Ascorbic Acid Accumulation and Expression of Genes Involved in Its Biosynthesis and Recycling in Developing Apple Fruit

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ABSTRACT. The objective of this study was to investigate ascorbic acid (AsA) accumulation, mRNA expression of genes involved in AsA biosynthesis as well as recycling, activity of key enzymes, and the relationship of them to AsA levels during the development of apple fruit (*Malus ×domestica* cv. Gala). AsA concentration, which mainly depends on biosynthesis, was the highest in young fruit post-anthesis and then decreased steadily toward maturation. However, AsA continued to accumulate over time because of the increase in fruit mass. Transcript levels of guanosine diphosphate (GDP)-L-galactose phosphorylase, GDP-mannose pyrophosphorylase, D-galacturonate reductase, and the post-transcriptionally regulated L-galactono-1,4-lactone dehydrogenase were not correlated with AsA accumulation in apple. In contrast, patterns of expression for L-galactose dehydrogenase, L-galactose-1-phosphate phosphatase, and GDP-mannose-3',5'-epimerase showed a pattern of change similar to that of AsA accumulation. Although activities and expression levels of monodehydroascorbate reductase and dehydroascorbate reductase in fruit, which had less capacity for AsA recycling, were much lower than in leaves, they were not clearly correlated with AsA level during fruit development.

AsA, also known as vitamin C or ascorbate, is important for all living eukaryotic cells. AsA, the most abundant water-soluble antioxidant in higher plants, is involved in the detoxification of reactive oxygen species and has an important role in promoting resistance to numerous environmental stresses such as ozone (Sanmartin et al., 2003), water stress (Wang et al., 2010), excess light (Yabuta et al., 2007), and pathogen infection (Pavet et al., 2005). It also participates in the regulation of several fundamental cellular processes [e.g., photoprotection, the cell cycle, and cell expansion (Davey et al., 2000)]. Moreover, a loss of the ability to synthesize AsA has occurred in primates and some other animals, and humans must secure it through dietary uptake of vitamin C, primarily through fruits and vegetables.

Although several biosynthesis pathways have been proposed in plants (Agius et al., 2003; Lorence et al., 2004; Wheeler et al., 1998; Wolucka and van Montagu, 2003), the most widely accepted route is through the L-galactose or Smirnoff–Wheeler pathway (Hancock and Viola, 2005; Wheeler et al., 1998), in which AsA can be synthesized from D-mannose-1-phosphate

through GDP-mannose and GDP-L-galactose (GDP-L-Gal). Free L-Gal is released from GDP-L-Gal through the action of GDP-L-Gal phosphorylase [GGP [Enzyme Commission number (EC) 2.7.7.69]; Dowdle et al., 2007] and L-Gal-1-phosphate phosphatase [GPP (EC 3.1.3.25); Conklin et al., 2006] and is then oxidized by L-Gal dehydrogenase [GalDH (EC 1.1.1.117)] to form L-galactono-1,4-lactone (L-GalL; Wheeler et al., 1998). L-GalL is oxidized to AsA by L-galactono-1,4-lactone dehydrogenase [GalLDH (EC 1.3.2.3)].

Phenotypic analyses of mutant or transgenic plants have provided strong supporting evidence for the operation of the L-galactose pathway as a main AsA biosynthetic route in plants (Conklin et al., 1999, 2006; Dowdle et al., 2007; Gatzek et al., 2002; Linster et al., 2007; Tabata et al., 2001), whereas several alternative AsA biosynthetic pathways have been proposed. These include the D-galacturonic acid pathway that uses D-galacturonic acid for the synthesis of L-galactonic intermediates by D-galacturonate reductase [GalUR (EC 1.1.1.203)] and also requires GalLDH to make AsA a last step (Agius et al., 2003), L-gulose pathway that uses L-gulonic intermediates and it is a branch of the L-galactose pathway (Wolucka and van Montagu, 2003), and the myo-inositol pathway that synthesizes L-gulonic intermediates from myo-inositol (Lorence et al., 2004). These pathways could act by cooperating with the L-galactose pathway or working in different tissues or physiological conditions.

AsA is not a stable metabolic product and can be oxidized to the monodehydroascorbate radical (MDHA) and dehydroascorbate

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(DHA), functioning as a major antioxidant to scavenge reactive oxygen species or as enzymatic cofactors (Noctor and Foyer, 1998). The resulting MDHA and DHA can be enzymatically reduced to AsA by NADPH- or NADH-dependent monodehydroascorbate reductase [MDHAR (EC 1.6.5.4)] and dehydroascorbate reductase [DHAR (EC 1.8.5.1)], respectively (Noctor and Foyer, 1998). The importance of MDHAR and DHAR in controlling AsA levels has been demonstrated in transgenic plants by expressing enzymes involved in the recycling of oxidized AsA, including DHAR (Chen et al., 2003) and MDHAR (Eltayeb et al., 2007).

The amount of AsA in plant cells is mainly modulated by a balance between its synthesis and oxidation loss and can be affected by both internal and external factors. These include developmental processes (Badejo et al., 2009; Bulley et al., 2009; Davey et al., 2004; Imai et al., 2009; Ioannidi et al., 2009; Li et al., 2010), lighting conditions (Cheng and Ma, 2004; Li et al., 2008; Yabuta et al., 2007), and oxidative stress (Davey et al., 2004; Ioannidi et al., 2009). Although its biosynthesis and metabolism processes in plants have been studied in depth, especially in photosynthetic tissues, little is known about the molecular mechanisms involved in the perception and/or transduction of those signals to regulate AsA levels, particularly in sink organs. Expression profiling of AsA-related genes have been conducted in fruit (Badejo et al., 2009; Bulley et al., 2009; Imai et al., 2009; Ioannidi et al., 2009). However, our understanding of the mechanisms that control AsA levels in plants remains quite limited, especially the regulation of AsA accumulations and amounts in sink organs (e.g., fruit).

Apple is one of the most economically important fruit worldwide. Compared with others such as kiwifruit (*Actinidia* spp.), blackcurrant (*Ribes nigrum*), or strawberry (*Fragaria × ananassa*), AsA concentrations in apple are quite low, although a wide variety occurs among different genotypes (Planchon et al., 2004). Davey et al. (2004) have reported that apple seeds are capable of AsA biosynthesis but fruit tissues are not. In contrast, Razavi et al. (2005) have shown that apple fruit (at least young fruit) can synthesize AsA. Likewise, our previous experiments with the feeding of synthetic precursors have demonstrated that the peel and flesh from the 'Gala' apple are able to produce AsA through the L-galactose pathway (Li et al., 2008). We have also detected the expression and activities of GalDH and GalLDH—two key enzymes in AsA biosynthesis—in apple fruit (Li et al., 2009).

Nevertheless, our understanding is limited about the mechanism for regulating AsA accumulation and levels in fruit. To gain new insight into that process in apple, we systematically investigated here the accumulation of AsA, mRNA expression of genes involved in its biosynthesis as well as recycling, and the relationships among gene expression, enzyme activity, and AsA levels during fruit development. Our objective was to provide useful information to breeding programs that focus on improving AsA concentration in apple fruit and to elucidate the mechanisms that regulate AsA accumulation in plant cells.

Material and Methods

PLANT MATERIALS. Eight-year-old 'Gala' apple trees grafted onto rootstock *Malus sieversii* were trained as a central leader system and grown at a density of 2 × 4 m in an experimental orchard at the Horticultural Experimental Station of Northwest A & F University, Yangling, China (lat. 34°20' N, long.

108°24' E). Fruit samples were harvested at 15 ± 2-d intervals after anthesis at 1600 and 1700 hr. The ovaries that the flower had separated from were designed as 0 days after anthesis (DAA) fruit. Fruit samples were gathered from the sunny outside (south-facing) of the canopy top. Mature leaves located south-facing of the canopy top also were collected at each time point. On each collection date, six apples, as one replication, were harvested from three trees, and five replications were done, respectively. Samples were directly taken from the fruit using a 0.5-cm-diameter cork borer in the transverse center of fruit, weighed and immediately frozen in liquid nitrogen, and then stored at -80 °C until use. For the final harvest, at 120 DAA, the peel and flesh samples were directly sliced from the sunny side of fruit using a knife.

ASSAYS OF ASCORBIC ACID. Samples of skin or flesh (3 g each) or leaves (0.5 g) were homogenized in 8 mL of ice-cold 6% (v/v) HClO₄ and centrifuged at 12,000 g_n for 20 min at 2 °C. Hepes buffer (0.1 M; pH 7.0) was added to the extracts at a 1 buffer:5 extract ratio (v:v); K₂CO₃ (5 M) was then added in until the pH reached 5.6. Extracts were centrifuged again at 12,000 g_n for 2 min to allow the removal of precipitated KClO₄, and the supernatants were used to assay AsA and DHA as described by Cheng and Ma (2004). That assay is based on the oxidation of AsA by ascorbate oxidase in an acidic solution. AsA was calculated as the difference in absorption at 265 nm before and after the addition of ascorbate oxidase using a 1-cm-path-length spectrophotometer (ultraviolet-2550; Shimadzu, Kyoto, Japan). AsA concentration was quantified by comparing the difference in absorption relative to a standard curve.

MRNA EXPRESSION ANALYSIS. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was used to analyze expression of the genes involved in AsA biosynthesis and recycling. These included *GalLDH* (GenBank accession number FJ752244), *GalDH* (GQ131419), *GPP* (FJ752240), *GMP* (FJ752238), *GME* (FJ752242), *GMP* (GQ149071), *GalUR* (CN907651), *MDHAR* (FJ752239), *DHAR1* (DQ322706), and *DHAR2* (FJ752243). Total RNA was extracted from samples by the modified CTAB method (Gasic et al., 2004), and DNase was used to clean out DNA before reverse transcription. Gene-specific primers, as used by Li et al. (2010), were designed from the coding sequences of apple genes. qRT-PCR was performed with a PrimeScriptTMRT Reagent Kit (Takara, Dalian, China), plus oligo(dT)20 and random primers for cDNA synthesis, according to the manufacturer's protocol. The amplified PCR products were quantified by an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with the SYBR Premix Ex Taq kit (Takara). *Actin* (CN938023) transcripts were used to standardize the different gene cDNA samples. For all samples, four tubes of total RNA were respectively extracted from four replications and then mixed in a tube used to reverse transcription. qRT-PCR experiments were done with four technical replications. The data were analyzed using the ddCT method in iQ5 2.0 standard optical system analysis software (Bio-Rad Laboratories).

ASSAY OF L-GALACTONO-1,4-LACTONE DEHYDROGENASE ACTIVITY. Crude GalLDH (EC 1.3.2.3) enzyme extract was prepared according to the method of Ôba et al. (1995). The samples (3 g of peel or flesh; 1 g of leaves) were homogenized in 10 mL of 0.1 M potassium phosphate buffer (pH 7.5) containing 0.4 M sucrose, 10% (v/v) glycerol, 0.1 mM phenylmethanesulfonyl fluoride, 0.5% (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO), 0.3% (v/v) mercaptoethanol, and 3% (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 500 g_n

for 10 min at 2 °C to remove chloroplast and cell debris, and the supernatant was centrifuged again at 16,000 g_n for 30 min at 2 °C to obtain the pellet. Then the pellet was suspended in 2 mL of 0.1 M phosphate buffer (pH 8.0) that contained 5 mM glutathione, 1 mM EDTA, and 10% (v/v) glycerol. This suspended solution was again centrifuged at 2000 g_n for 10 min at 2 °C, and the supernatant, as crude mitochondria protein, was used to determine the activity of GalLDH after cytochrome c was reduced. A reaction mixture (1.5 mL), containing 60 μ M of cytochrome c, 1 mM sodium azide, 2.5 mM L-GalL, 0.1% (v/v) Triton X-100, and 0.1 mL of the enzyme extract in 50 mM Tris-HCl (pH 8.5), was pre-incubated at 27 °C for 5 min. Subsequently, reduction of cytochrome c was monitored by the increase in absorption at 550 nm using an ultraviolet-2550 spectrophotometer. Each replication measured at least twice. One unit of enzyme was defined as the reduction of 1 μ mol of cytochrome c per minute.

ASSAY OF L-GALACTOSE DEHYDROGENASE ACTIVITY. GalDH (EC 1.1.1.117) activity was assayed according to a method described by Gatzek et al. (2002) with some modifications. The samples (2 g of peel or flesh; 0.6 g of leaves) were extracted on ice in 8 mL of 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 mM phenylmethanesulfonyl fluoride, 0.5% (v/v) Triton X-100, 0.2% (v/v) 2-mercaptoethanol, and 2% (w/v) PVP. The homogenates were centrifuged at 16,000 g_n for 20 min at 2 °C and the supernatants were collected as enzyme extracts. A reaction mixture (1.5 mL), containing 0.5 mM NAD⁺, 1 mM L-Gal, and the supernatant (0.1 mL) in 100 mM Tris-HCl (pH 8.0), was pre-incubated at 27 °C for 2 min. Subsequently, reduction of NAD⁺ was monitored by the increase in absorption at 340 nm. Each replication measured at least twice. Activity was calculated in terms of micromoles of NAD⁺ reduced per minute.

ASSAY FOR ENZYME ACTIVITIES OF DEHYDROASCORBATE REDUCTASE AND MONODEHYDROASCORBATE REDUCTASE. DHAR (EC 1.8.5.1) and MDHAR (EC 1.6.5.4) were assayed using the method described by Cheng and Ma (2004). The samples (2 g of peel or flesh; 0.6 g of leaves) were extracted with 8 mL of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM DTT, 0.5% (v/v) Triton X-100, and 2% (w/v) PVP. The homogenates were centrifuged at 16,000 g_n for 20 min at 4 °C and the supernatants were collected to assay for enzyme activity. Protein measurements were performed according to the procedure of Bradford (1976) using bovine serum albumin as standard. MDHAR activity was assayed at 340 nm in a 3 mL reaction mixture containing 50 mM HEPES-KOH (pH 7.6), 0.1 mM NADH, 0.25 mM AsA, 0.25 units AsA oxidase, and 0.1 mL of the supernatants. The reaction was initiated by adding AsA oxidase. DHAR activity was measured at 265 nm in a 3 mL assay solution containing 100 mM HEPES-KOH (pH 7.0), 1 mM EDTA, 2.5 mM GSH, 0.2 mM DHA, and 0.1 mL of the supernatants. The reaction was initiated by adding DHA. Each replication measured twice at least. MDHAR activity was calculated in terms of micromoles of NADH oxidized per minute and DHAR activity as micromoles of AsA produced per minute.

STATISTICAL ANALYSIS. For each harvest, sample was replicated at least five times with each replication measured twice, except qRT-

PCR. Data were subjected to analysis of variance using SAS (Version 9.1; SAS Institute, Cary, NC). Significant differences of AsA levels and enzyme activities were calculated by a Duncan's test at $P < 0.05$.

Results

CHANGES IN ASCORBIC ACID LEVELS DURING FRUIT DEVELOPMENT OF APPLE. AsA levels for 'Gala' apple were monitored from young fruit (0 DAA) up to maturity. Based on fresh weights, fruit at 0 DAA had the highest concentrations of total AsA (AsA + DHA, T-AsA), DHA, and AsA, but those levels decreased steadily to approximately one-third of initial values before 75 DAA (Fig. 1A). In contrast, AsA/DHA had a clear increase after 60 DAA and averaged 2.7 to 4.3 (data not shown). After that time point, their contents remained nearly constant until maturity. The greatest rate of accumulation for T-AsA and AsA occurred from 75 to 105 DAA, rising steadily toward maturation as a result of increasing fruit mass (Fig. 1B).

RELATIVE MRNA EXPRESSION OF ASCORBIC ACID SYNTHESIS GENES DURING FRUIT DEVELOPMENT. qRT-PCR was used to investigate the degree of mRNA expression by *GalLDH*, *GalDH*, *GPP*, *GMP*, *GME*, *GMP*, and *GalUR* during fruit development with *Actin* serving as our internal standard. Relative expression levels for *GalLDH* were higher and showed little change before 30 DAA (Fig. 2A), thus reflecting the high amount of AsA in young fruit that then decreased until 75 DAA. The mRNA expression abundance of *GalDH* increased to the highest level in 30 DAA fruit followed by a obvious decline at 45 and 60 DAA before remaining largely unchanged as the fruit ripened (Fig. 2A). Compared with the highest peak in 0 DAA fruit, the relative mRNA expression level of *GPP* showed a clear decline at 15 DAA, and no apparent change was observed throughout maturation (Fig. 2B). Transcript levels for *GMP* rose slightly over time with a temporary drop at 60 DAA but then a more dramatic decrease at 105 and 120 DAA than at 90 DAA (Fig. 2B). Expression of *GME* had an overall pattern similar to *GPP*, being lower at 15 DAA than at 0 DAA (Fig. 2C). *GMP* transcripts reached the highest level at 60 DAA followed by a clear decline toward 105 DAA. The relative mRNA expression of *GalUR* was the highest at 0 DAA and then greatly decreased at 15 DAA where it remained except for temporary recoveries at 75 and 90 DAA (Fig. 2C). However, with the

Fig. 1. Changes in ascorbic acid (AsA) accumulation levels during fruit development. (A) Total AsA (T-AsA), AsA, and dehydroascorbate (DHA) concentrations based on fresh weight of fruit; (B) T-AsA and AsA contents per fruit. Average fresh weights per fruit were determined by five replications with six fruit. T-AsA and AsA contents per fruit were obtained by multiply average fruit weight by their concentration based on fresh weight, respectively. Values of AsA concentration and fresh weight are means of five replicates $\pm$ sd.

Fig. 2. Changes in relative mRNA expression for genes encoding enzymes involved in ascorbic acid synthesis and recycling during fruit development. (A) L-galactono-1,4-lactone dehydrogenase (*GalLDH*) and L-galactose dehydrogenase (*GalDH*); (B) L-galactose-1-phosphate phosphatase (*GPP*) and GDP-L-galactose phosphorylase (*GGP*); (C) GDP-mannose-3',5'-epimerase (*GME*), GDP-mannose pyrophosphorylase (*GMP*), and D-galacturonate reductase (*GalUR*); (D) monodehydroascorbate reductase (*MDHAR*), dehydroascorbate reductase 1 (*DHAR1*), and dehydroascorbate reductase 2 (*DHAR2*). Fruit samples were harvested at 15 $\pm$ 2-d intervals after anthesis at 1600 and 1700 hr. The ovaries that the flower had separated from were designed as 0 DAA (days after anthesis) fruit. Quantitative reverse transcription-polymerase chain reaction was performed with specific primers designed from the coding sequences of apple. For each sample, transcript levels were normalized with those of *Actin*, and the relative expression levels of each gene were obtained using the ddCT method during development, whereas expression at 0 DAA was designated as "1." Values are means of at least three replicates $\pm$ sd.

exception of *GME* and *GalUR* expression, changes were less than twofold throughout fruit development.

Relative expression of *MDHAR* mRNA had its highest peak in 0 DAA fruit followed by a marked decline at 15 DAA and then a slower decrease until 75 DAA (Fig. 2D). The overall expression pattern for *DHAR1* slightly decreased during this developmental period. The transcript level of *DHAR2* increased obviously after anthesis, reaching its highest level at 45 DAA (Fig. 2D). This was followed by an evident drop at 75 DAA, but then no further changes were apparent until maturity.

CHANGES IN L-GALACTONO-1,4-LACTONE DEHYDROGENASE, L-GALACTOSE DEHYDROGENASE, MONODEHYDROASCORBATE REDUCTASE, AND DEHYDROASCORBATE REDUCTASE ACTIVITIES DURING FRUIT DEVELOPMENT. *GalLDH* activities showed a similar developmental pattern as that of relative mRNA expression levels (Figs. 2 and 3). From its highest peak at 30 DAA, *GalLDH* activity (based on fresh weight) decreased gradually at 45 and 60 DAA before slowing toward maturity. Its activity (based on protein content) had a clearer peak at 30 DAA than did values that had been based on fresh weight (Fig. 3A). No clear difference in *GalDH* activity, based on fresh weight, was found among fruit at 0 and 15 DAA, and such activity decreased gradually over time toward 105 DAA (Fig. 3B).

Similar to our observations for *GalDH*, *MDHAR* activities (based on fresh weight) declined gradually from 30 to 75 DAA but showed no further changes during ripening. In contrast, when

MDHAR activity was based on protein, the highest level was achieved at 30 DAA followed by a slower decrease (Fig. 3C). *DHAR* activity (based on fresh weight) also peaked at 30 DAA and then gradually decreased toward maturity (Fig. 3D). In contrast, when activity was based on protein, no marked differences were found from 30 to 120 DAA.

DIFFERENCES BETWEEN FRUIT AND LEAVES FOR RELATIVE mRNA EXPRESSION OF GENES INVOLVED IN SYNTHESIZING AND RECYCLING ASCORBIC ACID. To learn how AsA synthesis is controlled in apple, AsA levels in mature leaves and in the flesh and peel of mature fruit were compared based on mRNA expression levels. T-AsA and AsA concentrations in the flesh were 25.0% of that in the peel and $\approx$ 2.5% of that in the leaves, but the leaves had a higher ratio of AsA/DHA than did the other tissue types (Table 1).

Compared with the flesh, the peel had noticeably greater expression for the genes investigated here with mRNA abundances of *GGP* and *GME* being fourfold higher (Table 2). In the leaves, levels of *GME* and *GGP* were 15.40- and 7.69-fold higher than in the flesh, and *GalDH* and *GPP* were also clearly higher than in the flesh. The two major exceptions in the leaves were *GalLDH* and *GMP* in the L-galactose pathway, having approximately half and no clear difference compared with those in the flesh, respectively. *GalLDH*, *GMP*, and *GalUR* also had the greatest expression in the peel. Transcript levels for the *MDHAR* and *DHAR* were higher in the leaves than in the flesh; differences in expression abundance between leaves and flesh were greater for *DHAR1* than for *DHAR2*.

Similar to our findings for patterns of change in AsA levels, the activities of enzymes, including *GalLDH*, *GalDH*, *MDHAR*, and *DHAR*, were greater in the peel than in the flesh, but values for the leaves were still higher, being $\approx$ 10.40-, 12.79-, 31.70-, and 29.80-fold greater, respectively, than those found in the other tissue types (Table 3).

Discussion

AsA concentration in plant cells is highly regulated by developmental processes. This regulation is distinct for different genotypes, tissues, or cells. Davey et al. (2004) have verified that AsA levels in apple fruit are indeed controlled by those processes. Our young fruit post-anthesis had the highest amounts of T-AsA, DHA, and AsA, but those values then decreased steadily up to maturity (Fig. 1). This suggests that, during fruit development, the rate of AsA synthesis, based on fresh weight, is less than the rate of oxidation loss, because AsA accumulation is mainly determined by the balance between synthesis and oxidation loss of DHA in plant cell (Hancock and Viola, 2005). Higher AsA concentration also has been reported in the young fruit of acerola [*Malpighia glabra* (Badejo et al., 2009)], kiwifruit (Bulley et al., 2009), and peach [*Prunus persica* (Imai et al., 2009)]. By comparison, AsA levels tend to increase over

Fig. 3. Changes in enzyme activities of (A) L-galactono-1,4-lactone dehydrogenase (GalLDH), (B) L-galactose dehydrogenase (GalDH), (C) monodehydroascorbate reductase (MDHAR), and (D) dehydroascorbate reductase (DHAR) based on protein content and fresh weight during fruit development. Fruit samples were harvested at 15 ± 2 -d intervals after anthesis at 1600 and 1700 HR. The ovaries which the flower had separated from were designed as 0 DAA (days after anthesis) fruit. Values are means of at least five replicates $\pm$ SD.

Table 1. Comparison of total ascorbic acid (T-AsA), ascorbic acid (AsA), and dehydroascorbate (DHA) concentration and AsA:DHA ratio among mature leaves and the peel and flesh of mature 'Gala' apple fruit.^z

	T-AsA concn	AsA concn	DHA concn	AsA:DHA (ratio)
	[mean $\pm$ SD ($\mu\text{mol}\cdot\text{g}^{-1}$ fresh wt)]			
Flesh	0.64 ± 0.08 c ^y	0.49 ± 0.05 c	0.15 ± 0.01 c	3.27 ± 0.36 b
Peel	1.57 ± 0.12 b	1.15 ± 0.08 b	0.42 ± 0.05 b	2.74 ± 0.22 b
Leaf	24.9 ± 2.17 a	20.9 ± 1.37 a	3.90 ± 0.41 a	5.38 ± 0.47 a

^zAll samples were harvested at 120 d after anthesis. Flesh and peel were from sun side (red side) of fruit. Values are means of five replicates.

^yDifferent letters within the same column indicate significant difference at $P < 0.05$ by Duncan's test.

time in the fruit of tomato [*Solanum lycopersicum* (Ioannidi et al., 2009)]. We found that AsA steadily accumulated up to maturity as a result of increased fruit mass, which was especially rapid between 75 to 105 DAA (Fig. 1). This pattern has also been observed in the fruit of tomato (Ioannidi et al., 2009), whereas for kiwifruit (Bulley et al., 2009) and blackcurrant (Hancock et al., 2007), such accumulations mainly occur at the young fruit stage when biosynthetic capability is greater.

Our results demonstrated that six key genes involved in AsA synthesis through the L-galactose pathway were expressed during apple fruit development and that both GalLDH and GalDH were active (Figs. 2 and 3). This further supports our earlier reports that apple fruit can synthesize AsA by itself through the L-galactose pathway (Li et al., 2008, 2009). Rokni Zadeh et al. (2007) also have detected the expression of *GalLDH*, *GalDH*, *GPP*, and *GME* in young and mature apple fruit as well as that of *GalUR* and *myo-inositol oxygenase*, which could be involved in other possible pathways. D-GalUA

in apple leaves (Davey et al., 2004) and fruit peels (Li et al., 2008) can elevate AsA concentration. However, we found that expression levels of *GalUR* were higher in leaves and peel than in flesh (Table 2), and its expression abundance was clearly related to AsA concentration at the young fruit stage. Nevertheless, at 75 DAA, the large increase in *GalUR* expression did not lead to more AsA production. In different tissues of apple plants, roots have a very low AsA concentration but the highest expression of *GalUR* (Rokni Zadeh et al., 2007).

Within the major L-galactose or Smirnoff-Wheeler pathway for AsA biosynthesis in plant cells, GalLDH catalyzes oxidation of the last precursor L-GalL to AsA, and GalLDH is required in a possible D-galacturonic acid pathway (Hancock and Viola, 2005). During apple fruit formation, we observed much greater transcription and activity of GalLDH in young fruit (Figs. 2 and 3), a finding that concurs with our earlier report (Li et al., 2008) that young fruit have a stronger ability to transmit GalL to AsA. However, here we noted that slightly increased mRNA expression of *GalLDH* in mature fruit did not lead to a change in its enzyme activity or AsA concentration (Figs. 2 and 3), and the fruit that had a very low AsA concentration and GalLDH activity showed higher expression than the leaves (Table 2). Some researchers have proposed that *GalLDH* is post-transcriptionally regulated and is not related to AsA concentration in different organs or under stress (Bartoli et al., 2005; Loscos et al., 2008).

GalDH is a key enzyme that determines whether a plant can synthesize AsA through the L-galactose pathway (Wheeler et al., 1998). Here, we found that patterns of change in its transcript and activity were correlated with AsA accumulation in developing fruit and other organs (Figs. 2 and 3; Tables 2 and 3). However, because there is a substantial accumulation of AsA after L-Gal feeding (Wheeler et al., 1998) and a lack of effect by *Arabidopsis thaliana* GalDH over-expression on AsA concentration in tobacco under natural conditions (Gatzek et al., 2002), the role of *GalDH* becomes less of a possible factor in controlling AsA synthesis through the L-galactose pathway.

During the growth and development of apple fruit, the mRNA expression abundance of *GPP*, which catalyzes L-galactose-1-P to L-Gal (Conklin et al., 2006), closely followed the trend for AsA production (Fig. 2). We also observed this among other tested tissues as well as in separate experiments with developing apple leaf (Li et al., 2010). These relationships have also been found in immature and ripening tomato fruit, suggesting that *GPP* might play an important role in regulating AsA accumulation (Ioannidi et al., 2009). Further study is necessary for identifying the functioning of *GPP* in controlling AsA synthesis, especially research that involves characterization and transformation.

Table 2. Levels of relative mRNA expression for genes encoding enzymes involved in ascorbate synthesis [L-galactono-1,4-lactone dehydrogenase (*GalLDH*); L-galactose dehydrogenase (*GalDH*); L-galactose-1-phosphate phosphatase (*GPP*); GDP-L-galactose phosphorylase (*GPP*); GDP-mannose-3',5'-epimerase (*GME*); GDP-mannose pyrophosphorylase (*GMP*); D-galacturonate reductase (*D-GalUR*)] and recycling [monodehydroascorbate reductase (*MDHAR*); dehydroascorbate reductase 1 (*DHAR1*); dehydroascorbate reductase 2 (*DHAR2*)].^z

	Relative mRNA expression (mean $\pm$ SD)				
	<i>GalLDH</i>	<i>GalDH</i>	<i>GPP</i>	<i>GPP</i>	<i>GME</i>
Flesh	1.00 $\pm$ 0.10	1.00 $\pm$ 0.13	1.00 $\pm$ 0.09	1.00 $\pm$ 0.14	1.00 $\pm$ 0.05
Peel	1.46 $\pm$ 0.08	2.02 $\pm$ 0.14	1.53 $\pm$ 0.16	5.11 $\pm$ 0.35	4.06 $\pm$ 0.28
Leaf	0.52 $\pm$ 0.05	2.86 $\pm$ 0.25	2.16 $\pm$ 0.16	7.69 $\pm$ 1.08	15.4 $\pm$ 1.77
	<i>GMP</i>	<i>GalUR</i>	<i>MDHAR</i>	<i>DHAR1</i>	<i>DHAR2</i>
Flesh	1.00 $\pm$ 0.11	1.00 $\pm$ 0.11	1.00 $\pm$ 0.08	1.00 $\pm$ 0.11	1.00 $\pm$ 0.08
Peel	1.48 $\pm$ 0.09	2.98 $\pm$ 0.16	2.13 $\pm$ 0.15	2.54 $\pm$ 0.28	1.38 $\pm$ 0.15
Leaf	1.25 $\pm$ 0.19	2.42 $\pm$ 0.13	3.43 $\pm$ 0.35	4.96 $\pm$ 0.38	1.76 $\pm$ 0.13

^zComparisons were made among mature leaves and the peel and flesh of mature 'Gala' apple fruit. All samples were harvested at 120 d after anthesis. Flesh and peel were from sun side (red side) of fruit. Quantitative reverse transcription-polymerase chain reaction was performed with specific primers designed from the coding sequences of apple. For each sample, transcript levels were normalized with those of Actin, and the relative expression levels of each gene were obtained using the ddCT method during development while expression in flesh was designated as "1." Values are means of at least three replicates.

Table 3. Differences in activities of L-galactono-1,4-lactone dehydrogenase (*GalLDH*), L-galactose dehydrogenase (*GalDH*), monodehydroascorbate reductase (*MDHAR*), and dehydroascorbate reductase (*DHAR*) among mature leaves and the peel and flesh of mature 'Gala' apple fruit.^z

	<i>GalLDH</i>	<i>GalDH</i>	<i>MDHAR</i>	<i>DHAR</i>
	[mean $\pm$ SD (U/g fresh wt)]			
Flesh	0.58 $\pm$ 0.15 c ^y	1.28 $\pm$ 0.14 c	0.76 $\pm$ 0.06 c	1.32 $\pm$ 0.12 c
Peel	0.95 $\pm$ 0.20 b	2.61 $\pm$ 0.19 b	2.90 $\pm$ 0.12 b	4.97 $\pm$ 0.20 b
Leaf	6.05 $\pm$ 0.84 a	16.4 $\pm$ 1.54 a	24.1 $\pm$ 2.45 a	39.3 $\pm$ 2.61 a

^zAll samples were harvested at 120 d after anthesis. Flesh and peel were from sun side (red side) of fruit. Values of activities are means of five replicates.

^yDifferent letters within the same column indicate significant difference at $P < 0.05$ by Duncan's test.

The gene for GGP (VTC2/GDP-L-Gal phosphorylase) also might play an important role in controlling AsA biosynthesis (Bulley et al., 2009; Linster and Clarke, 2008). However, we found here that *GGP* expression levels were lowest in 0 DAA young fruit that had the highest amount of AsA (Fig. 2). Although GGP might be a main factor in regulating AsA level in kiwifruit (Bulley et al., 2009), such a function has not been reported with the fruit of tomato (Ioannidi et al., 2009) or apple leaves (Li et al., 2010). The higher level of expression that we observed in leaves (Table 2) supports previous findings of greater expression in green tissues (Dowdle et al., 2007).

GME converts GDP-D-mannose not only to GDP-L-galactose, but also to GDP-L-gulose (Wolucka and van Montagu, 2003). Wolucka et al. (2005) have suggested that *GME* might be a control point in AsA biosynthesis. From our present data, the *GME* transcript level showed a high correlation with AsA concentration during fruit development (Fig. 2). Compared with the peel and flesh, expression in the leaves was higher for *GME* than for any other genes tested here (Table 2). However, previous expression profiling of AsA-related genes has sug-

gested that *GME* is not a control point for AsA synthesis in fruit of peach (Imai et al., 2009), tomato (Ioannidi et al., 2009), kiwifruit (Bulley et al., 2009), or acerola (Badejo et al., 2009).

A mutation in the encoding of *GMP* causes an ozone-sensitive arabidopsis mutant to accumulate only 30% of the normal amount of AsA (Conklin et al., 1999). In our apple fruit, the *GMP* transcript level increased steadily over time but then declined after 60 DAA, a trend that did not follow that of AsA concentration (Fig. 2). We also found no clear difference in its expression abundance between flesh and leaf tissue (Table 2). Although the mRNA levels of *GMP* are correlated with AsA levels in acerola (Badejo et al., 2009), this is not true for fruit of tomato (Ioannidi et al., 2009) and kiwifruit (Bulley et al., 2009). Given that *GMP* is required for the biogenesis of cell walls and protein glycosylation (Hancock and Viola, 2005), it is possible that those two processes also are associated with the high *GMP* transcript level in young fruit.

The capacity for AsA turnover also plays an important role in maintaining AsA levels (Hancock and Viola, 2005). For example, *MDHAR* activity is correlated with AsA levels in tomato fruit under chilling stress (Stevens et al., 2008). *MDHAR* has also been implicated in the increment of DHA (Bermudez et al., 2008) as a fruit metabolite locus in tomato. Our results lend further support to these observations; here, greater *MDHAR* transcripts and activity levels in young fruit were correlated with a higher AsA concentration and the ratio of DHA concentration (Figs. 2 and 3). Their amounts were diminished in ripening fruit with high levels of AsA/DHA following the pattern suggested by Ioannidi et al. (2009) for the later stages of tomato fruit development. Because DHA cannot be quickly and completely reduced to AsA through *DHAR*, using GSH as the reducing substrate, it is further oxidized to 2,3-diketogulonic acid or non-renewable oxalic acid and/or tartaric acid and then lost (Debolt et al., 2007). Thus, *DHAR* is important to maintaining AsA levels and redox status. We found no clear relationship among AsA concentration, *DHAR* activity, and the mRNA expression levels of *DHAR1* and *DHAR2* during fruit development, but leaves have marked higher *DHAR* activity and expression levels of *DHAR1* and *DHAR2* than fruit, especially *DHAR1* (Figs. 2 and 3). Moreover, *DHAR1* and *DHAR2* have different expression patterns during fruit development; that difference was the greater *DHAR* activity and *DHAR2* transcription in young fruit with a higher DHA concentration but without any clear change in *DHAR1* over time.

In conclusion, the AsA concentration in plant cells is highly regulated by developmental processes. This regulation may differ according to genotype, tissue, or cell type. Our present data indicate that levels of AsA, which mainly depend on biosynthesis, are the highest in young fruit post-anthesis and then decrease steadily up to the time of full maturity. However, AsA continues to accumulate over time as a result of increasing fruit mass. Expression abundances of *GGP*, *GMP*, *GalUR*, and post-transcriptionally regulated *GalLDH* are not correlated with AsA concentration. Therefore, we believe that those genes are not

the main control points for AsA production. In contrast, transcript levels for *GalDH*, *GPP*, and *GME* do follow a pattern of change similar to that of AsA accumulation. We suggest that systematical research is still required that will focus on biosynthesis through main and alternative pathways. Other studies should involve controlling, including key factors that regulate AsA levels in apple plants (e.g., *GPP*).

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