one and two copies of *MaASR1*, respectively, while overexpression in transgenic lines provided evidence for the role of *MaASR1* to salt stress tolerance.
Conclusions: This study demonstrated that overexpression of *MaASR1* in *Arabidopsis* confers salt stress tolerance by reducing the expression of ABA/stress-responsive genes, but does not affect the expression of the ABA-independent pathway and biosynthesis pathway genes.

1. Introduction

High salt levels affect the growth and development of most plant species and cause significant losses in crop yield; however, plants also self-regulate to cope with the negative responses of such salt stress [1,2,3]. The major response of plants to salt stress includes abscisic acid (ABA) stress signals, perception, and transduction that involve a complex network of both positively and negatively regulating genes, including ABA biosynthesis, signaling, and transcriptional regulation [4]. However, genes involved in ABA reception and downstream transduction have not been well characterized.

An ABA-, stress-, and ripening-induced (ASR) protein acting as a downstream component of a common transduction pathway for ABA signals was first screened from tomato (*Solanum lycopersicum*) ripe fruit [5]. Subsequently, at least 24 *ASR* genes were identified in various species of gymnosperms [6,7], monocots [8,9], and dicots [10] plants but lacked orthologs in *Arabidopsis thaliana* [11]. All known *ASR* genes were shown to possess sequence-specific Zn^{2+}-dependent DNA binding activity at the N-terminus and a nuclear localization signal at the C-terminus [12]. Subcellular fractionation experiments indicated that the ASR protein is located in the nucleus [10] and cytoplasm [13].

The expression of *ASR* in various species is not only involved in plant development, but also responds to abiotic stresses [10,11,14,15]. The high expression of the litchi *LcASR* (accession no. JX291143) from 0 h at harvest time to 24 h is involved in fruit senescence, ripening, and dehydration [10]. A lily *ASR* (accession no. ACF57792) is preferentially expressed in the vegetative cell for pollen maturation [14], whereas its expression was induced by ABA, NaCl, or dehydration stress treatment [15]. In wheat, the *TaASR1* transcript level increased after treatments with PEG6000, ABA, and H_2O_2 [16]. Moreover, heterologous or homologous expression of *ASR* genes from litchi [10], lily [15], wheat [16], maize [17], tobacco [18], rice [19], and tomato [20] conferred abiotic stress resistance by altering the expression of ABA/stress-responsive genes in transgenic plants. However, it is not known whether banana *ASR* genes confer abiotic stress tolerance by altering the expression of ABA/stress-responsive genes.

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Banana (*Musa acuminata* L.) plays important roles in tropical and subtropical fruit production and agricultural economy. However, banana plant has shallow roots and a permanent green canopy, and is especially sensitive to unfavorable conditions, such as high salt, drought, and cold [21,22,23,24]. Therefore, understanding the molecular mechanisms of the abiotic stress response is necessary for genetic improvement of stress resistance in banana. Although studies in *M. acuminata* L. A. Colla and *Musa balbisiana* L. A. Colla have highlighted *mAsr* members' role in sucrose stress and wounding [21], the expression patterns and functional characterization of *ASR* genes in *M. acuminata* L. AAA group, cv. 'Dwarf Cavendish' (a commercially important Cavendish cultivar) under salt stress remain unknown.

In this study, we obtained a full-length ABA-, stress-, and ripening inducible gene named *MaASR1* from banana based on a cDNA fragment that originated from a single clone of a forward suppression subtractive hybridization (SSH) cDNA library of banana fruit [8]. We showed that *MaASR1* expression was induced in banana plants under salt stress and overexpression of *MaASR1* in *A. thaliana* improves its tolerance to salt stress. These results suggested that *MaASR1* plays an important role in salt stress tolerance.

2. Materials and methods

2.1. Plant materials

The ex vitro plants of banana (*M. acuminata* L. AAA group, cv. 'Dwarf Cavendish') (ITC 0002) ('Dwarf Cavendish' as known as 'Brazilian') were obtained from the banana tissue culture center (Institute of Banana and Plantain, Chinese Academy of Tropical Agricultural Sciences, Danzhou, Hainan, China). Ex vitro banana plants were grown at 28°C with 70% humidity, 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity, and 16 h light/8 h dark cycle. Ex vitro plants with uniform growth at the five-leaf stage were selected and twelve were divided into four groups for salt treatment. Banana grown in soil were irrigated with half-strength Hoag land's solution [25] supplemented with 300 mM NaCl for 0 h, 2 h, 4 h, and 6 h. All samples were separately frozen in liquid N_2 and stored at -70°C for RNA extraction and expression analysis.

The wild-type *A. thaliana* (Columbia ecotype) seeds were purchased from the Arabidopsis Biological Resource Center (ABRC, Ohio University, Columbus, OH, USA). The DH5α *Escherichia coli* and the LBA4404 *Agrobacterium tumefaciens* strains were provided by Professor Jiaming Zhang from the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences. All *Arabidopsis* seeds were sown on a 1:1:8 mixture (by weight) of vermiculite, perlite, and peat moss, respectively. *Arabidopsis* plants were grown at 22°C with 70% humidity and 16 h light/8 h dark cycle (Sylvania GRO LUX fluorescent lamps; Utrecht, The Netherlands).

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from the roots, leaves, rhizomes, flowers, as well as fruits of banana, leaves and roots after NaCl treatments using the RNeasy kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. First strand cDNA was synthesized from 2 μg total RNA from each sample using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA).

2.3. Cloning and sequence analysis of MaASR1

The full-length gene encoding *MaASR1* was amplified from a banana fruit 2 d after postharvest with the primers (5′-caagcatcccacactcaatac-3′ and 5′-cacaagcacaagatcgagg-3′) based on the cDNA sequence of *MaASR1* isolated from a banana fruit cDNA library [8] with the adapter primers Ptr5′ (ctccgagatctggacgagc) and Ptr3′ (taatacgactcactcactataggg). The *MaASR1* cDNA sequences were submitted to GenBank using the web-based submission tool "BankIt" from the NCBI home page (http://www.ncbi.nlm.nih.gov/Banklt/index.html). A comparison of the similarity of the full-length cDNA sequence of the *MaASR1* gene was performed in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Amino acid sequences were compared using the DNAMAN software package (Version 5.2.2, Lynnon Biosoft, Canada). A homology tree was constructed by the neighbor-joining method with a Poisson correction model using MEGA 5.05 software (Arizona State University, Tempe, AZ, USA). The number for each interior branch is the percent bootstrap values calculated from 1000 replicates.

2.4. Expression analysis of MaASR1 in different banana tissues and under salt treatment

Expression levels of *MaASR1* were assayed by quantitative real-time PCR (qPCR) performed in an iQ5 real-time PCR detection system (Bio-Rad, USA) using the SYBR ExScript RT-PCR kit (Takara, Otsu, Shiga, Japan). The reaction of 25.0 μL contained 12.5 μL SYBR Premix Ex*Taq*, 1.0 μL of each primer at 10.0 μM, 8.5 μL ddH_2O and 2.0 μL cDNA (40 ng). *MaActin*-F and *MaActin*-R primers (Table 1) were used as a loading control to normalize samples in separate tubes. The qPCR was performed in triplicate for each sample using the primers of *MaASR1*-F and *MaASR1*-R (Table 1). The relative expression level of *MaASR1* gene was calculated using the $2^{-\Delta\Delta CT}$ method [26].

All data were analyzed using IQ5 software in an iQ5 real-time PCR detection system (Bio-Rad, USA).

2.5. Subcellular localization of the MaASR1 protein

The cDNA encoding the ORF of *MaASR1* was digested with *Nco* I and *Spe* I restriction enzymes and inserted into pCAMBIA1304-GFP expression vector to generate a MaASR1-GFP fusion protein under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The recombinant pCAMBIA1304-MaASR1-GFP plasmid was transferred to the *A. tumefaciens* strain LBA4404 and introduced into *Nicotiana benthamiana* leaves as previously described by Goodin et al. [27]. After 48 h incubation on MS at 25°C, fluorescence was examined by fluorescence microscopy (LSM700, Carl Zeiss, Germany).

2.6. Plant transformation and generation of transgenic plants

The *MaASR1* coding region was inserted into the pBI121 vector by replacing the β-glucuronidase following digestion with *Bam*H I and *Sac* I. The pBI121-MaASR1 was transferred into *A. tumefaciens* strain LBA 4404. Transgenic *Arabidopsis* plants were generated using the floral dip-mediated infiltration method [28]. Seeds from T_0 transgenic plants were plated in kanamycin selection medium (50 $mg \cdot L^{-1}$). Homozygous T_3 lines were used for functional investigation of *MaASR1*.

2.7. Blot analyses

Two kanamycin-resistant transgenic lines from the T_3 generation were used to determine the integration of *MaASR1* to *A. thaliana*

Table 1
Primers used for qPCR analyses.

Gene name	Sequence (5′ to 3′)
MaASR1	F: agaagcatcaccatcatctc; R: caagcatcccacactcaacac
RD29A	F: gataacgttggaggaagagtcggc; R: cagctcagctcctgattcactacc
RD29B	F: gtgaagatgactatctcggtggtc; R: gcctaactctccggtgtaacctag
RAB18	F: atgacgagtacggaaatccgatgg; R: tatgtatacacgattgttcgaagc
DREB2A	F: aaggtaaaggaggaccagag; R: acacaaccaggagtctcaac
ABI1	F: agagtgtgcctttgtatggtttta; R: catcctctctctacaatagttcgct
AAO3	F: gaaggtcttggaaacacgaagaa; R:gaaatacacatccctggtgtac
MaActin	F: cgaggctcaatcaaaga; R: accagcaaggtccaaac
AtActin	F: catcaggaaggacttgtacgg; R: gatggacctgactcgtcatac

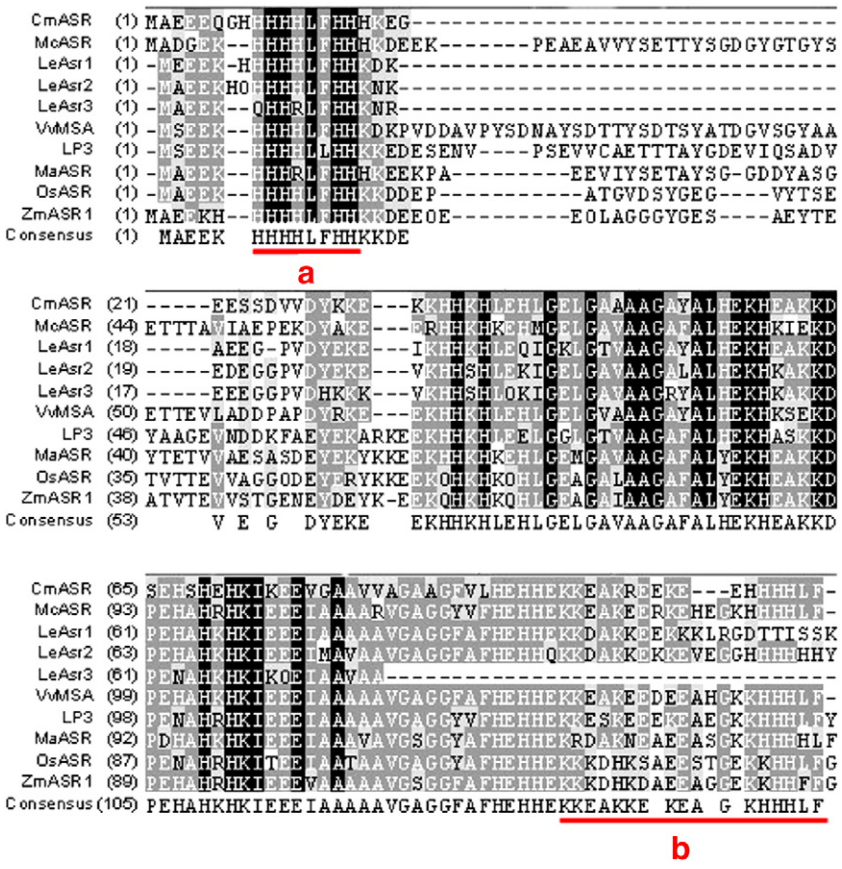

Fig. 1. Alignment of predicted amino acid sequences of *ASR* genes from different plants. (a) Domain: Zn^{2+}-dependent DNA binding site in the N-terminal, (b) domain: a conserved nuclear localization signal in the C-terminal.

genome by Southern blotting analysis. Probes from a partial region (389 bp) of the *MaASR1* gene for hybridization were prepared from the PCR product by using the primers (5′-ccgaggagaagcaccaccac-3′ and 5′-gccaccgct gcagcgatctcctc-3′) and used in DIG-dUTP according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). Northern blotting was performed according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). Probe of northern blotting was labeled using a random primer labeling system (Cat.1093657, Roche Applied Science, Mannheim, Germany). After hybridization, the membrane was washed and exposed to X-ray film (Kodak BioMax MS system) according to the methods of Miao et al. [29].

2.8. Salt stress and ABA treatment in wild-type and transgenic plants

For salt stress tolerance analysis, 4 week old plants were irrigated with 300 mM NaCl and survival rates were assessed after 15 d. For expression analysis of ABA/stress-responsive genes in wild-type and transgenic plants, 15 d old seedlings were transferred to 1/2 MS agar plates supplemented with 300 mM NaCl for 12 h or 100 μM ABA for 6 h.

The expression patterns of three ABA/stress-responsive genes (*RD29a*, *RD29b*, and *RAB18*), one ABA-independent pathway gene (*DREB2A*), one upstream element of ABA signaling pathway (*ABI1*), and an ABA biosynthesis rate-limiting enzyme gene (*AAO3*) in the leaves of *MaASR1* overexpressing transgenic plants and wild-type *A. thaliana* after NaCl or ABA treatments were detected by qPCR using corresponding primers (Table 1) and the *AtActin* as a control. The amplification program consisted of one cycle of 95°C for 1 min, followed by 40 cycles of 95°C for 10 s, 55°C–58°C for 15 s, and 72°C for 30 s. The expression levels of these genes were verified in triplicate and calculated using the $2^{-\Delta\Delta CT}$ method [26].

3. Results

3.1. Isolation and sequence analysis of banana MaASR1

A full-length *ASR* gene was obtained from the banana fruit and designated as *MaASR1*. The gene was deposited in GenBank under the Accession number AAT35818. Sequence analysis revealed that the full-length *MaASR1* cDNA has a 432 bp open reading frame (ORF) (Phred scores > 20) that encodes 143 amino acids. The deduced amino acid sequence of *MaASR1* contained the conserved N-terminal DNA binding site and a putative nuclear C-terminal localization signal

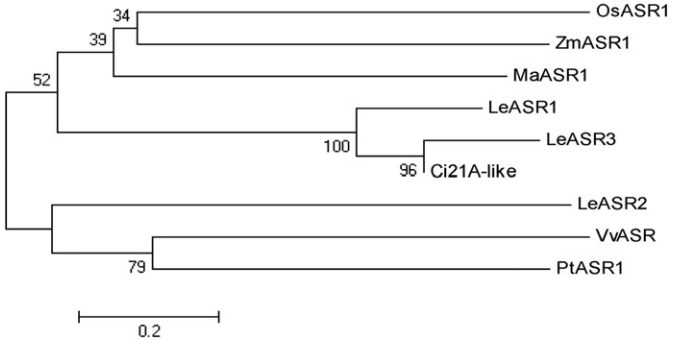

Fig. 2. Phylogenetic tree analysis of the deduced amino acid sequences of *ASR* genes in different species. *OsASR1* (accession no. AAB96681), *ZmASR1* (accession no. CAA72998), *MaASR* (accession no. AY628102), *LeAsr1* (accession no. AAA34137), *LeAsr3* (accession no. CAA52874), *Ci21A*-like (accession no. AAD00255), *LeAsr2* (accession no. CAA52873), *VvASR* (accession no. AAK69513), *PtASR1* (accession no. AAB03388).

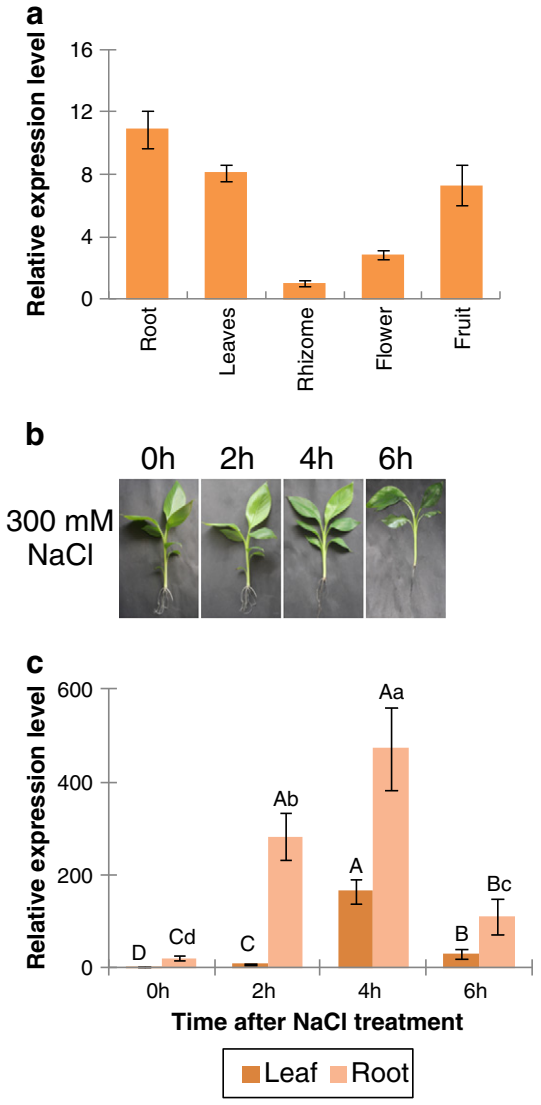

Fig. 3. *MaASR1* expression analysis in different tissues and under salt stress. (a) *MaASR1* expression in different tissues by qPCR, (b) phenotypes of banana leaves and roots under salt stress, (c) *MaASR1* expression analysis in banana leaves and roots under salt stress. Data are means ± SE of biological replicates (n = 3). Means, denoted by the same letter, do not significantly differ, when set at $P < 0.05$, as determined by Duncan's multiple range tests.

(Fig. 1), and it shared 62 and 66% identities with rice (*OsASR1*, AAB96681) and maize (*ZmASR1*, CAA72998), respectively (Fig. 2).

3.2. Differential expression of MaASR1 in various banana tissues

There were significant differences in the *MaASR1* expression in different banana tissues. *MaASR1* expression was detected in roots, leaves, rhizomes, flowers, and fruits. The roots showed the highest gene expression level, together with leaves, fruits, and flowers; the lowest level was found in rhizomes. The *MaASR1* expression level in roots was approximately 11-fold higher than that in rhizomes (Fig. 3a).

3.3. Phenotype and expression analysis of MaASR1 in banana plants under salt stress

The phenotype and expression of *MaASR1* in banana plants at different times under salt treatments were examined to determine the transcriptional response of *MaASR1* to salt stress. The results showed that banana roots and leaves exhibited different phenotypes at different times under salt stress. At 6 h under salt stress, the banana roots were black and leaves exhibited obvious brown spots (Fig. 3b). Significant differences in *MaASR1* expression were detected in the roots and leaves under salt stress. The expression levels of *MaASR1* quickly increased and reached its maximum levels in roots and leaves at 4 h. The expression in roots was approximately 3-fold higher than that in leaves at 4 h, and then gradually decreased over time (Fig. 3c). These results indicated that *MaASR1* expression was obviously induced in banana roots and leaves while roots may be more sensitive to salt stress.

3.4. MaASR1 localizes to the nucleus and plasma membrane

To determine the subcellular localization of the MaASR1 protein, its ORF was introduced into the pCAMBIA1304-GFP vector upstream of the *GFP* gene to create a MaASR1-GFP translational fusion construct. The recombinant pCAMBIA1304-MaASR-GFP fusion was infiltrated into the leaves of *N. benthamiana*. We observed that the green fluorescence MaASR1-GFP was confined to the nucleus and plasma membrane (Fig. 4). These results indicated that MaASR1 is targeted to the nucleus and plasma membrane.

3.5. Blot analyses of MaASR1 overexpressing transgenic lines

To examine the function of *MaASR1* in plants, *MaASR1* was introduced into a pBI121 vector under the control of a 35S promoter. After a floral-dip

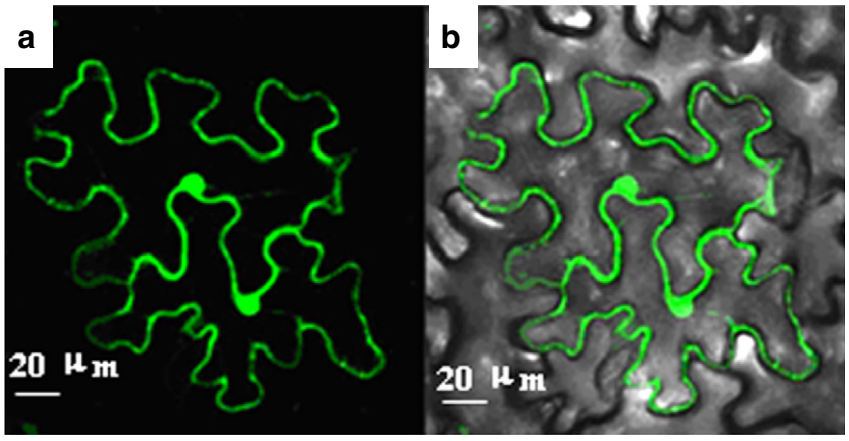

Fig. 4. Subcellular localization of the MaASR1 fused with GFP. The recombinant pCAMBIA1304-MaASR1-GFP plasmid was transferred to the *A. tumefaciens* strain LBA4404 and introduced into *N. benthamiana* leaves as previously described by Goodin et al. [27]. The fluorescence was examined by fluorescence microscopy (LSM700, Carl Zeiss, Germany). (a) Green fluorescence in dark field, (b) Green fluorescence in bright field.

transformation of *Arabidopsis*, two kanamycin-resistant transgenic lines from the T_3 generation were obtained. The copy number of these two transgenic lines was investigated by Southern blotting analysis. These results showed that the L14 line integrated two copies of *MaASR1*, while the L38 line integrated one copy of *MaASR1* (Fig. 5a). Northern analysis confirmed that the *MaASR1* transcripts were present in the leaf tissue of two transgenic lines compared to that no expression was detected in wild-type plants (Fig. 5b).

3.6. Overexpression of MaASR1 enhances tolerance to salt stress

When mature *Arabidopsis* plants were subjected to 300 mM NaCl treatment for 15 d, the transgenic plants exhibited better growth and a higher survival rate than that of the wild-type (Fig. 5c and Fig. 5d), where the survival rate of wild-type, L14, and L38 was 10.7, 87.3, and 82.7%, respectively (Fig. 5d). These results showed that overexpressing of *MaASR1* in *Arabidopsis* plants were more tolerant to salt stress than the wild-type.

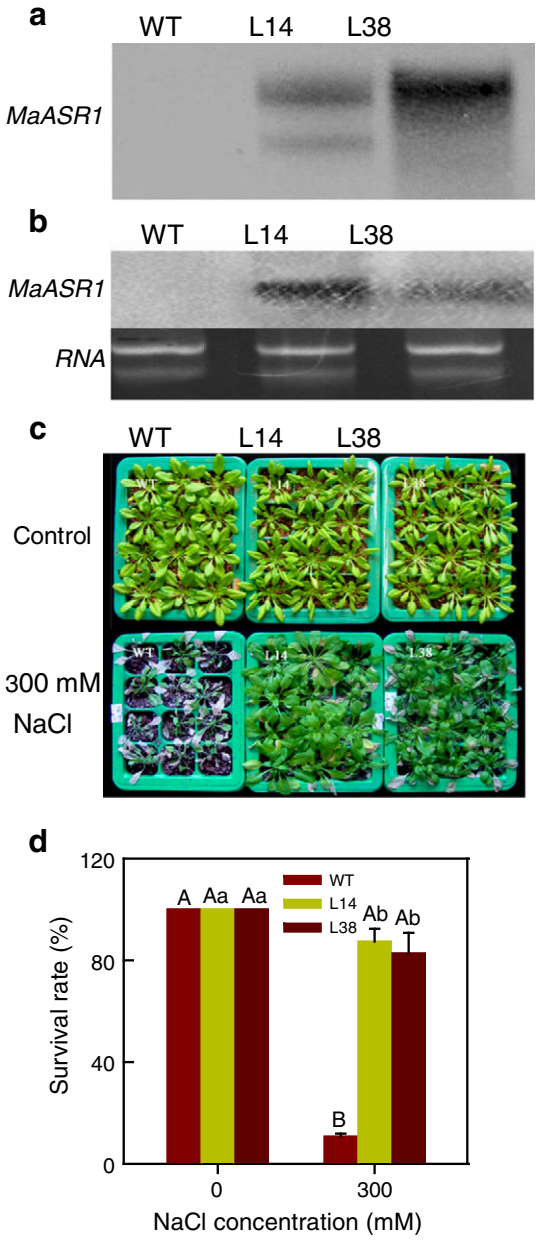

Fig. 5. Blot analysis and responses to salt stress of *MaASR1* transgenic lines in *Arabidopsis*. (a) The copy number of *MaASR1* transgenic lines by Southern blotting, (b) the expression of *MaASR1* transgenic lines by northern blotting, (c) photographs of wild-type and transgenic lines under normal or saline conditions, (d) survival rates of wild-type and transgenic lines under saline conditions. WT: wild-type; L14, L38: *MaASR1* transgenic lines. Data are means ± SE of biological replicates (n = 4). Means denoted by the same letter do not significantly differ, when set at $P < 0.05$, as determined by Duncan's multiple range tests.

3.7. Overexpression of MaASR1 decreases the expression of ABA/stress-responsive genes by NaCl treatment

To improve our understanding of *MaASR1* function during salt stress tolerance, the expression of several ABA/stress-responsive genes was examined in the wild-type plants and the *MaASR1* overexpressing transgenic plants (Fig. 6). Without the addition of NaCl, no significant difference was observed in the transcription of tested genes (*RD29a*, *RD29b*, *RAB18*, *DREB2A*, *ABI1*, and *AAO3*) in the *MaASR1* overexpressing transgenic plants compared to wild-type plants. Under salt stress, however, transgenic plants exposed to 6 h or 12 h of salt treatment exhibited reduced expression of *RD29A*, *RD29b*, and *RAB18* compared to wild-type plants that were similarly treated (Fig. 6). The expression of *DREB2A*, *ABI1*, and *AAO3* revealed similar trends at 6 h between wild-type and transgenic plants under salt stress (Fig. 6). This result indicated that *MaASR1* overexpression led to the down-regulation of ABA/stress-responsive genes under salt stress conditions, but didn't affect the expressions of ABA-independent pathway and biosynthetic pathways genes.

3.8. Overexpression of MaASR1 enhances the response of plants to ABA by exogenous ABA treatment

Under exogenous ABA treatment, the expressions of several ABA/stress-responsive genes, such as *RD29a*, *RD29b*, *RAB18*, and *ABI1*, were obviously increased in *MaASR1* overexpressing transgenic plants compared to wild-type plants that were similarly treated (Fig. 7). However, the expression of ABA-independent pathway gene *DREB2A* and ABA biosynthetic pathway gene *AAO3* revealed similar trends between wild-type and transgenic plants by ABA treatment (Fig. 7). This result suggested that overexpression of *MaASR1* might enhance the response of plants to ABA/stress signal pathway, but was no involved in ABA-independent and ABA biosynthetic pathways.

4. Discussion

In this study, *MaASR1* was identified in banana. *MaASR1* contains an ORF encoding 143 amino acids and two highly conserved regions, including Zn^{2+}-DNA binding sites at the N-terminus and a nuclear localization signal at the C-terminus (Fig. 1), whose structure was similar to *ASR* genes from lily [15] and tomato [18], and therefore was characterized as a potential *ASR* family member. Compared with other banana cultivars, the *MaASR1* from the *M. acuminata* L. AAA group, cv. 'Dwarf Cavendish' (accession no. AAT35818) shared 97 and 86% similarity with *Asr* amino acid sequences from the *M. acuminata* L. AAA group cultivars 'Mbwazirume' (accession no. ACZ60129) and 'Williams' (accession no. ACZ50751). Amino acid sequence differences may be because these *ASR* genes are from different banana cultivars or different *ASR* family members. Although another *mAsr1* (accession no. ACZ60119) was reported in different banana (*M. acuminata* subsp. burmannicoides) cultivar [21], amino acid sequence differences exist in the N-terminus, which suggests that *MaASR1* is different from *mAsr1*. Compared with other species, *MaASR1* shares higher similarities with rice *OsASR1* (accession no. AAB96681) and maize *ZmASR1* (accession no. CAA72998) (Fig. 2), which indicates that *MaASR1* might be a relatively conserved gene.

Distinct *ASR* family members exhibit variable responses to abiotic stress [21,30]. In tomato, *Asr1* and *Asr2* are members of the family preferentially induced by desiccation in leaves; *Asr2* is the only one activated in the roots from water-deficit-stressed plants [30]. Wheat

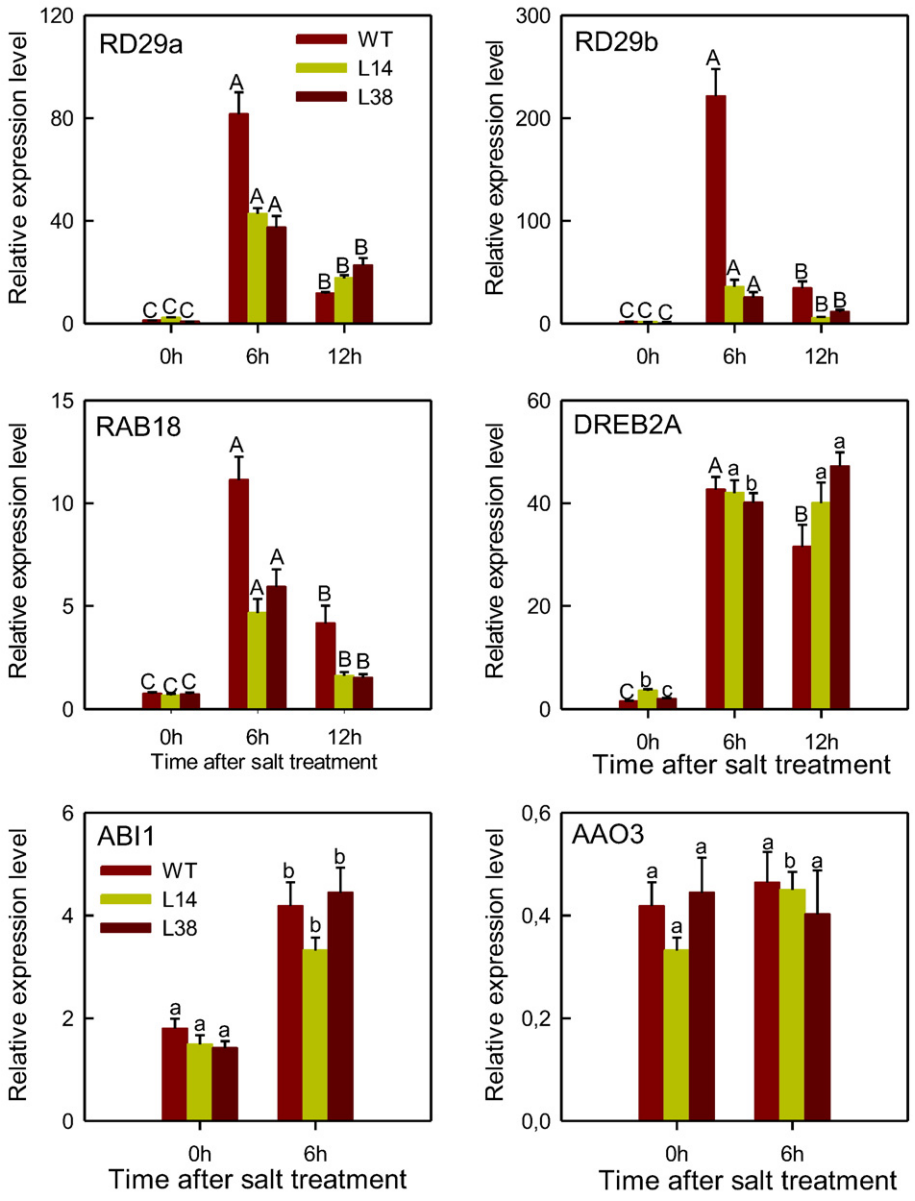

Fig. 6. Expression analysis of ABA/stress-responsive genes in wild-type and *MaASR1* overexpressing transgenic plants by NaCl treatment. WT: wild-type; L14, L38: *MaASR1* transgenic lines. Data are means ± SE of biological replicates (n = 4). Means denoted by the same letter do not significantly differ when set at $P < 0.05$ as determined by Duncan's multiple range tests.

TaASR1 transcript levels increase after treatments with PEG6000, ABA, and H_2O_2 [16]. The expression of lily *LLA23* is induced following the application of ABA, NaCl, or dehydration [15]. *LcAsr* was expressed in postharvest uncovered litchi fruit [10]. In banana meristems, *mAsr1* and *mAsr3* were induced by sucrose stress and wounding, while *mAsr3* and *mAsr4* were induced by exposure to ABA [21]. In this study, the expression of *MaASR1* was induced by salt stress (Fig. 3b and Fig. 3c), consistent with that reported for the tomato *Asr1* [18, 31], but we found that the expression level of *MaASR1* in roots was approximately 3-fold higher than in leaves at 4 h under salt treatment (Fig. 3c). These results indicated that the expression of *MaASR1* might be induced in leaves and roots by salt stress but banana roots might be more sensitive to salt stress.

Different ASR proteins' subcellular distribution patterns were observed in tomato [5], litchi [10], wheat [16], and lily [15]. The ASR1 from tomato was first reported as a nuclear protein [5]. The result supported the fact that most ASR proteins, such as lily [15], litchi [10], and wheat [16] were found in the nucleus. However, Kalifa et al. [18] reported that tomato ASR1 was localized in both the cytosol and the nucleus. During the early stages of pollen maturation, the ASR protein from lily translocates from the cytosol into the nucleus [14]. In this study, we demonstrated that the MaASR1 protein localized to the nucleus and plasma membrane (Fig. 1c), suggesting that it acts as a part of a transcription-regulating complex. However, further experiments are needed to determine the specific functions and cellular mechanisms of MaASR1 in the nucleus and plasma membrane.

ASRs are involved in abiotic stress tolerance [9,15,16,18]. In our study, to investigate the function of *MaASR1*, two *MaASR1* overexpressing *Arabidopsis* transgenic lines were generated and confirmed by Southern blot and northern blot (Fig. 5a and Fig. 5b). Under salt stress, the *MaASR1* transgenic plants exhibited increased tolerance to salt stress compared to the wild-types (Fig. 5c). The survival rates of *MaASR1* overexpressing transgenic plants were higher than that in the wild-type (Fig. 5d). These results demonstrated that overexpression of *MaASR1* genes confers salt stress tolerance to transgenic plants by enhancing the survival rates. The finding will have important theoretical and practical significance for improving the

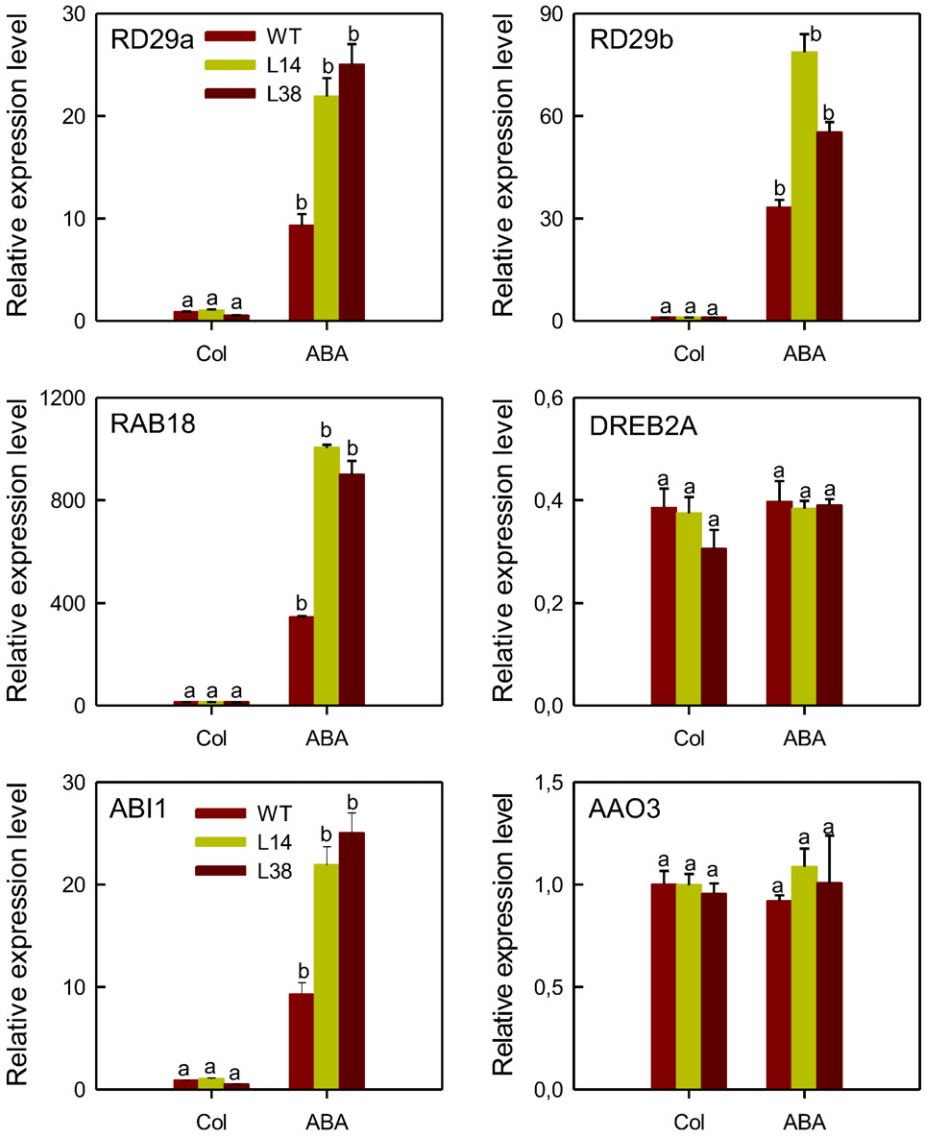

Fig. 7. Expression analysis of ABA/stress-responsive genes in wild-type and *MaASR1* overexpressing transgenic plants by ABA treatment. WT: wild-type; L14, L38: *MaASR1* transgenic lines. Data are means ± SE of biological replicates (n = 4). Means denoted by the same letter do not significantly differ when set at $P < 0.05$ as determined by Duncan's multiple range tests.

adaptability of banana plants to salt stress, breeding new varieties, and expanding the cultivation area of banana.

ABA-dependent signal transduction pathways play a crucial role in the adaptation of plants to abiotic stress [32]. The expression of several ABA/stress-responsive marker genes, including *RD29a*, *RD29b*, and *RAB18D*, are induced by abiotic stresses [24,33,34]. *DREB2A* is a DRE/CRT-binding transcription factor; its expression is not induced by ABA and abiotic stresses [35]. The *ABI1* and *AAO3* belonged to the upstream element of the ABA signaling pathway and ABA biosynthesis rate-limiting enzyme, respectively [33]. Under water stress, some ABA/stress-responsive genes, such as *RD29a*, *RD29b*, and *RAB18*, were up-regulated in wild-type *Arabidopsis* plants, indicating that the injury which resulted from water stress induces the expression of ABA/stress-responsive genes [36]. We examined the expression of ABA/stress-responsive genes (*RD29a*, *RD29b*, and *RAB18*), *DREB2A*, *ABI1* and *AAO3* in *MaASR1* overexpressing transgenic plants and wild-type plants. The expression of *DREB2A*, *ABI1* and *AAO3* presented similar trends between wild-type and transgenic lines under salt stress; however, the ABA/stress-responsive genes (*RD29a*, *RD29b*, and *RAB18*) were down-regulated in the transgenic plants subjected to salt treatments in comparison to similarly treated wild-type plants (Fig. 6). This result suggests that the *MaASR1* overexpressing transgenic plants might be involved in enhancing salt stress tolerance through reducing expression of the ABA/stress-responsive genes, but didn't affect the expression of ABA-independent pathway gene, the upstream element of ABA signaling pathway gene, and an ABA biosynthesis pathway gene.

ABA plays important regulatory roles in plant growth, development [37], and fruit ripening [38], particularly in the ability to respond to various unfavorable environmental stresses, including drought, salt [39], and cold [40]. In lily, constitutive expression of *LLA23* in transgenic plants significantly reduced ABA sensitivity and enhanced drought and salt resistance [15]. The constitutive overexpression of *OsASR1* also involved ABA signaling and increased high salinity stress tolerance in rice [19]. However, it is not known whether overexpression of *MaASR1* is involved in ABA signaling and enhances salt stress tolerance. In this study, we compared the expression of *RD29a*, *RD29b*, *RAB18D*, *DREB2A*, *ABI1*, and *AAO3* in *MaASR1* transgenic plants and wild-type by ABA treatment. *DREB2A* and *AAO3* exhibited similar trends between wild-type and transgenic plants under ABA treatment. However, the expression levels of *RD29a*, *RD29b*, *RAB18D*, and *ABI1* were higher in *MaASR1* overexpressing transgenic plants than that in the wild-type (Fig. 7). These results suggest that *MaASR1* overexpression is likely to

involve ABA signaling and enhances the salt stress tolerance by altering the expression of the ABA/stress-responsive genes (*RD29a*, *RD29b*, and *RAB18D*) and the upstream element of ABA signaling pathway (*ABI1*).

5. Concluding remarks

A full-length cDNA of *MaASR1* (accession number: AAT35818) was obtained from banana (*M. acuminata* L. AAA group, cv. 'Dwarf Cavendish') and it was obviously induced under salt stress. *MaASR1* overexpression resulted in enhanced tolerance to salt stress by reducing expression of the ABA/stress-responsive genes, but didn't affect the expression of ABA-independent pathway and biosynthesis pathway genes. Further studies are required to identify the direct target genes of *MaASR1* using Chromatin Immunoprecipitation (ChIP). This will enhance our understanding of the molecular interaction mechanisms of *MaASR1* in enhancing salt stress tolerance, improving the adaptability of plants to salt stress.

Conflict of interest

All the authors do not have any possible conflicts of interest.

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Author contributions

Proposed the theoretical frame: ZJ, BX. Conceived and designed the experiments: BX, HM. Contributed reagents/materials/analysis tools: JL, CJ, WH. Wrote the paper: HM. Performed the experiments: YW, HM, JL. Analyzed the data: HM, PS.

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