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The improvement of salt tolerance in transgenic tobacco by overexpression of wheat F-box gene *TaFBA1*

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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.

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

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

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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 ($O_2\cdot^-$), 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 $O_2\cdot^-$ 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