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Light regulates ascorbic acid accumulation and ascorbic acid-related genes expression in the peel of eggplant

M. Jiang [a,b], Y. Liu [b,*], L. Ren [b], X. She [a,**], H. Chen [b]

[a] Institute of Vegetable Science, Suzhou, Academy of Agricultural Sciences, Jiangsu 215155, PR China
[b] School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, PR China

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ABSTRACT

Ascorbic acid (AsA) is one of the important metabolites and beneficial to human health. First of all, we found the phenomenon that light could promote AsA accumulation in the peel of eggplant. To further understand the regulatory mechanism of light on the formation of AsA in fruits, we isolated seven novel L-galactose pathway related genes (*SmGMP*, *SmGME1*, *SmGME2*, *SmGGP*, *SmGPP*, *SmGalDH* and *SmGLDH*) in AsA biosynthesis from eggplant. Correlation analysis suggested that AsA content showed significant positive correlation with the expression levels of *SmGME2*, *SmGGP*, *SmGPP*, *SmGalDH* and *SmGLDH*. There were many putative light responsive elements in all the seven promoters of genes, and the expression analysis confirmed that the seven genes were induced by light. Then we overexpressed the seven genes in *Arabidopsis* and observed that the AsA content were significantly increased in the leaves of transgenic plants. Moreover, the transgenic plants also displayed an increase in low temperature tolerance. The present study provides insight into light regulation of AsA biosynthesis in eggplant and may facilitate genetic engineering for improvement of the AsA content and stress tolerance in plants.

1. Introduction

Ascorbic acid (AsA), commonly known by its popular name vitamin C, is the major water-soluble antioxidant in plant cells. It protects plants from oxidative damage by scavenging reactive oxygen species (ROS) that are generated by photosynthesis, oxidative metabolism, and environmental stresses (Noctor and Foyer, 1998). AsA is a cofactor for many important enzymes and also has been proposed to function as a regulator of plant growth, flowering, and hormone signalling (Conklin and Barth, 2004; Kotchoni et al., 2009; Foyer and Noctor, 2011; Kerchev et al., 2011). Moreover, AsA is essential for human survival. Besides the well-known role of AsA in preventing scurvy, AsA also play an important role in protecting the body against oxidative stress-related illnesses such as cancers, cardiovascular diseases and aging (Li and Schellhorn, 2007). Since human have lost the ability to synthesize AsA, this vitamin must be mainly acquired through the consumption of fruits and vegetables (Nishikirni et al., 1994).

The biosynthetic pathway of AsA was first proposed by Wheeler et al. (1998). Since then, many studies have addressed the mechanisms of AsA biosynthesis and four distinct pathways for AsA biosynthesis have been proposed in plants, including L-galactose, L-glucose pathway, galacturonate pathway and myo-inositol pathway (Huang et al., 2014). Among them, L-galactose pathway is thought to be the major route in higher plants (Linster and Clarke, 2008; Imai et al., 2009). GDP-D-mannose pyrophosphorylase (GMP) is an enzyme catalyzing the initial step of AsA biosynthesis to form GDP-D-mannose. Then, GDP-D-mannose 3′,5′-epimerase (GME) catalyses the conversion of GDP-D-mannose to GDP-L-galactose, and GDP-L-galactose is converted to L-galactose-1-P by GDP-L-galactose phosphorylase (GGP). Subsequently, L-galactose-1-phosphate phosphatase (GPP) converts L-galactose-1-P to L-galactose, and then oxidized by L-galactose dehydrogenase (GalDH) to form L-galactono-1,4-lactone. Finally, L-galactono-1,4-lactone is oxidized to AsA by L-galactono-1,4-lactone dehydrogenase (GLDH) and accompanied by electrons migrated to cytochrome *c* (Bartoli et al., 2000).

Light is an essential environmental factor for plant growth and development. Light regulation of AsA biosynthesis and metabolism has been principally studied in leaves (Li et al., 2013a). Light induce the

Abbreviations: AsA, ascorbic acid; ROS, reactive oxygen species; GMP, GDP-D -mannose pyrophosphorylase; GME, GDP-D-mannose 3′,5′-epimerase; GGP, GDP-L-galactose phosphorylase; GPP, L-galactose-1-phosphate phosphatase; GalDH, L-galactose dehydrogenase; GLDH, L-galactono-1,4-lactone dehydrogenase; qRT-PCR, quantitative real time-PCR; MDA, malondialdehyde; REC, relative electric conductivity.

* Correspondence to: Y. Liu, School of Agriculture and Biology, Shanghai Jiao Tong University, NO. 800, Rd. Dong Chuan, Shanghai 200240, PR China.
** Correspondence to: X. She, Institute of Vegetable Science, Suzhou Academy of Agricultural Sciences, Wangting Town, Xiangcheng District, Suzhou, Jiangsu 215155, PR China.
E-mail addresses: liuyangtl@sjtu.edu.cn (Y. Liu), shxdsz@126.com (X. She).

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expression of AsA synthesis-related genes and enhance AsA accumulation in leaves of *Arabidopsis* (*Arabidopsis thaliana*) and tomato (*Solanum lycopersicum*) (Huang et al., 2005; Dowdle et al., 2007; Massot et al., 2012). In tobacco (*Nicotiana tabacum*), low-light conditions decrease the transcript levels of genes involved in AsA biosynthesis, such as *GMP* and *GLDH* (Tabata et al., 2002). Gene expression level depends on a balance between activating and repressing the *cis*-acting elements in the promoter regions. Light-responsive promoter elements are the most predominant promoter motif present in the promoter sequences of AsA-related gene in tomato (Ioannidi et al., 2009).

Normally, the generation and elimination of ROS are in a dynamic balance state. However, various abiotic and biotic stresses lead to a higher ROS generation (Ishikawa and Shigeoka, 2008). Excessive ROS is harmful to cell membranes, resulting in membrane lipid peroxidation (Kranner et al., 2010). MDA is one of the end products of lipid peroxidation and can be used as an indicator of lipid peroxidation degree (Berger et al., 2001). To protect against ROS, plants have developed a highly efficient defense system, with both enzymatic and non-enzymatic antioxidants. The enzymatic antioxidants include superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) (Ma et al., 2011; Mostafa et al., 2011). The non-enzymatic antioxidants are generally small molecules, such as AsA, glutathione, carotenoid and tocopherol (Smirnoff and Wheeler, 2000).

Eggplant (*Solanum melongena* L.) is an agronomically important vegetable, and is cultivated and consumed in many countries (Cericola et al., 2014). In the practical production, weak light condition is frequently encountered. However, there have been no reports regarding the biosynthesis and regulation of AsA by light in eggplant. In this study, seven cDNA clones which involved in AsA biosynthesis were isolated from eggplant 'Lanshan Hexian', including *SmGMP*, *SmGME1*, *SmGME2*, *SmGGP*, *SmGPP*, *SmGalDH* and *SmGLDH*. Subsequently, the expression profiles of these AsA biosynthesis genes were investigated in different tissues and bagging treatment in the peel of eggplant. Then AsA content was analyzed to uncover the relationship with the structural genes expression. In addition, an investigation on the promoter structures of the seven genes was conferred. Furthermore, the seven genes were transformed into *Arabidopsis* Col-0 to verify the function of these genes, and the effects of increased AsA accumulation in transgenic *Arabidopsis* plants on tolerance to low temperature stress were investigated.

2. Materials and methods

2.1. Plant materials and treatments

The eggplant (*Solanum melongena* L.) cultivar 'Lanshan Hexian' was grown in the greenhouse of Shanghai Jiao Tong University, Shanghai, China. Throughout the experiments, the mean light intensity was 390 μmol m^{-2} s^{-1}, and the temperature was 15–27 °C with a relative humidity of approximately 70%. Young leaves from fifteen-day-old seedlings were collected for gene isolation. Tissues from roots, stems, leaves, petals, peel and flesh from the same plants were collected at the 14 days after flowering for determining tissue-specific gene expression. The fruit bagging experiment included three groups: (a) one group was bagged after the full bloom, and the bagging was continuous; (b) another group was bagged the same but for 14 days and on day 14 the bag was removed at 8:00 am which was considered time 0 of sampling and peel was collected at 0, 0.5, 4 and 8 h from 8:00 am; (C) third group was never encapsulated. The peel of one and third groups was collected on day 14 at 12:00 am. The bags were completely light-impermeable. All eggplants in a same treatment have the same degree of development and the same range of irradiance. Samples were immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Genomic DNA and Total RNA isolation

Genomic DNA and total RNA was extracted from 300 mg of each tissue with Genomic DNA Isolation Kit and Total RNA Isolation Kit (Sangon, China) following the manufacturer's instructions. The qualities and concentrations of DNA and RNA were determined by agarose gel electrophoresis and NanoDrop 2000 spectrophotometer (Thermo, USA), respectively. First strand cDNA was synthesized using *Primescript II* 1st strand cDNA synthesis Kit (Takara, Japan).

2.3. Gene isolation and sequence analysis

In order to isolate $_{L}$-galactose pathway-related genes from eggplant, we designed degenerate primers based on the conserved cDNA region from other plant species (Table S1). The PCR products were cloned into pMD19-T Vector (Takara) and sequenced by the Invitrogen Company (Shanghai, China).

According to cDNA sequence, primers were designed to obtain the genomic DNA sequence (Table S1). The amplification conditions were as follows: 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 5 min at 72 °C, and a final extension for 15 min at 72 °C. Then PCR products were cloned into pMD19-T vector and sequenced.

Homology searches were performed using the basic local alignment search tool (BLAST) from NCBI (http://www.ncbi.nlm.nih.gov/). The conserved domain was predicted by CDD software (http://www.ncbi.nlm.nih.gov/cdd), and theoretical isoelectric point (pI) and molecular mass were predicted on the ExPASy website (http://www.expasy.org/tools). Multiple sequence alignments were performed using DNAman 6.0 (Lynnon Biosoft, Canada). Then the phylogenetic trees were constructed by MEGA 6.0, as a consensus of 1000 bootstrap replicates by the Neighbor-Joining method (Tamura et al., 2013).

2.4. Expression analysis

qRT-PCR was used to analyse the gene expression patterns. Total RNA was treated with DNase to remove traces of DNA. 1 μg RNA was synthesized into cDNA with the PrimeScript RT Master Mix Perfect Real Time Kit (Takara). According to the manufacturer's instructions of SYBR Premix Ex Taq II Kit (Takara), qRT-PCR was performed on FTC-3000 real-time PCR System (Funglyn Biotech, Canada) using the following program: 95 °C for 30 s, follow ed by 40 cycles of 95 °C for 20 s, 58 °C for 30 s, and 72 °C for 30 s. The *Actin* gene (GU984779.1) from eggplant was amplified in parallel as an internal reference gene (Liu et al., 2012). The relative expression levels of the amplified products were analyzed using the comparative C_T method based on C_T values (Livak and Schmittgen, 2001).

2.5. Promoter isolation and cis-acting elements analysis

Promoter sequences of seven AsA biosynthesis structural genes were cloned using specific primers (Table S1) with a genome walking kit (Takara). The *cis*-acting elements were predicted using PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) databases.

2.6. Agrobacterium-mediated transformation of Arabidopsis and low temperature stress treatment

The full length ORF of each gene was subcloned into pCAMBIA1302 vector under the control of CaMV35S promoter. Transformation of *Arabidopsis thaliana* (ecotype Col-0) was carried out by floral dip method (Clough and Bent, 1998). Seeds from each surviving T_1 plant were harvested individually. The T_2 seeds were placed on 1/2 strength MS agar plates containing 50 mg l^{-1} hygromycin, and the transgenic lines with a 3:1 segregation ratio (resistant:sensitive) were selected to