

Research Article

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Overexpression of Zinc Finger Transcription Factor ZAT6 Enhances Salt Tolerance

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Abstract: The purpose of the present investigation is to examine the function of the C2H2-type zinc finger transcription factor of *Arabidopsis thaliana* 6 (*ZAT6*) in salt stress tolerance in cells of rice (*Oryza sativa* L.), cotton (*Gossypium hirsutum* L.) and slash pine (*Pinus elliottii* Engelm.). Cells of *O. sativa*, *G. hirsutum*, and *P. elliottii* overexpressing *ZAT6* were generated using *Agrobacterium*-mediated genetic transformation. Molecular and functional analysis of transgenic cell lines demonstrate that overexpression of *ZAT6* increased tolerance to salt stress by decreasing lipid peroxidation and increasing the content of abscisic acid (ABA) and GA₈, as well as enhancing the activities of antioxidant enzymes such as ascorbate peroxidase (APOX), catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD). In rice cells, *ZAT6* also increased expression of Ca²⁺-dependent protein kinase genes *OsCPK9* and *OsCPK25* by 5-7 fold under NaCl stress. Altogether, our results suggest that overexpression of *ZAT6* enhanced salt stress tolerance by increasing antioxidant enzyme activity, hormone content and expression of Ca²⁺-dependent protein kinase in transgenic cell lines of different plant species.

Keywords: NaCl stress tolerance; *Agrobacterium*-mediated genetic transformation; Ca²⁺-dependent protein kinase; Transcription factor; *ZAT6* gene

1 Introduction

Salt stress causes decreased growth, development, and reduced productivity in many plant species [1-5]. Plants respond to salt stress by increasing expression of stress-

inducible genes and activating expression of transcription factors [6-11]. Understanding the molecular mechanisms of salt stress tolerance in plants is essential for molecular biologists to improve plant growth and production of crop plant [12-18]. Although different mechanisms can be adapted by plant in response to salt stress, transcription factors have been reported to play a crucial roles in salt tolerance in many plant species [19-26].

Among different transcription factors (TFs) investigated in plant salt tolerance, basic region/leucine zipper (bZIP) TFs [27-29], AP2/ERF TFs [30-32], WRKY TFs [33-35], and MYB TFs [36-38] have been reported to enhance salt tolerance in a large number of plant species. The bZIP TFs are very important in regulating salt stress signaling, as well as modulating plant growth and development [29, 39-41]. AP2/ERF TFs interact with a cis-acting DRE (dehydration-responsive element)/CRT (C-repeat) DNA sequence and activate the expression of downstream genes that are involved in salt stress tolerance in plants [24,31,42,43]. WRKY TFs could bind to the W-box (TTGAC) and function on the stress-induced genes to increased sensitivity and confer salt and drought tolerance [35, 44-46]. MYB TFs improve salt and osmotic stress tolerance by affecting the expression of genes such as SOD, POD and P5CS to increase reactive oxygen species scavenging level and reduce water loss [15, 16, 36, 37, 47].

The C2H2-type zinc finger proteins (ZFPs) represent a large family of eukaryotic TFs [48-52]. A total of 176 proteins that contain one or more zinc finger domains have been reported in *Arabidopsis* [50]. It has been reported that the ZFP transcription factors play important roles in stress responses [49, 53, 54]. Expression of *Zinc finger of Arabidopsis thaliana 6* (*ZAT6*) is regulated by phytohormones and low concentration of phosphate in *Arabidopsis* [49, 55]. Recently, *ZAT6* has been reported to be involved in regulating responses of plants to abiotic stress including drought and freezing stresses [5, 48, 56]. It has been reported that overexpression of *ZAT6* improves seed germination of *Arabidopsis* under salt and osmotic stress and that phosphorylation of *ZAT6* by MPK6 is required for the enhanced salt and osmotic stress

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tolerance [57]. However, the possible mechanisms of ZAT6 in salt stress tolerance is not reported in slash pine (*Pinus elliottii* Engelm.), rice (*Oryza sativa* L.), and cotton (*Gossypium hirsutum* L.)”.

In this study, we have investigated the function of ZAT6 in plant responses to salt stress in transgenic cells overexpressing the ZAT6 gene. Cells of three different plant species slash pine (*P. elliottii* Engelm.), rice (*O. sativa* L.), and cotton (*G. hirsutum* L.), were genetically engineered via *Agrobacterium tumefaciens* LBA4404 harboring pBI-ZAT6. Increased cell growth was observed in transgenic cells of all three species that overexpress the ZAT6 transcription factor under the condition of salt stress. In cells overexpressing the ZAT6 gene, lipid peroxidation was decreased and the content of abscisic acid (ABA) and GA₃, as well as the activities of antioxidant enzymes ascorbate peroxidase (APOX), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT) were increased. In rice cells overexpressing the ZAT6 gene, expression of Ca²⁺-dependent protein kinase genes *OsCPK9* and *OsCPK25* was increased under treatment of NaCl. These results showed that overexpression of the Arabidopsis ZAT6 transcription factor in plant cells of angiosperm and gymnosperm improved salt stress tolerance through multiple mechanisms.

2 Materials and methods

2.1 Plasmid constructs

The cDNA of ZAT6 and the pBI121 binary vector were used to generate the expression vector. After the pBI121 vector and the ZAT6 DNA were digested by *Kpn* I and *Xba* I (Promega, Madison, WI, USA) at 37°C, the digested DNA was purified using QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA). The 717-bp protein encoding fragment of the ZAT6 gene was inserted into the vector pBI121 [58] to produce the expression vector pBI-ZAT6. Vector pBI-ZAT6 was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation.

2.2 *Agrobacterium*-mediated transformation

Transgenic cell lines of *O. sativa*, *G. hirsutum*, and *P. elliottii* were generated as described before [59], using *Agrobacterium tumefaciens* strain LBA4404 harboring the pBI-ZAT6 expression vector to transform cultured cells. To generate large amount of transformed cell cultures for molecular and enzyme measurements, cells from different

species were cultured on a liquid proliferation medium. Six weeks after culture, the cell cultures of different species were growing 50–70 mg of cells/L each week. These cell cultures were used for PCR, Southern, and Northern blot analysis.

2.3 Polymerase chain reaction and Southern blot analyses of transgenic cells of rice, cotton, and pine

Polymerase chain reaction (PCR) and Southern blot analysis of transgenic cells were conducted as previously described [58]. Five hundred mg cells of control and putative transgenic cell lines of rice, cotton, and pine were used to extract DNA using a Genomic DNA Isolation Kit (Sigma), by following the manufacturer’s protocol. PCR was performed with a PTC-100TM Programmable Thermal Controller (MJ Research, San Francisco, CA, USA). The primers used are the transcription factor ZAT6 forward primer (*zf*) 5'-GTCGACATGGCGGAGGAATTTGGAAGCATAG-3' the reverse primer (*zr*) 5'-CCATGGTAGACTCCTGCTTCGACATCATGG-3', *nrp* and *nfp* for NPTII gene [58]. The DNA template was 300 ng and the reaction mix 50 ml. The PCR mixture, the PCR conditions, and Southern blot analysis was carried out as described previously [58]. Five grams of control cells and transgenic cells of rice, cotton, and pine were used to isolate genomic DNA, using a Genomic DNA Isolation Kit (Sigma). Twenty-five micrograms of DNA were digested for 16 hours with the enzymes *Xba* I (Boehringer Mannheim) at 37°C. The molecular probes (717 bp fragment of ZAT6) were labeled by Digoxigenin (DIG) (Roche Diagnostics, Indianapolis, IN, USA).

2.4 RNA isolation and Northern blot analysis

Five grams of fresh cultures of transgenic and control cells were used to isolate total RNA, using a RNeasy Mini Plant Kit (Germantown, MD, USA) by following the manual. Six micrograms of total RNA were used for northern blotting as described before [58]. The hybridization probe is the Digoxigenin (DIG)-labelling ZAT6 DNA (717 bp) (Roche Diagnostics). The tobacco 25SrRNA was used as the loading control of RNA samples. After Southern and Northern blotting analyses, 6 cell lines of rice (Os1, Os2, Os3, Os4, Os5, and Os6), 6 cell lines of cotton (Gh1, Gh2, Gh3, Gh4, Gh5, and Gh6), and 6 cell lines of slash pine (Pe1, Pe2, Pe3, Pe4, Pe5, and Pe6), each carrying only one copy of the pBI-ZAT6 T-DNA, were used for salt-induced oxidative damage experiments.

2.5 Salt treatment of transgenic cell lines

Salt treatment was applied by adding different concentrations of NaCl (50, 100, 150, 200, 250, and 300 mM) to the media used for transgenic cells, which consisted of TE medium [58] supplemented with 0.5 mM indole-3-butyric acid, 8.9 mM BA. The influence of NaCl on cell growth of rice (Os1, Os2, Os3, Os4, Os5, and Os6), cotton (Gh1, Gh2, Gh3, Gh4, Gh5, and Gh6), and slash pine (Pe1, Pe2, Pe3, Pe4, Pe5, and Pe6) were examined by culture of cell on medium supplemented with different concentrations of NaCl, as previously described [59]. The average growth was expressed as mg/g FW/day. The control cells (non-transgenic cultures) were proliferated for 3 weeks and then transferred to media containing different concentrations of NaCl (0, 50, 100, 150, 200, 250, and 300 mM). The growth rate of cells was determined 7 days after salt stress treatment. The results demonstrated that 50–100 mM NaCl did not significantly decrease the growth rate. The 150 mM NaCl significantly reduced the growth rate. The 200–300 mM NaCl leads to no cell growth (Fig. 3). On medium without NaCl, rate of cell growth does not change significantly.

2.6 Thiobarbituric acid reactive substances (TBARS) determination

Lipid peroxidation was determined as the amount of thiobarbituric acid reactive substances (TBARS) measured by the thiobarbituric acid (TBA) reaction as described previously (Tang and Page 2013). Cell cultures (1 g) of rice (Os1, Os2, Os3, Os4, Os5, and Os6), cotton (Gh1, Gh2, Gh3, Gh4, Gh5, and Gh6), and slash pine (Pe1, Pe2, Pe3, Pe4, Pe5, and Pe6) were homogenized in 3 ml of 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 5,000 rpm for 20 min and mixed with 20% TCA containing 0.5% (w/v) TBA and 100 ml 4% butylated hydroxytoluene (BHT) in ethanol at 1:1. After the extracts of cell cultures were heated at 95°C for 30 min, they were cooled on ice for 5 minutes, centrifuged at 10,000 x g for 15 min. The absorbance of extracts from different cell lines was measured at 532 nm. The control of non-specific absorption at 600 nm was subtracted from the samples. The value of TBARS was calculated using the method described previously [59].

2.7 Determination of the antioxidant enzymes glutathione reductase (GR), ascorbate peroxidase (APOX), superoxide dismutase (SOD), and CAT activity

The activities of APOX, GR, SOD, and CAT were determined as described previously [59]. Two grams of control and transgenic cells of rice (Os1, Os2, Os3, Os4, Os5, and Os6), cotton (Gh1, Gh2, Gh3, Gh4, Gh5, and Gh6), and slash pine (Pe1, Pe2, Pe3, Pe4, Pe5, and Pe6) were homogenized under ice-cold conditions in 3 ml of extraction buffer, consisting of 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP, and 0.5% (v/v) Triton X-100 at 4°C. The extracts were centrifuged at 10,000 x g for 20 min. The supernatant was used to determine the enzyme activity. APOX activity was measured immediately in fresh extracts and was assayed as described [59]. GR activity was determined by following the decrease in absorbance at 340 nm due to NADPH oxidation [58, 60]. SOD activity was measured by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT), as described previously [58, 60]. CAT activity was determined in a 3 ml 50 mM potassium phosphate buffer (pH 7.8) containing 3 mM H₂O₂, as described previously [60].

2.8 Determination of the endogenous ABA and GA8 levels

The ABA content was determined using the radioimmunoassay method as described previously [58, 59, 61]. The GA₈ content was determined as previously described by Okamoto et al. [62]. The homogenates of 100 g transgenic cells of rice (Os1, Os2, Os3, Os4, Os5, and Os6), cotton (Gh1, Gh2, Gh3, Gh4, Gh5, and Gh6), and slash pine (Pe1, Pe2, Pe3, Pe4, Pe5, and Pe6) were centrifuged at 12,000 x g for 10 min at 4°C. The supernatant of different cell lines was used to determine ABA concentration. Fifty microliters of supernatant derived from different cell lines were mixed with 200 µl phosphate-buffered saline (pH 6.0), 100 µl [³H] ABA solution, and 100 µl diluted antibody solution. After the mixture was incubated at 4°C for 45 min, the bound radioactivity in each sample of different plant species was determined with a liquid scintillation counter.

2.9 Measurement of the *OsCPK* gene expression

Expression of *OsCPK9* and *OsCPK25* in different cell lines was examined using Northern blotting by the method of Tang et al. [59, 60]. Twenty micrograms of total RNA of rice (Os1, Os2, Os3, Os4, Os5, and Os6) was applied. The PCR-amplified fragments of *OsCPK9* (amplified by forward primer 5'-AAGTCGACACCGACAAGGAT-3' and reverse primer 5'-TCTCAAAGCCTGAATCGACT-3') and *OsCPK25* (amplified by forward primer 5'-ACGTACTCCATCGGCAAAGT-3' and reverse primer 5'-GATGATGCGGTCGAAGAGTT-3') were labeled by Digoxigenin (DIG) (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN, USA). The labeled fragments were used as a hybridization probe for Northern blotting analysis.

2.10 Statistical analyses

Statistical analysis was performed using the General Linear Model procedure of SAS (Cary, NC, USA), employing ANOVA models. The significant differences between mean values of different cell lines of *O. sativa*, *G. hirsutum*, and *P. elliptii* were made at 5% level of probability. Each value of different cell lines of *O. sativa*, *G. hirsutum*, and *P. elliptii* is presented as mean \pm standard deviations of the mean.

3 Results

3.1 Generation of transgenic cell lines

A. tumefaciens (Strain LBA4404) mediated genetic transformation was used to generate transgenic *O. sativa*, *G. hirsutum*, and *P. elliptii* lines (Fig. 1a), as described previously by Tang et al. [58]. Thirty-six *O. sativa* cell lines, thirty-eight *G. hirsutum* cell lines, and thirty-nine *P. elliptii* cell lines were infected with the *A. tumefaciens* strain LBA4404 containing pBI-ZAT6 (Fig. 1a). Transgenic cell lines were selected by kanamycin. Integration of the T-DNA into the genome was confirmed by PCR analysis (Fig. 1b). Seven, nine, and eight cell lines each with one copy of the ZAT6 gene were generated from *G. hirsutum*, *O. sativa*, and *P. elliptii*, respectively. After molecular analysis by PCR (Fig. 1b and c), Southern (Fig. 1d), and Northern blotting analysis (Fig. 1e), eighteen stable transgenic cell lines each containing only one copy of the pBI-ZAT6 T-DNA were obtained from rice, cotton, and slash pine and used for salt stress experiments.

3.2 Influence of ZAT6 overexpression on NaCl tolerance

Among different concentrations of NaCl tested, NaCl at 50–100 mM did not significantly decrease the rate of cell growth of transgenic lines in *O. sativa*, *G. hirsutum*, and *P. elliptii*, compared to the control (Fig. 2). NaCl at 150–300 mM significantly decrease the rate (Fig. 2). On medium supplemented with 150 mM NaCl, the rate of *O. sativa* cell growth reduced 49% (Fig. 2a), the rate of *G. hirsutum* cell growth decreased 49% (Fig. 2b), and the rate of *P. elliptii* cell growth decreased 82% (Fig. 2c). On medium supplemented with 300 mM NaCl, the rate of cell growth has the highest reduction in transgenic cell lines (Fig. 2a-c) of *O. sativa*, *G. hirsutum*, and *P. elliptii*.

3.3 Influence of ZAT6 overexpression on lipid peroxidation

To examine salt stress-induced oxidative damage in transgenic *O. sativa*, *G. hirsutum*, and *P. elliptii* cells, TBARS content that acts as marker of lipid peroxidation was measured in salt stressed cells and in medium. Compared to the control, the total amounts of TBARS (cell cultures + incubation medium) were significantly reduced in transgenic cells overexpressing ZAT6 in *O. sativa*, *G. hirsutum*, and *P. elliptii* on medium supplemented with 150–300 mM NaCl (Fig. 2d-f). TBARS were not significantly reduced in transgenic *O. sativa*, *G. hirsutum*, and *P. elliptii* cells under 50–100 mM NaCl (Fig. 2d-f). Significant reduction in the products of lipid peroxidation in transgenic *O. sativa*, *G. hirsutum*, and *P. elliptii* cells demonstrated that the protection on membranes in transgenic *O. sativa*, *G. hirsutum*, and *P. elliptii* cells with overexpression of ZAT6 was demonstrated by the decrease of TBARS (Fig. 2d-f).

3.4 Effect of ZAT6 overexpression on ABA and GA8 content

Among different concentrations of NaCl applied, NaCl at 50–100 mM did not significantly decrease ABA content of transgenic cell lines in cotton, rice, and slash pine, compared to the control (Fig. 3). Compared to the control, NaCl at concentration of 150–300 mM significantly reduce ABA content in *O. sativa*, *G. hirsutum*, and *P. elliptii* (Fig. 3), on medium supplemented with 150 mM NaCl, ABA content of *O. sativa* cells decreased 49% (Fig. 3a), ABA content of

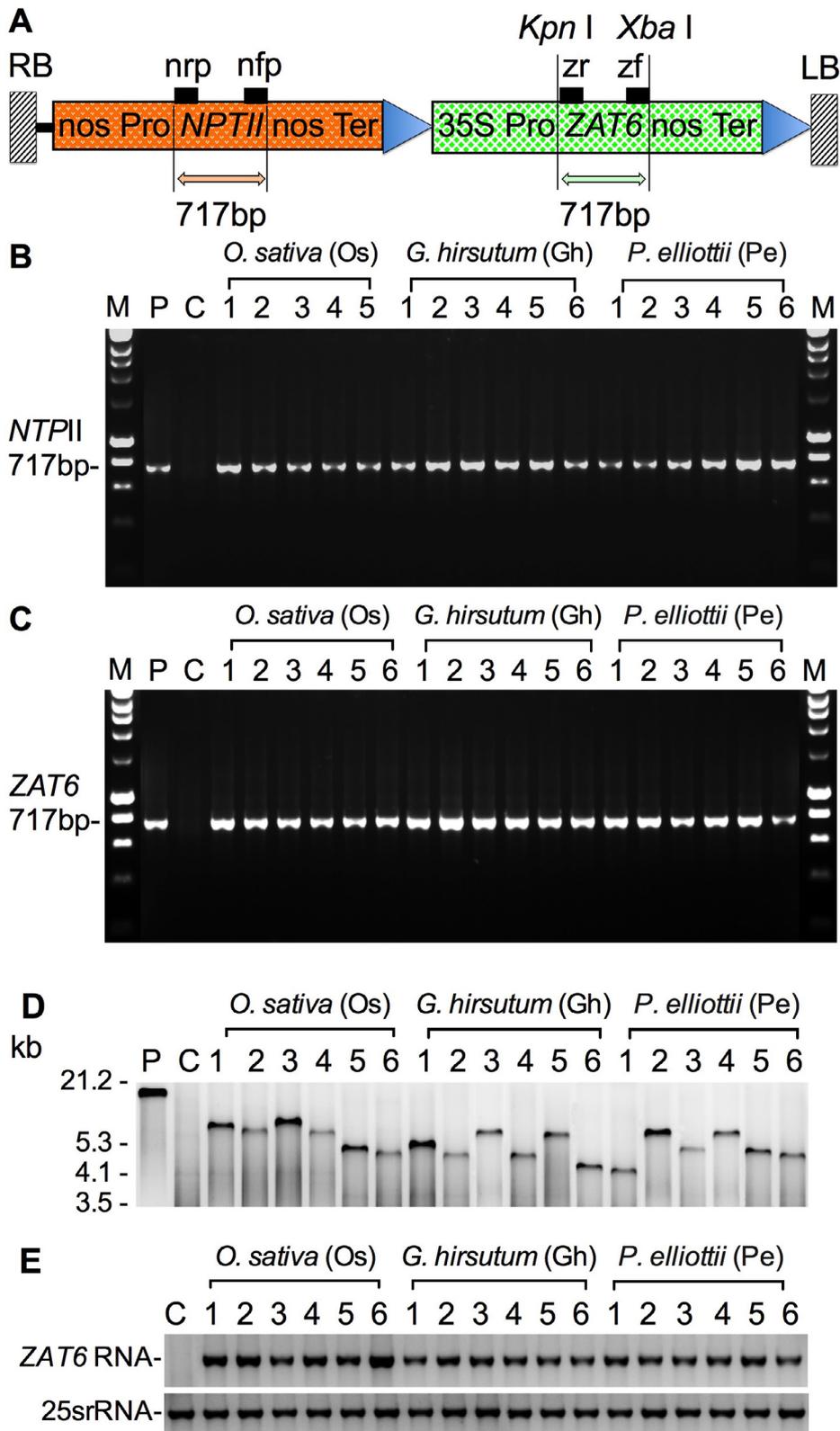


Fig. 1 Plasmid map and molecular analyses of transgenic cell lines. (A) A linear plasmid map of pBI-ZAT6. (B) PCR analysis of *NPTII* gene in transgenic cell lines. (C) PCR analysis of *ZAT6* gene in transgenic cell lines. (D) Southern blot analysis of transgenic cell lines. (E) Northern blot analysis of transgenic cell lines.

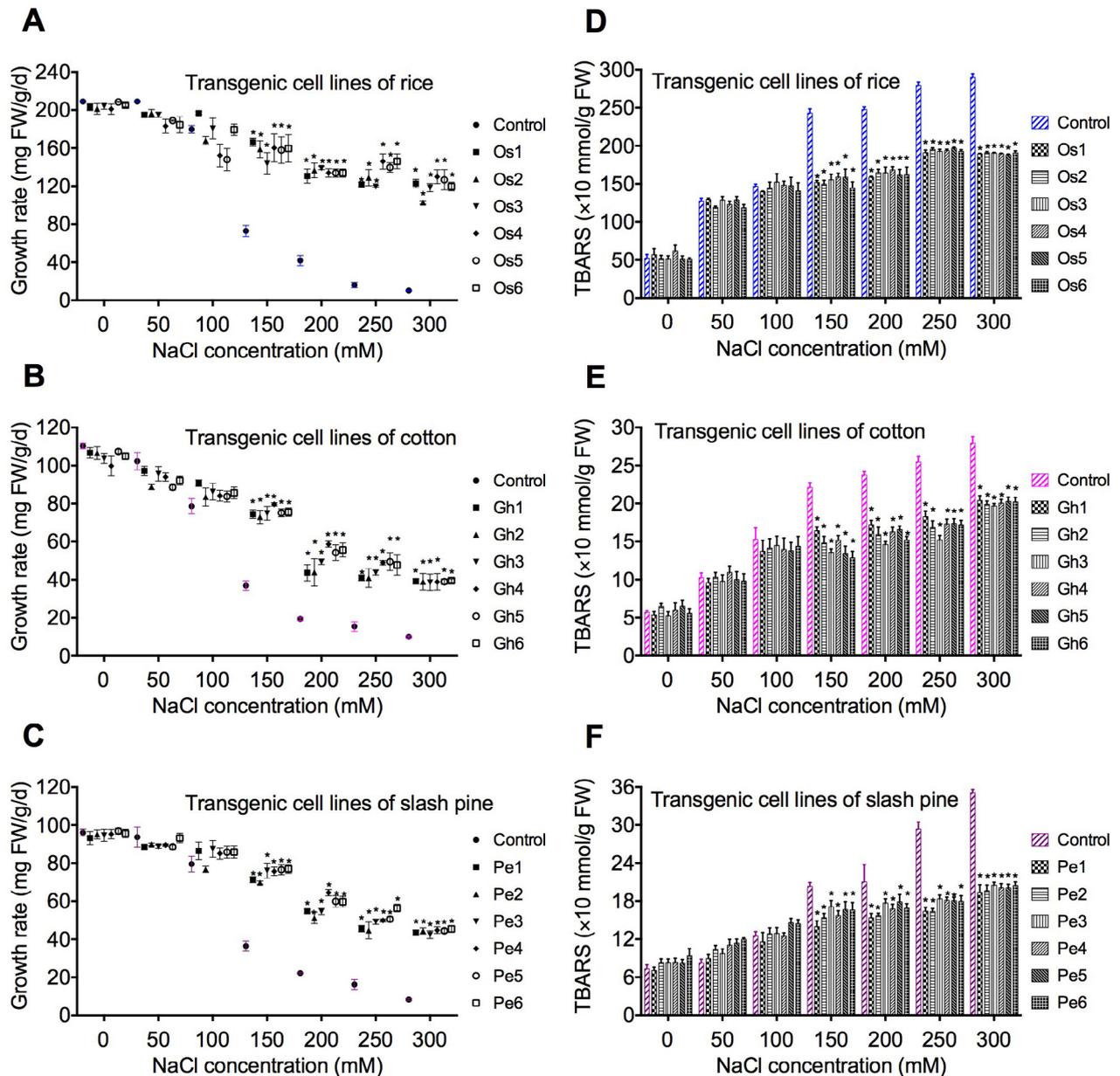


Fig. 2 Growth of cell lines under different concentrations of NaCl and thiobarbituric acid reactive substance changes (TBARS). (A-C) Effect of different concentrations of NaCl (50, 100, 150, 200, 250, and 300 mM) on the growth of transgenic cell lines of rice (Os1, Os2, Os3, Os4, Os5, and Os6), cotton (Gh1, Gh2, Gh3, Gh4, Gh5, and Gh6), and slash pine (Pe1, Pe2, Pe3, Pe4, Pe5, and Pe6) were measured 21 days after callus transgenic cell cultures were transferred into media containing NaCl. (D-F) TBARS changes in transgenic cell lines of rice (Os1, Os2, Os3, Os4, Os5, and Os6), cotton (Gh1, Gh2, Gh3, Gh4, Gh5, and Gh6), and slash pine (Pe1, Pe2, Pe3, Pe4, Pe5, and Pe6). The growth and TBARS were measured 21 days after cell cultures were transferred into media containing different concentrations of NaCl (50, 100, 150, 200, 250, and 300 mM). Each experiment was replicated three times, and each replicate consisted of five to ten 250-ml flasks of transgenic cell cultures. Values represent the means \pm S.D.

G. hirsutum cell cultures decreased 49% (Fig. 3b), and ABA content of *P. Elliottii* cell cultures decreased 82% (Fig. 3c). Under stress of 300 mM NaCl, ABA content has the highest increase (Fig. 3a-c) in *O. sativa*, *G. hirsutum*, and *P. Elliottii* cells. A similar change of GA_3 was obtained (Fig. 3a-c) in *O. sativa*, *G. hirsutum*, and *P. Elliottii* cells.

3.5 Antioxidant enzymes APOX, GR, SOD, and CAT activities

Antioxidant enzymes APOX (Fig. 4a-c), GR (Fig. 4d-f), SOD (Fig. 5a-c), and CAT (Fig. 5d-f) were selected to evaluate the oxidative damage caused by NaCl stress in

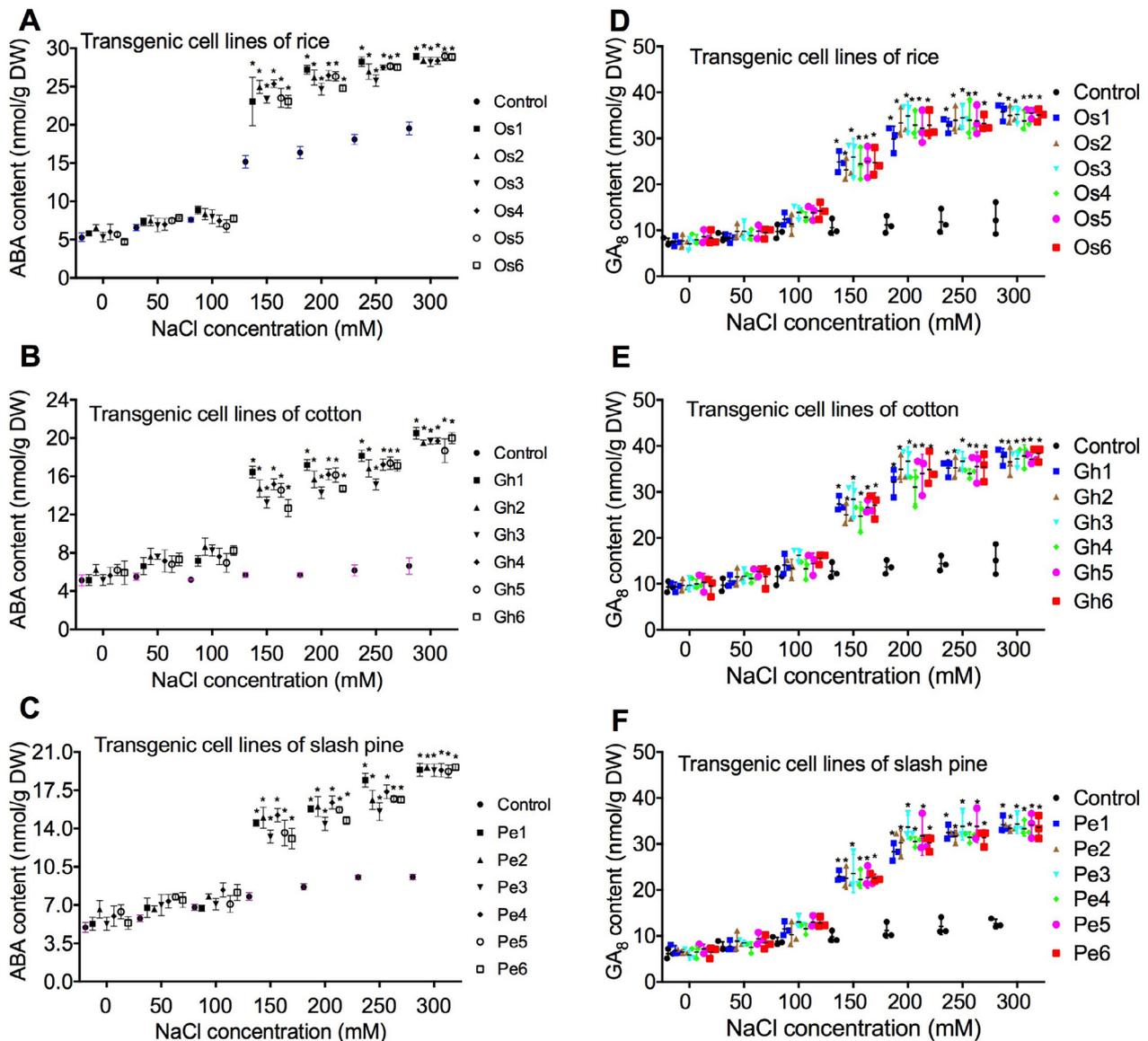


Fig. 3 Effect of ZAT6 overexpression on ABA and GA8 content. ABA content (A-C) and GA8 (D-F) content changes in transgenic cell lines of rice (Os1, Os2, Os3, Os4, Os5, and Os6), cotton (Gh1, Gh2, Gh3, Gh4, Gh5, and Gh6), and slash pine (Pe1, Pe2, Pe3, Pe4, Pe5, and Pe6) were measured 21 days after cell cultures were transferred into media containing different concentrations of NaCl (50, 100, 150, 200, 250, and 300 mM). Each experiment was replicated three times, and each replicate consisted of five to ten 250-ml flasks of transgenic cell cultures. Values represent the means \pm S.D.

transgenic cell lines of rice, cotton, and slash pine. APOX, GR, SOD, and CAT activities were significantly reduced from the concentration point 150mM to 300 mM of NaCl in non-transgenic control (Figs. 4 and 5), however, the enzyme activity was stable in transgenic cells of *O. sativa*, *G. hirsutum*, and *P. elliptii*. Compared to controls, the levels of APOX, GR, SOD, and CAT activities were not significantly decreased from 50 to 150 mM NaCl in rice, cotton, and slash pine transgenic cell lines (Figs. 4 and 5).

3.6 Effect of ZAT6 on expression of Ca²⁺-dependent protein kinase genes

The Ca²⁺-dependent protein kinase genes *OsCPK9* and *OsCPK25* have been reported to be associated with stress tolerance in rice (Wan et al. 2007). To examine the effect of the ZAT6 on expression of *OsCPK9* and *OsCPK25*, we have analyzed the expression of *OsCPK9* and *OsCPK25* in ZAT6 transgenic cells of *O. sativa* (Os1, Os2, Os3, Os4, and

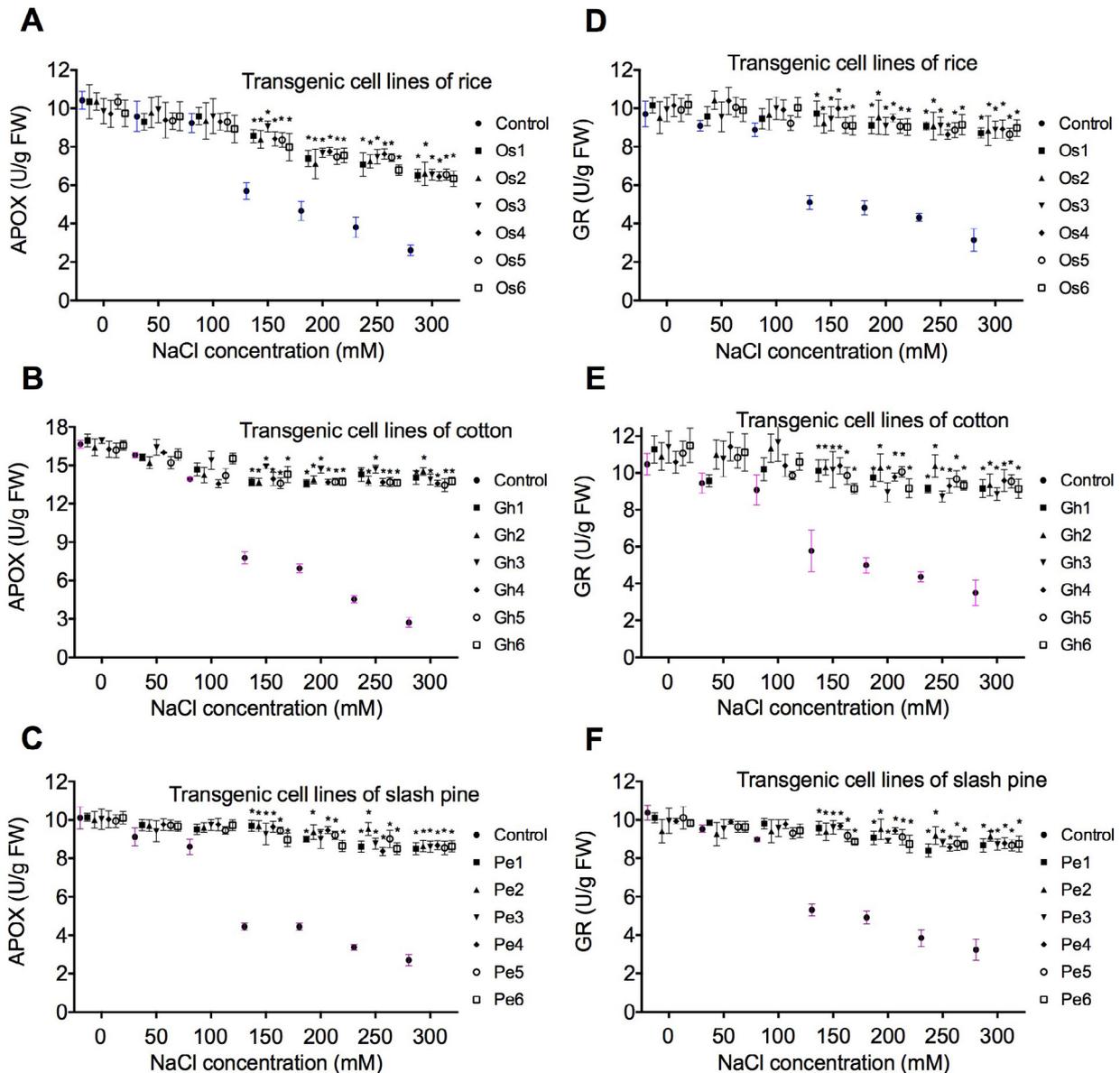


Fig. 4 Effect of ZAT overexpression on APOX and GR activity. APOX (A-C) and GR (D-F) changes in transgenic cell lines of rice (Os1, Os2, Os3, Os4, Os5, and Os6), cotton (Gh1, Gh2, Gh3, Gh4, Gh5, and Gh6), and slash pine (Pe1, Pe2, Pe3, Pe4, Pe5, and Pe6) were measured 21 days after cell cultures were transferred into media containing different concentrations of NaCl (50, 100, 150, 200, 250, and 300 mM). Each experiment was replicated three times, and each replicate consisted of five to ten 250-ml flasks of transgenic cell cultures. Values represent the means \pm S.D.

Os5) on medium supplemented with 150 mM NaCl (Fig. 6). These results showed that the expression level of OsCPK9 (Fig. 6 a-f) and *OsCPK25* (Fig. 6g-l) increased 5 to 7-fold in transgenic cells of *O. sativa*, *G. hirsutum*, and *P. elliotii*, compared to the control.

4 Discussion

TFs regulate NaCl stress response through different molecular mechanisms in a large number of plant species.

It has been reported that overexpression of bZIP, AP2/ERF, MYB, WRKY TFs and other stress related genes in plant cells could increase NaCl stress tolerance [24, 25, 27, 34, 36, 38, 45, 63]. TF-regulated signaling transduction pathways and metabolic changes are essential for plant cells to counteract damages caused by NaCl stress [24, 27, 36, 38, 64, 65]. Overexpression of transcription factors in cells lead to improvement of NaCl stress tolerance in many plant species including Arabidopsis, wheat, rice, and *Brachypodium distachyon* [47, 66-68]. In potato, soybean, poplar, and pine, transcription factors have

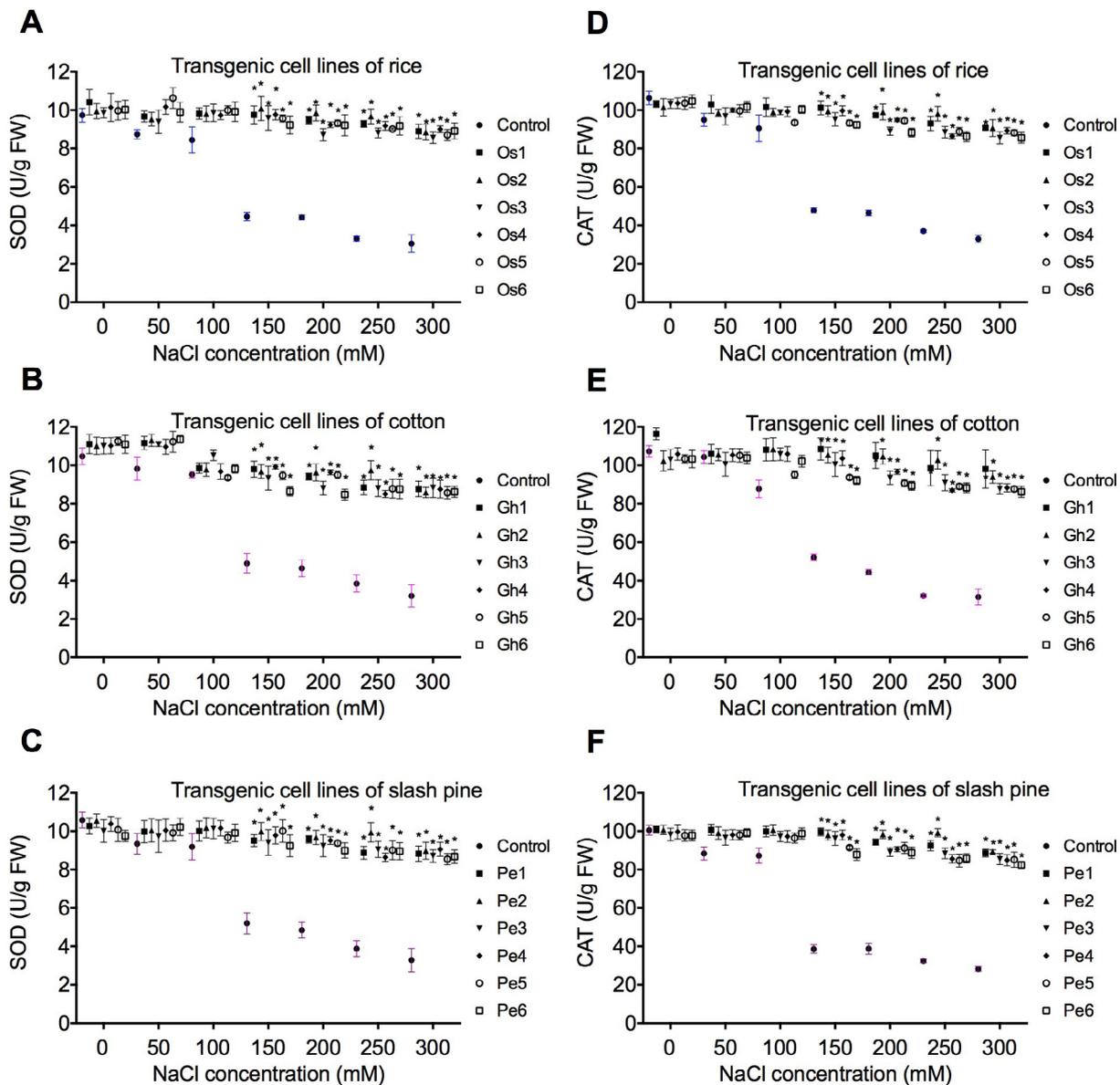


Fig. 5 Effect of ZAT6 overexpression on SOD (A-C) and CAT (D-F) activity. SOD and CAT changes in transgenic cell lines of rice (Os1, Os2, Os3, Os4, Os5, and Os6), cotton (Gh1, Gh2, Gh3, Gh4, Gh5, and Gh6), and slash pine (Pe1, Pe2, Pe3, Pe4, Pe5, and Pe6) were measured 21 days after cell cultures were transferred into media containing different concentrations of NaCl (50, 100, 150, 200, 250, and 300 mM). Each experiment was replicated three times, and each replicate consisted of five to ten 250-ml flasks of transgenic cell cultures. Values represent the means \pm S.D.

been reported to protect cells from NaCl stress through the decrease of oxidative damage and the increase of auxin signaling [43, 69-71]. Overexpression of some TFs lead to enhanced expression of antioxidant enzyme genes, induced metabolic reprogramming, and changes of stomatal closure in different plant species including Arabidopsis, tomato, tobacco, and pine, which resulted in increased NaCl tolerance [27, 43, 72, 73]. However, molecular mechanisms associated with TF ZAT6 increased NaCl stress tolerance is elusive.

Overexpression of stress-related transcription factor genes regulates salt stress response through regulation of ABA and GA metabolism and signaling [1, 74, 75]. It has been reported that, *RAS1* (*Response to ABA and Salt 1*) enhance salt stress tolerance and ABA sensitivity as a negative regulator during early seedling growth [76], *AtSAT32* [salt tolerance32 (*SAT32*)] functions on both salinity tolerance and ABA signaling as a positive regulator in Arabidopsis [1, 74, 77]. Endogenous ABA and GAs influence salt stress and endophytic fungal association in cucumber [78, 79]. GAs

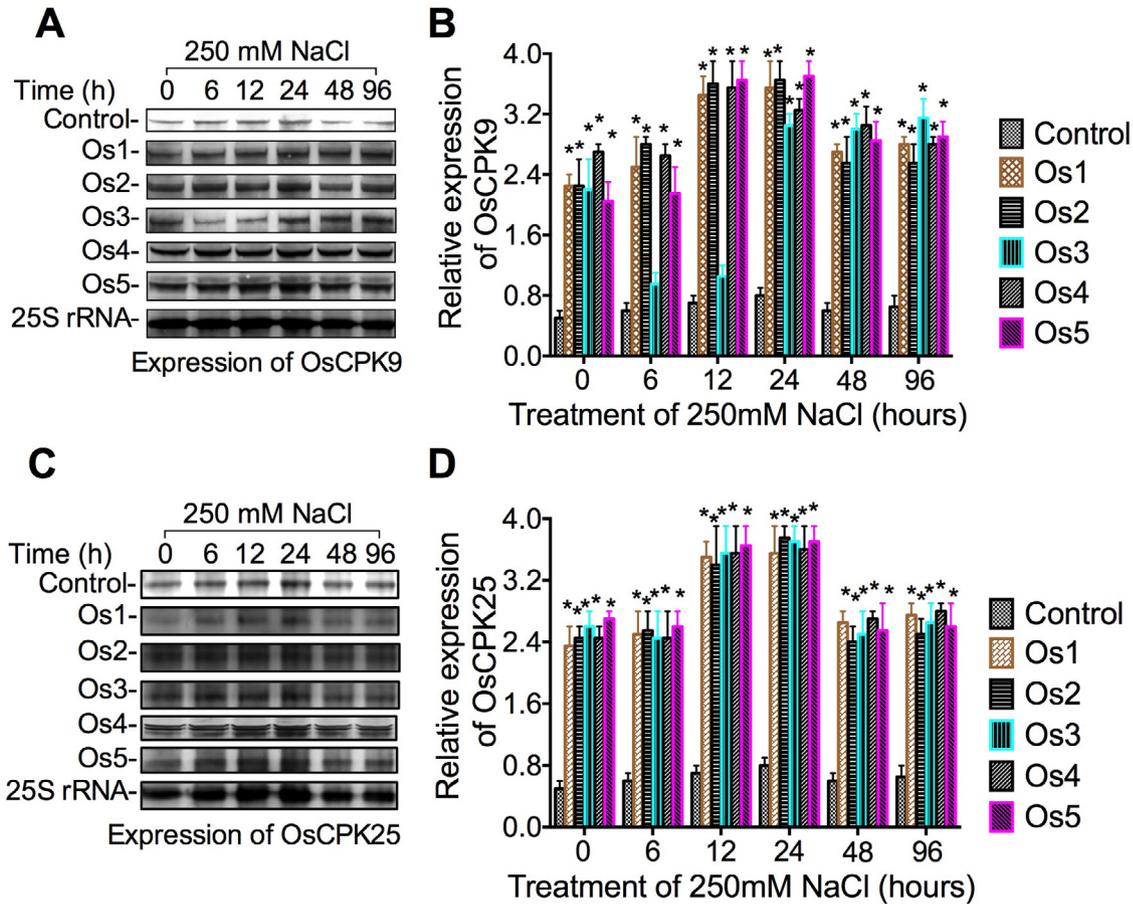


Fig. 6 Influence of NaCl on expression of rice Ca²⁺-dependent protein kinase gene *OsCPK9* and *OsCPK25* in ZAT6 overexpression cells. (A) Northern blotting analysis of expression of *OsCPK9* in ZAT6 transgenic cell lines Os1, Os2, Os3, Os4, and Os5 under 200 mM NaCl treatment at 6, 12, 24, 49, and 96 h. (B) Quantitative analysis of expression of *OsCPK9*. (C) Northern blotting analysis of expression of *OsCPK25* in ZAT6 transgenic cell lines Os1, Os2, Os3, Os4, and Os5 under 200 mM NaCl treatment at 6, 12, 24, 49, and 96 h. (D) Quantitative analysis of expression of *OsCPK25*. Each experiment was replicated three times. Values represent the mean ± SD.

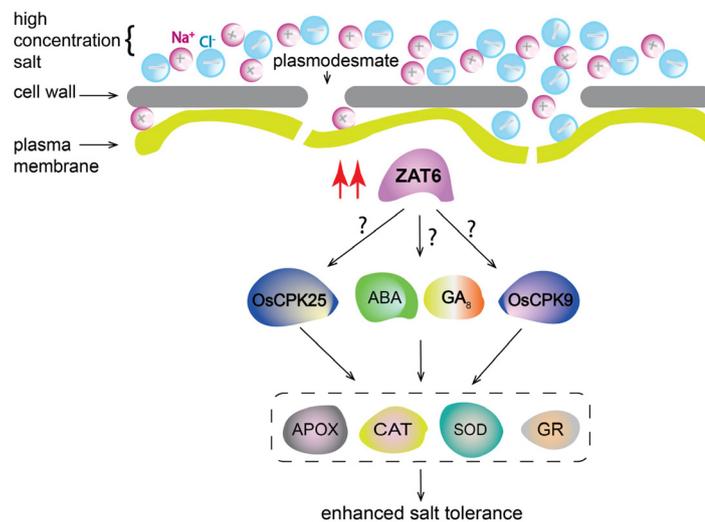


Fig. 7 Molecular mechanism of ZAT6 overexpression enhanced salt tolerance. ZAT6 overexpression enhances expression of *OsCPK25* and *OsCPK9*, as well as contents of ABA and GA₃ in cells of angiosperm and gymnosperm, which may contribute to increased activity of Antioxidant enzymes APOX, CAT, SOD, and GR. Increased activity of APOX, CAT, SOD, and GR enhance salt tolerance in cells of angiosperm and gymnosperm.

in fungal culture filtrate are involved in salinity induced oxidative stress in soybean plants through reduction of lipid peroxidation and regulation of the activities of the antioxidant enzymes [75, 80]. To examine if the enhanced salt stress via overexpression of *ZAT6* is related to ABA and GA metabolism, we examined the content of ABA and GA_8 in *ZAT6* transgenic cell lines of *O. sativa*, *G. hirsutum*, and *P. elliptii* under treatment of different concentrations (50, 100, 150, 200, 250, and 300 mM) of NaCl (Fig. 3). Our experimental results showed that the content of ABA and GA_8 was increased in transgenic *O. sativa*, *G. hirsutum* and *P. elliptii* cells, compared to the controls (Fig. 3). Our experimental results demonstrated that the increased ABA and GA_8 could be contributed to the increased NaCl stress tolerance in transgenic *G. hirsutum*, *O. sativa*, and *P. elliptii* cells.

NaCl stress affects the activity of antioxidative enzymes and lipid peroxidation in plants. In maize, NaCl stress increase the activities of superoxide dismutase (SOD), ascorbate peroxidase (APOX), and glutathione reductase (GR) to protect cells from oxidative damage, compared to the controls [81, 82]. In *Broussonetia papyrifera*, NaCl stress increases the activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) in the leaves, stems and roots. The changed activities of antioxidant defense enzymes were associated with distinct SOD and POD isoenzymes [60, 61]. It has been reported that overexpression of *ZAT6* in Arabidopsis enhances seed germination under salt and osmotic stress and that phosphorylation of *ZAT6* by MPK6 is required for the increased salt and osmotic stress tolerance [60, 61]. To determine if the *ZAT6*-overexpression increased salt stress tolerance is related to the reduced oxidative damage, we have examined the activities of antioxidant enzymes APOX, GR, SOD, and CAT in *ZAT6* transgenic *O. sativa*, *G. hirsutum*, and *P. elliptii* cells under treatment of NaCl (Figs. 4 and 5). Our results demonstrated that the activities of antioxidant enzymes APOX, GR, SOD, and CAT was increased in transgenic cells of rice, cotton, and pine, compared to the controls (Figs. 4 and 5), indicating that the increased activities of antioxidant enzymes could be related to the increased tolerance to NaCl stress in examined transgenic *O. sativa*, *G. hirsutum* and *P. elliptii* cell lines. Overexpression of *ZAT6* may decrease oxidative damage, at least in part, through the increase of the activity of antioxidant enzymes.

Ca^{2+} dependent protein kinases (CPKs) are involved in NaCl stress tolerance through regulating expression of transcriptional activators or transducing stress signals to other signaling molecular. Cross talk between CPKs-mediated signal transduction and ABA signaling may

regulate cellular homeostasis for NaCl stress tolerance. In rice, *OsCPK9* improves NaCl stress tolerance by increasing the osmotic adjustment ability, expression of stress-responsive genes, and stomatal closure in plants [83]. In Arabidopsis, CPKs can phosphorylate the biotinylated peptide at the threonine residue for stress-response signaling pathway, regulate expression of several ABA responsive genes, be involved in oxidative stress and lipid metabolism [83]. To examine if the increasing salt stress tolerance through overexpression of *ZAT6* is related to certain Ca^{2+} dependent protein kinases, we have investigated the expression of *OsCPK9* and *OsCPK25* in *ZAT6* overexpressing transgenic cells of Os1, Os2, Os3, Os4, and Os5 under treatment of 250 mM NaCl (Fig. 6). Our experimental results showed that the expression of *OsCPK9* and *OsCPK25* was increased 5 to 7 fold in transgenic cells, compared to the non-transgenic control cells (Fig. 6), indicating that *ZAT6* overexpression-enhanced expression of *OsCPK9* and *OsCPK25* could be associated with the increased NaCl stress tolerance in transgenic *O. sativa*, *G. hirsutum* and *P. elliptii* cell lines and that overexpression of *ZAT6* may contribute to CPK-mediated signaling through cross talk between CPKs and *ZAT6* under NaCl stress condition.

Although enhanced salt tolerance via overexpression of TFs has been documented in many different plant species [66, 67, 71, 84], molecular mechanisms of transcription factor-enhanced salt stress tolerance are not fully understood [70, 72, 73]. Overexpression of *ZAT6* enhanced salt stress tolerance is not reported in cells of *P. elliptii*, *O. sativa*, and *G. hirsutum*. Different mechanisms could be contributed to salt tolerance in different plant species (Fig. 7). Overexpression of stress-responsive transcription factors plays important roles in the response to salt stress by coordinating the phytohormone signaling networks [15, 16, 85]. Overexpression of the *TabHLH1* gene plays critical roles in plant tolerance to osmotic stresses through an ABA-dependent pathway [67]. Although cells use multiple strategies to improve salt tolerance, NaCl induces adverse effects by producing oxidative damage [5, 29, 34, 37, 86, 87]. In this study, we showed that increased tolerance to NaCl stress in cells of different plant species expressing transcription factor *ZAT6* was related to the increased content of ABA and GA_8 , the increased activities of antioxidant enzymes, the increased expression of Ca^{2+} -dependent protein kinase genes, and the decreased lipid peroxidation.

Compared to use whole plants, it is easy to get consistent phenotypes of transformed cells. This is one reason that we use transgenic cell lines to examine the function of *ZAT6* in *P. elliptii*, *O. sativa*, and *G. hirsutum* in

the present investigation. Although we demonstrated that overexpression of *ZAT6* enhanced salt stress tolerance and that the salt stress-associated changes of phytohormones, antioxidant enzymes, and CPKs in *ZAT6* overexpression transgenic cell lines of *P. elliptii*, *O. sativa*, and *G. hirsutum* extended the discovery of *ZAT6*-improved seed germination under salt and osmotic stress in Arabidopsis, further investigation is needed to examine the function of *ZAT6* in transgenic plants of *P. elliptii*, *O. sativa*, and *G. hirsutum* for practical application of transcription factor *ZAT6* in agriculture and forestry

Although it has been reported that overexpression of *ZAT6* improves seed germination under salt and osmotic stress and that phosphorylation of *ZAT6* by MPK6 is required for the enhanced salt and osmotic stress tolerance in Arabidopsis [54, 57], overexpression of *ZAT6* enhanced salt stress tolerance is not reported in crop plants and conifers. To explore the possible molecular mechanisms of *ZAT6* overexpression-enhanced salt stress tolerance in crop plants and conifers, we overexpressed *ZAT6* in cell lines of *P. elliptii*, *O. sativa*, and *G. hirsutum* and analyzed the changes of phytohormones, antioxidant enzymes, and CPKs in transgenic cell lines under salt stress. Overexpression of *ZAT6* enhanced salt stress and the salt stress-associated changes of phytohormones, antioxidant enzymes, and CPKs in *ZAT6* overexpression transgenic cell lines of *P. elliptii*, *O. sativa*, and *G. hirsutum* extended the discovery of *ZAT6* improved seed germination under salt and osmotic stress in Arabidopsis. Overexpression of stress-related transcription factor genes regulates salt stress response [74, 77, 88]. It has been reported that, *RAS1* (*Response to ABA and Salt 1*) enhance salt stress tolerance and ABA sensitivity as a negative regulator during early seedling growth [74, 77, 88], *AtSAT32* [salt tolerance32 (*SAT32*)] functions on both salinity tolerance and ABA signaling as a positive regulator in Arabidopsis [1, 74]. Endogenous ABA and GAs influence salt stress and endophytic fungal association in cucumber (Khan et al. 2012). GAs in fungal culture filtrate are involved in salinity induced oxidative stress in soybean plants through reduction of lipid peroxidation and regulation of the activities of the antioxidant enzymes [89]. Cross talk among phytohormones signaling, activity of antioxidant enzymes, and expression of *CPKs* genes may be associated with *ZAT6* overexpression-enhanced salt stress tolerance.

In conclusion, we have investigated the rate changes of cell growth, the content of ABA and GA_8 , the activities of antioxidant enzymes APOX, GR, SOD, and CAT, and lipid peroxidation in *ZAT6* transgenic *G. hirsutum*, *O. sativa*, and *P. elliptii* cells, as well as expression of *OsCPK9* and *OsCPK25* in *O. sativa*. Our experimental results showed

that overexpression of *ZAT6* decreased NaCl-induced oxidative damage by elevating the expression level of Ca^{2+} -dependent protein kinases and by elevating the activities of enzyme APOX, GR, and SOD and reducing lipid peroxidation (Fig. 7). The protection of transcription factor *ZAT6* overexpression against salt stress-induced oxidative damage was related to the increasing expression of Ca^{2+} -dependent protein kinase genes *OsCPK9* and *OsCPK25* in transgenic *O. sativa* cells. Overexpression of the *ZAT6* transcription factor could be valuable approach for engineering plant abiotic stress tolerance.

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