L-Ascorbate Biosynthesis in Peach Fruit: Change in L-Ascorbate Content during Fruit Development in Relation to L-Galactose Pathway-related Gene Expression

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Summary

L-Ascorbate (Asc) is an important nutritional component, known as vitamin C, in human diet. Because fruits and vegetables are two main sources for this vitamin, high vitamin C content is a valuable feature for these agricultural products. To gain the knowledge of vitamin C synthesis in fruit tissues, peach (*Prunus persica* (L.) Batsch) fruit development and fluctuation of Asc level from early developing to well-ripened fruit were studied. Fluctuation of Asc was divided into four periods based on the overall total Asc (T-Asc) content per fruit: Asc I, 0–36 days after full bloom (DAFB); Asc II, 37–65 DAFB; Asc III, 66–92 DAFB; and Asc IV, 93–112 DAFB. Asc III was a lag period for Asc accumulation, but did not coincide with the lag phase, stone hardening stage (Stage II) for fruit development. The T-Asc content was highest at the early stage until 21 DAFB [2–3 μmol per gram of fresh weight (g^{-1} FW)], and decreased to 1/4 and 1/15 of this value at 50 and 92 DAFB, respectively. T-Asc then remained at 0.15–0.20 μmol g^{-1} FW until harvest at 112 DAFB. More than 90% of the T-Asc was in the reduced form until 21 DAFB. The proportion of reduced form of Asc then decreased concomitantly with the decrease in Asc content. To determine the main pathway of Asc biosynthesis and the Asc biosynthetic capacity of peach flesh, several precursors were incubated with immature whole fruit (59 DAFB) or fruit discs prepared from mature fruit (112 DAFB). The Asc content increased markedly with L-galactono-1,4-lactone or L-galactose (Gal), but D-galacturonate and L-gulono-1,4-lactone failed to increase Asc, indicating dominance of the Gal

pathway and potent Asc biosynthetic capability in immature peach flesh. Although results obtained from mature fruit showed similar to that from immature fruit, it was not statistically significant. The genes encoding the last six step enzymes in the Gal pathway were cloned from peach: i) *GDP-D-mannose pyrophosphorylase* (*GMPH*, DDBJ accession AB457581); ii) *GDP-D-mannose-3',5'-epimerase* (*GME*, AB457582); iii) *GDP-L-galactose phosphorylase* (*GGP*, AB457583); iv) *L-galactose-1-phosphate phosphatase* (*GPP*, AB457584); v) *L-galactose-1-dehydrogenase* (*GDH*, AB457585) and vi) *L-galactono-1,4-lactone dehydrogenase* (*GLDH*, AB457586). The clones for *GMPH*, *GME*, *GPP* and *GDH* contain entire protein coding regions. Genomic Southern blotting suggested *GMPH*, *GME*, *GGP*, *GPP* and *GDH* are single copy, whereas *GLDH* seems to be two copy gene in the peach genome. Expression analyses of these genes were performed using RNA samples prepared at 21, 43, 69, 91 and 112 DAFB. *GMPH*, *GME* and *GGP* showed similar expression patterns that peaked at 43 DAFB. *GPP*, *GDH* and *GLDH* also had similar expression patterns that peaked twice at 21 and 91 DAFB, although the expression of *GDH* was quite low. High level of T-Asc conctent was roughly correlated with the high level of gene expression in the early period of fruit development (Asc I), whereas no such relationships were apparent in the other periods (e.g., Asc III and IV). Based on these findings, the regulation of Asc biosynthesis in peach fruit was discussed.

Abbreviations

AldLN – aldonolactonase

Asc – L-ascorbate

Asc-Na – sodium Asc

CDS – coding sequence

DAA – days after anthesis

DAFB – days after full bloom

DHA – dehydro-L-ascorbate

DHAR – DHA reductase

DIG – digoxigenin

DTT – dithiothreitol

EF-1α – elongation factor 1α

EST – expressed sequence tag

Gal – L-galactose

GalL – L-galactono-1,4-lactone

GalUA – D-galacturonate

GalUAR – GalUA reductase

GDH – L-galactose dehydrogenase

g^{-1} FW – per gram of fresh weight

GGP – GDP-L-galactose phosphorylase

GLDH – GalL dehydrogenase

GLO – L-gulono-1,4-lactone oxidase

GME – GDP-D-mannose-3’,5’-epimerase

GMPH – GDP-D-mannose pyrophosphorylase

GPP – L-galactose-1-phosphate phosphatase

GulL – L-gulono-1,4-lactone

MDA – monodehydroascorbate

MDAR – MDA reductase

MI – *myo*-inositol

PMM – phosphomannomutase

ROS – reactive oxygen species

RT-PCR – reverse transcription PCR

SMP – senescence marker protein

T-Asc – total Asc

UTR – untranslated region

WT – wild type

Chapter 1

General introduction

L-Ascorbate in peach flesh

Peach (*Prunus persica* (L.) Batsch) is an important economic fruit crop in temperate regions and has been the subject of many biochemical studies such as investigations of sugar accumulation, polyamine content and fruit softening (e.g., Moriguchi and Yamaki 1988; Hayama et al. 2006; Liu et al. 2006). Although L-ascorbate (Asc) is also an important nutritional constituent, little attention has been paid on Asc content in peach; there have been no reports regarding the changes in Asc content during fruit development and ripening. It was reported that mature peach flesh contained about 0.4–1.5 μmol per gram of fresh weight (g^{-1} FW) of total Asc (T-Asc) (Davey et al. 2000). Its value is slightly higher than that in apple (0.1–0.5 μmol g^{-1} FW) or pear (0.15–0.25 μmol g^{-1} FW), but lower than orange or strawberry (3–4 μmol g^{-1} FW). Therefore, from the nutritional viewpoint, peach fruit with high Asc content may have some impact of additional value in this species. Gil et al. (2002) also reported similar range values of T-Asc (0.22–1.1 μmol g^{-1} FW) in 5 white-flesh peach cultivars together with 5 yellow-flesh ones. From the results obtained, there seem some differences in T-Asc content among cultivars, suggesting contribution of genetic control. This was also suggested in 15 populations of peach and nectarine breeding progenies reported recently (Cantín et al. 2009). Gil et al. (2002) also demonstrated that skin contained 2– to 3–fold T-Asc as compared to flesh

tissues.

Peach fruit flesh develops brown color very easily, when fruit come in touch with something or by handling improperly. Relationship between Asc content and the degree of browning during cold storage of cut lettuce leaves were reported (Degl'Innocenti et al. 2005): Asc and its oxidized form dehydro-L-ascorbate (DHA) totally disappeared in browning susceptible cultivar ‘Green Salad Bowl’ whereas it remained, although the level was low, in browning resistant cultivar ‘Red Salad Bowl’. Polyphenoloxidase activity is one of the main causes for browning and its activity may be modified by Asc content (Mayer and Harel 1979) or by application of Asc in combination with citrate and heat treatment (Almeida and Nogueira 1995). Understanding the relationships between Asc content and polyphenoloxidase activity in peach may be useful for better handling of this fruit not only for food industries but also for consumers.

Occurrence of Asc and its proposed function

Asc is a pivotal low molecular weight compound exists in almost all multicellular organisms. Asc is involved in many important cellular processes (Davey et al. 2000; Smirnoff 2001), among which scavenging of reactive oxygen species (ROS) is one of the important missions for Asc in living cells (Noctor and Foyer 1998; Davey et al. 2000), because generation of ROS such as superoxide radicals or hydroxyl radicals are indispensable for organisms in the process of generating chemical energy by oxidation of organic compounds or transducing light energy in photosynthetic electron transport reactions in plants

(Mano et al. 2004). Asc is also important for redox homeostasis and signaling in plant cells (Foyer and Noctor 2005) that modulate cellular metabolic status. Furthermore, Asc serves as a cofactor in many dioxygenase enzyme reactions such as oxidation of 1-aminocyclopropane-1-carboxylate producing a plant hormone ethylene (Rocklin et al. 2004); and/or proryl- or lysyl-residue hydroxylation of extracellular structural protein, collagen biosynthesis in animals (Linster and Van Schaftingen 2007).

Asc promotes cell expansion (Kato and Esaka 1999, 2000) and affects root architecture and cell structure (Olmos et al. 2006). More recently, Asc is modulating hormone signaling pathways (Pastori et al. 2003), defence reactions (Pavet et al. 2005), flowering and senescence (Barth et al. 2006).

Asc function largely relies on its chemical structure of acidic hydroxyl group (p*K*a=4.2) (Davey et al. 2000; Linster and Van Schaftingen 2007). Therefore, at neutral pH, Asc exist as monoanionic form, that can be written with resonance of carbon 1 and 3 (Fig. 1). One electron-oxidized form of Asc, monodehydro- (or semidehydro-) ascorbate (MDA) radical also having resonance form that delocalizes radical electron on carbon 1, 2 or 3, and oxygen atoms covalently bound to these carbons (Fig. 1). Accordingly, MDA radical is relatively stable so that it is much less reactive than highly ROS such as superoxide or hydroxyl radical, functions as scavenger of these harmful molecules with other antioxidant enzymes (Noctor and Foyer 1998; Linster and Van Schaftingen 2007). MDA produced in leaves suffered from oxidative stress treatment was directly monitored by electron paramagnetic resonance spectroscopy (Heber et al. 1996). Two molecules of MDA radical spontaneously disproportionate to

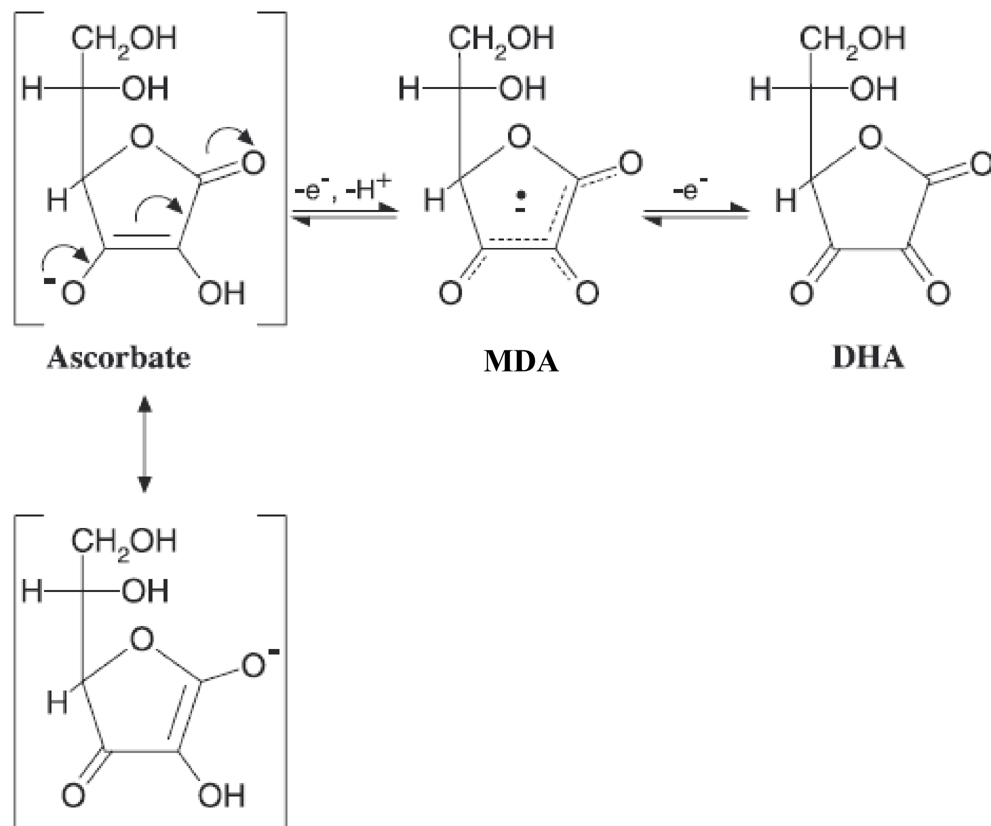


Fig. 1 Three redox forms of Asc: Ascorbate: fully reduced form, MDA: mono-oxidized form (monodehydroascorbate radical), DHA: di-oxidized form (dehydroascorbate). [Quoted from Linster and Van Schaftingen (2007)]

form Asc and DHA, two electron oxidized form of Asc (Fig. 1). MDA and DHA can be reduced back to Asc by enzymic or nonenzymic reactions (Noctor and Foyer 1998; Arrigoni and De Tullio 2002).

Asc concentration in plants varies depending on the species, ranging from the sub-micromolar to over 50-micromolar per gram of fresh weight (g^{-1} FW) [equivalent to 0.1 to 50 mM based on rough assumption of 1 g fresh weight corresponded to 1 mL of cellular solution] (Davey et al. 2000; Hancock and Viola 2005a). This is a quite larger value comparing to that in human plasma, which contained only 10–40 μM Asc (Levine et al. 1996). Human must rely on dietary intake of this vitamin, as lost the last step of enzyme in Asc biosynthesis (Nishikimi and Yagi 1996; Smirnoff 2001; Linster and Van Schaftingen 2007). Therefore, food materials from plant origin such as vegetables and fruits are important source for vitamin C for human.

On the other hand, in fungal species such as yeast (*Saccharomyces cerevisiae*), *Candida albicans*, *Phycomyces blakesleeanus*, a five carbon analogous molecule of Asc, D-erythroascorbate (Fig. 2) is biosynthesized (Huh et al. 1994; Kim et al. 1996; Spickett et al. 2000; Arrigoni and De Tullio 2002; Baroja-Mazo et al. 2005) and exhibited similar antioxidant function as Asc (Huh et al. 1998; Spickett et al. 2000). Biosynthetic conversion of D-arabinose to D-erythroascorbate resembles the last two steps of Asc biosynthesis in plants (Fig. 2).

It is interesting that bacteria *Escherichia coli* do not biosynthesize Asc itself, hence use other molecule for protection from oxidative stress. Neverthless, it possesses some of enzymes for Asc degradation (Linster and Van Schaftingen

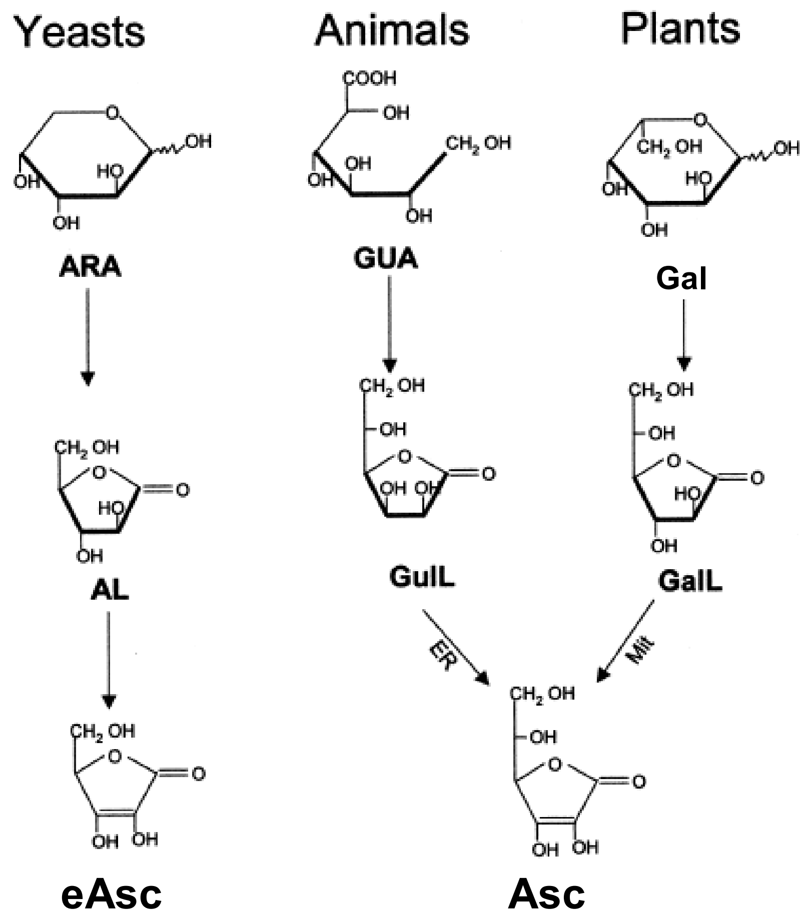


Fig. 2 Comparison of biosynthetic pathways of D-erythroascorbate (eAsc) in fungi and Asc in animals/plants. ARA: D-arabinose, AL: D-arabinono-1,4-lactone, GUA: L-gulonate, GulL: L-gulono-1,4-lactone, Gal: L-galactose, GalL: L-galactono-1,4-lactone, ER: endoplasmic reticulum, Mit: mitochodria. [Modified from Arrigoni and De Tullio (2002)]

2007) leading to produce D-xylulose-5-phosphate, a pentose phosphate intermediate (Fig. 3). Significance of this Asc degradation pathway in *E. coli* should need further evaluation.

Asc biosynthesis in animals

The biosynthetic pathway of Asc is different among plants, algae and animals (Nishikimi and Yagi 1996; Smirnoff 2001; Arrigoni and De Tullio 2002; Hancock and Viola 2005b; Ishikawa et al. 2006a, b, 2008; Linster and Van Schaftingen 2007; Wolucka and Van Montagu 2007). Asc biosynthesis in animals were well elucidated (Fig. 4) by a series of tracer, biochemical and molecular studies (Nishikimi and Yagi 1996; Smirnoff 2001; Linster and Van Schaftingen 2007). Early progress was made using specifically labeled D-glucose and found inversion of the carbon skeleton during biosynthesis (Horowitz et al. 1952; Horowitz and King 1953; Smirnoff 2001).

The last step enzyme, namely L-gulono-1,4-lactone oxidase (GLO; EC 1.1.3.8) was highly purified from rat and goat liver or chicken kidney (Nishikimi 1979; Kiuchi et al. 1982). GLO contains covalently-bound flavin adenine dinucleotide as a prosthetic group and localizes in microsome. A cDNA for rat GLO was cloned (Koshizaka et al. 1988); using this clone as a hybridization probe, genomic clones containing orthologous region (*GULO* locus) for rat gene was isolated from human (Nishikimi et al. 1994). It was revealed that many nucleotide substitution/insertion/deletion have been accumulated in the pseudogene of human *GULO* locus; mutated gene wreckage corresponding only to 3’-part of the rat gene was identified (Nishikimi et al. 1994; Nishikimi and

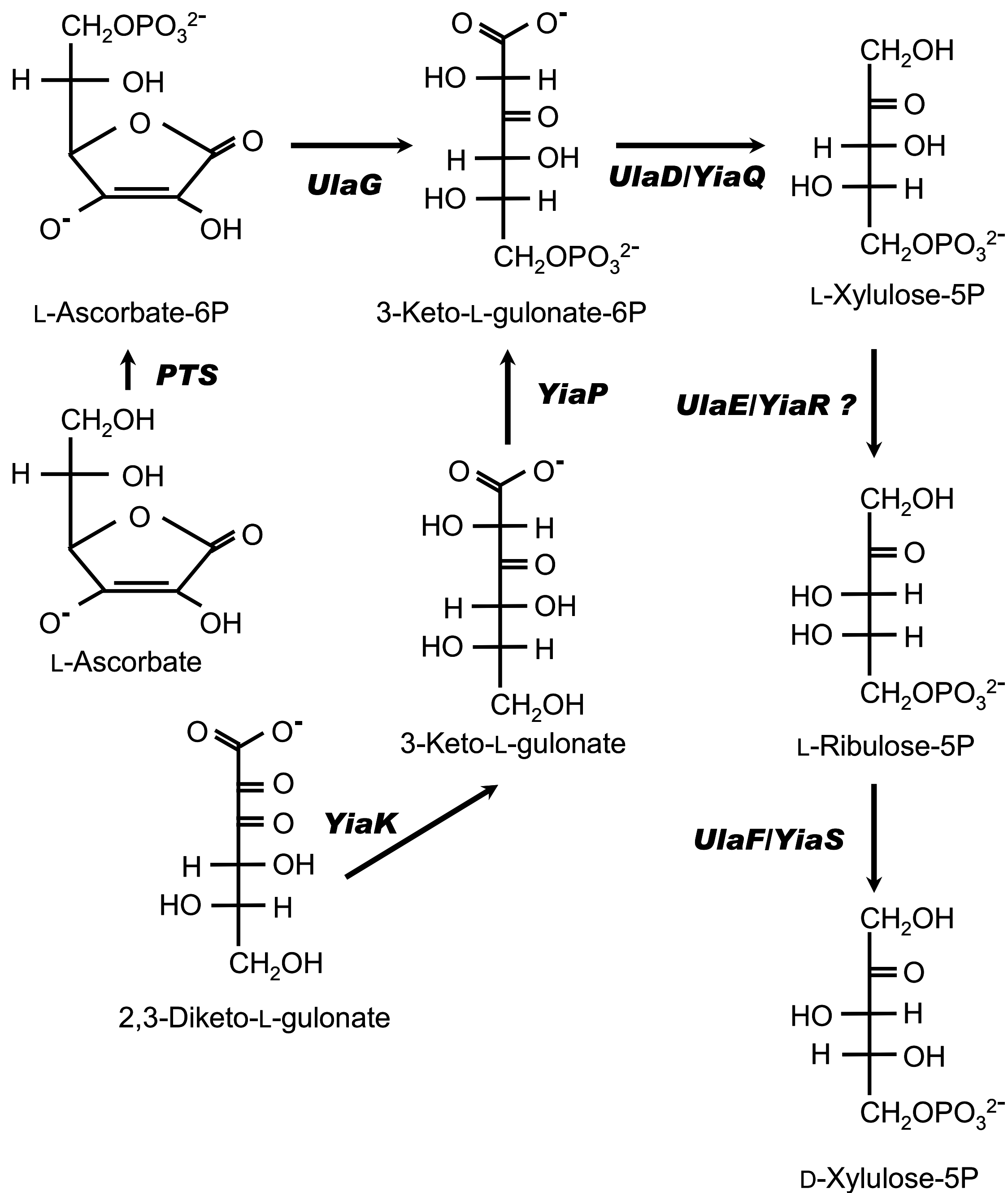


Fig. 3 Asc degradation pathway in *E. coli*. Gene symbols are indicated by the reactions catalyzed by each gene product. [Modified from Linster and Van Schaftingen (2007)]

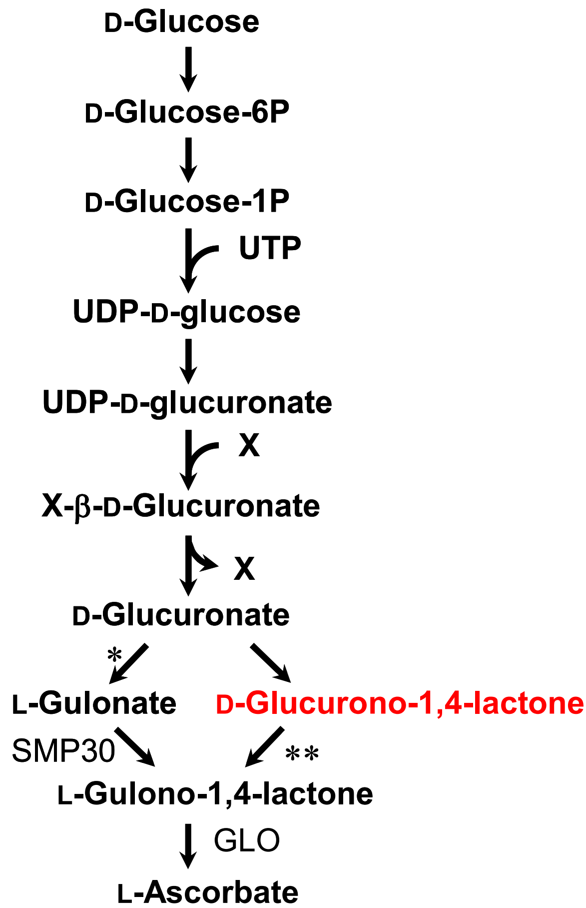


Fig. 4 The pathway of Asc biosynthesis in animals. X: conjugating molecule for glucuronidation. SMP30: senescence marker protein 30. GLO: L-gulono-1,4-lactone oxidase. Asterisks (* and **) indicate the reactions catalyzed by D-glucurono-1,4-lactone reductase. [Modified from Kondo et al. (2006)]

Yagi 1996). Accordingly, molecular basis for the lack of Asc biosynthetic activity in human was elucidated.

The second last step is catalyzed by aldonolactonase (AldLN; EC 3.1.1.17) (Chatterjee et al. 1960). Purification of the enzyme has achieved from rat liver with 110-fold in the activity (Bublitz and Lehninger 1961). Although biochemical characterization of the enzyme reaction has been reported, molecular basis of the lactonase protein was not known for more than 30 years. Recently, it was revealed that senescence marker protein 30 (SMP 30), known as a protein marker for aging, has AldLN activity (Kondo et al. 2006). Knockout mice for this gene (SMP30Y/-) exhibited i) scurvy-like symptoms under Asc deficient diet, ii) Asc content in liver or kidney at the time of death was < 1.6% of those in wild type (WT) and iii) body weight did not increase as in WT mice after 40 days from the onset of feeding with Asc-deficient food and died earlier than WT mice. The results suggested the reaction catalyzed by SMP30 is essential for Asc biosynthesis. Kondo et al. (2006) also demonstrated that occurrence of alternative pathway via D-glucurono-1,4-lactone (Fig. 4, shown in red) *in vivo*, although its flux was very small.

Glucuronidation step is not well understood. Reduction of D-glucuronate is catalyzed by aldo-keto reductase family protein; Hayashi et al. (1984) reported purification of NADPH-dependent D-glucurono-1,4-lactone reductase (EC 1.1.1.20) from rat kidney to a homogeneiety on SDS polyacrylamide gel electrophoresis. The enzyme reduces both D-glucuronate and its 1,4-lactone form in a similar activity (Fig. 4, indicated by asterisks), produced L-gulonate and L-gulono-1,4-lactone (GulL), respectively. The gene for this enzyme has

not been reported yet.

Asc biosynthesis in Euglena gracilis

In photosynthetic green algae, *Euglena gracilis*, Asc biosynthetic pathway via uronate has recently been confirmed (Fig. 5) by functional analysis of two enzymes, D-galacturonate reductase (GalUAR; EC 1.1.1.19) and AldLN (Ishikawa et al. 2006b, 2008). The pathway comprises animal pathway for the former part, and plant salvage pathway (Wolucka and Van Motagu 2007) for the latter part. The step for connecting these pathways is the reaction catalyzed by UDP-D-glucose-4-epimerase (Fig. 5).

Purified *Euglena* GalUAR exhibited similar catalytic efficiencies on both D-galacturonate (GalUA) and D-glucuronate; which was different from GalUAR from strawberry, the enzyme was GalUA-specific (Agius et al. 2003). N-terminal amino acid sequence (16 residues) analysis of *Euglena* GalUAR revealed unexpected high sequence similarity with malate dehydrogenases of both plant and animal species (Ishikawa et al. 2006b). cDNA cloning for *Euglena GalUAR* has not been published yet.

AldLN gene from *Euglena* was identified by BLAST search with similarity to gluconolactonase from rat. The recombinant *Euglena* AldLN protein expressed in *E. coli* revealed lactonase activity. RNA interference of *AldLN* gene expression resulted in growth arrest and recovered by the addition of L-galactono-1,4-lactone (GalL); suggesting AldLN is essential for Asc biosynthesis in *Euglena* (Ishikawa et al. 2008). Although the last step enzyme GalL dehydrogenase (GLDH; EC 1.3.2.3) has not been highly purified from

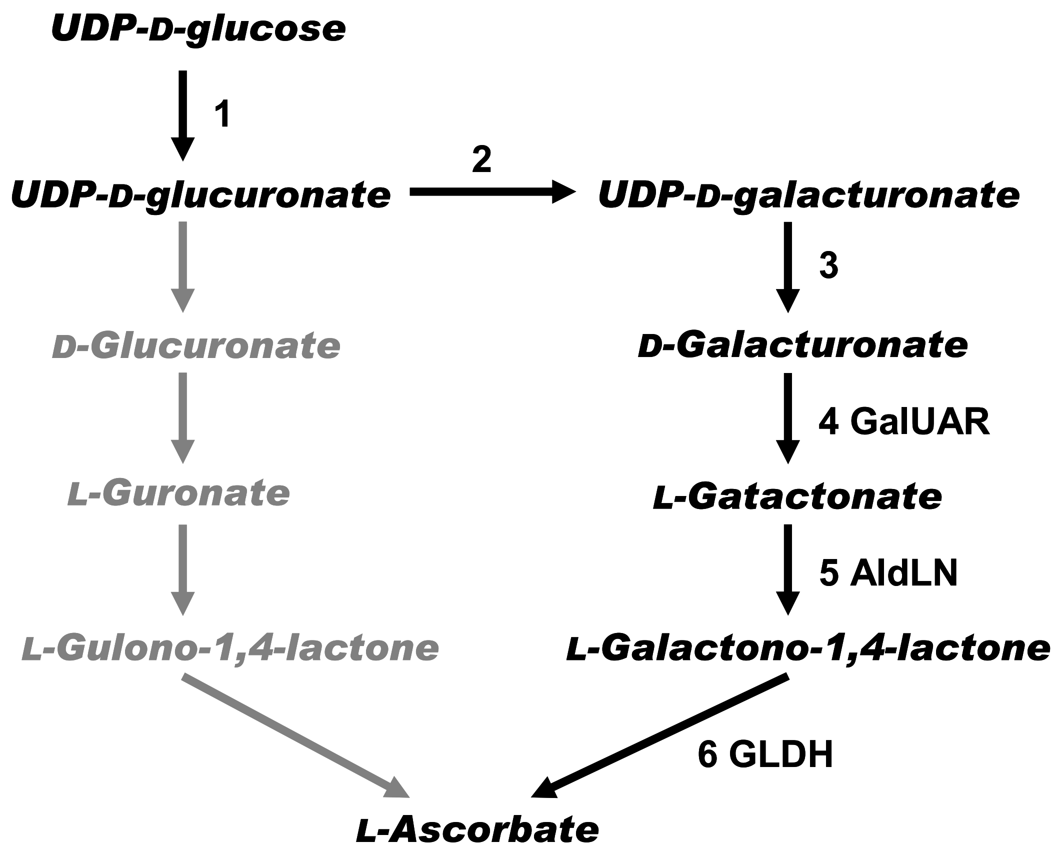


Fig. 5 The pathway of Asc biosynthesis in *Euglena gracilis*. Enzymes involved in the pathway is: 1, UDP-D-glucose dehydrogenase; 2, UDP-D-glucose-4-epimerase; 3, UDP-D-galacturonate pyrophosphatase; 4, D-galacturonate reductase (GalUAR); 5, aldono lactonase (AldLN); 6, L-galactono-1,4-lactone dehydrogenase (GLDH). Biosynthetic pathway functioning in animals is also shown in gray.

Euglena, involvement of GalL as Asc precursor was suggested strongly by feeding this compound to illuminated *Euglena* cells resulted in 32 times increase in Asc content to untreated cells (Shigeoka et al. 1979).

Euglena is the only species that have been established its Asc biosynthetic pathway via uronate; research on other photosynthetic algae species will help to understand the significance of this pathway.

One the other hand, colorless microalga *Prototheca moriformis*, exhibited similar biosynthetic enzyme activities in plants (Running et al. 2003). The amount of Asc produced was correlated with the GDP-D-mannose-3',5'-epimerase (GME; EC 5.1.3.18) activity in many *P. moriformis* mutant strains. Accordingly, it was suggested that *P. moriformis* biosynthesize Asc from fructose-6-phosphate via GDP-D-mannose and GDP-L-galactose (Running et al. 2003).

Asc biosynthesis in plants

With the exception of the last step, biosynthetic pathway of Asc in plant tissue was just elucidated in the past ten years. In the early progress accomplished in 1950s, studies were conducted by feeding with specifically labeled sugars to plant tissues. From the results obtained, a distinct pathway of Asc synthesis in plants was proposed: unlike in animals, there was no inversion of the glucose carbon skeleton (Loewus et al. 1956; Isherwood and Mapson 1961). Although the details of this plant pathway were not known for more than 30 years, Jackson et al. (1961) demonstrated the conversion of GalL, a stereoisomer of GulL, into Asc by the feeding of GalL in nine plant species. In addition,

GLDH was partially purified, and its ability to convert GalL into Asc in an GalL-specific manner was confirmed (Mapson and Breslow 1958). After a long lag of progress, a new pathway involving the GDP-D-mannose / GDP-L-galactose [L-Galactose (Gal) pathway, Fig. 6] has been proposed based on biochemical and genetic studies (Wheeler et al. 1998; Smirnoff et al. 2001; Wolucka and Van Montagu 2007). The pathway accounts for the non-inversion of the carbon skeleton suggested by tracer studies. Furthermore, other alternative pathways, namely GalUA, L-gulose and *myo*-inositol (MI) pathways have been suggested (Fig. 6, Davey et al. 1999; Agius et al. 2003; Wolucka and Van Montagu 2003; Lorence et al. 2004). However, as discussed in *Chapter 3*, experimental evidence of downstream reactions has not been elucidated yet (dotted line reactions, Fig. 6). Currently, it seems that Gal pathway functions ubiquitously in plants; other pathways may be optional routes for Asc biosynthesis. The different biosynthetic pathways for Asc among animals, algae and plants indicate these Asc biosynthetic pathways may be evolved independently after the divergence of each ancestral organism.

Aims of this research

As aforementioned, Asc is an important vitamin for humans, and fruits are important source for this vitamin. Accordingly, enhancement of Asc is one of the goals for breeding in some fruit crop species such as strawberry and tomato. Beside improvement of nutritional value for the consumers, high level of Asc may contribute resistance for biotic or abiotic stresses. Because Asc probably is the only antioxidant buffer in the apoplast, Asc level and its redox status in

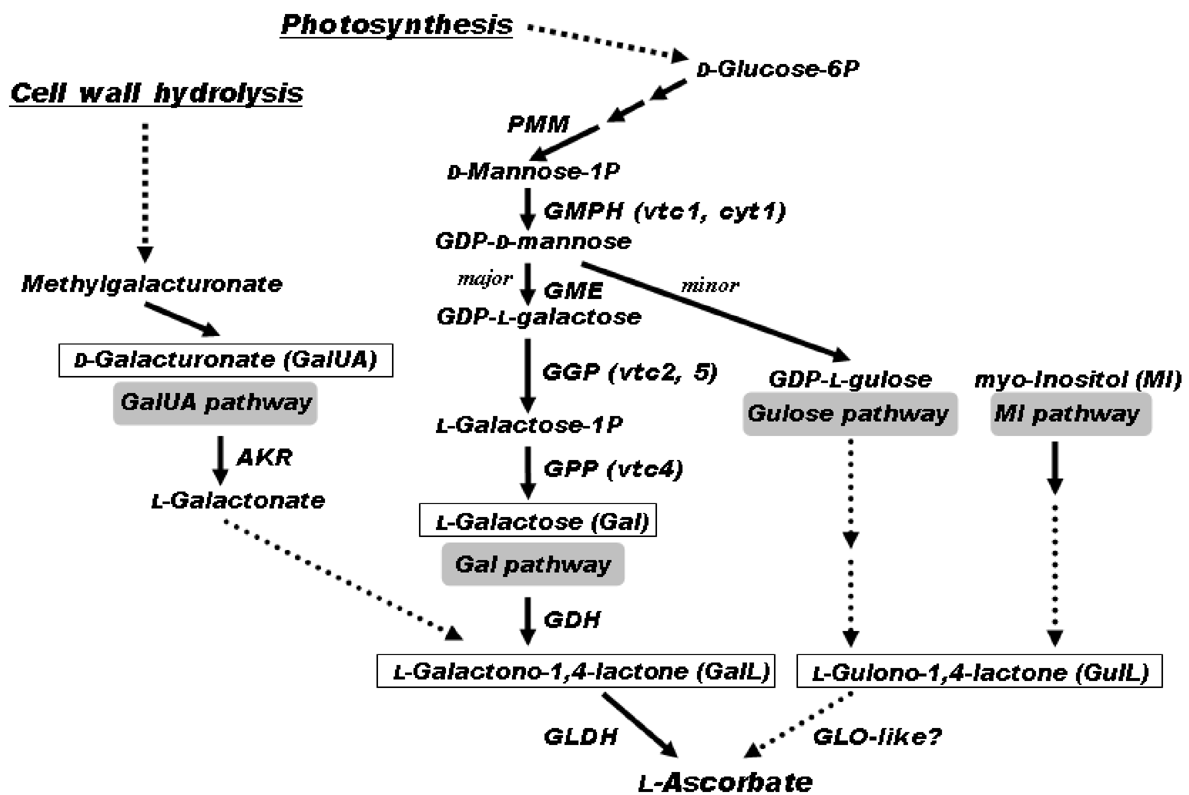


Fig. 6 Asc biosynthetic pathways in plants. The four possible pathways include the GalUA, Gal, L-gulose, and MI pathways. PMM: phosphomannomutase, GMPH: GDP-D-mannose pyrophosphorylase, GME: GDP-D-mannose-3',5'-epimerase, GGP: GDP-L-galactose phosphorylase, GPP: L-galactose-1-phosphate phosphatase, GDH: L-galactose-1-dehydrogenase, AKR: aldo-keto reductase, GLDH and GLO: see Fig. 5 and 4, respectively. GalUA, Gal, GalL and GulL were used for the feeding study (in *Chapter 3*); indicated by the boxes. The *vtc1*, *2*, and *4* in parentheses indicate the steps encoded by genes mutated in AsA-deficient *Arabidopsis* lines (Conklin et al. 1999, 2000, 2006); *vtc 5* indicates T-DNA insertion mutant *Arabidopsis* lines in another *GGP* gene (Dowdle et al. 2007). *cyt1* indicates *GMPH* is the gene also mutated in *Arabidopsis* line (*cyt1* mutant) manifested by embryonic lethality with severe defect in cell wall biogenesis. Dashed lines indicate possible sources for the substrate. Dotted lines indicate that the step is hypothetical in plants.

apoplast may play an important role in regulation of defense reactions or cell growth (Pignocchi and Foyer 2003). Indeed, ascorbate peroxidase activity was found in apoplastic compartment of apricot and peach (Diaz-Vivancos et al. 2006) and the activity was actually raised against plum pox virus infection. It is regrettable that Diaz-Vivancos et al. (2006) did not measure Asc content in apoplastic fluid studied for ascorbate peroxidase activity.

A lot of reports are published on Asc function and metabolism. Regulation of Asc level in cells is however, still largely unknown at present. Why some fruits can accumulate large quantity of Asc? How is Asc level controlled?

In the present study, we tried to answer these questions using peach fruit as a model case. Firstly, changes in Asc content along with fruit development was studied (*Chapter 2*). Secondary, possible precursors for Asc were fed to peach fruit in order to elucidate Asc biosynthetic pathway (*Chapter 3*). cDNAs encoding Asc biosynthetic enzymes for the last six steps were then cloned, their transcript abundances were also studied along with fruit development (*Chapter 4*). Finally, based on the results obtained, modulation of Asc level in fruit tissue including peach was discussed (*Chapter 5*).

Chapter 2

Changes of L-ascorbate content in relation to peach fruit development

Introduction

Three stages of fruit growing pattern in peach were first reported by Connors (1919): Stage I, rapid growth mainly owing to seed part increase; Stage II, apparent rest period in relation to stone hardening, and Stage III, rapid growth of fruit flesh to maturity. Using five cultivars for ripening at different seasons, Tukey (1933) reported that the first stage was lasted for about 50 days irrespective of cultivar. Accordingly, difference in duration of Stage II is mainly (in some cultivar that of Stage III also) affected total days need to reach maturity. From the observation using microscope, Zanchin et al. (1994) estimated cell division occurred only within the first 4 weeks [corresponding to 0–28 days after full bloom (DAFB)], and after that, increase in fruit size was entirely depended on cell size enlargement together with enlargement in intercellular spaces. To get insight in peach fruit development under our cultivated conditions, we decided to follow increase in latitudinal diameter and fresh weight.

The Asc content varies depending on the species, ranging from the sub-micromolar to 100-micromolar order g^{-1} FW (Davey et al. 2000; Hancock and Viola 2005a). High Asc-accumulating fruit such as acerola (*Malpighia glabra* L.) contain three to five times higher total Asc levels than that in leaf

tissue (Badejo et al. 2007). Two types of Asc accumulation (expressed as g^{-1} FW) pattern in fruit have been reported: i) the Asc content is basically unchanged or increases slightly during fruit development, or ii) the Asc decreases with fruit development. Fruits that exhibit the first type of pattern include melon, tomato, strawberry, kiwifruit and blackcurrant (Agius et al. 2003; Andrews et al. 2004; Pateraki et al. 2004; Alhagdow et al. 2007; Hancock et al. 2007; Bulley et al. 2009), whereas those that exhibit the second type include apple, orange and acerola (Davey et al. 2000, 2004; Badejo et al. 2007). To date, there has been no report on Asc fluctuation in regarding fruit development in peach.

Materials and methods

Measurement of peach fruit growth

Peach fruit 'Akatsuki' was sampled from four 20-year-old trees in the experimental orchard at the National Institute of Fruit Tree Science, Tsukuba, Ibaraki, Japan, in 2007. For growth evaluation, the latitudinal diameter was measured weekly for 20 fruits, and the weight was determined weekly for 10–25 fruits taken from 21 to 112 DAFB.

For Asc measurement, fruits were picked from 21 to 112 DAFB at roughly 2-week intervals, along with young fruitlets at 9 DAFB. During early fruit development, whole fruit at 9, 21 and 36 DAFB were used. After then, flesh tissues were sampled using a cork borer (6 mm in diameter), the flesh disc of which contained skin, flesh, embryo, and surrounding pre-stone tissues for the samples collected at 50 DAFB, whereas only flesh tissues (skin and flesh) without stone were collected after 65 DAFB. All samples were immediately frozen in liquid nitrogen and stored at –80°C until use.

Measurement of Asc and T-Asc content

Asc and T-Asc were measured using the Asc oxidase assay as described previously (Andrews et al. 2004; Imai et al. 2009b). Briefly, frozen samples were ground to a fine powder and extracted in 1 M perchloric acid then centrifuged. Aliquots of the supernatant (0.2 mL) were mixed with 40 μL of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (HEPES) -KOH (pH 7.0), neutralized with 5 M potassium carbonate until pH of the mixture came to 5 or 6,

and centrifuged again. Aliquots of 5–20 μL of neutralized extract were mixed with 0.1 M sodium phosphate buffer (pH 5.6), fill up to 95 μL. The absorbance at 265 nm was followed before and after the addition of 0.5 units of ascorbate oxidase (0.1 units μL^{-1}, Sigma-Aldrich, Saint Louis, MI). A known amount of sodium Asc (Asc-Na) was treated in the same manner and used as a calibration standard. For T-Asc measurement, dithiothreitol (DTT) was added to the buffer-diluted neutralized extract at a final concentration of 0.1 mM for the Asc-Na standard and 5.0 mM for tissue extracts (Imai et al. 2009a). DHA was estimated as the difference between T-Asc and Asc.

Results

Measurement of peach fruit growth

Latitudinal diameter and fresh weight of 'Akatsuki' peach fruit were measured from 21 to 112 DAFB. Based on the increase in these parameters, growth could be divided into three stages, Stage I, Stage II and Stage III (Fig. 7A, B; Table 1). Stage I lasted for 50 days, from 0 to 50 DAFB, in which the latitudinal diameter increased quickly; percentage increment per week was 34–65%. On the other hand, percentage increment in fresh weight was 84–364%, a peak value of 364% was observed at the period of 30–36 DAFB; percentage increment in latitudinal diameter at the same period was 59%. Stage II lasted for 28 days, from 51 to 78 DAFB. In this stage, the latitudinal diameter increased very slowly, 3–14% per week. Percentage increment in fresh weight was 11–56% per week in this period. Stage III lasted for 34 days, from 79 to 112 DAFB, exhibiting the second fast growth period. Percentage increment in this period was 7–15% per week and that in fresh weight was 20–45%. Overall increase, from 21 to 112 DAFB in latitudinal diameter was 11.4–fold and that in fresh weight was 940–fold (Table 1).

Measurement of Asc and T-Asc content

Asc and T-Asc was measured and its content was calculated on fresh weight basis. It was about 2.5–3.0 μmol g^{-1} FW, relatively high at the early developmental stage until 21 DAFB (Fig. 8A). After then, it decreased rapidly until 92 DAFB: 1/4 and 1/15 of initial value at 50 and 92 DAFB, respectively.

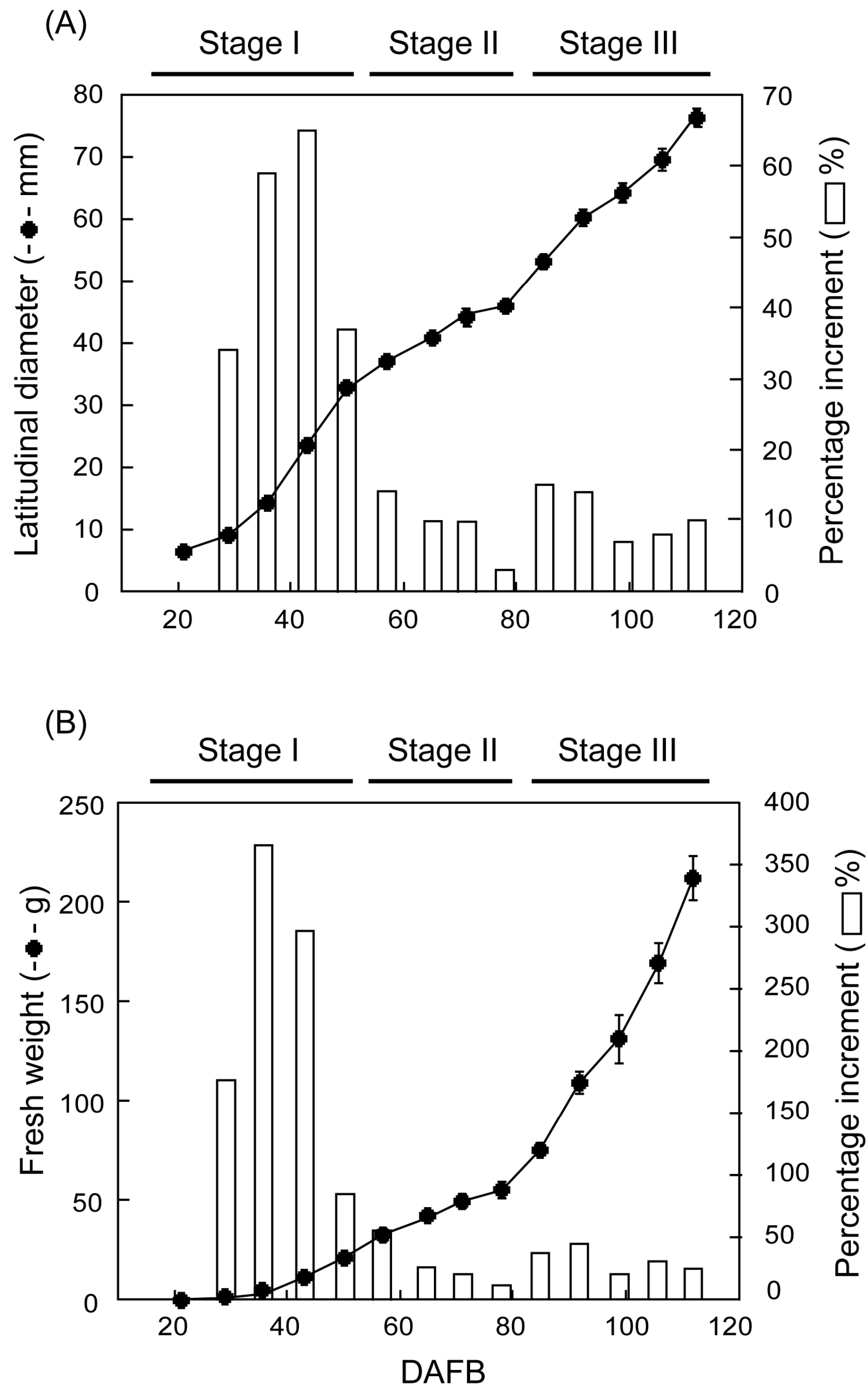


Fig. 7 (A) Growth curve based on the latitudinal diameter of fruit (black circles) and percentage latitudinal increment (white columns). (B) Growth curve based on the fresh weight of fruit (black circles) and percentage fresh weight increment (white columns). Peach fruit growth was measured weekly, and expressed as days after full bloom (DAFB). In both panels, the three distinct developmental stages (Stages I to III) and standard errors are indicated.

Table 1 Fruit growth followed by measurement of latitudinal diameter and fresh weight. Three developmental stages are indicated in different colors in the DAFB and the date columns: stage I for yellow, II for orange and III for rose pink, respectively. ND: not determined.

DAFB	21	29	36	43	50	57	65	71	78	85	92	99	106	112
Date	07/4/23	07/5/1	07/5/8	07/5/15	07/5/22	07/5/29	07/6/6	07/6/12	07/6/19	07/6/26	07/7/3	07/7/10	07/7/17	07/7/23
Latitudinal diameter														
Increment (%)	--	34	59	65	37	14	10	10	3	15	14	7	8	10
Average (mm)	6.74	9.06	14.4	23.7	32.6	37.1	40.8	44.8	45.9	53.0	60.2	64.3	69.5	76.8
S.E. (mm)	0.25	0.38	0.52	0.44	0.52	0.49	0.66	0.79	0.78	0.92	1.11	1.30	1.33	1.07
Fold	1.0	1.3	2.1	3.5	4.8	5.5	6.1	6.6	6.8	7.9	8.9	9.5	10.3	11.4
Fresh weight														
Increment (%)	--	173	364	296	84	56	26	20	11	37	45	20	30	25
Average (g)	0.225	0.616	2.86	11.3	20.8	32.5	41.0	49.4	54.9	75.1	109	131	169	212
S.E. (g)	ND	ND	ND	0.32	0.76	3.0	1.5	3.6	3.9	3.3	5.6	12.3	10.3	11.2
Fold	1.0	2.7	12.7	50.3	92.6	144.5	182.3	219.6	243.9	333.7	484.2	581.2	752.8	940.4

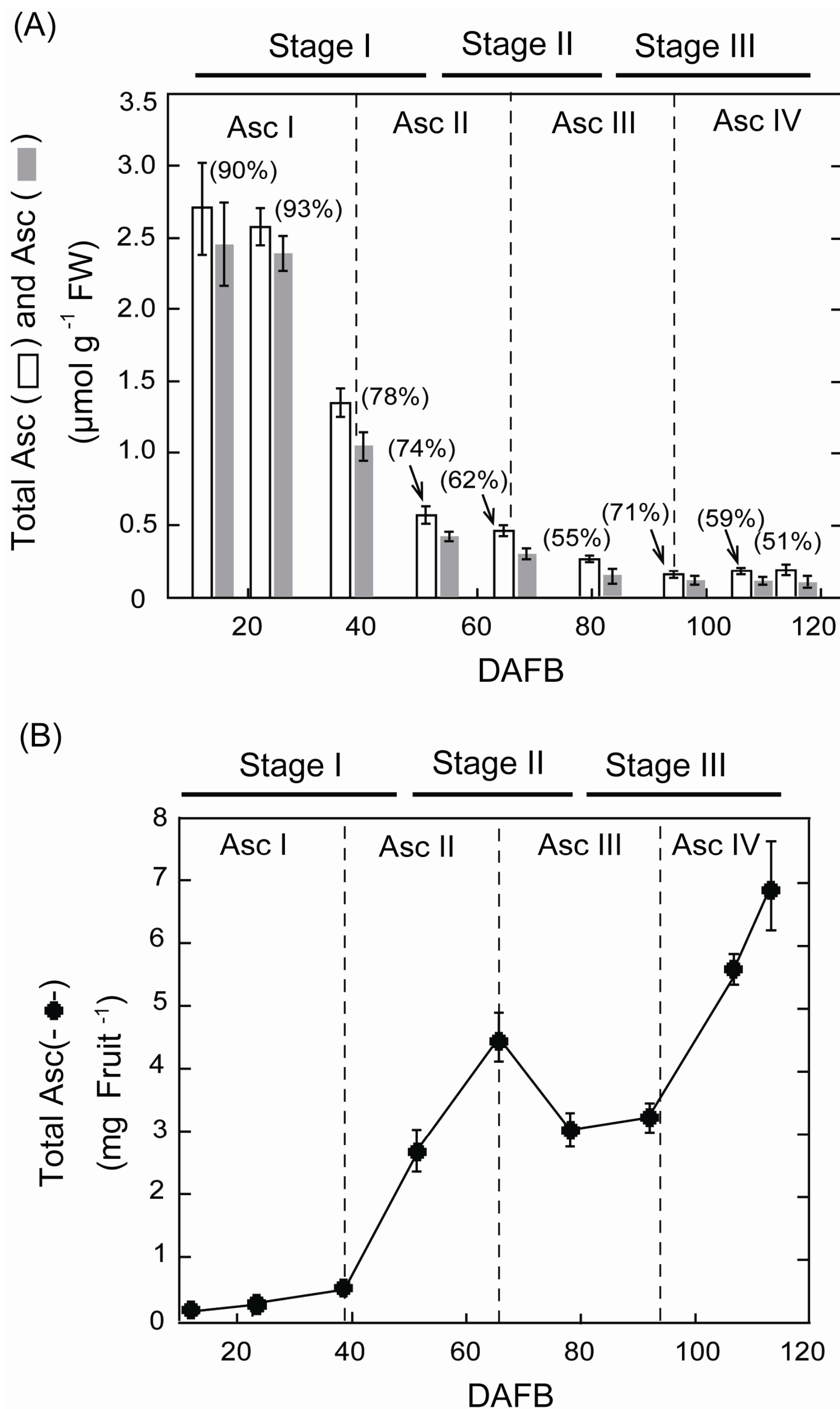


Fig. 8 Changes in total Asc (T-Asc) (white columns) and Asc (gray columns) content per gram of fresh weight (g^{-1} FW) (A) and overall T-Asc content per fruit (B) during fruit development. Parentheses indicate the percentage of the reduced form to the T-Asc. Standard errors (n=3–5) are indicated. In both panels, four characteristic Asc accumulation periods (Asc I to Asc IV) and three distinct developmental stages (Stages I to III) are indicated.

During the final maturation and ripening stage from 93 to 112 DAFB, it remained constant value of 0.15–0.20 μmol g^{-1} FW until harvest (Fig. 8A).

Based on the T-Asc content and total fruit weight, T-Asc amount per fruit was calculated. From the fluctuation in this value, Asc accumulation in peach fruit was divided into four phases Asc I to Asc IV (Fig. 8B), consisting of one slow and two rapid accumulation phases together with one lag phase. Asc I lasted for 36 days, from 0 to 36 DAFB. In this period, T-Asc amount per fruit increased steadily, 24 μg at 9 DAFB to 335 μg at 36 DAFB. Asc II was characterized as the first rapid increase period lasted from 37 to 65 DAFB. T-Asc amount was increased 13–fold in this period. T-Asc amount then transiently decelerated and retained its level about 3 mg per fruit until 92 DAFB (Asc III, Fig. 8B), before re-acceleration in the final stage of development starting from 93 DAFB (Asc IV). T-Asc amount decreased 29% from 65 DAFB to 92 DAFB during the period of Asc III. Asc III was a lag phase for Asc accumulation, but did not coincide with the lag phase for fruit development, i.e. Stage II. T-Asc amount was increased by 2.2–fold in Asc IV, its level at harvest was 6.8±0.7 mg $fruit^{-1}$. Overall increase in T-Asc from 9 DAFB to 112 DAFB in mature fruit was 283–fold.

The proportion of the reduced form of Asc was approximately 90% at the early developmental stage (former half of Asc I). It decreased to 55% from 22 to 78 DAFB (latter half of Asc I, Asc II, and former half of Asc III); increased transiently to 70% at 92 DAFB; and finally decreased to 51% at harvest during Asc IV (Fig. 8A).

Discussion

Fruit development was divided into three stages: Stage I for 50 days; Stage II for 28 days; and Stage III for 34 days (Fig. 7A, B) in our experimental condition. It was confirmed that the period of Stage I was invariable, i.e., 50 days as proposed by Tukey (1933). Stage II was characterized as apparent slow development stage associated with stone hardening. Similar results were reported previously by Zanchin et al. (1994) and Liu et al. (2006), in particular, the first two stages. But they divided Stage III further into two stages (S3 and S4): S3 was the second rapid growth stage lasted from 81 to 105 DAFB; and S4 was slowdown of increment in diameter following final maturation lasted from 106 to 120 DAFB (Zanchin et al. 1994). Based on our data, the slowing of the increase in latitudinal diameter in final ripening was unclear (Fig. 7A, B). All the data suggested double sigmoid growth curve in peach.

The inverse correlation between T-Asc content per fruit (fruit^{-1}) and g^{-1} FW along with fruit development was a characteristic feature in peach fruit. In blackcurrant, Hancock et al. (2007) reported that T-Asc amount in a fruit rapidly increased in the early stage of fruit development, while T-Asc content (expressed as μmol g^{-1} FW) exhibited a trend of slight decrease. With the exception of the final maturation stage (93–112 DAFB), characterized by the second rapid Asc accumulation stage (Asc IV) together with a rather constant T-Asc content in fruit flesh, no clear correlations were observed among fruit growth, amount of T-Asc (fruit^{-1} basis) and T-Asc content (g^{-1} FW basis). Similar decreases in Asc content expressed as g^{-1} FW basis with fruit development were reported in apple

(Davey et al. 2004; Li et al. 2008) and acerola (Badejo et al. 2007), but the reduction in the Asc content was less marked in these fruits than in peach, with reductions of approximately one-half or one-third of the initial Asc level in apple and acerola, respectively. Therefore, peach belongs to the class of fruit that exhibits decreases in Asc content (g^{-1} FW basis) with its development. After a marked decrease, the T-Asc level in mature peach fruit was approximately 0.18 μmol g^{-1} FW, which is relatively low in comparison to other fruits such as tomato, apple, or strawberry (Agius et al. 2003; Andrews et al. 2004; Davey et al. 2004).

Chapter 3

Feeding of precursors for L-ascorbate on peach fruit

Introduction

As described in *Chapter 1*, four possible pathways are proposed for Asc biosynthesis in plant tissues (Fig. 6). Among them, Gal pathway seems to be operating in various species. Low Asc content *Arabidopsis* mutant *vtc* lines suggested dominance of Gal pathway (see *Chapter 4*, Introduction). Although transgenic *Arabidopsis* plants exhibiting high Asc content were successfully produced by overexpressing i) aldo-keto reductase (AKR) from strawberry for the GalUA pathway, ii) *myo*-inositol oxygenase from *Arabidopsis* for the MI pathway, downstream enzymatic reactions were not confirmed by biochemically (indicated by dotted lines, Fig. 6). To clarify the issue in peach fruit, four possible precursors, namely GalL, Gal, GalUA and GulL were fed to immature whole fruit at 59 DAFB or mature fruit disc at 112 DAFB, and increase in Asc content were investigated.

Materials and methods

Feeding with immature whole fruit

Intact fruits collected at 59 DAFB were placed in 100-mL beakers either containing water, 25 mM GalL, 50 mM Gal, 50 mM GalUA or 25 mM GulL at a depth of 5–7 mm, immersing the end of the fruit stem (Fig. 9A). Beakers were sealed with Parafilm and left to stand for 18 h at room temperature. Three fruits were used per treatment, except for GulL for which two fruits were used. Fruit weight was measured before and after incubation. Tissues without stone were collected using a cork borer (6 mm in diameter), weighed and frozen in liquid nitrogen. Asc was measured as described in *Chapter 2*. Statistical significances were evaluated by t-test performed with Microsoft Excel software.

Feeding with fruit discs from mature fruit

Fruit tissues including skin were taken from mature fruit at 112 DAFB using a cork borer (6 mm in diameter) and incubated with either 10 mM GalL, 20 mM or 50 mM Gal, 20mM or 50 mM GalUA for 6 h. To avoid melting-like breakdown of mature fruit flesh tissues, a short incubation period was adopted. After incubation, excess solution was removed by paper towel, weighed and frozen in liquid nitrogen.

(A)

(B)

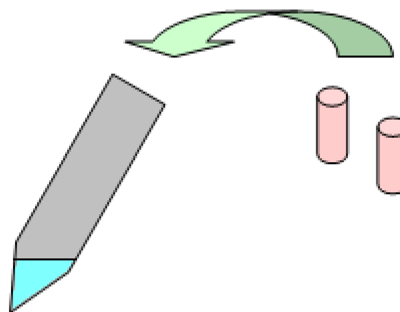


Fig. 9　Feeding of precursors for Asc to immature whole fruit (A) or discs of mature fruit (B). A: Photograph of incubated fruit. Details are described in the text. B: Schematic illustration of incubation with fruit discs.

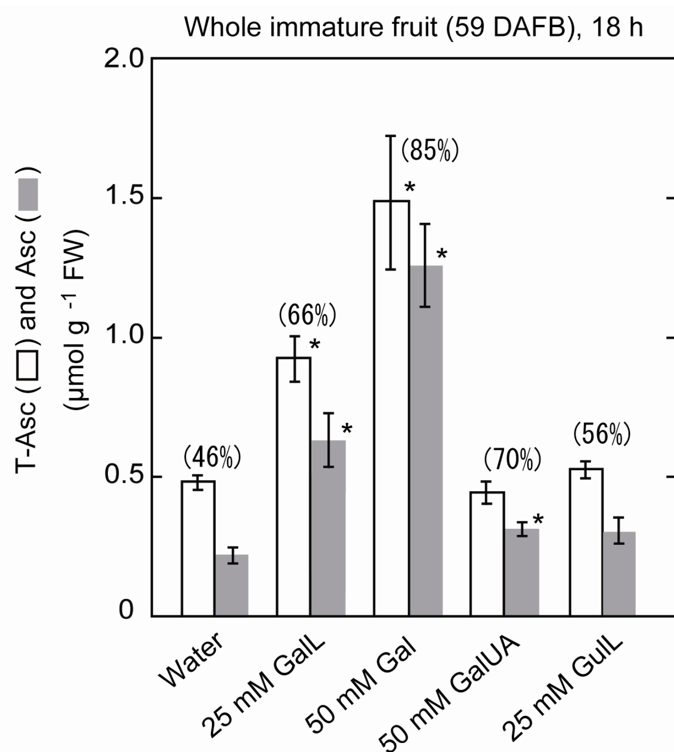
Results

Feeding with immature whole fruit

Feeding experiments using substrates for GDH, GLDH and AKR, as well as GulL, a precursor of Asc in animals, were performed. At this stage, fruit weight ranged from 18 to 25 g. A solution containing each precursor was fed through the stem end for 18 h (Fig. 9A). To confirm the obvious increase in T-Asc content, higher concentrations of precursors were tested for upstream substrates such as Gal (50 mM) or GalUA (50 mM). The contents of T-Asc and the reduced form of Asc increased significantly on incubation with 25 mM GalL and 50 mM Gal ($P < 0.05$; Fig. 10A). No significant increases in T-Asc were observed on incubation with GalUA or GulL, although significant increase in Asc was observed with 50 mM GalUA (Fig. 10A).

Feeding with fruit discs from mature fruit

Similar experiments were performed using mature fruit at 112 DAFB (fruit weights of approximately 300 g) with exogenous application of the precursors to fruit discs. As shown in Fig. 10B, a trend of increase (40–60%) was observed after 6 h incubation with Gal or GalL, but those values were not statistically significant, possibly because of shorter incubation time and relatively low activity of biosynthesis.



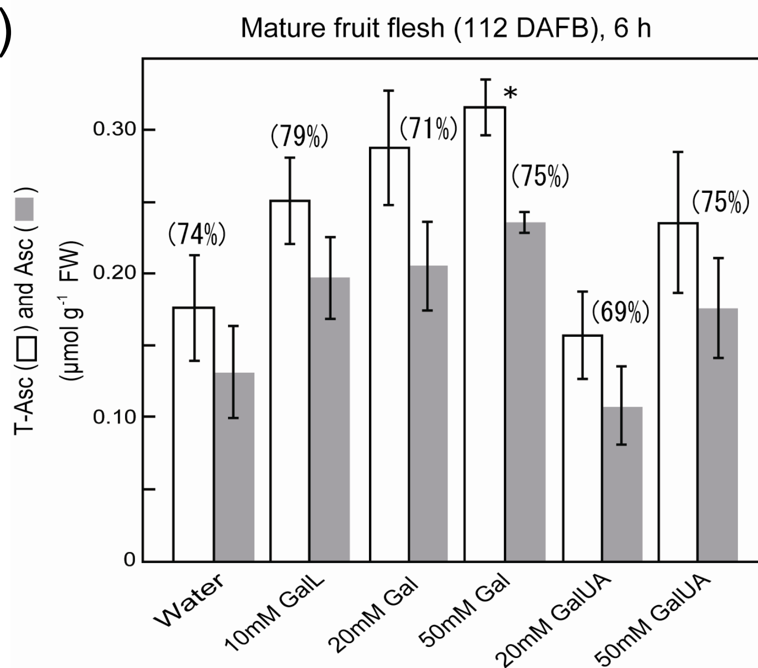


Fig. 10 Feeding of potential precursors for Asc biosynthesis. (A), Whole immature fruit incubated for 18 h. (B), Mature fruit disc incubated for 6 h. Asc and T-Asc were then measured. Asterisks (*) indicated significance (P<0.05) of the value. Parentheses indicate the percentage of the reduced form to the T-Asc. Standard errors (n=3) are indicated.

Discussion

The feeding of immature intact whole fruit with potential Asc precursors at 59 DAFB indicated that GalL and Gal were used for Asc biosynthesis, whereas GulL and GalUA were not (Fig. 10A). No clear results were obtained when mature fruit were used, although a similar tendency for an increase in T-Asc was observed (Fig. 10B). Nevertheless, the results suggest that the Gal pathway is dominant in peach fruit tissue and can biosynthesize Asc at least in the early developmental stage at 59 DAFB. Similar results have been reported in apple: Li et al. (2008) demonstrated that incubation with Gal or GalL caused an increase in Asc in seeds, peel and flesh. Li et al. (2008) also demonstrated in flesh tissue, the increase was clear with immature fruit (40 DAFB) but relatively ambiguous with mature fruit (105 DAFB), the results were in agreement with the present study in peach. In addition, almost the same results were reported by Hancock et al. (2007) using blackcurrant fruits: i) Gal and GalL were effectively increase Asc content while L-Gulose, GulL and GalUA did not, ii) flower and early stage fruit exhibited clear increase in Asc content, however later (mid and ripe) stages of fruit exhibited rather ambiguous or almost no increase. Laing et al. (2004) detected clear GDH activity in mature kiwifruit, although its activity was approximately one-fifth of that in young fruit at 4 weeks after anthesis. Taken together, the Gal pathway is dominantly operating in fruit flesh of these species. The activities of GDH and GLDH seemed relatively high in young immature fruit. Mature fruit flesh may be biosynthesize Asc although the biosynthetic activity was relatively low,

indicating developmental regulation of Asc biosynthesis in fruit tissues.

The results obtained in this study and from similar feeding experiments reported, it was concluded that Gal pathway is ubiquitously functions in plants. Other proposed pathways (Fig. 5) may be optional routes for Asc biosynthesis.

Chapter 4

Cloning and expression analysis of L-ascorbate biosynthesis-related genes in peach

Introduction

As described in *Chapter 1*, the biosynthetic pathway of Asc in plants was uncertain, except for the last step, until just 10 years ago. The last step, oxidation of GalL, a stereoisomer of GulL, was elucidated as early as 1950s (Mapson and Breslow 1958; Jackson et al. 1961). The step is catalyzed by GLDH, which localized mainly in mitochondria (Mapson and Breslow 1958; Ôba et al. 1994), was highly purified (Ôba et al. 1995), and its gene was first cloned from cauliflower (Østergaard et al. 1997). Of the six last steps in the Gal pathway in Asc biosynthesis, three steps were established by chemically mutated *Arabidopsis* lines originally identified their increased ozone sensitivity (Conklin et al. 1996, 2000), in which *vtc1*, *vtc2* and *vtc4* are mutated in the biosynthetic pathway genes namely GDP-D-mannose pyrophosphorylase (GMPH; EC 2.7.7.13), GDP-L-galactose phosphorylase (GGP) and L-galactose-1-phosphate phosphatase (GPP), respectively (Conklin et al. 1999, 2006; Dowdle et al. 2007; Laing et al. 2007; Linster et al. 2007). The mutated position in each gene was elucidated and reduction of enzyme activity in each biosynthetic step in the mutants was confirmed. Other two steps, catalyzed by GME and L-galactose dehydrogenase (GDH), were also studied by purified enzyme from *Arabidopsis* cultured cells (GME) and pea seedlings (GDH),

followed by cloned a cDNA from *Arabidopsis* (Wolucka et al. 2001; Gatzek et al. 2002; Wolucka and Van Montagu 2003).

Although the genes were cloned, expression studies of Asc biosynthesis-related genes were limited. Expression of *GMPH* in acerola exhibited good correlation with Asc content (Badejo et al. 2007): much higher in fruit than leaf and higher in the young fruit than mature one. *VTC2* gene (At4g26850) product accounts for 90 % activity of GGP in *Arabidopsis* (Dowdle et al. 2007), and its expression is highly light-dependent (Dowdle et al. 2007; Müller-Moulé 2008). When *VTC2* promoter was fused to GUS coding gene (*VTC2::GUS*) and transformed into *vtc2* mutant, GUS activity was mainly detected in leaf tissues (Müller-Moulé 2008). *GDH* was evenly expressed in leaf, stem and root tissues in spinach (Mieda et al. 2004). The last step enzyme gene, *GLDH* was preferentially expressed in rapidly dividing cells such as apical meristem, root meristem, flower bud and young fruit at 7 days after bloom (Alhagdow et al. 2007) revealed by *in situ* hybridization. Recently, light dependence of Gal pathway genes were reported using two week-old *Arabidopsis* seedlings: i) *GMPH*, *GGP* and *GPP* were light inducible, ii) *GLDH* was not light inducible but its expression reduced by dark treatment, and iii) *GME* and *GDH* were independent from light condition (Yabuta et al. 2007). However, relationships between Asc content and biosynthetic gene expressions are largely unknown. Accumulating these data together with more than 80,000 expressed sequence tag (EST) sequences from peach in the public DNA database, and from the results of feeding experiments described in *Chapter 3*, we decided to isolate peach orthologs of cDNA clones encoding biosynthetic

enzymes of GMPH, GME, GGP, GPP, GDH and GLDH in the Gal pathway.

In this chapter, cDNA cloning of above mentioned six steps is described. Using cloned sequences, genomic Southern blotting was performed to estimate copy number for each gene in peach. Also, RNA expression of biosynthetic genes was studied using RNA samples prepared from peach fruit at 21, 43, 69, 91 and 112 DAFB. This is the first report on the gene expression study of Gal pathway genes in peach.

Materials and methods

Cloning of cDNA fragments and homology search results

We have created a cDNA library from immature 'Akatsuki' fruits at 25 DAFB. Double strand cDNA was synthesized using TimeSaver kit (Amersham Biosciences, Piscataway, NJ) with a *Not*I site-containing oligo dT primer according to manufacturer's protocol. After digestion with *Not*I restriction endonuclease, *Eco*RI linker (Amersham Biosciences) was ligated, followed by ligated into *Eco*RI / *Not*I-treated lambda ExCell phage (Amersham Biosciences). Recombinant phage DNA was packaged using Ready-To-Go packaging kit according to manufacturer's protocol (Amersham Biosciences). Plasmid cDNAs were obtained from excision from phage clones according to manufacturer's protocol.

Random sequencing of cDNA clones and subsequent homology search using FastA program (http://www.dna.affrc.go.jp/fasta/prefastadna.pl) revealed a cDNA clone designated PF12044 was found to be a 5'-truncated sequence encoding GMPH (*VTC1* ortholog). The 5' portion of cDNA [668 base pairs (bp)] was amplified using *GMPH*-specific primers (Pp_GMPH_p09 and Pp_GMPH_p19, Table 2); which were designed based on the cDNA sequences of *Arabidopsis* (GenBank accession no. AF076484), potato (AF022716) and human (AF135421) using 1:10 diluted cDNA solution prepared from immature fruit at 25 DAFB as a template. The amplified fragment was ligated into the pCR2.1-TOPO vector (Invitrogen, San Diego, CA) and transformed into *E. coli* TOP10 (Invitrogen). Nucleotide sequences were determined

Table 2 Primers used for this study. Mixed bases are indicated in parenthesis. Gene symbles of *GMPH*, *GME*, *GGP*, *GPP*, *GDH* and *GLDH* were the same as in Fig. 6. *EF-1α*: elongation factor 1α. Primers for *GGP*, *GPP*, *GDH* and *GLDH* were also used for DIG labeling. Primers for *GDH* were also used for RT-PCR.

Target gene	Primers name	Primers sequence (5' to 3')	Product bp	Target region
for cDNA cloning				
GMPH	GMPH_p09	AAGATGAAGGCACT(A/C/G/T)AT(C/T)CT	668	5'UTR+CDS
	GMPH_p19	TG(C/G)CCAATGTCCATCCAAAA		
GGP	VTC2_p02	CCTTCTCTTTCTTGCTGGTG	1064	5'UTR+CDS
	VTC2_p13	AGTTCAGAAGCTCAGAGACC		
GPP	VTC4_p01	TCATCAACATCACCCATCAAA	896	5'UTR+CDS +3'UTR
	VTC4_p12	GATAGAGGCTGTCTCATTC		
GDH	GDH_p01	CCTGAACCAAAATCCCACTC	1029	5'UTR+CDS
	GDH_p11	TCAGCTCTGCTGGA(G/T)TCC		
GLDH	GLDH_p01	AAATGCTCAGAGCTCTCAC	1768	5'UTR+CDS
	GLDH_p11	AG(C/T)TTTTCCAGCATGTTATT		
for DIG labeling				
GMPH	GMPH_p01	TCCATAAATCCCATGGAGGA	680	CDS
	GMPH_p11	GATTTCTTTGTGGGGCAAAA		
GME	GME_p01	TGGACATGACTACGGTGCAT	623	CDS
	GME_p11	CACCTTTCCAGGTTCCAAAA		
for RT-PCR				
EF-1α	EF1α p01	ATCAACAT(C/T)GTGGTCATTGG	454	CDS
	EF1α p11	T(A/G)GTGGCATCCATCTTGTTA		

using DyeTerminator cycle sequencing kit v1.1 (Applied Biosystems, Foster City, CA) and model 377 or 3130xl DNA sequencer (Applied Biosystems).

Another cDNA clone, designated PF10988 in the cDNA library, was identified as a transcript for *GME* based on a homology search result using Fastx (http://www.dna.affrc.go.jp/fasta/prefastx.pl).

The cDNA clone for *GGP* (*VTC2* ortholog) and *GPP* (*VTC4* ortholog) were amplified by PCR using gene-specific primers (Table 2). The primers were designed from EST sequences in the Genome Database for *Rosaceae* (http://www.bioinfo.wsu.edu/gdr/). EST sequences were obtained by searching using key words "*vtc*2" and "L-galactose-1-phosphate phosphatase", respectively.

For *GDH*, primers were designed based on the EST sequences of peach (GenBank accession nos. DY639014 and DY647900) and a cDNA sequence of apple (AY264803) (Table 2). Similarly, for *GLDH*, primers were designed (Table 2) based on the EST sequences of peach (DY640431) and apple (EF660744).

Genomic Southern blotting

Genomic DNA was isolated from mature leaves of 'Akatsuki' trees using a modified CTAB-based method (Hasebe and Iwatsuki 1990). Aliquots of 10 μg of genomic DNA were digested with *Dra*I, *Eco*RI, *Kpn*I, or *Pst*I. Two sets of the restriction enzymes were used for digestion; the cutting sites of respective enzymes are not present in the probe regions of each cDNA sequence. Digested DNA was subjected to electrophoresis in 0.8% (w/v) agarose gels and

transferred onto nylon Hybond N membranes (Amersham Biosciences) by capillary transfer. Digoxigenin (DIG)-labeled DNA probes were generated by PCR using a DIG DNA labeling kit (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. For labeling of *GGP*, *GPP*, *GDH* and *GLDH* fragments, the same primers were used for cloning (Table 2). For labeling of *GMPH* and *GME*, new primers to amplify fragments of 680 bp and 623 bp, respectively, were designed (Table 2) and used as probes. Prehybridization (1 h) and hybridization (overnight) were carried out in high-SDS hybridization buffer containing 50% formamide, 5×SSC (75 mM NaCl and 7.5 mM trisodium citrate, pH 7.0), 2% blocking solution (Roche Diagnostics), 0.1% lauroylsarcosine, and 7% SDS at 42°C. After hybridization, the membranes were washed twice with 2×SSC and 0.1% SDS at room temperature for 5 min, followed by washing twice with 0.1×SSC and 0.1% SDS at 68°C for 15 min. Detection was performed using a DIG-CDP star system according to the manufacturer's instructions (Roche Diagnostics), and the membranes were exposed to X-ray film (Fuji Photo Film, Tokyo, Japan).

Gene expression analyses

For Northern blotting, we used RNA isolated from fruits at 21, 43, 69, 91 and 122 DAFB by hot-borate method (Wan and Wilkins 1994). Aliquots of 5 μg of RNA were separated in 1.0 % agarose gels containing 0.62 M formaldehyde. Hybridization, washing and detection were carried out under the same conditions as described for Southern blotting.

Semiquantitative reverse transcription (RT)-PCR

Semiquantitative RT-PCR was carried out for *GDH*. A housekeeping gene, elongation factor 1α (*EF-1α*), was used for normalization. Total RNA was diluted to 4 ng μL^{-1}, and aliquots of 1.5 μL (6 ng) were subjected to RT-PCR using SuperScript III One Step RT-PCR Platinum Taq Hifi (Invitrogen). Thermal control for amplification was as follows: 56°C for 12 min (reverse transcription); 94°C for 2 min (reverse transcriptase inactivation); defined cycles of 94°C for 15 s, 55°C for 30 s, and 68°C for 70 s; and a final extension at 68°C for 5 min. The number of cycles was set before saturation of amplification, i.e., 48 cycles for *GDH* and 28 cycles for *EF-1α*; these were determined from amplification results using a series of different numbers of cycles (data not shown).

Results

Cloning of cDNA fragments and homology search results

A 668 bp fragment was successfully amplified using *GMPH*-specific primers against cDNA template prepared from fruits at 25 DAFB. Comparing the sequence with that of PF12044, complete overlapping of 212 nucleotides was confirmed (not shown). Combining these sequences built up a 1311 bp sequence (GenBank accession no AB457581) consisting of 3 bp of 5’-untranslated region (UTR), 1086 bp of protein coding sequence (CDS) and 222 bp of 3’-UTR.

PF10988 encoding GME contained an entire CDS of 1131 bp together with 5’-UTR of 76 bp and 3’-UTR of 326 bp (GenBank AB457582).

The cDNA fragments encoding GGP, GPP, GDH and GLDH were amplified successfully from cDNA template prepared from immature fruit. A 1051 bp fragment for *GGP* (GenBank AB457583) contained 270 bp of 5’-UTR and 781 bp of partial CDS. The *GPP* fragment (GenBank AB457584) contained 70 bp of 5’-UTR, 813 bp of CDS and 13 bp of 3’-UTR. The *GDH* fragment (GenBank AB457585) contained 53 bp of 5’-UTR and 975 bp of complete CDS. The *GLDH* fragment (GenBank AB457586) contained 2 bp of 5’-UTR and 1766 bp of partial CDS. As a result from the deduced amino acid sequences, peach enzymes for GMPH, GME, GPP and GDH are consisted of 361, 376, 270 and 324 amino acid residues, respectively.

Homology search results of these cloned sequences using Fastx showed high degrees of similarity to the expected biosynthetic enzymes (Table 3). It is

Table 3 Fastx homology search results for cloned sequences. Top hit results are shown. Percentage of homology and length of homologous region in deduced amino acid (aa) sequence is shown under 'Homology' column.

Accsession no. for peach clone	Gene product	Top hit ortholog			opt score	Homology
		Accsession	Organism	Annotation		
AB457581	GMPH	AY639647	Alfalfa	GDP-D-mannnose pyrophophorylase	2217	92.0% / 361 aa
AB457582	GME	DQ229167	Acerola	GDP-D-mannose-3',5'-epimerase	2436	94.4% / 376 aa
AB457583	GGP	EF379384	Kiwifruit	VTC2-like protein	1518	82.6% / 276 aa
AB457584	GPP	AY787586	Apple	L-galactose-1-phosphate phosphatase	1587	89.6% / 270 aa
AB457585	GDH	AY264803	Apple	L-galactose dehydrogenase	1965	92.3% / 324 aa
AB457586	GLDH	AY643403	Chestnut rose	L-galactono-1,4-lactone dehydrogenase	3269	83.8% / 593 aa

suggested that the cloned sequences are peach orthologs that encoded AsA biosynthetic enzymes and could therefore be used as probes for expression analyses.

Genomic Southern blotting

On hybridization under high stringency conditions, a few bands were detected for five genes except for *GLDH* (Fig. 11), indicating that *GMPH*, *GME*, *GGP*, *GPP* and *GDH* were present as single copies, whereas *GLDH* may consist of two copies in the peach genome.

Gene expression analyses

Expression patterns of *GMPH*, *GME*, *GGP*, *GPP*, *GDH* and *GLDH* during fruit development were examined using the same probes as used for Southern blotting. The expression of *GMPH*, *GME* and *GGP* showed roughly similar patterns, they were highest at 43 DAFB and then decreased progressively toward harvest (Fig. 12A). In contrast, the expression patterns of *GPP* and *GLDH* were similar, with two peaks at 21 and 91 DAFB. For *GDH*, the signal was almost undetectable by Northern blotting (Fig. 12A); semiquantitative RT-PCR was therefore performed to confirm *GDH* transcript levels during fruit development (Fig. 12B). RNA samples were normalized by amplification of *EF-1*α. *GDH* transcript was observed at all stages of fruit tested, albeit with fluctuations in intensity; the patterns of *GDH* were roughly similar to those for *GPP* and *GLDH.* Thus, T-AsA content (Fig. 8A) roughly correlated with the levels of gene expression in the early stage of fruit development (Asc I),

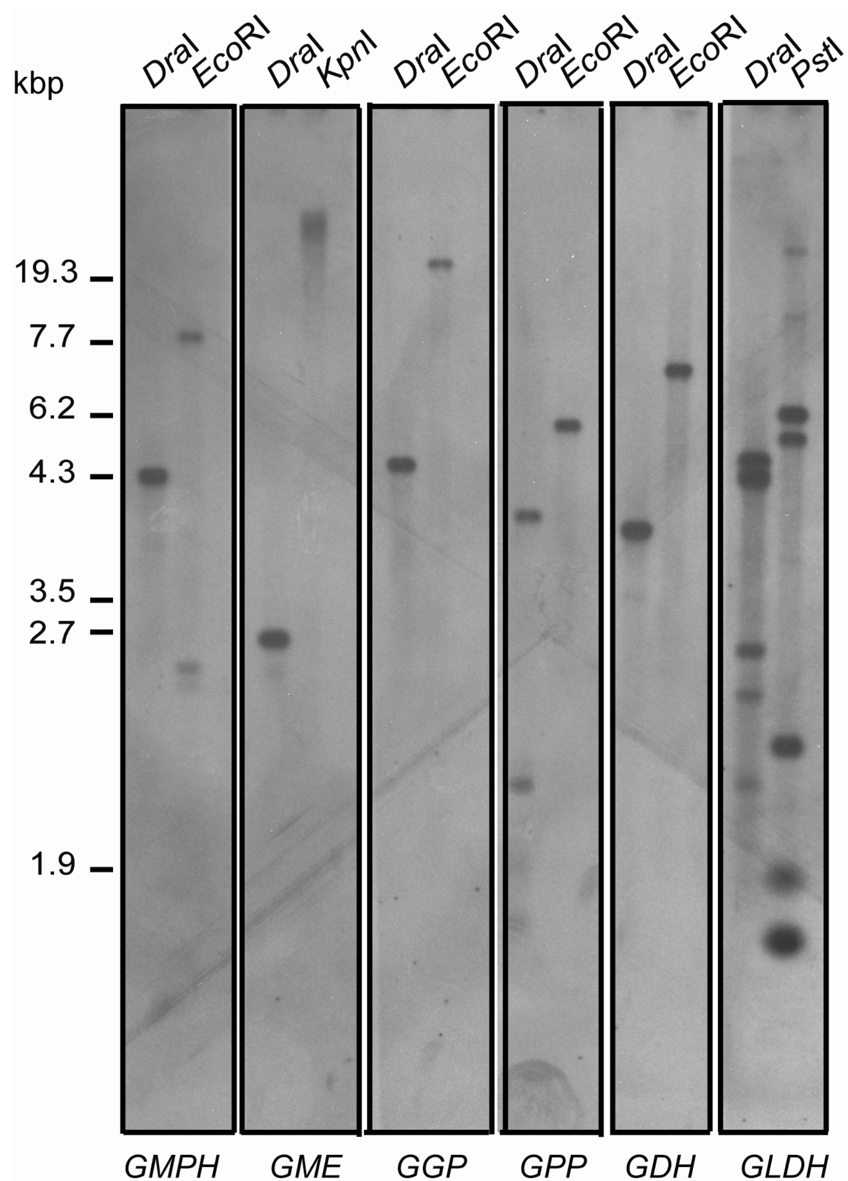


Fig. 11 Genomic Southern blotting of six Gal pathway-related genes. Gene symbols are the same as in Fig. 6. Two sets of the indicated restriction enzymes (*Dra*I, *Eco*RI, *Kpn*I, or *Pst*I) for each gene were used to digest 10 μg aliquots of total DNA from 'Akatsuki' peach leaves. Positions of size marker fragments are indicated in left.

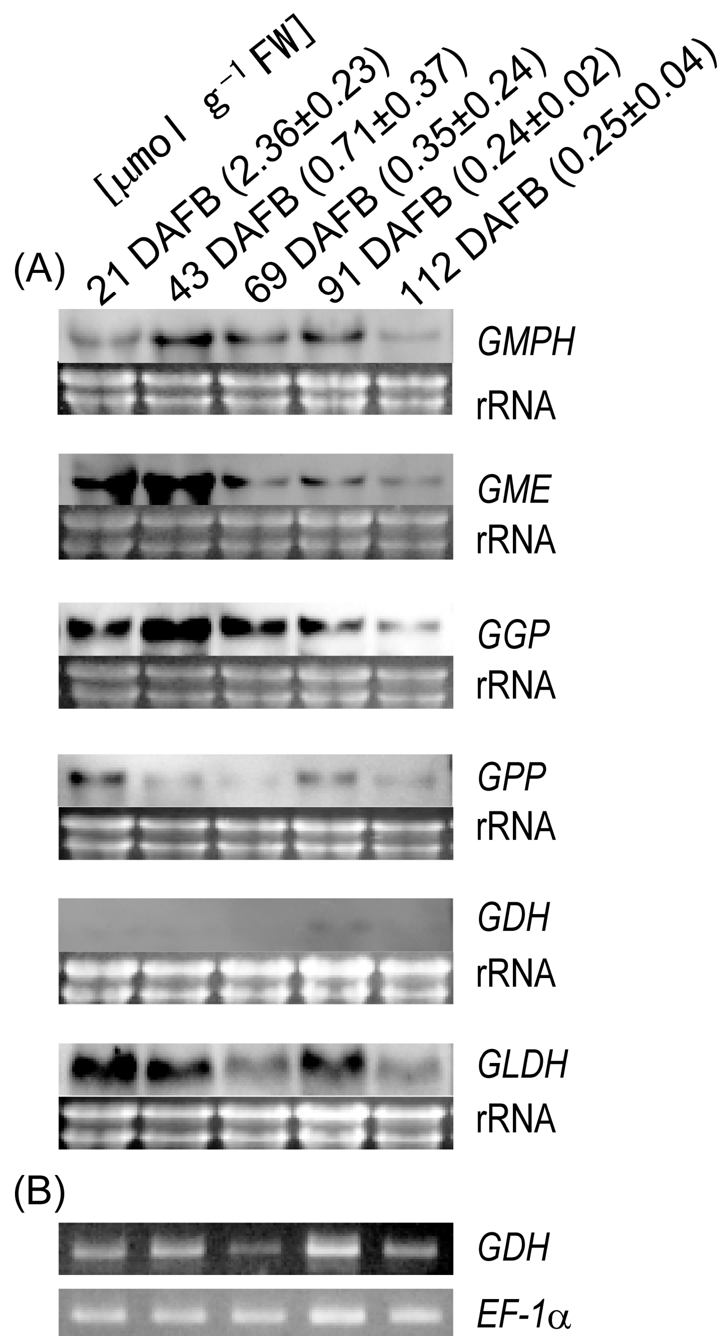


Fig. 12 (A) RNA blotting of six Gal pathway-related genes. Gene symbols are the same as in Table 2. Each lane contained 5 µg of total RNA from 'Akatsuki' peach fruit. The rRNA bands are also shown as a loading control. (B) RT-PCR for *GDH* and *EF-1α*. *EF-1α* was used as a loading control. The number of cycles was set before the saturation of amplification, i.e. 48 cycles for *GDH* and 28 cycles for *EF-1α*. The T-Asc content (µmol g^{-1} FW) is indicated in parentheses.

whereas such relationships were not apparent during other stages, especially in the final ripening (Asc IV).

Discussion

cDNA fragments encoding six Gal pathway-related genes were isolated from peach and their expression during fruit development were studied. Similar research using other fleshy-fruited plants such as acerola, tomato and kiwifruit have been published recently (Badejo et al. 2009b; Bulley et al. 2009; Ioannidi et al. 2009). In tomato, cDNA clones encoding all the six step enzymes analyzed in this study were isolated, while *GPP* in acerola and *GLDH* in kiwifruit were not cloned (Badejo et al. 2009b; Bulley et al. 2009). In this sense, peach is the second instance, in which all the six cDNA clones were isolated and analyzed their expression in fruit tissues. Based on the registered sequences in the DNA database, identities in the deduced protein amino acid sequences are summarized in Table 4. It is suggested that the amino acid sequences are highly conserved, with the exception of N-terminal regions of *GGP* and *GLDH* (not shown). Of the six genes, *GMPH* and *GME* are highly conserved: identities among the three plant species were over 88 %. The results indicated amino acid sequences of Asc biosynthetic enzymes are highly conservative.

Occurrence of single copy gene was suggested for *GMPH*, *GME*, *GGP*, *GPP* and *GDH*, while at least two copies of *GLDH* were appeared (Fig. 11). This is different from the results obtained in tobacco and melon (Yabuta et al. 2000; Pateraki et al. 2004), suggested to possess only a single copy gene for *GLDH*. In contrast, there are two copies of *GLDH* genes, i.e., Os11g0143500 and Os12g0139600, in the rice genome. The gene products of which show > 98%

Table 4 Identities (%) of deduced amino acid sequences of Gal pathway-related genes with that in peach. NA: Sequence not available. Note that only partial sequences corresponding to peach cDNA were compared for GGP (260 residues) and GLDH (589 residues). *Two unigene sequenses are registered in the tomato gene database.

Enzyme	*Arabidopsis*	Tomato	Acerola	Kiwifruit
GMPH	89.8	88.4 / 91.4 *	91.7	NA
GME	91.5	94.4	94.4	NA
GGP	72.4	72.5	74.6	76.4
GPP	77.5	81.9	NA	84.4
GDH	78.7	79.3	79.5	85.2
GLDH	76.0	77.2	79.8	NA

identity, both are transcribed as confirmed by EST analysis. For GGP, there are two isofunctional enzymes are reported in *Arabidopsis* (Dowdle et al. 2007; Linster et al. 2008). The gene for each isozyme has been identified, namely At4g26850 (*VTC2*) and At5g55120 (*VTC5*), and confirmed phosphorylase activity against GDP-L-galactose (Dowdle et al. 2007). By analyzing homozygous T-DNA insertion mutant in *VTC5* gene, Dowdle et al. (2007) concluded that *VTC2* accounts for 90% of biosynthetic activity of leaf Asc, *VTC5* accounts for the rest of 10%. Identities between *VTC2* and *VTC5* genes are less than 70% (Dowdle et al. 2007), it is probable that simultaneous detection of both genes by Southern blotting using one of the gene probe seems difficult. Therefore, although it was suggested that *GGP* is a single gene in the peach genome, there still may be a possibility that *VTC5* ortholog functions in Asc biosynthesis in peach.

Comparing biosynthetic gene expression in kiwifruit (Bulley et al. 2009), tomato (Ioannidi et al. 2009), acerola (Badejo et al. 2009b) and peach (this study), in generally, a similar tendency of strong expression in the early fruit developmental stage was observed. This was probably a reflection of high Asc demand for rapid cell division. Following stage was characterized as cell and intercell space enlargement (Zanchin et al. 1994). The expressions of biosynthetic gene were gradually decreased in this stage: one half to one fourth in kiwifruit (Bulley et al. 2009), one third to about one tenth in tomato (Ioannidi et al. 2009) with the exception for *GPP* and up to one fifth in acerola (Badejo et al. 2009b). In acerola, slightly different expression peak was observed in the mid stage of developing (10–17 days post anthesis) in *GDH* and *GLDH*. In

peach, reduction in the gene expression seemed approximately one-third to one-fifth (Fig. 12A).

Which is the important Asc biosynthetic step that affects Asc level in fruits? Ioannidi et al. (2009) pointed out that expression of *GPP* was best fitted in Asc content in tomato fruit. In this research, Ioannidi et al. (2009) reported Asc level in developing green fruit was rather low, increased after pink stage. But this was not coincided with the former report (Jimenez et al. 2002; Alhagdow et al. 2007), who observed rather constant level of Asc using the same cultivar as Ioannidi et al. (2009), i.e., ‘Ailsa Craig’ (Jimenez et al. 2002), or another cherry tomato cultivar ‘West Virginia 106’ (Alhagdow et al. 2007), respectively. In addition, we also confirmed relatively constant Asc content in developing and ripening tomato using ‘MicroTom’ (Fig. 13, Imai, unpublished data). The same method for quantification of Asc was used based on the spectrophotometric assay (A_{265}) using Asc oxidase in this study and reported by Ioannidi et al. (2009). Jimenez et al. (2002) and Bulley et al. (2009) used different HPLC-based method. Alhagdow et al. (2007) determined Asc by colormetric assay (A_{524}) using 2,6-dichloroindophenol. Although different methods were used for Asc measurement, Asc content in tomato fruits reported by Jimenez et al. (2002), Alhagdow et al. (2007) and this study were in good agreement with each other. Therefore, the reason for the discrepancy reported by Ioannidi et al. (2009) is still unknown.

It was noteworthy that *GPP* expression was quite different from other biosynthetic genes (Ioannidi et al. 2009); therefore they concluded only *GPP* expression was correlated with Asc level in tomato fruit. In kiwifruit (Bulley et

A

B

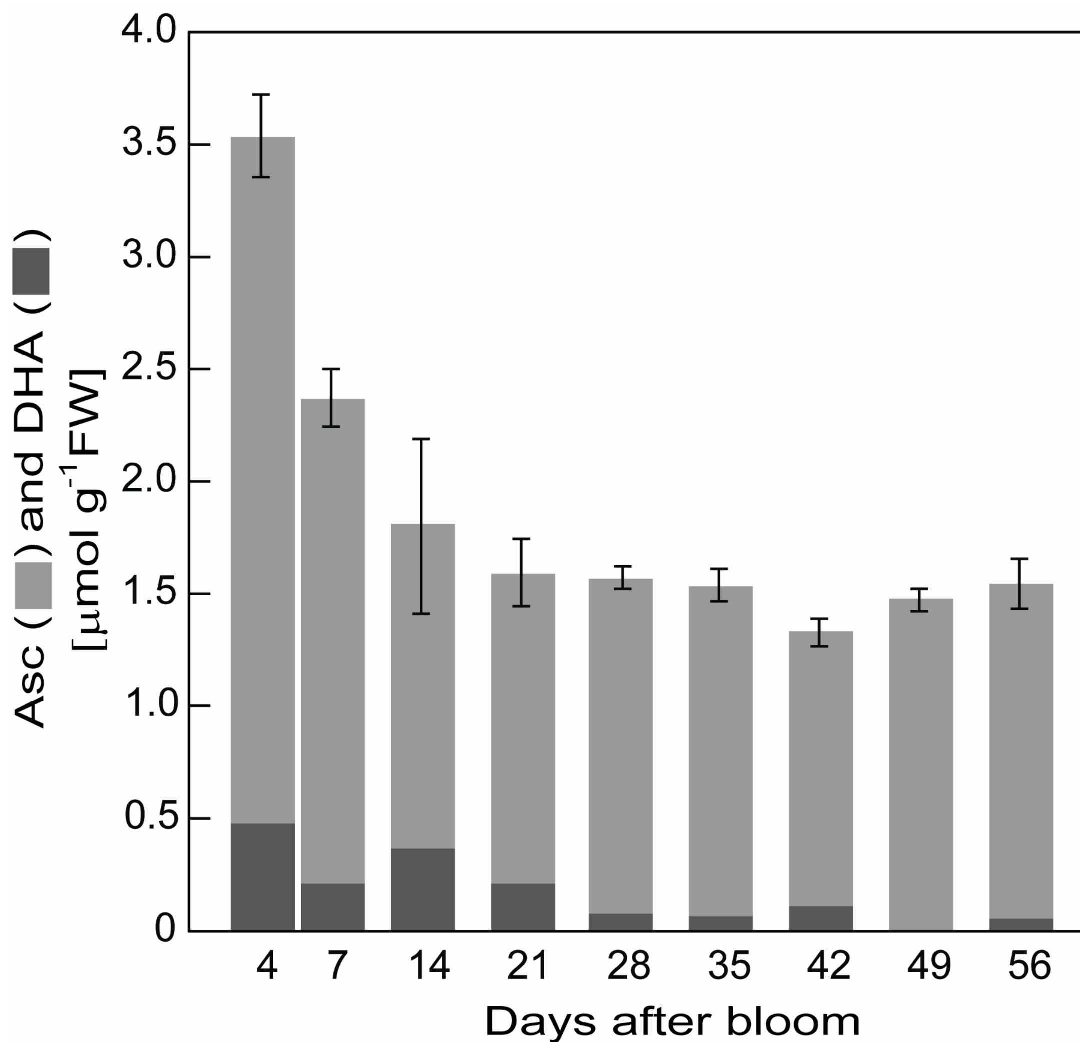


Fig. 13 Asc and DHA content in developing, ripening and ripened tomato fruits cv "MicroTom". (A) Photograph of fruits. Harvest day is indicated below. (B) Asc and DHA content (*n*=3 or 4). Standard errors for T-Asc are indicated.

al. 2009) and peach (this study), a trend of decrease in *GPP* expression was observed along with fruit development/ripening instead of explosive expression in ripening stage of tomato reported by Ioannidi et al. (2009).

In kiwifruit, based on the relatively high gene expression of *GGP* in high Asc genotype (*Actinidia eriantha*) in the early developmental period (4-6 weeks after anthsis), Bulley et al. (2009) pointed that the reaction catalyzed by GGP is the most important. *GME* gene expression was also high, although not as much as that in *GGP*. To confirm the significance of these gene expressions with Asc pool size, Bulley et al. (2009) conducted two types of transgenic research; i) stable transformation of *Arabidopsis* plants with *GGP* gene under the control of constitutive promoter, ii) transient gene-injection assay in tobacco leaves with either of *GGP* or *GME* gene, or both. Enhancement of Asc pool size was confirmed in both transgenic studies; that is, i) up to 4–fold in the third generation of transgenic *Arabidopsis* lines and ii) up to 7–fold in *GME* and *GGP* co-expressesd tobacco leaves. Accordingly, Bulley et al. (2009) concluded the reaction step catalyzed by GGP is rate-limiting.

In acerola, Asc content in fruit flesh was well correlated with biosynthetic gene expressions (Badejo et al. 2009b). Similar to the case in kiwifruit or peach, Asc content and Gal pathway-related gene expressions were higher in young developing fruit [6–13 days after anthesis (DAA)], lower in mature fruit (18–21 DAA), although peak gene expression in *GLDH* was slightly later (14–17 DAA). Fruits of acerola contain 3–4 fold Asc (60–80 μmol g^{-1} FW) comparing to leaf tissue and similar relationship was found in *GMPH* gene expression (Badejo et al 2007). Comparing Asc content and Gal

pathway-related gene expression between in leaves of *Arabidopsis* and acerola, Asc pool size was 8–fold larger in acerola while gene expression ratio varied from 5–fold (*GLDH*) to 700–fold (*GGP*) (Badejo et al. 2009b).

It was notable that lowest gene expression step was different among the species. As shown in Fig. 12, *GDH* was the lowest in peach while *GLDH* was the case in acerola (Badejo et al. 2009b). It may be also true in kiwifruit, as Bulley et al. (2009) could not found any EST sequences related to *GLDH* in a large collection of cDNA clones.

Taken together, generally, Asc levels and its biosynthetic gene expressions are relatively high in young developing fruits irrespective of species. However, developmental control of Gal pathway genes differs among species. No obvious relationship between Asc level and biosynthetic gene expression was observed in fruit tissues. This indicates although the same Gal pathway operates for Asc biosynthesis in plant tissues, Asc level control may largely depend on post-transcriptional control or modulation of enzyme protein.

Chapter 5

General discussion

Based on accumulating knowledge about Asc biosynthesis in plants, a study was conducted on the change in Asc content during fruit development in peach to get insight of its accumulation mechanism. A sharp decrease of Asc content on g^{-1} FW basis was observed for the first time in peach fruit. Dominance of Gal pathway was suggested by feeding potential precursors to peach fruit. RNA blotting suggested no obvious correlation between Gal pathway-related gene expressions and Asc content change. To find key regulation point of Asc content in peach fruit, the results are discussed once again.

Decrease of Asc content during peach fruit development

As shown in *Chapter 2*, Asc and DHA content in peach fruit was drastically decreased from 20 to 90 DAFB. During this period, T-Asc content fell from 2–3 to 0.2–0.3 μmol g^{-1} FW. However, due to the increase in fresh weight of the fruit, T-Asc in a fruit (mg $fruit^{-1}$) progressively increased with the exception of one period Asc III (65–91 DAFB, Fig. 8B). On the other hand, T-Asc content (μmol g^{-1} FW) during the final ripening period of Asc IV (92–112 DAFB) remained constant level of 0.15–0.20 μmol g^{-1} FW (Fig. 8A), but the amount of T-Asc in a fruit increased rapidly due to the second rapid fruit growth in this period (Figs. 7A, B; 8B). This is different from the case in tomato or blackcurrant, where T-Asc amount in a fruit was almost unchanged during the

latter half of fruit development (Jimenez et al. 2002; Hancock et al. 2007).

The Gal pathway as a main route for Asc biosynthesis in peach

In *Chapter 3*, it was suggested that Asc biosynthesis is mainly owing to Gal pathway in peach. In fruits, similar result has also been reported with using blackcurrant (Hancock et al. 2007) or apple (Li et al. 2008). When tissues were incubated with Gal or GalL, excellent increase in Asc content was also observed in pea embryonic axes (Pallanca and Smirnoff 1999) or leaves and seeds of apple (Davey et al. 2004). These experimental evidences suggested that the Gal pathway is functioning in many plant species. On the other hand, GalUA and MI pathways are confirmed only in one case in each: in strawberry (Agius et al. 2003) and in *Arabidopsis* (Lorence et al. 2004), respectively. Also, L-gulose pathway is proposed based on the occurrence of minor equilibration product of GDP- L-gulose: about 13% with *Arabidopsis* enzyme and about 5% with rice enzyme in the GME reaction (Wolucka and Van Montagu 2003; Watanabe et al. 2006). In addition, the most challenging points for these pathways are, as described in *Chapter 3*, lack of experimental evidence of downstream reaction steps, i.e., lactonization of L-galactonate in the GalUA pathway, and oxididation of GulL in both L-gulose and MI pathways (Fig. 6). Therefore, the Gal pathway may be ubiquitously functioning in all plant species; other pathways may be optional routes and still need experimental evidence for downstream reaction to establish the pathway.

Asc biosynthesis-related gene expression in relation to Asc content

As discussed in *Chapter 4*, the elucidation of the regulatory steps affecting the Asc pool size in fruit was difficult by RNA blotting analysis. Therefore, other approaches such as generation of transgenic plants could be required. Imai et al. (2009b) found that overexpression of *GLDH* did not lead to elevation of the T-Asc pool size in leaves of transgenic tobacco lines. In addition, in the present study, *GDH* expression was low as assessed by Northern blotting (Fig. 12A). A low level expression of *GDH* was also suggested by a low abundance of EST in apple or kiwifruit cDNA clone populations (approximately 0.002%; Laing et al. 2004). Therefore, it could be hypothesized that this low level of *GDH* gene expression could be insufficient for the biosynthesis of GalL. However, Gatzek et al. (2002) demonstrated that overexpression of *GDH* did not augment the Asc pool size in leaves of transgenic tobacco lines. In addition, Asc accumulation in leaves by exogenous Gal application was similar between *GDH*-overexpressing- and vector control-transgenic lines (Gatzek et al. 2002). These results suggest that both the activities of GDH and GLDH in nontransformed plants are sufficient to meet normal Asc demands. Thus, we assume that the reactions catalyzed by GLDH and GDH are not limiting steps for Asc biosynthesis and that the supply of Gal is the key factor. Although many metabolic differences may exist between fruit and leaf tissues, similar assumptions can be made from the result of Gal feeding to immature fruit. If upstream steps leading to an increase in Gal supply could be regulated by upstream gene expression(s), it would be possible to elevate the T-Asc content in peach fruit.

In addition, it has been clearly demonstrated that the Asc pool in the

Arabidopsis seedlings is enlarged by continuous light (Yabuta et al. 2007). Based on recent observations that the *VTC2* transcript encoding GGP is highly inducible by light (Müller-Moulé 2008) and exhibits diurnal variations (Dowdle et al. 2007), this step may also be critical for the regulation of Asc biosynthesis in photosynthetic tissues. In fruit tissue, metabolic flow may be different from leaves, but *GGP* expression may also affect the regulation of Asc biosynthesis. Although transcriptional control by light is evident, translational control or modulation of GGP activity by some factor is unknown at this moment. Following the change in GGP activity together with *VTC2* transcript level in relation to day/night regime in fruit tissue will be clarified the significance of this step as a biosynthetic control point.

Of the six steps examined in this study, the first one, catalyzed by GMPH seemed also influenced Asc pool size. In acerola, *GMPH* gene expression was well fitted to Asc content in respective tissue or developmental change in the fruit (Badejo et al. 2007). In addition, transgenic tobacco plants overexpressing *GMPH* gene from acerola driven by its native promoter exhibited comparable level of gene expression driven by a constitutive 35S promoter (Badejo et al. 2008). Consequently, Asc pool size in *GMPH*-overexpressing tobacco leaves was approximately 2–fold compared to nontransformed plants. The results indicated that transcriptional enhancement of *GMPH* expression could elevate cellular Asc pool size.

Recently, experimental evidence for phosphomannomutase (PMM; EC 5.4.2.8) reaction, catalyzing interconversion between D-mannose-6-phosphate and D-mannose-1-phosphte, in Asc biosynthesis has been reported (Qian et al.

2007). This step comprises one earlier step in the Gal pathway (Fig. 6) proposed by Wheeler et al. (1998), providing D-mannose-1-phospate, the substrate for GMPH. Qian et al. (2007) demonstrated, virus-inducing gene silencing of *PMM* expression resulted in about 50 % reduction of Asc content in the *Agrobacterium*-infiltrated *Nicotiana benthamina* leaves. Qian et al. (2007) also demonstrated that in *PMM*-overexpressing transgenic *Arabidopsis* plants homozygous for the *PMM* transgene exhibited more than 10–fold of PMM activity in the leaves of 4-week-old transgenic plants. Asc concentration in these plants was however, only up to 30 % comparing to nontransformed control plants (Qian et al. 2007). Badejo et al. (2009a) also reported that *PMM* gene expression was in good correlation with Asc concentration in fruits and leaves in acerola. This is also the case in the leaves of *PMM*-overexpressing transgenic tobacco plants: there were good correlations among *PMM* transcript level, PMM activity (up to 3–fold) and Asc content (up to 2–fold) (Badejo et al. 2009a). These results suggested that *PMM* gene expression may contribute modulation in Asc level in plant cells, suggesting the supply of D-mannose-1-phospate to Gal pathway is an important step for regulation.

Further research will be required for enhancement of Asc content in plants

Although we focused on the Asc biosynthesis in this study, the Asc pool size may be affected by three other factors, i) the regeneration capacity of Asc by enzymatic and nonenzymatic reactions, ii) Asc translocation from other tissues and iii) Asc consumption due to biosynthesis of tartarate or oxalate (DeBolt et al. 2007). The apparent Asc pool size is therefore balanced by these factors and

biosynthetic regulation. At present, it is largely unknown about the influence of Asc regeneration capacity and importing capacity on Asc pool size in developing fruit tissues. Further studies will be required for elucidating these points.

Combining the results reported, it seems difficult to enlarge Asc pool size over 3–fold by overexpressing Asc biosynthetic gene(s) (Ishikawa et al. 2006a; Wolucka and Van Montagu 2007), which may indicate homeostatic control of Asc content. Based on many proposed functions of Asc on human health, however, high Asc-content fruits or vegetables are appreciated. Besides understanding genetic control of Asc, cultivation techniques and postharvest control should also be important for improving Asc content in plant-derived food materials. The results obtained in this study could provide some important insights for such future research.

Concluding remarks

In conclusion, peach fruit exhibited marked decrease in Asc content g^{-1} FW basis during its development. The expression patterns of genes in the Gal pathway were not correlated with T-Asc content in fruits. Supply of Gal, however, directly correlated with Asc accumulation in immature peach fruit, suggesting that upstream steps of the Gal pathway (i.e., *GMPH*, *GME*, *GGP* and/or *GPP*) may be rate limiting steps for Asc biosynthesis. Considering the results obtained from the similar studies on the gene expression analyses of Gal pathway in fruit tissues of acerola, tomato and kiwifruit, together with transgenic or mutant studies on Gal pathway genes, it is suggested that a

complex tuning mechanism, yet unknown at present, control Asc content in plant tissues.

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