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Ascorbate peroxidase from *Jatropha curcas* enhances salt tolerance in transgenic *Arabidopsis*

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ABSTRACT. Ascorbate peroxidase (APX) plays a central role in the ascorbate-glutathione cycle and is a key enzyme in cellular H_2O_2 metabolism. It includes a family of isoenzymes with different characteristics, which are identified in many higher plants. In the present study, we isolated the *APX* gene from *Jatropha curcas L,* which is similar with other previously characterized APXs as revealed by alignment and phylogenetic analysis of its deduced amino acid sequence. Real-time qPCR analysis showed that the expression level of *JcAPX* transcript significantly increased under NaCl stress. Subsequently, to elucidate the contribution of *JcAPX* to the protection against salt-induced oxidative stress, the expression construct *p35S*: *JcAPX* was created and transformed into *Arabidopsis* and transcribed. Under 150-mM NaCl stress, compared with wild type (WT), the overexpression of *JcAPX* in *Arabidopsis* increased the germination rate, the number of leaves,

and the rosette area. In addition, the transgenic plants had longer roots, higher total chlorophyll content, higher total APX activity, and lower H_2O_2 content than the WT under NaCl stress conditions. These results suggested that higher APX activity in transgenic lines increases the salt tolerance by enhancing scavenging capacity for reactive oxygen species under NaCl stress conditions.

Key words: Oxidative stress; Ascorbate peroxidase; Salt stress; H_2O_2; *Jatropha curcas*

INTRODUCTION

Soil salinity is one of the most important environmental stress factors that significantly decreases the yield of a wide variety of crops worldwide (Zhu, 2001). Salt stress may alter biochemical pathways and physiological responses mediated by an increased generation of reactive oxygen species (ROS) (Zhu et al., 2013). Accumulation of high levels of ROS perturbs or overwhelms anti-oxidative defense, which could lead to severe damages such as lipid peroxidation, protein denaturation, and DNA mutation (Imlay, 2008). To prevent damage to cellular components by ROS, plants have developed a complex antioxidant system comprised of non-enzymatic antioxidants such as ascorbic acid, glutathione, and carotenoids, as well as of antioxidative enzymes such as superoxide dismutase, ascorbate peroxidase (APX), and glutathione reductase. These systems scavenge ROS and regenerate the active forms of antioxidants (Zhu et al., 2013).

APX (EC 1.11.1.11) catalyzes the conversion of H_2O_2 to H_2O and O_2 using ascorbate as specific electron donor (Foyer and Halliwell, 1976). Thus, APX has a key function in scavenging ROS and in protection against toxic effects of ROS in higher plants (Asada, 1992). A relationship between salt tolerance and increased activation of APX has been demonstrated in *Arabidopsis* (Lu et al., 2007), tobacco (Liu et al., 2014), tomato (Wang et al., 2005), and other plants. Lu et al. (2007) also found that both of two cytosols (c) APXs (OsAPXa and OsAPXb) in rice can improve salt tolerance in transgenic *Arabidopsis*, but to a different extent, i.e. the *OsAPXb* gene is more responsive to salt than the *OsAPXa* gene. Therefore, isolation and characterization of new cDNA of *APX* is utilized to further characterize the APX gene and provide new insights into the physiological roles and regulation of these enzymes by their overexpression in transgenic plants.

Recently, because of the high seed oil content and quality, low seed cost, rapid growth, adaptation to a wide range of agro-climatic conditions, and a bushy/shrub-like nature, *Jatropha curcas* has received special attention. It originates from arid and semiarid areas of South America, and it is distributed in all tropical regions. It grows in environments with unfavorable conditions, such as drought, salinity, and fluctuations in temperature, where most of the agriculturally important plant species are not able to thrive (Divakara et al., 2010). Previous studies have indicated that the antioxidant response might be one of the most important factors in *J. curcas* for the tolerance of abiotic stress (Gao et al., 2010). However, in contrast to other plants, the key antioxidative enzymes of *J. curcas* have not been well characterized at the molecular level.

In the present study, a novel APX gene was cloned from *J. curcas* by rapid amplification of cDNA end (RACE). The expression of *JcAPX* was examined in different tissues and under salt stress conditions. Additionally, the gene was introduced into *Arabidopsis* by *Agro-*

bacterium-mediated transformation to characterize the function of *JcAPX* under salt stress conditions. Subsequently, the differences between the wild type (WT) and transgenic plants were analyzed under NaCl stress conditions and optimal conditions. This research demonstrated that ectopic expression of the gene enhanced salt tolerance in transgenic *Arabidopsis* and suggested its possible application in improving the tolerance abilities in crops by the way of genetic modification.

MATERIAL AND METHODS

Plant material and growth conditions

Flowers, stems, leaves, and roots of *J. curcas* were gathered in the summer from Panzhihua city, Sichuan Province, China, and quickly frozen in liquid N_2. The mature seeds were surface sterilized with 1% NaClO for 10 min and washed with distilled water. Then their cotyledons were removed and placed in flasks containing Murashige and Skoog (MS) medium. Four days later, the rooted cotyledons were transferred to pots with a mixture of soil and vermiculite (2:1) in a chamber with photosynthetic active radiation of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$, a photoperiod of 16 h light/8 h dark, and temperature of 28°C. The WT *Arabidopsis thaliana* seed (ecotype Col-0) were surface sterilized and plated on a solid MS medium. After three days of cold (4°C) treatment, the plates were stored at 22°C in an incubator for propagation. The seedlings were transferred from the plates to a vermiculite-peat medium (1:1, v/v) and grown to maturity at 22°C under 16-h-light/8-h-dark cycle in a growth chamber.

JcAPX gene cloning and analysis

For cloning of cDNA of *JcAPX* from *J. curcas*, total RNA was isolated from young leaves using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was prepared from 1 μg of total RNA using a SMART™ RACE cDNA Amplification kit (Clontech Laboratories Inc., Mountain View, CA, USA). A fragment of *JcAPX* was amplified with two degenerate primers: JcAPX 1, 5'-ACATT(T/C)CTG(G/A)CTTCTG(C/A)TATCC-3' and JcAPX2, 5'-CTCG(G/A)CTG(C/T)G(T/G)TGG(T/A)ACTTCT-3', which were designed based on the conserved regions of the corresponding genes from other vascular plants. The 5'- and 3'-ends of *JcAPX* were amplified by a BD SMART RACE cDNA Amplification Kit (Clontech Laboratories Inc.) as recommended by the manufacturer and corresponding primers. DNA sequencing was carried out on an ABI Prism 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA) at Huada Gene Laboratory (Shengzhen, China). Amino acid sequences were aligned with ClustalX (Larkin et al., 2007), and phylogenetic trees were constructed using the MEGA4 software (Tamura et al., 2011).

Real-time PCR

Total RNA was extracted from different tissues or leaves subjected to 200 mM NaCl stress with an RNA prep pure Plant kit (Tiangen Biotech Co., Ltd., Beijing, China). Each cDNA was synthesized using 1 μg total RNA with a PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Tokyo, Japan). Real time-PCR was performed on a CFX Connect™ Real-Time PCR Detection System (Bio-RAD, Hercules, CA, USA) using an iQ™ SYBR® Green Supermix

(Bio-RAD). Data were obtained using the CFX Manager™ software (Bio-RAD). *JcAPX* fragment (156 bp) was amplified with gene-specific primers: JcAPX3, 5'-AGCTGTTATGTCGGCGTT CCT-3' and JcAPX4, 5'-GTTTCTCGGCTGCGTTGGTAC-3'. The *J. curcas actin* gene, 180-bp long, which was amplified with primers Actin-F, 5'-ATGAGCTTCGAGTTGCACCA -3' and Actin-R, 5'-AGCATCAGTGAGATCACGAC-3', was used as a reference for normalizing the *JcAPX* cDNA amounts.

Plasmid construction and *Arabidopsis* transformation

For *JcAPX* stable expression in *Arabidopsis*, the full length cDNA sequence was amplified and cloned into the *Bam*HI-*Sac* I restriction enzyme sites of the pBI121 vector, in which the expression of *JcAPX* was controlled by the CaMV 35S promoter. Transformation of *Arabidopsis* was performed by the floral dip method using *Agrobacterium tumefaciens* strain EHA105 (Clough and Bent, 1998). T1 seeds were germinated on MS plates containing 50 mg/mL kanamycin, and resistant plants were transferred to soil to obtain T2 seeds. Three independent lines of homozygous T3 plants containing a single insertion of each construct were used in the following analysis.

Anti-salinity analysis

For the germination salt tolerance assay, seeds were surface sterilized and sown on MS medium with or without the addition of 150 mM NaCl for 10 days under normal growth conditions (22°C, 16-h-white light/8-h-dark cycle). Germinated seeds were cultured on MS medium with or without the addition of 125 mM NaCl under the same growth conditions to analyze the salt tolerance in seedlings.

APX activity and H_2O_2 content assay

APX activity was assayed according to Gest et al (2013). The reaction mixture (1.0 mL) contained 50 mM potassium phosphate buffer, pH 7.0, 0.1 mM H_2O_2, 0.5 mM ascorbate, and 15 µL protein extract. The reaction was initiated by the addition of H_2O_2, and ascorbate oxidation was measured at 290 nm for 1 min by using UV-VIS spectrophotometer (Hitachi U-2910, Tokyo, Japan). One unit of APX was defined as the amount of enzyme that oxidized 1 µM of ascorbate per min at 25°C. Protein was determined by a Protein Assay Kit (Bio-RAD) using bovine serum albumin as a standard.

The histochemical detection of H_2O_2 in leaves was performed by *in situ* endogenous peroxidase-dependent staining. Whole explants of WT and transgenic *Arabidopsis* were submerged in 50 mM Tris-acetate buffer (pH 5.0) with 0.1 mg/mL 3, 3-diaminobenzidine (DAB) (Clemente-Moreno et al., 2012) and incubated at 25°C in the dark for 2 h. The leaves were then rinsed in 80% ethanol for 5 min at 60°C and photographed. For H_2O_2 content analyses, leaves (0.5 g) were homogenized with 2 mL Tris-acetate buffer (50 mM), pH 5.0, in the presence of 5 mM KCN. The measurement of H_2O_2 was based on the peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange (Bellicampi et al., 2000).

RESULTS

Cloning and characterization of *JcAPX* gene

Based on sequence homology among the APX genes or transcripts from other plants, the full-length cDNA (GenBank accession No.: KF792062) obtained by 5'- and 3'-RACE amplification consists of 1041 nucleotides and encodes a polypeptide of 346 amino acid residues with a calculated molecular mass of 37,810 Da and theoretical isoelectric point of 8.40. The alignment of the deduced amino acid sequence of *JcAPX* with other *APX*s indicated that it was homologous to sequences from *Vitis vinifera*, *Populus trichocarpa*, and *Citrus clementina*. Additionally, highly conserved regions were in the C-terminal end, whereas non-conserved region existed at the N-terminal end (Figure 1). Moreover, phylogenetic analysis of the amino acids of JcAPX group showed that JcAPX clustered with VvAPX from *Vitis vinifera* (Figure 2).

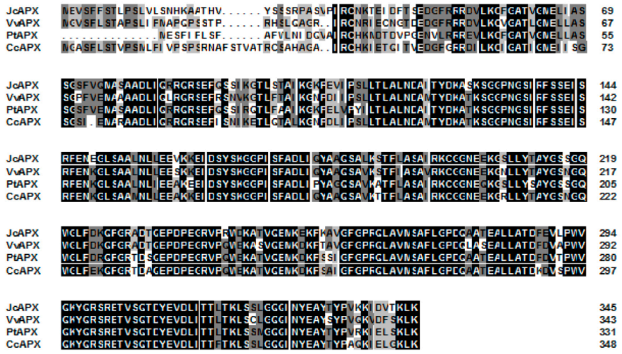

Figure 1. Alignment of the JcAPX sequence and its homologs using ClustalX. GenBank accession number and name of the species are as follows: JcAPX, *Jatropha curcas* APX (KF792062); VvAPX, *Vitis vinifera* APX (cbi19198.3); PtAPX, *Populus trichocarpa* APX (XP_002306565.1); CcAPX, *Citrus clementina* APX (XP_006433930). Identical amino acid residues are highlighted in black; conserved and semiconserved residues are highlighted in gray; dashes indicate gaps in the sequence introduced to optimize the alignment.

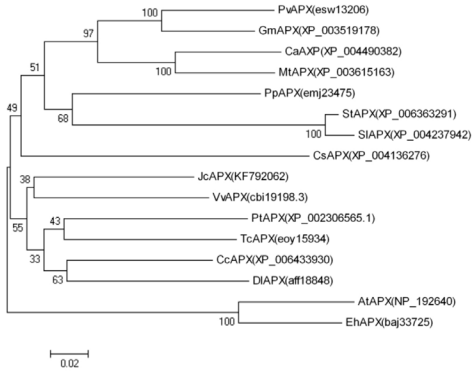

Figure 2. Phylogenetic tree based on amino acid sequences of APX inferred using the neighbor-joining method implemented in MEGA4. The sequences were obtained from GenBank and aligned with that of JcAPX. GenBank accession numbers are given in brackets. *Pv*, *Phaseolus vulgaris*; *Gm*, *Glycine max*; *Ca*, *Cicer arietinum*; *Mt*, *Medicago truncatula*; *Pp*, *Prunus persica*; *St*, *Solanum tuberosum*; *Sl*, *Solanum lycopersicum*; *Cs*, *Cucumis sativus*; *Jc*, *Jatropha curcas*; *Vv*, *Vitis vinifera*; *Pt*, *Populus trichocarpa*; *Tc*, *Theobroma cacao*; *Cc*, *Citrus clementina*; *Dl*, *Dimocarpus longan*; *At*, *Arabidopsis thaliana*; *Eh*, *Eutrema halophilum*.

Comparison of mRNA expression levels of *JcAPX*

The results of the spatial expression pattern tests indicated that *JcAPX* was expressed in all *J. curcas* tissues, but the level of expression varied considerably among the tissues. The greatest level of expression of *JcAPX* mRNA was in the leaf, and the lowest was in the stem (Figure 3A).

Prolonged treatment with 200 mM NaCl upregulated the *JcAPX*'s expression in a time-dependent manner. The gene expression significantly increased after 6 h (Figure 3B), which is probably closely related to salt stress.

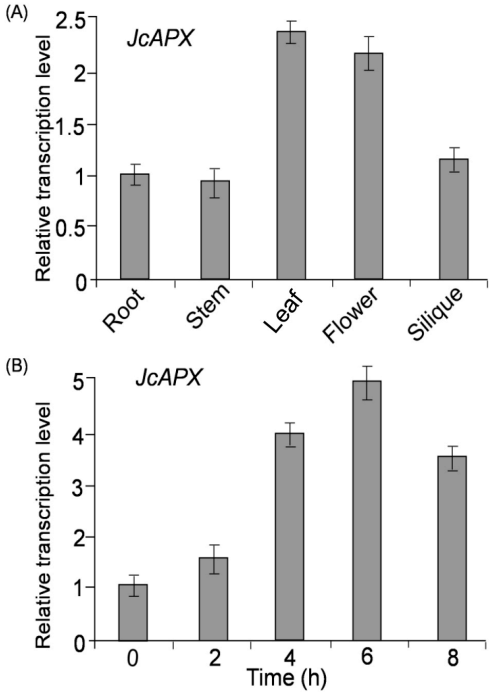

Figure 3. Expression pattern of *JcAPX*. (**A**) Real-time PCR analysis of *JcAPX* gene expression in different plant organs. Total RNA was extracted from the root, stem, leaf, flower, and silique. Expression level of *JcAPX* was monitored by real-time PCR against *Actin.* The level of expression in the root was set to 1. The data are means of three separate runs and SDs are indicated. (**B**) qPCR analysis of *JcAPX* gene expression under salt stress conditions. Three-week-old seedlings were treated with 200 mM NaCl. Total RNA was extracted at the indicated times and analyzed by qPCR. *Actin* was used as an internal control. The data are reported as means of three separate experiments and SDs are indicated.

Molecular characterization of transgenic *Arabidopsis* plants

The full-length *JcAPX* sequence was placed under the control of CaMV-35S promoter. The resulting p35S: *JcAPX* construct was successfully transformed into *A. thaliana*, and transgenic plants were detected with PCR after the first screening with 50 μg/ mL kanamycin. Furthermore, the expression of the exogenous *JcAPX* was confirmed by semi-quantitative real time-PCR. *JcAPX* transcripts were detected in transgenic lines T5, T12, and T19, which were chosen for further analysis (Figure 4).

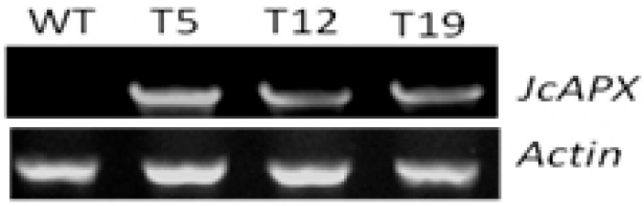

Figure 4. The transcript levels of *JcAPX* in different overexpression lines were analyzed by real-time PCR. Total RNA was extracted from leaf samples collected from T_2 transgenic plants grown under normal conditions. The actin housekeeping gene was selected as an internal control.

JcAPX transgenic plants display tolerance to salt stress

Seeds of three JcAPX-overexpressing lines and WT plants were germinated on MS plates supplemented with different concentration of NaCl. Transgenic lines grown on MS plates without NaCl did not show any significant difference from the control group (Figure 5A and C). However, the germination rates of *JcAPX*-overexpressing lines sawn on the medium containing 150 mM NaCl were significantly higher than the germination rates in the WT (Figure 5B and D).

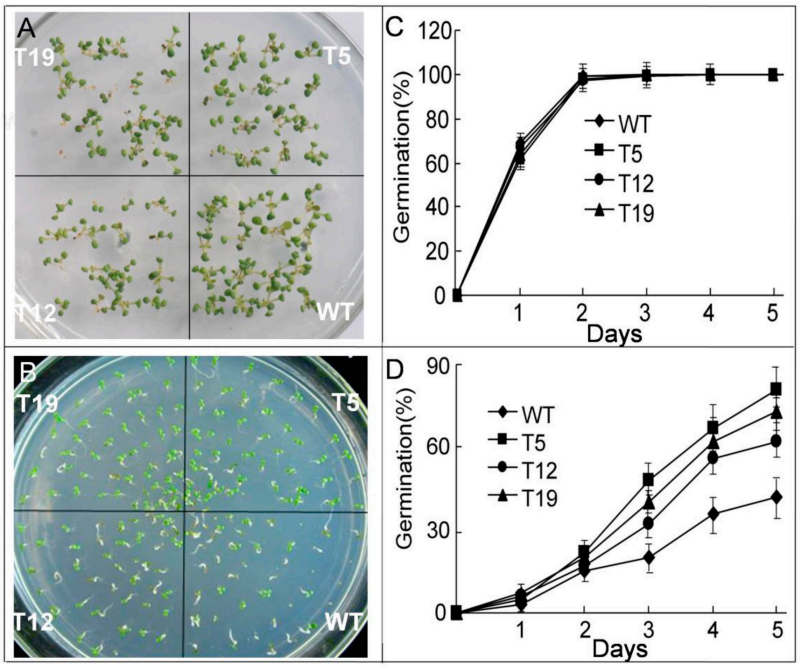

Figure 5. Salt stress response of wild-type and *JcAPX* transgenic plants during seed germination stage**.** Seeds were sown on MS medium without (**A**) and with 150 mM NaCl (**B**). Photos were taken five days after stratification. Percentage of germinating seeds grown for a period as indicated under normal condition (**C**) and NaCl treatment (**D**). Data are reported as means ± SD (n > 100) from three independent experiments. WT, wild type; T5, T12, and T19, are independent *JcAPX* transgenic lines.

After a 5-day treatment with 150 mM NaCl on MS medium, the germination ratio was 78, 82, and 73% in the T5, T12, and T19 *JcAPX* transgenic lines, respectively, while the ratio decreased to 36% in the untreated control groups. To further investigate the effects of high salinity on postgermination growth, root lengths were compared between the WT and transgenic seedlings under high salinity conditions. There were no obvious discrepancies in the morphology and growth characteristics when the plants were grown on normal MS medium (Figure 6A). However, in the presence of 150 mM NaCl, the transgenic plants were more vigorous than the WT, forming longer primary root and more lateral roots (Figure 6B).

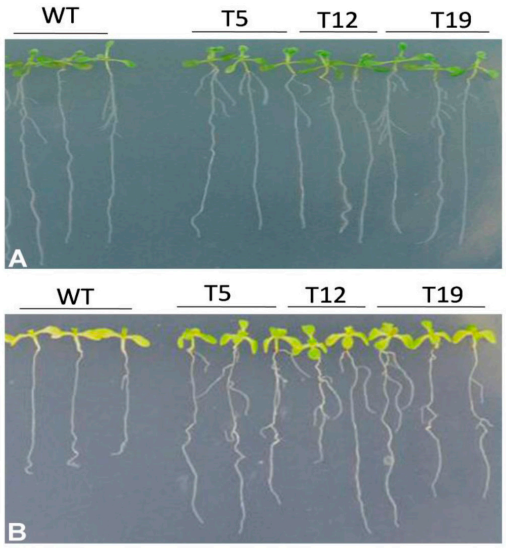

Figure 6. Transgenic plants are more tolerant than the wild-type plants to NaCl treatment. Root growth of germinated seedlings on MS without (**A**) and with 150 mM NaCl (**B**). After 48 h of stratification, seeds were grown on MS medium for 3 days, and then transferred to a medium containing 150 mM NaCl and growing vertically for additional 9 days. WT, wild type; T5, T12, and T19, are independent *JcAPX* transgenic lines.

APX activity and H_2O_2 levels under salt stress conditions

To identify the influence of salt on APX activity, we investigated the APX activity in all lines tested. As shown in Figure 7, under controlled conditions, the APX activity in the three transgenic lines was higher than in the WT plants. When exposed to 150 mM NaCl, the difference was more evident. After 24 h of treatment, the APX activity in transgenic lines increased about 0.9 times (T5), 0.4 times (T12), and 0.6 times compared with the APX activity in the WT (Figure 7).

Since the main function of APX is scavenging H_2O_2, it is necessary to detect the endogenous H_2O_2 content in transgenic plants and the WT. Therefore a histochemical staining with DAB was performed after the salt stress treatment. The results showed that, after the treatment with 150 mM NaCl, only a weak and non-specific brownish staining was detected in transgenic lines T5 and T19. DAB staining in transgenic line T12 was visible only in the internodes, whereas this coloration in the WT was very intense in the entire seedling (Figure 8A). H_2O_2 content was also quantified in plant leaves. Lines T5, T12, and T19 exhibited approxi-

mately 26, 19, and 37% lower H_2O_2 concentration, respectively, than the non-transformed control (Figure 8B). These results are consistent with those from DAB staining.

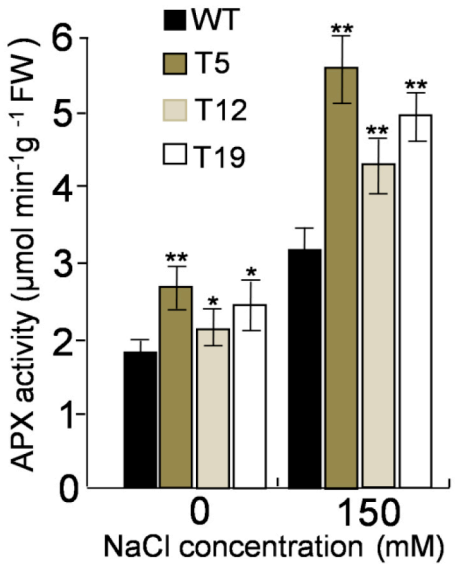

Figure 7. Activity assay of APX in transgenic and WT plants. Values are reported as means (n = 3) ± SD of four independent experiments. * and ** represent significant differences determined by the Student *t*-test at $P < 0.05$, and $P < 0.01$, respectively.

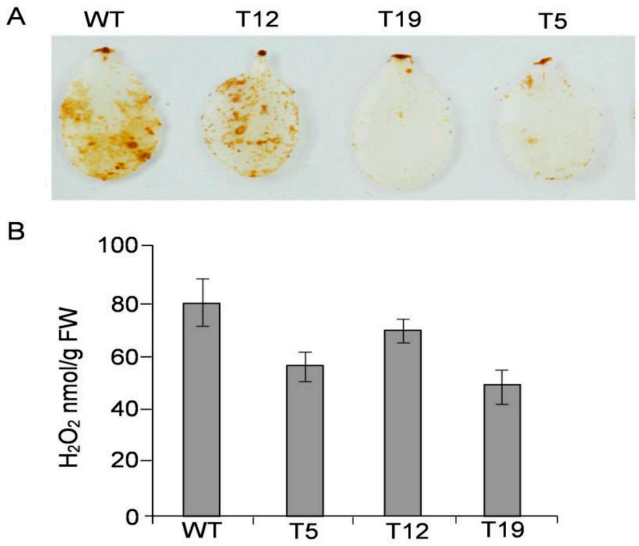

Figure 8. Effect of *JcAPX* transformations on H_2O_2 content. **A.** Detection of H_2O_2 accumulation in DAB-stained *Arabidopsis* leaves. **B.** Endogenous H_2O_2 contents in leaves. Data are reported as means (n = 3) ± SD of four independent experiments.

DISCUSSION

APXs, especially the cytosolic APX, are thought to be involved in the protection of plants against oxidative stress conditions (Shigeoka et al., 2002). It has been reported that the overexpression of *APX* is essential for effective protection of plants against oxidative damage caused by salt stress (Diaz-Vivancos et al., 2013). In the present study, we isolated a novel APX gene from *J. curcas* and examined its potential function in transgenic *Arabidopsis* under salt stress conditions. Sequence alignment of JcAPX with other plants suggests almost identical function with other reported homologous proteins. Our present data indicate that *JcAPX* is gradually and strongly induced by NaCl stress (Figure 3B). Similarly, the expression of *APX* genes from other species is upregulated under salt stress (Sun et al., 2010; Liu et al., 2014; Singh et al., 2014).

Although growth in all tested lines was suppressed under the salt stress, transgenic *Arabidopsis* lines showed significantly higher seed germination rate and evidently reduced sensitivity to NaCl in seedling stage under salt stress conditions (Figures 5 and 6). This was concurrent with previous reports of transgenic plants overexpressing the pepper APX or *Populus* pAPX genes (Sarowar et al., 2005; Sun et al., 2010; Wu et al., 2014). These results suggested that overexpression of JcAPX in transgenic lines with higher APX activity plays an effective role in alleviating this adverse stress conditions in *Arabidopsis* growth and development.

Whether under NaCl stress or not, APX activity was much higher in transgenic lines than in WT (Figure 7). High levels of APX activity can serve to directly scavenge H_2O_2 and maintain higher photosynthetic activity when plants are exposed to salt stress (Diaz-Vivancos et al., 2013). A notable symptom of oxidative damage is the marked accumulation of ROS such as H_2O_2 when plants are subjected to salt stress (Negrão et al., 2013). The accumulation of H_2O_2 due to salt stress has been reported in rice, marine macroalgae, and *Medicago sativa* (Luo and Liu, 2011; Hu et al., 2012; Mishra et al., 2013; Guo et al., 2014). Our results showed that WT plants accumulate large amounts of H_2O_2, far more than the transgenic plants under salt stress conditions (Figure 8). This phenomenon further confirmed that APX is a central component in the H_2O_2 scavenging mechanism. Furthermore, Davletova et al. (2005) also demonstrated that H_2O_2 levels increase in the absence of the cytosolic ascorbate peroxidase 1 (APX1).

In summary, the expression of *JcAPX* conferred the transgenic *Arabidopsis* seedlings to salt tolerance contents. Moreover, plants transformed displayed a greater increase in APX activity than the control plants when exposed to NaCl. Transgenic plants suppressed the accumulation of H_2O_2, providing evidence for a pivotal role of JcAPX in H_2O_2 scavenging. Therefore, *JcAPX* is a potential candidate for improving abiotic stress tolerance and could be used to engineer salt stress tolerance in crop plants.

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