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Physiological responses to nitrate stress of transgenic tobacco plants harbouring the cucumber mitogen-activated protein kinase gene

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Abstract: The physiological responses to nitrate stress of 2 transgenic tobacco lines containing the cucumber mitogen-activated protein kinase (*CsNMAPK*) gene were investigated. Seed germination rates of the transgenic tobacco lines were higher than that of the wild type (WT) tobacco under 150 mM nitrate treatment. The transgenic seedlings had higher root fresh weight (FW) and dry weight (DW) than the WT plants after 98 mM and 182 mM nitrate treatment. The malondialdehyde (MDA) content, electrolytic leakage (EL), and H_2O_2 content were higher in the WT than they were in the transgenic plants after 7-day nitrate stress treatment. The antioxidant enzyme (superoxide dismutase [SOD], catalase [CAT], peroxidase [POD], ascorbate peroxidase [APX]) activities increased with the increasing of nitrate concentration and the transgenic plants exhibited higher activities than the WT did. Excess nitrate stress induced more proline accumulation in the transgenic plants than in the WT plants. These results suggested that the tolerance of overexpressing-*CsNMAPK* tobacco plants to nitrate stress might partly be attributed to higher antioxidant enzyme activities and enhanced osmotic regulation capacity.

Key words: Mitogen-activated protein kinase, cucumber, nitrate stress, antioxidant enzymes

1. Introduction

Abiotic stress, such as drought, high salinity, extreme temperature, and flooding, is a major cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Bray et al., 2000). Proper physiological and biochemical responses to such stresses are controlled by an array of stress-dependent signal transduction pathways (Xiong et al., 2002). Mitogen-activated protein kinase (MAPK) cascades are known to be one of the major pathways by which extracellular signals such as growth factors, hormones, and stress stimuli are transduced into intracellular responses in yeast and mammalian cells as well as in plants (Emerling et al., 2005; Sumbayev & Yasinska, 2005).

MAPK cascades are signalling modules that minimally consist of a MAPK kinase kinase (MAPKKK/MEKK), a MAPK kinase (MAPKK/MKK), and MAPK. Upon a stimulus-triggered activation of a MAPKKK, the signal is transduced via phosphorylation-mediated activation of a corresponding downstream MAPKK, which in turn phosphorylates and thereby activates a specific MAPK. The phosphorylated (activated) MAPK interacts with and alters the phosphorylation status of target proteins, including transcription factors, enzymes, and other proteins, ultimately influencing gene expression, metabolism, cell division, and growth. Plant MAPKs have been involved in the regulation of certain aspects of plant growth and development, including not only cell division, hormone action, and pollen development, but also stress tolerance (Hirt, 2000; Zhang et al., 2001). Many data indicated that MAPK was rapidly activated in plants exposed to a variety of abiotic and biotic stresses including salt, cold, drought, UV-irradiation, wounding, and pathogens (Ichimura et al., 2000; Fu et al., 2002; Cheong et al., 2003; Blanco et al., 2006; Shoresh et al., 2006). In *Arabidopsis*, MAPK cascades are known to be involved in a number of stress response signalling pathways (Colcombet & Hirt, 2008; Pitzschke et al., 2009).

Cucumber is one of the most important vegetables in the greenhouses of China. Currently, there are over 1.5 million hectares of protected vegetables in China for which secondary salinisation is an ever-present threat to the yield and quality of vegetables (Ouyang et al., 2007). According to previous studies, accumulation of ions in protected

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farmland is greatly different from that at the seaside. Chen and Yang (1995) discovered that the excessively accumulated cations and anions in the soil of greenhouses were Ca^{2+}, K^+, and NO_3^-. The content of NO_3^- was about 580 mg kg^{-1} in some greenhouse soil in Liaoning Province and about 800 mg kg^{-1} in Jiangsu Province in China (Yu et al., 2007). The large accumulation of salt and salt ions might induce other limiting factors of greenhouse cropping systems, such as nutritional disorders, acidification of soil, and short supply of CO_2 (Yu et al., 2007). In past years, a lot of research was done about salt stress, but most of these studies focused on NaCl stress (Zhu, 2002; Stepien & Johnson, 2009). So far, there have been few investigations about nitrate stress in vegetables.

Recently, we have reported the transformation of cucumber *CsNMAPK* into tobacco and discovered that the transgenic plants T1-4 and T1-7 have enhanced NaCl stress tolerance during seed germination. The germination rate of T1-7 (87.5%) was significant higher than that of T1-4 (40.0%) after 200 mM NaCl treatment for 20 days (Xu et al., 2010). Antisense expression *CsNMAPK* cucumber plants have higher MDA content and lower SOD activity and proline than wild type (WT) cucumber plants under NaCl stress (Xu et al., 2011). Shi et al. (2004) compared iso-osmotic stress of $Ca(NO_3)_2$ (80 mM) and NaCl (120 mM) to tomato and observed that the MDA and proline were all significantly accumulated, indicating that excess nitrate stress to plants shared similar defence pathways with NaCl stress. Therefore, we hypothesise that overexpression *CsNMAPK* tobacco plants might have higher nitrate stress tolerance as the NaCl stress tolerance with the same defence pathway.

To test our hypothesis, seed germination rates and some physiological parameters of the seedlings of T1-4, T1-7, and WT tobacco plants under nitrate stress treatment were investigated. The results indicated that the tolerance of overexpressing-*CsNMAPK* tobacco plants to nitrate stress might partly be attributed to higher antioxidant enzyme activities and enhanced osmotic regulation capacity.

2. Materials and methods

2.1. Germination assay under nitrate stress

Seeds of WT, T1-4, and T1-7 tobacco plants were germinated in sterile agar medium containing Murashige and Skoog (MS) salts supplemented with 30 g L^{-1} sucrose. Germination assays were carried out on 3 replicates of 40 seeds. To determine the effect of excess nitrate stress on germination, MS medium was supplemented with 0 and 150 mM NO_3^-. A seed was regarded as germinated when the radical protruded through the seed coat. Seeds were geminated in controlled environment chambers at an irradiance of 140 µmol photons m^{-2} s^{-1}, 22 °C, and relative humidity of 60%.

2.2. Plant growth and stress treatments

Seedlings of T1-4 and T1-7 transgenic tobacco lines and WT plants were grown hydroponically in a plastic tank in the greenhouse of Shandong Agricultural University with 10 L nutrient solution of pH 6.0–6.5 containing aerated full nutrient solution: $Ca(NO_3)_2$ 3.5 mmol L^{-1}, KNO_3 7 mmol L^{-1}, KH_2O_4 0.78 mmol L^{-1}, $MgSO_4$ 2 mmol L^{-1}, H_3BO_3, 29.6 µmmol L^{-1}, $MnSO_4$ 10 µmmol L^{-1}, Fe-EDTA 50 µmmol L^{-1}, $ZnSO_4$ 1.0 µmmol L^{-1}, H_2MoO_4 0.05 µmmol L^{-1}, $CuSO_4$ 0.95 µmmol L^{-1}. The experiment was carried out under natural conditions with an air temperature of 25–30 °C during the day and 18–25 °C during the night. The WT tobacco (*Nicotiana tabacum* cv. NC 89) was used as a control.

When the tobacco seedlings were at the 6-leaf stage, KNO_3 and $Ca(NO_3)_2$ were added to the nutrient solution to form the final NO_3^- concentration of 98 and 182 mM (KNO_3 and $Ca(NO_3)_2$ provide the same mol of NO_3^-) and the normal NO_3^- concentration of 14 mM in the nutrient solution was used as a control. Measurements were taken after 7 days of treatment. Plants were divided into shoots and roots. Their fresh weight (FW) of roots were directly determined. For dry weight (DW) determination, the roots were dried at 80 °C for 48 h and then weighed. For determination of antioxidant enzyme activities and lipid peroxidation, root samples were harvested, weighed, and stored at –80 °C for analysis. The electrolyte leakage and proline content were measured with fresh root samples.

2.3. Electrolyte leakage assay

The electrolyte leakage was assayed according to the method described by Lutts et al. (1996). Root samples were washed 3 times with deionised water to remove surface-adhered electrolytes. Then 0.5-g fresh root samples were cut into 1-cm length and placed in test tubes containing 20 mL of distilled deionised water. The tubes were covered with plastic caps. After 4 h, the initial electrical conductivity levels of the medium (EC_1) and deionised water (EC_0) were measured using an electrical conductivity meter (ORION conductivity TDS meter, Japan). The samples were heated afterwards at 100 °C for 15 min to completely kill the tissues and release all electrolytes. Samples were then cooled to 25 °C and the final electrical conductivity (EC_2) was measured. The electrolyte leakage (EL) was expressed using the formula $EL\ (\%) = (EC_1 - EC_0)/(EC_2 - EC_0) \times 100$.

2.4. Lipid peroxidation assay

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) formation using the thiobarbituric acid method described by Madhava Rao and Sresty (2000). MDA is a product of lipid peroxidation by thiobarbituric acid reaction. The concentration of MDA was calculated from the absorbance at 532 nm by using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.5. H_2O_2 content assay

H_2O_2 content was determined according to Patterson et al. (1984). The assay was based on the absorbance change of the titanium peroxide complex at 415 nm. Absorbance values were quantified using standard curve generated from known concentrations of H_2O_2.

2.6. Antioxidant enzyme assays

Root samples of both transgenic and WT plants after treatment with excess nitrate for 7 days were used for enzyme analysis. First 0.5 g of root was homogenised in 4 mL of 0.05 M sodium phosphate buffer (pH 7.8) including 1 mM EDTA and 2% (w/v) PVP. The homogenate was centrifuged at 10,000 × *g* for 20 min at 4 °C. Supernatant was used for enzyme activity. All steps in the preparation of the enzyme extract were carried out at 4 °C. All spectrophotometric analyses were conducted on a Shimadzu (UV-2450PC) spectrophotometer.

SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm (Madhava Rao & Sresty, 2000). The reaction mixture consisted of 0.3 mL each of 0.75 mM NBT, 130 mM methionine, 0.1 mM EDTA-Na_2, 0.02 mM riboflavin, and sterilised water, and 1 mL of 50 mM Na-phosphate buffer (pH 7.8). The reaction was started by adding 0.5 mL of enzyme extract and was carried out for 20 min at 25 °C under a light intensity of 300 $\mu mol^{-1}\ m^{-2}\ s^{-1}$. One unit of enzyme activity was defined as the quantity of SOD required to produce a 50% inhibition of reduction of NBT and the specific enzyme activity was expressed as unit mg^{-1} protein g FW.

CAT activity was measured as the decline in absorbance at 240 nm due to the decline of extinction of H_2O_2 using the method described by Patra et al. (1978). The reaction mixture contained 25 mM sodium phosphate buffer (pH 7.0), 10 mM H_2O_2, and 0.1 mM enzyme extract. The reaction was initiated by adding H_2O_2.

POD activity was measured by the increase in absorbance at 470 nm due to guaiacol oxidation (Nickel & Cunningham, 1969). The reaction mixture contained 25 mM guaiacol, 10 mM H_2O_2, and 0.1 mL enzyme extract. The reaction was started by adding H_2O_2.

APX activity was measured according to Nakano and Asada (1981). The assay depends on the decrease in absorbance at 290 nm as ascorbate is oxidised. The reaction mixture contained 25 mM sodium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2, 0.1 mM EDTA, and 0.1 mL of enzyme extract. The reaction was started by adding H_2O_2.

2.7. Proline content assays

To determine free proline level, 0.5-g root samples from each group were homogenised in 3% (w/v) sulphosalicylic acid and then homogenate filtered through filter paper (Bates et al., 1973). The mixture was heated at 100 °C for 1 h in a water bath after addition of acid ninhydrin and glacial acid. The reaction was then stopped by the ice bath. The mixture was extracted with toluene. The absorbance of the upper phase was spectrophotometrically determined at 520 nm. Proline concentration was determined using a calibration curve and expressed as μmol proline g^{-1} FW.

2.8. Statistical analysis

The data were analyzed with OriginPro8 (Version8E, OriginLab Corporation, Massachusetts, USA) and presented as means of 3 replicates ± standard errors. For statistical analysis, one-way ANOVA and the t-test were used to determine the significance at $P < 0.05$.

3. Results

3.1. Seed germination rates of transgenic tobacco plants under nitrate stress

Figure 1 shows changes in the seed germination rates of transgenic tobacco plants in MS medium supplemented

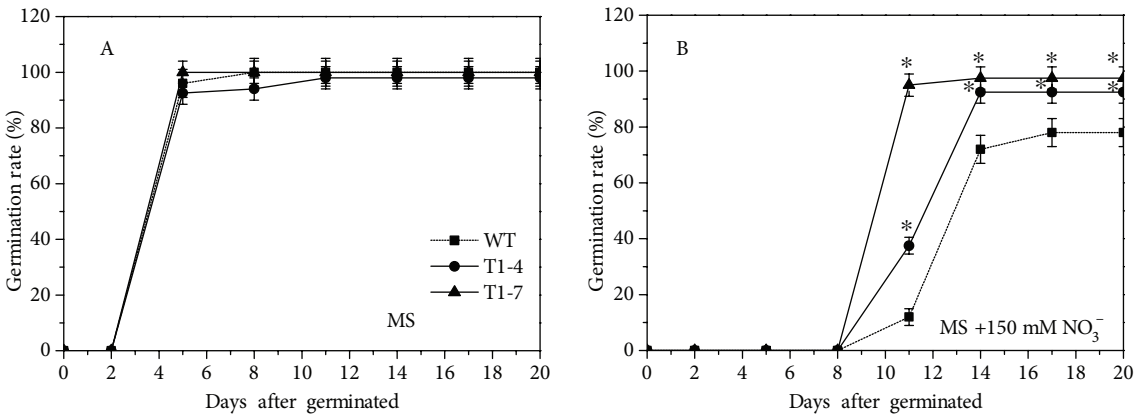

Figure 1. Comparison of germination rates between transgenic lines and wild type (WT) plants under excess nitrate for 20 days. The seeds of WT and transgenic plants of T1 generation were planted on MS agar medium adding 0 and 150 mM NO_3^-. Values shown are means ± S.E. (n = 3) of 3 independent experiments. An asterisk (*) indicates significant difference with respect to WT at $P < 0.05$.

with 0 and 150 mM NO_3^-. There was no significant difference in germination rates between the WT and T1-4 and T1-7 transgenic plants grown on MS medium. Compared with the control, 150 mM NO_3^- resulted in a great delay of germination time and serious inhibition of seed germination. On day 11, the germination rates of T1-4 and T1-7 were 37.5% and 95.0%, while the germination rate of the WT plants was 12.0%. At the end of the treatment course, germination rates of T1-4 and T1-7 were 92.5% and 97.5%, which were significantly higher than that of WT (78.0%) ($P < 0.05$). These results indicate that overexpression of *CsNMAPK* positively regulates plant tolerance to nitrate stress.

3.2. Effect of nitrate stress on fresh and dry weight of transgenic tobacco root

The effect of nitrate stress on the growth of transgenic tobacco and WT plants is shown in Figure 2. The growth of the tobacco plants was inhibited with increasing nitrate concentration and the growth of transgenic plants was better than that of the WT plants. There was no significant difference in fresh weight or dry weight of WT and transgenic tobacco plant seedlings under normal growth conditions. In the presence of nitrate stress, both the WT and the transgenics showed growth retardation in a dose-dependent manner, but the retardation was greater in the WT plants. After 98 mM nitrate treatment for 7 days, the fresh weight of root of WT plants decreased by 55.2%, while that of T1-4 and T1-7 decreased by 45.7% and 43.0%, respectively ($P < 0.05$). The root dry weight of WT, T1-4, and T1-7 decreased by 69.3%, 65.3%, and 63.2%, respectively, after 182 mM NO_3^- treatment for 7 days.

3.3. Effect of nitrate stress on electrolytic leakage and MDA content of transgenic tobacco plants

Electrolyte leakage (EL) of plants indicates the extent of membrane damage under various stress conditions. Malondialdehyde (MDA), an end product of lipid peroxidation, was used as an indicator of free radical production and membrane injury. For all lines, low values of EL and MDA content were recorded under normal conditions (Figure 3) and there were no significant differences in electrolyte leakage or MDA content between WT and transgenic lines. After 98 and 182 mM nitrate

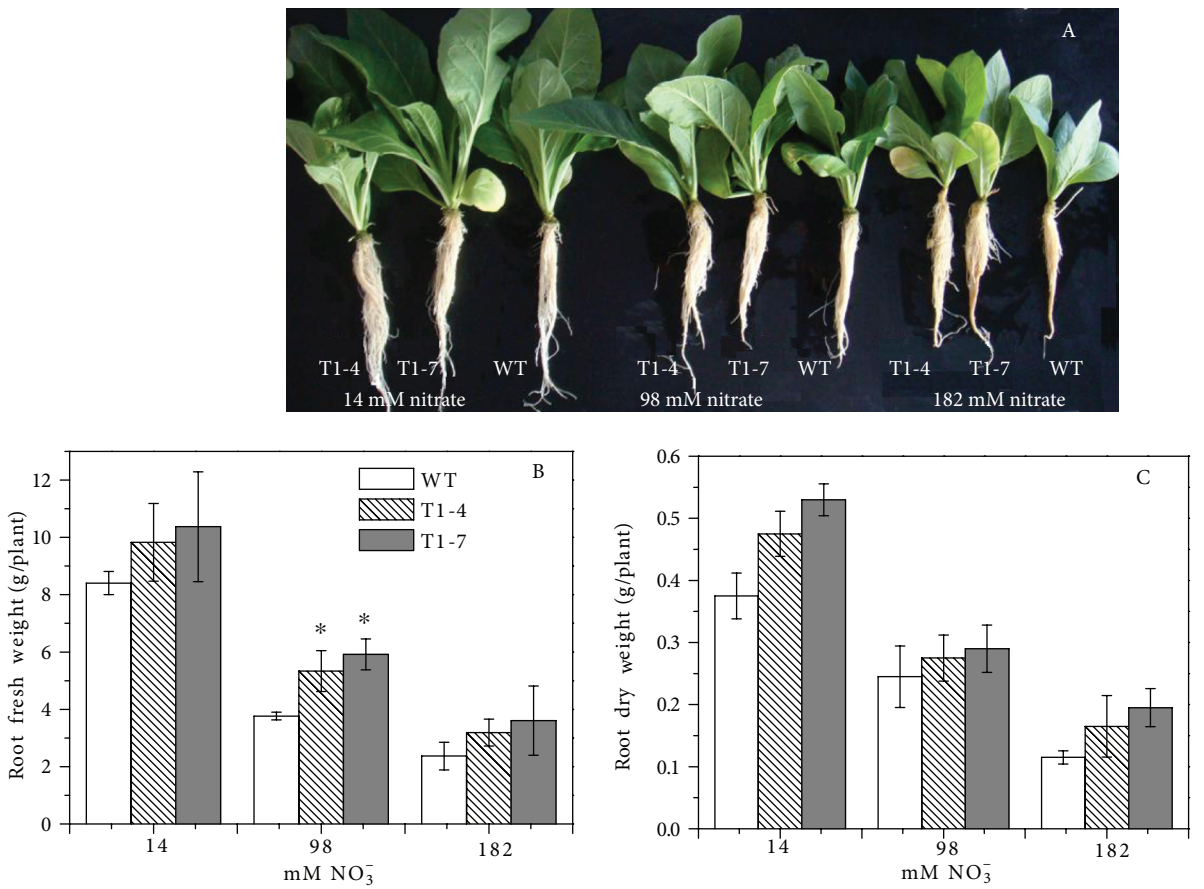

Figure 2. The morphology (A), root fresh weight (B) and root dry weight(C) of T1-4, T1-7 transgenic plants and WT plants after 98 mM and 182 mM nitrate treatment for 7 days. The values are mean ± S.E. of 3 independent experiments. An asterisk (*) indicates significant difference with respect to WT at $P < 0.05$.

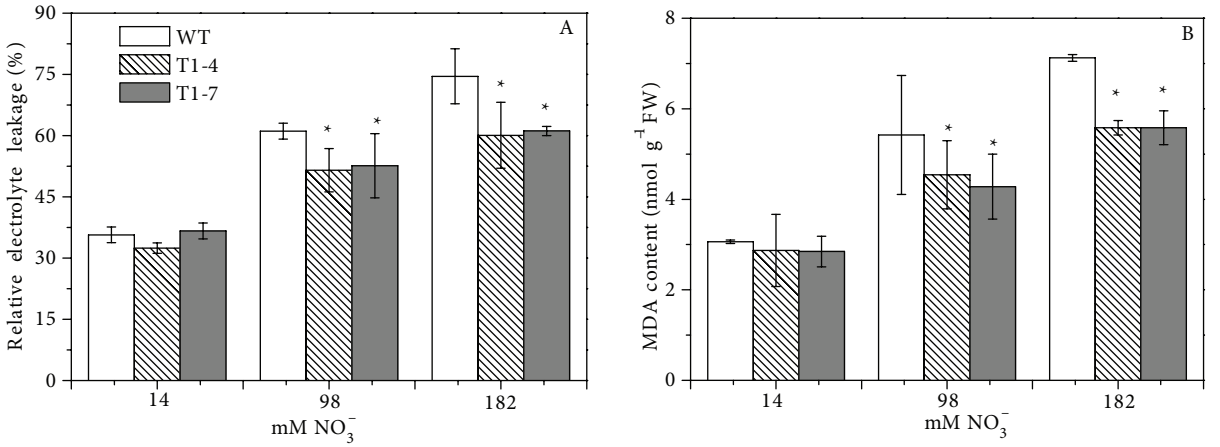

Figure 3. Effect of excess nitrate on the relative electrolyte leakage (A) and MDA content (B) of root tissue of transgenic and WT plants. Plants were treated with 98 mM and 182 mM NO_3^- for 7 days. The values are mean ± S.E. of 3 independent experiments. An asterisk (*) indicates significant difference with respect to WT at $P < 0.05$.

stress treatment for 7 days, the EL of all tobacco plants increased significantly and the increment of WT was higher than that of T1-4 and T1-7 transgenic tobacco plants ($P < 0.05$). After 98 mM nitrate treatment, the EL increments of WT, T1-4, and T1-7 were 17.1%, 15.9%, and 14.4%, respectively, while the increments of MDA content of WT, T1-4, and T1-7 were 43.5%, 36.8%, and 33.5%, respectively ($P < 0.05$). These results indicate that the expression of *CsNMAPK* in tobacco plants provided increased tolerance to nitrate stress related to membrane lipid peroxidation.

3.4. Effect of nitrate stress on H_2O_2 content of transgenic tobacco plants

Since high salinity is reported to induce oxidative stress, the levels of H_2O_2 in both transgenic and WT plants were measured after 98 and 182 mM nitrate stress treatment. As shown in Figure 4, excess nitrate increased H_2O_2 accumulation in the root of both WT and transgenic tobacco plants, especially in the WT. The H_2O_2 content in the transgenic tobacco plants was significantly lower than that in the WT plants ($P < 0.05$). After 182 mM NO_3^- treatment for 7 days, the increment of H_2O_2 content in WT, T1-4, and T1-7 was 3.40-, 1.63-, and 1.60-fold compared to the normal growth conditions (14 mM NO_3^-).

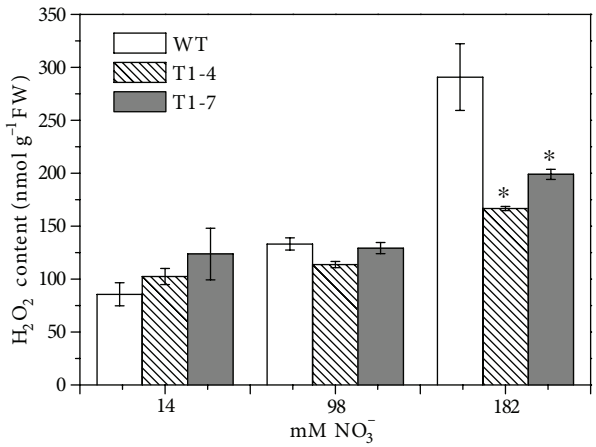

Figure 4. Effect of excess nitrate on H_2O_2 content of root tissue of transgenic and WT plants. Plants were treated with 98 mM and 182 mM NO_3^- for 7 days. The values are mean ± S.E. of 3 independent experiments. An asterisk (*) indicates significant difference with respect to WT at $P < 0.05$.

3.5. Effect of nitrate stress on antioxidant enzyme activities of transgenic tobacco plants

To understand the response of some of the antioxidant enzymes to nitrate stress, 4 enzymes, namely superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX), were monitored. As shown in Figure 5, there was no significant difference in the enzyme activities of WT or transgenic tobacco plants under normal growth conditions. After 98 mM nitrate treatment for 7 days, the enzyme activities of SOD, CAT, POD, and APX were all increased and the enzyme activities decreased after 182 mM nitrate treatment; however, the enzyme activities of the transgenic tobacco plants were still higher than that of the WT.

The SOD activity of the WT, T1-4, and T1-7 was 1.5-, 1.7-, and 1.7-fold compared to the normal growth conditions after 98 mM nitrate treatment for 7 days (Figure 5A). After 182 mM nitrate treatment, the activities of T1-4 and T1-7 decreased by 80.1% and 88.2%, compared to the control, which were higher than that of the WT (57.1%). CAT, POD, and APX are important enzymes in scavenging H_2O_2 in plants. As shown in Figure 5B, the CAT activities of T1-4 and T1-7 (1.6- and 2.3-fold) were higher than

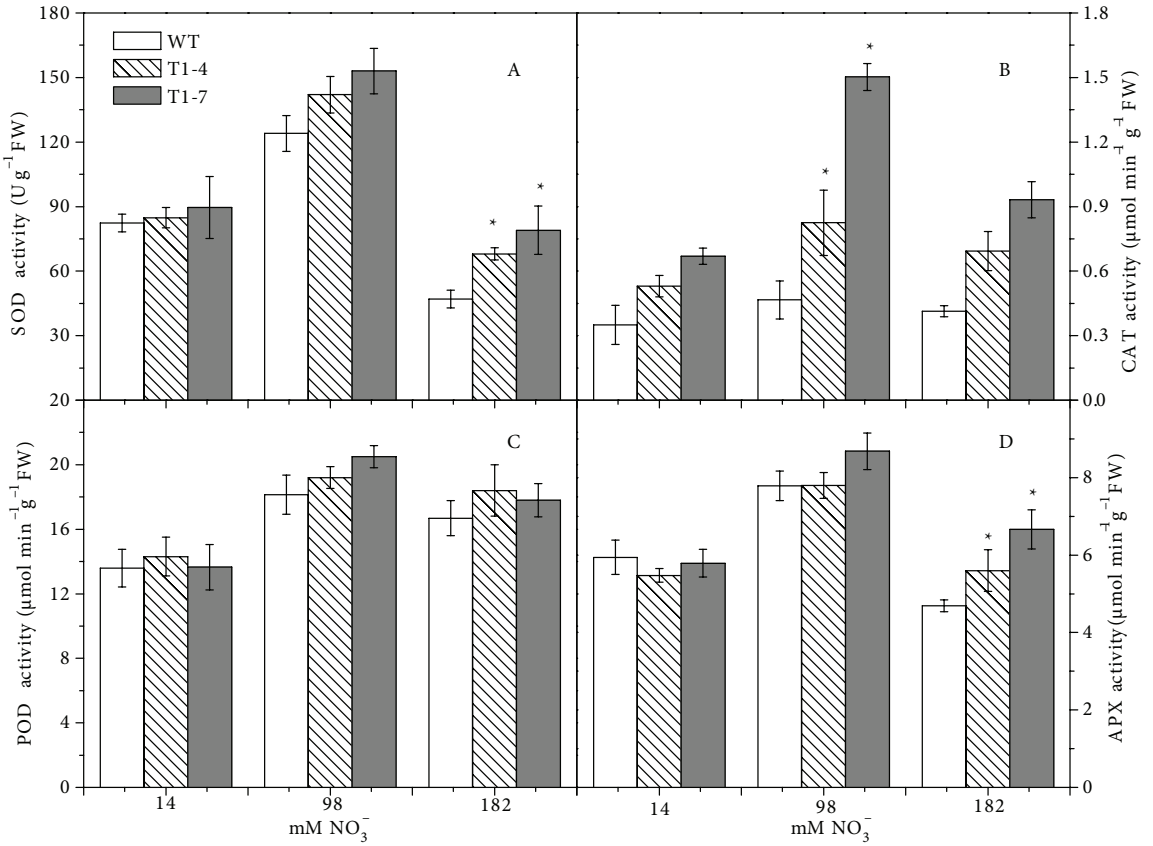

Figure 5. Effect of excess nitrate on the activities of SOD, CAT, POD, and APX of root tissue of transgenic and WT plants. Plants were treated with 98 mM and 182 mM NO_3^- for 7 days. The values are mean ± S.E. of 3 independent experiments. An asterisk (*) indicates significant difference with respect to WT at $P < 0.05$.

that in WT (1.33-fold) after 98 mM NO_3^- treatment. After 182 mM nitrate treatment, the CAT activities of the transgenic plants were still higher than that of the WT plants. As shown in Figure 5C and Figure 5D, there were no significant differences in POD or APX activities between WT and transgenic tobacco plants, although the activities in the transgenic plants were higher than that in the WT after nitrate stress treatment. After 98 mM nitrate treatment the APX activities in WT, T1-4, and T1-7 increased to 1.3-, 1.5-, and 1.5-fold compared to the control. After 182 mM nitrate treatment, the APX activity in WT was decreased, while in T1-4 and T1-7 the APX activities were still increased compared to the control.

3.6. Effect of nitrate stress on proline content of transgenic tobacco plants

As shown in Figure 6, the proline content of transgenic lines and WT tobacco plants were all increased with the increasing nitrate concentration. The increase in proline content of T1-4 and T1-7 was 2.3- and 2.6-fold in comparison to the normal plants, which was higher than the content of WT plants (1.8-fold) after 98 mM NO_3^- treatment for 7 days. After 182 mM NO_3^- treatment, the

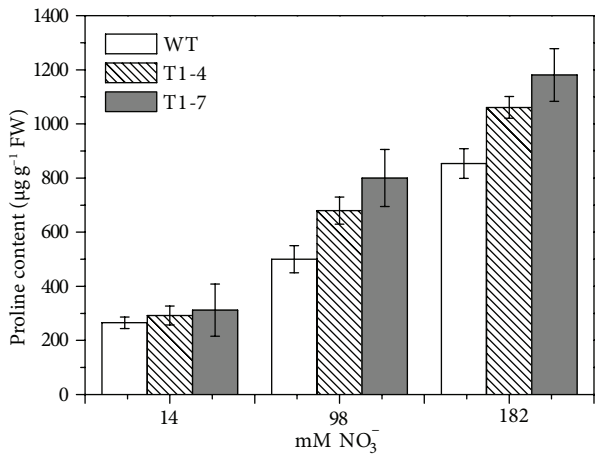

Figure 6. Effect of excess nitrate on the proline content of root tissue of transgenic and WT plants. Plants were treated with 98 mM and 182 mM NO_3^- for 7 days. The values are mean ± S.E. of 3 independent experiments.

transgenic lines still had higher proline content than the WT plants. The proline content in T1-4, T1-7, and WT was increased to 3.2-, 3.8-, and 2.6-fold, respectively, compared to the normal plants.

4. Discussion

As sessile organisms, plants have evolved a complex signalling network that mediates the perception of and responses to different environmental cues. Recent studies have shown that MAPK cascades are evolutionarily conserved signalling modules that play a pivotal role in plant responses to multiple biotic and abiotic stresses. A lot of research indicated that MAPKs may play positive or negative roles in plant stress tolerance. OsBWMK1 phosphorylates transcription factor OsEREBP1 in vitro and positively regulates PR genes in tobacco plants. Silencing of this MAPK caused a reduction in pathogen-induced Phe ammonia-lyase (PAL) and OsBWMK1 mRNAs and an increase in the mRNA of another MAPK of rice, OsMAPK5a (Cheong et al., 2003). Another group has reported that a multiple stress-responsive MAPK (OsMAPK5a) inversely modulates abiotic stress and disease resistance (Xiong & Yang, 2003). Rice plant lines overexpressing OsMAPK5a exhibited increased OsMAPK5a kinase activity and increased tolerance to drought, salt, and cold stresses, whereas OsMAPK5a-silenced plants had a significant reduction in abiotic stress tolerance, but enhanced resistance to fungal and bacterial pathogens. Xing et al. (2007) showed that overexpression of AtMEK1 in *Arabidopsis* increased plant resistance to drought or salt stress. It has been demonstrated that AtMEK1 was a crucial signal mediating the regulation of the antioxidative system under stress conditions, and thereby played important roles in both drought and salt tolerance in *Arabidopsis*. In our experiments, transgenic tobacco plants containing *CsNMAPK* had higher germination rates and better seedling growth after nitrate stress treatment, indicated that MAPK may positively regulate tobacco tolerance to nitrate stress.

Cell membranes are the first target of attack under various stress conditions. Salt stress can destroy the integrity of the cell membrane, resulting in the leakage of more solute. Ürek and Tarhan (2012) found that under nitrate supplemented conditions the levels of lipid peroxidation significantly decreased between days 8 and 13 and then increased in the following incubation days. In the present study, although the electrolytic leakage and production of lipid peroxide of overexpressing *CsNMAPK* plants increased, the increment was much lower than that of WT tobacco plants after 7-day nitrate stress treatment (Figure 3), suggesting that transgenic plants have higher nitrate stress tolerance. These results were substantially in agreement with those of other authors who reported a lower decrease in membrane stability index in tolerant genotypes than in salt-sensitive ones under salt stress (Ruiz et al., 2005; Sairam et al., 2005).

Much of the injury to plants imposed by stress exposure is associated with oxidative damage at the cellular level. Plants possess a sophisticated ROS scavenging network, comprising antioxidants and antioxidative enzymes, which allow them to keep ROS levels under tight control. As part of these systems, SOD, CAT, POD, and APX play a key role in defence reactions. Increasing the antioxidant activity plays an important role in scavenging oxidants (Çekiç et al., 2012). Moreover, as shown in the research of the past few years, plants have developed efficient strategies for targeted production of ROS. Mitogen-activated protein kinase (MAPK) cascades are key players in ROS signalling (Pitzschke & Hirt, 2009). In our previous study, we found that *CsNMAPK* was involved in positive regulation of ROS scavenage and osmotic adjustment in cucumber under NaCl stress (Xu et al., 2011). In our experiment, the H_2O_2 content in the transgenic tobacco plants was significantly lower than that in the WT plants ($P < 0.05$). The 4 antioxidant enzyme activities in the transgenic plants were all higher than that in the WT plants after nitrate stress.

In addition to antioxidant systems, osmotic adjustment is an important mechanism for plants to acclimate themselves to salt stress. Proline is considered the main substance for osmotic adjustment in plants under salt stress (Zhou et al., 2004). When water potential outside decreases due to salt stress and other factors, the concentration of proline in plant tissue increases and is involved in osmotic adjustment to prevent excess loss of water in vivo (Soussi et al., 1999). After treatment with 98 mM and 182 mM nitrate, proline accumulated to slightly higher levels in transgenic than in WT tobacco plants (Figure 6). Therefore, it is possible that the elevated concentration of proline in transgenic plants helps to protect antioxidative enzyme, thus alleviating the negative effects imposed by salt on transgenic *CsNMAPK* plants. Based on these results, we conclude that the tolerance of overexpressing-*CsNMAPK* tobacco plants to nitrate stress might partly be attributed to higher antioxidant enzyme activities and enhanced osmotic regulation capacity.

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References

Bates LS, Waldren RP & Teare I (1973). Rapid determination of free proline for water-stress studies. *Plant Soil* 39: 205–207.

Blanco FA, Zanetti ME, Casalongué CA & Daleo GR (2006). Molecular characterization of a potato MAP kinase transcriptionally regulated by multiple environmental stresses. *Plant Physiology and Biochemistry* 44: 315–322.

Bray EA, Bailey-Serres J & Weretilnyk E (2000). Responses to abiotic stress. In: Buchanan B, Gruissem W, Jones R, eds. Biochemistry and molecular biology of plants. *The American Society of Plant Physiologists* 1158–1203.

Çekiç FÖ, Ünyayar S & Ortaş İ (2012). Effects of arbuscular mycorrhizal inoculation on biochemical parameters in *Capsicum annuum* grown under long term salt stress. *Turkish Journal of Botany* 36: 63–72.

Chen DM & Yang JS (1995). The condition and nutrient manage of soil salinization. *Progress in Soil Science* 23: 7–13 (in Chinese, with English abstract).

Cheong YH, Moon BC, Kim JK, Kim CY, Kim MC, Kim IH, Park CY, Kim JC, Park BO, Koo SC, Yoon HW, Chung WS, Lim CO, Lee SY & Cho MJ (2003). *BWMK1*, a rice mitogen-activated protein kinase, locates in the nucleus and mediates pathogenesis-related gene expression by activation of a transcription factor. *Plant Physiology* 132: 1961–1972.

Colcombet J & Hirt H (2008). *Arabidopsis* MAPKs: A complex signalling network involved in multiple biological processes. *Biochemical Journal* 413: 217–226.

Emerling BM, Platanias LC, Black E, Nebreda AR, Davis RJ & Chandel NS (2005). Mitochondrial reactive oxygen species activation of p38 mitogen-activated protein kinase is required for hypoxia signaling. *Molecular and Cellular Biology* 25: 4853–4862.

Fu SF, Chou WC & Huang DD (2002). Transcriptional regulation of a rice mitogen-activated protein kinase gene, *OsMAPK4*, in response to environmental stresses. *Plant & Cell Physiology* 43: 958–963.

Hirt H (2000). Connecting oxidative stress, auxin, and cell cycle regulation through a plant mitogen-activated protein kinase pathway. *Proceedings of the National Academy of Sciences* 97: 2405–2407.

Ichimura K, Mizoguchi T, Yoshida R, Yuasa T & Yamaguchi-Shinozaki K (2000). Various abiotic stresses rapidly activate *Arabidopsis* MAP kinases *AtMPK4 and AtMPK6*. *Plant Journal* 24: 655–666.

Lutts S, Kinet JM & Bouharmont J (1996). NaCl-induced senescence in leaves of rice (*Oryza sativa* L.) cultivar differing in salinity resistance. *Annals of Botany* 78: 389–398.

Madhava Rao KV & Sresty TVS (2000). Antioxidative parameters in the seedlings of pigeonpea (*Cajanus cajan* L. Millspaugh) in response to Zn and Ni stresses. *Plant Science* 157: 113–128.

Nakano Y & Asada K (1981). Hydrogen peroxide scavenged by ascorbate-specific peroxidase in spinach chloroplast. *Plant & Cell Physiology* 22: 867–880.

Nickel RS & Cunningham BA (1969). Improved peroxidase assay method using Ieuco 2,3,6-trichlcroindophenol and application to comparative measurements of peroxidase catalysism. *Annual Review of Biochemistry* 27: 292–299.

Ouyang B, Yang T, Li HX, Zhang L, Zhang YY, Zhang JH, Fei ZJ & Ye ZB (2007). Identification of early salt stress response genes in tomato root by suppression subtractive hybridization and microarray analysis. *Journal of Experimental Botany* 58: 507–520.

Patra HL, Kar M & Mishre D (1978). Catalase activity in leaves and cotyledons during plant development and senescence. *Comparative Biochemistry and Physiology* 1972: 385–390.

Patterson BD, MacRae EA & Ferguson IB (1984). Estimation of hydrogen peroxide in plant extracts using titanium (IV). *Analytical Biochemistry* 139: 487–492.

Pitzschke A & Hirt H (2009). Disentangling the complexity of mitogen-activated protein kinases and reactive oxygen species signaling. *Plant Physiology* 149: 606–615.

Pitzschke A, Schikora A & Hirt H (2009). MAPK cascade signalling networks in plant defence. *Current Opinion in Plant Biology* 12: 421–426.

Ruiz JM, Blasco B, Rivero RM & Romero L (2005). Nicotine-free and salt-tolerant tobacco plants obtained by grafting to salinity-resistant rootstocks of tomato. *Physiologia Plantarum* 124: 465–475.

Sairam RK, Srivastava GC, Agarwal S & Meena RC (2005). Differences in antioxidant activity in response to salinity stress in tolerant and susceptible wheat genotypes. *Biologia Plantarum* 49: 85–91.

Shi QH, Zhu ZJ, Al-aghabary K, Liu HY &Yu JQ (2004). Effects of isoosmotic salt stress on the activities of antioxidative enzymes, H^+-ATPase and H^+-PPase in tomato plants. *Journal of Plant Physiology and Molecular Biology* 30: 311–361. (in Chinese, with English abstract)

Shoresh M, Gal-On A, Leibman D & Chet I (2006). Characterization of a mitogen-activated protein kinase gene from cucumber required for Trichoderman-conferred plant resistance. *Plant Physiology* 142: 1169–1179.

Soussi M, Lluch C & Ocaña A (1999). Comparative study of nitrogen fixation and carbon metabolism in two chick-pea (*Cicer arietinum* L.) cultivars under salt stress. *Journal of Experimetal Botany* 50: 1701–1708.

Stepien P & Johnson GN (2009). Contrasting responses of photosynthesis to salt stress in the glycophyte *Arabidopsis* and the halophyte *Thellungiella*: role of the plastid terminal oxidase as an alternative electron sink. *Plant Physiology* 149: 1154–1165.

Sumbayev VV & Yasinska IM (2005). Regulation of MAP kinase dependent apoptotic pathway: implication of reactive oxygen and nitrogen species. *Archives of Biochemistry and Biophysics* 436: 406–412.

Ürek RÖ & Tarhan L (2012). The relationship between the antioxidant system and phycocyanin production in *Spirulina maxima* with respect to nitrate concentration. *Turkish Journal of Botany* 36: doi:10.3906/bot-1106-1.

Xing Y, Jia WS & Zhang JH (2007). AtMEK1 mediates stress-induced gene expression of CAT1 catalase by triggering H_2O_2 production in *Arabidopsis*. *Journal of Experimental Botany* 1–13.

Xiong L, Schumaker KS & Zhu J (2002). Cell signaling during cold, drought, and salt stress. *Plant Cell* 14 (Suppl): S165–S183.

Xiong LZ & Yang YN (2003). Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. *Plant Cell* 15: 745–759.

Xu HN, Li KZ, Yang FJ, Shi QH & Wang XF (2010). Overexpression of *CsNMAPK* in tobacco enhanced seed germination under salt and osmotic stresses. *Molecular Biology Reports* 37(1): 3157–3163.

Xu HN, Sun XD, Wang XF, Shi QH, Yang XY & Yang FJ (2011). Involvement of a cucumber MAPK gene (*CsNMAPK*) in positive regulation of ROS scavengence and osmotic adjustment under salt stress. *Scientia Horticulturae* 127: 488–493.

Yu HY, Li TX & Zhou JM (2007). Salt accumulation, translocation and ion composition in greenhouse soil profiles. *Plant Nutrition and Fertilizer Science* 13: 642–650. (in Chinese, with English abstract).

Zhang SQ & Klessig DF (2001). MAPK cascades in plant defense signaling. *Trends in Plant Science* 6: 520–527.

Zhou W, Sun QJ, Zhang CF, Yuan YZ, Zhang J & Lu BB (2004). Effect of salt stress on ammonium assimilation enzymes of the roots of rice (*Oryza sativa*) cultivars differing in salinity resistance. *Acta Botanica Sinica* 46: 921–927.

Zhu JK (2002). Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology* 53: 247–273.