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Title: The Improvement of Salt Tolerance in Transgenic Tobacco by Overexpression of Wheat F-box Gene *TaFBA1*

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Highlights

Overexpression of *TaFBA1* in tobacco increased seed germination rate and root elongation compared with WT under salt stress. Under long time salt stress, transgenic plants have greater biomass accumulation than WT, and the photosynthesis was more stable than WT. The transgenic plants had higher activity of antioxidant enzymes and less accumulation of ROS under salt stress. Further, the transgenic plants had lower Na^+ content and higher K^+ content than WT in leaves and roots. At the same time, H^+-ATPase on Plasma membrane in the transgenic plants were higher than those of WT, accompanied by the net of Na^+ efflux rate under salt stress. In tonoplast, the activities of V-ATPase and PPase were also higher than WT, helpful to Na^+ maximize intracellular compartmentation. The expression of some salt stress related genes were upregulated under salt stress. These results suggested that the improvement of *TaFBA1* in plant salt stress tolerance may be associated with the improvement of antioxidative compete and Na^+/K^+ ion regionalization.

Abstract: F-box protein is a major subunit of the Skp1-Cullin-F-box (SCF) complex. We previously isolated an F-box gene from wheat, *TaFBA1*, and here we show that overexpression of *TaFBA1* in transgenic plants under salt stress increases germination rate, root elongation, and biomass accumulation compared with WT plants. Improvements in the photosynthetic rate and its corresponding parameters were also found in the transgenic plants. These results suggest that overexpression of *TaFBA1* improves salt stress tolerance in transgenic tobacco. Further, the transgenic plants displayed less membrane damage, higher antioxidant enzyme activity, and less accumulation of ROS under salt stress. The transgenic plants also had lower Na^+ content and higher K^+ content than WT plants in leaves and roots. The activity of H^+-ATPase on the plasma membrane in the transgenic plants was higher than in WT plants, and was accompanied by a net Na^+ efflux. In the tonoplast, the activity levels of V-ATPase and PPase were also higher in the transgenic plants, thus helping to maximize intracellular Na^+ compartmentalization. The expression of some stress-related genes was upregulated by salt stress. This suggests that the enhancement of plant salt stress tolerance may be associated with an improvement in antioxidative competition and Na^+/K^+ ion regionalization.

Abbreviations: DAB, 3′,3′-diaminobenzidine; H^+-ATPase, protontranslocating ATPase; MDA, malondialdehyde; NBT, nitro blue tetrazolium; NMT, non-invasive micro-test ~~technique~~; PM, plasma membrane; PPase, proton-translocating pyrophosphatase; qRT-PCR, quantitative real-time polymerase chain reaction; RM, resuspension medium; ROS, Reactive oxygen species; RWC, Relative water content; SCF, Skp1–Cullin–F-box; Ub, Ubiqui-tin; UPS, Ubiquitin (Ub)-26S proteasome pathyway; V-ATPase, vacuolar H^+-ATPase

Keywords: E3 Ligase • F-box protein; salt stress • *Nicotiana tabacum*; antioxidant ability • ion regionalization.

1. Introduction

Soil salinity is an important factor in plant growth, development, and productivity [1]. When exposed to salt, plants take up Na^+ from the environment and distribute it to the cells in different tissues and organs. Excess salt rapidly and intensely affects phenotypic development and photosynthesis both directly and indirectly [2]. Alterations induced by salt stress on the photosynthesis machinery affects the modulation of many essential proteins, inducing decreases or increases in abundance associated with changes in the redox state, phosphorylation, and degradation synthesis [3]. Secondary stresses, such as oxidative damage and photosynthetic system damage, also often occur in plants under salt stress [1]. In some situations, the secondary effects of salt stress are more serious than ionic toxicity at the level of the whole plant [4].

In response to environmental challenges, plants have developed complex cellular signaling mechanisms to sense and respond to unfavorable conditions [5]. These mechanisms include water content control by regulating stomatal closure [6], osmotic adjustments by increasing the levels of osmolytes [7], enhancements to the activity of antioxidant enzymes to counter oxidative stress [8], and reductions in Na^+ content and the compartmentalization of ions in subcellular organelles to reduce the toxicity [9].

The ubiquitin 26S proteasome system (UPS) is important for the quality control of intracellular proteins and it is known to respond to abiotic stresses [10]. In the UPS, three key enzymes are involved: E1 (Ub-activating enzyme), E2 (Ub-conjugating enzyme), and E3 (Ub-protein ligases). In this process,

the Ub molecule is attached to its target protein through sequential actions [11]. The most important enzyme for conferring substrate selectivity in the Ub-mediated protein degradation pathway is E3 ligase [12]. Based on known E3 ligase motifs, the E3 ligase group can be divided into different families: the anaphase promoting complex (APC), Skp1-Cullin-F-box complex (SCF), homologous to E6-APC terminus (HECT), and Ring/U-box [12]. F-box proteins, in the SCF E3 ligase group, are known to play an important role in responses to abiotic stresses [13].

Some F-box protein genes respond to salt stress [14]. Under salt treatment, *AtPP2-B11* was remarkably increased in terms of both transcript and protein levels [15]. Overexpression of *AtPP2-B11* enhanced the salt stress tolerance of transgenic tobacco plants, whereas an RNA interference line was more sensitive to salt stress than WT plants [15]. SDIR1, a RING finger E3 ligase, is involved in abiotic stress responses in *Arabidopsis* [16].

In our previous work, we cloned the F-box gene *TaFBA1* from wheat, and we found that its expression can be induced by salt stress [17]. Overexpression of *TaFBA1* improved oxidative stress and drought resistance in transgenic tobacco. In this paper, we report on the responses of *TaFBA1*-overexpression tobacco plants to salt stress, and discuss and analyze the physiological, biochemical, and molecular mechanisms involved.

2. Materials and methods

2.1. Screening of transgenic plants

We appraised our transgenic tobacco plants using multiple methods. First, we used a *TaFBA1* special-primer to screen transgenic tobacco lines with RT-PCR. The sequences are listed in Supplementary Table S1.

Next, TaFBA1 protein from leaves extraction was conducted as described by Lee et al. [18]. For

immunoblotting, total plant proteins separated by SDS-PAGE were electrophoretically transferred to polyvinylidene fluoride membranes and then detected with antibody following the methods described in our previous work [17].

Finally, the activity of E3 ligase was measured with a kit from Shanghai Enzyme-linked Biotechnology Co., Ltd. (http://www.mlbio.cn).

2.2. Plant materials and growth conditions

Tobacco plants NC 89 (*Nicotiana tabacum* cv) were used as the wild type (WT). *TaFBA1*-overexpressing (OE) lines were generated in our lab [17]. WT and OE lines were grown in vermiculite under greenhouse conditions with a photoperiod of 16-h light/8-h dark at a temperature of 25 °C. Two-week-old WT and OE plants were potted in vermiculite and treated with 200 mM NaCl or water (control). Samples were collected at the indicated time points. All samples were frozen in liquid nitrogen immediately after collection and stored at -80 °C for use.

The seeds of the *Arabidopsis* plants used were surface-sterilized in 75% ethanol for 30 s, followed by 10% NaClO for 10 min, and then washed with sterile distilled water five to six times. After stratification at 4 °C in darkness for 3 d, they were put on plates containing MS medium.

2.3. Seed germination and growth assays

Approximately 25 surface-sterilized seeds from each OE and WT line were germinated on ½ MS medium with different concentrations of NaCl under a photoperiod of 16-h light/8-h dark at 25 °C. Seed germination rates were evaluated by measuring root emergence after germination for 14 d.

The WT and OE seeds were germinated and allowed to grow on normal ½ MS medium under comfortable environmental conditions for 6 d. They were then transferred to the different concentrations of NaCl for 10 d, when photographs were taken and physiological parameters were

measured.

Arabidopsis plants were grown at 22 °C in a controlled environmental growth chamber with a 16-h light/8-h dark photoperiod after treatment at 4 °C in darkness for 3 d. The young seedlings were then transferred into soil, and every 2 d the plants were irrigated with NaCl concentrations of 50 mM, 100 mM, or 200 mM. Control plants were irrigated with water only. Pictures were taken 5 d after salt treatment.

2.4 Relative water content, chlorophyll content, and proline contents, malondialdehyde content, electrolyte leakage examinations and photosynthesis-related parameter assays

Two-week-old plants grown in vermiculite were treated with 200 mM NaCl for 14 days, pictures were taken in three plants, and the leaves were used to measure the chlorophyll, proline contents, and relative water content (RWC). RWC was detected according to Virginia et al. [19]. The proline content was determined according to Ryu et al. [20]. The chlorophyll content was measured by UV spectrophotometric method as described by Yang et al. [21]. The malondialdehyde (MDA) content was detected according to Zhou et al. [17]. Electrolyte leakage was measured as described by Lutts et al. [22].

Eight-week-old plants grown in vermiculite were treated with 200 mM NaCl for 20 days, and the leaves were used for the measurements. The net photosynthetic rate (Pn), intercellular CO_2 concentration (Ci), and stomatal conductance (Gs) were measured according to Wang et al. [23]. OJIP Chlorophyll α fluorescence was detected according to Appenroth et al. [24].

2.5 Histochemical staining for H_2O_2 and superoxide anion radical ($O_2^{\bullet-}$), detecting for the activity of antioxidative enzymes, measurement of Na^+ distribution and visualization of Na^+/K^+ content

Three-week-old transgenic and WT plants, subjected to 200 mM NaCl for 14 days and these

plants were used for the measurements. The histochemical ROS staining and determination of H_2O_2 and $O_2^{\bullet-}$ content was performed as perthe method described by Zhou et al. [17]. The activities of peroxidase (POD), catalase (CAT), super oxide dismutase (SOD), and ascorbate peroxidase (APX) were measured as described in a previous study Wang et al. [25]

To confirm the distribution of Na^+ in different plant tissues, two-week-old transgenic and WT plants were subjected to 200 mM NaCl for 4 h, and then Na^+ distribution was detected according to the method described by Jia et al. [15]. For Na^+ and K^+ content measurements, the plants were treated with 200 mM NaCl for 7 d. The Na^+ and K^+ ion content was determined following Shukla et al. [26].

2.6. Assays of H^+-ATPase, V-ATPase, and PPase enzyme activity

The PM and tonoplast were separated and used for enzyme activity determination. Microsomal membranes were prepared as described by Bennett et al. [27]. To purify the tonoplast, the tonoplast fraction, which is at the 16/27% Suc interface, was collected according to Bennett and Spanswick [28]. We used two-phase partitioning to purify the PM of the tobacco cells [29]. The PM partitioned into the PEG-rich upper phase, while the intracellular membranes partitioned at the interface and in the dextran-rich lower phase [30].

The activity of PM H^+-ATPase was calculated as the difference in Pi released in the presence or absence of vanadate [31]. Azide and molybdate were added to the reaction mixture to inhibit mitochondrial and phosphatase activity, respectively [32]. V-ATPase activity was measured according to Smart et al. [33]. Enzyme activity was expressed as stimulated K^+ and total PPase activity [34]. Calculations were based on the release of Pi as described by Lin and Morales [35].

2.7. Gene expression analysis by real-time PCR

To analyze gene expression in response to salt stress, 3-week-old transgenic and WT plants grown

in vermiculite were treated with water or 200 mM NaCl for 3 d. Total RNA extraction, cDNA synthesis, and semiquantitative reverse-transcription PCR (RT-PCR) were performed following the procedure described by Zhou et al. [17]. The detected Na^+/H^+ antiporter-related genes, ROS-related genes, and stress-responsive genes in tobacco are listed in Supplementary Table S1. The expression of a specific gene versus a control reference was normalized with the formula $2^{-\Delta\Delta CT}$.

2.8. Na^+ and K^+ flux recording in plant roots and leaves

We used the non-invasive micro-test ~~technique~~ (NMT-YG-100, Younger USA LLC, Amherst, MA, USA) to measure the steady-state fluxes of Na^+ and K^+ (the method for both was the same; not shown). Two-week-old seedlings were treated with 200 mM NaCl for 24 h. Apices (1-2 cm) and tender leaves were used to measure steady Na^+ flux profiles. Na^+ micro-electrodes were prepared and calibrated as previously described by Lang et al. [36]. Roots and tender leaves were then tested in measuring solution following 30 min of equilibration. A continuous flux recording was taken for 600 s for each plant in each of the different tissues. Ionic flux rates were obtained using the non-invasive Micro-test Technology (NMT) of ~~Xu~~ (http://cn.xuyue.net/).

2.9 Statistical analysis

Data were analysed using one-way ANOVA implemented in the SPSS software 13. Significant difference was detected at $P<0.01$. The data was presented as the mean ± standard error (SE) of three independent experiments.

3. Results

3.1. Identification of the transgenic lines

To test the biological functions of *TaFBA1* in plants, we regenerated the *TaFBA1*-overexpression transgenic lines we developed previously [17]. In this paper, three transgenic lines with overexpression

of *TaFBA1* were used (Supplementary Fig. S1A). As shown in Fig. 1A, the antibody of TaFBA1 recognized a protein with a molecular mass of approximately 36.6 kDa in the samples obtained from the transgenic lines, but no signal was observed in WT plants. After salt stress treatment, the TaFBA1 protein levels declined, although mRNA accumulation was induced by salt stress in wheat [17]. Under normal conditions, the activity levels of E3 ligase exhibited no significant differences between the transgenic lines and WT plants. However, when treated with 200 mM NaCl, E3 ligase activity declined in all tobacco plants, but the E3 ligase activity in *TaFBA1*-overexpressing lines was much higher than it was in WT plants (Fig. 1B). These results indicate that the transgene *TaFBA1* was successfully expressed, both transcriptionally and translationally, in the transgenic tobacco plants.

3.2. TaFBA1-*overexpression enhances salt tolerance in transgenic tobacco*

Sterilized seeds of OE3, OE6, OE9, and WT plants were plated on ½ MS growth medium or ½ MS medium containing either 50 or 150 mM NaCl. As shown in Figs. 2A and B, the seed germination rates of the transgenic tobacco exhibited no significant differences from WT plants under normal conditions, and no significant changes were observed under 50 mM salt stress. However, under salt stress conditions with 150 mM NaCl, the germination rates decreased drastically in all seeds (Fig. 2C), although the germination rates of the *TaFBA1*-overexpressing lines decreased much less than the WT rates, especially at 9 d after germination (Figs. 2C and D).

Next, 6-day-old WT and OE seedlings grown on normal ½ MS medium were transferred to ½ MS medium with or without 150 mM NaCl for another 10 d. As shown in Fig. 2E, no significant differences were observed between the transgenic and WT plants on MS medium without NaCl. However, after growing on the MS medium with 150 mM NaCl for 10 d, the root length of the OE lines was much longer than in the WT plants (Figs. 2E and F). These results demonstrate that

overexpression of *TaFBA1* confers salt stress tolerance on the transgenic tobacco during seed germination and seedling growth stages.

In addition, we plated tobacco seeds on ½ MS for 6 d and then transferred them to vermiculite for 2 weeks. Healthy and uniform transgenic and WT plants were selected for further treatment with or without 200 mM NaCl for 2 weeks (Fig. 3A). Under normal growth conditions, the transgenic lines and WT plants showed no significant developmental abnormalities. However, when treated with 200 mM NaCl, the growth of all the tobacco plants was inhibited and the leaves turned yellow, and this phenotypic change was more serious in WT plants than in transgenic lines (Fig. 3). As shown in Fig. 3C, after exposure to salt stress, all the transgenic plants had higher RWC in their leaves than the WT plants. The results shown in Fig. 3D indicate that the proline content in all the plants increased under 200 mM NaCl, but this increase was much greater in the transgenic plants than in the WT plants.

3.3. Effects of TaFBA1-*overexpression on the biomass and photosynthetic characteristics of mature transgenic tobacco lines*

In another experiment, 10-week-old tobacco plants were treated with 200 mM NaCl for 20 d. As shown in Fig. 4, salt stress restrained the growth of the transgenic lines and the WT plants (Figs. 4A and B), but compared to the WT plants, the biomass decrease was less in the transgenic plants.

We also evaluated the effects of salt stress on photosynthetic gas exchange parameters. As shown in Fig. 4C, under normal conditions there were no significant differences in net photosynthesis rate (Pn) between transgenic and WT plants. Salt stress resulted in a significant decrease in Pn, but this value was still maintained at a higher level in transgenic lines than in WT plants under salt stress. Similar results were observed in transpiration rate (E; Fig. 4D) and stomatal conductance (Gs; Fig. 4E). However, the intercellular CO_2 concentration (Ci; Fig. 4F) was higher in transgenic plants than in WT

plants, although higher Pn values were also observed in the transgenic plants, which may be related to their high Gs (Fig. 4E).

The chlorophyll *a* fluorescence transients evidenced important changes in PS II photochemical efficiency under salt stress in the different lines. As shown in Fig. 4G, the WT and OE lines had almost the same dynamic states of their OJIP curves. While under salt stress, higher levels of J transient and I transient were exhibited by the WT plants in comparison to the transgenic plants (Fig. 4H). These results imply that the photosynthetic apparatus of WT plants was subjected to more damage by salt stress compared to the OE plants.

All the results shown in Figs. 2-4 demonstrate that overexpression of *TaFBA1* enhanced the salt stress tolerance of the transgenic tobacco plants.

3.4. ROS accumulation and antioxidative competence in WT and OE plants under salt stress

WT and transgenic lines exhibited similar staining in the presence of NBT and DAB under normal conditions. However, after treatment with 200 mM NaCl, WT leaves accumulated more blue (indicator of $O_2^{\bullet-}$) and brown-colored spots (indicator of H_2O_2) in comparison with transgenic plants (Figs. 5A and B). Similarly, in the presence of salinity, $O_2^{\bullet-}$ production rate and H_2O_2 content were significantly lower in transgenic plants compared to WT plants (Figs. 5C and D).

We quantified electrolyte leakage as a measure of cellular damage in response to salt stress. After 200 mM NaCl treatment for 2 weeks, transgenic plants showed remarkably lower electrolyte leakage levels compared to WT plants, while they showed no significant differences under normal conditions (Fig. 5E). MDA content displayed a pattern similar to electrolyte leakage (Fig. 5F), suggesting that the transgenic plants experienced less lipid peroxidation and maintained more membrane stability than WT plants under salt stress.

We also examined the activity of antioxidative enzymes, including superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) in WT plants and *TaFBA1*-overexpressing lines. Under normal conditions, no significant differences were observed in the activity levels of any of the antioxidative enzymes examined, but salt stress changed these activity levels in different ways. For instance, salt stress induced the activity of SOD. A similar trend was found in the activity levels of POD and APX (Figs. 5G-K). The activity levels of CAT, however, were decreased by salt stress. In all cases, however, the activity levels of these antioxidative enzymes were higher in the transgenic plants than in the WT plants under salt stress.

3.5. Na^+ and K^+ content in roots and leaves of transgenic lines and WT plants

The accumulation of Na^+ was visualized by confocal microscopy using CoroNa Green dye (a green fluorescent indicator of Na^+). Under normal conditions, no differences were observed in Na^+ fluorescence between transgenic lines and WT plants in either roots or leaves. Under 200 mM NaCl stress for 4 h, the fluorescence in the root was weaker in the transgenic lines than in the WT lines. However, in the leaves, no obvious difference was observed in Na^+ fluorescence between transgenic lines and WT plants (Figs. 6A and B). The quantitative results of Na^+ levels in roots and leaves, shown in Figs. 6C and F, were consistent with the Na^+ fluorescence.

In response to NaCl stress, the *TaFBA1*-transgenic lines showed higher K^+ content in roots and leaves compared to WT plants. There were no significant differences between the transgenic lines and WT plants (Figs. 6D and G) under normal conditions. As a result, the transgenic plants showed lower Na^+/K^+ ratios than WT plants under salt stress (Figs. 6E and H).

3.6. Responses of Na^+ transport-related enzyme activity and cellular ion fluxes to salt stress

Na^+ transport is important for ion partitioning in different tissues and organs of plants in response to

the salt environment.

We assayed the activity of PM H^+-ATPase and the V-ATPase in the PM and tonoplast-enriched fractions using a published procedure that relies on the differential sensitivity of the V-type and P-type ATPases to inhibitors [33]. The pyrophosphatase activity of the PPase was also measured in the microsomes and tonoplast-enriched fractions as described by Rea et al. [34]. As shown in Fig. 7, H^+-ATPase activity displayed higher levels in the transgenic lines than in WT plants under treatment with 200 mM NaCl. However, under normal conditions no difference between them was observed (Fig. 7A). A similar trend was found in the activity levels of V-ATPase and PPase (Figs. 7B and C) under 200 mM NaCl stress.

The expression levels of several stress-responsive genes were detected with qRT-PCR. These genes encode enzymes involved in Na^+ antiporter genes (*NtHKT521*, *NtHKT555*, and *NtHKT586*), plasmalemma Na^+/H^+ antiporter (*NtSOS*), proline (*NtP5CS*), and ABA (*NtNCED1*) metabolism, stress defense proteins (*NtERD10C*, *NtERD10D*, and *NtLEA5*), and regulatory proteins (*NtDREB*) (Figs. 8A-J). Salt stress exposure caused upregulation of transcript levels of all the analyzed genes in all plants, and three OE lines had higher expression levels in comparison with the WT plants (Fig. 8). What is more, the antioxidant-related genes (*NtPOD* and *NtSOD*) (Figs. 8K and L) were also upregulated. Steady-state fluxes of Na^+ and K^+ were measured using the non-invasive micro-test ~~technique~~ (NMT-YG-100, Younger USA LLC, Amherst, MA, USA). Figs. 9 and 10 show the different response patterns of Na^+ and K^+ fluxes in roots and leaves of the OE3 and WT plants when exposed to 200 mM NaCl for 24 h.

Under normal conditions, the roots of all the studied plants had a net Na^+ efflux (Figs. 9A and B), but their leaves displayed Na^+ absorption (Figs. 9C and D). When treated with 200 mM NaCl, both leaf

and root cells exhibited an enhanced net Na^+ efflux compared to normal conditions (Figs. 9E-H). The Na^+ extrusion in salt stressed plants may result from the active Na^+/H^+ antiporters across the PM (Fig. 7). After treatment with 500 μM sodium orthovanadate (inhibitor of the PM H^+-ATPase), the dynamic curves displayed a continuous decrease in net Na^+ efflux in both roots and leaves in OE3 and WT plants (Figs. 9J-M).

Cellular fluxes of K^+ in roots and leaves of OE3 and WT plants were also recorded. Under normal conditions, the net K^+ flux out of OE3 and WT roots exhibited no significant differences (Figs. 10A-B), but the net K^+ flux out of leaves did differ (Figs. 10C-D). After 200 mM NaCl treatment, as shown in Figs. 10E-H, the dynamic curves displayed a continuous increase in net K^+ influx in the roots and leaves, and this increase was greater in the transgenic lines than in WT plants.

To confirm the function of *TaFBA1* in salt stress tolerance, an *Arabidopsis* homologous gene mutant was used. In *Arabidopsis*, a mutation of FBW2 was identified in the SALK collection of T-DNA insertions, Salk_144548 (*atfbw2-4*), and we found that *AtFBW2* exhibited high homology with wheat *TaFBA1* (Fig. 11A) [37, 38]. Our analysis in this paper showed that the *AtFBW2* and *TaFBA1* genes have similar expression patterns (Fig. 11B). When treated with 200 mM NaCl, the growth and biomass of *Arabidopsis* plants was inhibited and the leaves turned yellow, and this phenotypic change was more serious in mutant plants than in WT plants (Figs. 11C-G).

4. Discussion

4.1. Overexpression of wheat TaFBA1 *enhanced the salt tolerance of transgenic tobacco plants*

We used PCR and western blot analysis to identify transgenic plants, and as shown in Supplementary Fig. S1 and Fig. 1A, we obtained homogenous transgenic tobacco lines with overexpression of *TaFBA1*. We also found that E3 ligase activity exhibited no significant differences

under normal conditions in the transgenic lines and WT plants. When treated with 200 mM NaCl, E3 ligase activity decreased, but that decrease was less in transgenic lines than in WT plants (Fig. 1B). We suggest that salt stress damaged E3 activity, but the damage was less in transgenic lines than in WT plants, and this was consistent with the high level of TaFBA1 proteins overexpressed by *TaFBA1* as indicated in Fig. 1A, and the improved salt stress tolerance (Figs. 2-4). But the mechanisms underlying the increased E3 ligase activity under salt stress in the OEs plants need to study further.

The improved germination, increased root growth, and high chlorophyll content of transgenic plants under salinity (Figs. 2 and 3) suggested that the *TaFBA1*-overexpressing transgenic tobacco plants showed improvement in salt stress tolerance. The ability to maintain photosynthetic stabilization is essential to salt acclimation and involves phenotypic plasticity mechanisms [39, 40]. The poor Pn values under salt stress were positively related to the observed decrease in Gs [41]. As shown in Fig. 4, tobacco plants exposed to increasing levels of NaCl in the soil showed a diminished net photosynthetic rate (Pn), accompanied by a significant decrease in stomatal conductance (Gs) and transpiration rate (E). But the transgenic plants showed higher photosynthetic rates under salt stress than the WT plants, which may be due to the high levels of photosynthetic pigments and RWC (Fig. 2). The high photosynthetic rate also contributed to the increased accumulation of biomass (Figs. 4A and B). The OJIP curves under salt stress in Fig. 4H also suggest there was less damage to the photosynthetic apparatus in the transgenic plants.

4.2. Improved antioxidant levels may be involved in the enhanced salt tolerance of the transgenic plants

High salinity enhances the production of ROS and causes ROS-associated injury [42-44]. In the present study, salt stress resulted in ROS accumulation as indicated in Figs. 5A-D, which led to cell

membrane damage and membrane lipid peroxidation as indicated by electrolyte leakage and changes in MDA content (Figs. 5E-F). However, overexpressing *TaFBA1* decreased ROS accumulation, lipid peroxidation, and cell membrane damage. In addition, the increased proline levels (Fig. 3D) may also play an important role in protecting cells against increased ROS levels under stress [8].

Plants have evolved various mechanisms to decrease the production of ROS during salt stress. These mechanisms involve enzymatic and non-enzymatic systems [45]. The improved salt tolerance of the transgenic plants might be related to the enhanced activities of antioxidant enzymes, including SOD, POD, CAT, and APX (Fig. 5) [46]. The upregulated expression levels of *NtPOD* and *NtSOD* may be involved (Figs. 8K and L).

4.3. Na^+/K^+ ion regionalization may also be involved in the improved salt tolerance of the transgenic plants

When exposed to salt, plants takes up Na^+ from the environment, which is then distributed in different tissues of the plant and in different regions within the cells. Exclusion of Na^+ is an important mechanism for plants growing on saline substrates to maintain reasonable leaf Na^+ concentrations and to avoid toxicity to photosynthetic tissues [2]. The weaker Na^+ fluorescence in the root of the transgenic lines suggested there was less accumulation of Na^+ there than in WT plants. In the leaves, however, no obvious difference was observed in Na^+ fluorescence between transgenic and WT plants (Figs. 6A and B). By contrast, the transgenic lines contained higher K^+ content than the WT plants in root and leaf tissues (Figs. 6D and G), resulting in a decreased Na^+/ K^+ ratio in their root and leaf tissues under salt stress (Figs. 6E and H).

Plants have evolved a variety of mechanisms to deal with salt stress, and compartmentalization of Na^+ into the vacuole is one of the most important mechanisms for maintaining a low Na^+ content in the

cytoplasm [47]. Vacuolar Na^+ compartmentalization not only keeps Na^+ away from the sites of metabolism but also increases the osmolarity of the cell and regulates cytoplasmic pH [48]. In the vacuole, the driving force for the transport and accumulation of ions is provided by two types of electrogenic, proton-translocating pumps, one that hydrolyzes ATP (V-ATPase) and another that hydrolyzes PPi (PPase) [33]. Membrane potential across the PM can be generated by an H^+-ATPase, which is structurally quite different from V-ATPase [33]. Movement of anions, which serve as osmotica, across the tonoplast and the PM occurs through various carriers and channels [49]. As shown in Fig. 7, the activity of the H^+-ATPase on the PM increased under salt stress, and this increase was greater in transgenic lines than in WT plants (Fig. 7A), moving Na^+ out of the plants and reducing intracellular Na^+ accumulation. Similar to H^+-ATPase, the activity levels of V-ATPase and PPase also increased under salt stress, and they increased more in the transgenic plants (Figs. 7B and C), contributing to the movement of Na^+ into the vacuole, and reducing ion poisoning.

It is well established that Na^+ can enter plant cells through ion channels and carrier-type transporters. In addition, Na^+ uptake can be mediated by carrier-type transporters on the plasma membrane, particularly those of the high-affinity potassium transporter (HKT) family [50]. *SOS1* expression is prominent in root tip cells and also occurs in the xylem parenchyma [51]. As shown in Fig. 8, *HKT521*, *HKT555*, *HKT586*, and *SOS* expression was upregulated in transgenic plants after salt stress treatment (Figs. 8A-D). *ERD10* (*C*/*D*) encodes group 2 late embryogenesis abundant (LEA) proteins, which partially bind water, stabilize labile enzymes, protect cellular and macromolecular structures, and reduce extensive membrane damage [52]. As shown in Figs. 8E-F, the expression of *ERD10* (*C*/*D*) was also higher in the transgenic lines than in WT plants. The delta1-pyrroline-5-carboxylate synthetase (*P5CS*) gene encodes an enzyme that is involved in catalysis

of the first two steps in proline biosynthesis [53]. Increased expression of *NtP5CS* may lead to further increased proline production in transgenic tobacco (Fig. 8G). Circumstantial evidence suggests that LEA5 proteins function in osmotic stress tolerance, in stabilizing labile enzymes, and in protecting macromolecule and membranes structures [54, 55]. Our results show that *NtLEA5* expression was upregulated in transgenic plants after salt stress treatment (Fig. 8H). Moreover, *DREB*, which encodes dehydration-responsive element-binding proteins, also exhibited higher expression in transgenic lines than in WT plants (Fig. 8I). Biochemical and genetic evidence reveals that many enzymes are involved in the ABA biosynthetic pathway; *NCEDs*, for example, play a pivotal role in ABA accumulation [56]. As shown in Fig. 8J, the transcript level of *NtNCED1* was enhanced in transgenic lines as compared with WT plants after salt treatment.

Na^+ efflux resulted from active Na^+ exclusion across the plasma membrane [57]. NMT data show that the roots of the transgenic lines have a higher capacity to exclude Na^+ under salt stress (Figs. 9E-H). Inhibition of Na^+/H^+ antiporter by sodium orthovanadate (a PM H^+-ATPase inhibitor) in roots and leaves verified the involvement of Na^+/H^+ antiporters and H^+ pumps (PM H^+-ATPase) in Na^+ exclusion (Figs. 9J-M). The great capacity to exclude Na^+ in the roots of the transgenic lines may restrict salt transport and its subsequent accumulation in aerial tissues. It is obvious that the roots of the transgenic lines have a higher capacity than WT plants to absorb K^+ under salt stress (Figs. 10E-F). At the same time, net Na^+ movement out of plants was promoted. This helps to maintain the balance of ions in the transgenic plants.

How and why did the overexpressed TaFBA1 affect so many plant responses to salt stress? *TaFBA1* encodes an F-box protein that is a major subunit of the SCF complex. The SCF complex functions as a group of E3 ligases, which are the key enzymes that define the specificity of target

proteins for degradation by the ubiquitin-26S proteasome system (UPS) [58, 59]. In this process, F-box proteins interact with specific other proteins. For instance, the late embryogenesis abundant (LEA) proteins play an important role in multiple stress responses [60]. *LEA14* is upregulated by cold, drought, and salt stress [61], and it can physically interact with AtPP2-B11, an F-box E3 ligase [62]. AtPP2-B11 positively regulates salt responses in *Arabidopsis* by AtLEA14 to maintain the stability of AtPP2-B11 [62]. F-box proteins have also been found to be involved in ABA signal transduction [63]. The F-box protein EDL3 functions as a positive regulator in ABA signal transduction and regulates seed germination, root growth, and etiolated seedlings turning green [64]. Our recent data (not shown) also demonstrate that TaFBA1 can interact with other proteins, which could result in multifunctionality for TaFBA1. However, although F-box protein family has been reported to regulate many different target proteins, as a particular F-box, TaFBA1 is not likely to be involved in so many aspects in salt tolerance, some characteristics of the OEs plants under salt stress in this paper might be the consequence of salt tolerance, rather than the reason of it. Further experiments should be conducted to clarify the detailed regulatory mechanism of TaFBA1 in salt stress tolerance.

In conclusion, plants employ three different strategies to avoid and adapt to high Na^+ concentrations: (1) active Na^+ efflux, (2) Na^+ compartmentalization in vacuoles, and (3) Na^+ influx prevention [65, 66]. We suggest the following model based on our results (Fig. 12): *TaFBA1*, an SCF E3 ligase, responds to salt stress. Overexpression of *TaFBA1* enhances the salt stress tolerance of transgenic tobacco plants, and this may be associated with (1) repressed ROS accumulation, and (2) improved Na^+ homeostasis under salt stress.

Author contribution statement

The paper presented here was carried out in collaboration between all authors. W. Wang defined

the research theme and designed most of the methods and experiments; Zhongxian Zhao performed the laboratory experiments with the help of Guangqiang Zhang and Yuanqing Ren; Shumei Zhou executed the gene transformation; Zhongxian Zhao wrote the paper. All authors discussed the results and approved the article.

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Legends to figures

Fig. 1 Screening of transgenic tobacco lines and the activity of E3 ligase for salt stress. (A) Western blot analysis TaFBA1 protein abundance in transgenic tobacco lines. (B) The activity of E3 ligase, 8-week-old plants with 200 mM NaCl for 2 weeks. Double Antibody Sandwich Method. Asterisks indicate significant difference between transgenic and WT plants (*P < 0.05, **P < 0.01).

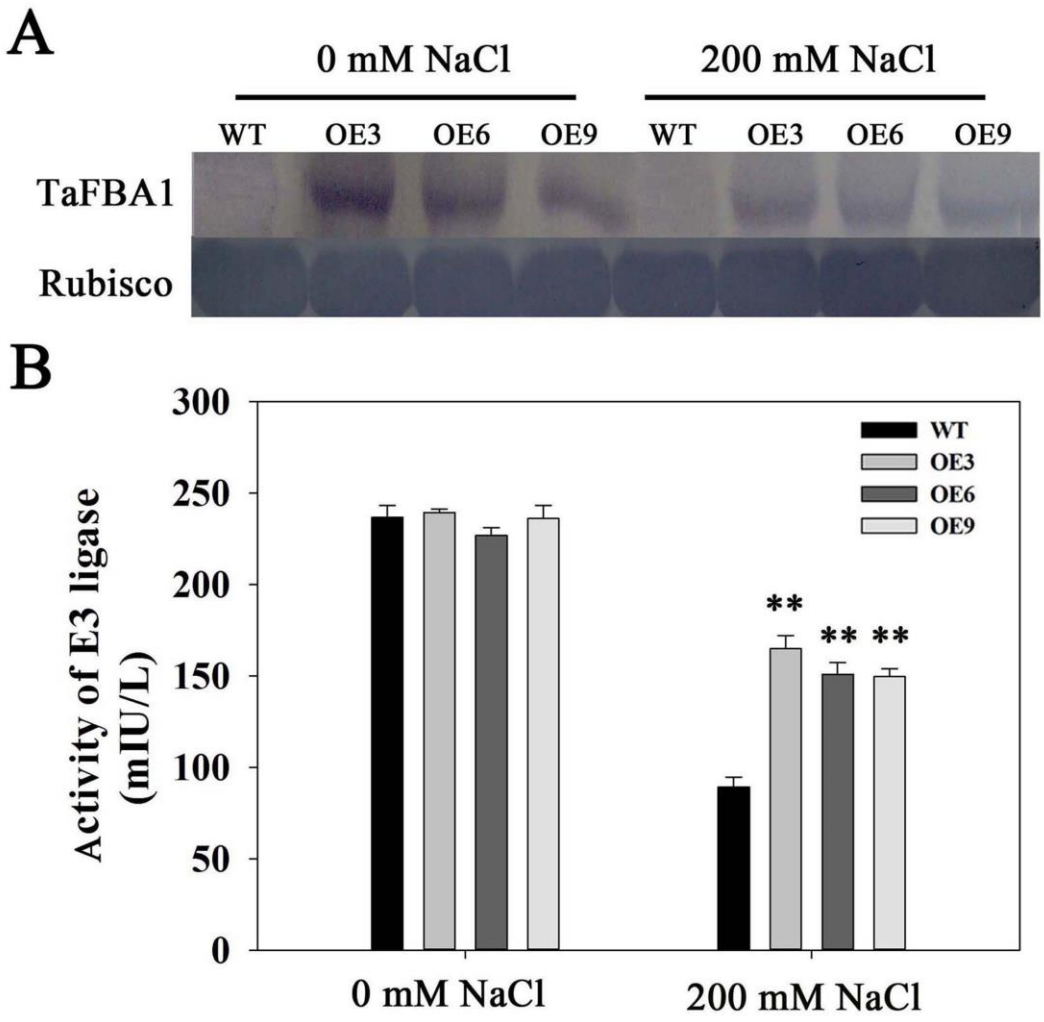

Fig. 2 Under different concentrations of NaCl, germination and phenotype of transgenic lines and WT on MS medium. (A) Seed germination phenotypes on MS medium containing different concentrations of NaCl. (B, C, D) Germination rates of the OE lines (OE3, OE6 and OE9) and WT under normal and NaCl treatment conditions. (D) Germination rates of the WT and OE lines under normal and NaCl treatment conditions on 9th day. The presented data are the means±SE of three independent experiments (n=3). (E) The seedlings of the OE lines and WT under normal and NaCl treatment conditions. The seeds sown on MS medium that showed radicle emergence after 6 d were transferred to MS medium containing different concentrations of NaCl. The plates were oriented vertically, with seedlings kept upside down, and a photograph was taken 10 d after transfer. (F) Primary root lengths of the seedlings at 10 d after germination in the presence of different NaCl concentrations. The presented data are the means±SE of three independent experiments (n=6). Asterisks indicate significant difference between transgenic and WT plants (*P < 0.05, **P < 0.01).

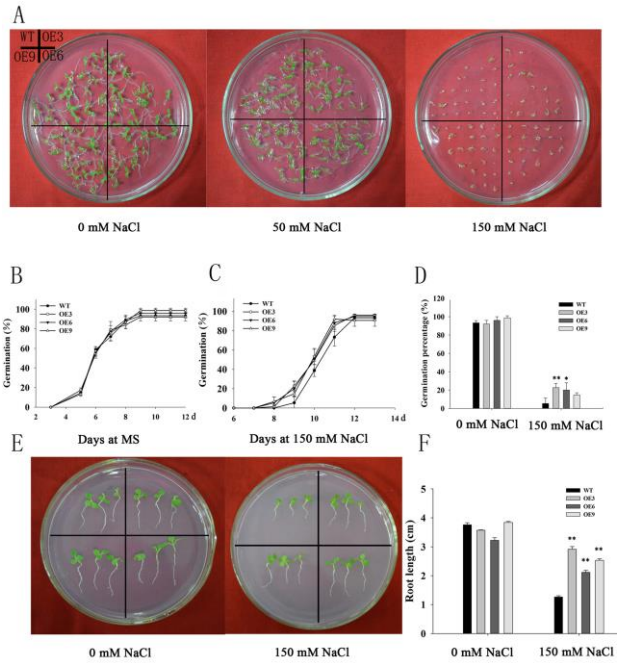

Fig. 3 Responses of *TaFBA1*-transgenic and WT plants to salt stress at the seedling stage. (A, B) Photographs of *TaFBA1*-transgenic and WT plants after watering with 200 mM NaCl. After transferred to normal conditions for 2 weeks, the transgenic and WT plants were watered with 200 mM NaCl for 2 weeks. RWC (%) (C), Proline content (D), Chlorophyll a (E) and b (F) levels of *TaFBA1*-transgenic and WT plants before and after watering with 200 mM NaCl for 2 weeks at the seedling stage. Data showed the mean±SD of at least three replicates. Asterisks indicate significant difference between transgenic and WT plants (*P < 0.05, **P < 0.01).

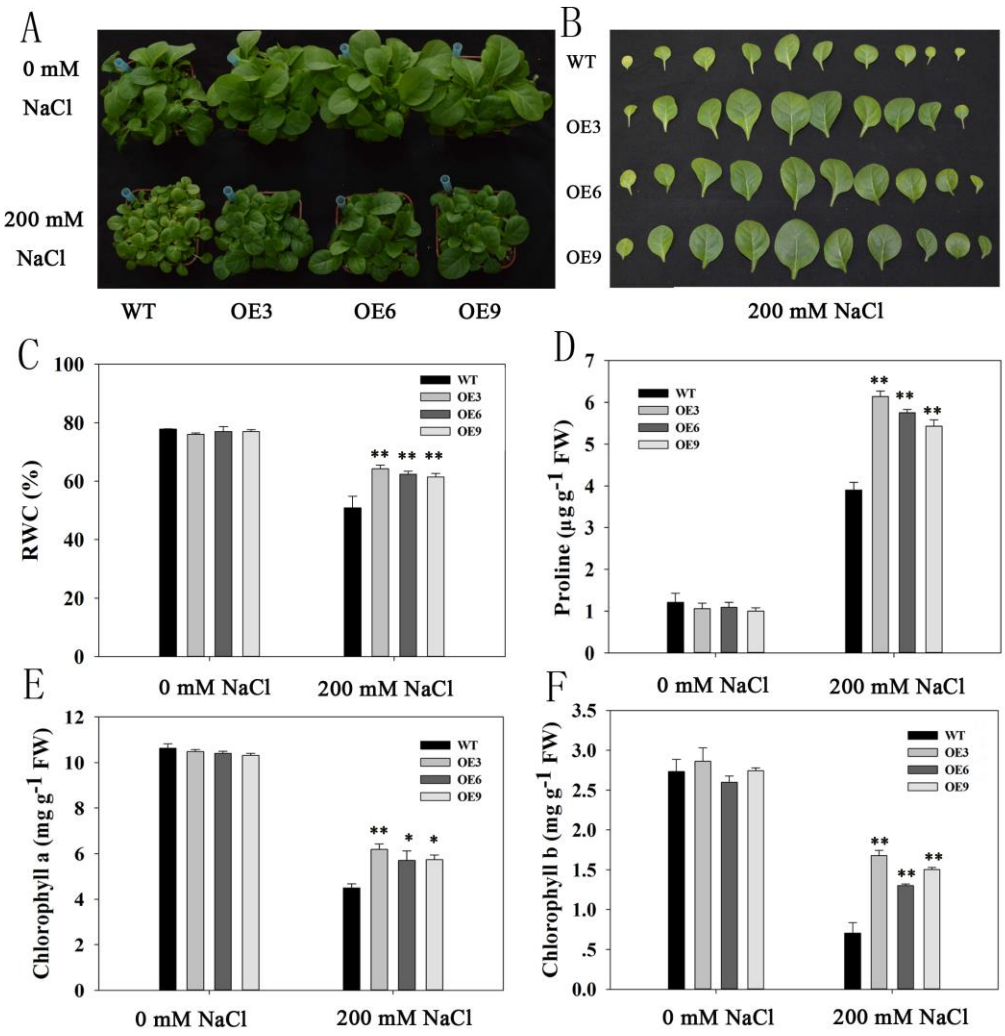

Fig. 4 Photosynthetic performance of *TaFBA1*-transgenic and WT tobacco plants in response to salt stress. (A) 8-week-old plants with 200 mM NaCl for 20 d. (B) The biomass that were irrigated with different concentrations of NaCl for 20 d. (C) Net photosynthetic rate (Pn, μmol CO_2 m^{-2} s^{-1}), (D) Transpiration rate (E, mmol H_2O m^{-2} s^{-1}), (E) stomatal conductance (Gs, mmol H_2O m^{-2} s^{-1}), (F) Intercellular CO_2 concentration (Ci, μmol CO_2 mol^{-1}), (G, H) Chorophyll a fluorescence transients in leaves of WT(G) and OE plants(H) before and after 200 mM NaCl for 20 d, (Vt=(Ft-Fo)/(Fm-Fo)). The presented data are the means ±SE of at least three independent experiments. Asterisks indicate significant difference between transgenic and WT plants (*P < 0.05, **P < 0.01).

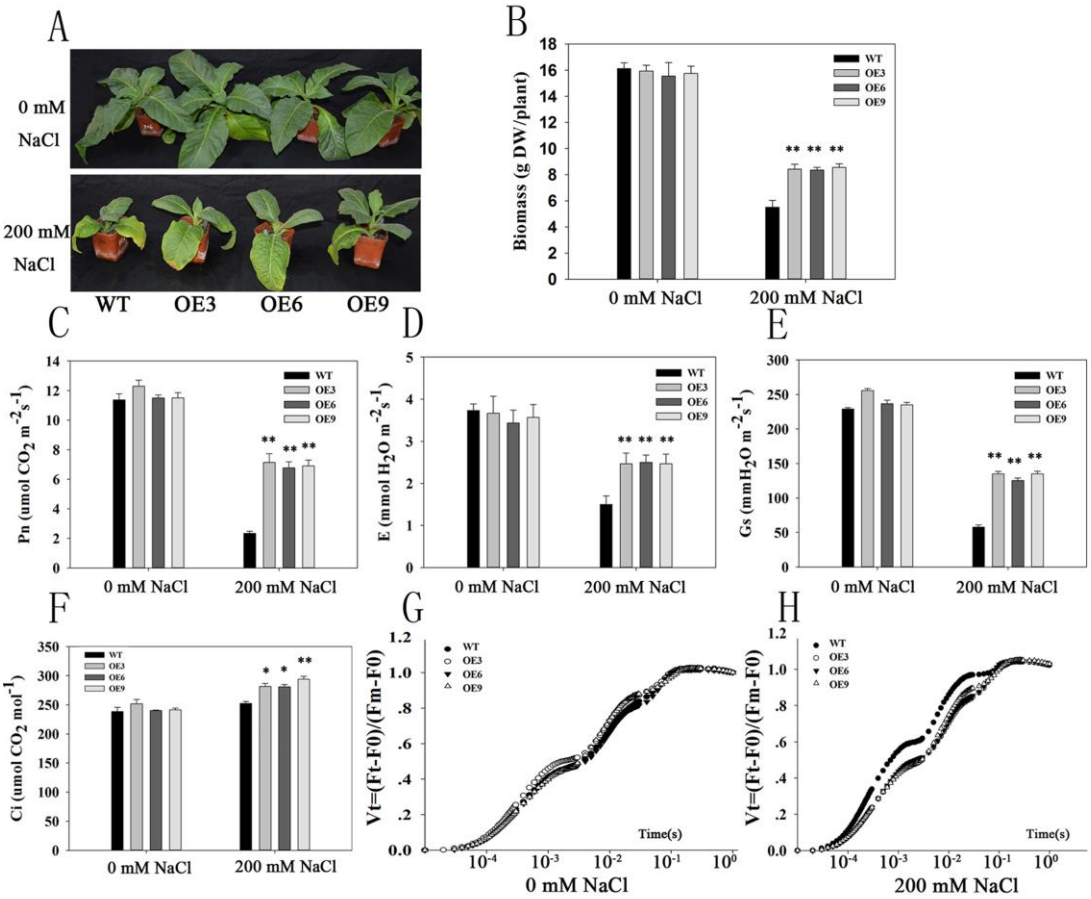

Fig. 5 The responses of ROS accumulation and antioxidative competence in WT and OE plants to salt stress. H_2O_2 (A) and $O_2^{\bullet-}$(C) production rate detected by DAB and NBT staining, respectively. Quantification of H_2O_2 (B) and $O_2^{\bullet-}$ (D) production rate. The presented data are the means±SE of three independent experiments. (E) Electrolyte leakage, (F) MDA content, (G) SOD activity, (H) CAT activity, (J) POD activity and (K) APX activity in 3-week-old WT and *TaFBA1*-overexpressing lines after spraying with 200 mM NaCl or water (control, CK) for 2 weeks. Each column represents the average of three replicates, and error bars represent the standard deviation. The statistical significance of the difference was confirmed by Student's t-test, *P<0.05. **P<0.01.

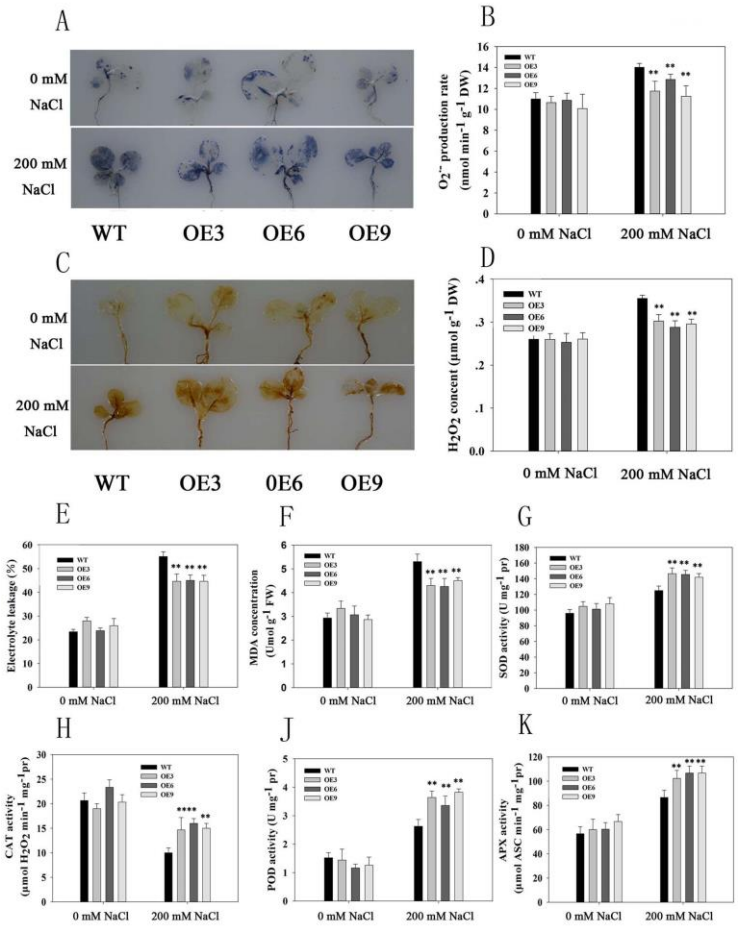

Fig. 6 Na^+ and K^+ accumulation in root and leaf tissues of transgenic and WT lines in response to salt stress. (A, B) Na^+ accumulation in root and leaf tissues of 2-week-old *TaFBA1* transgenic and WT seedlings treated with 200 mM NaCl for 4 h. Na^+ was visualized using CoroNa Green dye by laser-scanning confocal microscope. (C) Na^+ contents in root, (D) K^+ contents in root, (F) Na^+ contents in leaf, (G) K^+ contents in leaf. (E) Na^+/K^+ ratios in root, (H) Na^+/K^+ ratios in leaf. Asterisks indicate significant difference between transgenic and WT plants (*P < 0.05, **P < 0.01).

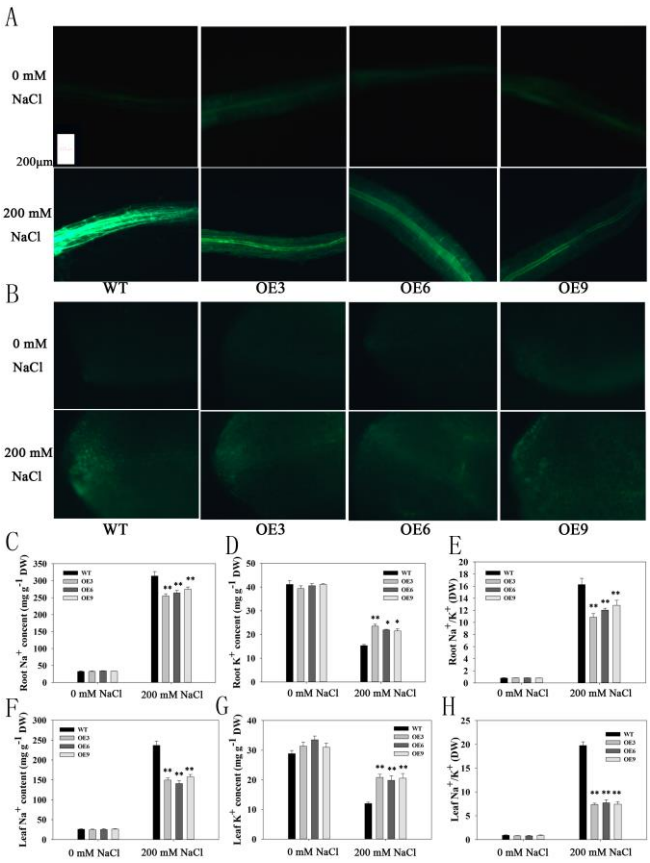

Fig. 7 Na^+ transport activities in the WT and transgenic plants in response to salt stress. (A) Cell membrance H^+-ATPase activity was determined from microsomal membranes of 6-week-old plants of WT and transgenic lines treated with 200 mM NaCl for 2 weeks. (B) Vacuolar H^+-ATPase, (C) Vacuolar PPase activity; data are mean ± SE of four independent assays; Asterisks indicate significant difference between transgenic and WT plants (*P < 0.05, **P < 0.01).

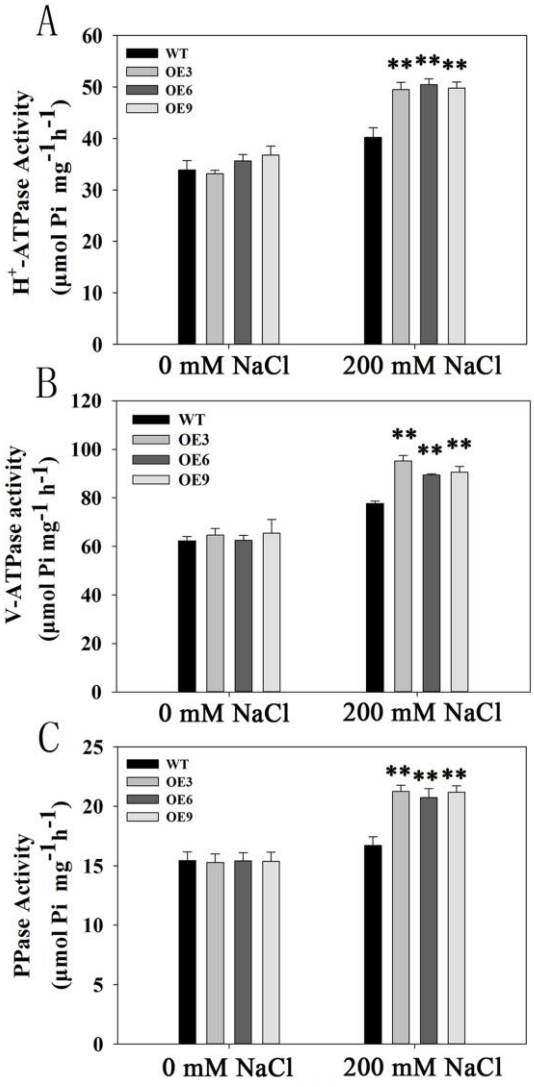

Fig. 8 Relative expression levels of some Na^+ antiporter genes and defense-related genes in the leaves from the WT and transgenic tobacco plants before and after salt treatment. (A-D) Relative transcript expression of Na^+ antiporter genes *NtHKT521*, *NtHKT555*, *NtHKT586* and *NtSOS*; (E-J) Relative transcript expression of defense-related genes *NtERD10C*, *NtERD10D*, *NtP5CS*, *NtLEA5*, *NtDREB* and *NtNCED1*; (K-L) Relative transcript expression of antioxidant related genes genes *NtPOD* and *NtSOD*. The levels in WT plants (set at 1) referring to the transcript of actin in the same samples. Data are mean ± SE of four independent assays; Asterisks indicate significant difference between transgenic and WT plants (*P < 0.05, **P < 0.01).

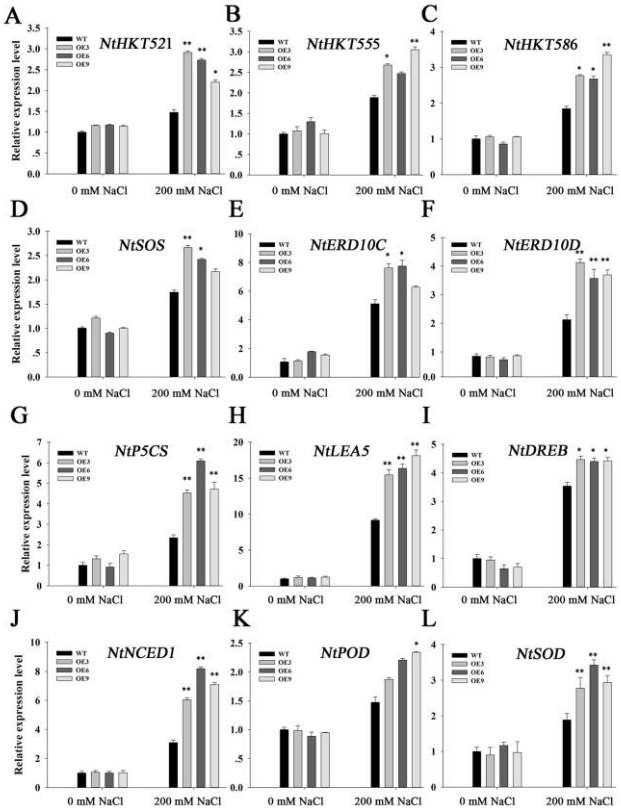

Fig. 9 Net fluxes of Na^+ in roots and leaves from WT and transgenic lines under normal (A-D) and salt stress conditions (E-H). (B, D, F, H, J and L) Mean of net Na^+ fluxes corresponding to (A, C, E, K and M) during the measuring periods. Add sodium orthovanadate (500 μM) (J-M), the flux rates of Na^+ within the measuring period of 600 s. Data are mean ± SE of four independent assays; Asterisks indicate significant difference between transgenic and WT plants (*P < 0.05, **P < 0.01).

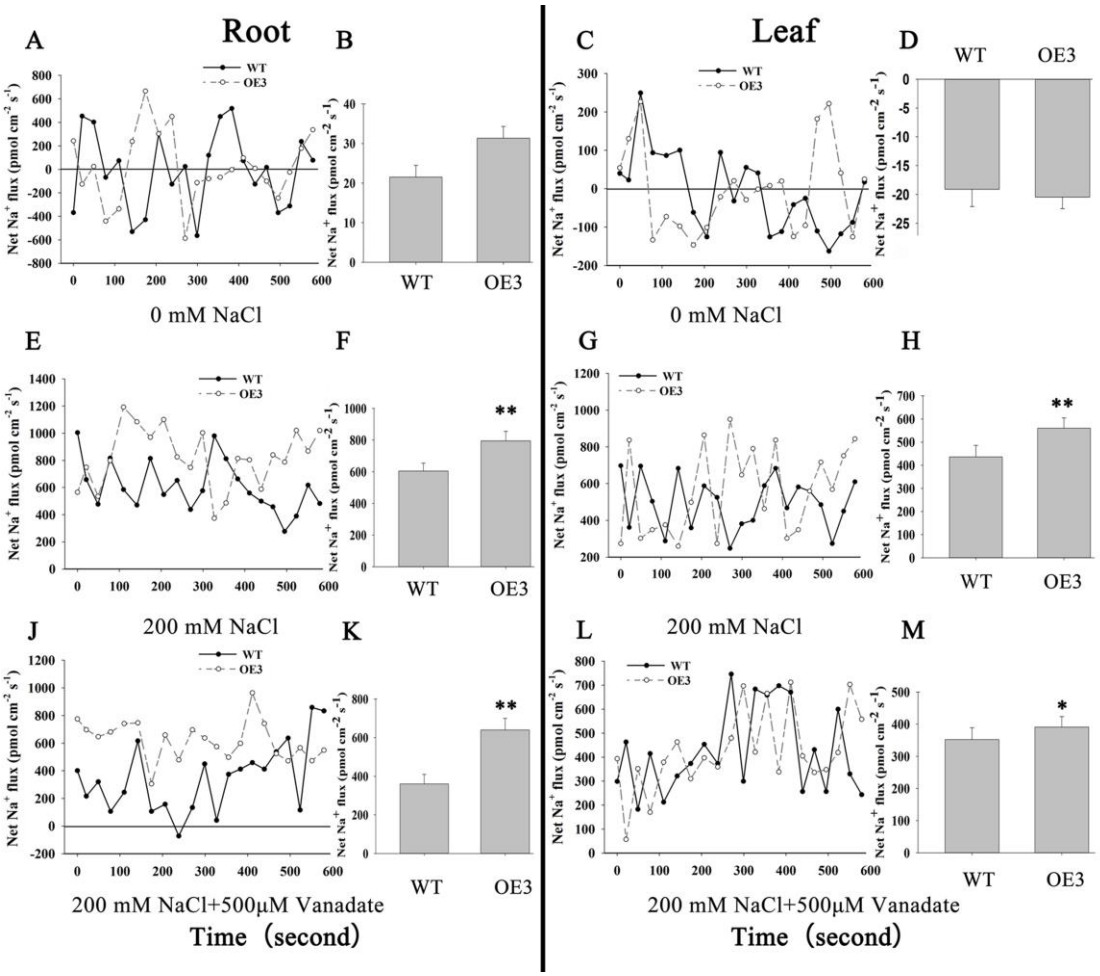

Fig. 10 Effects of salt stress on net K^+ fluxes in roots and leaves of the transgenic and WT plants under normal (A-D) and salt stress conditions (E-H). The flux rates of Na^+ within the measuring period of 600 s. (B, D, F and H) Mean of net K^+ fluxes corresponding to (A, C, E and G) during the measuring periods. Data are mean ± SE of four independent assays; Asterisks indicate significant difference between transgenic and WT plants (*P < 0.05, **P < 0.01).

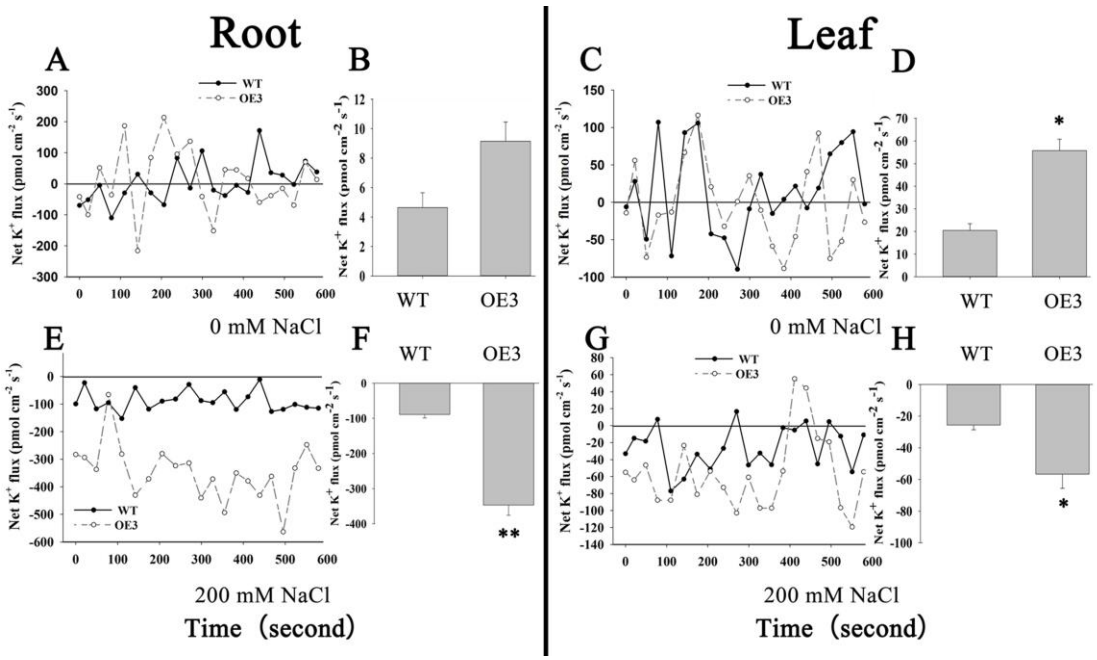

Fig. 11 Identifying the salt tolerance of *atfbw2-4* mutant. (A) The T-DNA was inserted into the F-box domain of FBW2. PCR genotyping shows that *atfbw2-4* mutant is homozygous for the T-DNA insertion. (B) Changes in AtFBW2 mRNA levels in response to exogenous NaCl treatment measured by real-time RT-PCR. *Arabidopsis* seedlings were collected after exposure to 200 mM NaCl treatment. Seedlings incubated in sterile water were used as the control. The expression data correspond to means of triplicates, and using a control sample from 0 h as the calibrator. Experiments were independently replicated at least three times and with at least 20 plants per experiment. (C) Phenotypes of WT and *atfbw2-4* mutant before and after salt treatment in soil. (D) and (E) Root lengths of WT and *atfbw2-4* mutant plants under salt stress conditions (n=20 plants from three individual plates. (F) The plant biomass that was irrigated with different concentrations of NaCl for 5 d. (G) Number of leaves before and after salt treatment in WT and *atfbw2-4* mutant. Statistical significance between samples was indicted by different letters according to the Student t-test.

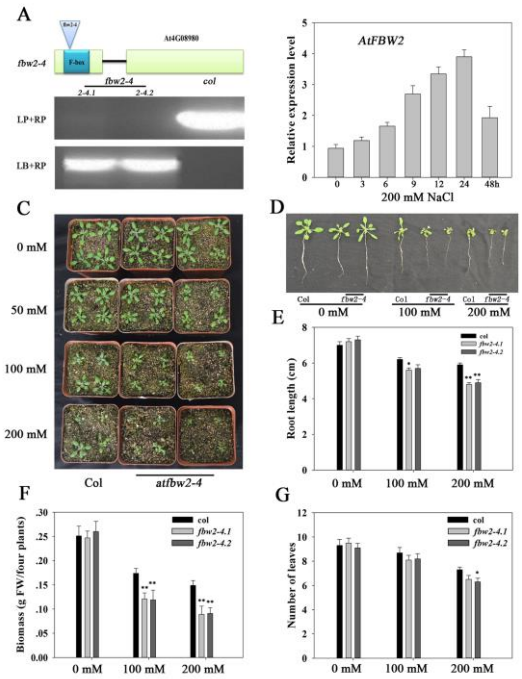

Fig. 12 Model of *TaFBA1* involvement in the salt stress response. Overexpressing *TaFBA1* repressed ROS accumulation, upregulated the activities of Na^+ antiporters, and affected Na^+ homeostasis under salt stress conditions to elevated salt stress tolerance.