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# Antisense-mediated depletion of tomato GDP-L-galactose phosphorylase increases susceptibility to chilling stress

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ABSTRACT

The GDP-L-galactose phosphorylase (GGP), which converts GDP-L-galactose to L-Gal-1-phosphate, is generally considered to be a key enzyme of the major ascorbate biosynthesis pathways in higher plants, but experimental evidence for its role in tomato is lacking. In the present study, the GGP gene was isolated from tomato (*Solanum lycopersicum*) and transient expression of SlGGP-GFP (green fluorescent protein) fusion protein in onion cells revealed the cytoplasmic and nucleus localization of the protein. Antisense transgenic tomato lines with only 50–75% ascorbate level of the wild type (WT) were obtained. Chilling treatment induced lower increase in AsA levels and redox ratio of ascorbate in antisense transgenic plants compared with WT plants. Under chilling stress, transgenic plants accumulated more malendialdehyde (MDA) and more $O_2^{\bullet -}$, leaked more electrolytes and showed lower maximal photochemical efficiency of PSII (Fv/Fm), net photosynthetic rate (Pn), and oxidizable P700 compared with WT plants. Furthermore, the antisense transgenic plants exhibited significantly higher $H_2O_2$ level and lower ascorbate peroxidase (APX) activity. Our results suggested that GGP plays an important role in protecting plants against chilling stress by maintaining ascorbate pool and ascorbate redox state.



## Introduction

Temperature stress severely affects the growth, yield and quality of plants. Reactive oxygen species (ROS) tend to increase if plants were exposed to low or high temperature stress conditions. Plants have developed antioxidant mechanisms to cope with this changing temperature environment. These mechanisms employ antioxidant enzymes, such as ascorbate peroxidase (APX, EC 1.11.1.11), superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6), as well as non-enzymatic antioxidants, such as reduced glutathione, phenolic compounds and ascorbic acid (AsA) (Asada, 1999).

In higher plants, ascorbate is the most abundant water-soluble antioxidant acting to scavenge reactive oxygen species including singlet oxygen, superoxide and hydroxyl radicals that are generated during photosynthesis, oxidative metabolism and abiotic or biotic stresses (Padh, 1990; Conklin, 2001). Ascorbate is also an important co-substrate of many enzymes, such as APX and AsA-dependent dioxygenases (Arrigoni and De Tullio, 2002). It is also a cofactor for violaxanthin deepoxidase (VDE) in the xanthophyll cycle, a process contributing to dissipate absorbed light as heat (Horemans et al., 2000; Smirnoff and Wheeler, 2000; Müller-Moulé et al., 2002). In addition, ascorbate has been implicated in processes including growth (Pignocchi and Foyer, 2003), pathogen responses (Barth et al., 2004), programmed cell death (de Pinto et al., 2006), flowering (Kotchoni et al., 2009) and senescence (Barth et al., 2004), as well as protecting plants against environmental stress including ozone (Conklin and Barth, 2004), UV radiation (Gao and Zhang, 2008), high light intensity (Müller-Moulé et al., 2004), high temperature, and low temperature (Larkindale et al., 2005).

It was not until recently that the major plant pathway (the L-galactose pathway) for AsA biosynthesis was described (Linster and Van Schaftingen, 2007), which is different from the animal L-ascorbate synthesis pathway. L-Galactose pathway, also named as the Smirnoff/Wheeler or D-mannose pathway, is the only physiologically significant source of ascorbate in *Arabdopsis thaliana* (Dowdle et al., 2007). The Smirnoff/Wheeler pathway of ascorbate synthesis consists of the formation of ascorbate from guanosine diphosphate-mannose (GDP-Man) with the intermediates GDP-L-galactose (GDP-L-Gal), L-Gal-1-phosphate, L-Gal, and L-galactone-1,4-lactone (Wheeler et al., 1998). Recently, Laing et al.

*Abbreviations:* APX, ascorbate peroxidase; AsA, ascorbate; DAB, diaminobenzidin; DHA, dehydroascorbate; Fv/Fm, the maximal photochemical efficiency of PSII; GFP, green fluorescence protein; HL, high light; LL, low light; MDA, malendialdehyde; MDHA, monodehydroascorbate radical; MDHAR, monodehydroascorbate reductase; MV, methyl viologen; NBT, nitroblue tetrazolium; NLS, nuclear localization sequence; PFD, photon flux density; Pn, the net photosynthetic rate; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; TBA, thiobarbituric acid; VDE, violaxanthin deepoxidase; WT, wild type.

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(2007) and Linster et al. (2007) elucidated the last unknown enzyme of GDP-Man pathway, GDP-L-Gal phosphorylase (GGP) (EC 2.7.7.69), which converts GDP-L-galactose to L-Gal-1-phosphate, having attracted considerable interest in recent years.

Since the discovery of GGP, many researchers have focused on whether it is the key enzyme of AsA synthesis. As the first six steps of the AsA synthesis pathway are used to synthesize activated nucleotide sugars that are also precursors of cell wall polysaccharides and glycoproteins, the committed pathway of L-ascorbate biosynthesis then consists of the sequential conversion of GDP-L-galactose to L-galactose-1-P, L-galactose, L-galactono-1, 4-lactone, and L-ascorbate (Smirnoff, 2000; Reuhs et al., 2004). Previous studies showed that the GDP-L-galactose phosphorylase (GGP) step may play a key role in controlling ascorbate biosynthesis. VTC2 (encoded GGP in Arabidopsis) expression and GDP-L-galactose phosphorylase activity rapidly increase on transfer to high light, but the activity of other enzymes in the GDP-mannose pathway is affected little, except for a small (two-fold) increase in L-galactono-1, 4-lactone dehydrogenase activity (Dowdle et al., 2007). The induction of VTC2 mRNA has also been confirmed by additional study. VTC2 mRNA expression was induced strongly by exposing dark-grown seedlings to light (Müller-Moulé, 2008). Furthermore, jasmonates could induce ascorbate biosynthesis (Sasaki-Sekimoto et al., 2005; Wolucka et al., 2005). Both VTC2 and VTC5 transcription in Arabidopsis are increased by jasmonates (Sasaki-Sekimoto et al., 2005). Evidence for the key function of GGP is also proved by metabolite regulation. L-Ascorbate, L-galactono-1, 4-lactone and L-galactose had no effect on GGP activity in Arabidopsis, indicating no feed back regulation of the enzyme by these metabolites (Dowdle et al., 2007). However, L-ascorbate supplementation decreased VTC2 expression in Arabidopsis plants, suggesting feed back inhibition by L-ascorbate at the transcriptional level (Dowdle et al., 2007).

Previous reports about GGP mostly focus on Arabidopsis. Little research has been carried out on the tomato. Gilbert et al. (2009) has confirmed that repression of *GME* genes in tomato increases the transcript abundance of *SlGGP*. In a recent study, significant decrease in transcript abundance of *SlGGP* was found in the *SlGME2*-over expressing plants, indicating that GGP may play an important role in ascorbate synthesis in tomato (Zhang et al., 2010). However, experimental evidence in support of this hypothesis is still lacking. In order to investigate the role of GGP in plant ascorbate biosynthesis and tolerance to chilling stress, we obtained antisense transgenic plants with decrease in *SlGGP* expression and measured the change of photosynthesis, growth, and oxidative damage, which indicated the enhanced sensitivity to chilling stress in transgenic plants relative to WT plants. The results reveal a crucial role for GGP in the regulation of ascorbate biosynthesis, and give new insights between ascorbate biosynthesis and chilling tolerance in tomato.

## Materials and methods

### *Plant growth and treatments*

Tomato seeds of cultivar (*Solanum lycopersicum* cv. Zhongshu 6) were germinated on Murashige-Skoog (MS) agar media and transgenic plants were cultivated on MS agar media with kanamycin (50 $\mu g\,mL^{-1}$) in close glass bottles for 7 days at 25 °C. Sprouted seedlings were transplanted into sterilized soil and grew at 25/20 °C (day/night) with a 16/8 h photoperiod, 300–500 $\mu mol\,m^{-2}\,s^{-1}$ photon flux density (PFD), and 50–60% relative humidity in the greenhouse. The 6-week-old wild type (WT) and transgenic plants were used for subsequent abiotic stress and quantitative real-time RT-PCR assays.

For temperature treatment, the whole plants were exposed to low temperature (4 °C) for 0, 3, 6, and 12 h respectively in an illuminated incubation chamber (GXZ-260C) with PFD of 200 $\mu mol\,m^{-2}\,s^{-1}$ [25/20 °C (day/night) with a 16/8 h photoperiod], 50–60% relative humidity. All the physiological and biochemical measurements were carried out on the youngest, fully expanded leaves. For the oxidative stress test, plants were sprayed with 100 $\mu mol\,L^{-1}$ methyl viologen (MV) for 24 h with spraying water as control. For NaCl stress, the plants were watered with Hogland nutrition solution containing 200 $mmol\,L^{-1}$ NaCl with Hogland nutrition solution as control.

### *Isolation of SlGGP*

Total RNA was extracted from 0.10 g of fresh tomato leaves with total RNA isolation reagent (Tiangen, China) following the manufacturer's instructions. The first-strand cDNAs were synthesized using First-Strand cDNA Synthesis kit (Fermentas, Glen Burnie, MD, USA). To clone the conserved cDNA region of the *SlGGP*, a pair of primers, Pf1 (GGATCCTTCCGACCTCCTCTTCCTT *Bam*HI site underlined) and Pr1 (GAGCTCATGAACACCAGAGCTGGG *Sac*I site underlined), were designed according to the predicted *S. lycopersicum* GGP gene (SGN-U312646 and SGN-U579800) (Dowdle et al., 2007; Zhang et al., 2010). The cDNA amplification products were cloned into the pMD18-T vector and sequenced. The primers were synthesized from Shanghai Bioasia Bio-engineering Limited Company. Nucleotide and deduced amino acid sequences were analyzed using DNAman version 5.2 (Lynnon Biosoft, USA).

### *Plasmid construction and Agrobacterium mediated transformation of tomato plants*

The coding sequence of *SlGGP* cDNA was amplified with primers (Pf2 GAGCTCTTCCGACCTCCTCTTCCTT SacI site underlined Pr2. GGATCCATGAACACCAGAGCTGGG BamHI site underlined). Then this fragment was sub-cloned into the rebuilt pBI121 with cauliflower mosaic virus 35S (35S-CaMV) promoter upstream to form antisense constructs (pBI-*SlGGP*). The antisense 35S-CaMV-*SlGGP* recombinants were introduced into *Agrobacterium tumefaciens* LBA4404 via freezing transformation method. Leaf disk transformation was performed as described by Horsch et al. (1985). Leaf disks of WT plant infected with *A. tumefaciens* were cultured on medium to induce shoots. After 4–8 weeks, regenerated shoots were transferred to medium to induce roots. Both of the media above contained cefotaxime sodium (250 $\mu g\,mL^{-1}$) to suppress *A. tumefaciens* and kanamycin (50 $\mu g\,mL^{-1}$) as selection pressure. As a consequence, from tissue culture obtained 21 individual kanamycin resistant lines in which 18 lines were screened out as $T_0$ generation of transgenic plants by PCR amplification to integrated sequence in their genomic DNA.

### *Sub-cellular localization of SlGGP-GFP (green fluorescent protein) fusion protein*

The coding region of *SlGGP* without termination codon in the pMD18-T simple vector about 821 bp was digested with XbaII and KpnI and inserted between the upstream constitutive CaMV35S promoter and the downstream GFP in a pBINmGFP5-ER expression vector. The recombinant plasmid was transformed into living onion epidermal cells by plasmid bombardment as described (Kinkema et al., 2000). The sub-cellular location of the *SlGGP* was detected by monitoring the transient expression of GFP in onion epidermal cells. The transformed cells were incubated in MS medium at 25 °C for 24–48 h and then were observed with a fluorescence