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ORIGINAL RESEARCH

Expression of Yeast *YAP1* in Transgenic Arabidopsis Results in Increased Salt Tolerance

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Abstract Soil salinity is a major abiotic stress that lowers agricultural production around the world. Mainly caused by elevated levels of NaCl, it induces a wide range of responses in plants. In addition to ion toxicity, high salt levels can induce oxidative stress with the formation and accumulation of reactive oxygen species (ROS). We introduced the transcription factor *YAP1*, originally from yeast (*Saccharomyces cerevisiae*), into *Arabidopsis thaliana* (ecotype Columbia). When treated with various NaCl concentrations, transgenic plants showed increased activities of antioxidant enzymes catalase, superoxide dismutase, ascorbate peroxidase, peroxidase, glutathione *S*-transferase, and glutathione reductase compared with the wild-type Arabidopsis. This demonstrated that an active oxygen scavenging system was enhanced to protect plants from salt stress by equilibrating ROS metabolism. Transgenic Arabidopsis maintained higher photosynthesis levels and lower amounts of H_2O_2, suggesting that ROS production was reduced. Physiological analysis implied that transgenic Arabidopsis might employ multiple mechanisms to improve its salt tolerance.

Keywords Salt tolerance · Transcription factor · Transgenic Arabidopsis · *YAP1*

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Introduction

Salinity severely limits growth and crop yield by imposing both ionic and osmotic stresses on plants. High levels of salt enhance the production of reactive oxygen species (ROS) [31]. A common feature among the different ROS types is their capacity to cause oxidative damage to membrane lipids, proteins, and nucleic acids [20]. However, the improved ability of plants to scavenge ROS may increase their salt tolerance.

The *Saccharomyces cerevisiae* transcription factor *YAP1* is a functional homolog of mammalian activator protein 1 (AP-1) because of its ability to bind an AP-1 recognition element. Upon exposure to oxidative stress, *YAP1* becomes localized in the nucleus where it induces the expression of antioxidant proteins [12, 13, 27]. *YAP1* contains a basic leucine zipper domain similar to that of Jun [32], which is a component of mammalian AP-1 transcription factor complexes. Two cysteine-rich domains—carboxy terminal and amino terminal—are critical for *YAP1*-mediated resistance to oxidative stress and for appropriate subcellular *YAP1* localization. The activity of *YAP1* is controlled by redox-sensitive nuclear export; redox signals cause intramolecular disulfide bonds to form, resulting in changes in protein formation that mask its nuclear export sequence [27]. Because the transcriptional activity of this factor is prompted by oxidative stress, there is an increased expression of cellular target genes of *YAP1*, including several that are involved in combating the adverse effects of such stress. Nuclear *YAP1* regulates the expression of up to 70 genes that are related to oxidative stress [15, 26].

High salinity is known to cause oxidative stress [5, 19]. Several ROS, e.g., superoxide radicals $\left(O_2^{\cdot-}\right)$, alkoxyl

radicals ($RO_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), and perhydroxyl radical ($^{\cdot}HO_2$), are produced when salt stress occurs [1, 24]. These can damage almost every macromolecule [24]. Plant cells contain various defense systems that help detoxify ROS. These include both enzymatic (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; guaiacol peroxidase, GPX; and glutathione reductase, GR) and non-enzymatic antioxidants (ascorbate, glutathione, and α-tocopherol). The homolog of *YAP1 in plants has not yet been found. Here, we introduced that gene into Arabidopsis to examine its role in plant tolerance responses to salt.*

Materials and Methods

Plasmid Construction and Plant Transformation

The 1953-bp *YAP1* open reading frame was amplified using *S. cerevisiae* cDNA as template and primers 5′-TCTAGAATGAGTGTGTCTACCGCCAA-3′ and 5′-GAGCTCGTTCCCGCTTTAGTTCATATGC-3′. Polymerase chain reaction (PCR) conditions included 94°C for 3 min; followed by 35 cycles of 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min and 30 s; then 72°C for 10 min. *YAP1* PCR products were fused into a T-vector and confirmed by sequence analysis on an Applied Biosystem 3730 Automated DNA sequencer (ABI/Perkin-Elmer, Foster City, CA, USA). Fragments were then cut with *Xba*I and *Sac*I and ligated into the *Xba*I–*Sac*I site of the binary plant vector pROK2, between the cauliflower mosaic virus 35S promoter and the octopine synthase terminator. The resulting recombinant plasmid, named pROK-*YAP1*, was mobilized to *Agrobacterium tumefaciens* strain GV3101 and used for transformation. Adult plants (5 weeks old) of Arabidopsis (ecotype Columbia type) were infected with *Agrobacterium* by the floral dipping method [10] and grown in a greenhouse. Their seeds were harvested (T_0) and plated on an MS medium [33] supplemented with 50 μg mL^{-1} kanamycin before the transgenic lines (T_1) were selected.

Northern Blot Analysis

Northern-blotting experiments were performed with approximately 30 μg of total RNA per track. Samples were prepared according to the method of Chomczynski and Sacchi [9], and transferred to a Hybond N^+ nylon membrane (Amersham, Buckinghamshire, UK). The full length of *YAP1* was used as a probe, and hybridizations (55°C) were carried out as described by Maniatis et al. [28].

Growth Parameters and Germination Studies

Arabidopsis plants were grown in the greenhouse at 22°C, under a 16-h photoperiod. For the germination study, wild-type (wt) and transgenic Arabidopsis seeds were surface-sterilized and sown in 9-cm Petri dishes (approximately 100 seeds each) that contained 30 mL of an MS medium and 3% (*w*/*v*) sucrose, plus 50, 100, or 150 mM NaCl. Germination was scored after 7 days.

For stress treatments and growth measurements, 4-day-old seedlings reared on MS media in vertical plates were transferred and their roots placed downward on vertical agar plates supplemented with different levels of NaCl. Each plate contained about 10 wt and transgenic seedlings. Three replicate plates were used for each treatment. Increases in root lengths were measured after 7 days.

Salt-Stress Treatment of Arabidopsis

Seeds of transgenic (T_3 generation) and non-transgenic Arabidopsis were grown on selection and non-selection media, respectively. One-week-old seedlings were then transplanted to 10×10 cm plastic pots (three seedlings each) that were filled with nutrient soil and irrigated with half-strength Hoagland nutrient solution every other day. In the second week, NaCl treatments began on these seedlings under the same conditions. NaCl was dissolved in half-strength Hoagland solution for salt treatment. These NaCl concentrations were stepped up by 50 mM at 2-day increments to final concentrations of 0, 100, 150, or 200 mM, respectively. After treated at those final concentrations for 1 week, the seedlings were used for further physiological analysis.

Membrane Permeability Measurement

The membrane permeability of transgenic and non-transgenic Arabidopsis was examined with a conductance method, defined by the relative electrolyte exudation ratio. A DDS-12 type digital conductance apparatus was used.

Determination of H_2O_2 Content

Hydrogen peroxide levels were measured as described by Patterson et al. [36]. Leaf tissues (0.5 g) were homogenized in an ice bath with 5 mL of ice-cold acetone. The homogenate was centrifuged at 3,000×*g* for 10 min, then 0.1 mL of 20% $TiCl_4$ in HCl and 0.2 mL of ammonia were added into the 1.0-mL supernatant. After this mixture was centrifuged at 3,000×*g* for 10 min, the precipitate was washed five times with acetone and dissolved in 3 mL of H_2SO_4 before the absorbance was recorded at 410 nm.

Assays of SOD, POD, CAT, GR, APX, and GST Activities

Leaf tissues (0.5 g) were homogenized in 5 mL of 0.1 mM potassium phosphate buffer (pH 7.0) that included 1% (*w*/*v*) polyvinylpyrrolidone. After two-step centrifugation at 12,000×*g* for 5 min and 26,900×*g* for 15 min, the supernatant was prepared for determination of enzyme activities.

SOD activity was assessed based on its capacity to inhibit reduction of nitro-blue tetrazolium (NBT) by super-oxide radicals that were generated by xanthine–xanthine oxidase [4]. One SOD unit was defined as the amount of extract that caused 50% inhibition of NBT reduction at 560 nm. POD activity was measured according to the methods of Dias and Costa [14]. This reaction was done by adding 100 mmol L^{-1} H_2O_2 at 479 nm optical density. CAT activity was determined directly by the decomposition of H_2O_2 at 240 nm, utilizing the protocol of Kato and Shimizu [22]. The activity of GR was measured as described by Rao et al. [38] and was obtained by monitoring the oxidation of NADPH at 340 nm. APX activity was assayed by estimating the decreasing rate of ascorbate oxidation at 290 nm, according to the method of Nakano and Asada [34]. GST activity was assayed per Davis and Swanson [11]. This reaction was initiated by adding CDNB substrate, and the change in absorbance at 340 nm was monitored. Finally, protein was determined by the method of Bradford [6], using bovine serum albumin as a standard.

Measurement of Malonyldialdehyde Content

To measure lipid peroxidation in Arabidopsis, we performed a thiobarbituric acid (TBA) test, which determines malonyldialdehyde (MDA) as an end product [18]. Absorbance of the supernatant was read at 532 nm and the value for nonspecific absorption at 600 nm was subtracted. The amount of MDA–TBA complex was calculated from the extension coefficient of 155 mmol L^{-1} cm^{-1}.

Measurement of Net Photosynthesis

For obtaining net photosynthetic rates (Pn), we applied the same growing conditions as for our NaCl treatments. Pn was measured with an automatic photosynthetic measuring apparatus (Ciras-2; PP Systems, Hitchin, Hertfordshire, UK) as described by Qiu et al. [37].

Results

Identification of Transgenic Arabidopsis

The *YAP1* gene from yeast *S. cerevisiae* was introduced into Arabidopsis (Columbia type) through *A. tumefaciens*, which mediated transformation under control of the cauliflower mosaic virus (CaMV) 35S promoter. Kanamycin-resistant independent lines were generated and those transgenic plants showed no obvious morphological or developmental differences from the wild-type (wt) plants. In all, 24 transgenic lines carrying the *YAP1* gene were identified through PCR amplification. An intense 1.95-kb band corresponding in size to the *YAP1* product was obtained from the transgenics whereas nothing was detected from the wild type (Fig. 1). PCR-Southern hybridization was performed using the *YAP1* gene as the probe. All kanamycin-resistant plants had strong positive signals, but no signal was present in wt plants (data not shown). Four of these transgenic lines homozygous at T_2—S3-12, S4-2, S5-2, and S6-7—were sampled in extracting total RNA samples from their leaves. Our northern blots showed that all four had hybridization signals while the wt line had none (Fig. 2). This indicated that the transgenic lines accumulated *YAP1* transcripts, although they varied in their intensity of expression. We then examined salt tolerance in Lines S3-12 and S4-2, which had similar levels of *YAP1* activity.

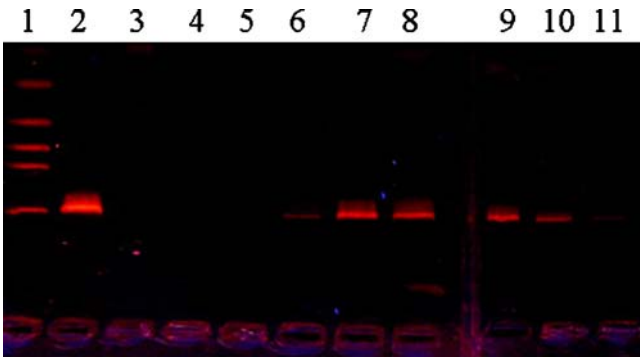

Fig. 1 PCR for detection of *YAP1* in transgenic Arabidopsis lines

Improving Early Salt Tolerance in Transgenic Arabidopsis Seedlings

To test whether overexpression of *YAP1* in Arabidopsis could enhance salt tolerance during the early seedling stage,

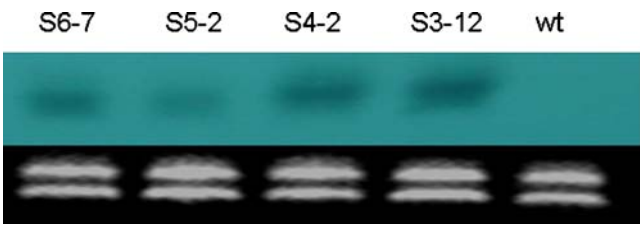

Fig. 2 RNA-blot analysis of *YAP1* expression in T_2 transgenic Arabidopsis plants. Total RNA (30 μg) was analyzed by RNA gel blotting; full length of *YAP1* was used as probe. *wt*: nontransformed Arabidopsis; *S3-12*, *S4-2*, *S5-2*, *S6-7*: *YAP1*-transformed *Arabidopsis* lines

seeds from the wild type and the T_2 generation of two transgenic lines were germinated on MS media supplied with different NaCl concentrations. The rate of germination was higher for the transgenic seeds (Fig. 3). After 15 days of cultivation on the MS medium with 100 mM NaCl, wt seedlings showed inhibited growth while the transgenic plants continued to grow normally. Wild-type roots were shorter than those from the transgenics. When exposed to 150 mM NaCl, the wt plants turned yellow and ceased growing after 15 days, but the transgenic plants remained green, albeit with slowing development. We also compared root growth among plants from S3-12, S4-2, and the wt under salt stress. Seeds of the three genotypes were placed on an MS medium for 4 days then transferred to MS plates with different levels of NaCl. Under normal conditions, all three types showed nearly the same pattern of root growth but, under salt stress, the extent of such development was somewhat less inhibited in the transgenic lines than in the wt plants (Fig. 4). This indicated that overexpression of *YAP1* resulted in enhanced early tolerance by seedlings to salt stress.

Improving the Stability of Cytoplasm Membrane

The cell membrane, especially the cytoplasm structure, is very sensitive to salt stress [29]. Here, 3-week-old seedlings from transgenic Arabidopsis Lines S3-12 and S4-2 and the wt were treated with different concentrations of NaCl applied in half-strength Hoagland solution. After the first week, we measured plasma membrane permeability (Fig. 5). Relative conductance was lower in both S3-12 and S4-2 than in the wt, indicating that the latter incurred greater harm to its cytoplasm membrane. Plants produce significant levels of ROS and hydroxyl radicals under salt stress. This increase causes lipid peroxidation and brings remarkable enhancement of MDA content, a parameter that can be used to assess damage to the cytoplasm membrane. Here, MDA contents were lower in the transgenic lines than in the wt Arabidopsis when under salt stress (Fig. 6). Therefore, overexpression of *YAP1* improved the plasma membrane permeability of Arabidopsis.

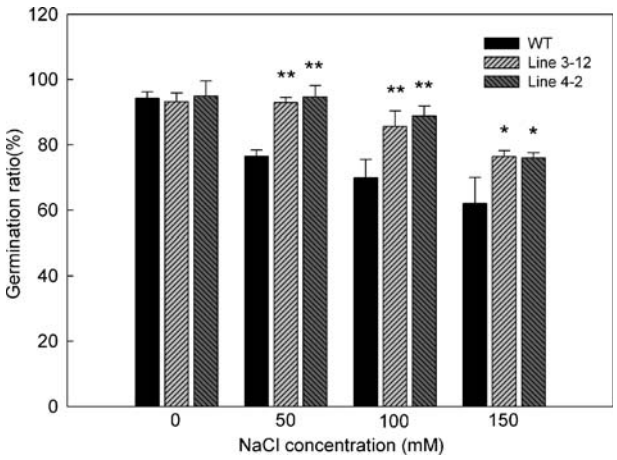

Fig. 3 Germination of wt and two transgenic plants 7 days after sowing on Murashige and Skoog medium with different concentrations of NaCl (0, 50, 100, or 150 mM). Results are means ± SE (n=3). Significantly different from wt at *P<0.05 and **P<0.01, respectively, by Student's t test

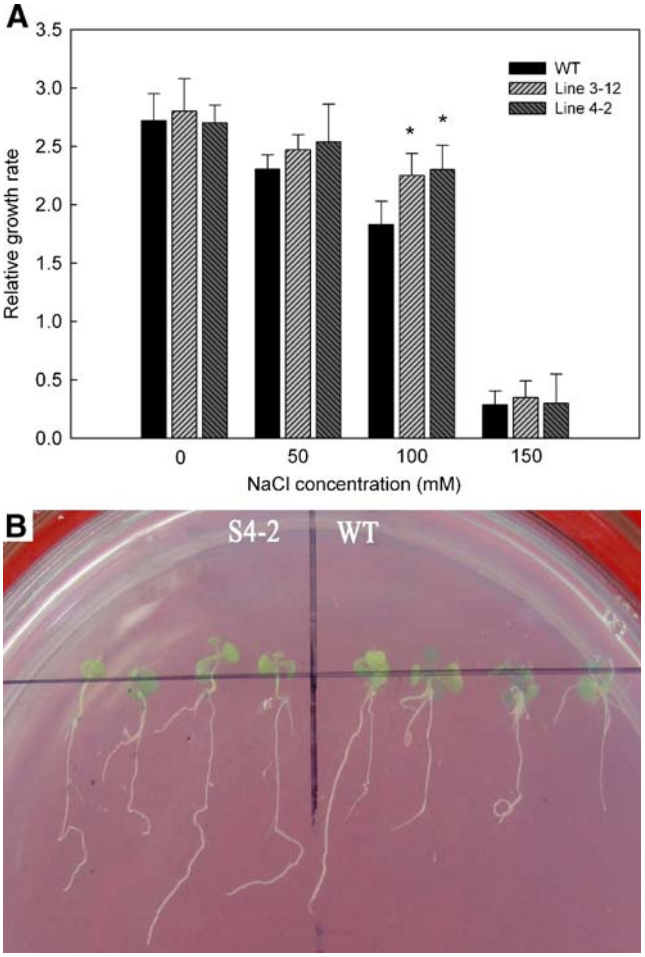

Fig. 4 **A** Overexpression of *YAP1* showed impacts on root growth under different concentration of NaCl (0, 50, 100, or 150 mM). Data are means ± SE (n=3). Significantly different from wt at *P<0.05 and **P<0.01, respectively, by Student's t test. **B** Root growth phenotype of wt and transgenic Line S4-2 on MS medium with 100 mM NaCl (pH=5.8)

Maintaining Higher Photosynthetic Activity

Stress-induced generation of ROS leads to a lower biochemical yield of photosynthesis in plant leaves. Reductions in growth due to salt stress are often related to diminished photosynthetic activity [17]. NaCl has both osmotic and ionic effects, which decrease the amount of

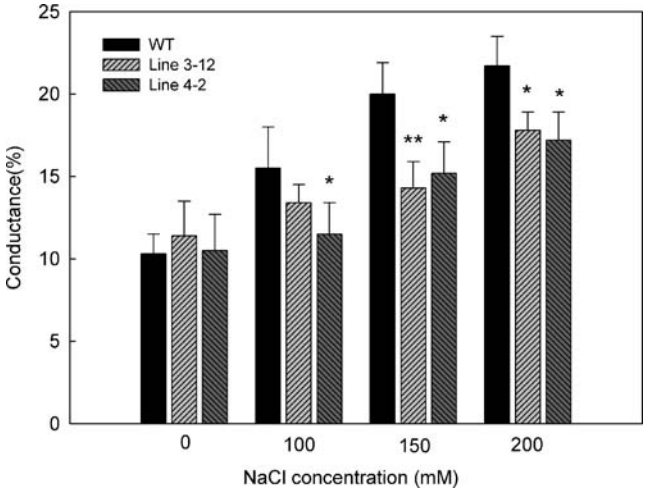

Fig. 5 Relative conductance of transgenic and non-transgenic plants in different concentrations of NaCl (0, 100, 150, or 200 mM). Data are means ±SE (n=3). Significantly different from wt at *P<0.05 and **P<0.01, respectively, by Student's t test

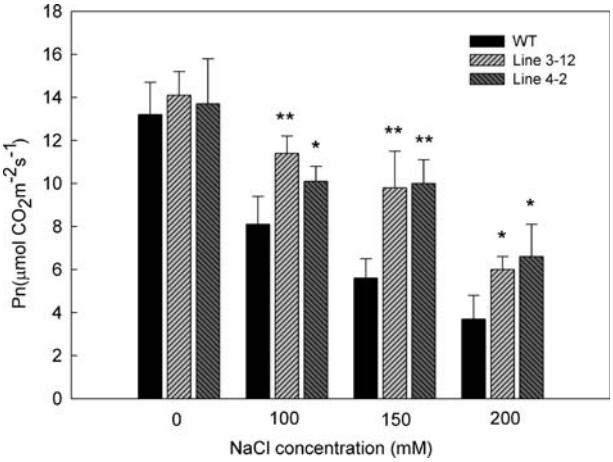

Fig. 7 Net photosynthetic rates of transgenic and non-transgenic plants in different concentrations of NaCl (0, 100, 150, or 200 mM). Data are means ± SE (n=3). Significantly different from wt at *P< 0.05 and **P<0.01, respectively, by Student's t test

water and increase the concentration of salt in the cytosol, thereby irreversibly inactivating PSI and PSII [2]. Photosystem II plays an important role in the photosynthesis response of higher plants to environmental perturbations and stresses [3]. We further confirmed the enhanced salt tolerance by *YAP1* transgenic plants by measuring changes in their net photosynthetic rate (Pn). When 3-week-old T_3 transgenic (S3-12, S4-2) and non-transgenic plants were treated with different concentrations of NaCl for 1 week in the greenhouse, Pn values declined as salt concentration rose in all lines, although this drop was less in the transgenics than in the wt Arabidopsis (Fig. 7).

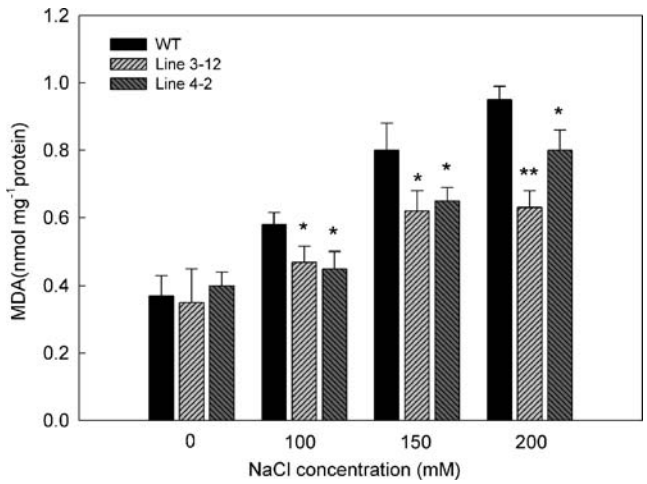

Fig. 6 MDA content of transgenic and non-transgenic plants in different concentrations of NaCl (0, 100, 150, or 200 mM). Data are means ± SE (n=3). Significantly different from wt at *P<0.05 and *P<0.01, respectively, by Student's t test

Increased Activity of Some ROS Scavenger Enzymes in Transgenic Arabidopsis

Salt stress causes oxidative stress, which then leads to the production of ROS, e.g., superoxide radicals $\left(O_2^{\cdot-}\right)$, alkoxyl radicals $\left(RO^{\cdot-}\right)$, hydroxyl radical $\left(^{\cdot}OH\right)$, and perhydroxyl radical $\left(^{\cdot}HO_2\right)$. In plant cells, both enzymatic and non-enzymatic antioxidant defense systems help in detoxifying these ROS. We measured the activity of those enzymes from transgenic and non-transgenic Arabidopsis and found almost no differences among the two transgenic lines and the wt under normal conditions. However, the activities of SOD, POD, CAT, GST, APX, and GR declined in all treatments as the NaCl concentration increased, although this decrease was less in the transgenic lines (Fig. 8).

Under different salt concentrations, the activities of various enzymes were not similar. For example, at the lower end of the range, differences were insignificant among all genotypes, except for GR. At higher concentrations, however, these differences were much more obvious for all enzymes. The enhancement of such activity may have contributed to the improvement in salt tolerance by transgenic plants, as evidenced by the growth phenotypes for all three genotypes (Fig. 9).

Reduced Concentration of H_2O_2

To test whether H_2O_2 is involved in these changes in enzymatic activity, we measured its content in plants exposed to different concentrations of NaCl. Accumulations were greater as the concentration increased in either wt or transgenic plants (Fig. 10), although the H_2O_2 content was lower in the transgenic lines.

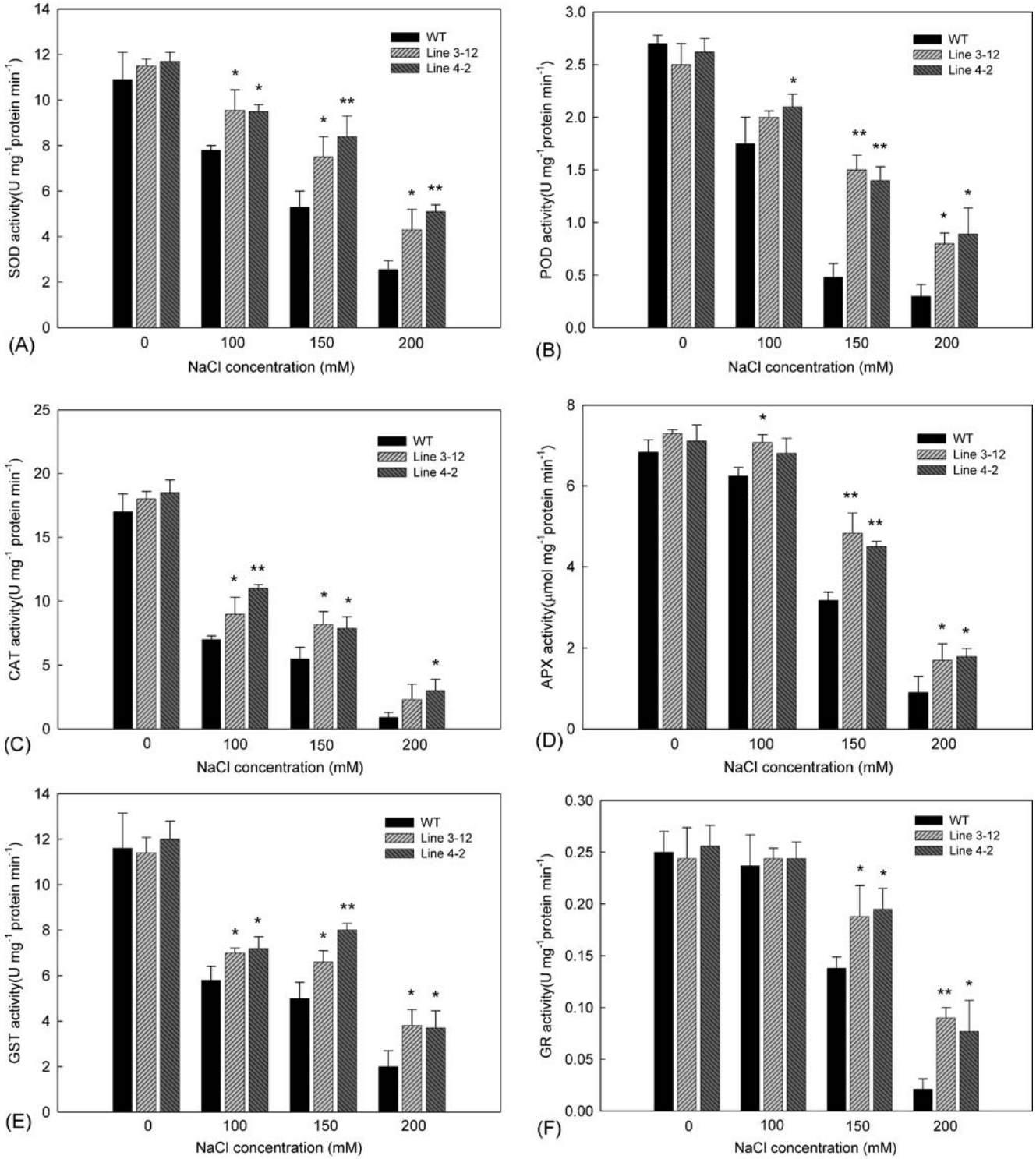

Fig. 8 Enzyme activity of **A** SOD, **B** POD, **C** CAT, **D** APX, **E** GST, and **F** GR of transgenic and non-transgenic plants in various concentrations of NaCl (0, 100, 150, and 200 mM). Results as means ± SE

Discussion

The adverse effects of salinity on plant growth may be due to ion cytotoxicity and osmotic stress [7]. Therefore, in a high-salt environment, one important survival strategy is to reestablish homeostasis via a more efficient and complete gene network. High NaCl concentrations also induce the accumulation of ROS [43]; plants utilize a ROS

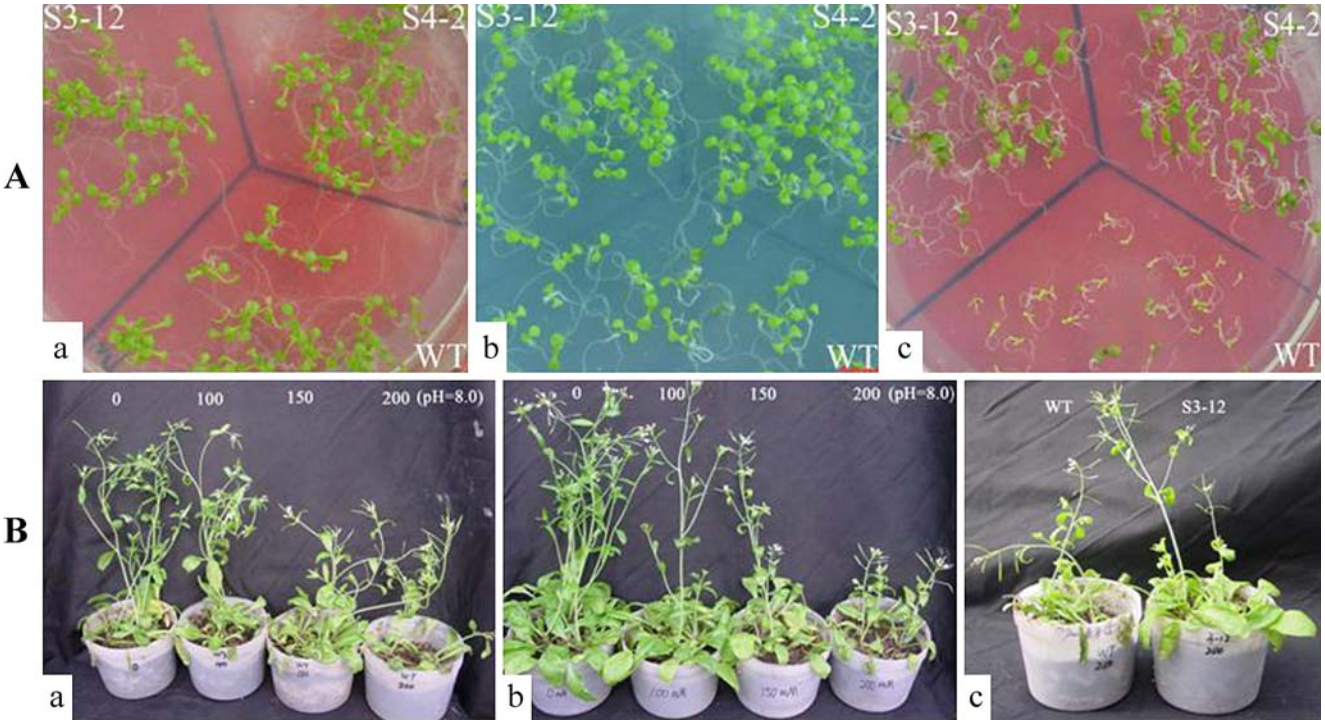

Fig. 9 Increased resistance to NaCl stress by transgenic plants overexpressing *YAP1*. **A** Growth phenotypes of wild type and transgenics from Lines S3-12 and S4-2 after 10 days of cultivation on: *a* MS+0 mM NaCl, *b* MS+100 mM NaCl, or *c* MS+150 mM NaCl (pH=5.8). **B** Phenotypes of wt *a* and T_3 transgenic plants from Line S3-12 *b* after treatment with, left to right, 0, 100, 150, and 200 mM NaCl. *c* Phenotypes of wild type and transgenic S3-12 after 200 mM NaCl treatment. All plants were grown in soil and watered every 3 days to induce salt stress

scavenging system to deal with such stress. Thus, studies of plant tolerance must include examinations of the network for scavenger genes and mechanisms for ROS tolerance.

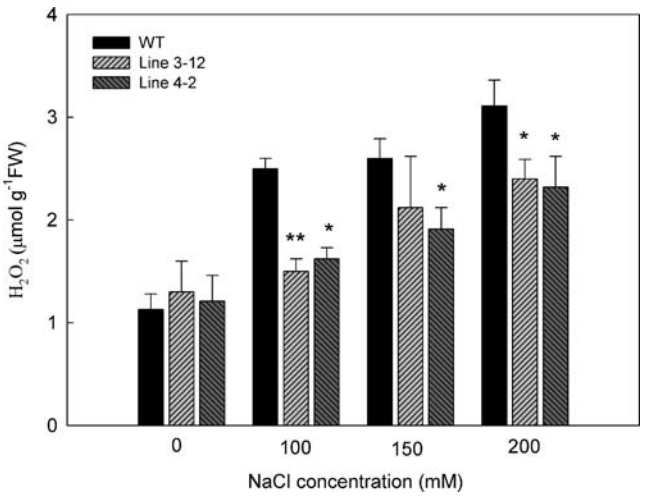

Fig. 10 H_2O_2 content of transgenic and non-transgenic plants at various concentrations of NaCl (0, 100, 150, or 200 mM). Data are means ±SE (n=3). Significantly different from wt at *P<0.05, **P< 0.01, respectively, by Student's t test

The complex nature of plant responses to salt stress makes it difficult to understand those molecular mechanisms and the roles of numerous ROS scavenging-related genes already known, including those for SOD, POD, CAT, GSH, and GPX. Arabidopsis contains more than 150 genes in that network [30].Transgenic plants that over-express ROS-scavenging enzymes, e.g., superoxide dismutase, ascorbate peroxidase, and glutathione *S*-transferase or glutathione peroxidase, all have increased stress tolerance. Overexpression of the tobacco *NtGST*/*GPX* gene in transgenic tobacco plants leads to improved salt and chilling tolerance because of enhanced ROS scavenging and the prevention of membrane damage (reviewed by [8]). Although some knowledge is available about the possible functions for these genes, information on the coordination, degree of redundancy, and cross talk between different branches of the ROS network and especially the ROS-response transcription factor is still incomplete. Research on stress-responsive transcription factors has focused on abiotic-stress tolerance; some stress-responsive transcription factors, such as in the CBF/DREB family, that confer drought tolerance have been extensively analyzed in Arabidopsis [16, 23] and NFYB [35]. This family shows constitutive expression of their downstream responsive genes. Nonetheless, little is known about transcription

factors involved in the ROS response in higher plants or about how manipulation of a single gene related to oxidative stress can enhance stress tolerance.

Work on yeasts has shown that, in response to different stress conditions, the induction of *YAP1* results in increased expression of crucial target genes that are important in the yeast cell response. Those genes include GSH_1 (encoding γ-glutamylcysteine synthetase, which catalyzes the first, rate-limiting step of glutathione synthesis; [40, 42]), and GSH_2 (encoding glutathione synthetase, the second enzyme involved in glutathione biosynthesis; [41]). Results from our current investigation, which demonstrated that *YAP1* is expressed in a higher plant, Arabidopsis, are consistent with those reported in yeast [21, 25, 39]. Therefore, we can conclude that *YAP1* also acts as an upstream regulating element in both Arabidopsis and yeast, and is up-regulated for downstream gene expression. Although a homolog of *YAP1* has not yet been found in plants, we assume that it exists and that it may have a pivotal role in improving the ROS-scavenging ability of plants.

In conclusion, ectopic expression of *YAP1* can upregulate a set of downstream ROS-scavenging genes. The reduction in H_2O_2 content and an increase in activity by those enzymes, together with other physiological characters in *YAP1*-transformed Arabidopsis, suggest that expression of the yeast *YAP1* gene can promote a high level of salt tolerance in transgenic Arabidopsis. As with yeast, plants may contain a transcription factor that improves their capacity to react to abiotic stress, especially from ROS. Research on the plant homolog of *YAP1* shows promise toward understanding the molecular mechanism and improving ROS tolerance in crops via genetic engineering approaches.

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