



Research article

Ascorbic acid formation and profiling of genes expressed in its synthesis and recycling in apple leaves of different ages

Mingjun Li, Fengwang Ma*, Chunmiao Guo, Jun Liu

College of Horticulture, Northwest A&F University, Yangling, Shaanxi 712100, China

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ABSTRACT

Ascorbic acid (AsA), as a unique antioxidant and enzyme cofactor, has multiple roles in plants. However, there is very limited information on the mechanism of AsA accumulation and controlling in leaves. In this study, we determined AsA accumulation levels, analyzed expression patterns of the genes involved in synthesizing via L-galactose pathway and recycling as well as enzyme activities in apple (*Malus domestica* Borkh) leaves with different age. AsA content was found to increase with leaf development, reaching the highest level in 20-day-old leaves. This level was maintained in mature leaves until the dropping in senescent leaves. Comparing with young and senescent leaves, mature leaves had higher capability for AsA synthesis with high expression levels and activity of L-galactose dehydrogenase and L-galactono-1,4-lactone dehydrogenase. The mRNA expression of genes involved in AsA synthesis also showed highest abundance in 20-day-old leaves, though GDP-mannose-3',5'-epimerase and L-galactose-1-phosphate phosphatase expression reached the highest levels before 20 days old. These results suggest that AsA accumulation in apple leaves mainly occurs during the transition phase from young to mature leaves with high rates of synthesis and recycling, and that L-galactose-1-phosphate phosphatase could play an important role in regulating AsA biosynthesis via the L-galactose pathway.

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1. Introduction

L-Ascorbic acid (AsA), also known as vitamin C or ascorbate, is important for all living eukaryotic cells. It is also well known that AsA, as the most abundant water-soluble antioxidant in higher plants, is involved in the detoxification of reactive oxygen species and has an important role in resistance to a number of environmental stresses, such as pathogen infection [33], hypoxia stress [15], high light and UV-B radiation [39]. In addition, AsA also participates in the regulation of several fundamental cellular processes such as photo-protection, the cell cycle and cell expansion [11,34]. Although the roles of AsA have been well documented, the mechanism by

which AsA levels are controlled during plant developmental processes is not clearly established.

AsA biosynthesis is a main source of AsA accumulation in plant cells. Since the 1960s, the AsA synthesis pathway in animals has been completely characterized [7]. It involves D-glucose as the initial precursor and the last step is catalysed by an L-gulonono-1,4-lactone oxidase, which oxidizes L-gulonono-1,4-lactone (L-GulL) to produce AsA. In plants, the AsA synthesis pathway is different from animals and has remained unknown until recently, when convincing evidence in support of a novel pathway was established [36]. AsA can be synthesized from D-mannose-1-phosphate via GDP-mannose and GDP-L-galactose (GDP-L-Gal). Free L-Gal is released from GDP-L-Gal via the action of GDP-L-Gal phosphorylase (GGP) [13] and L-Gal-1-phosphate phosphatase (GPP) [9], and then oxidized by L-Gal dehydrogenase (GalDH) to form L-galactono-1,4-lactone (L-GalL) [36]. L-GalL is oxidized to AsA by L-galactono-1,4-lactone dehydrogenase (GalLDH) (Fig. 1). To date, phenotypic analysis of mutant or transgenic plants have provided strong evidence for operation of the L-galactose or Smirnoff–Wheeler pathway as a major AsA biosynthetic route in plants (Fig. 1) [9,10,13,17,25], while other suggested pathways [18] are lacking in strong evidence. Although the L-galactose pathway appeared as the most relevant so far, the galacturonic or myoinositol pathways could be relevant in other species still uncharacterised.

Abbreviations: APX, ascorbate peroxidase; AsA, reduced ascorbic acid; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; L-Gal, L-galactose; GalDH, L-galactose dehydrogenase; L-GalL, L-galactono-1,4-lactone; GalLDH, L-galactono-1,4-lactone dehydrogenase; D-GalUA, D-galacturonic acid; GGP, GDP-L-galactose-1-phosphate phosphorylase; GME, GDP-mannose-3',5'-epimerase; GMP, GDP-mannose pyrophosphorylase; GPP, L-galactose-1-phosphate phosphatase; D-GluA, D-glucuronic acid; D-Glc, D-glucose; L-GulL, L-gulonono-1,4-lactone; H₂O₂, hydrogen peroxide; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; MI, myoinositol; PVP, polyvinylpyrrolidone; T-AsA, total ascorbic acid (AsA + DHA); T-GSH, total glutathione (GSH + GSSG).

* Corresponding author. Tel./fax: +86 29 87082648.

E-mail addresses: fwm64@sina.com, fwm64@nwsuaf.edu.cn (F. Ma).

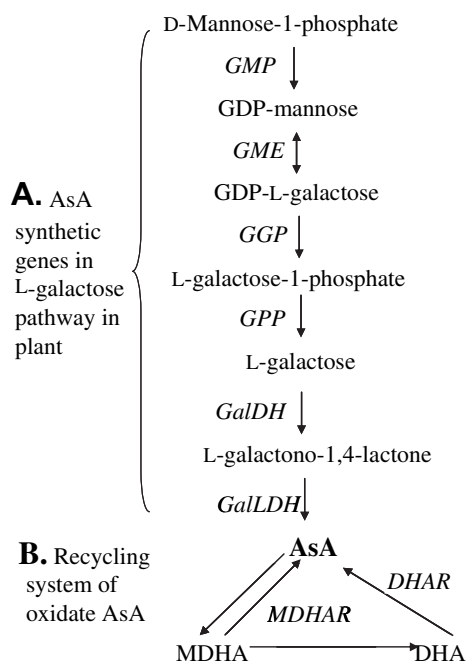


Fig. 1. The main AsA synthetic (A) and recycling pathway (B) (Hancock et al., 2005; Linster et al., 2007) in plants. GMP, GDP-mannose pyrophosphorylase; GME, GDP-mannose-3',5'-epimerase; GGP, GDP-L-galactose-1-phosphate phosphorylase; GPP, L-galactose-1-phosphate phosphatase; GalDH, L-galactose dehydrogenase; GalLDH, L-galactono-1,4-lactone dehydrogenase; AsA, ascorbic acid; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase.

AsA is not a stable metabolic product and can be oxidized to the monodehydroascorbate radical (MDHA) and dehydroascorbate (DHA), while AsA functions as a major antioxidant to scavenge reactive oxygen species [18]. The resulting MDHA and DHA can be enzymatically reduced to AsA by monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and dehydroascorbate reductase (DHAR, EC 1.8.5.1), respectively [11]. The importance of MDHAR and DHAR in controlling AsA levels in plant tissues has been demonstrated in transgenic plants by overexpressing these two enzymes [8,14]. This is judged to have led to a huge increase of AsA content through improving the rate of AsA turnover.

In plant cells, AsA content is modulated by both its synthesis and oxidation loss, and can be affected by a great number of factors, either internal or external to plants [18]. These include developmental processes [1,6,12,20], light conditions [23,39] and oxidative stress [12,20]. Although AsA biosynthesis and metabolism processes in plants have been studied deeply, especially in photosynthetic tissues [18,21], very little is known regarding the molecular mechanisms involved in the perception and/or transduction of internal or external signals in regulation of AsA levels. Identification of biosynthetic routes along with the cloning and expression of genes involved in AsA synthesis and recycling could bring new insights into control mechanisms. Lately, some attentions [1,6,20] were given to expression profiling of AsA-related genes in fruits which are a major source of dietary vitamin C for humans. But no such information is available for leaves, where AsA is most commonly used in plants. It is not clear when AsA accumulates in leaves and how AsA content is controlled during leaf development.

Apple (*Malus domestica* Borkh) is one of the most economically important fruit crops grown worldwide. Apple leaves generally maintain their photosynthetic function for several months after leaf expansion. They can be divided into several growth stages according to the period of growth and physiological data. To understand the

regulatory mechanisms of AsA accumulation, we systematically investigated AsA levels, enzyme activities and mRNA expression of genes involved in AsA biosynthesis and recycling in leaves of different ages. Our results should provide useful information on the regulatory mechanisms of AsA accumulation in photosynthetic organs and breeding programs aiming at the improvement of AsA levels in plants.

2. Materials and methods

2.1. Plant material

Leaves were harvested from 2-year-old apple (*M. domestica* Borkh. cv. Gala) plants grafted onto rootstock *Malus sieversii* (Ledeb.) Roem. The plants had no lateral branches, so all the leaves were well-exposed to sunlight during growth. Plants were grown at a spacing of 0.5 × 0.6 m in pots (30 × 26.5 × 22 cm) with a mixture of local sandy top soil, humus and composted manure (5:1:1 (v/v)) at the experimental orchard of the Northwest A & F University, Yangling (34°20'N, 108°24'E), China. Standard horticultural practices were used for disease and pest control.

The first folded-leaf at the tip of a growing shoot was designated as 1-day-old. On April 16th, 2008 (when shoots had at least grown out of the 6th leaf), then 1-day-old leaves were marked with marking continuing at about 5-day intervals until shoots had almost stopped growing by September 15th. During 5–6 pm of September 15th (a sunny day), 1-, 5-, 11-, 20-, 31-, 40-, 105-, 125- and 152-day-old leaves, respectively, were harvested at the same time. On June 1st, most shoots began to stop spring growth. From the end of spring shoot growth to the beginning of autumn shoot growth (August), plants did not produce good leaves, so no new leaves were harvested during this period. Each time point was designed five replications from 10 individual trees at least. The samples were immediately frozen in liquid nitrogen and then stored at –72 °C until analysis of AsA content, enzyme activity and mRNA expression of genes. Another set of samples was at the same time to measure leaf area, chlorophyll content and chlorophyll fluorescence.

2.2. Assay of AsA levels

Samples (0.5 g) were homogenized in 7 mL of ice-cold 6% (v/v) HClO₄ and centrifuged at 12,000 × g for 20 min at 2 °C. Hepes buffer (0.1 M, pH 7.0) was added at a ratio of 1:5 (buffer: extract v/v) to the extracts and K₂CO₃ (5 M) was then added until its pH reached 5.6. The extracts were centrifuged again at 12,000 × g for 2 min to allow the removal of precipitated K₂ClO₄, and supernatants were then used to assay AsA and DHA, as described by Logan and coworkers [27]. The assay was based on oxidation of AsA by ascorbate oxidase in an acidic solution. AsA was calculated as the difference in absorbance at 265 nm before and after the addition of ascorbate oxidase. AsA concentration was quantified by comparison of difference of absorbance relative to a standard curve.

2.3. AsA biosynthesis by feeding with candidate precursors

Young (5 days old), mature (36 days old) and senescent leaves (152 days old) were used following the procedure of Davey et al. [12]. Leaf discs were pre-incubated for 1 h in an aerated buffer consisting of 25 mM MES (pH 5.5), 300 mM mannitol, 5 mM MgCl₂, 2 mM KCl, 1 mM CaCl₂ and 1 mM CaSO₄ in Petri dishes. These were subject to rotary shaking at 100 rpm for 1 h. Then precursors was added, including D-glucose (D-Glc), L-galactono-1,4-lactone (L-GalL), L-galactose (L-Gal), L-gulono-1,4-lactone (L-GulL), D-glucuronic acid (D-GluA), D-galacturonic acid (D-GalUA) and myo-inositol (MI), to achieve a final concentration of 5 mM. Sucrose was used as the

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