Electronic Journal of Biotechnology 25 (2017) 1–8

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Over-expression of dehydroascorbate reductase enhances oxidative stress tolerance in tobacco

Lingmo Chang [1], Huan Sun [1], Hua Yang, Xuehua Wang, Zhizhe Su, Fang Chen, Wei Wei *

Key Laboratory of Bio-Resources and Eco-Environment, Ministry of Education, College of Life Sciences, Sichuan University, Chengdu 610064, China

ARTICLE INFO

Article history:
Received 27 March 2016
Accepted 24 October 2016
Available online 16 November 2016

Keywords:
Antioxidants
Ascorbic acid
DHAR
H_2O_2 stress
Jatropha curcas L.
Metabolic recycling of ascorbic acid
Oxidative damage
Quantitative RT-PCR
ROS
Salt stress
PEG stress

ABSTRACT

Background: Ascorbic acid (Asc) is one of the most abundant antioxidants and it serves as a major contributor to protect plants against oxidative damage. Plants use two enzymes that participate in the metabolic recycling of Asc. One of these two enzymes is dehydroascorbate reductase (DHAR). It directly regenerates Asc from its oxidized state and thus prevents Asc from being irreversibly hydrolyzed to 2, 3-diketogulonic acid. This study aimed to examine whether over-expression of DHAR leads to an enhanced oxidative stress tolerance in tobacco plants.
Results: In this study, we functionally characterized a novel JcDHAR gene from *Jatropha curcas* and found via quantitative RT-PCR analysis that JcDHAR can be induced with H_2O_2, salt and PEG stresses. The DHAR activities of transgenic tobacco plants increased from 2.0 to 5.3 fold compared to wild-type plants. As a result, the transgenic plants displayed enhanced tolerance to oxidative stress.
Conclusions: Our results indicate that JcDHAR expression can effectively enhance the tolerance to oxidative stress in plants.

1. Introduction

Plants are constantly exposed to an adverse environment that is typically associated with the production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), and hydroxyl radicals (OH^-) [1]. Enzymatic and non-enzymatic scavenging systems effectively reduce the damage caused by these oxide toxicities. Ascorbic acid (Asc), a potent antioxidant, is synthesized in the mitochondria of plants and then transported to other cell compartments to eliminate ROS [2,3]. In addition, various ways exist to regenerate Asc from its oxidative forms. In the chloroplast or cytosol, Asc is oxidized to the monodehydroascorbate (MDHA), which can be reduced to Asc via monodehydroascorbate reductase (MDHAR) in an NAD(P)H-dependent reaction [4]. Moreover, in the chloroplast, MDHA can also be reduced to Asc via thylakoid-associated reduced ferredoxin via an effective and rapid disproportionation to Asc and dehydroascorbate (DHA) when the pH of the lumen is low [4,5]. Furthermore, DHA is reduced to Asc by DHA reductase (DHAR) in the presence of reduced glutathione (GSH), which in turn is regenerated by Glutathione reductase (GR).

Asc represents about 10% of the total soluble carbohydrate pool and acts as an electron donor for the detoxification of ROS in plants [6,7,8]. The role that Asc plays in responding to oxidative stresses has been shown in *Arabidopsis thaliana* vtc mutants, which are impaired in Asc biosynthesis [9,10,11]. Moreover, Asc is a cofactor for some hydroxylase enzymes and regulates cell growth and division [12,13], it modulates growth and defense gene expression through hormones [14,15], and affects the flowering time in *Arabidopsis* [16].

The level of Asc is strongly controlled by synthesis, recycling, degradation and transport, while the Asc recycling pathway is particularly important for the stress response and adaptation [17,18]. For the recycling of Asc, MDHAR and DHAR are the components that are crucial to maintain the reduced Asc pool [19]. So far, several DHAR genes were successively cloned from plants such as rice [20], tobacco and wheat [21]. Asc content of plants can be increased via enhancing the expression of DHAR [21]. Over-expressing the wheat DHAR gene increased protection against oxidative damage in tobacco [22]. Eltayeb et al. [6] reported that the elevation of Asc levels via expression of *A. thaliana* DHAR provided a significantly enhanced oxidative stress tolerance to drought and salt. Over-expression of rice DHAR increased the salt tolerance of *Arabidopsis* plants [23]. The recycling of Asc via wheat DHAR expression can enhance the protection against photo

* Corresponding author.
E-mail address: wwscuwz@163.com (W. Wei).
[1] These authors contributed equally to this work.
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2016.10.009

oxidative stress [24]. Over-expression of *A. thaliana* cytosolic DHAR conferred enhanced tolerance to Al stress in tobacco [18]. Elevating ascorbate contents of potatoes via over-expression of *A. thaliana* DHAR led to higher tolerance to herbicide, drought and salt stresses [25].

Jatropha curcas L., a tropical energy plant, prefers arid environment and thus could be used to reclaim lands and many other ways. Due to its excellent anti-stress properties in several aspects, it became a focus of attention. In our study, we cloned a full-length cDNA sequence of *J. curcas* L. *DHAR* (*JcDHAR*) from *J. curcas* L. leaves. JcDHAR-expressed tobacco plants showed a significantly enhanced stress tolerance compared to wild-type plants.

2. Material and methods

2.1. Plant material and treatments

J. curcas L. seeds were germinated and grown in a growth chamber at 25°C with a 16-h light/8-h dark photoperiod. To investigate responses of *JcDHAR* to different abiotic stresses, we sprayed four-week-old seedlings or watered with 1% H_2O_2, 300 mM NaCl, 30% PEG respectively. For RNA analysis, we collected every alternative leave of seedlings at 0, 1, 6, 12 and 24 h and stored them at −80°C.

2.2. Isolation of JcDHAR cDNA and expression analysis

We isolated full-length *JcDHAR* cDNA via rapid amplification of cDNA ends (RACE) from young leaves of *J. curcas* seedlings. We used the sequence of *Nicotiana tabacum DHAR* (GenBank: AY074787) as a query sequence for homologous BLAST on NCBI. We chose two high similarity sequences, *Ricinus communis DHAR* (GenBank: XM_002522984) and *Populus trichocarpa DHAR* (GenBank: XM_002316281) for analysis with the DNAMAN software. For 3′RACE, we designed two degenerated primers (L1: 5′-GGAAYTRAAGG CMTTGGA-3′, L2: 5′-GAKGTKGCTCTTGSCCATT-3′) according to the conserved sequence regions. For 5′RACE, we designed specific primers (S1: 5′ AGATTCGAGGGAGAACAG-3′, S2: 5′-GAAATGAGTCAAGTTT TCGG-3′) according to 3′-end sequence obtained via 3′RACE. For the open reading frame (ORF), we obtained specific primers (D-S: 5′-GCATAACTTCGTATAGCAT-3′, D-R: 5′-ATAATATAAACTAGTTCTG GT-3) from the aligned sequences of 3′ and 5′ PCR products.

To assay the *JcDHAR* expression profiles, we treated one-month-old seedlings with 1% H_2O_2, 300 mM NaCl, and 30% PEG respectively. We collected leaves at 0, 1, 6, 12, and 24 h for RNA extraction. We performed quantitative RT-PCR (qRT-PCR) using *JcDHAR* gene-specific primers (F: 5′-GGAGATTGCCCATTTTGCC-3, R: 5′-GGCGTCTGGAGGAG TAACAAG-3′) and used the primers (18s-F: 5′-CAACCATAAACGATGC CGACC-3, 18s-R: 5′-CAGCCTTGCGACCATACTCCC-3′) for 18s rRNA as reference.

2.3. Plant transformation and western blot analysis

We inserted the cDNA that encodes *JcDHAR* into the binary vector pBI121 under the control of the cauliflower mosaic virus 35S promoter via the *Xba I* and *Sac I* sites. We generated transgenic tobacco plants (*Nicotiana benthamiana*) that express JcDHAR via *A. tumefaciens* transformation [26]. We closed the full-length *JcDHAR* cDNA upstream of 6xHis-tag sequence between the *NcoI* and *XhoI* sites of the pET-28 vector. The His-tagged recombinant JcDHAR protein was over-expressed and purified by Ni-NTA agarose system (QIAexpress, Qiagen) according to the manufacturer's instructions. Injecting this protein into rabbit raised antibodies against the purified recombinant JcDHAR and we used the Anti-JcDHAR antiserum for our western blot analysis. We ground plant leaves in liquid nitrogen and transferred to extraction buffer (100 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, 10 mM β-mercaptoethanol, 200 mM sucrose) followed by centrifugation at 13,000 rpm for 20 min to obtain the soluble protein. We separated the protein extracts via 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane (PVDF) via electroblotting. Following the transfer, we blocked the PVDF in 5% milk in TPBS (0.1% Tween 20, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4), then incubated with anti-JcDHAR antiserum diluted 1:5000 in TPBS with 5% milk for 1 h at room temperature. After three times of washing with TPBS every 10 min, we incubated the membrane with goat anti-rabbit horseradish peroxidase-conjugated antibodies (ZSGB-BIO, China) diluted to 1:10,000 for 1 h at 37°C. We washed the blots five times with TPBS and used chemiluminescence (Bio-Rad, USA) for the signal detected between 1 to 3 min. We estimated the protein concentration using Bradford's [28] method via bovine serum albumin as standard.

2.4. Enzyme assays

We assayed DHAR activity following the method as described in Hossain and Asada [29]. We ground 0.2 g plant leaves in liquid nitrogen and quickly transferred the powders to 5-mL centrifuge tubes containing 1 mL pre-cooled extraction buffer (50 mM KH_2PO_4/K_2HPO_4, pH 7.8, 2 mM Asc, 5 mM EDTA). We obtained the soluble protein following a 20-min centrifugation at 12,000 rpm after short vortex. Enzyme activity was followed by an increase at A_{265} after we added the matching volume of soluble protein into the reaction buffer (50 mM KH_2PO_4/K_2HPO_4, pH 7.0, 0.2 mM DHA, 2.5 mM GSH and 0.1 mM EDTA). H_2O_2 is able to pass through cell membranes and reach cell locations remote from its site of formation [30]. In order

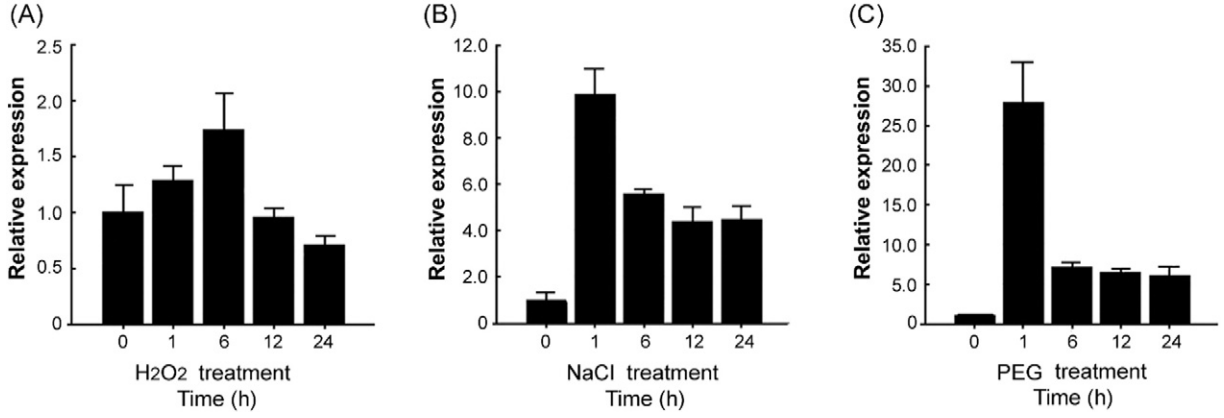

Fig. 1. Expression profile of *JcDHAR* under the treatment of 1% H_2O_2 (A), 300% NaCl (B), and 30% PEG (C) measured via quantitative real-time PCR in four-week-old *J. curcas* seedlings. Relative expression levels are shown as fold change values. Data are expressed as means ± SD.

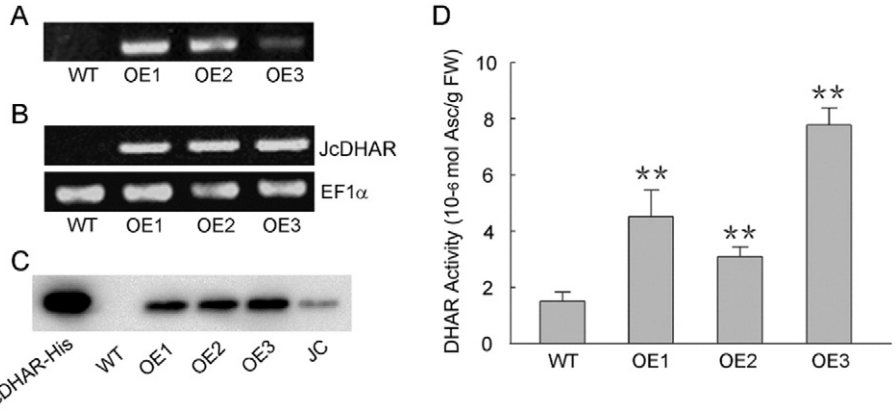

Fig. 2. Identification of JcDHAR transgenic *N. benthamiana*. (A) Identification of JcDHAR transgenic *N. benthamiana* by genome PCR with 35S-F and JcDHAR-R primers. (B) Identification of JcDHAR transgenic *N. benthamiana* by RT-PCR with JcDHAR-F1 and JcDHAR-R1. WT, wild-type. 1–3, transgenic lines. (C)Western blot of DHAR transgenic, control plants and *J. curcas* protein samples (25 mg) were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (PVDF), and applied to an immunoblotting assay. DHAR: His-tagged recombinant protein. (E) DHAR enzyme activity. WT: control plant. DHAR transgenic lines are represented by OE1, OE2, OE3. JC, *J. curcas* L. Data are expressed as means ± SD, n = 6. ** indicates significant differences in comparison to the wild type at $P < 0.01$.

to detect the activities of antioxidant enzymes under H_2O_2 stress, we treated 4-week-old tobacco plants with 1% H_2O_2 for 12 h. We collected 0.2 g of leaves for CAT, APX, GR and SOD measurements. We measured APX activity as described via measuring the decrease in absorbance at 290 nm as ascorbate is oxidized [31]. We determined GR activity from the decrease in absorbance of NADPH at 340 nm [32]. We performed SOD and CAT measurements as described in Yang et al. [33].

2.5. Analysis of ROS scavenging via H_2O_2 andO_2^- staining

To observe H_2O_2 andO_2^- accumulation in plant leaves, we treated 3-week-old tobacco leaves with 1% H_2O_2, 150 mM NaCl, and 20% PEG for 5 h, smearing the solutions onto the leaves with cotton buds. For the H_2O_2 staining, we collected leaves and infiltrated them in the DBA solution (1 mg/mL, pH 3.8) for 24 h at 25°C in the dark. After staining, we soaked the leaves in ethanol for 2 d to remove all chlorophyll. For O_2^- detection, we collected the treated seedling leaves and incubated them in the substate NBT (0.1 mg/mL) for 24 h at 25°C in the dark, and then soaked them in ethanol for 2 d to remove all chlorophyll. We used seedlings that were treated with water as control.

2.6. The response of tobacco leaves to oxidative stress

For our oxidative damage experiments, we detached leaf disks of 1.5-cm diameter from healthy and fully expanded leaves of wild-type and transgenic plants. We let all disks float in solutions of three different concentrations of H_2O_2 (0, 1% and 2%) for 72 h, and then dipped them in 95% alcohol for 48 h to detect the concentrations of chlorophyll a and b [34]. This experiment was repeated for three times. We assessed lipid peroxidation via the TBARS method described by Heath and Packer [35]. For our MDA assay [36], we ground leaf samples from plants supplemented with or without 300 mM NaCl in 5 mL of 0.1% trichlorocetic acid (TCA) and mixed with 5 mL of 0.5% thiobarbituric acid at 4°C. We boiled the homogenate for 10 min and followed with 3000 rpm centrifugation at 4°C for 10 min. We then used the cleared supernatant for our MDA assay at A_{532} and A_{600}.

2.7. Salt stress tests

We surface sterilized wild-type and transgenic seeds with 75% alcohol for 30 s, and washed with sterile water for five times. We

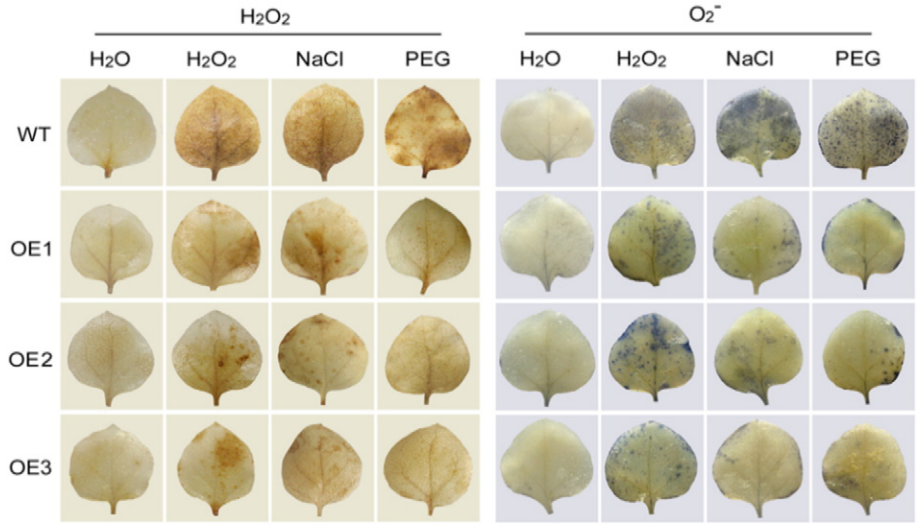

Fig. 3. Analysis of ROS accumulation in WT and transgenic plants (OE1,OE2 and OE3) in response to abiotic stress. We detected abiotic stress-induced H_2O_2 and O_2^- accumulation for 5 h via NBT and DAB staining, respectively. Three-week-old tobacco leaves smeared with H_2O_2, NaCl and Polyethylene glycol (PEG) for 5 h followed by H_2O_2 and O_2^- staining. Smeared with water, almost no H_2O_2 or O_2^- staining was detected in either WT or transgenic tobaccos (OE1, OE2, OE3). Following H_2O_2, salt and PEG treatments, H_2O_2 and O_2^- staining were notably higher in OE1, OE2, OE3 compared to WT plants.

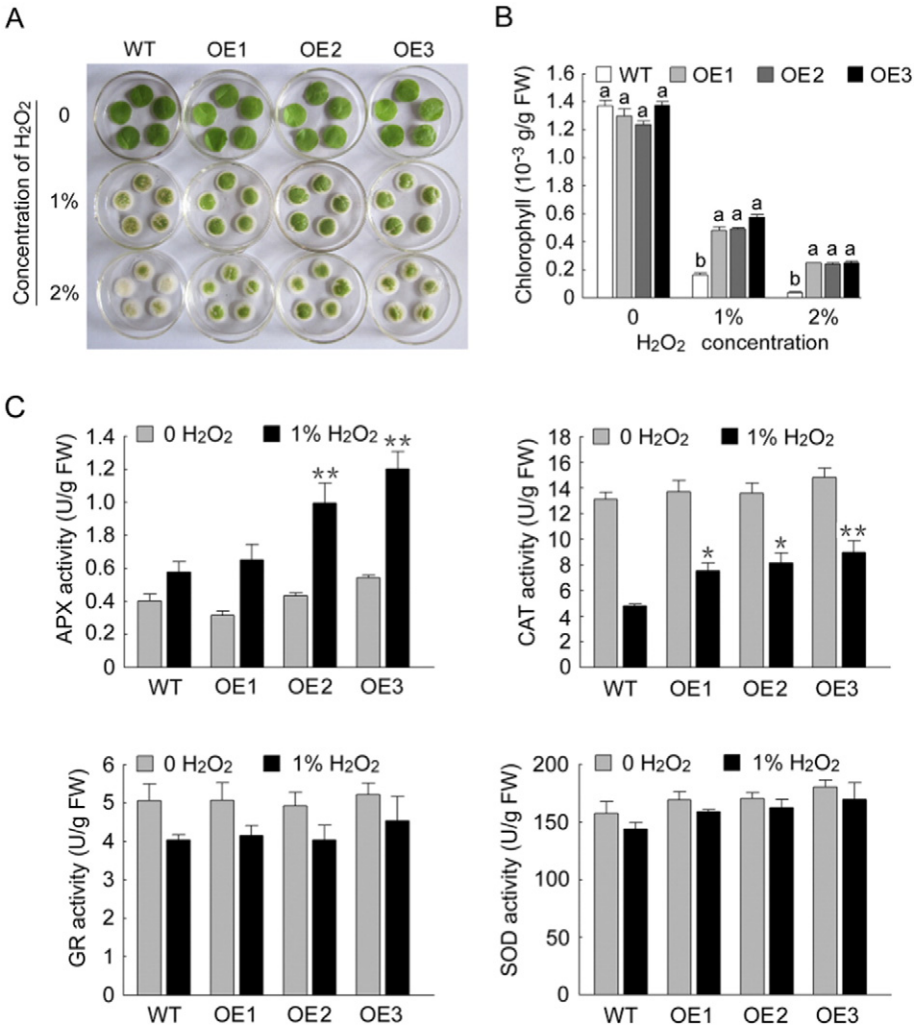

Fig. 4. Effects of oxidative stress on the performance of WT and transgenic tobacco plants (OE1, OE2, OE3). (A) Leaf disks were incubated in water and different concentrations of H_2O_2 (1% and 2%, respectively) under white light for a total of 72 h. (B) Chlorophyll content was determined in leaf disks of WT and transgenic plants following 72 h treatment of H_2O_2. Leaf disks floated in water served as control. Data are expressed as means ± SD, n = 3. Different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. (C) The influence of antioxidant enzymes in transgenic tobacco plants. The total activities of the antioxidant enzymes APX, CAT, GR and SOD in the tobacco plants treated with 1% H_2O_2 for 12 h. Data are expressed as means ± SD, n = 6. * and ** indicate significant differences in comparison to the wild type at $P < 0.05$ and $P < 0.01$, respectively.

let the sterilized transgenic seeds germinate on kanamycin-containing 1/2 MS plates and the WT seeds on antibiotic-free 1/2 MS plates. For root growth measurements, we transferred the just germinated seeds to solid 1/2 MS medium supplemented with different concentrations of NaCl (0, 100, 200, 300 mM). We oriented all plates vertically with seedlings upside down. We measured the root length after 7 d of treatment. We performed three replicates for each experiment. For our salt tolerance experiment, we transferred one-week-old seedlings to pots containing soil. We watered all plants weekly and cultured them at 25°C under 16-h light/8-h dark photoperiod with white fluorescent light (~125 μmol m^{-2} s^{-1}). We used two different growth stage plants for our study. We treated four-week-old plants with 300 mM NaCl per week for two weeks, and watered six-week-old plants with 800 mM NaCl per week for four weeks. We collected leaves from the same part of plants to analyze chlorophyll a and b contents. We weighed the plant roots after slightly washing them with water and subsequent drying in oven at 65°C. We used plants supplied with water as control.

2.8. Drought tolerance experiment

To detect the drought tolerance, we sterilized WT and transgenic plant seeds and let them germinate as described above. We transferred germinated seedlings on the plates into soil and watered them weekly. One-month-old and two-month-old healthy plants of identical size were not watered anymore, respectively. After two weeks, we photographed the plants.

3. Results

3.1. Isolation of JcDHAR full-length cDNA

For the *JcDHAR* Full-length cDNA isolation, we used the *NtDHAR* sequence to blast on NCBI and chose two homologous *DHAR* sequences of *Ricinus communis* and *Populus trichocarpa* from the blasting results. Based on the resulting three sequences, we amplified the 3′ end of a putative *JcDHAR* fragment from *J. curcas* leaves with degenerated primers using 3′-RACE, and obtained the 5′ end sequence using 5′-RACE with gene specific primers designed according to the sequence of the3′ RACE fragment. Via analyzing the aligned sequences, we obtained an 809 bp *JcDHAR* (GenBank: JF518880.1) fragment containing a 639 bp complete open reading frame (ORF), that encodes a putative protein of 212 amino acids with a predicted protein pI of 5.75 and molecular weight of 23.5 KDa.

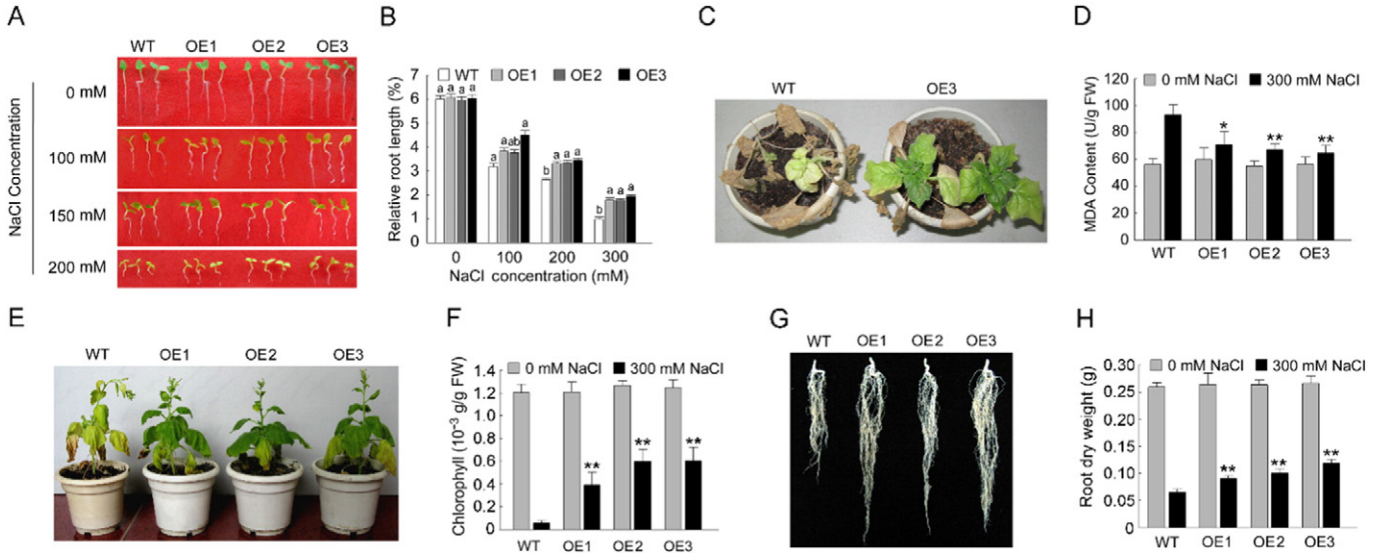

Fig. 5. *Jc*DHAR over-expression in transgenic plants leads to significant increase in the tolerance for high concentrations of NaCl. (A) Phenotypes of seedlings treated with different concentrations of NaCl (0 mM, 100 mM, 150 mM, and 200 mM, respectively). (B) We measured the corresponding relative root lengths after 7 d of NaCl treatment. Data are expressed as means ± SD, n = 10. Different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. (C) Symptoms of plants that were treated with NaCl for two weeks. (D) MDA contents of plants treated with or without NaCl after 3 d. Data are expressed as means ± SD, n = 10. * and ** indicate significant differences in comparison to the wild-type at $P < 0.05$ and $P < 0.01$, respectively. (E) Phenotypes of tobacco plants treated with 800 mM NaCl. (F) Leaf chlorophyll concentration of treated or untreated tobacco plants. Data are expressed as means ± SD, n = 10. ** indicate significant differences in comparison to the wild type at $P < 0.01$, respectively. (G) Root length of tobacco plants treated with 800 mM NaCl. (H) Root weight of treated or untreated tobacco plants. Data are expressed as means ± SD, n = 10. ** indicate significant differences in comparison to the wild type at $P < 0.01$, respectively.

3.2. Expression profiles of JcDHAR under salt, H_2O_2, PEG treatment

To identify whether *JcDHAR* could respond to several stresses, we treated one-month-old *J. curcas* seedlings with NaCl, H_2O_2 and PEG respectively, and extracted the total RNA from the leaves for qRT-PCR analysis. Fig. 1A shows *JcDHAR* transcripts gradually increasing from 0 h to 6 h and decreasing over the following hours under H_2O_2 treatment. Adding PEG or NaCl, strongly induced *JcDHAR* at 1 h, and then it quickly reduced and maintained the level over the following hours (Fig. 1B and Fig. 1C). These results indicate that *JcDHAR* responds to these three stresses.

Fig. 6. Effect of drought stress on plant performance. Photograph of a representative wild type (WT) and transgenic line OE3 grown in nutrient soil without supplying water for two weeks.

3.3. Constitutive expression of JcDHAR in transgenic tobacco

To obtain tobacco that over-expressed JcDHAR, we introduced *JcDHAR* ORF, driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter, into the genome of tobacco via *Agrobacterium tumefaciens*-mediated transformation [26]. We identified a total amount of 20 independent transgenic lines via genomic PCR and RT-PCR (data not shown) and randomly selected three lines (OE1, OE2, and OE3) of the T1 progeny for further study. Western blotting detected JcDHAR proteins in transgenic lines and *J. curcas*, while these were absent in wild type (WT) plants (Fig. 2A). A DHAR activity assay showed that transgenic lines exhibited highly increased DHAR activities of up to 3.0, 2.0 and 5.3 fold compared with WT plants, respectively (Fig. 2B). These results showed that JcDHAR was not only successfully introduced but also highly expressed in our transgenic tobaccos.

3.4. JcDHAR in transgenic tobaccos decreases the accumulation of ROS

The generation of ROS such as H_2O_2 and O_2^- usually follows stress conditions in plants [23]. To detect whether JcDHAR results in enhanced tolerance of transgenic tobaccos, we first observed the phenotype characteristics of three-week-old tobacco leaves smeared with H_2O_2, NaCl and polyethylene glycol (PEG) for 5 h followed by H_2O_2 and O_2^- staining. As major members of ROS, the accumulation of H_2O_2 and O_2^- could be discriminated via staining with diaminobenzidine (DAB) and nitro blue tetrazolium (NBT), respectively. When smeared with water, almost no H_2O_2 or O_2^- was detected in either WT or transgenic tobaccos. Following H_2O_2, salt and PEG treatments, the accumulation of H_2O_2 was notably lower in transgenic lines compared to WT plants (Fig. 3). Similar to the H_2O_2 staining, the concentration of O_2^- was significantly decreased in transgenic plants compared to wild-type plants (Fig. 3). As expected, the lower amount of ROS in transgenic plants suggests that over-expression of JcDHAR could lead to the enhanced scavenging ability of transgenic plants.

3.5. Transgenic tobaccos displayed increased tolerance to H_2O_2 stress

Having demonstrated that JcDHAR over-expressing plants can reduce ROS accumulation during H_2O_2, NaCl and PEG stress, we next focused on the H_2O_2 tolerance of leaf disks from 6-week-old plants. As shown in Fig. 4A and Fig. 4B, disks incubated in water that was free of H_2O_2 showed no significant change. When exposed to 1% or 2% concentrations of H_2O_2 for 72 h, the bleached border of wild-type disks were damaged more severely than that of transgenic disks. However, damages in wild-type plants were more severe than in the transgenic lines. To further explore the mechanism of antioxidant ability in transgenic plants, we next investigated the ROS-scavenging enzymes SOD, CAT, APX, and GR under treatment with 1% H_2O_2 (Fig. 4C). SOD and GR activities were reduced slightly without any notable difference in WT and transgenic plants. Although CAT activity decreased significantly, it was still higher in transgenic plants than in WT plants. Different to other enzymes, APX activity was increased in both transgenic plants and WT plants, and a more apparent elevation was observed in transgenic plants. These results suggest that over-expression of JcDNAR in transgenic tobacco leads to increased tolerance to H_2O_2 stress. It furthermore affected some ROS-scavenging enzyme activities to resist ROS stresses as well.

3.6. Expression of JcDHAR increases salt tolerance of transgenic tobacco plants

JcDHAR-expressing tobaccos are known for their ROS scavenging ability and strong tolerance to H_2O_2 stress. Because salt stress is a common factor for ROS production, we studied transgenic tobacco tolerance to salt stress in different growth periods. We transferred the germinated seeds of wild-type and transgenic tobaccos to 1/2 MS medium containing different concentrations of NaCl. The root lengths were similar between WT and transgenic lines when cultured on normal 1/2 MS medium, while we observed more evident growth retardation in WT compared to transgenic seedlings with increasing concentrations of NaCl (Fig. 5A and Fig. 5B). We supplemented one-week-old transgenic tobaccos with 300 mM NaCl once per week. In addition to growth retardation, WT plants lost almost all chlorophyll and some began to die in contrast to JcDHAR expressing tobaccos that performed much better (Fig. 5C). Lipid hydroperoxidation is an effective indicator for cellular oxidative damage [27]. We determined the MDA content for the amount of lipid hydroperoxide production induced by oxidative stress. After 3 d of treatment, the concentration of MDA increased significantly in WT plants compared to transgenic lines (Fig. 5D). In addition, six-week-old transgenic tobaccos were supplemented with 800 mM NaCl once per week. The extreme concentration of 800 mM NaCl first led to quick leaf wilting due to severe osmotic pressure on the plant roots in both WT and transgenic plants, but distinct differences gradually became apparent until the fourth week (Fig. 5E). WT plants almost turned yellow, while we observed a smaller intensity of symptoms in transgenic plants. We quantified these symptoms via chlorophyll content detection of the leaves (Fig. 5F). Even though the growth of both the transgenic plant roots and the wild-type plant roots, was inhibited, the roots of the transgenic plants developed better than those of the wild-type plants (Fig. 5G and Fig. 5H). These results suggest that over-expression of JcDHAR can significantly increase tolerance to salt stress in transgenic tobacco plants.

3.7. JcDHAR expressing tobaccos showed high drought tolerance

Young transgenic seedlings have effective ROS scavenging abilities that are stimulated by PEG exposure in drought. We tested the effect of water shortage on tobacco plants. Subsequent to two weeks of drought, WT plants aged one-month-old and two-month-old were not watered anymore, respectively. After two weeks, we photographed the plants wilted completely, while transgenic plants were almost not affected (Fig. 6). This indicates that JcDHAR can enhance drought tolerance in tobacco.

4. Discussion

In the present study, we cloned and described a novel gene *JcDHAR* from *J. curcas*. Expression pattern analysis and transgenic tobacco functional analysis showed that JcDHAR has the potential to increase the resistance of H_2O_2, salt and drought stress in transgenic tobacco plants.

ROS are involved in the damaging effects of various environmental stressors and they are produced in chloroplasts and mitochondria [37]. We established that H_2O_2, NaCl and PEG induce *JcDHAR* in *J. curcas* seedlings (Fig. 1), indicating *JcDHAR* as response to these stresses. In comparison with WT tobaccos, JcDHAR-expressing tobacco lines showed increased DHAR activities (Fig. 2B). According to these results, we analyzed the scavenging ability of ROS. We found remarkable differences in young plant leaves via DAB staining following smearing with H_2O_2, NaCl and PEG (Fig. 2C). In addition to self-oxidation–reduction reactions and elimination by SOD, O_2^- can also be reduced to H_2O_2 by Asc through a non-enzymatic reaction [38]. We observed O_2^- staining in the same treatments (Fig. 2C). Furthermore, as shown in Fig. 3A, leaf disks of transgenic plants displayed resistance to H_2O_2-contained water. We also detected relative enzymes actives and our results show that higher APX and CAT activities were monitored when the water was mixed with a concentration of 1% H_2O_2 (Fig. 3C). We thus speculate that JcDHAR could increase the tolerance of tobacco to oxidative stresses via eliminating H_2O_2 over time. We obtained evidence to support this hypothesis from stress tolerance experiments with DHAR over-expressing plants [18,21,24].

Plants resistance to oxidative stress induced by salt was previously reported in several studies. In strawberry leaves, NaCl stress resulted in H_2O_2/O_2^- accumulation, an increase in lipid peroxidation and carbonyl-groups content [39]. H_2O_2 accumulation was observed in the chloroplasts of leafs that were treated with NaCl [40]. In order to avoid the toxicity of H_2O_2, maintaining the level of Asc via synthesis [3] and regeneration [29] is necessary. Under salinity stress, the salt tolerant *Phaseolus aureus* showed higher levels of Asc than salt sensitive plants [41]. In Fig. 4, we show that transgenic seedlings had better root elongation at different salt concentrations. Young plants had lower MDA content, and mature transgenic plants before flowering at the condition of 800 mM NaCl could still display a better state than WT. The improved performance of DHAR transgenic plants can be attributed to the elevated level of Asc, which effectively removes H_2O_2 and is induced by salt stress. Similar conclusions were confirmed by Kwon et al. [42] and Ushimaru et al. [23].

We found *JcDHAR* to be induced by PEG (Fig. 1C) and enhanced JcDHAR supplies transgenic plants with better protection against drought stress (Fig. 2C). Rice DHAR activity was higher during drought stress [43]. Drought significantly increased the efficiency of antioxidant systems and the levels of Asc content [44]. Eltayeb et al. [6] reported that the *DHAR* transgenic plants exhibited an enhanced tolerance to drought and PEG stresses. However, it has been reported that suppressing *DHAR* expression confers drought tolerance while higher Asc redox states in plants exhibited greater water loss when exposed to drought [45]. An increase in the Asc redox state has been shown to reduce guard cell responsiveness to H_2O_2, which was the trigger of stomatal closure exhibiting lower CO_2 assimilation after severe water stress. This caused leaf wilting, whereas an increased net photosynthesis was monitored in

transgenic plants [6] and no wilted leafs appeared in our JcDHAR expressing plants during drought stress. These seem to be conflicting results. However, Chen and Gallie [22] reported that decreasing foliar Asc limits ROS diffusion by increasing guard cell responsiveness (i.e. enhanced avoidance) while decreasing the ability of leaf cells to detoxify the ROS that enter (i.e. reduced tolerance). In addition, transpiration of plants is not only affected by stomatal movements but is also limited by the condition of root growth. In this study, the root growth of WT plants was more affected under salt stress than in transgenic plants (Fig. 4G). This means that over-expression of JcDHAR enhances plant root tolerance against oxygen toxicity and well-developed root systems have a stronger capacity of water absorption to counter the water loss due to transpiration.

In summary, we characterized the functions of *JcDHAR* in enhancing tolerance to different stressors. Our results suggest that *JcDHAR* effectively increases the resistance to H_2O_2, salt and drought stress in plants. We furthermore suggest that future studies focus on the impact of the whole plant growth under stress conditions.

Financial support

This study was supported by grants from the National Science Foundation of China (no.31071448), and Application of Sichuan Province Based Project (no. 2014JY0051).

Conflicts of interest

The authors declare no conflict of interest.

References

[1] Scandalios JG. Oxygen stress and superoxide dismutases. Plant Physiol 1993; 101:7–12.
[2] Horemans N, Foyer CH, Asard H. Transport and action of ascorbate at the plant plasma membrane. Trends Plant Sci 2000;5:263–7. http://dx.doi.org/10.1016/S1360-1385(00)01649-6.
[3] Wheeler GL, Jones MA, Smirnoff N. The biosynthetic pathway of vitamin C in higher plants. Nature 1998;393:365–9. http://dx.doi.org/10.1038/30728.
[4] Asada K. The water–water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol 1999;50: 601–39. http://dx.doi.org/10.1146/annurev.arplant.50.1.601.
[5] Miyake C, Asada K. Ferredoxin-dependent photoreduction of the monodehydroascorbate radical in spinach thylakoids. Plant Cell Physiol 1994;35:539–49.
[6] Eltayeb AE, Kawano N, Badawi GH, Kaminaka H, Sanekata T, Morishima I, et al. Enhanced tolerance to ozone and drought stresses in transgenic tobacco overexpressing dehydroascorbate reductase in cytosol. Physiol Plant 2006;127: 57–65. http://dx.doi.org/10.1111/j.1399-3054.2006.00624.x.
[7] Noctor G, Foyer C. Ascorbate and glutathione: Keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 1998;49:249–79. http://dx.doi.org/10.1146/annurev.arplant.49.1.249.
[8] Smirnoff N, Wheeler GL. Ascorbic acid in plants: Biosynthesis and function. Crit Rev Biochem Mol Biol 2000;35:291–314. http://dx.doi.org/10.1080/10409230008984166.
[9] Conklin PL, Williams EH, Last RL. Environmental stress sensitivity of an ascorbic acid-deficient *Arabidopsis* mutant. Proc Natl Acad Sci U S A 1996;93:9970–4. http://dx.doi.org/10.1073/pnas.93.18.9970.
[10] Huang CH, He W, Guo JK, Chang XX, Su PX, Zhang LX. Increased sensitivity to salt stress in an ascorbate-deficient *Arabidopsis* mutant. J Exp Bot 2005;56:3041–9. http://dx.doi.org/10.1093/jxb/eri301.
[11] Muller-Moule P, Golan T, Niyogi KK. Ascorbate-deficient mutants of *Arabidopsis* grow in high light despite chronic photooxidative stress. Plant Physiol 2004;134: 1163–72. http://dx.doi.org/10.1104/pp.103.032375.
[12] Arrigoni O, De Tulio MC. The role of ascorbic acid in cell metabolism: Between gene-directed functions and unpredictable chemical reactions. J Plant Physiol 2000;157: 481–8. http://dx.doi.org/10.1016/S0176-1617(00)80102-9.
[13] Matamoros MA, Loscos J, Coronado MJ, Ramos J, Sato S, Testillano PS, et al. Biosynthesis of ascorbic acid in legume root nodules. Plant Physiol 2006;141: 1068–77. http://dx.doi.org/10.1104/pp.106.081463.
[14] Kerchev PI, Pellny TK, Vivancos PD, Kiddle G, Hedden P, Driscoll S, et al. The transcription factor ABI4 is required for the ascorbic acid-dependent regulation of growth and regulation of jasmonate-dependent defense signaling pathways in *Arabidopsis*. Plant Cell 2011;23:3319–34. http://dx.doi.org/10.1105/tpc.111.090100.
[15] Pastori GM, Kiddle G, Antoniw J, Bernard S, Veljovic-Jovanovic S, Verrier PJ, et al. Leaf vitamin C contents modulate plant defense transcripts and regulate genes that control development through hormone signaling. Plant Cell 2003;15:939–51. http://dx.doi.org/10.1105/tpc.010538.
[16] Kotchoni SO, Larrimore KE, Mukherjee M, Kempinski CF, Barth C. Alterations in the endogenous ascorbic acid content affect flowering time in *Arabidopsis*. Plant Physiol 2009;149:803–15. http://dx.doi.org/10.1104/pp.108.132324.
[17] Stevens R, Page D, Gouble B, Garchery C, Zamir D, Causse M. Tomato fruit ascorbic acid content is linked with monodehydroascorbate reductase activity and tolerance to chilling stress. Plant Cell Environ 2008;31:1086–96. http://dx.doi.org/10.1111/j.1365-3040.2008.01824.x.
[18] Yin L, Wang S, Eltayeb AE, Uddin MI, Yamamoto Y, Tsuji W, et al. Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminum stress in transgenic tobacco. Planta 2010;231:609–21. http://dx.doi.org/10.1007/s00425-009-1075-3.
[19] Eltayeb AE, Kawano N, Badawi GH, Kaminaka H, Sanekata T, Shibahara T, et al. Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses. Planta 2007; 225:1255–64. http://dx.doi.org/10.1007/s00425-006-0417-7.
[20] Urano J, Nakawaga T, Maki Y, Masumura T, Tanaka K, Murata N, et al. Molecular cloning and characterization of a rice dehydroascorbate reductase. FEBS Lett 2000; 466:107–11. http://dx.doi.org/10.1016/S0014-5793(99)01768-8.
[21] Chen Z, Young TE, Ling J, Chang SC, Gallie DR. Increasing vitamin C content of plants through enhanced ascorbate recycling. Proc Natl Acad Sci U S A 2003;100:3525–30. http://dx.doi.org/10.1073/pnas.0635176100.
[22] Chen Z, Gallie DR. Increasing tolerance to ozone by elevating foliar ascorbic acid confers greater protection against ozone than increasing avoidance. Plant Physiol 2005;138:1673–89. http://dx.doi.org/10.1104/pp.105.062000.
[23] Ushimaru T, Nakagawa T, Fujioka Y, Daicho K, Naito M, Yamauchi Y, et al. Transgenic *Arabidopsis* plants expressing the rice dehydroascorbate reductase gene are resistant to salt stress. J Plant Physiol 2006;163:1179–84. http://dx.doi.org/10.1016/j.jplph.2005.10.002.
[24] Chen Z, Gallie DR. Dehydroascorbate reductase affects non-photochemical quenching and photosynthetic performance. J Biol Chem 2008;283:21347–61. http://dx.doi.org/10.1074/jbc.M802601200.
[25] Eltayeb AE, Yamamoto S, Habora MEE, Yin LN, Tsujimoto H, Tanaka K. Transgenic potato overexpressing *Arabidopsis* cytosolic *AtDHAR1* showed higher tolerance to herbicide, drought and salt stresses. Breed Sci 2011;61:3–10. http://dx.doi.org/10.1270/jsbbs.61.3.
[26] Clough SJ, Bent AF. Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 1998;16:735. http://dx.doi.org/10.1046/j.1365-313x.1998.00343.x.
[27] Yoshimura K, Miyao K, Gaber A, Takeda T, Kanaboshi H, Miyasaka H, et al. Enhancement of stress tolerance in transgenic tobacco plants overexpressing *Chlamydomonas glutathione* peroxidase in chloroplasts or cytosol. Plant J 2004;37: 21–33. http://dx.doi.org/10.1046/j.1365-313X.2003.01930.x.
[28] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54. http://dx.doi.org/10.1016/0003-2697(76)90527-3.
[29] Hossain MA, Asada K. Purification of dehydroascorbate reductase from spinach and its characterization as a thiol enzyme. Plant Cell Physiol 1984;25: 85–92.
[30] Foyer CH, Lopez-Delgado H, Dat JF, Scott IM. Hydrogen peroxide-and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. Physiol Plant 1997;100:241–54. http://dx.doi.org/10.1111/j.1399-3054.1997.tb04780.x.
[31] Nakano YAK. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 1981;22:867–80.
[32] Foyer CH, Halliwell B. The presence of glutathione and glutathione reductase in chloroplasts: A proposed role in ascorbic acid metabolism. Planta 1976;133:21–5. http://dx.doi.org/10.1007/BF00386001.
[33] Yang L, Tang R, Zhu J, Liu H, Mueller-Roeber B, Xia H, et al. Enhancement of stress tolerance in transgenic tobacco plants constitutively expressing AtIpk2β, an inositol polyphosphate 6-/3-kinase from *Arabidopsis thaliana*. Plant Mol Biol 2008; 66:329–43. http://dx.doi.org/10.1007/s11103-007-9267-3.
[34] Xue X, Wu Y. Chlorophyll content determination and its relationship with spad value in wheat. Hubei Agric Sci 2010;49:2701–2.
[35] Heath RL, Packer L. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 1968;125:189–98. http://dx.doi.org/10.1016/0003-9861(68)90654-1.
[36] Qin YR, Nong YC, Huang JB, Pan ZX. The comparison of contents of malondialdehyde and proline in area of karst in northweastern Guang province. Agric Sci Technol 2011;12:469–73.
[37] Apse MP, Blumwald E. Engineering salt tolerance in plants. Curr Opin Biotechnol 2002;13:146–50. http://dx.doi.org/10.1016/S0958-1669(02)00298-7.
[38] Arrigoni O, De Tullio M. Ascorbic acid: Much more than just an antioxidant. Biochim Biophys Acta Gen Subj 2002;1569:1–9. http://dx.doi.org/10.1016/S0304-4165(01)00235-5.
[39] Tanou G, Molassiotis A, Diamantidis G. Induction of reactive oxygen species and necrotic death-like destruction in strawberry leaves by salinity. Environ Exp Bot 2009;65:270–81. http://dx.doi.org/10.1016/j.envexpbot.2008.09.005.
[40] Yamane K, Taniguchi M, Miyake H. Salinity-induced subcellular accumulation of H_2O_2 in leaves of rice. Protoplasma 2012;249:301–8. http://dx.doi.org/10.1007/s00709-011-0280-7.

[41] Misra N, Ansari MS, Gupta AK. Differential response of scavenging of reactive oxygen species in green gram genotype grown under salinity stress. Am J Plant Physiol 2006;1:41–53. http://dx.doi.org/10.3923/ajpp.2006.41.53.
[42] Kwon SY, Choi SM, Ahn YO, Lee HS, Lee HB, Park YM, et al. Enhanced stress-tolerance of transgenic tobacco plants expressing a human dehydroascorbate reductase gene. J Plant Physiol 2003;160:347–53. http://dx.doi.org/10.1078/0176-1617-00926.
[43] Sharma P, Dubey RS. Drought induces oxidative stress and enhances the activities of antioxidant enzymes in growing rice seedlings. Plant Growth Regul 2005;46:209–21. http://dx.doi.org/10.1007/s10725-005-0002-2.
[44] Lei YB, Yin C, Li CY. Differences in some morphological, physiological, and biochemical responses to drought stress in two contrasting populations of *Populus przewalskii*. Physiol Plant 2006;127:182–91. http://dx.doi.org/10.1111/j.1399-3054.2006.00638.x.
[45] Chen Z, Gallie DR. The ascorbic acid redox state controls guard cell signaling and stomatal movement. Plant Cell 2004;16:1143–62. http://dx.doi.org/10.1105/tpc.021584.