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The rice Aux/IAA transcription factor gene *OsIAA18* enhances salt and osmotic tolerance in *Arabidopsis*

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Abstract

In plants, auxin/indoleacetic acid (Aux/IAA) proteins are transcriptional regulators, which regulate developmental process and responses to phytohormones and stress treatments. A previous study has shown that the rice Aux/IAA transcription factor gene *OsIAA18* is induced by salt and osmotic stresses. However, little is known about the regulatory functions of this gene. In this study, the *OsIAA18* gene was successfully cloned from rice. Subcellular localization analysis in onion epidermal cells indicated that OsIAA18 was localized to the nucleus. Expression analysis in yeast showed that the full length OsIAA18 exhibited transcriptional activation. Heterologous expression of *OsIAA18* significantly enhanced salt and osmotic tolerance in transgenic *Arabidopsis* plants. Real-time quantitative PCR analysis showed that constitutive expression of *OsIAA18* up-regulated genes involved in abscisic acid (ABA) biosynthesis, proline biosynthesis, stress responses, and reactive oxygen species scavenging under salt and osmotic stresses. Enzymatic analyses found that the transgenic plants had higher 9-*cis*-epoxycarotenoid dioxygenase, pyrroline-5-carboxylate synthase, superoxide dismutase, and peroxidase activities than wild-type plants under salt and osmotic stresses. In the transgenic plants, ABA and proline content significantly increased, whereas H_2O_2 and malondialdehyde content significantly decreased. In addition, the transgenic plants had also a lower electrolyte leakage and water loss rate. These overall results indicate that the *OsIAA18* gene is involved in enhancing salt and osmotic tolerance in transgenic *Arabidopsis* plants. The *OsIAA18* gene has a potential to be used to enhance the tolerance to abiotic stresses in other plant species.

Additional key words: abscisic acid, onion, *Oryza sativa*, MDA, POD, proline, ROS, SOD, yeast.

Introduction

Growth and development of crops worldwide have been adversely impacted by salt and osmotic stresses, which are becoming serious threats to agricultural efforts to provide and satisfy the needs of a rapidly growing human population (Munns and Tester 2008, Wang *et al*. 2016a-d). It has been reported that approximately 20 % of the irrigated soils are suffering from salt stress (Zhao *et al*. 2013). Meanwhile, the problems of global water scarcity and worldwide climate change are threatening sustainable crop farming (Yang *et al*. 2010). Therefore, it is necessary to enhance salt and osmotic tolerance of crops. The studies indicate that some plants have evolved counter-mechanisms, such as growth and development regulation, detoxification, ion homeostasis, and osmotic adjustment, in order to adapt to abiotic stresses (including salinity, drought, and osmotic stress) (Bohnert *et al*. 1995, Zhu 2001, 2002).

Auxin plays a very important role in a wide variety of plant developmental and physiological processes (Friml 2003, Song *et al*. 2009). It has been shown that auxin signalling, recruiting specific transcription factors to regulate the expression of down-stream genes, performs the required responses (Vogler and Kuhlemeier 2003).

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Abbreviations: ABA - abscisic acid; Aux/IAA - auxin/indoleacetic acid; GFP - green fluorescent protein; Hyg - hygromycin; MDA - malondialdehyde; NCED - 9-*cis*-epoxycarotenoid dioxygenase; ORF - open reading frame; PEG - polyethylene glycol; POD - peroxidase; P5CS - pyrroline-5-carboxylate synthase; ROS - reactive oxygen species; SOD - superoxide dismutase; WT - wild-type.
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Auxin/indoleacetic acid (Aux/IAA) and auxin response factor (ARF) are two important protein families in developmental process and responses to phytohormones and stress treatments by controlling auxin-responsive transcription (Berleth *et al*. 2004, Hagen and Guilfoyle 2002, Liscum and Reed 2002, Song *et al*. 2009).

The Aux/IAA-encoding genes have been cloned and identified in mung bean (Yamamoto 1994), *Arabidopsis* (Reed 2001), and rice (Thakur *et al*. 2001, Nakamura *et al*. 2006). In rice, 31 Aux/IAA genes (*OsIAA*) was identified and their sequences were analyzed (Jain *et al*. 2006, Wang *et al*. 2007). The first rice *OsIAA* gene, named *OsIAA13*, was cloned by Thakur *et al*. (2001, 2005), while the first Aux/IAA protein OsIAA31 was characterized in rice by Nakamura *et al*. (2006). The rice plants overexpressing *OsIAA31* show insensitivity to auxin and gravitropic stimuli and exhibit short leaf blades, reduced crown root formation, and abnormal leaf formation (Nakamura *et al*. 2006). *OsIAA23* plays an important role in postembryonic maintenance of quiescent center (Jun *et al*. 2011). *OsIAA13* is involved in lateral root initiation (Kitomi *et al*. 2012). *OsIAA11* affects lateral root development (Zhu *et al*. 2012). Overexpression of *OsIAA4* in rice induces morphological changes and reduces responsiveness to auxin (Song and Xu 2013). Song *et al*. (2009) found that most *OsIAA* genes are responsive to various abiotic stresses, indicating an interaction between plant growth and abiotic stress. However, the role of the *OsIAA* genes in enhancing tolerance to abiotic stresses has not been reported.

The *OsIAA18* gene is induced by salt, osmotic, IAA, and abscisic acid (ABA) treatments in rice (Song *et al*. 2009). However, little work has been devoted to the regulatory functions of this gene. Therefore, we isolated the *OsIAA18* gene (Genebank accession No. NP_001046357) from rice and estimated its roles in transgenic *Arabidopsis*. The aim was to confirm our hypothesis that heterologous expression of *OsIAA18* can significantly enhance salt and osmotic tolerance in transgenic *Arabidopsis*.

Materials and methods

Plants: Rice (*Oryza sativa* L.) cultivar Nipponbare (Huaiyin Institute of Agricultural Sciences of Xuhuai Region, Huai'an, China) was employed for the *OsIAA18* gene cloning in this study. One expressed sequence tag (EST) encoding OsIAA18 protein was selected from the cDNA-AFLP library for cloning this gene. *Arabidopsis thaliana* [ecotype Columbia-0, wild type (WT)] (Huaiyin Institute of Technology, Huai'an, China) was used as a model plant to identify the functions of this gene.

Cloning and sequence analysis of the rice *OsIAA18* gene: Total RNA was extracted from the leaves of cv. Nipponbare with the *RNAprep Pure* kit (*Tiangen Biotech*, Beijing, China). RNA samples were reverse-transcribed according to the instructions of *Quantscript* reverse transcriptase kit (*Tiangen Biotech*). Based on the sequence of *OsIAA18*, we designed one gene-specific primer of reverse transcription PCR (RT-PCR) (Table 1 Suppl.) to obtain its full-length cDNA sequence. PCR was performed with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 10 min. PCR products were separated on a 1.0 % (m/v) agarose gel. Target DNA bands were recovered by gel extraction, then cloned into PMD19-T (*TaKaRa*, Beijing, China), and finally transformed into competent cells of *Escherichia coli* strain DH5α. White colonies were checked by PCR and the positive colonies were sequenced (*Invitrogen*, Beijing, China).

The full-length cDNA of *OsIAA18* was analyzed by an online *BLAST* at the *National Center for Biotechnology Information* (*NCBI*) website (http://www.ncbi.nlm.nih.gov/, 06-10-2017). For the multiple sequence alignment analysis, the amino acid sequence of *OsIAA18* and other *IAA* homologs from different plant species retrieved from *NCBI* were aligned using the *DNAMAN* software (*Lynnon Biosoft*, Quebec, Canada). The phylogenetic analysis was conducted with the *MEGA4* software (http://www.megasoftware.net/). Theoretical molecular mass and isoelectronic point (pI) were calculated using *ProtParam* tool (http://web.expasy.org/protparam/). The conserved domain of OsIAA18 protein was scanned by *InterProScan* program (http://www.ebi.ac.uk/Tools/pfa/iprscan/). The nuclear localization signal of OsIAA18 protein was predicted by *cNLS Mapper* program (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi).

Subcellular localization and transactivation assay of OsIAA18: Subcellular localization of OsIAA18 in onion (*Allium cepa*) epidermal cells was analyzed as described by Wang *et al*. (2016b). Transactivation assay of OsIAA18 in yeast (*Saccharomyces cerevisiae*) was conducted according to the method of Jiang *et al*. (2014). GUS activities were assayed by the fluorometric method (Jefferson *et al*. 1987).

Transformation of *Arabidopsis* with *OsIAA18*: The coding region of the synthesized *OsIAA18* gene was cloned with terminal *Bam*H I and *Sac* I restriction sites (Table 1 Suppl.) and then inserted into the same restriction sites in vector pCAMBIA1301 to create expression vector pCAMBIA1301-*OsIAA18* under the control of CaMV 35S promoter and NOS terminator of the expression box. This vector also contained β-glucuronidase (*gusA*) and hygromycin (*hpt* II) genes driven by a CaMV 35S promoter, respectively. The recombinant vector was transformed into the *Agrobacterium tumefaciens* strain LBA4404 cells according to the methods described previously (Lou *et al*. 2007), and transgenic plants were produced according to methods described in another publication (Zhang *et al*. 2006). Transformants were selected based on their resistance to hygromycin (Hyg). Putative transformed seeds were germinated on agar-solidified Murashige and Skoog (1962; MS) medium containing 25 mg dm^{-3} Hyg. Positively transgenic seedlings were grown in pots containing a mixture of soil, *Vermiculite*, and humus (1:1:1, v/v/v) to obtain T_2 and T_3 seeds. The incubation and growth conditions of *Arabidopsis* were the same as

described previously (Zhang *et al*. 2006).

Analysis of transgenic plants by PCR: The presence of the *OsIAA18* expression construct in positive plants was assessed by PCR analysis using specific primers (Table 1 Suppl.). DNA was extracted from *Arabidopsis* leaves according to the instructions of *EasyPure* plant genomic DNA kit (*Transgen*, Beijing, China). PCR amplifications were performed with an initial denaturation at 94 for 3 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 10 min. PCR products were separated by electrophoresis on a 1.0 % (m/v) agarose gel.

Reverse transcription PCR analysis: Total leaf RNA was extracted from the transgenic plants and WT using the *RNAprep Pure* plant kit (*Tiangen Biotech*). RNA samples were reverse-transcribed using *Quantscript* reverse transcriptase kit (*Tiangen Biotech*). The cDNA solution was used as templates for PCR amplification with specific primers of the *OsIAA18* gene (Table 1 Suppl.). *Arabidopsis AtActin* gene (Genebank accession No. NM112764) was used as an internal control and amplified by the specific primers (Table 1 Suppl.) (Li *et al*. 2013). PCR amplifications were performed as described above, and the PCR products were separated by electrophoresis on a 1 % agarose gel. The same results were obtained for three independent experiments.

Immunobot analysis: OsIAA18 antibody (full-length) was obtained from *Beijing Protein Innovation*. Total proteins were extracted from leaves of transgenic and WT plants in extraction buffer [50 mM Tris-HCl (pH of 8.0), 150 mM KCl, 1 mM EDTA, 0.5 % (m/v) *Triton X-100*, 1 mM dithiothreitol (DTT), 1 mM phenyl methyl sulfonyl fluoride (PMSF), and a 1× protease inhibitor cocktail tablet (*Applygen Technologies*, Beijing, China)]. The content of extracted protein was determined by bicinchoninic acid method (Gao *et al*. 2011a). Immunobot analysis was performed according to the method of Gao *et al*. (2011a). Fifty micrograms of protein were separated by dodecyl sodium sulfate-polyacrylamide gel electrophoresis [SDS-PAGE; 15 % (m/v) separation gel and 5.4 % stacking gel]. After electrophoresis, the protein on the gel was transferred to a nitrocellulose transfer membrane (*Whatman*, Braunschweig, Germany) by semi-dry electrophoretic transfer cell and immunostaining was performed as described by Wang and Fang (2002). The protein extracts were mixed with SDS-PAGE sample buffer and detected by immunoblotting using OsIAA18 antibody (Wang and Fang 2002).

Assay for salt and osmotic tolerance: *In vitro* assay for salt and osmotic tolerance was conducted as described by Liu *et al*. (2015b). Transgenic *Arabidopsis* T_3 and WT seeds were sown on Murashige and Skoog (MS) medium with 200 mM NaCl and 25 % (m/v) PEG6000 and grown at a temperature of 22 °C, a 13-h photoperiod and an irradiance of 54 μmol m^{-2} s^{-1} for 2 weeks. Then their root length and fresh mass were measured.

In vivo assay for salt and osmotic tolerance was based on the method of Wang *et al*. (2016b). Two-week-old transgenic *Arabidopsis* T_3 and WT seedlings were grown in 6-cm diameter pots containing a mixture of soil, *Vermiculite*, and humus (1:1:1, v/v/v) in a greenhouse, with nine plants per pot. All pots were irrigated sufficiently with half-Hoagland solution for 2 weeks and then each pot was irrigated with a 200 cm^3 of 300 mM NaCl solution every 2 d for 4 weeks, or subjected to drought (without watering) for 6 weeks. After treatment, the survival rate of these plants was observed immediately. All treatments were performed in triplicate.

Expression analysis of *OsIAA18* and the related genes: The expressions of genes related to ABA biosynthesis, proline biosynthesis, stress responses, and ROS scavenging, were analyzed by real-time quantitative PCR (qPCR) as described Wang *et al*. (2016b). Transgenic or WT plants were grown in pots for 2 weeks under 200 mM NaCl or for 4 weeks under 25 % PEG. Specific primers designed from conserved regions of genes were listed in Table 1 Suppl. *Arabidopsis AtActin* gene was used as an internal control. Quantification of the gene expression was done with comparative C_T method (Schmittgen and Livak 2008).

Analyses of 9-cis-epoxycarotenoid dioxygenase, pyrroline-5-carboxylate synthase, superoxide dismutase, and peroxidase activities: The activities of pyrroline-5-carboxylate synthase (P5CS), superoxide dismutase (SOD), and peroxidase (POD) in the transgenic plants and WT grown in pots and incubated for 4 weeks under optimum growth condition, for 2 weeks under 300 mM NaCl, or for 4 weeks under 25 % PEG were measured according to the methods of Hayzer and Leisinger (1980), Gao *et al*. (2011b), and Lurie *et al*. (1997), respectively. The 9-cis-epoxycarotenoid dioxygenase (NCED) activity was measured with *Plant NCED ELISA* kit (*USCN Life Science*, Shanghai, China). One unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes conversion of one micromole of substrate per minute under the specified conditions of the assay method.

Measurements of the content of ABA, proline, malondialdehyde, and H_2O_2, and electrolyte leakage: The content of ABA, proline, malondialdehyde (MDA), and H_2O_2 in the transgenic *Arabidopsis* plants and WT grown in pots and incubated for 4 weeks under optimum growth condition, for 2 weeks under 300 mM NaCl stress, or for 4 weeks under 25 % PEG were measured according to the methods of Gao *et al*. (2011b) and Alexieva *et al*. (2001).

Electrolyte leakage rates in the transgenic and WT plants were determined according to Ben-Amor *et al*. (1999). Water loss rate was measured according the method of Liu *et al*. (2015b).

Statistical analysis: The experiments were repeated three times and the data presented are the means ± standard errors (SEs). Where applicable, data were analyzed by

the Student's *t*-test in a two-tailed analysis using *SPSS v.17.0*. Values of $P < 0.05$ or < 0.01 were considered to be statistically significant.

Results

The *OsIAA18* gene was cloned by RT-PCR. The ORF was 903 bp in size and encoded a polypeptide that is 300 amino acids in length. The theoretical molecular mass of the protein was 32.21 kDa, and the theoretical isoelectric point (pI) was 6.34. A putative nuclear localization signal sequence was also identified at amino acid residues 274 - 284 (Fig. 1 Suppl.). Sequence analysis *via* the *InterProScan* program showed that OsIAA18 protein contained an Aux/IAA protein domain (Fig. 1 Suppl.).

A *BLAST* search indicated that the amino acid sequence of OsIAA18 showed a high amino acid identity with the predicted protein products of *Setaria italica* (XP_004951397, 67.75 %), *Brachypodium distachyon* (XP_003571137, 64.47 %), *Zea mays* (ADX60088, 59.93 %), *Solanum tuberosum* (XP_006343143, 42.07 %), *Brassica napus* (XP_013655539, 41.14 %), *Cucumis sativus* (XP_004150768, 40.61%), *Cucumis melo* (XP_008447559, 38.79 %), *Camelina sativa* (XP_010504392, 38.78 %), *Nicotiana tomentosiformis* (XP_009628748, 38.26 %), *Glycine max* (XP_003543621, 38.22 %), *Populus trichocarpa* (XP_002298246, 37.90 %), *Arabidopsis thaliana* (AAM65282, 37.62 %), and *Gossypium raimondii* (XP_012445590, 36.24 %) (Fig. 1 Suppl.). Phylogenetic analyses revealed that OsIAA18 had a close relationship with the predicted protein products of *S. italica* (Fig. 1 Suppl.).

To provide further evidence for the potential role of OsIAA18 in transcriptional regulation, we examined subcellular localization of OsIAA18 in onion epidermal cells. The GFP fluorescence of OsIAA18-GFP was exclusively located in the nuclei of the cells, whereas the GFP control was distributed throughout the whole onion cells (Fig. 1). These results indicate that OsIAA18 is a nuclear-localized protein.

To test whether OsIAA18 has transcription activity, OsIAA18 was fused in-frame to GAL4 DNA-binding domain in the pGBKT7 vector and the fusion constructs pBD-OsIAA18 were transformed into the yeast strain AH109. The results showed that yeast transformants harbouring pGAL4 and pBD-OsIAA18 grew normally on SD/Trp$^-$/His$^-$ medium exclusively and exhibited the activity of β-galactosidase reporter gene upon addition of X-gal on *Whatman* filter paper (Fig. 2 Suppl.). Thus these results confirm that OsIAA18 is a transcription activator.

The ORF of *OsIAA18* was ectopically expressed in *Arabidopsis* (Col-0, WT) using the binary vector pCAMBIA1301-*OsIAA18* (Fig. 2*A*). Multiple lines were obtained from Hyg resistance selection. Two independent

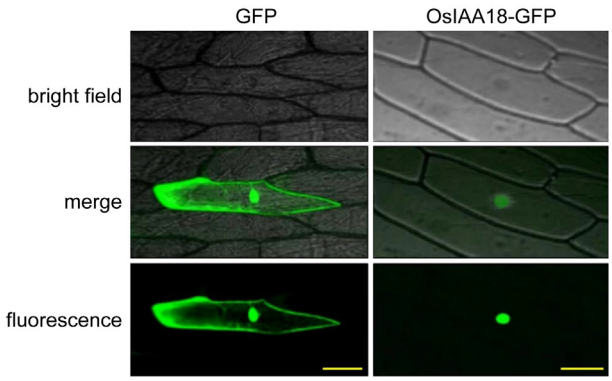

Fig. 1. Subcellular localization of the OsIAA18 protein in onion epidermal cells. The OsIAA18-GFP fusion protein was localized to the nucleus. *Scale bars* are 100 μm.

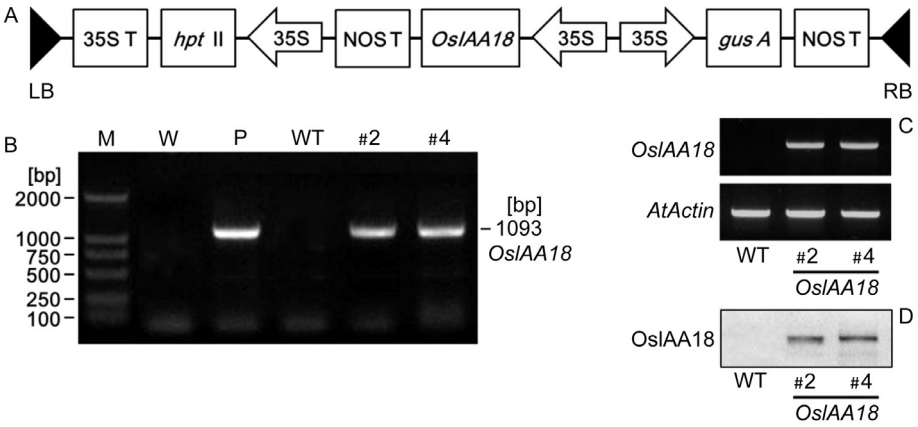

Fig. 2. Molecular analyses of the *OsIAA18*-expressing *Arabidopsis* plants. *A* - Aschematic diagram of the T-DNA region of the binary plasmid pCAMBIA1301-*OsIAA18*. LB - left border, RB - right border, *hpt* II - hygromycin phosphotransferase II gene, *OsIAA18* - rice Aux/IAA transcription factor gene, *gusA* - β-glucuronidase gene, 35S - cauliflower mosaic virus (CaMV) 35S promoter, 35S T - CaMV 35S terminator, NOS T - nopaline synthase terminator. *B* - Analysis of transgenic plants by PCR. *Lane M* - DL2000 DNA marker, *lane W* - water as a negative control, *lane P* - plasmid pCAMBIA1301-*OsIAA18* as a positive control, *lane WT* - wild type, *lanes* #2 and #4 - transgenic plants. *C* - Expression of *OsIAA18* in the *OsIAA18*-expressing *Arabidopsis* plants. The *Arabidopsis AtActin* gene was used as an internal control. *D* - Protein content of OsIAA18 in the *OsIAA18*-expressing *Arabidopsis* plants. Total proteins of 60 μg from leaves were used for immunobot analysis.

transgenic lines of *Arabidopsis* expressing *OsIAA18* gene were obtained (T_1 generation), named #2 and #4, and their progenies (T_3 generation) were generated. PCR analysis of genomic DNA indicated that they were transgenic (Fig. 2*B*). These transgenic plants were further confirmed to reveal enhanced expression of *OsIAA18* gene by RT-PCR and immunoblot analyses (Fig. 2*C*,*D*), thus they were selected for further experiments.

The two transgenic lines (#2 and #4) and WT seedlings were cultured on MS medium supplemented with 200 mM NaCl and 25 % PEG6000 for 2 weeks, respectively. The transgenic plants exhibited significantly higher root length and fresh mass than WT under salt and osmotic stresses, while no differences in root growth were observed under optimum conditions (Fig. 3).

The two-week-old transgenic plants (#2 and #4) and WT were grown in pots under 300 mM NaCl stress or disruption of watering for further evaluation of their salt and osmotic tolerance. No differences in growth were observed between the transgenic plants and WT under optimum growth condition (Fig. 4). After 4 weeks of 300 mM NaCl stress or 6 weeks after disruption of watering, the transgenic plants showed good growth, while WT died (Fig. 4). These results demonstrated that the transgenic lines (#2 and #4) had significantly higher salt and osmotic tolerance compared to WT.

Expression of *OsIAA18*, ABA biosynthesis, proline biosynthesis, stress responses, and ROS scavenging genes in the transgenic (#2 and #4) and WT plants were analyzed by real time qPCR. The results showed that the expression of *OsIAA18* was significantly higher in the transgenic plants compared to WT (Fig. 5). Under salt and osmotic stresses, the expressions of well-known salt and osmotic stress-responsive genes encoding zeaxanthin epoxidase (*AtZEP*), 9-cis-epoxycarotenoid dioxygenase (*AtNCED*), xanthoxin dehydrogenase (*AtABA2*), aldehyde oxidase (*AtAAO*), pyrroline-5-carboxylate synthase (*AtP5CS*), pyrroline-5-carboxylate reductase (*AtP5CR*), and late embryogenesis abundant protein (*AtLEA*) were also higher in transgenic plants (Fig. 5). Up-regulation of the reactive oxygen species (ROS) scavenging associated genes (*AtSOD* and *AtPOD*) was observed (Fig. 5). The results indicated that *OsIAA18* might be involved in multiple regulatory pathways.

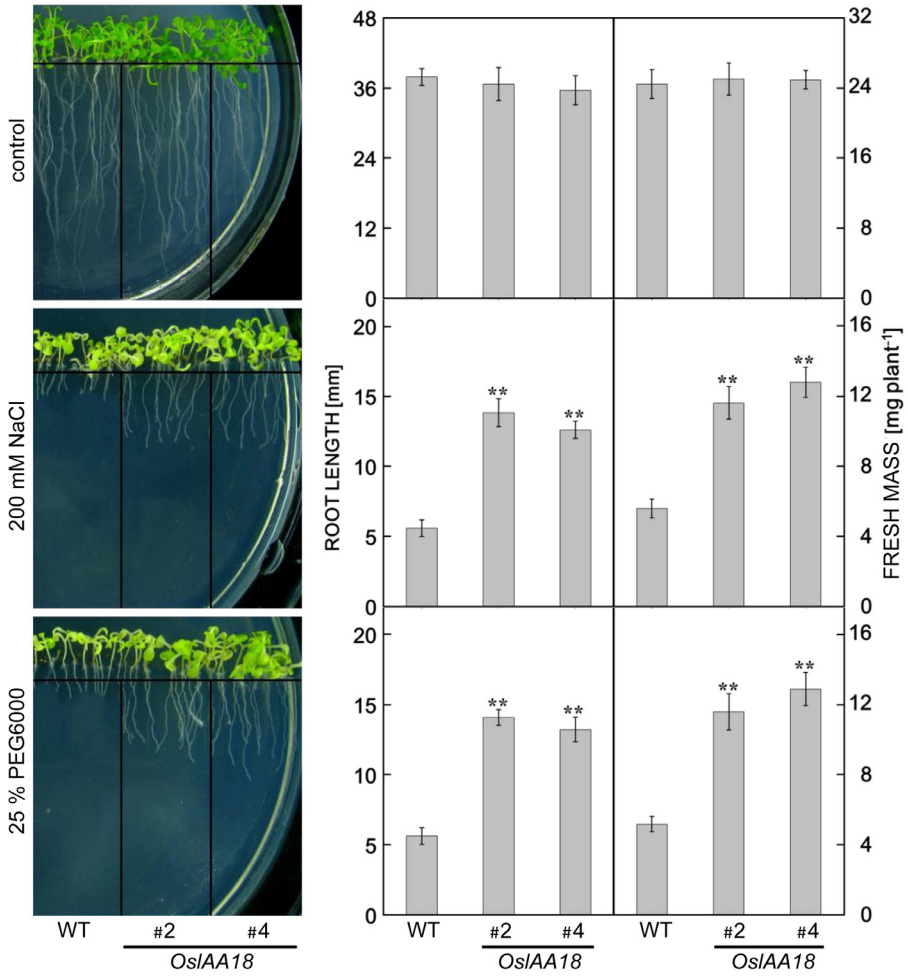

Fig. 3. Responses of transgenic and wild type *Arabidopsis* seedlings cultured on a Murashige and Skoog (MS) medium to salt and osmotic stresses. *Arabidopsis* seedlings were grown for 2 weeks on a pure MS medium (no stress), 200 mM NaCl, or 25 % (m/v) PEG6000. Root length and fresh mass were measured. Means ± SEs, $n = 3$, * and ** indicate significant differences at $P < 0.05$ and < 0.01, respectively, determined by the Student's *t*-test.

Abscisic acid and proline, as important physiological indices, play pivotal roles in evaluating the plant stress response. ABA and proline content in the transgenic *Arabidopsis* plants and WT grown in pots and incubated for 4 weeks under optimum growth condition, for 2 weeks under 300 mM NaCl stress or for 4 weeks under 25 % PEG6000 were analyzed. These results showed that ABA and proline content more increased in the transgenic plants compared to WT under salt and osmotic stresses (Fig. 6). Further we found that activities of NCED and P5CS (key rate limiting enzymes in ABA and proline biosynthesis pathways) were significantly higher in the transgenic plants compared to WT (Fig. 7).

The effects of salt and osmotic stresses on plant H_2O_2 content were examined in *OsIAA18*-expressing plants and WT. The results showed that the transgenic plants had significantly lower H_2O_2 content compared to WT under salt and osmotic stresses (Fig. 6). We also found that MDA accumulation was significantly decreased in the transgenic plants (Fig. 6). Meanwhile, we prompted to assay the major antioxidant enzyme (SOD and POD) activities in the transgenic and WT plants under salt and osmotic stresses. Under these stresses, the SOD and POD activities were significantly higher in the transgenic plants than in WT (Fig. 7). Thus, it is thought that overexpression of *OsIAA18* reduced the accumulation of H_2O_2 and MDA and enhanced the activities of antioxidant enzymes leading to the inhibited ROS-induced damage under salt and osmotic stresses.

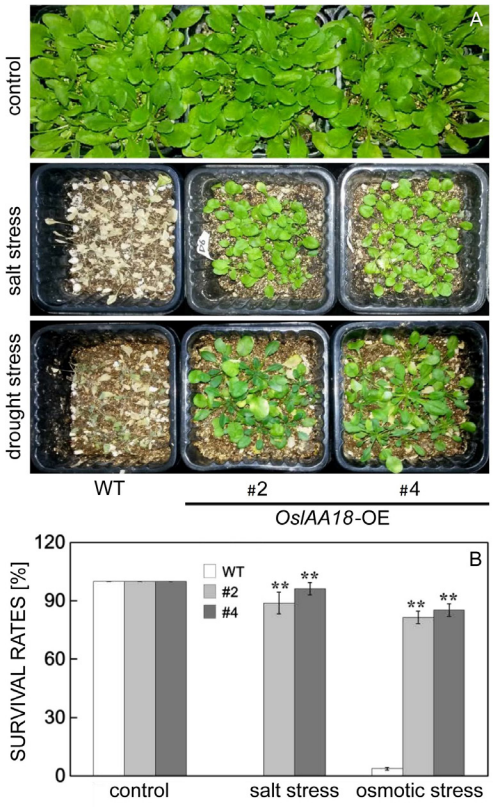

Fig. 4. Responses of the transgenic and wild type (WT) *Arabidopsis* plants grown in pots under salt and osmotic stresses. *A* - Transgenic and WT plants were grown in pots and incubated for 6 weeks under optimum growth conditions, for 4 weeks under 300 mM NaCl stress, and for 6 weeks under disruption of watering. *B* - Survival rates of transgenic and WT plants grown under salt and drought stresses. Means ± SEs, $n = 3$, * and ** indicate significant differences at $P < 0.05$ and < 0.01, respectively (the Student's *t*-test).

Electrolyte leakage measurements were performed to monitor membrane integrity. The transgenic plants had significantly lower electrolyte leakage rates compared to WT under NaCl and PEG treatments, whereas no significant differences in electrolyte leakage rates were observed among these plants under optimum growth condition (Fig. 8). Further analysis found that the transgenic plants had lower water loss rates compared to WT under dehydration for 2 to 6 h (Fig. 8). These results demonstrate some impact of the *OsIAA18* gene on the conservation of water under dehydration.

Discussion

The *OsIAA* genes play important roles in the developmental processes, stresses, and responses to phytohormones (Song *et al*. 2009, Song and Xu 2013). It was found that *OsIAA18* gene was induced by salt and osmotic stresses in rice (Song *et al*. 2009). However, little is known about the regulatory functions of this gene. In this paper, we cloned the *OsIAA18* gene from rice. The OsIAA18 protein was localized in the nucleus (Fig. 1). Further transcriptional activation analysis found that the OsIAA18 protein was a transcription activator (Fig. 2 Suppl.). Constitutive expression of *OsIAA18* significantly enhanced salt and osmotic tolerance in transgenic *Arabidopsis* plants (Figs. 3, 4).

The study of Song *et al*. (2009) revealed that the *OsIAA18* gene is involved in plants responses mediated by ABA. In this study, ABA content increased more in the *OsIAA18*-expressing *Arabidopsis* plants than in WT under salt and osmotic stresses (Fig. 6). Consistent with this phenomenon, we found the systematic up-regulation of the genes (*AtZEP*, *AtNCED*, *AtABA2*, and *AtAAO*) and a significant increase in activity of rate limiting enzyme (NCED) involved in ABA biosynthesis pathway in the transgenic plants under salt and osmotic stresses (Figs. 5, 7). Thus, it is suggested that constitutive expression of *OsIAA18* enhances salt and osmotic tolerance due to the up-regulation of genes involved in ABA biosynthesis, which increase the production of signalling molecules and further the expression of tolerance-responsive genes (Fig. 9).

Abscisic acid is a prime mediator of plant responses to abiotic stresses (such as salt, osmotic and cold stresses) and regulates the expression of abiotic stress-responsive genes (*P5CS*, *P5CR*, and the *late embryogenesis abundant*) in several plant species (Zhu 2002, Silva-Ortega *et al*. 2008, Dalal *et al*. 2009, Sripinyowanich *et al*. 2013, Zhai *et al*. 2016). In the present study, the tolerance-responsive genes mentioned above were significantly up-regulated in the *OsIAA18*-expressing *Arabidopsis* plants under salt and osmotic stresses (Fig. 5). Furthermore, the transgenic

plants had higher amount of P5CS protein under salt and osmotic stresses (Fig. 7). Our results support that high ABA content up-regulate these tolerance-responsive genes, leading to the enhanced salt and osmotic tolerance in the *OsIAA18*-expressing *Arabidopsis* plants (Fig. 9).

In plants, the up-regulation of *P5CS* and *P5CR* expression has been shown to increase proline content, resulting in the enhanced salt and osmotic tolerance (Krasensky and Jonak 2012, Liu *et al*. 2014, Zhai *et al*. 2016). Meanwhile, the *OsIAA18*-expressing *Arabidopsis* plants had significantly higher proline content compared to WT under salt and osmotic stresses (Fig. 6). Enhanced proline accumulation might maintain osmotic balance between the intracellular and extracellular environment and protect membrane integrity, resulting in enhanced salt and osmotic tolerance. Similar results were reported in some other studies (Zhang *et al*. 2012, Liu *et al*. 2014, 2015a, Zhai *et al*. 2016).

Salt and osmotic stresses induce ROS production in plant cells. It is important to maintain a stronger ROS-scavenging ability under salt stress to alleviate the induced oxidative damage, especially in plant leaves where photosynthesis is dramatically impacted (Gill and Tuteja 2010). In the present study, the *OsIAA18*-overexpressing *Arabidopsis* plants had significantly lower H_2O_2 accumulation compared to WT under salt and

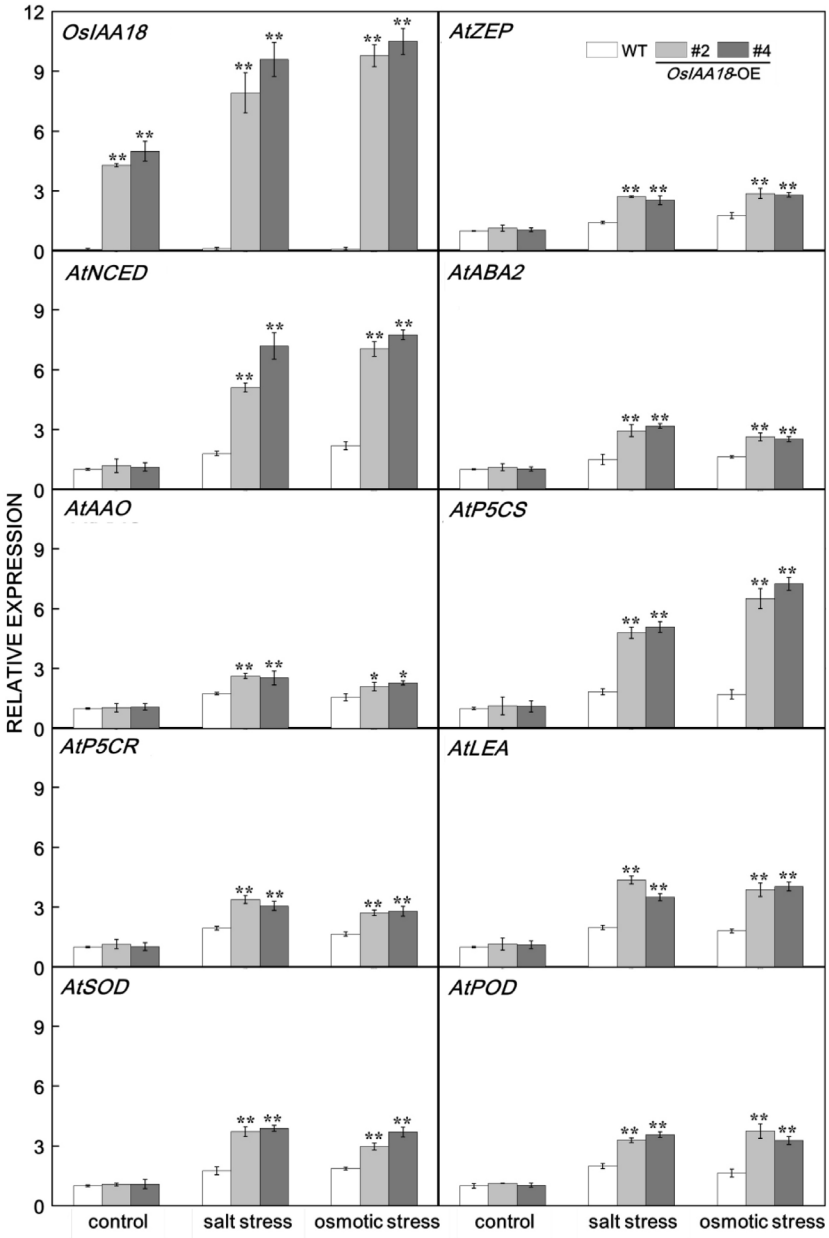

Fig. 5. Relative expressions of *OsIAA18* and its related genes in the leaves of transgenic and wild type *Arabidopsis* plants. Pot-grown plants were incubated for 4 weeks under optimum growth conditions, for 2 weeks under 300 mM NaCl, or for 4 weeks under 25 % (m/v) PEG6000. The *Arabidopsis actin* gene was used as an internal control. Means ± SEs, $n = 3$, * and ** indicate significant differences at $P < 0.05$ and < 0.01, respectively (the Student's *t*-test).

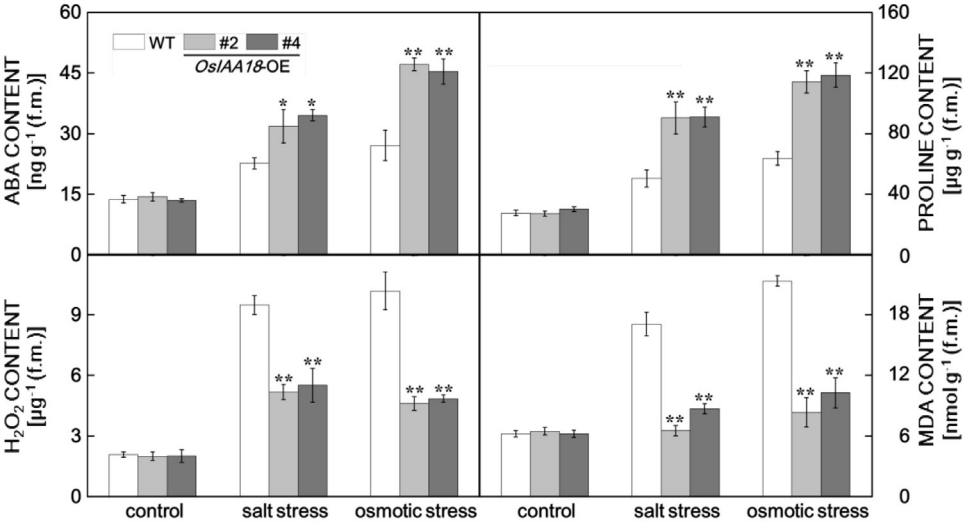

Fig. 6. The content of abscisic acid (ABA), proline, H_2O_2, and malondialdehyde (MDA) in the transgenic and wild type *Arabidopsis* plants. Plants grown in pots were incubated for 4 weeks under optimum growth conditions, for 2 weeks under 300 mM NaCl, or for 4 weeks under 25 % (m/v) PEG6000. Means ± SEs, $n = 3$, * and ** indicate significant differences at $P < 0.05$ and < 0.01, respectively (the Student's *t*-test).

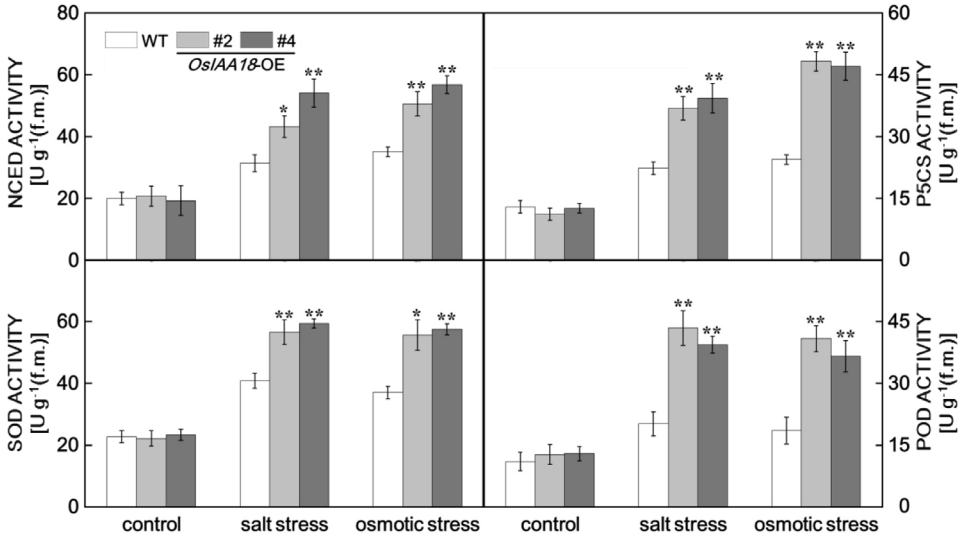

Fig. 7. The activities of 9-*cis*-epoxycarotenoid dioxygenase (NCED), pyrroline-5-carboxylate synthase (P5CS), superoxide dismutase (SOD), and peroxidase (POD) in the transgenic and wild type *Arabidopsis* plants. Plants grown in pots were incubated for 4 weeks under optimum growth conditions, for 2 weeks under 300 mM NaCl, or for 4 weeks under 25 % (m/v) PEG6000. Means ± SEs, $n = 3$, * and ** indicate significant differences at $P < 0.05$ and < 0.01, respectively (the Student's *t*-test).

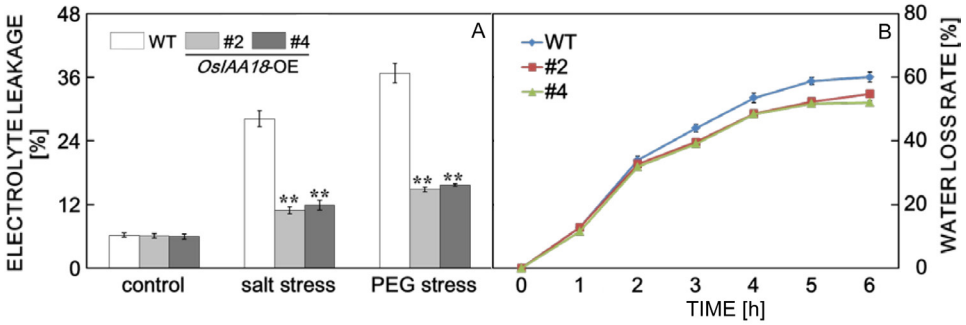

Fig. 8. Detection of electrolyte leakage and water loss rate. *A* - Comparison of electrolyte leakage rates in in transgenic *Arabidopsis* and wild type (WT) plants. Plants grown in pots were treated with water (control), 300 mM NaCl, and 25 % PEG6000 for 48 h. *B* - Analysis of water loss rates in WT and transgenic plants grown under control conditions. The *x*-axis shows the designated time length for plant desiccation. Means ± SEs, $n = 3$, * and ** indicate significant differences at $P < 0.05$ and < 0.01, respectively (the Student's *t*-test).

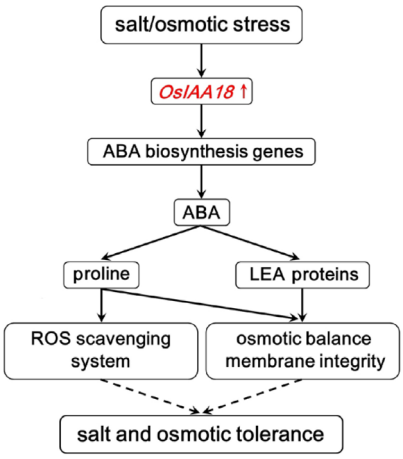

Fig. 9. The hypothesis of the regulatory network of the *OsIAA18* gene involved in salt and osmotic stresses responses. Constitutive expression of *OsIAA18* up-regulates genes involved in abscisic acid (ABA) biosynthesis, proline biosynthesis, and reactive oxygen species (ROS) scavenging, which results in significant physiological changes, including increased ABA and proline content and reduced ROS accumulation, leading to an enhanced salt and osmotic tolerance. LEA - late embryogenesis abundant.

osmotic stresses (Fig. 6). We also found that the systematic up-regulation of ROS scavenging genes (*AtSOD* and *AtPOD*) and a significant increase of antioxidant enzyme (SOD and POD) activities were observed in the transgenic plants under salt and osmotic stresses (Figs. 5, 7). Therefore, improved salt and osmotic tolerance of the transgenic plants might be due, at least in part, to the enhanced ROS scavenging capacity (Zhang *et al.* 2012, Liu *et al*. 2014, 2015a, Zhai *et al*. 2016, Wang *et al*. 2018). In addition, proline is an effective scavenger of singlet oxygen and hydroxyl radicals (Alia *et al*. 2001, Smirnoff and Cumbes 1989). Our results support that enhanced proline accumulation activates ROS scavenging system, leading to the enhanced salt and osmotic tolerance in the *OsIAA18*-expressing *Arabidops*is plants (Liu *et al*. 2014, 2015a, Zhai *et al*. 2016, Wang *et al*. 2018) (Fig. 9).

In addition, late embryogenesis abundant protein, as one of the most important stress-associated gene families, plays pivotal roles in stress tolerance, as osmotic adjustment material, as a protection material for cell membrane structure (Dalal *et al*. 2009, Jia *et al*. 2014, Banerjee *et al*. 2015). The *late embryogenesis abundant* genes have been shown to enhance tolerance to salt and osmotic stresses in plants (Park *et al*. 2011, Zhao *et al*. 2011, Ganguly *et al*. 2012, Muñoz-Mayor *et al*. 2012, Gao *et al*. 2013). In the present study, the expression of *AtLEA* gene was up-regulated in the *OsIAA18*-expressing *Arabidopsis* plants under salt and osmotic stresses, indicating the marked improvement of their salt and osmotic tolerance (Figs. 5 and 9).

Conclusions

In this study, *OsIAA18* gene has been successfully cloned from rice. Constitutive expression of *OsIAA18* significantly enhanced salt and osmotic tolerance in transgenic *Arabidopsis* plants. Our results suggest that the *OsIAA18* gene may enhance salt and osmotic tolerance of *Arabidopsis* transgenic plants by up-regulating the expression of ABA biosynthesis, proline biosynthesis, stress responses, and ROS scavenging genes. *OsIAA18* gene might be a hopeful candidate for enhancing tolerance to abiotic stresses also in other plants.

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