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Research article

The functional identification of glycine-rich TtASR from *Tetragonia tetragonoides* (Pall.) Kuntze involving in plant abiotic stress tolerance

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ABSTRACT

In this study, we reported on an *ASR* gene (*TtASR*) related to salt/drought tolerance from the edible halophyte *Tetragonia tetragonoides* (Pall.) Kuntze (Aizoaceae). A phylogenetic analysis revealed that TtASR was evolutionarily close to other two halophytic glycine-rich ASR members, SbASR-1 (from *Salicornia brachiate)* and SlASR (from *Suaeda liaotungensis*), with a typical abscisic acid (ABA)/water-deficit stress (WDS) domain at C-terminal. Quantitative RT-PCR analyses showed that *TtASR* was expressed in all tested different organs of the *T. tetragonoides* plant and that expression levels were apparently induced after salt, osmotic stress, and ABA treatments in *T. tetragonoides* seedlings. An induction of TtASR improved the growth performance of yeast and bacteria more than the control under high salinity, osmotic stress, and oxidative stress. TtASR was not a nuclear-specific protein in plant, and the transcriptional activation assay also demonstrated that TtASR could not activate reporter gene's expression in yeast. *TtASR* overexpressed Arabidopsis plants exhibited higher tolerance for salt/drought and oxidative stresses and lower ROS accumulation than wild type (WT) plants, accompanied by increased CAT, SOD activities, higher proline content, and lower MDA content *in vivo*. The results indicated that the *TtASR* was involved in plant responses to salt and drought, probably by mediating water homeostasis or by acting as ROS scavengers, and that it decreased the membrane damage and improved cellular osmotic adjustment that respond to abiotic stresses in microorganisms and plants.

1. Introduction

Tetragonia tetragonoides (Pall.) Kuntze, also called New Zealand spinach, belongs to Tetragonoideae, Aizoaceae. As an edible and pharmaceutical vegetable, *T. tetragonoides* is better adapted to high salinity than glycophytic vegetables and crops and can be planted in high-salinity paddy soil caused by seawater flowing backward to reduce the salt content and improve the soil on some coastal farms (Wilson et al., 2000; Wang, 2017). Salt-induced suppression of crop yield is a major issue in the agricultural economy. Halophytes are considered to be potential germplasms for saline agriculture since they possess a strong capacity to thrive under extremely saline conditions (Mishra and Tanna, 2017). As salinity has multiple effects on plants, plants have developed many mechanisms to tolerate this stress, which can be classified into three main categories: osmotic tolerance, ion exclusion, and tissue tolerance (Roy et al., 2014), or more broadly, as the osmotic stress response and the ionic stress response (Zhu, 2002; Munns and Tester, 2008). Several types of genes contributing to plant salt-tolerance have been identified (Zhu, 2002; Roy et al., 2014). In general, these genes trigger protective roles in plant cells from high salinity through Na^+/K^+ absorption, translocation/redistribution, and exclusion, mainly via transporters such as *NHX* (Jiang et al., 2010) and *HKT*

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(Hamamoto et al., 2015). Therefore, the genes can alleviate the toxic effects of high salt ions. Furthermore, some metabolic enzymes, protective proteins, and transcription factors have been proposed to be involved in salt tolerance or salt resistance (Roy et al., 2014).

The *ASR* (*ABA, stress, and ripening*) families that have been identified to date are plant-specific and usually contain a few members in a variety of plant genomes, including monocots, dicots, herbs, and xylophyta except Brassicaceae (including Arabidopsis) (González and Iusem, 2014; Li et al., 2017a). Typically, the ASR proteins often contain a highly conserved abscisic acid (ABA)/water-deficit stress (WDS) domain (PF02496 in PFAM) (Canel et al., 1995) and this domain has been suggested to play some specific biochemical roles in cellular activities due to its considerable accessibility to water molecules (Maskin et al., 2001). ASRs are often heat-stable, highly hydrophilic, and low in molecular weight. Most ASRs feature variable N-terminals possessing His-rich motifs and shorter C-terminals with putative nuclear targeting signals (Cakir et al., 2003). Decades of research has shown that ASRs can act not only as molecular chaperones to protect the activities of cellular components and maintain the basal metabolism under cellular water-deficit but also as transcription factors that regulate gene expression during stress response and improve plant stress resistance (Dominguez and Carrari, 2015). In general, the biological function of plant ASRs could be summarized as the following: (1) regulation of the fruit ripening (Picton et al., 1993) or pollen maturation (Wang et al., 1998), mainly involving in physiological water loss of plant tissue; (2) responding to abscisic acid (ABA) or abiotic stress, mainly salinity and drought (Yang et al., 2005), or cold stress (Hsu et al., 2011); alleviating metal toxicity by transcriptional regulation (Arenhart et al., 2016) or iron-chelation (Zhang et al., 2019); and (3) controlling metabolism of sugar and amino acids (Dominguez and Carrari, 2015) and reactive oxygen species scavenging (Li et al., 2013; Zheng et al., 2019).

There is increasing evidence to suggest that ASR proteins play important roles in halophyte or drought-enduring plant responses to multiple abiotic stresses, especially salt and drought tolerance (Fischer et al., 2011; González and Iusem, 2014), which is mainly involved in water deficits. ASR members from wild or special habitat plant species have received a huge amount of attention in plant abiotic stress tolerance studies in recent years. For example, a glycine-rich type ASR from the extreme halophyte *Salicornia brachiata*, *SbASR-1*, has been proven to enhance drought and high-salinity tolerance in transgenic tobacco and groundnut (Jha et al., 2012; Tiwari et al., 2015). The extreme halophyte *Suaeda liaotungensis* K. *SlASR* that is induced by water-deficit stress and ABA treatment and overexpression of *SlASR* in Arabidopsis has also been shown to enhance drought and salt tolerance in transgenic lines (Hu et al., 2014). Moreover, a foxtail millet ASR gene, *SiASR4*, was induced by high salinity/osmotic stresses and ABA challenges. When it was overexpressed in Arabidopsis and foxtail millet plants, *SiASR4* enhanced the drought and osmotic stress tolerance in transgenic plants by activating the antioxidant system and the expression levels of stress-responsive genes (Li et al., 2017a). A jujube *ASR* gene *ZnAsr1* was shown to improve the osmotic drought tolerance and survival of bacterium under PEG challenges under the heterologous expression of the recombinant ZnAsr1 in *Escherichia coli* (Padaria et al., 2016). Barley *ASR* gene *HvASR5* was able to confer drought and salt stress tolerance to *Arabidopsis thaliana* and to improve growth performance of rice plants under stress conditions (Pérez-Díaz et al., 2019). We previously isolated an *ASR* cDNA (*IpASR*) from the *Ipomoea pes-caprae* seedlings cDNA library (Zheng et al., 2018), and *IpASR* has been shown to play pivotal roles in the salt and drought tolerance of *I. pes-caprae*. In short, although the exact biological function of new *ASR* members remains to be established, most plant *ASRs* have been shown to play an important role in improving plant adaptability to different abiotic stresses, mainly those involved in water-deficit stresses.

There have been no reports to date about the salt tolerance mechanisms of the halophyte *T. tetragonoides*; in particular, no studies have been conducted on the participation of the glycine-rich ASR of *T. tetragonoides* in abiotic stress responses. Therefore, this study presented for the first time the functional characterization of an *ASR* member in *T. tetragonoides* (*TtASR*). The transcript patterns of *TtASR* in response to high salinity/osmotic stress and ABA treatment were characterized, and the transactivation activity and subcellular localization of TtASR protein was also studied. The overexpression of *TtASR* was used to evaluate its ability to provide salt and drought tolerance using morphological, molecular, and biochemical assessments. This was the first study describing the characterization of an *ASR* member from *T. tetragonoides*, and our results could pave the way for greater understanding of the potential applications of the glycine-rich *TtASR* in engineering drought- and salt-tolerant vegetables and crops.

2. Materials and methods

2.1. Plant materials, growth conditions, and stress treatments

The *T. tetragonoides* seeds were gathered from Wenchang city in the Hainan province of China. The *T. tetragonoides* seeds were dried in a lab drying oven under 37 °C, and the seedlings were cultivated with nutrient-rich soil until they flowered and their seeds matured indoors or outdoors. Briefly, the seeds were first sterilized with 70% ethanol and then placed into a Murashige & Skoog (MS) basal salt liquid medium. After 2 days of germination in an incubator (37 °C), the sprouted seeds were distributed into plates filled with sand and nutrient-rich soils in a greenhouse at 26 ± 1 °C and kept for 16 h in the light and 8 h in the dark, or outdoors with vermiculite and nutrient solution used as the growth matrix. The seedlings of *T. tetragonoides* were then used for stress treatment assays to check the expression patterns of *TtASR*. Subsequently, watering plants with 300 mM NaCl or 300 mM mannitol was adopted to simulate high salt or dehydration treatments, and ABA (0.1 mM, leaf spray and root soaking) was administered to the *T. tetragonoides* seedlings for hormone treatment, to detect the expression pattern of *TtASR* under the above challenges. The aerial part (leaves and stems) and roots of *T. tetragonoides* were then harvested, immediately treated with liquid nitrogen afterward, and then stored in an ultra-low temperature freezer for future use.

Arabidopsis thaliana (ecotype Col-0) plants were grown as previously described (Zheng et al., 2018). Transgenic plants were generated using the floral dip method as previously described (Clough and Bent, 1998). Transformed plants were selected using Basta (30 μL 13.5% Basta solution added to 100 mL MS medium, Bayer, Germany) media plates. Three T3 homozygous transgenic lines were selected for functional analysis, and all the seeds used to perform phenotypic assays, including the wild type (WT), were same-batch-harvested. To identify the physiological indicators in Arabidopsis, the transgenic lines and the WT (3-week-old) assays planted in soils were immersed in 200 mM NaCl or 300 mM mannitol solutions for 24 h. The leaves were then harvested until used for detecting the physiological indices.

2.2. Isolation of the full-length TtASR cDNA

A full-length cDNA library from *T. tetragonoides* was constructed using the SMART™ cDNA Library Construction Kit (Clontech, Takara Bio USA), with the *Saccharomyces cerevisiae* expression vector pYES-DEST52 used as the vector of library. Ninety-six single colonies were picked randomly and then sequenced, and we found that one of the cDNA sequences was homologous to other plant *ASRs*. This cDNA sequence was named *TtASR* (accession no.: MH454101).

2.3. Sequence analysis of TtASR gene

The full-length *TtASR* cDNA sequence was translated online via the ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/). A highly conserved ABA/WDS domain was identified (151–228) using the PFAM database (http://xfam.org/). The Phylogenetic Analysis by Maximum

Likelihood (PAML) method for phylogenetic tree reconstruction was performed with MEGA 6. The grand average of hydropathy (GRAVY) and other physical and chemical properties for TtASR were predicted with the Expasy program (http://www.expasy.org/tools).

The amino acid sequence of TtASR (accession no.: QBF58646.1) was aligned with several known plant ASR members using ClustalW software (http://clustalw.ddbj.nig.ac.jp/). The amino acid sequences of the plant ASRs used were as follows: SbASR-1 from *S. brachiata*; SlASR from *S. liaotungensis*; CqASR from *Chenopodium quinoa*; SoASR from *Spinacia oleracea*; CsoASR from *Calystegia soldanella*; InASR from *Ipomoea nil*; IpASR from *Ipomoea pes-caprae*; AcASR from *Ananas comosus*; CsiASR from *Camellia sinensis*; MiASR from *Musa itinerans*; ZmASR from *Ziziphus mauritiana*; PpASR from *Prunus persica*; PmASR from *Prunus mume*; PaASR from *Prunus armeniaca*; PaASR from *Prunus avium*; PtASR from *Pinus taeda*; VpASR from *Vitis pseudoreticulata*; CcASR from *Cajanus cajan*; ScASR from *Solanum chacoense*; SpASR from *Solanum peruvianum*; SlASR from *Solanum lycopersicum*; TuASR from *Triticum urartu*; GbASR from *Ginkgo biloba*; PsASR from *Picea sitchensis*; and PwASR from *Picea wilsonii*.

2.4. Functional identification of TtASR in microorganism

The recombinant plasmids TtASR-pYES-DEST52 and pYES2 (as a negative control) were then transformed into the *Saccharomyces cerevisiae* strains W303, *yap1Δ*, and *skn7Δ*. The yeast wild-type strain W303 was provided by Zhou et al. (2015). The H_2O_2-sensitive mutant yeast strains *yap1Δ* (Y00569) and *skn7Δ* (Y02900) were obtained from Euroscarf (http://www.euroscarf.de/index.php?name=News). The yeast spot assay for NaCl or H_2O_2 tolerance were performed as previously described (Zheng et al., 2018).

The GST-TtASR fusion protein was inductively expressed in *E. coli* to further identify the biological function of TtASR in unicellular organisms. The CDS of *TtASR* was PCR-amplified using the primer pair (TtASREPF and TtASREPR) listed in Table S1. In brief, the PCR fragments were subsequently inserted into the *Bam*HI site of pGEX 6p-1, and then the recombinant plasmid and pGEX 6p-1 (as control) were transformed into *E. coli* BL21 (DE3). The GST-TtASR or GST proteins were induced as previously described (Zheng et al., 2018). The *E. coli* spot assay and growth curve assay to test the abiotic stress tolerance were also performed as described in our previous study (Zheng et al., 2018).

2.5. Analysis of TtASR's expression pattern in T. tetragonoides

The total RNA's isolation and cDNA synthesis were performed according to our previous study (Zheng et al., 2018). Quantitative reverse transcription PCR (qRT-PCR) was conducted using a model 7500 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific Inc., USA) and TransStart Tip Green qPCR SuperMix (TransGen Biotech, Beijing, China). To examine the expression levels of *TtASR* in various organs of seedlings and adult *T. tetragonoides* plants, including the root, stem, leaf, flower, and unmatured seed, qRT-PCR was first performed. The *T. tetragonoides* seedling samples (roots, stems, and leaves) were treated with salt (300 mM NaCl) and osmotic stress (300 mM mannitol), and the ABA (0.1 mM ABA) was also checked to examine the expression changes of *TtASR*. All of the gene expression data obtained via qRT-PCR was normalized to the expression of *TtACT* (NCBI accession no.: MH33308). The primers (TtACTRTF/TtACTRTR and TtASRRTF/TtASRRTR) used for qRT-PCR are listed in Table S1.

2.6. Analysis of transcriptional activation activities in yeast

The full-length CDSs of TtASR and three truncated TtASRs were cloned into vector pGBKT7 (Clontech, USA) for transcription activation analysis. The primers used for plasmid construction are shown in Table S1. These constructs, along with the negative control pGBKT7 plasmid or positive control pGADT7-IpASR plasmid, were transformed into the *Saccharomyces cerevisiae* strain AH109. The β-galactosidase colony assay was performed according to our previous study (Zheng et al., 2018).

2.7. Generation of transgenic plants and subcellular localization analysis of TtASR

To generate the recombinant vector for the overexpression assay in Arabidopsis, the CDS of the *TtASR* cDNA was PCR-amplified using the primer pair TtASROXGF and TtASROXGR (Table S1). The PCR product was cloned into the *Eco*RI and *Bam*HI sites of plant expression vectors pEGAD (Cutler et al., 2000) to generate *TtASR*-pEGAD (Fig. S1) followed the in-fusion technique (BD In-Fusion PCR Cloning Kit, Takara Bio USA). After sequencing confirmation, the constructs (*TtASR*-pEGAD and pEGAD as a control) were transferred into *Agrobacterium tumefaciens* GV3101 and then transformed into Arabidopsis using the floral dip method (Clough and Bent, 1998). Positive transgenic plants were confirmed by genomic PCR with the primer pair TtASROXGF/TtASROXGR. Finally, T3 homozygous transgenic and WT seeds were germinated, and the plant tolerance assays were performed. GFP fluorescence was recorded with a camera fitted to a confocal laser scanning microscope (Zeiss LSM700) with T3 homozygous transgenic Arabidopsis (conferring *TtASR*-pEGAD and pEGAD) seedlings.

2.8. Phenotypic analysis of transgenic Arabidopsis plants

The seed germination rates of *TtASR* transgenic Arabidopsis (*TtASR OX3*, *TtASR OX5*, and *TtASR OX6*) were detected under the challenges of NaCl (200 mM and 300 mM), mannitol (200 mM and 300 mM), and H_2O_2 (2 mM and 4 mM) to detect the positive effect of *TtASR*'s overexpression in improving the salt/osmotic and oxidative tolerance of transgenic Arabidopsis seed germination. Moreover, the roots' length was also calculated to evaluate the influences of *TtASR*'s overexpression on transgenic Arabidopsis seedlings under the above abiotic stresses (150 mM and 200 mM NaCl; 200 mM, 300 mM, and 400 mM mannitol; 3 mM and 5 mM H_2O_2). The WT Arabidopsis plant was used as a control.

Salt and drought tolerance assays were also performed on transgenic Arabidopsis adult plants. Both the WT and transgenic seeds were grown on an MS medium. Ten-day-old seedlings were planted in sieve-like square pots filled with soil mixture and then adequately watered. Thirty plants of each genotype were cultured in a growth chamber as described above without watering for 14 days. Then, plants were started on the following assays. For the drought tolerance assays, WT and transgenic plants (*TtASR OX3*, *TtASR OX5*, and *TtASR OX6*) were kept in a continuous drought for 7 days and then re-watered for 7 days. For the salt tolerance assays, plants of each genotype were planted in sieve-like pots and then adequately watered as described for the drought tolerance treatment. Water was withheld for 14 days before irrigation with NaCl solution (150 mM) from the bottom of the plates. When the soil was completely saturated with salt water, then NaCl solution was removed, and the plants were cultured normally. The plants were grown in the salt-soused soils for 7 days and then photographed or collected for chlorophyll measurement.

2.9. Physiological and histochemical analysis of transgenic Arabidopsis plants

Rosette leaves were collected after high salinity treatment, and the total chlorophyll content was measured with a spectrophotometer according to the method described by Porra et al. (1989). The 4-week-old TtASR OX and WT plants were soaked with 200 mM NaCl or 300 mM mannitol for 24 h to simulate the high-salinity and drought stresses, and then the rosette leaves were harvested. We then performed the following physiological indices detection. Free proline content, malondialdehyde (MDA) content, and superoxide dismutase (SOD) and catalase (CAT) activities were determined using proline, MDA, SOD, and Catalase Assay Kits (Nanjing Jiancheng, China), according to the manufacturer's instructions, respectively.

The 4-week-old TtASR OX and WT seedlings were then transferred into 200 mM NaCl or 300 mM mannitol solutions for 24 h to simulate the high-salinity and drought stresses. *In situ* detection of H_2O_2 and O_2^- was determined with NBT or DAB staining as previously described (Zheng et al., 2018).

2.10. Statistical analysis

The experiments in this study were performed at least three times, and the numerical data from the three repetitions were subjected to statistical analyses in EXCEL 2010. The values were further analyzed by Student's *t*-test. Asterisks indicated a significant difference between the WT and the transgenic lines ($^*p < 0.05$; $^{**}p < 0.01$) based on Student's *t*-test.

3. Results

3.1. TtASR encoding a glycine-rich type ASR protein

The cDNA sequence of *TtASR* was 1112 bp in length, with a 75 bp 5′ untranslated region (UTR) and a 314 bp 3’ UTR (including polyA tail). The open reading frame (ORF) encoded a polypeptide of 240 amino acid residues with a predicted molecular mass of 25.29 kDa and a pI value of 5.26 (Fig. S2 and Table S2). The GRAVY value and the instability index of TtASR were -1.369 (< -1.0) and 30.51 (< 40), respectively (ExPASy ProtParam, http://web.expasy.org/protparam/). The N-terminal of TtASR contained an atypical histidine-rich region (HHNKKTEE) and glycine-rich region, and the C-terminal region revealed a highly conserved ABA/WDS domain, which was immediately followed by a nuclear localization signal (NLS) peptide (Fig. S3).

The amino acid content analyses indicated that TtASR was dominated by glycine residues (G, 21.25%), especially in the N-terminal. We also found that it was rich in hydrophilic amino acids, such as glutamate (E, 15%), serine (S, 7.08%), and threonine (T, 10.42%). Conversely, the hydrophobic amino acids, such as isoleucine (I, 0.83%), leucine (L, 1.67%), methionine (M, 0.42%), phenylalanine (F, 2.5%), and proline (P, 1.25%), contained lower, even lacking of tryptophan (W) and cysteine (C). The online blastp program search in NCBI showed that TtASR was highly homologous to two other halophyte ASR members: SlASR from *S. liaotungensis* and SbASR-1 from *S. brachiata*. A phylogenetic tree was constructed with ClustalW (MEGA 6.0) based on sequences of TtASR and other 25 ASR members using the maximum likelihood method. The TtASR protein shared the closest relationship with two halophyte ASRs (SlASR and SbASR-1), followed by CqASR and SoASR (Fig. S4).

3.2. TtASR improving salt and H_2O_2 tolerance assays in microorganism

The TtASR protein was induced in yeast by 2% galactose under the control of the galactose-inducible promoter P_{GAL}. The effect of TtASR accumulation in yeast cells involved in stress tolerance was studied through spot assays. As Fig. 1A shows, obvious differences in yeast salt stress tolerance were observed for the spot assay; the growth condition of colonies containing TtASR (W303) was better with NaCl stress treatments compared with yeast cells with an empty vector (pYES2) control. When assayed using a similar method under H_2O_2 stress conditions, the growth of yeast strains (*yap1Δ* and *skn7Δ*) expressing the TtASR grew significantly compared with yeast cells expressing the empty vector ((pYES2) control (Fig. 1A and C). This result suggested that TtASR expression yeast improves its survival capacity under salt and oxidative challenges.

We also verified the function test of the induced expression for TtASR in *E. coli*, with osmotic stress and oxidative stress challenges. As Fig. S5 shows, the similar spot assay with accumulated GST-TtASR cells grew much better than did the negative control *E. coli*, in which only the GST-tag was expressed (Fig. S5A). In addition, the *E. coli* growth curve assays with several stress factors in liquid culture mediums showed that the cells that expressed GST-TtASR were more tolerant to high salinity (2% and 3% NaCl), osmotic pressure (0.5 M and 0.7 M sorbitol), and the oxidant H_2O_2 (0.4 mM and 0.5 mM) than were the control cells that expressed the GST-tag only (Fig. S5B − H).

3.3. Expression patterns of TtASR in T. tetragonoides

T. tetragonoides seedlings were planted in greenhouse for 2 months, and then the whole roots and leaves were sampled to analyze the expression patterns in the vegetative stage. When the *T. tetragonoide* plants reached the reproductive stage, the stem, flower tissues, and developing seeds were also collected for *TtASR* expression pattern's analysis. Moreover, the stress responses of *TtASR* were tested to explore *TtASR* expression patterns under salt, mannitol, or ABA treatments and normal growth conditions in one-month-old *T. tetragonoides* seedlings. As Fig. 2A shows, our results demonstrated that *TtASR* was expressed in most *T. tetragonoides* tissues (Fig. 2A). The highest level of the *TtASR* transcript was detected in the immature seeds and stems, but the roots and leaves also showed a relative high expression of *TtASR*. When challenged by 300 mM NaCl of salt stress for 24 h, the transcript level of *TtASR* showed the greatest increase, by several dozen folds in the stems and leaves, which was accompanied by a relatively slight increase in roots (over 20 fold) (Fig. 2B). Under high osmotic stress (300 mM mannitol, simulate water-deficit stress), the *T. tetragonoides* seedling plants all showed an increased expression pattern of *TtASR*, and in the stem, the induced level reached almost several hundred folds (Fig. 2C). The ABA could also induce the expression of *TtASR* in *T. tetragonoides* seedlings, especially in the root and leaf. The root and leaf grew by 100–500 folds (Fig. 2D). Our results showed that high salinity/osmotic stresses and ABA treatment could greatly and rapidly increase the transcript level of *TtASR* (Fig. 2).

3.4. TtASRs exhibit no transcriptional activation activities in yeast

To assess the *transcriptional activation* activity of TtASR, the complete coding region of the *TtASR* cDNA was fused in-frame with the GAL4 DNA binding domain of the pGBKT7 vector in this study. Furthermore, three truncated TtASR fragments were also generated in the pGBKT7 vector to categorize the crucial region of TtASR to drive transcription (Fig. 3A). The yeast growth on the SD medium lacking tryptophan and histidine (SD/-Trp/-His), and the LacZ staining assay of β-galactosidase activity in Fig. 3B, indicated that the TtASRs showed no transactivation activities with IpASR as a positive control (Zheng et al., 2018).

3.5. Subcellular localization of TtASR

The subcellular localization of TtASR was determined through an *in vivo* targeting experiment in which soluble enhanced green fluorescent protein (GFP)-fused TtASR was constitutively expressed in Arabidopsis plants. In the root tips of transgenic Arabidopsis plants, the results showed that GFP-TtASR was primarily distributed in the entire cytoplasm and nucleus (Fig. 4, upper row), with a similar localization pattern with control GFP observed throughout the whole cells (Fig. 4, lower row). Based on these *in vivo* targeting results, we concluded that TtASR was predominantly localized in the whole cells. In addition, we also predicted the values of TtASR's subcellular localization through online programs. The Plant-PLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant/) showed that TtASR was mainly located in the cytoplasm, but the WoLF PSORT (http://wolfpsort.org/) indicated that TtASR had a relatively higher likelihood of distribution in the nucleus

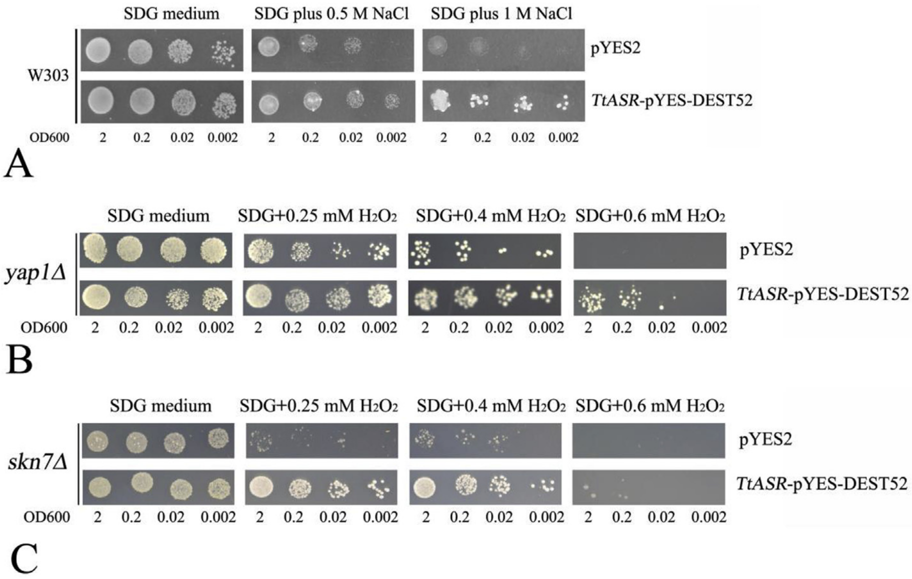

Fig. 1. Functional analysis of galactose-induced expression of *TtASR* for salt and H_2O_2 tolerance in yeast. (**A**) The growth performance of yeast strain W303 (pYES2, upper)/(TtASR-pYES DEST52, lower) on SDG plates containing high salinity (right, 0.5 M NaCl and 1 M NaCl). Control (left): SDG medium; (**B**) The growth performance of yeast H_2O_2-sensitive strains *yap1Δ* (pYES2, upper)/(TtASR-pYES DEST52, lower) on SDG plates containing H_2O_2 (right, 0.25 mM H_2O_2, 0.4 mM H_2O_2, and 0.6 mM H_2O_2). Control (left): SDG medium; (**C**) The growth performance of yeast H_2O_2-sensitive strains *skn7Δ* (pYES2, upper)/(TtASR-pYES DEST52, lower) on SDG plates containing H_2O_2 (right, 0.25 mM H_2O_2, 0.4 mM H_2O_2, and 0.6 mM H_2O_2). Control (left): SDG medium.

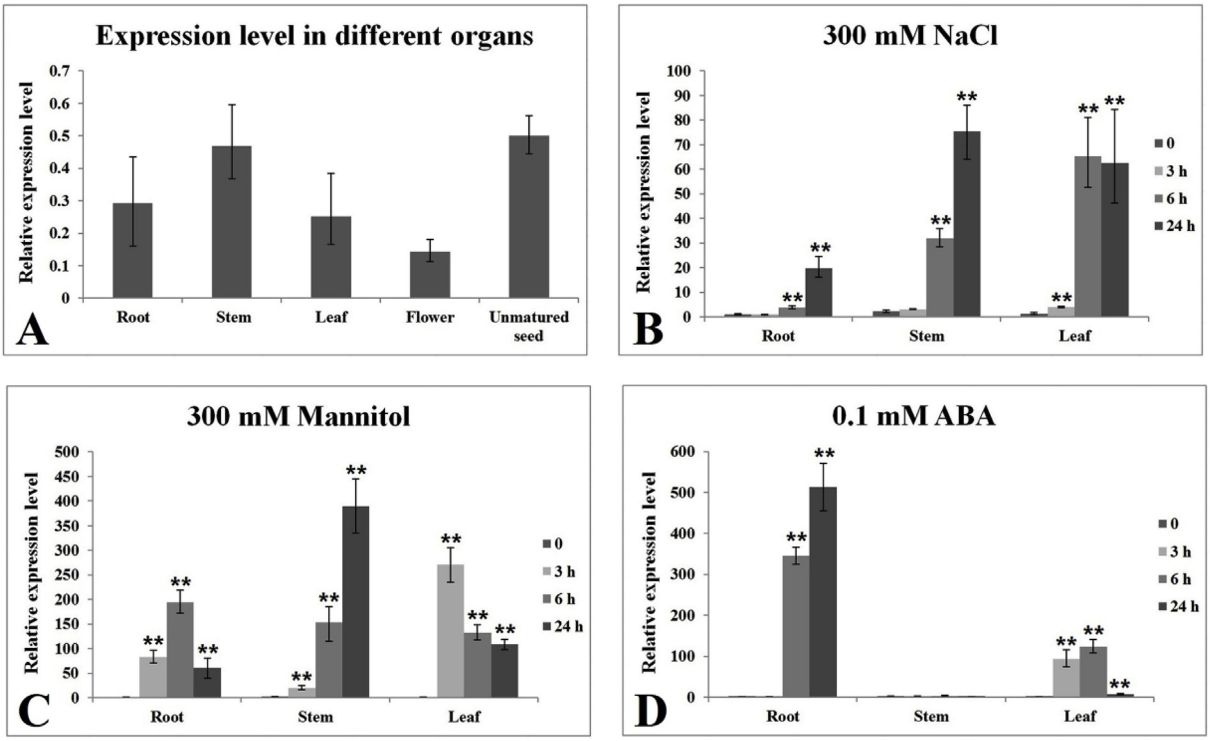

Fig. 2. Expression profiles of the *TtASR* in *T. tetragonioides* tissues. (**A**) Differential expression of *TtASR* in various tissues (root, stem, leaf, flower, and unmatured seed); (**B-D**) Time-course expression patterns of *TtASR* in response to different abiotic stresses: high salinity (**B**), osmotic stress (**C**), and ABA (**D**). Error bars indicate the ± SD based on three replicates. Asterisks indicate significant differences from the control (Student's t-test P values, $^*p < 0.05$ and $^{**}p < 0.01$).

(with a higher value of 8 for the nucleus and lower values of 3, 1, 1, and 1 for the cytoplasm, plastid, extracellular region, and peroxisome, respectively). Combining this observation with the experimental evidence of TtASR showing no transactivation activity in yeast, we concluded that TtASR played a greater role as a protective protein or a molecular chaperone rather than as a transcription factor.

3.6. *TtASR's overexpression improves tolerance to high-salinity and drought stresses in plant*

The *TtASR* gene was introduced into Arabidopsis plants to study its functions by exploring the phenotype changing under normal planting conditions or abiotic stress challenges. Three transgenic homozygous lines (T3 generation, *TtASR OX3*, *TtASR OX5*, and *TtASR OX6*) were obtained through Basta screening and used to perform further investigation. The DNA insertion and *TtASR*'s expression were assessed through qRT-PCR (Fig. S6). All of the three T3 homozygous transgenic lines showed no differences relative to WT plants during the whole regular growing period, which indicated that the overexpression of *TtASR* had no obvious growth-regulating effects on plant development under normal conditions. Here we emphasized the functions of *TtASR* involving in abiotic stress response. Firstly, we examined germination and seedling growth in WT and *TtASR OX* lines under salt/osmotic stresses to understand the abiotic stress response in *TtASR's*

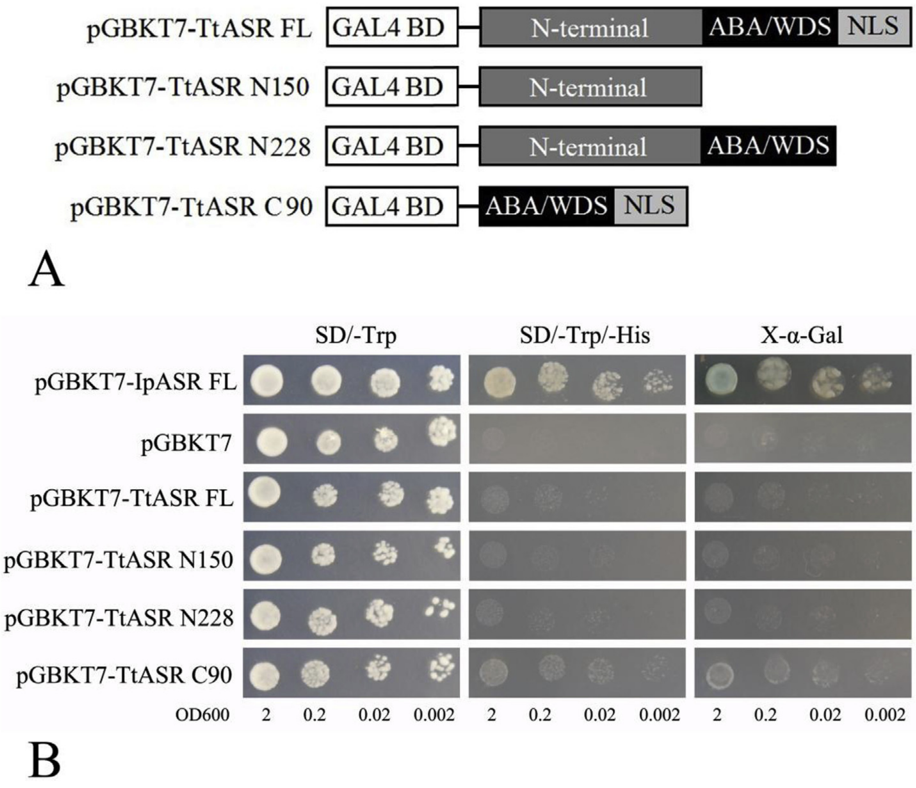

Fig. 3. GAL4 DNA binding domain (GAL4 BD)-TtASR fusion analyses for transactivation activity and α-galactosidase assay in yeast. (A) The diagrams of GAL4 BD was fused with different parts of TtASR and transformed into the yeast strain AH109 containing the *His3* and *MEL1* reporter genes; (B) Analysis of α-galactosidase activity of the relative yeast strains on plates. The yeast culture (OD_{600} to 1) was serially diluted to OD_{600} values of 0.1, 0.01 and 0.001, and then the 2 μL yeast liquid was spotted onto SD plates and cultured for 2 d at 30 °C. The full-length IpASR was used as a positive control (pGBKT7-IpASR FL, upper), and the empty vector pGBKT7 as negative control.

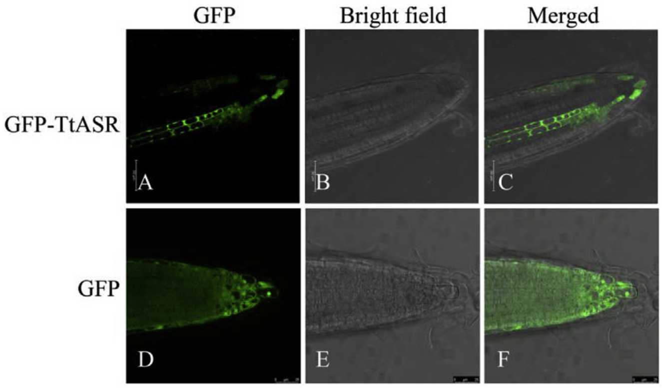

Fig. 4. Subcellular localization of TtASR protein. Arabidopsis roots expressing 35S:GFP-TtASR fusion proteins (**A, B, C**) and 35S:GFP (**D, E, F**) observed under a laser scanning confocal microscope.

overexpression in plants. Seeds from WT and *TtASR OX* lines were germinated and grown in an MS medium (as control) and MS media containing NaCl (200 mM and 300 mM) or mannitol (200 mM and 300 mM) to evaluate their stress tolerance. Under normal conditions, with an MS medium, the germination rate and seedling growth of the WT and transgenic lines were similar. However, in the presence of NaCl and mannitol, the seeds and seedlings (7 days after germination) from the transgenic line exhibited significantly higher germination rates and larger cotyledons (Fig. S7 and Fig. S8). In particular, under 200/300 mM NaCl and 200/300 mM mannitol (Fig. S7), the statistical analyses indicated that the effects of *TtASR* for salt/osmotic stress tolerances on seed germination rates were significant (Fig. S7B). To test the salt and osmotic tolerance of plant seedlings, 4-day-old seedlings from the WT and *TtASR OX* lines were transferred to an MS medium containing different concentrations of NaCl (150 mM and 200 mM) and mannitol (200 mM, 300 mM, and 400 mM), and the length of the root was measured after further cultivation for 7 days. When grown in a medium without NaCl or mannitol, no significant differences in root length were observed between the WT and transgenic lines of plants. In contrast, root lengths of all of transgenic plants were significantly longer than those of the WT plants when grown in a medium containing NaCl or mannitol (200 mM, 300 mM, and 400 mM) (Figs. S8A and B), suggesting that the overexpression of *TtASR* enhances the salt/osmotic tolerance of seedling growth (Fig. S8).

As the TtASR protein could enhance the anti-oxidative stress ability of microorganisms both in bacteria and in yeast (Fig. 1 and Fig. S5), we speculated that the overexpression of *TtASR* in Arabidopsis can also enhance the levels of antioxidation of plants. We particularly performed the assays on the antioxidation activity of plant seeds and seedlings with H_2O_2 as an exogenous oxidizing agent. Our results revealed that the germination rates of the transgenic lines were higher than those of the WT control (Fig. 5A and B); however, the differences were not as obvious as the phenotype under high salinity or osmotic stresses (Fig. S8). When the 4-day-old seedlings were transferred into the MS plates supplied with 3 mM and 5 mM H_2O_2, the H_2O_2 could significantly suppress the growth of both the WT and *TtASR*-overexpressed lines, especially for the elongation of roots. Conversely, the *TtASR*-overexpress lines showed better growth status than did the WT, since the growth of the WT roots was almost suppressed completely by H_2O_2 (Fig. 5A and C).

Adult plants from the WT and *TtASR OX* lines were also assessed to investigate the effect of *TtASR*'s overexpression in Arabidopsis plants. Ten-day-old Arabidopsis seedlings were transplanted into well-watered vermiculite with a nutrient solution from MS medium plates. For 24-day-old seedlings deprived of water for 14 days to gradually reduce the water content of vermiculite, the WT and *TtASR OX* lines plants seemed to grow well and retain similar conditions, and then the salt/drought tolerance assay was started. We chose a 150-mM NaCl treatment for the salinity stress challenge. Under normal conditions, there were no differences in the growth of the transgenic lines (*OX3*, *OX5*, and *OX6*) and the WT controls. However, after the induction of the salinity stress for 2 weeks, almost all the WT plants showed severe reductions in growth, and the leaves started to turn yellow. After re-watering them with the

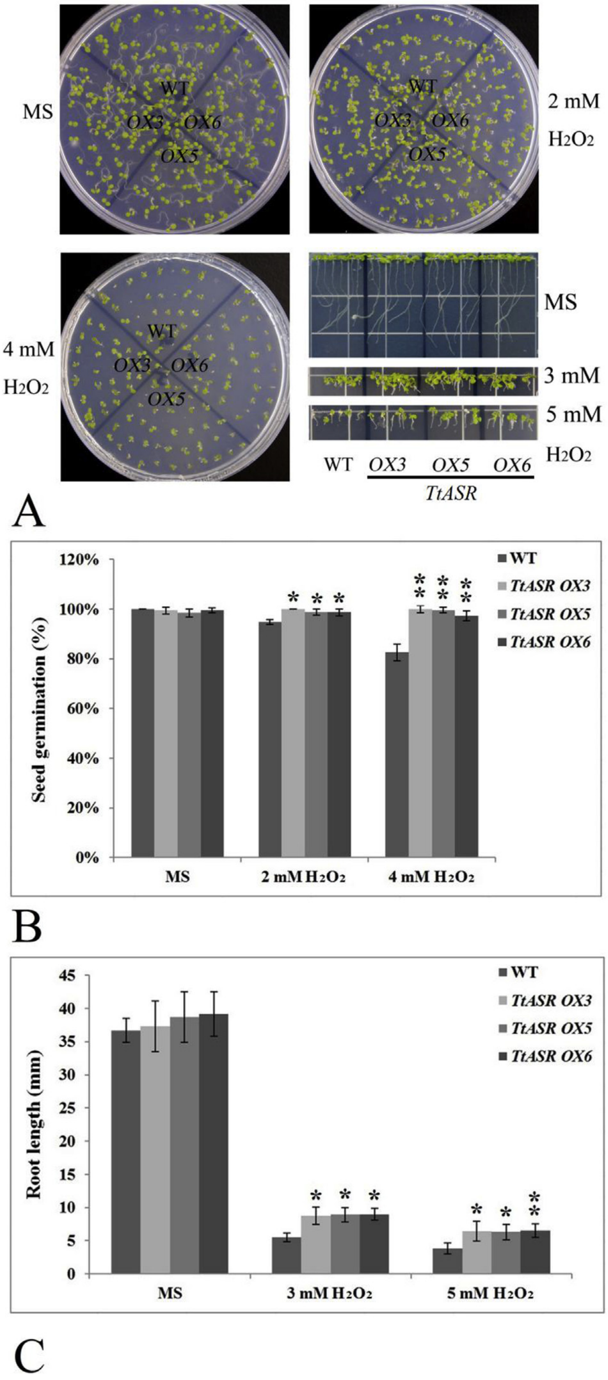

Fig. 5. Oxidative stress (tolerant to H_2O_2) analyses of three *TtASR* transgenic over-expression lines (*TtASR OX3*, *TtASR OX5*, and *TtASR OX6*) and wild type (WT) Arabidopsis with respect to the seed germination rate and seedling root length. (**A**) Photographs of three *TtASR* transgenic over-expression lines and WT seeds germinating and seedlings status on MS medium or MS medium with H_2O_2; (**B**) Seed germination rates were calculated for three *TtASR* transgenic over-expression lines and WT under H_2O_2 stress; (**C**) Seedling root length of three *TtASR* transgenic over-expression lines and WT under H_2O_2 stress. Error bars indicate the ± SD based on three replicates. Asterisks indicate significant differences from the WT (control, Student's t-test P values, $^*p < 0.05$ and $^{**}p < 0.01$).

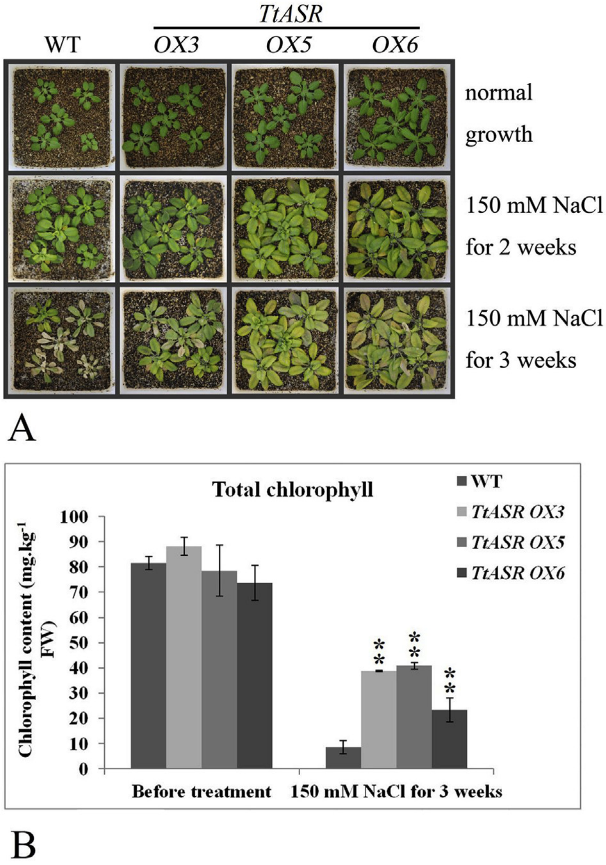

Fig. 6. Photographs of three *TtASR* transgenic over-expression lines (*TtASR OX3, TtASR OX5,* and *TtASR OX6*) and wild type (WT) Arabidopsis plants grown in vermiculite pots under normal and salt condition (150 mM NaCl). (**A**) The effects of 150 mM NaCl on three *TtASR* transgenic over-expression lines and WT; (**B**) The chlorophyll contents of three *TtASR* transgenic over-expression lines and WT plant were measured at the beginning and the end of salt stress treatment. Error bars indicate the ± SD based on three replicates. Asterisks indicate significant differences from the WT (control, Student's t-test P values, $^*p < 0.05$ and $^{**}p < 0.01$).

150 mM NaCl for another week, the transgenic lines grew slowly, whereas the WT lines seemed yellow and wilted even till death (Fig. 6A). At the beginning and end of these salt treatments, the chlorophyll contents were measured to evaluate the differences in the inhibition effect of high salinity between the WT and *TtASR OX* lines. Our results revealed that the TtASR OX lines showed better salt tolerance (Fig. 6B). Next, we moved to the drought resistance assay. In the first 2 weeks of the culture period, which were conducted under normal growth conditions, since the vermiculite contained some water, without irrigation after transplantation from the MS medium plates, the WT and *TtASR OX* lines showed no differences in the growth status. After another week of water deprivation, we could observe severe dehydration of the leaves. The whole plant had wilted, and anthocyanin had accumulated in the leaves of both the WT and *TtASR OX* lines. The WT plants showed retarded growth, whereas the transgenic lines still grew slowly (Fig. 7A). After moderate watering for another 7 days, the transgenic lines showed growth recovery, whereas some WT plants exhibited the death phenotype (Fig. 7B). These results suggested that *TtASR* may enhance the salt and drought stress resistance of transgenic Arabidopsis plants.

3.7. Overexpression of TtASR increased CAT/SOD activities and proline content and decreased MDA content under osmotic stress

To further clarify the possible physiological mechanisms involved in cellular protection mediated by TtASR, several physiological indices, including CAT/SOD activities, proline content, and MDA content (which are mainly related to a cellular osmotic stress tolerance active

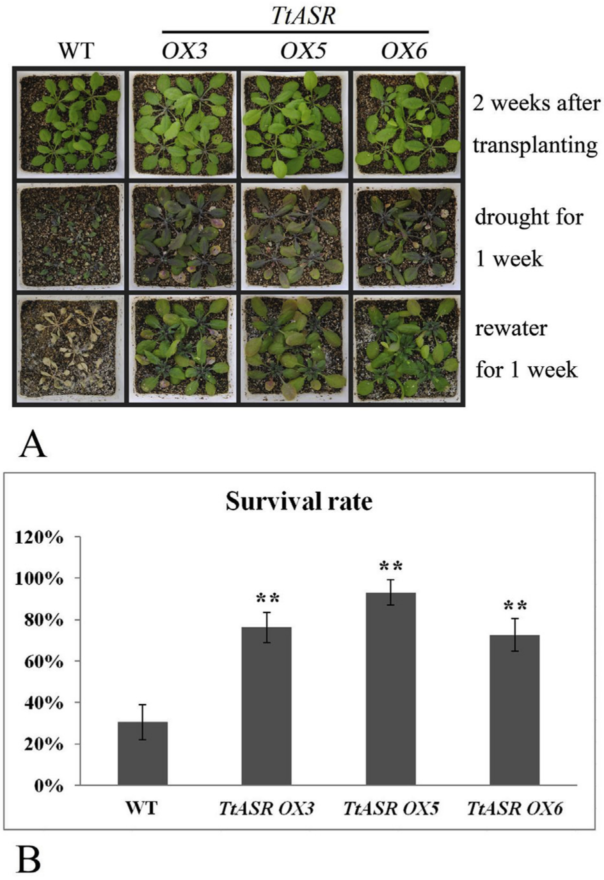

Fig. 7. Photographs of three *TtASR* transgenic over-expression lines (*TtASR OX3, TtASR OX5,* and *TtASR OX6*) and wild type (WT) Arabidopsis plants growning in vermiculite pots under drought for 1 week and re-watering for another 1 week. (**A**) The effects of drought and rewater treatment on three *TtASR* transgenic over-expression lines and WT; (**B**) The survival rate statistic of three *TtASR* transgenic over-expression lines and WT (each for 30 plants) after drought and rewatering treatment. Error bars indicate the ± SD based on three replicates. Asterisks indicate significant differences from the WT (control, Student's t-test P values, $*p < 0.05$ and $**p < 0.01$).

oxygen scavenging system in plants), were tested with the WT and *TtASR OX* lines under salt (200 mM NaCl, 1 day) and dehydration (300 mM mannitol, 1 day) treatments. Compared with the WT Arabidopsis plants, the CAT/SOD activities and proline content were higher in the *TtASR OX* lines when they were subjected to salt stress (Fig. 8A – C), which indicated that the cells in the transgenic lines demonstrated more active oxygen scavenging system and stress resistance than did the WT plants. Accordingly, the MDA levels also displayed lower patterns in the *TtASR OX* lines relative to the WT lines (Fig. 8D). These physiological indices demonstrated that the transgenic lines were more resistant to salt and osmotic stress, which hinted at the protective role of TtASR in the cellular membrane system, including for cell vitality under osmotic stress.

Reactive oxygen *in situ* staining assays were also performed with the WT and *TtASR OX* lines' seedlings under salt (200 mM NaCl, 1 day) and dehydration (300 mM mannitol, 1 day) treatments. The DAB and NBT staining indicated that the WT plants accumulated more H_2O_2 and O_2^- than did the *TtASR OX* lines (Fig. 9A and B). These results suggested that the overexpression of *TtASR* can reduce ROS accumulation by enhancing SOD and CAT activities under osmotic stress.

4. Discussion

As a halophytic functional vegetable, *T. tetragonoides* can grow in saltwater intrusion areas and improve the soil by absorbing saline ions (Wang, 2017), which suggests that there are some specific molecular mechanisms or highly efficient salt tolerance genes in *T. tetragonoides*. In the current study of *T. tetragonoides*, we first reported on a specific *ASR* gene, *TtASR*, which encoded a glycine-rich type of ASR protein involved in salt/drought stress tolerance in plants. The TtASR of *T. tetragonoides* shared high homology with other halophytes' ASR proteins, specifically SbASR-1 from *S. brachiata* (57%) and SlASR from *S. liaotungensis* (63%) (Fig. S2). In an effort to provide additional insights into the ASR protein involving in the ecological adaptability of *T. tetragonoides* to hypersaline special habitat, we mainly focused on the characterization of TtASR responding to abiotic stresses by its highly hydrophilic feature and possible antioxidant ability.

The first identification of the *ASR* was in relation to the ripening/maturity of tomato fruit ripening/maturity and the stress of water-deficit on the leaves, as its name suggests (Iusem et al., 1993). During the past two decades, an increasing number of previous studies have shown that many plant *ASR* homologs conferred tolerance to multiple stresses, responses to ABA signaling, as well as vegetative and reproductive tissues' development during cellular desiccation processes (González and Iusem, 2014; Dominguez and Carrari, 2015; Pérez-Díaz et al., 2019). Plant-specific ASRs are often small gene families comprising several members in identified plant species till now, but they are obviously absent from Brassicaceae plants (including Arabidopsis) (González and Iusem, 2014). It is worth noting that all of the identified ASR members contained highly conserved ABA/WDS domains (PF02496) in their C-terminus, which suggested that this group of proteins possessed specific and unique functions and roles in plant growth and development. Basically, most ASRs exhibit similar sequence conservation features, harboring an N-terminal zinc-binding domain (typical His-rich motif) and a C-terminal nuclear targeting signal (NLS). ASRs also have a high content of hydrophilic amino acids, and biophysical analysis indicated that they belong to intrinsically disordered protein (IDP) and can undergo structural transitions in the presence of zinc (Wetzler et al., 2018) or encountering dehydration (Hamdi et al., 2017). Here in our report, similar to SbASR-1 and SlASR, the TtASR also contained a glycine-rich region, and thereby was classified as a glycine-rich type ASR (Fig. S3). Several studies have shown that the heterogeneous overexpression of *SlASR* and *SbASR-1* could obviously elevate the salt- and drought-stress tolerance of transgenic plants (Jha et al., 2012; Hu et al., 2014; Tiwari et al., 2015), which suggested that these two specific glycine-rich type *ASR* members might be responsible for the highly salt-/drought-tolerant features of the halophytes *S. liaotungensis* and *S. brachiata*.

The expression level analysis of *TtASR* in different tissues in *T. tetragonoides* contributed to our understanding of the function of *TtASR* in plant seed development and vegetable organs' response to environmental insults and stresses, especially to water-deficit or ABA treatment (Fig. 2). In general, our sequence analyses and expression pattern verification for *TtASR* suggested that *TtASR* might contribute to alleviating abiotic stress responses as a key protective gene in the evolutionary process of *T. tetragonoides* as a halophyte.

Although the transcription activation assay in yeast showed that TtASR did not lead to reporter gene expression (Fig. 3), and subcellular localization analyses revealed that GFP-TtASR was not nuclear-specific and was distributed throughout the whole cell as GFP control (Fig. 3), we concluded that TtASR might act as a protective protein or molecular chaperone to maintain the basal cellular metabolisms and to stave off cellular activity disorders caused by all kinds of water deficits both in cytoplasm and in nucleus. To prove our speculation about the TtASR, a series of biological function verification assays were performed in both unicellular organisms (Fig. 1 and Fig. S5) and plants (Figs. 5–9 and Figs. S7 and S8). In this study, the presented evidence indicated that a high level of cellular *TtASR* expression was associated with the anti-oxidation system *in vivo*, which affirmed the viability of cells or plants under high-salinity or osmotic stress challenges.

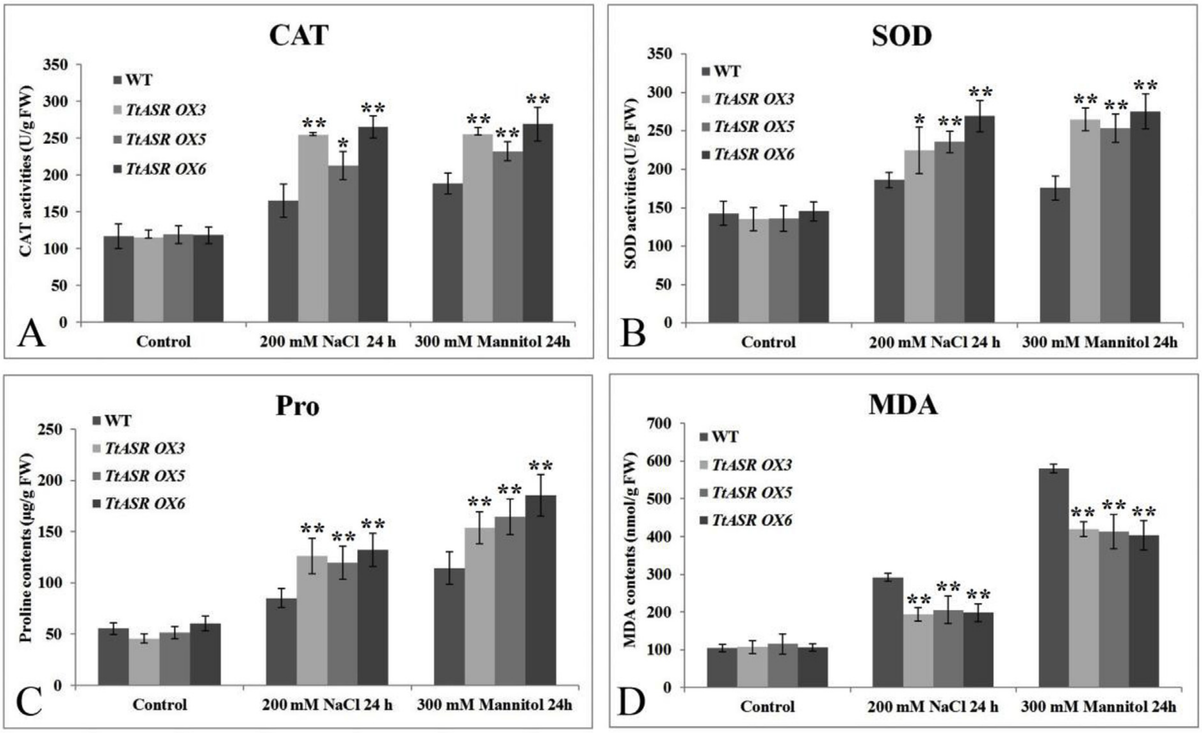

Fig. 8. Changes in physiological parameters of three *TtASR* transgenic over-expression OX lines (*TtASR OX3, TtASR OX5,* and *TtASR OX6*) and WT Arabidopsis seedlings (4 weeks) with 200 mM NaCl and 300 mM mannitol treatments for 24 h. (**A**) CAT activities; (**B**) SOD activities; (**C**) Free proline contents; and (**D**) MDA contents. All determinations were carried out on three biological replicates. Error bars indicate the ± SD based on three replicates. Asterisks indicate significant differences from the WT (control, Student's t-test P values, $^*p < 0.05$ and $^{**}p < 0.01$).

It has been well established that *ASR* responds to environmental stresses through its dual roles as a chaperone-like protein or a protective protein in the cytosol (Konrad and Bar-Zvi, 2008) and as a transcription factor in the nucleus (Hu et al., 2013; Ricardi et al., 2014; Arenhart et al., 2016). Several researches have indicated that the common Zn-binding site (His-rich motif) in the N-terminal of ASRs is crucial for their transcriptional regulation activity (Rom et al., 2006; González and Iusem, 2014), and many reports have revealed that the binding of Zn by this motif in ASRs could prompt a conformational transition of ASRs from an unfolded to a folded state due to a gain of more α-helix and β-strand domains, thereby enhancing the interaction between ASRs and DNA (Goldgur et al., 2007; Wetzler et al., 2018; Zhang et al., 2019). Some ASR member with typical His-rich motif (HHHHLFHH, OsASR5) didn't show transcriptional activation capability in yeast (Li et al., 2017b), while some ASRs were completely the opposite. For example, both BdASR1 and OsASR2 were lacking of typical His-rich motif and showed obvious transcriptional activation activity (Wang et al., 2016; Li et al., 2018). Generally, according to previous researches (Hu et al., 2013; Zheng et al., 2018), we speculated that the N-terminal of ASR is a key issue for the transcriptional activation in yeast, thereby played pivotal roles for ASRs acting as transcription factors in plants. We summarized the N-terminal sequence information and transcriptional activation activities of known plant ASRs in Table S3. For example, the transcriptional activation activity of SlASR was determined in the yeast cells, and the result showed that SlASR was not a transcriptional activator in yeast cells (Hu et al., 2014). Although the SbASR-1 with His-tag purified from exogenous expression system in *E. coli* has been proven to bind specific DNA probe (*ARBS-8*) *in vitro* by EMSA assay (Tiwari et al., 2015), questions remain as to whether this endogenous SbASR-1 without His-tag can act as a transcription factor solely *in vivo*. But meanwhile, even lacking of transcriptional activation activity in yeast cells, some ASRs, such SiASR4 (Li et al., 2017a) and BdASR4 (Yoon et al., 2019) both possessed His-rich motif and still were recognized as transcription factors in plant, basically due to their nuclear localization features. Conversely, we previously characterized an IpASR from *Ipomoea pes-caprae*, which contains an N-terminal typical His-rich motif (HHHHFGHH). The IpASR showed obvious transcriptional activation activity, whereas TtASR showed no activation activity in yeast (Fig. 3) (Zheng et al., 2018). Interestingly, research about OsASR5 (with typical His-rich motif in N-terminal) protein interaction assay in yeast indicated that OsASR5 could bind to other stress-related proteins and act as chaperone-like protein (Li et al., 2017b), and also OsASR5 showed nuclear and cytoplasmic localization and interacted with OsASR3 to regulate synergistically gene expression as complementary transcription factor (Arenhart et al., 2016). Combining the subcellular localization of GFP-TtASR distributed in the whole plant cytoplasm, not just in nucleus, additionally suggested that TtASR might probably serve as a chaperone-like protein or protective protein, rather than being a transcription factor solely.

In recent years, studies have focused on the biological functions of glycine-rich proteins (GRPs) under biotic and abiotic stresses in plants, and some reports have indicated that a part of plant GRPs are involved in cellular osmotic and salt stress responses and signaling (Mangeon et al., 2010; Czolpinska and Rurek, 2018). However, little knowledge exists regarding the role of glycine-rich type ASRs when plants are under salt and drought stress conditions, especially for their molecular mechanisms. According to the definition by Garay-Arroyo et al., proteins with high glycine content ($> 6\%$) and a high hydrophilicity index (< -1.0) can be classified into “hydrophilins” (Garay-Arroyo et al., 2000). In this study, we specifically pointed out three glycine-rich type ASRs that were isolated from halophytes. The glycine content of these three ASRs (SlASR, SbASR-1, and TtASR) was over 20% in all three cases, and their GRAVY values are −1.405, −1.198, and −1.369, respectively. The high hydrophilic features and their intrinsically disordered protein (IDP) characteristics implied that these ASRs can

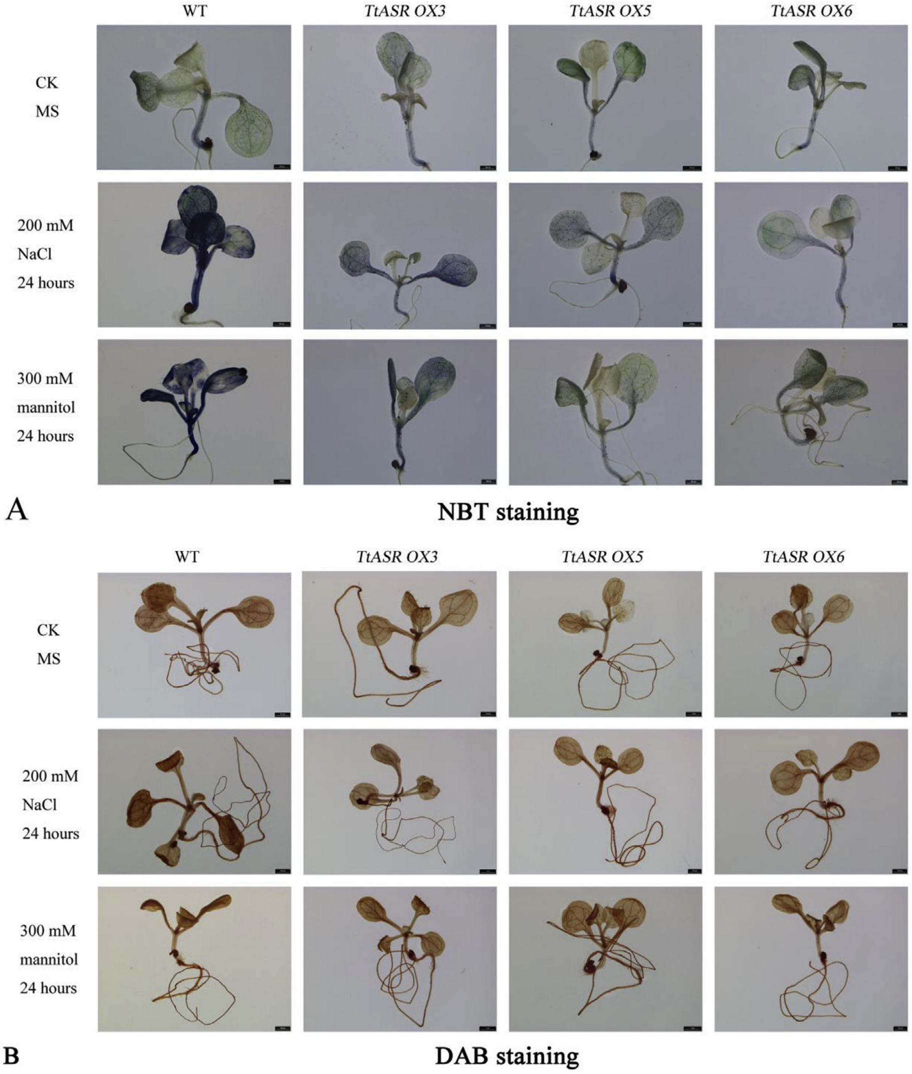

Fig. 9. Oxidative stress analyses of three *TtASR* transgenic over-expression lines (*TtASR OX3, TtASR OX5,* and *TtASR OX6*) and WT Arabidopsis plants. Histochemical staining assays were used to detect H_2O_2 and O_2^- in seedlings under challenges of 200 mM NaCl or 300 mM mannitol for 24 h by NBT (A) or DAB (B) staining, respectively.

undergo extensive conformational changes, protect other cellular proteins, and aid in water conservation in other organelle structures under water-deficit stress conditions.

In many plant species, GRPs are involved in plant biotic/abiotic stress responses and provide new and interesting insights on the molecular and cell biology of plant stresses and developmental responses (Sachetto-Martins et al., 2000; Mangeon et al., 2010). A handful of studies have suggested that plant *GRPs*' expression is mainly affected by environmental stresses, such as salt, ABA, high/low temperature, and osmotic stresses (Czolpinska et al., 2018). Accordingly, it can be inferred that the expression of plant *ASRs* may also be closely related to the response of plant tissues/organs to water-deficits and certain hormones (González and Iusem, 2014). In addition, the overexpression of plant *GRPs* or *ASRs* may yield elevated stress tolerance characteristics of heterogeneous plants, which might serve as a clue about abiotic stress tolerance in future plant biotechnological engineering. For example, a glycine-rich type *dehydrin-like* gene from *Physcomitrella patens*, *PpDHN*, has been proven to be crucial for salt and osmotic stress tolerance of *P. patens* (Saavedra et al., 2006). Recent research about maize glycine-rich ZmASR3 (over 20% glycine content) indicated that overexpression of *ZmASR3* in Arabidopsis conferred enhanced drought tolerance for transgenic plants (Liang et al., 2019). In this study, TtASR was recognized as more than an ASR protein and a plant GRP. In line with our assumption, we also performed the transgenic overexpression assay in an Arabidopsis plant, and the results were similar to those for SbASR-1 or SlASR (Jha et al., 2012; Hu et al., 2014). This phenomenon might lead to *TtASR*-mediated improved plant salt and drought tolerance in ASR members similar to SlASR or SbASR-1, mainly as cellular protective proteins when challenged by abiotic stresses. Our study showed that *TtASR* significantly improved tolerance to salt and drought stress in transgenic Arabidopsis plants, as evidenced by better growth performance and a higher survival rate under stress conditions (Figs. 6 and 7, Fig. S7, Fig. S8). Interestingly, *TtASR's* overexpression could also display enhanced resistance to H_2O_2 (Fig. 5), which exhibited a similar antioxidative function in yeast and *E. coli* (Fig. 1 and Fig. S5).

High salinity and drought impose osmotic stress, which leads to the production of ROS and then causes oxidative damage or impairment of normal plant metabolism. In our initial experiments testing the antioxidant capacity of TtASR, the TtASR has been induced both in *E. coli* and in yeast. Our results demonstrated clearly and directly that TtASR could lead to enhanced tolerance to oxidative stress in unicellular organisms (Fig. 1 and Fig. S5). In plant cells, the O_2^- and H_2O_2 are two main components of ROS caused by the cellular response to various stresses. They can be scavenged by enzymatic antioxidants, including

SOD, POD, CAT, APX, and proline, or by some other non-enzymatic antioxidants (You and Chan, 2015). In the present study, drought (simulated by mannitol) and salt stress in transgenic Arabidopsis plants overexpressing *TtASR* caused less accumulation of H_2O_2 and O_2^- then in WT, as indicated by DAB and NBT staining (Fig. 9), which implied that the presence of *TtASR* in the transgenic plants elevated ROS scavenging and improved cell activities, probably by maintaining the regularity and stability of the cells' antioxidant system. This conclusion was consistent with that of evaluation for physiological indicators, such as CAT/SOD activities, MDA/proline contents in the *TtASR* transgenic plants, and WT Arabidopsis plants that were challenged by NaCl and mannitol (Fig. 8). Here, the activity of the enzymatic antioxidants CAT and SOD in the *TtASR*-OX plants was higher than that in the WT plants under osmotic stresses (Fig. 8A and B). Similarly, the proline content was also higher in the *TtASR*-OX plants (Fig. 8C), but the MDA content was lower (Fig. 8D). These results indicated that the enhanced tolerance of the *TtASR-OX* plants to drought and salt stresses may have been attributed to the involvement of TtASR as a protective protein or a molecular chaperone in ROS scavenging.

5. Conclusions

Here in our report, we cloned and characterized a new glycine-rich *ASR* member, *TtASR*, from the halophyte *T. tetragonoides*. Our study demonstrated that *TtASR* was a stress-responsive gene that encoded high-hydrophilic proteins with a high content of glycine and conserved the ABA/WDA domain. The TtASR showed no transcriptional activation activity in yeast and could improve the yeast and *E. coli* cells' endurance in oxidizing H_2O_2. Our findings suggested that *TtASR*-overexpressing Arabidopsis plants exhibit important physiological functions in their drought and salt responses through ROS scavenging. Even lacking of experimental evidence about TtASR as chaperone or protective protein, here in our study we firstly presented the results of TtASR could directly enhance tolerance to oxidative stress in unicellular organisms and in plant. The results in this paper also suggested that *TtASR* is of great importance in improving the performance of plants under abiotic stresses and could pave the way for understanding the potential application of the *TtASR* gene in engineering drought- and salt-tolerant transgenic plants. However, the more specific molecular and biochemical mechanism of the glycine-rich region or the ABA/WDS domain of TtASR needs to be further explored in future experiments.

Author contributions

Conceived and designed the experiments: Lixiang Yang, Mei Zhang, Ming Luo, and Hongfeng Chen; Experiment Conducted: Yuyan Ye, Ruoyi Lin and Huaxiang Su; Analyzed the data: Yuyan Ye, Ruoyi Lin, and Mei Zhang; Plants acquisition and management: Hongfeng Chen; Draft and revise the manuscript: Lixiang Yang and Mei Zhang. All authors have read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.013.

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