Overexpression of an alfalfa GDP-mannose 3, 5-epimerase gene enhances acid, drought and salt tolerance in transgenic Arabidopsis *by increasing ascorbate accumulation*

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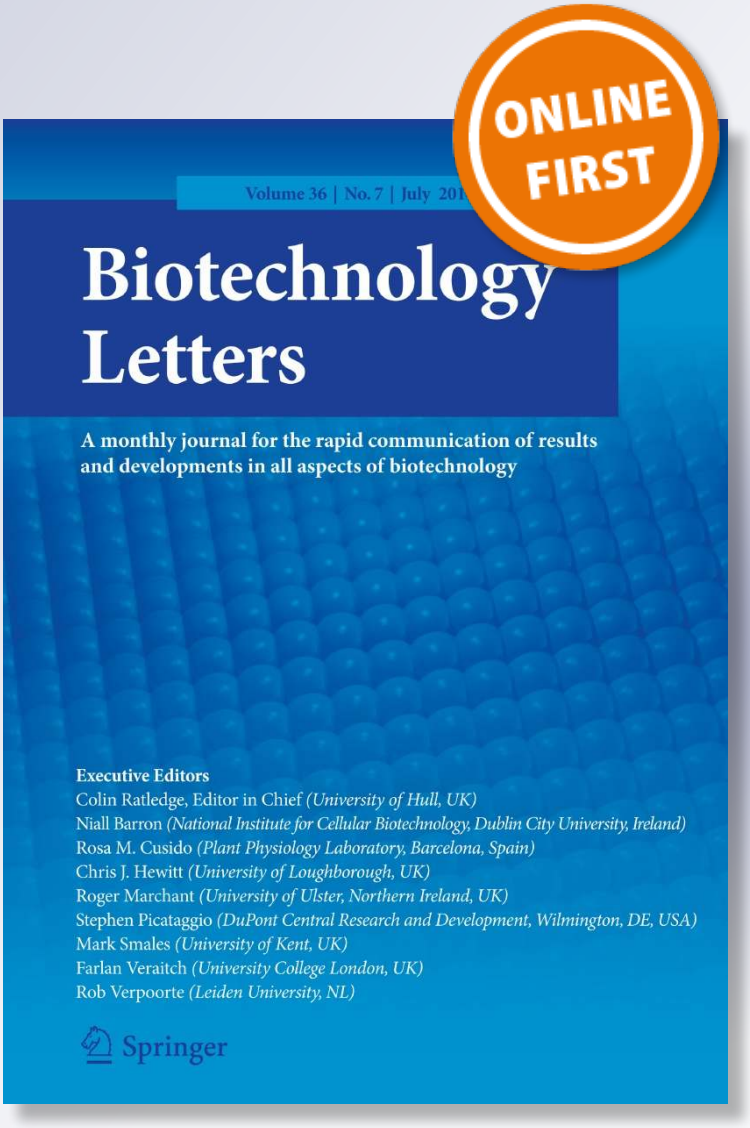

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ORIGINAL RESEARCH PAPER

Overexpression of an alfalfa GDP-mannose 3, 5-epimerase gene enhances acid, drought and salt tolerance in transgenic *Arabidopsis* by increasing ascorbate accumulation

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Abstract GDP-mannose 3′, 5′-epimerase (GME) catalyses the conversion of GDP-D-mannose to GDP-L-galactose, an important step in the ascorbic acid (ascorbic acid) biosynthetic pathway in higher plants. In this study, a novel cDNA fragment (*MsGME*) encoding a GME protein was isolated and characterised from alfalfa (*Medicago sativa*). An expression analysis confirmed that *MsGME* expression was induced by salinity, PEG and acidity stresses. *MsGME* overexpression in *Arabidopsis* enhanced tolerance of the transgenic plants to salt, drought and acid. Real-time PCR analysis revealed that the transcript levels of GDP-D-mannose pyrophosphorylase (GMP), L-galactose-phosphate 1-P phosphatase (GP) and GDP-L-galactose phosphorylase (GGP) were increased in transgenic *Arabidopsis* (T3 generation). Moreover, the ascorbate content was increased in transgenic *Arabidopsis*. Our results suggest that *MsGME* can effectively enhance tolerance of transgenic *Arabidopsis* to acid, drought and salt by increasing ascorbate accumulation.

Keywords Acid tolerance · Alfalfa · *Arabidopsis* · Ascorbic acid · Drought tolerance · GDP-mannose 3′, 5′-epimerase · Salt tolerance · Transgenic plants

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Introduction

GDP-mannose 3′, 5′-epimerase (GME) catalyses the conversion of GDP-D-mannose to GDP-L-galactose, which is an important step in the ascorbic acid biosynthetic pathway in higher plants. Ascorbic acid is a major antioxidant that protects plant cells against reactive oxygen species (ROS), the production of which is an unavoidable consequence of aerobic metabolism (Dutilleul et al. 2003; Moller and Sweetlove 2010). The most common ROS, such as H_2O_2, singlet oxygen, and superoxide anion radical (O_2^-), are generated by physiological processes and abiotic stresses (Chang et al. 2009; Moller and Sweetlove 2010). Although ROS are widely involved in plant metabolism, the imbalance between ROS production and scavenging leads to irreversible cellular damage through the reaction of ROS with cellular components,

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such as lipids, proteins, and nucleic acids (Cho and Park 2000; Girotti 2001; Moller and Sweetlove 2010). Redox homeostasis is mediated by a complex network of pro-oxidant and antioxidant systems. Ascorbic acid functions as an antioxidant and enzymatic cofactor and plays important roles in many physiological processes, including photosynthesis, photo-protection, stress resistance, cell growth, and the biosynthesis of hormones and cell wall constituents (Conklin and Barth 2004; Davey et al. 1999).

Wheeler et al. (1998) first proposed the biosynthetic pathway for ascorbate in higher plants, which involved GDP-D-mannose, GDP-L-galactose, L-galactose and L-galactono-1, 4-lactone. This pathway is known as the Smirnoff/Wheeler or D-mannose/L-galactose pathway (Wheeler et al. 1998). Recently, Laing et al. (2007) and Linster et al. (2007) elucidated the remaining unknown step, which involves the conversion of GDP-L-galactose to L-galactose- 1-P, thereby facilitating the identification of all of the genes encoding the enzymes involved in the D-mannose/L-galactose pathway in *Arabidopsis*. Although alternative ascorbate biosynthetic pathways in plants have been proposed (Agius et al. 2003; Jain and Nessler 2000; Lorence et al. 2004), most of the specific enzymes and their corresponding genes have not been identified. In the past few years, biochemical and molecular genetic methods have demonstrated that the D-mannose/L-galactose pathway is the main pathway for ascorbate biosynthesis.

GME, a key enzyme in the biosynthesis of ascorbate, catalyses the conversion of GDP-D-mannose to GDP-L-galactose in the D-mannose/L-galactose pathway. GME was first isolated and characterised from a green algae, *Chlorella pyrenoidosa* (Barber 1975, 1979; Hebda et al. 1979; Hebda and Barber 1982). In higher plants, *GME* has been cloned and characterised in *Arabidopsis* (Wolucka et al. 2001; Wolucka and Van Montagu 2003), rice (*Oryza sativa*) (Watanabe et al. 2006) and tomato (*Solanum lycopersicum*) (Gilbert et al. 2009), and the reaction catalysed by GME is possibly the rate-limiting step in the ascorbic acid biosynthetic pathway in plants. Indeed, experiments with an *Arabidopsis* cell suspension culture have indicated that the rate-limiting step in ascorbic acid biosynthesis occurs between D-mannose and L-galactose (Davey et al. 1999). Moreover, there is a strong correlation between the GME reaction and the accumulation of ascorbic acid in the colourless microalga, *Prototheca moriformis* (Running et al. 2003).

In the current study, an *MsGME* gene encoding a key enzyme in the biosynthesis of ascorbate was obtained from alfalfa (*Medicago sativa*) for the first time and overexpressed in *Arabidopsis*. Because ascorbic acid plays an important role as an antioxidant in the defence against various abiotic stresses, the effects of increased ascorbate accumulation in transgenic *Arabidopsis* plants on tolerance to acid, drought and salt stresses were investigated.

Materials and methods

Plant materials

Alfalfa and *Arabidopsis* plants were grown in a greenhouse with 16 h light/8 h darkness at 22 °C. Tissues from alfalfa roots, stems and leaves at various developmental stages and leaves from transgenic and wild-type *Arabidopsis* were collected. All samples were immediately frozen in liquid nitrogen and stored at −80 °C.

Isolation of the *MsGME* gene and transgenic plant generation

Total RNA was extracted from the roots, stems and leaves of alfalfa and used to synthesise single-strand cDNA using MMLV reverse transcriptase (TaKaRa, Japan) according to the manufacturer's instructions. The cDNAs were used as the templates for *MsGME* amplification by PCR using the primers *MsGME-Sma*I-F (TTTCCCGGGATGG GAAGTTCTGGAACAAAC) and *MsGME-Sac*I-R (ATAGAGCTCTTCATTGCCATCTGCAGCCC). The amplified products were isolated, gel-purified and cloned into a pEGM-T Easy vector (Promega, USA) to generate the T-*MsGME* vector and then further sequenced. The T-*MsGME* vector containing the complete ORF of *MsGME* was double-digested with *Sma*I and *Sac*I and ligated into the pMyc expression vector. The resulting expression vector harbouring a CaMV35S promoter and *MsGME* was designated pMyc-*35S*::*MsGME*. After sequence confirmation, pMyc-*35S*::*MsGME* was transformed into the *Agrobacterium tumefaciens* GV3101 strain by electroporation. To generate transgenic *Arabidopsis* plants expressing *MsGME*, *Agrobacterium* GV3101 transformed with the plasmid *35S*::*MsGME*

was used to infect *Arabidopsis* using the floral dip method (Clough and Bent 1998). Transformed *Arabidopsis* seeds were selected on MS medium containing 50 mg kanamycin l^{-1} and 0.7 % agar. Positive transgenic plants were confirmed by amplifying the 35S promoter using genomic PCR. Primers specific for the 35S promoter (35S-F (GATTGTGCGTCATCCCTTAC) and 35S-R (GATAGCTGGGCAATGGAATC)) were used and to amplified a 324-bp fragment. The selected T3 generation transgenic lines that displayed 100 % resistance to kanamycin were considered homozygous and thus were harvested individually for further analysis.

For semi-quantitative RT-PCR analysis, total RNA was extracted from the leaves of transgenic *Arabidopsis* and used to synthesise single-strand cDNA using MMLV reverse transcriptase (TaKaRa, Japan) according to the manufacturer's instructions. cDNA samples from 54 transgenic *Arabidopsis* plants were used for a semi quantitative RT-PCR analysis. The specific primers were *MsGME*-F (AATGTGAGCTTGAAGGAGGC) and *MsGME*-R (TGTTATGGAACCGCCCAATG). The *AtACTIN* gene was used as the internal control in the semi quantitative RT-PCR and amplified using *AtACTIN*-F (CTACGAGCAAGAGCTAGAGAC) and *AtACTIN*-R (GGATTCCAGGAGCTTCCATTC).

Real-time PCR analysis

Real-time PCR was used to analyse the expression levels of *MsGME* under salt, drought and acid stress in alfalfa and the expression of genes involved in ascorbic acid biosynthesis in wild-type and transgenic *Arabidopsis*, including *GMP*, *GP* and *GGP*. The two primers used for the expression levels of *MsGME* were *MsGME*-F (AATGTGAGCTTGAAGGAGGC) and *MsGME*-R (TGTTATGGAACCGCCCAATG). The *MsACTIN* gene was used as the internal control in the real-time PCR and amplified with primers *MsACTIN*-F (CTCTCAAGTACCCCATTGAGC) and *MsACTIN*-R (TATTGGCCTTTGGGTTAAGTG). The three pairs of primers used for the expression levels of *GMP*, *GP* and *GGP* were *AtGMP*-F (GGCCATCAATTATCAGCCAG) and *AtGMP*-R (GCTCTCCAGATCCATCAAGC), *AtGP*-F (GGAGAAGAAACTACAGCTGC) and *AtGP*-R (GGAAACACACACGAAAGGG), and *AtGGP*-F (CCGATTGAGTATGGGCATGTG) and *AtGGP*-R (AGCGCCTAGACTGTTGTATCC). The *AtACTIN* gene was used as the internal control in the real-time PCR and amplified with primers *AtACTIN*-F and *AtACTIN*-R.

Ascorbic acid assay

The ascorbic acid content was determined as described by Rizzolo et al. (1984). Briefly, samples were ground in liquid N_2 and homogenised in 5 ml cold 0.1 % (w/v) metaphosphoric acid. The homogenate was then centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant was filtered through a Millipore membrane (0.22 μm) and 300 μl was incubated with 300 μl 50 mM dithiothreitol for 15 min at room temperature. The extracts were then analysed by HPLC using an SB-aq column (Agilent) eluting with acetate buffer (0.2 M pH 4.5) at 1 ml min^{-1} to measure the total ascorbate. The eluates were detected at 254 nm with a linear response from 2 to 40 μg ascorbic acid ml^{-1}.

Abiotic stress treatments

For the abiotic stress treatments, 2-week-old alfalfa seedlings were transferred to half-strength MS liquid medium supplemented with 150 mM NaCl, 15 % (v/v) PEG 6000 or 50 μM $Al_2(SO_4)_3$ (pH 4.5) for 0, 1, 6, 12, 24, 36, 48 and 72 h. Aluminium toxicity is an important growth-limiting factor for plants in acidic soils. The pH value of the half-strength MS liquid medium for $Al_2(SO_4)_3$ stress was set to 4.5 using 1 M HCl. After each treatment, a mixture of leaves, stems and roots from 20 alfalfa seedlings were harvested and analysed by real-time PCR. In addition, 2-week-old transgenic and wild-type *Arabidopsis* seedlings were transferred to half-strength MS liquid medium supplemented with 150 mM NaCl, 15 % PEG 6000 or 50 μM $Al_2(SO_4)_3$ (pH 4.5). After one week, the fresh weight of the seedlings was measured. Prior to the abiotic stress treatments, 2-week-old transgenic and wild-type *Arabidopsis* seedlings were transferred to plastic culture pots (5 × 5 cm square pots that were 4 cm high) containing vermiculite and turf soil (1:1) under a photoperiod of 16/8 h (light/dark) at 22 °C and 60 % RH. The plants were watered every 3 days for 3 weeks, and the water was then supplemented with 150 mM NaCl, 15 % PEG 6000 or 50 μM $Al_2(SO_4)_3$ (pH 4.5) solution. After abiotic stress treatment for one week, leaves from the transgenic and wild-type *Arabidopsis* seedlings were harvested, and the ascorbic acid and malondialdehyde (MDA)

contents and relative membrane permeability (RMP) were determined.

Measurement of melondialdehyde (MDA)

Extraction was performed by homogenisation of 0.5 g leaf tissue with 10 ml 5 % (w/v) trichloroacetic acid and centrifugation at 10,000×*g* for 10 min. Then 2 ml 5 % trichloroacetic acid-containing 0.67 % (w/v) thiobarbituric acid was added to 2 ml of the supernatant. The mixture was boiled for 15 min, quickly cooled on ice and centrifuged at 10,000×*g* for 5 min. The supernatant was used for MDA determination according to Heath and Packer (1968).

Relative membrane permeability (RMP) measurement

The RMP of leaf cells was measured according to Bao et al. (2009) using a conductivity meter (EC215, Hanna, Italy). The RMP was calculated using the following equation: RMP (%) = S1/S2 × 100, where S1 and S2 refer to the conductivity of live and boiled *Arabidopsis* leaves, respectively.

Statistical analysis

The data generated in this study were subjected to a one-way analysis of variance (ANOVA) using SPSS 16.0 (SPSS Inc., USA) and Duncan's multiple range tests to detect significant differences between the means at a significance level of $P < 0.05$. All data are presented as the mean ± SD.

Results

Cloning of the *MsGME* gene

The *MsGME* gene was amplified from alfalfa variety Golden Empress. The nucleotide sequence analysis revealed that *MsGME* contains an 1,146-bp ORF that encodes a 381-amino acid polypeptide. The *MsGME* sequence has been submitted to GenBank under accession number KF935649. Comparison of the nucleotide and amino acid sequences of *MsGME* and its orthologues in *M. truncatula* showed that the *MsGME* and *MtGME* genes share 75 % nucleotide sequence identity, whereas the two putative amino acid sequences share 95 % similarity.

Stress-induced transcription of *MsGME* in alfalfa seedlings

GME is a key enzyme in the biosynthesis of ascorbate, which plays an important role as an antioxidant in the defence against various abiotic stresses. The transcript levels of *MsGME* in response to multiple abiotic stresses were measured by real-time PCR. Under NaCl treatment, the *MsGME* expression level did not change notably at 1 or 6 h after treatment but became substantially elevated and reached its maximum by 12 h (Fig. 1a). In response to PEG 6000 treatment, *MsGME* transcripts increased more than fivefold by 12 h, reached a maximum at 24 h and declined at 36 h (Fig. 1b). In the case of $Al_2(SO_4)_3$, the highest transcript accumulation was observed at 24 h, which then returned to the basal level at 48 h (Fig. 1c).

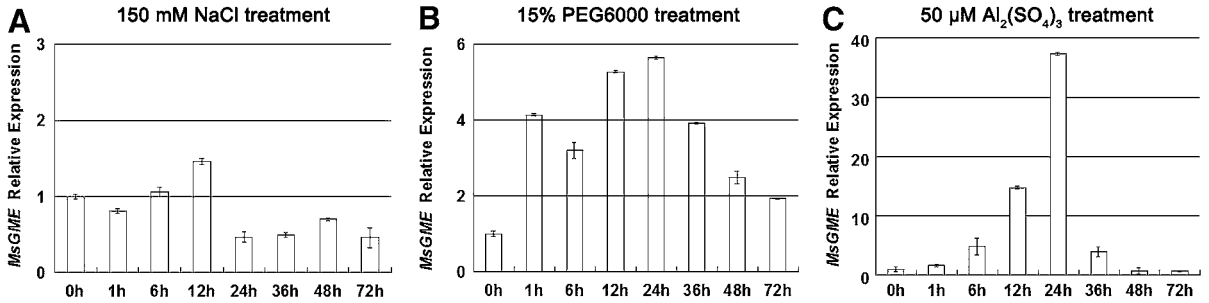

Fig. 1 Real-time PCR analysis of *MsGME* in alfalfa. **a** Transcript abundance in 2-week-old seedlings after 150 mM NaCl treatment for 72 h. **b** Transcript abundance in 2-week-old seedlings after 15 % PEG 6000 treatment for 72 h. **c** Transcript abundance in 2-week-old seedlings after 50 μM $Al_2(SO_4)_3$ treatment for 72 h

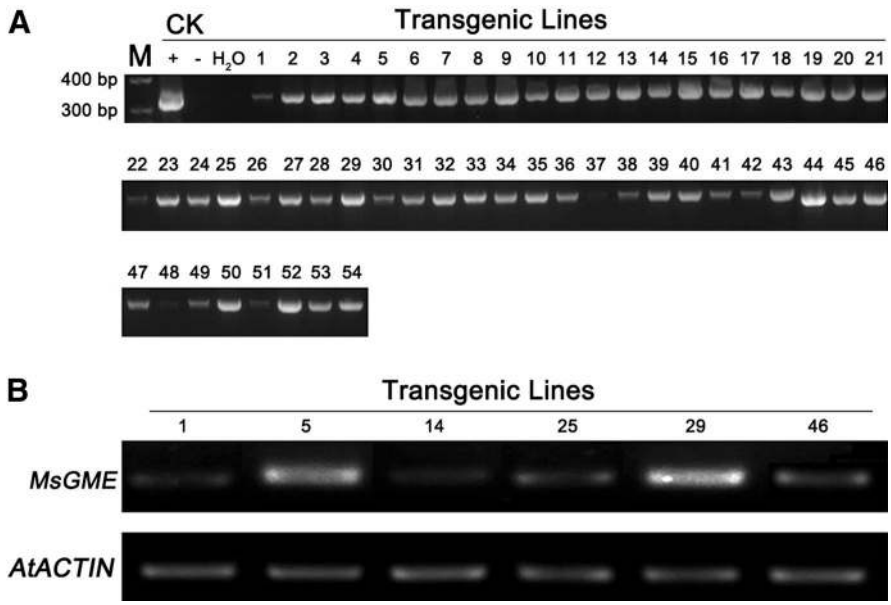

Fig. 2 Molecular analysis of transgenic *Arabidopsis* seedlings transformed with *MsGME*. **a** PCR confirmation of transgenic *lines 1* through *54*. CK-, non-transgenic plants. CK + , *Agrobacterium tumefaciens* GV3101 carrying the expression vector pMyc-*35S*::*MsGME*. **b** Semi-quantitative RT-PCR analysis of *MsGME* in transgenic *Arabidopsis*

Molecular characterisation of transgenic plants

A total of 54 putative transgenic *Arabidopsis* plants were screened for resistance to kanamycin using a kanamycin-supplemented culture medium, and all plants (T1) were confirmed to be *35S*::*MsGME*-transgenic plants by PCR analysis using primers annealing to the 35S promoter (Fig. 2a). Two lines with a high level of transgene expression were chosen for further analysis. The significant increase in *MsGME* transcript levels in lines 5 and 29 was detected by semi-quantitative RT-PCR (Fig. 2b). All of the selected transgenic plants exhibited no differences in growth compared to the wild-type plants.

Overexpression of *MsGME* altered the expression of ascorbate biosynthetic pathway genes in transgenic *Arabidopsis*

The transcript levels of several genes involved in the major ascorbate biosynthetic pathway were measured in the shoots of transgenic *Arabidopsis* by real-time PCR. The overexpression of *MsGME* resulted in changes in the expression of most of the ascorbate biosynthesis genes, including genes upstream of *GMP* but downstream of *GP* and *GGP*, and the overexpression of *MsGME* was related to the significant up-regulation of these genes. In transgenic lines 5 and 29, the expression of *GMP*, *GP* and *GGP* was increased by 1.28- and 1.26-fold, 1.29- and 1.35-fold, and 1.39- and 1.43-fold, respectively, compared with the wild-type plants (Fig. 3a). These findings showed that the overexpression of *MsGME* could affect ascorbate biosynthesis in transgenic *Arabidopsis*.

Overexpression of *MsGME* improved ascorbic acid content in transgenic *Arabidopsis*

The amount of ascorbic acid in the shoots of selected T3 transgenic lines and untransformed plants were measured by HPLC. Compared with the wild-type plants, the ascorbic acid levels increased by 1.69- and 1.52-fold in the shoots of transgenic lines 5 and 29, respectively, under control conditions. However, the shoot ascorbic acid levels increased by 1.76- and 1.65-fold under 150 mM NaCl, by 1.72- and 1.56-fold under 15 % PEG 6000, and by 1.75- and 1.58-fold under 50 μM $Al_2(SO_4)_3$ stresses in transgenic lines 5 and 29, respectively, (Fig. 3b), compared with the wild-type plants. These results indicated that the elevation of *MsGME* expression increased the ascorbic acid content in the shoots of transgenic *Arabidopsis*.

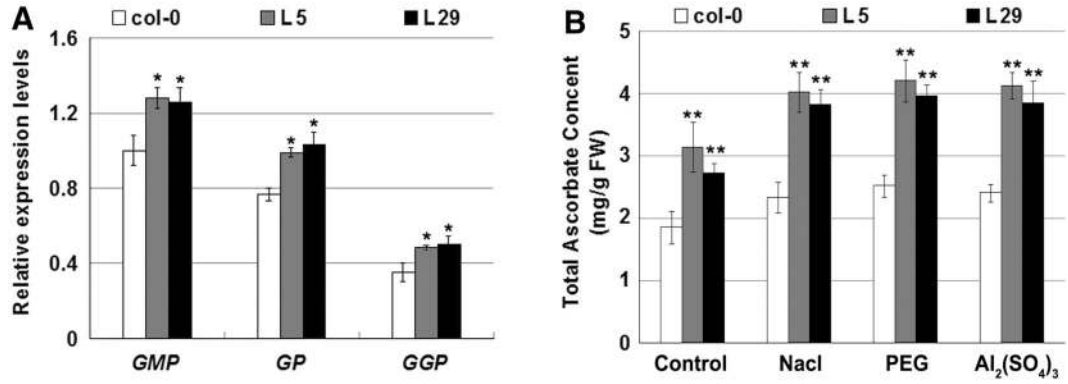

Fig. 3 Overexpression of the *MsGME* gene in *Arabidopsis* induced ascorbate biosynthesis-related gene expression and increased the total ascorbic acid content of transgenic lines. **a** The relative transcript abundances of ascorbate biosynthesis-related genes were assayed in the shoots of 5-week-old wild-type and transgenic plants (*lines 5 and 29*). Real-time PCR data were normalised against *AtACTIN* and are shown as a percentage of wild-type plants. **b** Total ascorbic acid content in the shoots of 5-week-old wild-type and transgenic plants (*lines 5 and 29*). The data are presented as the mean ± SD ($n = 10$) of triplicate independent measurements. The asterisks indicate values that are significantly different from those of the wild-type plants (Duncan test $P < 0.05$)

Overexpression of *MsGME* improved tolerance to abiotic stresses in transgenic *Arabidopsis*

To evaluate whether the overexpression of *MsGME* in *Arabidopsis* increased its tolerance to abiotic stresses, 2-week-old T3 homozygous transgenic lines 5 and 29 and wild-type *Arabidopsis* seedlings were exposed to 150 mM NaCl, 15 % PEG 6000 or 50 μM $Al_2(SO_4)_3$ (pH 4.5) for 1 week. The transgenic plants developed faster than the wild-type plants under these stresses (Fig. 4a). Although there was no significant difference in the fresh weight compared to the wild-type under control conditions, the fresh weight of the wild-type plants was significantly lower than that of the transgenic plants under the conditions of abiotic stress (Fig. 4b). For instance, in the presence of 150 mM NaCl, the fresh weights of lines 5 and 29 were 2.1-fold and 2.3-fold higher, respectively, than wild-type plants (Fig. 4b). Furthermore, 5-week-old T3 homozygous transgenic lines 5 and 29 and wild-type *Arabidopsis* seedlings were also subjected to abiotic stresses by irrigating with 150 mM NaCl, 15 % PEG 6000 or 50 μM $Al_2(SO_4)_3$ for one week. The wild-type plants displayed chlorosis and general growth inhibition, whereas both transgenic lines grew well (Fig. 4c).

Abiotic stress causes membrane-lipid peroxidation, leading to increased malondialdehyde (MDA) content. Under control conditions, the MDA content of the wild-type and transgenic plants showed no significant difference. However, after the 150 mM NaCl treatment, the MDA content in the wild-type plants increased to 76.17 %, whereas only 23.66 and 7.26 % increases were found for lines 5 and 29, respectively (Fig. 5a). In the 15 % PEG 6000 treatment, MDA in the wild-type plants increased 168 %, whereas only 47 and 40 % increases were found for lines 5 and 29, respectively (Fig. 5b). Regarding the 50 μM $Al_2(SO_4)_3$ treatment, MDA in wild-type plants increased 154 %, whereas only 21 and 3.9 % increases were observed for lines 5 and 29, respectively (Fig. 5c).These results demonstrated that the constitutively expressed *MsGME* gene in *Arabidopsis* increased its tolerance to abiotic stresses.

In addition, the relative membrane permeability (RMP) was measured to investigate membrane lipid stability in transgenic and wild-type plants challenged with abiotic stresses. RMP was 26, 24 and 23 % in col-0, line 5 and line 29, respectively. Under the stresses of 150 mM NaCl, 15 % PEG 6000 and 50 μM $Al_2(SO_4)_3$, RMP significantly increased in all of the plants but showed a much greater increase in the wild-type plants than in lines 5 and 29 (Fig. 6). In transgenic lines 5 and 29, RMP was 28 and 27 % with 150 mM NaCl (Fig. 6a), 28 and 33 % with15 % PEG 6000 (Fig. 6b), and 28 and 29 % with 50 μM $Al_2(SO_4)_3$ (Fig. 6c), respectively, whereas RMP was significantly lower in wild-type plants (60 % under 150 mM

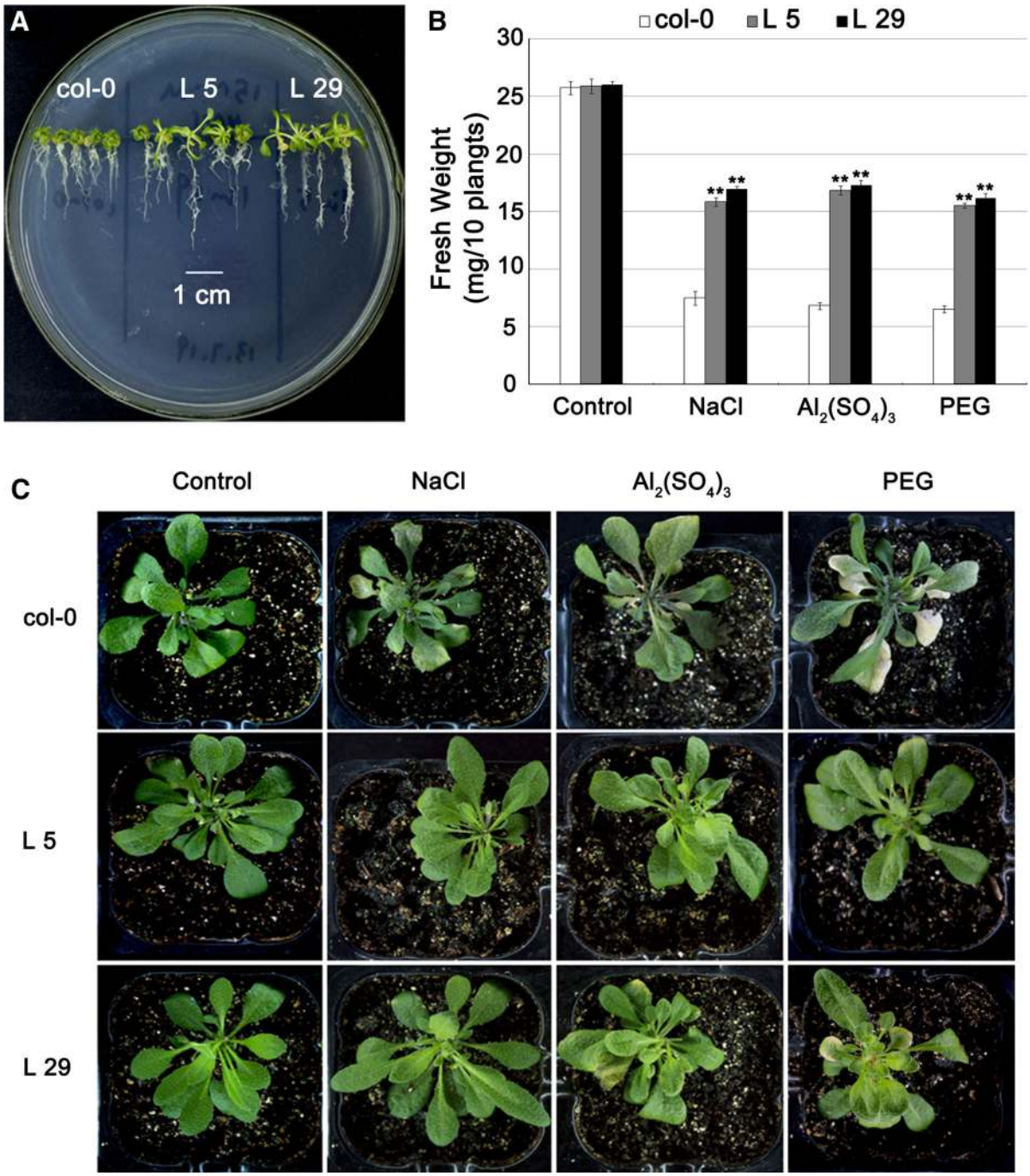

Fig. 4 Transgenic *Arabidopsis* plants showed enhanced salt, drought and acid tolerance. **a** Representative 2-week-old wild-type and transgenic plants (*lines 5 and 29*) exposed to 150 mM NaCl for 1 week. **b** The fresh weights of wild-type and two transgenic plants (*lines 5 and 29*) under 150 mM NaCl, 15 % PEG 6000 or 50 μM $Al_2(SO_4)_3$ (pH 4.5) treatments. **c** Representative 5-week-old wild-type and transgenic plants (*lines 5 and 29*) irrigated with 150 mM NaCl, 15 % PEG 6000 or 50 μM $Al_2(SO_4)_3$ for one week. The data are presented as the mean ± SD ($n = 10$) of triplicate independent measurements. The *asterisks* indicate values that are significantly different from those of the wild-type plants (Duncan test $P < 0.05$)

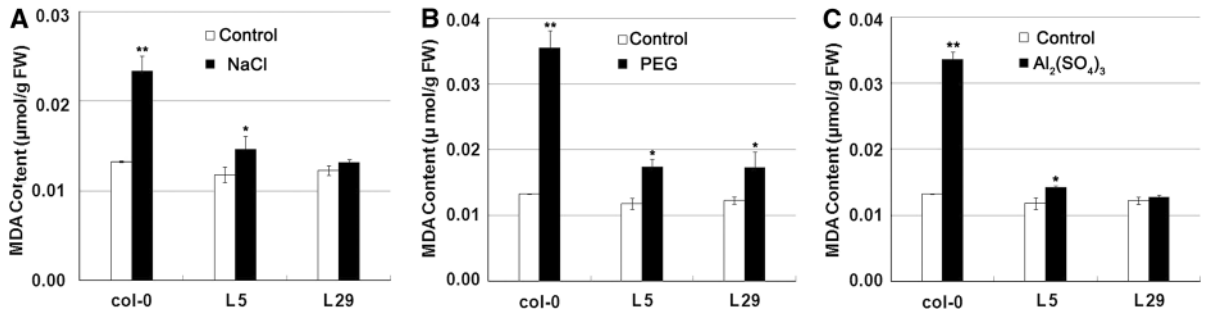

Fig. 5 MDA content of transgenic *Arabidopsis* under salt, drought and acid stresses. **a** 5-week-old wild-type and transgenic plants (*lines 5 and 29*) were subjected to 150 mM NaCl for 1 week. **b** 5-week-old wild-type and transgenic plants (*lines 5 and 29*) were subjected to 15 % PEG 6000 for one week. **c** 5-week-old wild-type and transgenic plants (*lines 5 and 29*) were subjected to 50 μM $Al_2(SO_4)_3$ for 1 week. The data are presented as the mean ± SD ($n = 10$) of triplicate independent measurements. The *asterisks* indicate values that are significantly different from those of the wild-type plants (Duncan test $P < 0.05$)

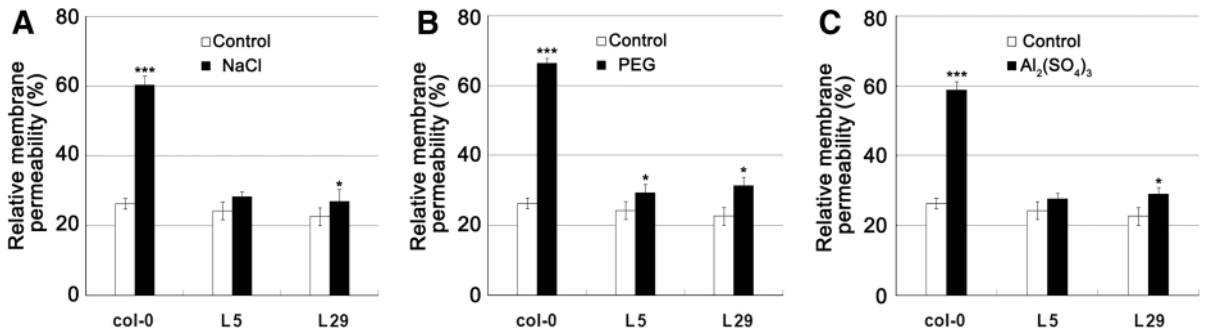

Fig. 6 Relative membrane permeability (RMP) of transgenic *Arabidopsis* under salt, drought and acid stresses. **a** Five-week-old wild-type and transgenic plants (*lines 5 and 29*) were subjected to 150 mM NaCl for one week. **b** Five-week-old wild-type and transgenic plants (*lines 5 and 29*) were subjected to 15 % PEG 6000 for one week. **c** Five-week-old wild-type and transgenic plants (*lines 5 and 29*) were subjected to 50 μM $Al_2(SO_4)_3$ for 1 week. The data are presented as the mean ± SD ($n = 10$) of triplicate independent measurements. The *asterisks* indicate values that are significantly different from those of the wild-type plants (Duncan test $P < 0.05$)

NaCl, 66 % under 15 % PEG 6000 and 58 % under 50 μM $Al_2(SO_4)_3$. These results suggested that the cell membranes of transgenic *Arabidopsis* plants were healthier and showed less damage under salt, drought and acid stress.

Discussion

The growth and development of plants and the productivity of crops are affected by various abiotic stresses, including acid, drought and high salinity. Under adverse conditions, plants will signal and then activate the expression of regulatory genes to ultimately induce resistance genes and improve plant stress tolerance (Fujimoto et al. 2000). The GME-catalysed formation of GDP-D-mannose is the initial step in the D-mannose/L-galactose pathway of ascorbic acid biosynthesis. GME may also regulate ascorbate synthesis under stress conditions and adjust the balance between ascorbate and cell wall monosaccharide biosynthesis (Wolucka and Van Montagu 2003). Thus, it has been hypothesised that GME, in association with the VTC2 (GDP-L-galactose phosphorylase) enzyme catalysing the subsequent step of ascorbate biosynthesis, constitutes a control point for the regulation of the ascorbate pathway in plants (Laing et al. 2007; Wolucka and Van Montagu 2007). Alfalfa is the most important leguminous forage plant, and abiotic stresses are the major limiting factors for

its growth and crop yield. To address this limitation, we isolated an alfalfa GME gene, *MsGME*, which encodes a nuclear protein of 381 amino acids. The expression of *MsGME* in alfalfa seedlings was induced by salt, drought and $Al_2(SO_4)_3$, suggesting that *MsGME* may be involved in various abiotic stress responses in alfalfa (Fig. 1).

In this study, the overexpression of *MsGME* in *Arabidopsis* affected the expression of ascorbate biosynthesis-related genes in the shoots of transgenic *Arabidopsis* (Fig. 3a), demonstrating that *MsGME* plays an important role in ascorbate biosynthesis in *Arabidopsis*. A previous report confirmed that repression of the *GME* gene in tomato (*S. lycopersicum*) increases the transcript abundance of *SlGMP* and *SlGGP* (tomato homologues of *VTC2*), but the effect on *SlGP* is not as clear (Gilbert et al. 2009). A VTC2 circle involving *GME* and *GGP* has been proposed to exist in plants (Laing et al. 2007; Wolucka and Van Montagu 2007; Linster and Clarke 2008). In the present study, significant increases in the transcript abundance of *GMP*, *GP* and *GGP* were found in the transgenic *Arabidopsis* plants (Fig. 3a). The *GMP* expression was elevated to compensate for the decreases in GDP-L-galactose, the precursor of GDP-D-mannose, and expression of *GP* and *GGP* were increased to consume GDP-L-galactose, possibly because the overexpression of *GME* in *Arabidopsis* increased the levels of GDP-L-galactose. Thus, biological processes maintain the balance of the biosynthetic pathway and a relative and stable ascorbate output by regulating the expression of *GMP*, *GME*, *GP*, *GGP* and other genes.

The ascorbic acid content was increased by overexpressing *MsGME* in transgenic *Arabidopsis*, demonstrating that endogenous *GME* in *Arabidopsis* is expressed at a moderate level and could be enhanced by regulation to promote ascorbic acid biosynthesis. However, the expression profiling of ascorbic acid-related genes has revealed that *GGP* might play an important role in the regulation of ascorbate accumulation (Ioannidi et al. 2009). These results indicate that the regulation of ascorbate synthesis in *Arabidopsis* might occur at more than one step and that the network regulating the ascorbate pathway might be extremely complex.

The ascorbate-deficient *Arabidopsis* mutant, *vtc1* (containing 30 % of wild-type ascorbate), is sensitive to ozone, SO_2 and UV-B radiation (Conklin et al. 1996), suggesting that ascorbate plays key roles as an antioxidant in the defence against different abiotic stresses. An increase in the ascorbic acid content could confer tolerance to various stresses, including salt, ozone and chilling (Chen and Gallie 2005; Eltayeb et al. 2007). In this study, the overexpression of *MsGME* led to an elevated ascorbic acid content in transgenic *Arabidopsis* and improved the tolerance to salt, drought and acid stresses. Our results revealed that the MDA content of the transgenic lines was significantly lower than that of the non-transgenic plants, suggesting that lipid peroxidation was lower in the *MsGME*-enhanced plants. The fact that the transgenic lines overexpressing *MsGME* accumulated more ascorbate suggests the hypothesis that the improved abiotic stress tolerance was closely related to the increased ability to scavenge ROS resulting from the higher ascorbate content. All of these results indicated that the expression of *MsGME* in *Arabidopsis* confers salt, drought and acid tolerance to the transgenic plants. Similar results were obtained in transgenic potato (*Solanum tuberosum*) plants expressing ascorbate biosynthesis-related genes, including the strawberry D-galacturonic acid reductase gene, with the plants exhibiting a better survival rate under various abiotic stresses (Upadhyaya et al. 2009). Therefore, the *MsGME* gene has the potential to be used as a candidate gene for enhancing tolerance to salt, drought and acid in breeding crops in the future. It should also be tested for its ability to enhance other forms of abiotic stress tolerance.

Conclusions

A novel cDNA fragment from alfalfa encoding an MsGME protein was isolated and characterised. The *MsGME* cDNA has an ORF of 1,146 bp that encodes a nuclear protein of 381 amino acids. Expression analysis confirmed that *MsGME* was induced by salinity, PEG and acidity stresses. Our results suggest that *MsGME* can effectively enhance acid, drought and salt tolerance in transgenic *Arabidopsis* by increasing ascorbate accumulation.

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