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Expression pattern and promoter analysis of the gene encoding GDP-D-mannose 3′,5′-epimerase under abiotic stresses and applications of hormones by kiwifruit

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ABSTRACT

In higher plants, ascorbic acid (AsA) plays important roles in antioxidative defenses against environmental stresses. GDP-D-mannose 3′,5′-epimerase (*GME*) catalyses a committed step in AsA biosynthesis. After isolating *GME* cDNAs from two species of *Actinidia* (kiwifruit), we analyzed expression abundance and promoter activities under abiotic stresses or applications of exogenous stress-signaling hormones. Exposure to ABA, SA, cold or light by kiwifruit leaves increased AsA contents, whereas application of GA_3, wounding, heat or darkness decreased those amounts. Expression of *GME* was up-regulated in the leaves by SA, wounding, cold, and heat, but down-regulated by GA_3. Promoter activities were enhanced by SA and heat, but were not affected by wounding, darkness, or light. *GME* promoters affected differentially by some stress conditions or stimuli between two kiwifruit genotypes used, although AsA levels are not linked with the expression of *GME*, indicating other factors are important for determining AsA levels. We speculated that AsA concentrations are under complicated regulation so that only one gene expression, here *GME* sole cannot determine AsA concentrations.

1. Introduction

L-Ascorbic acid (AsA) is a vital compound in plants with a range of functions as cellular anti-oxidant, stress response factor and enzyme co-factor. Researchers have proposed that it is also involved in many stress-induced oxidative processes, e.g., responses to water loss (Fotopoulos et al., 2008), pathogens and oxidizing agents (Fotopoulos et al., 2006), high temperature (Ma et al., 2008), photo-oxidative stress and ozone (Sanmartin et al., 2003; Müller-Moulé et al., 2004). AsA has roles in both detoxification from reactive oxygen species (ROS) and the maintenance of ROS levels that are required for signaling (Foyer and Noctor, 2005). In addition, AsA is active in many metabolic processes, including photosynthesis (Yabuta et al., 2007), cell wall formation (Barth et al., 2006) and plant growth (Dowdle et al., 2007).

GDP-D-mannose 3′,5′-epimerase (*GME*), an enzyme in the biosynthesis of AsA, catalyses the conversion of GDP-D-mannose to GDP-L-galactose in the D-mannose/L-galactose pathway (Wolucka and Van Montagu, 2003). In higher plants, *GME* has been reported in pea (*Pisum sativum*) and *Arabidopsis thaliana* (Wheeler et al., 1998), and has been recently cloned and characterized in *A. thaliana* (Wolucka and Van Montagu, 2003) and rice (*Oryza sativa*) (Watanabe et al., 2006). In four genotypes of kiwifruit (*Actinidia*), expression of *GME* genes has been shown to be correlated with a peak in AsA accumulation in fruits, whereas transient expression of *GME* alone resulted in little change in tobacco leaf AsA (Bulley et al., 2009). Biochemical studies on the cytosolic *GME* of *A. thaliana* have led to the discovery of unsuspected and novel 5′-epimerase activity responsible for the synthesis of GDP-L-gulose (Wolucka and Van Montagu, 2003). *GME* may interact with an Hsc70.3 heat-shock protein and undergo complex regulation that involves redox control and inhibition by GDP-L-fucose (Wolucka and Van Montagu, 2003). Leaves from transgenic tomato over-expressing *SlGME1* and *SlGME2* exhibit an increase in total AsA, and improved plant tolerance to oxidative, cold, and salt stresses (Zhang et al., 2011). Methyl jasmonate also enhances *GME* expression in *A. thaliana* and tobacco Bright Yellow-2 suspension cells (Goossens et al., 2003; Wolucka et al., 2005).

The kiwifruit (*Actinidia* spp.) are AsA-rich fruits and a tremendous variation of AsA content exists within the fruits of this genus (Bulley et al., 2009). For this reason, two kiwifruit genotypes in different AsA levels have been used for the experiment. Despite the high AsA content of kiwifruit, understanding the biosynthesis and metabolic regulation of this important antioxidant is far from complete in this plant. In previous studies, little information has been reported about *GME* expression under stress conditions

Abbreviations: ABA, abscisic acid; AsA, ascorbic acid; CaMV35S, cauliflower mosaic virus 35S promoter; GA_3, gibberellin; *GME*, GDP-D-mannose 3′,5′-epimerase; *GPP*, L-galactose-1-phosphate phosphatase; *GUS*, β-glucuronidase; MUG, 4-methylumbelliferyl-β-D-glucuronidehydrate; 4-MU, 4-methylumbelliferone; RT-PCR, reverse transcriptase-polymerase chain reaction; SA, salicylic acid.

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Table 1
Primer sequences used in study; underlined sequences show restriction enzyme sites.

Primer name	Primer sequence (5′–3′)
GME-F	AGAATGGGAAGCACCAGTGAATCT
GME-R	GTATCTGCTTCATTCTTTGCCATCA
Actin-F	GCTTACAGAGGCACCACTCAACC
Actin-R	CCGGAATCCAGCACAATACCAG
GME-RT-F	GGCATTGAATGTAGGATTGGAA
GME-RT-R	TATCAGTGGAGGTAAGGGTCT
SP1	TCTCTTAACACCATTGACCCTAGCT
SP2	GTCATGTGCTCGTTTTTCTTCCAGT
SP3	CAATGTGCGAGGCAATGAAC
PD-F	GGGTTATTACCTCAACTGGG
PD-R	CGGTCATGTGCTCGTTT
V-F	CCCAAGCTTGGGTTATTACCTCAACTGGGTTTTG
VR-R	CCGGAATTCTCTGCACACCACATCATTAAGGGAT
VD-R	TCCCCCGGGTCTGCACACCTCATCATTAAGAGAT

in kiwifruit. To gain new insights into the regulatory mechanisms for *GME* expression in two species of *Actinidia*, we performed a systematic investigation of AsA levels, mRNA expression, and promoter activities in response to various abiotic stress and hormonal treatments. The research, reported herein, details the results of the regulation of leaf *GME* expression and how that was related to stresses in two species of *Actinidia*.

2. Materials and methods

2.1. Plant materials

Eight-year-old vines of kiwifruit (*Actinidia rufa* and *Actinidia deliciosa* cv. "qinmei") were trained as a trellis system and grown at a 2 m × 3 m spacing in an experimental orchard at the Horticultural Experimental Station of Northwest A & F University. Two-year-old kiwifruit plants (*A. rufa* and *A. deliciosa* cv. "qinmei") were placed in plastic pots (250-mm diam.) filled with a 5:1 (v:v) medium of local topsoil sand. Six-week-old plants of tobacco (*Nicotiana tabacum* cv. NC89) were cultured in a controlled-environment growth chamber under a 16-h photoperiod, 65% relative humidity, and a 25 °C/21 °C (day/night) temperature cycle.

2.2. Nucleic acid extractions and RT-PCR analysis

Total RNA was isolated from leaf samples (0.1 g fresh weight) by the LiCl precipitation method (Asif et al., 2000). First-strand cDNA was synthesized from DNase-treated total RNA using M-MLV reverse transcriptase (Takara, Kyoto, Japan). To clone the entire cDNA sequence of the two *GME* genes – *AdGME* and *ArGME* – we designed primers *GME*-F and *GME*-R (Table 1). PCR products were cloned into the pMD-18T vector (Takara, Kyoto, Japan), and several clones were sequenced for each reaction.

2.3. Real-time quantitative PCR (RT-qPCR) analysis

RT-qPCR was performed on an iQ5.0 instrument (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Green RT-qPCR kits (Takara, Kyoto, Japan) according to the manufacturer's instructions. Primer (Table 1) specificity was determined by RT-PCR and melting-curve analysis. RT-qPCR was conducted with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. Constitutive *Actin* served as the endogenous control (Li et al., 2010b). Data from the individual runs were collated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Values for mean expression and standard deviation (SD) were calculated from the results of three independent experiments.

2.4. Measurement of ascorbic acid content

Frozen and homogenized kiwifruit leaf samples (0.1 g fresh weight) were suspended in 1 ml of cold 6.0% (v/v) $HClO_4$ and centrifuged at 12,000 rpm for 10 min at 4 °C. Their AsA contents were determined by the ascorbate oxidase method, essentially as described by Tokunaga and Esaka (2007).

2.5. Genomic DNA-walking

Genomic DNA was extracted with phenol/chloroform, precipitated with ethanol, and dissolved in sterile water. The fragment of the upstream sequence of *GME* was isolated from genomic DNA of *A. rufa* using an in vitro cloning kit (Takara, Kyoto, Japan). PCR amplifications were performed as described by the manufacturer. Primary PCR was used to amplify the 5′-regulatory region with cassette primer AP1 and three gene-specific primers, SP1, SP2, and SP3 (Table 1). The 5′-flanking region for *GME* from *A. deliciosa* was isolated with primers PD-F and PD-R (Table 1). PCR products were cloned into the pMD-18T vector (Takara, Kyoto, Japan), and several clones were sequenced for each reaction. The promoter sequences were analyzed via PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) database (Lescot et al., 2002).

2.6. Construction of ParGME::GUS and PadGME::GUS fusion vectors

Two expression vectors, pC0390*GUS* and pC35S*GUS* (Xu et al., 2010), were constructed for transient expression assays. The 1225-bp flanking regions of *ParGME* and *PadGME* were generated through PCR amplifications. A *Hind*III restriction enzyme site was introduced at the 5′ end of the forward primer V–F; an *Eco*RI site, was added at the 3′-end of the Reverse primer VR-R for the *ArGME* promoter; an *Sma*I site was added at the 3′-end of the reverse primer VD-R for the *AdGME* promoter (Table 1). The promoter fragment of *ParGME* was double-digested with *Hind*III/*Eco*RI and the promoter fragment of *PadGME* was double-digested with *Hind*III/*Sma*I. Each was then ligated into the corresponding site of vector pC0390*GUS* which were double-digested with the same restriction enzymes. Fusion constructs, verified by sequencing, were introduced into *Agrobacterium* strain EHA105 by the freeze–thaw method.

2.7. Agrobacterium-mediated transient assay and GUS activity analysis in tobacco plants

Agrobacterium-mediated transient assays were performed as described previously (Sparkes et al., 2006). Fully expanded, infiltrated tobacco leaves were collected from intact tobacco plants representing each construct and used to determine GUS activity.

The method of histochemical staining for tobacco leaves were performed as described previously (Xu et al., 2010). Quantitative GUS assays were performed as described previously (Jefferson, 1987), using 4-methyl umbelliferyl glucuronide (MUG; Sigma–Aldrich) as substrate. Fluorescence of the methyl umbelliferone products was quantified with a Hitachi 850 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The total concentration of protein extracts from tested samples was normalized by an established protocol (Bradford, 1976). GUS activity was expressed as nM of 4-methylumbelliferon (4-MU; Sigma–Aldrich) generated per minute per milligram of soluble proteins.