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ENHANCED SALT-MEDIATED OXIDATIVE STRESS TOLERANCE IN TRANSGENIC TOBACCO OVEREXPRESSING ACEROLA GDP-L-GALACTOSE PHOSPHORYLASE AND PHOSPHOMANNOMUTASE GENES

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ABSTRACT

Ascorbate (AsA) plays an important role in stress responses in plants as one of the highly abundant metabolites that scavenge reactive oxygen species (ROS). Under salt stress conditions, transgenic tobacco plants overexpressing acerola (*Malpighia glabra*) GDP-L-galactose phosphorylase (GGP) and phosphomannomutase (PMM) genes accumulated higher amount of AsA, exhibited reduced levels of lipid peroxidation, and maintained higher levels of chlorophyll compared with control plants. These results indicate that overexpressing of acerola GGP and PMM confers greater protection for transgenic plants under oxidative stress conditions mediated by salt.

INTRODUCTION

Salinity is one of the critical environmental stresses facing the growing plants. It is not only determines the geographical and regional distribution of crops, but also affects the productivity of the cultivated areas (Vaidyanathan *et al*., 2003). As other abiotic stresses, salinity triggers oxidative stress in plant tissues (Foyer and Noctor, 2003), enhances photoinhibition by inhibiting repair of PSII (Al-Taweel *et al*., 2007) and augments the production of reactive oxygen species (ROS) such as singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radical (Asada, 1999). ROS can damage essential membrane lipids as well as proteins and nucleic acids (Inzé and Van Montague, 1995; Noctor and Foyer, 1998). Plant cells are equipped with excellent antioxidant defense mechanisms to detoxify the harmful effects of ROS.

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Ascorbic acid (AsA) is the most abundant, water soluble, and powerful antioxidant acting to prevent or minimize the damage caused by ROS in plants. High level of endogenous AsA is essential to effectively maintain the antioxidant system that protects plants from oxidative damage (Shigeoka *et al*., 2002). AsA is also considered the most powerful ROS scavenger because of its ability to donate electrons in a number of enzymatic and non-enzymatic reactions. It interacts enzymatically and non-enzymatically with the damaging ROS (Davey *et al*., 2000). Exogenous AsA increased the percentage of tomato seedlings able to survive the toxic effects of exposure to NaCl (Shalata and Neumann, 2001). The levels of AsA can be increased by engineering AsA related genes to generate AsA-rich transgenic plants (Chen *et al*., 2003; Badejo *et al*., 2009a; Badejo *et al*., 2009b). Acerola (*Malpighia glabra*) is a tropical plant very rich in AsA (Davey *et al*., 2000). Recently, we have successfully generated AsA-rich transgenic tobacco (*Nicotiana tabacum*) plants overexpressing acerola GDP-L-galactose phosphorylase (*MgGGP*) and

phosphomannomutase (*MgPMM*) genes (Badejo *et al.*, 2009a; Badejo *et al.*, 2009b). To test whether the elevated AsA can improve the ability of plants to tolerate the oxidative stress, we have investigated the performance of transgenic tobacco overexpressing *MgGGP* (MgGGP transgenic plants) and transgenic tobacco overexpressing *MgPMM* (MgPMM transgenic plants) under salt stress conditions.

MATERIALS AND METHODS

Plant materials and growth conditions: Transgenic tobacco plants (*Nicotiana tabcum*) expressing acerola's (*Malpighia glabra*) GGP (Badejo *et al.*, 2009a) and PMM (Badejo *et al.*, 2009b) were self pollinated to produce T_1 seeds. The seeds were germinated on MS medium (Murashige and Skoog, 1962) containing 100 µg ml^{-1} kanamycin, while control plants seeds were germinated on antibiotic-free MS medium and maintained at 25 °C under 16/8 h light/dark photoperiod. Seven-weeks-old seedlings were then transplanted into soil and maintained under a 16-h photoperiod at 25 °C.

DNA isolation and PCR: Genomic DNA was extracted from tobacco leaves by the CTAB method described earlier (Eltelib *et al.*, 2011). The existence of *MgGGP* and *MgPMM* in T_2 plants was confirmed by PCR using genes specific primers (Badejo *et al.*, 2009a; Badejo *et al.*, 2009b). The amplified DNA fragments corresponding to *MgGGP* and *MgPMM* were detected by electrophoresis in 1% agarose gels.

RNA isolation and northern blot analysis: The expression of *MgPMM* and *MgGGP* in T_2 lines was confirmed using northern blot analysis described by Eltelib *et al*. (2011). Total RNA was isolated from the leaves of both control and transgenic plants as described earlier (Badejo *et al*., 2009b).

Salt stress treatment: Three independent MgGGP transgenic lines (G4, G6, and G7) and three independent MgPMM transgenic lines (P1, P6, and P14) in addition to control tobacco plants were subjected to salt stress treatment. Seedlings were irrigated daily with Hyponex nutrient solution (HYPONeX, Osaka, Japan). Thirty five days after transplanting, three replications were used in salt stress evaluation experiment. Salt stress was imposed by the application of 300 mM NaCl solution supplied with 1 ml/L nutrient solution (Hponex, Osaka, Japan), renewed every day for 9 days. The plants were grown under a light intensity of about 150 µmol m^{-2} s^{-1} and a 16/8-h light/dark photoperiod at 25 °C.

Chlorophyll determination: Chlorophyll content of the leaves of control and transgenic plants was determined according to the method of Porra *et al*. (1989). Leaves were homogenized in 1.0 mL of *N, N′*-dimethylformamide and centrifuged at 20,000 × *g* for 10 min at 4 °C. The supernatant was retained and the absorbance was recorded at 665 and 647 nm. Three plants of each line were used for sampling.

Malondialdehyde determination: Malondialdehyde (MDA) content was determined according to the method of Dionisio-Sese *et al.* (1998). Leaves were harvested and ground in liquid nitrogen. Finely ground leaves (0.1 g) were homogenized in 2 mL of 50 mM potassium phosphate buffer (pH 7.0) and centrifuged at 20,000 × *g* for 30 min at 4 °C. The extract was then mixed with the same volume of 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) trichloroacetic acid. The mixture was heated at 95 °C for 30 min and the reaction was stopped by placing the samples in an ice bath. The cooled mixture was then centrifuged at 10,000 × *g* for 10 min, and the absorbance was recorded at 532 and 600 nm. After subtracting the non-specific absorbance at 600 nm, the MDA concentration was calculated using the extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Ascorbate determination: AsA content was determined essentially as described by (Eltelib *et al.*, 2011).

Statistical analysis: Three replicates of each sample were used for statistical analysis. Data were analyzed using analysis of variance (ANOVA). Significant differences were calculated ($P < 0.05$) using least significant difference (LSD).

RESULTS AND DISSCUSSION

Transgenic tobacco plants expressing *MgGGP* and *MgPMM* genes showed considerable increase in AsA contents (Badejo *et al.*, 2009a; Badejo *et al*., 2009b). Before starting the stress treatment, the existence of *MgGGP* and *MgPMM* in T_2 plants was confirmed by PCR (Fig. 1A and C). In addition, the expression of *MgGGP* and *MgPMM* in the transgenic lines was confirmed using northern blot analysis. The two genes were expressed in the T_2 transgenic plants, but not in the control plants (Fig. 1B and D).

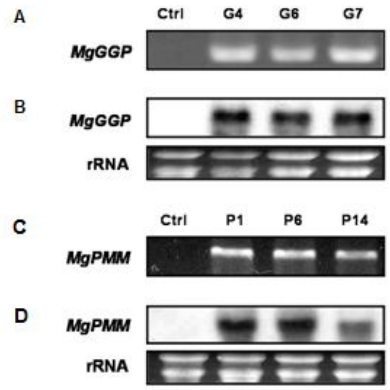
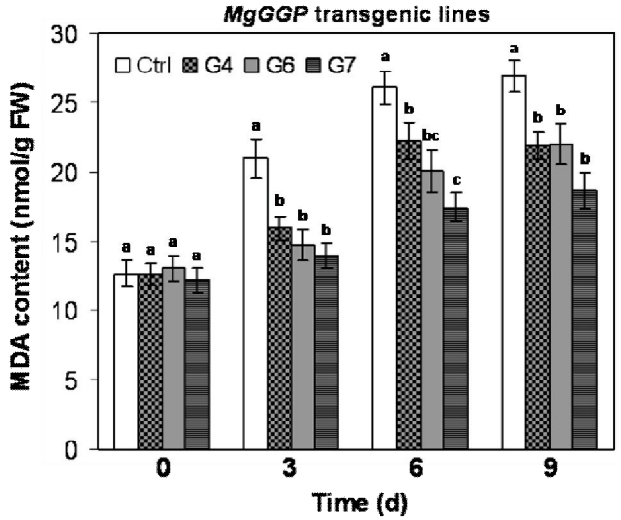


Figure 1. Expression of acerola GGP and PMM (*MgGGP* and *MgPMM*) in GGP transgenic lines (G4, G6 and G7) and PMM transgenic lines (P1, P6 and P14). (A and C) PCR using *MgGGP* and *MgPMM* gene specific primers for detection of the transgenes in the genome of the selected GGP and PMM transgenic lines. (B and D) Northern blot analysis for the mRNA expression levels of *MgGGP* and *MgPMM* in GGP and PMM transgenic lines. Total RNA (10 µg) was isolated from tobacco leaves, and probed using *MgGGP* and *MgPMM* cDNAs.

Plants exposed to abiotic stresses exhibit an increase in lipid peroxidation, which is considered as the most damaging process known to occur in every living organism (Gill and Tuteja, 2010). Malondialdehyde, a product of lipid peroxidation, is considered an indicator of oxidative damage. The levels of MDA were similar in both the control and transgenic tobacco plants before the application of salt treatment. However, the level of MDA increased dramatically under salt stress in the control plants (Fig. 2A, B). MgGGP

and MgPMM transgenic lines had significantly less MDA accumulation compared to control plants in response to salt stress. After 6 d of salt stress treatment, the average MDA contents of MgGGP and MgPMM transgenic lines were about 20 nmol/g FW compared with 26.1 nmol/g FW for control plants. This suggests that the extent of lipid peroxidation in the control plants was significantly higher than those of transgenic



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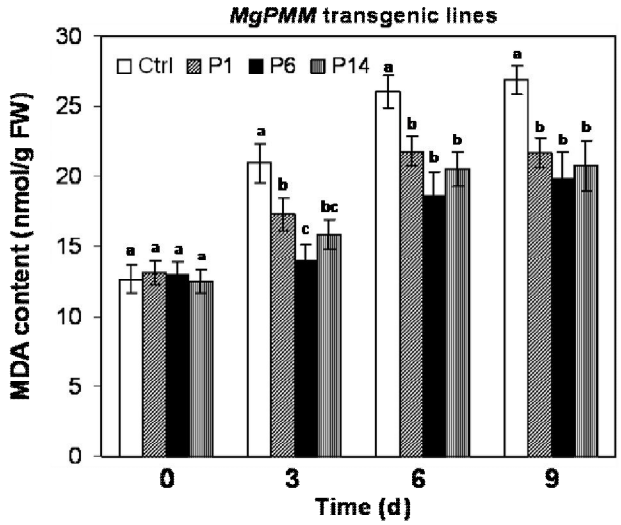

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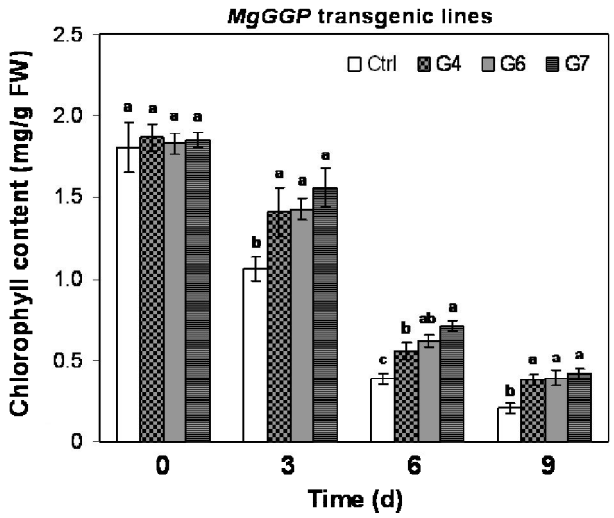
Figure 2. Malondialdehyde (MDA) contents of control and transgenic plants in response to salt stress. (A) MDA contents of the leaves of control (Ctrl) and MgGGP transgenic lines (G4, G6 and G7) irrigated with a nutrient solution containing 300 mM NaCl for 3, 6 and 9 d. (B) MDA contents of the leaves of control (Ctrl) and MgPMM transgenic lines (P1, P6 and P14) irrigated with a nutrient solution containing 300 mM NaCl for 3, 6 and 9 d. Three replicates of each sample were used for statistical analysis. Values followed by the same letter are not significantly different (*P*<0.05).

lines under salt stress conditions. Similar results were obtained in transgenic tobacco and transgenic *Arabidopsis* expressing ascorbate biosynthesis-related genes (Ma *et al.*, 2014; Wang *et al.*, 2014), and transgenic tobacco expressing ascorbate recycling-related gene (Eltelib *et al.*, 2012). The lower level of MDA in MgGGP and MgPMM transgenic lines compared with control plants can be attributed to the elevated levels of AsA in transgenic lines, since AsA prevents or minimizes the damage caused by ROS generated by salt stress in plants. We have also examined the effect of salt stress on the chlorophyll contents of control and transgenic plants. No significant difference in chlorophyll content was recorded between the control and transgenic plants before applying the salt stress treatment. However, application of salt stress treatment markedly reduced the chlorophyll content of control and transgenic plants (Fig. 3A and B), indicating that the loss of chlorophyll is part of the damage caused by salt stress.



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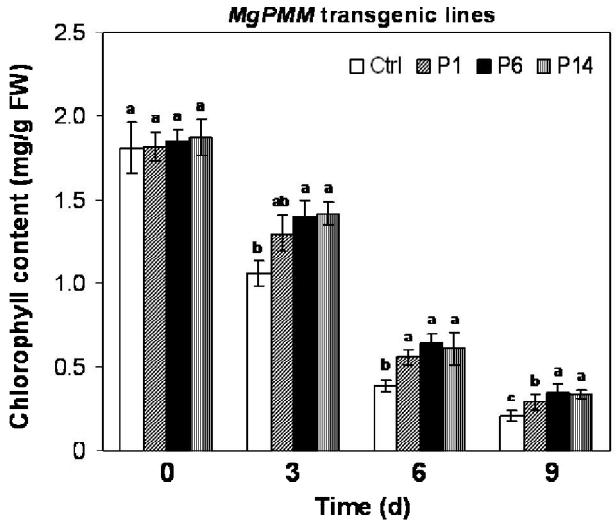

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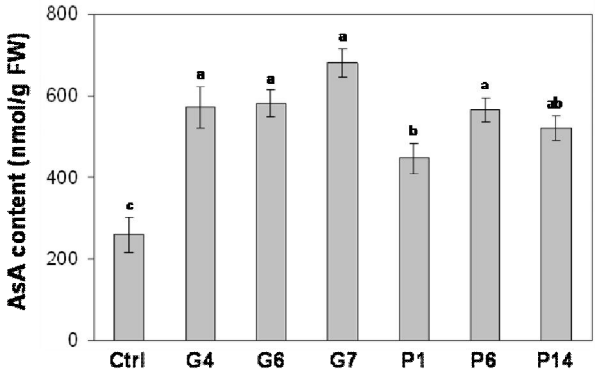
Figure 3. Chlorophyll contents of control and transgenic plants in response to salt stress. (A) Chlorophyll contents of the leaves of control (Ctrl) and MgGGP transgenic lines (G4, G6 and G7) irrigated with a nutrient solution containing 300 mM NaCl for 3, 6 and 9 d. (B) Chlorophyll contents of the leaves of control (Ctrl) and MgPMM transgenic lines (P1, P6 and P14) irrigated with a nutrient solution containing 300 mM NaCl for 3, 6 and 9 d. Three replicates of each sample were used for statistical analysis. Values followed by the same letter are not significantly different (*P*<0.05).

Despite the reduction in total chlorophyll contents, MgGGP and MgPMM transgenic lines were able to retain significantly higher levels of total chlorophyll compared to control plants. Six days after the initiation of salt treatment, the average chlorophyll content of MgGGP and MgPMM transgenic lines was 0.63 and 0.61 mg/g FW, respectively, compared with 0.39 mg/g FW for the control plants. These results were consistent with the results of Chen and Gallie (2005) who reported that tobacco plants with increased levels of AsA exhibited higher chlorophyll levels compared to the control under ozone oxidative stress conditions. The results also coincided with the recent report of Wang *et al*. (2014) who concluded that transgenic tobacco plants expressing tomato GGP gene showed elevated levels of AsA and higher chlorophyll contents than wild type plants under chilling stress condition. Our results also indicated that plants with larger ascorbate pool are able to maintain high chlorophyll content under salinity stress conditions. The substantial reduction in the chlorophyll content of control plants compared to transgenic lines during salt stress treatment suggests that these plants were less able to detoxify ROS. Chlorophyll is the main pigment responsible for photosynthesis. Thus, the high levels of chlorophyll in the transgenic lines may reflect positively on its photosynthetic performance.

On the ninth day of salt stress treatment, MgGGP and MgPMM transgenic plants were able to maintain higher AsA levels compared with the control plants (Fig. 4A). Under salt stress condition, there was a considerable reduction in the growth of the control as well as the transgenic plants. However, MgGGP and MgPMM transgenic plants with elevated AsA levels maintained substantially better growth. As a result, the shoot dry weight of the transgenic lines was significantly higher than the control plants at the end of the stress treatment (Fig. 4B). After 9 days of salt stress treatment, the average shoot dry weight of the transgenic lines G7 and P6 was 3.53 and 3.21 g/plant, respectively, compared with 2.21 g/plant for the control plants. This result is in conformity with the finding of Ma *et al*. (2014) who reported that transgenic *Arabidopsis* expressing alfalfa GDP-mannose 3, 5-epimerase gene, a key enzyme in the biosynthesis of AsA, exhibited elevated AsA levels and higher fresh weight than the wild-type plants under salt stress conditions. Previously, Kato and Esaka (2000) reported the importance of AsA in the regulation of cell expansion. In another report, Tabata *et al*. (2001) also observed that the decline in AsA levels of transgenic tobacco cells expressing antisense RNA for AsA-biosynthesis related gene has an adverse effect on the division, growth and structure of the cell.

Taken together, the previous results along with our presented data, we propose that the higher shoot dry weight of the transgenic plants can be attributed, in part, to the higher levels of AsA, which might allow the plants to grow better under salt stress conditions. Moreover, transgenic lines with elevated AsA levels during stress, which led to reduced oxidative damage and reduced chlorophyll degradation, are expected to grow well and produce greater biomass compared to control plants. Chen and Gallie (2005) reported that increasing the level of AsA through enhanced AsA recycling provided greater protection against oxidative damage. Our results indicated that the level of AsA may determine the level of tolerance to salt-mediated oxidative stress. The results also suggested that the elevated levels of AsA in transgenic lines provided greater protection against salt-mediated oxidative stress and confirmed the important protective role of AsA as an antioxidant. Finally, we conclude that the increase in AsA contents of the transgenic plants is associated with the ability of these plants to maintain higher levels of total chlorophyll and to reduce the level of lipid peroxidation under salt stress. We thus demonstrate the improvement of salt tolerance through engineering AsA biosynthesis-related genes of acerola.



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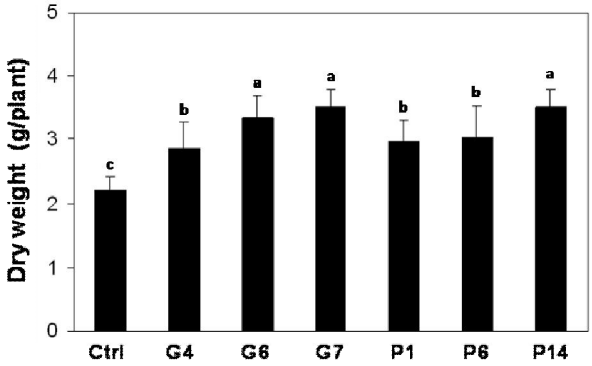

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Figure 4. AsA contents and dry weight of control and transgenic plants after 9 d of salt stress treatment. (A) AsA contents of the leaves of control (Ctrl), MgGGP transgenic lines (G4, G6 and G7) and MgPMM transgenic lines (P1, P6 and P14). Plants were irrigated with a nutrient solution containing 300 mM NaCl for 9 d. (B) Shoot dry weight of control (Ctrl), MgGGP transgenic lines (G4, G6 and G7) and MgPMM transgenic lines (P1, P6 and P14). Plants were harvested after 9 d of irrigation with a nutrient solution containing 300 mM NaCl. Three replicates of each sample were used for statistical analysis. Values followed by the same letter are not significantly different ($P<0.05$)

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