Chemistry and Metabolism of Ascorbic Acid in Plants

Kazuya Yoshimura and Takahiro Ishikawa

Abstract Ascorbic acid (ascorbate/AsA/vitamin C) is one of the most abundant and versatile biomolecules in plants and animals. In particular, AsA is present in high concentrations in the chloroplasts and cytosol of plant species. In plants, AsA serves as a major antioxidant and enzyme cofactor and regulates various physiological processes including stress tolerance as well as growth, development, and signal transduction. To accomplish crucial physiological roles adequately, intracellular levels of AsA in plant cells must be tightly regulated. Recent studies have revealed the pathways for AsA biosynthesis and their regulation in plants. In addition, AsA is known to be utilized as a biosynthetic precursor in the formation of several organic acids. This chapter presents up-to-date information on the metabolic processes of AsA, including biosynthesis and degradation, which influence the intracellular concentration of AsA.

Keywords Ascorbic acid · Biosynthesis · Antioxidant · VTC · Redox

1 Introduction

Ascorbic acid (ascorbate/AsA) is a ubiquitous molecule in eukaryotes. AsA is an important antioxidant and has various metabolic functions owing to its redox potential in humans. However, AsA is not synthesized by humans and, therefore, it must be incorporated into the diet as "vitamin C." In plants, which are the main source of vitamin C for humans, AsA is the most abundant and naturally essential compound.

K. Yoshimura (✉)
Department of Food and Nutritional Science, College of Bioscience and Biotechnology, Chubu University, Kasugai, Aichi, Japan
e-mail: k-yosi@isc.chubu.ac.jp

T. Ishikawa
Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane, Japan

M. A. Hossain et al. (eds.), *Ascorbic Acid in Plant Growth, Development and Stress Tolerance*, https://doi.org/10.1007/978-3-319-74057-7_1

AsA plays an important role in many aspects of control not only of the cellular redox state and anti-oxidative activity in plant cells, but also of cell division and cell expansion, and plant development and growth as an enzyme cofactor (Noctor and Foyer 1998; Loewus 1999; Arrigoni and De Tullio 2000; Davey et al. 2000; Smirnoff 2000a; Smirnoff and Wheeler 2000; Conklin 2001; Smirnoff et al. 2001; Smirnoff and Gatzek 2004; Shigeoka and Maruta 2014). In addition, AsA has recently been recognized as having specific functions in redox signaling, such as the response to pathogen insults, the determination of flowering time, and the regulation of the expression of various genes involved in plant growth, hormonal signaling pathways, and stress defense networks (Pastori et al. 2003; Conklin and Barth 2004; Barth et al. 2006; Noctor 2006; Gao et al. 2011).

AsA is present in plant species in concentrations that range from an estimated 300 mM in the chloroplast stroma to less than 20 mM in other organelles (Smirnoff 2000b). Therefore, AsA mostly accumulates in photosynthetic organs but can also reach high concentrations in non-photosynthetic tissues. However, the subcellular concentration of AsA remains controversial since the concentrations in plant organelles have differed in respective reports owing to different methods utilized (Foyer et al. 1983; Rautenkranz et al. 1994; Zechmann et al. 2011). The levels of AsA varies between not only plant species but also different cultivars of the same plant species (Gest et al. 2013; Bulley and Laing 2016). In addition, AsA levels are different between the respective plant tissues and are influenced with a wide range of values by the growth environment. For example, AsA content is generally high in meristematic tissues, flowers or young fruits, root tips, and apices of stolons or tubers (Gest et al. 2013). Light is the most important environmental cue for regulating the biosynthesis of AsA and the accumulation of AsA in plant leaves is enhanced depending on light intensity and is suppressed in the shade (Bartoli et al. 2006; Dowdle et al. 2007; Yabuta et al. 2007). To accomplish its crucial physiological roles adequately and to prevent waste of the carbon source required for AsA synthesis, the levels of AsA in plant cells must be rapidly and tightly regulated. This is achieved at various levels, such as the regulation of gene expression and modulation of enzyme activities involved in the biosynthesis, degradation, and recycling of AsA depending on types of tissues and/or in response to developmental and environmental cues. In addition, subcellular compartmentation and intercellular transport of AsA maintain AsA concentrations in each organelle and tissue. This chapter focuses on the processes of biosynthesis and degradation of AsA that vastly influence the intracellular concentrations of AsA.

2 Chemical Properties and Redox Reaction of Ascorbic Acid

Structurally, AsA is related to hexose sugars and has the conjugated structure of the five-carbon lactone ring, containing an enediol group on C2 and C3. Delocalization of the π-electrons over the C2-C3 enediol group stabilizes the molecule and causes dissociation of the hydrogen of the C3 hydroxyl to become a monovalent anion at physiological pH values since pKa_1 is 4.13 ($pKa_2 = 11.6$). AsA can donate electrons

to a wide range of electron receptors (Gest et al. 2013). There are two experimental values for the redox potential of AsA, +0.06 V (Loach 1976) and +0.35 V (Zhang and Dong 2004). Matsui et al. (2015) computed that the redox potential for AsA ranges from +0.40 to +0.50 V, thus supporting the experimental value of +0.35 V. The redox potential of AsA means it interacts with hydroxyl radicals, singlet oxygen, superoxide, and also with oxidized glutathione and tocopherol radicals (Noctor and Foyer 1998; Gest et al. 2013). AsA can act directly to neutralize such reactive oxygen species (ROS) and also repair oxidized organic molecules, in certain cases more efficiently than glutathione (Tsuchiya et al. 1985; Niki 1991; Sturgeon et al. 1998). The first oxidation product, monodehydroascorbate (MDA), is very stable arising from the way that MDA can delocalize spare electrons around a central carbon ring and its three carbonyl groups (Bielski 1982). A MDA radical also undergoes spontaneous disproportionation with another MDA radical to create an AsA molecule and a non-radical dehydroascorbate (DHA) molecule (Smirnoff 2000b). AsA is regenerated from its oxidized forms by the action of specific reductases and electrons from glutathione, NADH, or NADPH in the AsA–glutathione cycle (Foyer and Noctor 2011) (Molecular structure of DHAR and MDHAR and their roles in modulating abiotic stress tolerance in plants).

3 Metabolism of Ascorbic Acid

3.1 Biosynthetic Pathways of Ascorbic Acid

All animals except for humans, non-human primates, and a few other mammals generate AsA. The committed step in the biosynthesis of AsA consists of a branch of the D-glucuronate pathway via the pentose phosphate pathway in which the stereochemistry of the carbon skeleton of the primary substrate glucose is inverted for AsA via uronate, D-glucuronate, L-gulonate, and L-gulono-1,4-lactone (Burns 1967; Nishikimi and Yagi 1996) (Fig. 1). In this pathway, the microsome-localized enzyme, L-gulono-1,4-lactone oxidase (L-GulLO), catalyzes the final step to produce AsA (Nishikimi and Yagi 1996). Compared to animal cells, plant cells can accumulate much higher levels of AsA (Foyer and Shigeoka 2011). Specifically, the AsA levels in leaf cells often exceed those in chlorophyll and represent over 10% of soluble carbohydrates (Noctor and Foyer 1998). After the first investigations of AsA biosynthesis in plants during the 1950s, the biosynthetic pathway of AsA has created a notable gap in our understanding of carbon metabolism in plants (Smirnoff and Wheeler 2000). However, since 1999 the AsA biosynthetic pathways in plants have been expeditiously uncovered using a combination of genetic and biochemical approaches (Conklin et al. 1999). Until now, three different pathways for AsA biosynthesis via D-mannose/L-galactose, D-glucuronate, and D-galacturonate in plant cells have been proposed (Wheeler et al. 1998; Valpuesta and Botella 2004; Hancock and Viola 2005a, b; Ishikawa et al. 2006a; Ishikawa and Shigeoka 2008) (Fig. 1), which implies that the biosynthetic pathway for AsA differs between animals, plants, and possibly certain species of algae.

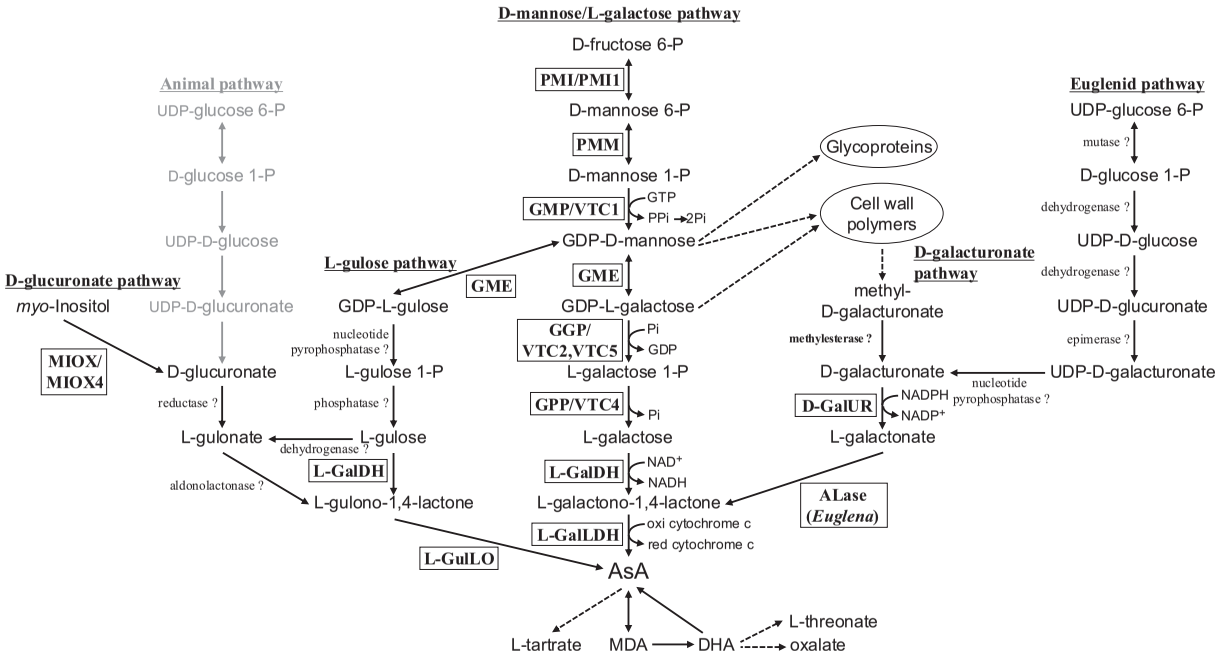

Fig. 1 The metabolisms of AsA in photosynthetic organisms. The D-mannose/L-galactose, D-galacturonate, euglenid, and D-glucuronate pathways, and the degradation pathways are shown. Animal pathway is shown by gray color. The names of enzymes/genes in the AsA biosynthetic pathways are indicated in rectangular boxes. ALase has been identified only from *Euglena*. Question marks indicate possible reactions where gene and specific enzyme have not yet been identified. Dotted line with arrowhead indicates that this reaction is composed of multistep. Among the enzymes involved in the AsA biosynthesis, L-GalLDH is localized to mitochondrial inner membrane, while others are in the cytosol. The conversion of AsA to oxalate via the formation of DHA occurs in the apoplast of cultured *Rosa* sp. cells. *PMI* phosphomannose isomerase; *PMM* phosphomannose mutase; *GMP* GDP-D-mannose pyrophosphorylase; *GME* GDP-D-mannose-3′,5′-epimerase; *GGP* GDP-L-galactose phosphorylase; *GPP* L-galactose-1-P phosphatase; *L-GalDH* L-galactose dehydrogenase; *L-GalLDH* L-L-galactono-1,4-lactone dehydrogenase; *L-GulLO* L-gulono-1,4-lactone oxidase; *MIOX myo*-inositol oxygenase; *D-GalUR* D-galacturonic acid reductase; *ALase* aldonolactonase

3.2 D-Mannose/L-Galactose Pathway

Since the sensitivity of plants to the oxidant ozone are correlated with levels of AsA, in previous studies, vitamin C-deficient (*vtc*) mutants were isolated from ethyl methanesulfonate (EMS)-mutagenized Arabidopsis seedlings that had ozone-sensitive phenotypes (Conklin et al. 2000; Dowdle et al. 2007; Kerchev et al. 2011). When several *vtc* mutants were identified, information on the properties and functions of the enzymes for the D-mannose/L-galactose pathway in a model plant, Arabidopsis, were uncovered (Conklin et al. 2000). The D-mannose/L-galactose pathway has been recognized as the predominant pathway in leaves of higher plants. In addition, the existence of D-mannose/L-galactose pathway has been reported for many fruit-bearing plants such as kiwifruits, acerola, apple, peach, citrus, and tomato (Badejo et al. 2009; Bulley et al. 2009; Imai et al. 2009; Ioannidi et al. 2009; Li et al. 2009) although the contribution of this pathway to the control of AsA levels during fruit ripening is still under debate. Therefore, it is most likely that the other pathways contribute to the tissue- and/or species-specific alternative AsA biosynthesis.

The homologs of all genes involved in the D-mannose/L-galactose pathway are also found in other plant species such as kiwifruit, acerola, tomato, rice, and maize (Badejo et al. 2008; Bulley et al. 2009; Ioannidi et al. 2009; Wheeler et al. 2015). Although AsA biosynthesis in non-vascular plants is poorly understood, it is known that bryophytes and green algae may contain 100-fold less AsA than higher plants contain (Gest et al. 2013; Wheeler et al. 2015), and green algae, bryophytes, and pteridophytes also probably use the D-mannose/L-galactose pathway (Running et al. 2003; Urzica et al. 2012; Wheeler et al. 2015). In this pathway, D-mannose is synthesized from hexose phosphates, such as D-glucose 1- or 6-phosphate and D-fructose 6-phosphate, produced by hexokinase and phosphoglucose isomerase in glycolysis, and proceeds via GDP-D-mannose and L-galactose, with the final aldonolactone precursor of AsA being L-galactono-1,4-lactone. This process does not involve inversion of the carbon chain of the primary substrate for AsA. The D-mannose/L-galactose pathway is composed of eight reaction steps catalyzed by phosphomannose isomerase (PMI), phosphomannose mutase (PMM), GDP-D-mannose pyrophosphorylase (GMP), GDP-D-mannose-3′,5′-epimerase (GME), GDP-L-galactose phosphorylase/L-galactose guanylyltransferase (GGP), L-galactose-1-phosphate phosphatase (GPP), L-galactose dehydrogenase (L-GalDH), and L-galactono-1,4-lactone dehydrogenase (L-GalLDH) (Fig. 1). Except for L-GalLDH, which is localized in the inner membrane of the mitochondria, all remaining enzymes are localized in the cytosol, indicating that almost all predominant AsA biosynthesis steps occur in the cytoplasm of plants. The first four steps of the D-mannose/L-galactose pathway are shared with the synthesis of nucleotide sugars since GDP-D-mannose and GDP-L-galactose are precursors of cell wall polysaccharides and glycoproteins. Therefore, the committed pathway to AsA biosynthesis consists of four subsequent steps, by which GDP-L-galactose is sequentially converted to L-galactose-1-phosphate, L-galactose, L-galactono-1,4-lactone, and

AsA. The molecular and enzymatic properties and functions of these enzymes in higher plants, mainly in a model plant, Arabidopsis, are summarized below.

PMI—For the first step of this pathway in Arabidopsis, there are two enzymes, PMI1 and PMI2, which can catalyze reversible isomerization between D-fructose 6-phosphate and D-mannose 6-phosphate (Dowdle et al. 2007; Maruta et al. 2008). The K_m and V_{max} values for mannose 6-phosphate of the recombinant PMI1 have been shown to be 41.3 μM and 1.89 μmol min^{-1} mg^{-1} protein, respectively, whereas those of the recombinant PMI2 are 372 μM and 22.5 μmol min^{-1} mg^{-1} protein, respectively (Maruta et al. 2008). Both PMI1 and PMI2 are inhibited by EDTA, Zn^{2+}, Cd^{2+}, and AsA. The expression of PMI2 (also named DARK INDUCIBLE 9) has been detected in leaves only 24 h after dark treatment (Fujiki et al. 2001) although the levels of AsA increased under light as described above. On the other hand, the expression levels of PMI1 changed largely in parallel with leaf AsA levels during dark and light periods (Maruta et al. 2008). The analyses of Arabidopsis plants suppressing or depleting their expression have clearly shown that PMI1 is the sole enzyme for the AsA biosynthesis; that is, the approximately 70% decrease in PMI activity by the suppression of *PMI1* expression using the RNAi method caused an approximately 50% decrease in leaf AsA levels although the complete inhibition of *PMI2* expression was not affected (Maruta et al. 2008). However, conditional overexpression of the *PMI1* expression did not result in an increase in AsA levels (Yoshimura et al. 2014), indicating that the reaction catalyzed by this enzyme is not the limiting step in the D-mannose/L-galactose pathway. Conversely, since PMI was inhibited by high concentrations of AsA, feedback inhibition at this step of AsA biosynthesis has been shown to be involved in regulating intracellular AsA levels (Maruta et al. 2008).

PMM—PMM catalyzes reversible isomerization between D-mannose-6-phosphate and D-mannose-1-phosphate. The recombinant Arabidopsis PMM is able to catalyze the conversion of not only mannose 1-phosphate into mannose 6-phosphate but also glucose 1-phosphate into glucose 6-phosphate (Qian et al. 2007). However, the former reaction (V_{max}, 14.4 μmol min^{-1} mg^{-1} protein; K_m, 29.7 μM) was more efficient than the latter reaction (V_{max}, 1.40 μmol min^{-1} mg^{-1} protein; K_m, 65.4 μM). A decrease in the expression levels of *PMM* using a gene silencing method caused a substantial decrease in the AsA levels in the leaves of *Nicotiana benthamiana* (Qian et al. 2007). In contrast, an increase in the *PMM* expression levels led to a 20–50% increase in the AsA levels in *N. benthamiana* and Arabidopsis, suggesting that the step catalyzed by PMM has little effect on the overall rate of AsA biosynthesis. However, the activity of PMM in Arabidopsis leaves was remarkably high even under low light conditions (Dowdle et al. 2007).

GMP—GMP catalyzes the reversible conversion of GTP and D-mannose 1-phosphate into diphosphate and GDP-D-mannose; however, the substrate, diphosphate, is readily degraded into two phosphates and, therefore, this reaction is irreversible. The crystal structure of the recombinant GMP from Arabidopsis has been investigated (Zhao and Liu 2016). The *GMP* gene was first identified as the gene responsible for AsA-deficiency from the Arabidopsis *vtc* mutants and, therefore, was named *VTC1* (Conklin et al. 1996, 1999). This mutant had missense mutation

in the *GMP/VTC1* gene leading to an approximately 40% decrease in GMP activity and thereby accumulated only approximately 25% of the normal AsA levels. Similar to the result in Arabidopsis (Keller et al. 1999), the expression levels of *GMP/VTC1* were correlated with the AsA levels in several plant species (Badejo et al. 2007, 2008). These results suggest that some control of the AsA biosynthesis could occur at this step. However, conditional overexpression of the *GMP/VTC1* gene did not result in an increase in AsA levels (Yoshimura et al. 2014). The photomorphogenic factor, COP9 signalosome subunit 5B, has been shown to promote ubiquitination-dependent GMP/VTC1 degradation via the 26S proteasome pathway, which suppressed the overaccumulation of AsA under both light and dark conditions (Wang et al. 2013). This suggests the importance of degradation of the GMP/VTC1 enzyme in the regulation of AsA biosynthesis.

Since GDP-D-mannose is also an important intermediate in cell wall synthesis, protein *N*-glycosylation, and glycosylphosphatidylinositol-anchoring in plants (Lukowitz et al. 2001), the modulation of intracellular levels of GDP-D-mannose could be important for maintaining various cellular processes. In fact, the Arabidopsis mutant *cyt1*, which is a mutant allele of the *vtc1* mutants, showed not only lower AsA levels, but also more severe phenotypes, such as ectopic accumulation of callose and occurrence of incomplete cell walls, than the *vtc1* mutants (Lukowitz et al. 2001). Therefore, it is clear that the reaction catalyzed by this enzyme results in many downstream consequences. A relationship has been reported between the metabolism of GDP-D-mannose and sensitivity to ammonium (NH_4^+), a major nitrogen source for plants. The root growth of *vtc1* mutants was stunted in the presence of NH_4^+, whereas they developed roots similar to those of the wild-type plants in the absence of NH_4^+ (Barth et al. 2010). The *vtc1* mutants were also found to have *N*-glycosylation defects, enhanced programmed cell death, and some cell cycle defects in the presence of NH_4^+ (Qin et al. 2008; Kempinski et al. 2011). An Arabidopsis GDP-D-mannose pyrophosphohydrolase, AtNUDX9, which hydrolyzes GDP-D-mannose to guanosine monophosphate and mannose 1-phosphate, has been identified (K_m = 376 ± 24 μM; V_{max} = 1.61 μmol min^{-1} mg^{-1} protein) (Tanaka et al. 2015). Therefore, it is suggested that AtNUDX9 and GMP/VTC1 constitute a futile cycle and function cooperatively in fine-tuning the GDP-D-mannose levels. Although the kinetic parameters of GMP/VTC1 have not yet been demonstrated, it is likely that the production capacity of GDP-D-mannose by the action of GMP/VTC1 is much higher than its hydrolysis by AtNUDX9, at least under normal conditions. In fact, no difference was observed in the levels of AsA in the wild-type and KO-*nudx9* plants, whereas a marked increase in *N*-glycoprotein levels and enhanced growth were detected in the KO-*nudx9* plants in the presence of NH_4^+ (Tanaka et al. 2015).

GME—GDP-D-mannose and GDP-L-galactose are precursors of cell wall polysaccharides and glycoproteins, which implies the importance of the equilibrium constant of the epimerization by GME. GME catalyzes the 3′ and 5′ epimerization of GDP-D-mannose, by which GDP-L-galactose is produced (Wolucka et al. 2001). However, GDP-L-galactose is not the only epimerization product. The native and recombinant GME from Arabidopsis also produces GDP-L-gulose as an

interconversion compound, which results from 5′-epimerization of the GDP-D-mannose (Wolucka and Van Montagu 2003). By the reaction of GME, GDP-L-gulose was in equilibrium with GDP-D-mannose and GDP-L-galactose (0.2:1.0:0.4). The K_m and V_{max} values for GDP-D-mannose of the native and recombinant GME in Arabidopsis have been shown as 4.5–31 μM and 0.31–1.76 μmol min^{-1} mg^{-1} protein, respectively (Wolucka and Van Montagu 2003). GDP-L-gulose can initiate an alternative branch (the L-gulose pathway) of the D-mannose/L-galactose pathway, suggesting that GME may impact on the flow of intermediates into these pathways. It has been demonstrated that exogenous L-gulose and L-gulono-1,4-lactone serve as direct precursors to AsA in certain plant species (Wolucka and Van Montagu 2003). In addition, the intermediate L-gulonate has been detected in plant cells (Wagner et al. 2003). As described below, L-GalDH can convert L-gulose into L-gulono-1,4-lactone, which might be sequentially oxidized to AsA by the reaction of L-GulLO. However, it remains unclear how the relative contribution of the L-gulose pathway to AsA biosynthesis occurs in vivo. The activity of GME in Arabidopsis leaves was remarkably low even under high light conditions (Dowdle et al. 2007). As described above, the recombinant GME has very low V_{max} compared to the other enzymes involved in the D-mannose/L-galactose pathway (Wolucka and Van Montagu 2003; Dowdle et al. 2007). In addition, a feedback inhibition of this enzyme by AsA has been observed (Wolucka and Van Montagu 2003). Therefore, it is suggested that this step is important in the limitation of subcellular AsA levels. However, conditional overexpression of the *GME* did not result in an increase in AsA levels (Yoshimura et al. 2014). On the other hand, transient co-expression of both the *GGP/VTC2* (known as the rate-limiting enzyme in the D-mannose/L-galactose pathway) and *GME* genes in tobacco leaves have been shown to result in an approximately eightfold increase in AsA levels, while the *GGP/VTC2* gene only resulted in an approximately fourfold increase. This suggests the importance of the step catalyzed by GME as the second rate-limiting step following GGP/VTC2 in the D-mannose/L-galactose pathway (Bulley et al. 2009).

GGP—GGP catalyzes the first committed step in the AsA biosynthesis via the D-mannose/L-galactose pathway. This enzyme converts GDP-L-galactose to L-galactose 1-phosphate. Among the ozone-sensitive AsA-deficient Arabidopsis *vtc* mutants, three mutants had a mutation in the gene encoding GGP, which was named *vtc2* (Conklin et al. 2000). Originally, it had been reported that one of the three mutant alleles, the *vtc2-1* that has complete loss of the GGP activity, had approximately 20–30% wild-type AsA levels and showed decreased growth under normal conditions, suggesting that 70–80% loss of AsA levels may affect plant growth. However, subsequent analyses using the other mutant (KO-*vtc2/vtc2-4*) with T-DNA insertion into the *GGP/VTC2* gene and segregation analysis of F2 progeny from crossing between the wild-type and *vtc2-1* mutants revealed that the *vtc2-1* mutants have an independent cryptic mutation affecting growth, and a 70–80% decrease in AsA levels (at least in Arabidopsis) does not cause growth inhibition (Lim et al. 2016).

There has been some controversy regarding whether the physiological guanylyl acceptor of the reaction by GGP/VTC2 is inorganic phosphate, which leads to a phosphorylase activity that generates GDP, or a hexose 1-phosphate such as D-

mannose 1-phosphate, which leads to a transferase activity that generates the corresponding GDP-hexose (Laing et al. 2007; Linster et al. 2007; Wolucka and Van Montagu 2007; Linster and Clarke 2008). Laing et al. (2007) and Wolucka and Van Montagu (2007) have proposed the VTC2 cycle, whereby L-galactose 1-phosphate is produced without the reactions by PMI, PMM, and GMP. However, suppression of the expression of *PMM* and *GMP* caused a decrease in AsA levels (Conklin et al. 1999; Qian et al. 2007), indicating the involvement of the steps producing GDP-L-galactose from D-fructose 6-phosphate during AsA biosynthesis. A dual radiolabeling experiment in Arabidopsis cell cultures indicated the in vivo involvement of PMI in GDP-D-mannose synthesis (Sharples and Fry 2007). In addition, GGP/VTC2 was discovered to be more than 100-fold more efficient as a GDP-L-galactose phosphorylase than as a GDP-L-galactose-D-glucose 1-phosphate guanylyltransferase (Linster et al. 2008). Therefore, at present, there is general agreement that GGP/VTC2 acts predominantly as a phosphorylase.

Arabidopsis also possesses the other gene (*VTC5*) that encodes the GGP enzyme. Judging from the results that AsA levels in the T-DNA insertion mutants, KO-*vtc2* and KO-*vtc5*, were approximately 20% and 80%, respectively, it is clear that GGP/VTC2 is predominant over GGP/VTC5 for AsA biosynthesis (Dowdle et al. 2007). The double KO mutant of *GGP/VTC2 and GGP/VTC5* genes showed seedling lethal without supplementation with AsA or L-galactose, indicating that the reaction catalyzed by GGP is indispensable for biosynthesis of AsA. The fact that the expression levels of *GGP/VTC2* and *GGP/VTC5* genes were relatively high in leaves and roots, respectively, suggests that GGP/VTC2 and GGP/VTC5 are important for AsA biosynthesis in photosynthetic and non-photosynthetic tissues, respectively (Dowdle et al. 2007).

The step catalyzed by GGP appears to have a major role in the regulation of AsA biosynthesis. The activity of GGP in Arabidopsis leaves was relatively low under both low and high light conditions (Dowdle et al. 2007). The expression of *GGP/VTC2* and *GGP/VTC5* is demonstrated to be in parallel with the subcellular AsA levels. For example, levels of both *GGP/VTC2* gene expression and AsA increased under light irradiation and their degree was facilitated depending on light intensity (Dowdle et al. 2007; Gao et al. 2011). On the other hand, there is feedback suppression of *GGP/VTC2* expression at post-transcriptional levels under high concentrations of AsA (Laing et al. 2015). The GGP/VTC2 mRNA has an upstream open reading frame (uORF) encoding a peptide, which may function in the inhibition of translation. Studies have shown that disruption of the uORF abolished the feedback regulation of GGP/VTC2 translation and increased the subcellular AsA levels, and high levels of ribosomes were colocated with both the uORF and the downstream open reading frame of GGP/VTC2. Therefore, it was proposed that the uORF translated preferentially rather than the downstream open reading frame of GGP/VTC2 under high AsA levels, leading to suppression of *GGP/VTC2* expression (Laing et al. 2015). The uORF is conserved in *GGP/VTC2* genes from mosses to angiosperms, implying its ubiquity. Yoshimura et al. (2014) reported that conditional overexpression of the *GGP/VTC2* gene without uORF markedly increased AsA levels in Arabidopsis seedlings, while that of the other

genes encoding GMP/VTC1, GPP/VTC4, GME, and PMI did not. In addition, the constitutive overexpression of the *GGP/VTC2* gene enhanced AsA levels in the leaves of tomato, strawberry, and potato plants (Bulley et al. 2012). These findings clearly indicate that GGP/VTC2 catalyzes the rate-limiting step in the D-mannose/L-galactose pathway, and the expression of *GGP/VTC2* is regulated to maintain proper levels of AsA in the cell.

As described above, all enzymes except for L-GalLDH are localized in the cytosol. However, Müller-Moulé (2008) demonstrated that a VTC2:YFP fusion protein is found not only in the cytosol where AsA biosynthesis occurs, but also in the nucleus, implying that VTC2 might have a dual function as the GGP enzyme and as a regulatory factor. Orthologs of the *GGP/VTC2* gene are found in invertebrates, vertebrates, and plants, and these genes show a low similarity with members of the histidine triad (HIT) protein superfamily, which are characterized as nucleotide-binding proteins and hydrolases (Linster et al. 2007; Müller-Moulé 2008).

GPP—GPP catalyzes a dephosphorylation reaction, by which L-galactose 1-phosphate is converted into L-galactose and inorganic phosphate. Originally, this gene had been annotated as a *myo*-inositol-1-phosphate phosphatase in GenBank. However, partially purified GPP from young kiwifruit (*Actinidia deliciosa*) berries and its recombinant proteins expressed by *E. coli* were very specific to L-galactose 1-phosphate. The activity was completely dependent on Mg^{2+}. The K_m value for L-galactose 1-phosphate of the partially purified enzyme was demonstrated to be 20–40 μM and a K_a for Mg^{2+} was 0.2 mM (Laing et al. 2004). Similar results were observed in the recombinant GPP from tobacco (*Nicotiana tabacum*) (Sakamoto et al. 2012). The *VTC4* gene, initially identified from the Arabidopsis *vtc* mutants (Conklin et al. 2000), encodes the GPP enzyme (Laing et al. 2004; Conklin et al. 2006). However, the *vtc4* mutants, which have a missense mutation in the *GPP/VTC4* gene causing complete loss of production of the encoded protein, is reported to be only partially decreased in AsA levels (by approximately 60%) as well as GPP activity (by approximately 40%) (Conklin et al. 2006; Torabinejad et al. 2009). In addition, GPP activity did not change under high light conditions although the expression levels of GPP/VTC4 mRNA as well as the AsA levels were correlated with light intensity (Dowdle et al. 2007; Yabuta et al. 2007). These facts suggest that GPP/VTC4 is not the sole enzyme catalyzing the GPP reaction in Arabidopsis. The purple acid phosphatase, AtPAP15, is a candidate for the remaining GPP activity in Arabidopsis since overexpression of the *AtPAP15* gene has been shown to increase AsA levels in the leaves of Arabidopsis (Zhang et al. 2008).

L-*GalDH*—L-GalDH catalyzes the conversion of L-galactose and NAD^+ into L-galactono-1,4-lactone and NADH. Antisense suppression of L-GalDH in Arabidopsis has been reported to result in a 70% decrease in expression levels and thereby the AsA levels decrease by 50% (Gatzek et al. 2002). In addition, the activity of L-GalDH in Arabidopsis leaves was remarkably high even under low light conditions (Dowdle et al. 2007). These results imply that this reaction is not a limiting step *in planta*. On the other hand, L-GalDH from spinach exhibited reversible competitive inhibition by AsA with a K_i of 0.13 mM (Mieda et al. 2004), sug-

gesting a feedback inhibition of AsA biosynthesis at this step. However, substantial accumulation of AsA was observed after L-galactose feeding (Wheeler et al. 1998) and overexpression of Arabidopsis L-GalDH in tobacco did not influence AsA levels (Gatzek et al. 2002). These results suggest that the inhibition of L-GalDH by AsA is absent or attenuated in vivo.

L-*GalLDH*—L-GalLDH is a flavoprotein and catalyzes the last step of the D-mannose/L-galactose pathway where AsA is produced from L-galactono-1,4-lactone (Leferink et al. 2008). The enzymatic properties of this enzyme in various plant species have been studied extensively (Oba et al. 1995; Østergaard et al. 1997; Imai et al. 1998; Yabuta et al. 2000). For example, the K_m values for L-galactono-1,4-lactone of the native and recombinant enzymes from tobacco have been stated as 0.08 mM and 0.06 mM, respectively, while the values for cytochrome *c* as the electron donor are 3.6 μM and 3.7 μM, respectively (Yabuta et al. 2000). The tobacco L-GalLDH has been shown to be extremely inhibited by incubation with *N*-ethylmaleimide, *p*-chloromercuribenzoate, and divalent cations such as Cu^{2+} and Zn^{2+}. Notably, the reaction of L-GalLDH involved electron transfer to cytochrome *c*, suggesting its association with the mitochondrial respiratory chain (Smirnoff et al. 2001; Hancock and Viola 2005a, b; Leferink et al. 2008). In fact, unlike the other enzymes in the D-mannose/L-galactose pathway, L-GalLDH has been reported to localize to the mitochondrial inner membrane as an integral protein (Siendones et al. 1999; Bartoli et al. 2000); however, it has also been suggested to be a peripheral protein (Leferink et al. 2008). Importantly, it has been reported that L-GalLDH is detected in a smaller form of complex I (NADH:ubiquinone oxidoreductase) (Heazlewood et al. 2003; Schertl et al. 2012; Schimmeyer et al. 2016). In addition, L-GalLDH has been demonstrated to be required for the accumulation of complex I since complex I was not detected in the KO mutants of the L-*GalLDH* gene (Pineau et al. 2008). From these results, it has been proposed that L-GalLDH is a dual-function protein that has a second, non-enzymatic function in complex I assembly as a plant-specific assembly factor (Schimmeyer et al. 2016).

The expression level and enzyme activity of L-GalLDH have been observed to positively correlate with AsA levels in various plant tissues (Smirnoff 2000b; Ishikawa et al. 2006a; Dowdle et al. 2007; Yabuta et al. 2007). However, an 80% decrease in L-GalLDH activity using the RNAi method in tomato did not cause any decrease in AsA levels, even though growth rates and fruit development of the RNAi plants were decreased and inhibited, respectively (Alhagdow et al. 2007). These effects on growth rates and fruit development have been demonstrated to be accompanied by significant changes in mitochondrial function and altered redox state of AsA. A combination of transcriptomic and metabolomic approaches have shown that several primary metabolic pathways, such as the tricarboxylic acid cycle, as well as secondary metabolic pathways related to stress response are modified in leaves and fruit of RNAi plants. These results suggest that the role of L-GalLDH in the complex I assembly is linked to plant metabolism rather than to AsA biosynthesis.

3.3 D-Galacturonate Pathway

The D-galacturonate pathway, which has been identified in strawberry fruits (*Fragaria x ananassa*), involves inversion of the carbon skeleton of the primary substrate glucose (Li et al. 2010) (Fig. 1). All the gene encoding enzymes that are involved in this pathway have not been fully identified yet. The strawberry D-galacturonate reductase (D-GalUR), encoded by *AKR2* that shared significant homology to cytosolic NAD(P)H-dependent aldo-keto reductases (AKRs), catalyzes production of L-galactonate from D-galacturonate (Agius et al. 2003). Among diverse cultivars of strawberry and different *Fragaria* species, a correlation between D-GalUR and AsA content was apparent in most cases (Cruz-Rus et al. 2011). The proteins most similar to AKR2 are a codeinone reductase from the opium poppy (*Papaver somniferum*) and chalcone reductases from soybean (*Glycine max*), alfalfa (*Medicago sativa*), and a tropical legume (*Sesbania rostrata*). However, this similarity is mainly restricted to the consensus motif characteristic of AKRs (Agius et al. 2003). Aldonolactonase (ALase) that is required to catalyze the conversion of L-galactonate to L-galactono-1,4-lactone has not been found. L-galactono-1,4-lactone is a substrate of L-GalLDH, which is the enzyme catalyzing the final step of the D-mannose/L-galactose pathway as described above. This pathway was activated during fruit ripening, demonstrating its importance in the accumulation with considerably higher levels of AsA in fruits. The precursor to the D-galacturonate pathway is likely to be either cell wall pectin or UDP-glucuronate epimerization to UDP-galacturonate (Agius et al. 2003; Di Matteo et al. 2010; Badejo et al. 2012). That is, this pathway could constitute a carbon salvage mechanism after the breakdown of cell walls in ripening fruits. However, the importance of the D-galacturonate pathway in the fruits of other plant species is largely unknown. A positive correlation between the AsA levels and the expression levels of genes involved in the D-mannose/L-galactose pathway has been found in the fruits of some plant species. For example, the levels of AsA and expression of *GMP*, *GGP/VTC2*, L-*GalDH*, and L-*GalLDH* in orange (*Citrus sinensis Osb.*) during fruit ripening were higher than those in Satsuma mandarin (*Citrus unshiu Marc.*) (Yang et al. 2011). Similarly, in the fruits of kiwifruit species (*Actinidia eriantha*, *A. chinensis*, and *A. deliciosa*), the rise in AsA levels during ripening occurred close to the peak in expression of *GGP/VTC2* and *A. eriantha* showed highest levels of both AsA and *GGP/VTC2* expression (Bulley et al. 2009). Therefore, it is reasonable that this pathway shares the control of fruit AsA levels with the D-galacturonate pathways depending on ripening stage and/or plant species (Melino et al. 2009; Cruz-Rus et al. 2011; Badejo et al. 2012; Gest et al. 2013). In fact, it has been demonstrated that tomato fruits (Micro-Tom) could switch between different sources for AsA supply depending on their ripening stages (Badejo et al. 2012). L-galactose feeding, but neither D-galacturonate nor L-gulono-1,4-lactone, enhanced the levels of AsA in immature green fruit. In contrast, L-galactose and D-galacturonate, but not L-gulono-1,4-lactone, resulted in an increase in the AsA levels of red ripened fruits. In addition,

crude extract prepared from insoluble fractions of green and red fruits showed the D-GalUR and ALase activities involved in the D-galacturonate pathway. Translocation of the precursor of AsA and/or AsA itself from leaves, in which AsA is synthesized predominantly by the D-mannose/L-galactose pathway, also impacts on the AsA levels of fruits (Badejo et al. 2012) (Ascorbate transporter in plants). Overexpression of *GalUR* in Arabidopsis resulted in a two- to threefold increase in leaf AsA levels, implying the existence of this pathway in plant leaves.

3.4 Euglenid Pathway

In the unicellular alga, *Euglena gracilis*, the biosynthesis of AsA is proceeded via the conversion of UDP-D-glucuronate to UDP-D-galacturonate, with production of D-galacturonate and reduction to L-galactonate (Shigeoka et al. 1979a) (Fig. 1). *Euglena* D-galacturonate reductase (D-GalUR) was demonstrated to catalyze the reduction of D-galacturonate to L-galactonate, which is subsequently converted to L-galactono-1,4-lactone (Shigeoka et al. 1979a; Ishikawa et al. 2006b). In addition, *Euglena* cells have ALase that shows significant sequence identity with rat gluconolactonase, a key enzyme for the production of AsA via D-glucuronate in animals (Ishikawa et al. 2008). *Euglena* ALase catalyzed the reversible reaction of L-galactonate and L-galactono-1,4-lactone. Although D-GalUR and ALase had almost the same catalytic efficiency with uronates, *Euglena* accumulated greater than two-fold amounts of AsA on supplementation with D-galacturonate than with D-glucuronate (Ishikawa et al. 2008) and *Euglena* ALase had higher activity with L-galacturonate than with L-glucuronate (Shigeoka et al. 1979a, b; Ishikawa et al. 2008). These findings indicate that the pathway via D-galacturonate/L-galactonate, which is analogous to both the animal D-glucuronate pathway and the plant D-mannose/L-galactose pathway and a hybrid of them, is predominantly utilized to produce AsA in *Euglena* cells. A similar pathway appears to be functional in some stramenopile algae (Helsper et al. 1982; Grün and Loewus 1984). This pathway involves inversion of the carbon chain of primary substrate. Wheeler et al. (2015) proposed that the host for certain eukaryote lineages, such as diatoms, haptophytes, and euglenids initially synthesized AsA via an animal-type pathway (involving inversion of the chain and L-GulLO) and that the red or green algal symbiont used a plant-type pathway (involving non-inversion of the carbon chain and L-GalLDH). However, neither pathway appears to operate in photosynthetic eukaryotes with secondary plastids, including *Euglena*, which instead uses a euglenid-type D-galacturonate/L-galactonate pathway. In such organisms, the endosymbiotic gene transfer of L-*GalLDH* from the symbiont may have resulted in the functional replacement of L-*GulLO* in the animal-type pathway of the host leading to a hybrid biosynthetic pathway involving inversion of the carbon chain that employed D-galacturonate rather than D-glucuronate as the intermediate to provide L-galactono-1,4-lactone as a substrate for L-GalLDH.

3.5 D-Glucuronate Pathway

The precursor to the D-glucuronate pathway without inversion of the carbon skeleton is likely to be either from UDP-glucose or from *myo*-inositol, which are converted to (UDP-) D-glucuronate (Lorence et al. 2004; Li et al. 2010) (Fig. 1). All enzymes involved in this pathway have not been fully identified yet; however, the Arabidopsis *myo*-inositol oxygenase (MIOX4) that catalyzes the production of D-glucuronate from *myo*-inositol has been characterized (Lorence et al. 2004). By this pathway, D-glucuronate appears to be converted to L-gulono-1,4-lactone, which is subsequently converted to AsA by L-GulLO, a homolog of an animal enzyme (Maruta et al. 2010). However, contribution of this pathway to the AsA biosynthesis *in planta* is controversial although overexpression of the *MIOX4* and L-*GulLO* genes have increased AsA levels of the transgenic plants (Jain and Nessler 2000; Agius et al. 2003; Lorence et al. 2004; Maruta et al. 2010).

3.6 Degradation of Ascorbic Acid

The balance between synthesis and degradation could affect the intracellular levels of AsA. There is evidence of a relatively high turnover rate of AsA in some plant tissues (Pallanca and Smirnoff 2000). AsA is not a stable metabolic end-product nor is limited to oxidation–reduction reactions that alter the balance of AsA to DHA. That is, one of the multifunction of AsA is as a biosynthetic precursor. It has been demonstrated that AsA is catabolized to form oxalate, L-threonate, and L-tartrate in certain plants (Loewus 1999; Bánhegyi and Loewus 2004; Debolt et al. 2007; Melino et al. 2009) (Fig. 1). Cleavage of AsA between carbon atoms 2 and 3 results in the formation of oxalate from carbon atoms 1 and 2, and L-threonate from carbon atoms 3 to 6 via L-idonate (Wagner and Loewus 1973; Debolt et al. 2007). L-Threonate may be further oxidized to form L-tartrate. The cleavage reactions may follow a number of steps involving delactonization, oxidation, and reduction. In the majority of plants, AsA degradation has been demonstrated to occur via DHA, yielding oxalate and L-threonate (Yang and Loewus 1975; Green and Fry 2005; Helsper and Loewus 1982; Saito et al. 1997; Bánhegyi and Loewus 2004; deBolt et al. 2004). It appears that the cleavage pathways of AsA are different depending on plant species; however, the entire cleavage pathways remain unresolved at present.

In Arabidopsis mature leaves, the loss or turnover of AsA has been demonstrated to be only about 2.5% of the pool per hour, while in embryonic axes of pea seedlings, the turnover is about 13% per hour (Conklin et al. 1997; Pallanca and Smirnoff 2000; Melino et al. 2009). In blackcurrant (*Ribes nigrum L.*), AsA turnover of fruits was low at 1.41% of the total AsA pool per hour during the early stage of ripening and was increased to excess of 3% per hour during late ripening stage (Hancock et al. 2007). The rate of AsA turnover in high oxalate or tartrate accumulating plants, such as in grape berries, is yet to be established (Melino et al. 2009).

L-Tartrate does not commonly accumulate in plants and the *in planta* function of accumulation of this compounds remains unclear. However, it is clear that L-tartrate is produced as the dominant organic acid in the vacuoles of grape berries (*Vitis vinifera*) although the berries do not accumulate large amounts of AsA in comparison to other fruits where fruit acidity is conferred by malic acid, citric acid, or AsA. L-Tartrate in grape berries has been found to increase during the initial 4 weeks post-anthesis and remain unchanged until maturation (Iland and Coombe 1988). Economically, L-tartrate plays a critical role in determining the suitability of grapes for use in winemaking; berry L-tartrate is largely responsible for controlling juice pH through L-tartrate addition during vinification, the winemaker can minimize oxidative and microbial spoilage, thereby promoting both organoleptic and aging potentials of the finished wine (Debolt et al. 2007). In grape berries, cleavage of the intermediate 5-keto-D-gluconate between carbon atoms 4 and 5 leads to L-tartrate formation, with the 2-carbon fragment of atoms 5 and 6 putatively recycled into central metabolic pathways (Saito and Kasai 1969, 1982; Saito et al. 1997; DeBolt et al. 2006; Melino et al. 2009). The synthesis of oxalate and L-tartrate has been found to occur within these organs (DeBolt et al. 2004).

While the accumulation of L-tartrate is limited to a handful of plant genera, oxalate is widely distributed and often forms crystals of calcium oxalate (Stafford 1959; Debolt et al. 2007). Oxalate-producing plants accumulate oxalate in the range of 3–80% (w/w) of their dry weight. Members of more than 215 plant families including many crop plants accumulate crystals within their tissues (McNair 1932; Nakata 2003). Crystals have been observed in virtually all the tissues of a plant, and they most often accumulate within the vacuoles of specialized cells called crystal idioblasts (Horner and Wagner 1995). In plants that accumulate significant amounts of calcium oxalate, this compound has been found to form raphides, needle-shaped crystals often clustered into bundles; this has been proposed to allow toxins to be secreted into the wounds produced upon contact with the crystal tips (Franceschi and Nakata 2005). In addition, accumulation of calcium oxalate crystals in plant tissues may be involved in regulation of cellular calcium levels and sequestration of toxic metals, and resistance to herbivory (Nakata 2003; Franceschi and Nakata 2005).

Green and Fry (2005) have demonstrated that the conversion of AsA to oxalate via the formation of DHA occurs in the apoplast of cultured *Rosa* sp. cells (Fig. 1). This pathway produces oxalate and L-threonate via the uncharacterized intermediate 4-*O*-oxalyl-L-threonate, which is formed from DHA and involves at least one new soluble esterase having hydrolysis activity toward the intermediate. The pathway can also operate non-enzymatically, accounting for approximately 50% of total AsA loss. Four steps in this pathway may generate peroxide; this may contribute to the role of AsA as a pro-oxidant, which is potentially capable of loosening the plant cell wall accompanying physiological processes such as fruit ripening and softening, and/or triggering an oxidative burst.

4 Conclusions

This chapter has highlighted recent knowledge of the biosynthetic and degradation pathways of AsA. The control of intracellular AsA levels in cells potentially involves regulation of biosynthesis, degradation, recycling, and transport of this compound. Current knowledge about recycling and transport of AsA, and regulation of its biosynthesis has been reviewed in other chapters.

There is no clear evidence for AsA biosynthesis in prokaryotes, suggesting that the ability to synthesize AsA evolved in eukaryotes. Wheeler et al. (2015) proposed that ancestral eukaryotes developed multiple antioxidant mechanisms to protect themselves from ROS derived from organelles. However, evolutionary events such as the transition to an aerobic environment and to life on land have exposed organisms to new and challenging environments. The requirement of a large amount of energy by aerobic metabolism in eukaryotes might lead to increased requirements for cellular antioxidants to protect the host cell from ROS. In addition, acquisition of a photosynthetic cyanobacterial endosymbiont in photosynthetic eukaryotes has resulted in a greatly increased requirement for cellular antioxidants to protect the host cell from ROS secreted by the plastid. Especially, plants must show particularly good adaptation capacity as they are unable to move and must withstand various environmental conditions. Via evolutionary processes, photosynthetic eukaryotes might have potentiated the capability of AsA biosynthesis to maintain proper redox states in the cells and evolved multiple functions of AsA. In addition, by accumulating in fruits and seeds, AsA can affect a plant's reproductive capacity via roles during the development of these organs and the seed dormancy and germination, in addition to ensuring seedling and plant survival in different environments (Gest et al. 2013).

Acknowledgement This work was partially supported by the Ministry of Education, Culture, Sports, Science, and Technology of Japan [Grant-in-Aid for Scientific Research (B) (to K.Y and T.I: 17H03807)].

References

Agius F, Gonzalez-Lamothe R, Caballero J, Munoz-Blanco J, Botella M, Valpuesta V (2003) Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. Nat Biotechnol 21:177–181

Alhagdow M, Mounet F, Gilbert L, Nunes-Nesi A, Garcia V, Just D, Petit J, Beauvoit B, Fernie AR, Rothan C, Baldet P (2007) Silencing of the mitochondrial ascorbate synthesizing enzyme L-galactono-1,4-lactone dehydrogenase affects plant and fruit development in tomato. Plant Physiol 145:1408–1422

Arrigoni O, De Tullio MC (2000) The role of ascorbic acid in cell metabolism: between gene-directed functions and unpredictable chemical reactions. J Plant Physiol 157:481–488

Badejo AA, Jeong ST, Goto-Yamamoto N, Esaka M (2007) Cloning and expression of GDP-D-mannose pyrophosphorylase gene and ascorbic acid content of acerola (*Malpighia glabra* L.) fruit at ripening stages. Plant Physiol Biochem 45:665–672

Badejo AA, Tanaka N, Esaka M (2008) Analysis of GDP-D-mannose pyrophosphorylase gene promoter from acerola (*Malpighia glabra*) and increase in ascorbate content of transgenic tobacco expressing the acerola gene. Plant Cell Physiol 49:126–132

Badejo AA, Fujikawa Y, Esaka M (2009) Gene expression of ascorbic acid biosynthesis related enzymes of the Smirnoff-Wheeler pathway in acerola (*Malpighia glabra*). J Plant Physiol 166:652–660

Badejo AA, Wada K, Gao YS, Maruta T, Sawa Y, Shigeoka S, Ishikawa T (2012) Translocation and the alternative D-galacturonate pathway contribute to increasing the ascorbate level in ripening tomato fruits together with the D-mannose/Lgalactose pathway. J Exp Bot 63:229–239

Bánhegyi G, Loewus FA (2004) Ascorbic acid catabolism: breakdown pathways in animals and plants. In: Asard H, May JM, Smirnoff N (eds) Vitamin C: functions and biochemistry in animals and plants. BIOS Scientific Publishers Ltd, Oxford, pp 31–48

Barth C, De Tullio M, Conklin PL (2006) The role of ascorbic acid in the control of flowering time and the onset of senescence. J Exp Bot 57:1657–1665

Barth C, Gouzd ZA, Steele HP, Imperio RM (2010) A mutation in GDP-mannose pyrophosphorylase causes conditional hypersensitivity to ammonium, resulting in Arabidopsis root growth inhibition, altered ammonium metabolism, and hormone homeostasis. J Exp Bot 61:379–394

Bartoli CG, Pastori GM, Foyer CH (2000) Ascorbate biosynthesis in mitochondria is linked to the electron transport chain between complexes III and IV. Plant Physiol 123:335–343

Bartoli CG, Yu JP, Gomez F, Fernandez L, McIntosh L, Foyer CH (2006) Inter-relationships between light and respiration in the control of ascorbic acid synthesis and accumulation in *Arabidopsis thaliana* leaves. J Exp Bot 57:1621–1631

Bielski BHJ (1982) Chemistry of ascorbic acid radicals. In: Seib PA, Tolbert BM (eds) Ascorbic acid: chemistry, metabolism and uses, vol 200. American Chemical Society, Washington, DC, pp 81–100

Bulley S, Laing W (2016) The regulation of ascorbate biosynthesis. Curr Opin Plant Biol 33:15–22

Bulley SM, Rassam M, Hoser D, Otto W, Schünemann N, Wright M, MacRae E, Gleave A, Laing W (2009) Gene expression studies in kiwifruit and gene over-expression in Arabidopsis indicates that GDP-L-galactose guanyltransferase is a major control point of vitamin C biosynthesis. J Exp Bot 60:765–778

Bulley S, Wright M, Rommens C, Yan H, Rassam M, Lin-Wang K, Andre C, Brewster D, Karunairetnam S, Allan AC, Laing WA (2012) Enhancing ascorbate in fruits and tubers through over-expression of the L-galactose pathway gene GDP-L-galactose phosphorylase. Plant Biotechnol J 10:390–397

Burns JJ (1967) Ascorbic acid. In: Greenberg DM (ed) Metabolic pathways, vol 1, 3rd edn. Academic Press, New York, pp 394–411

Conklin PL, Barth C (2004) Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. Plant Cell Environ 27:959–970

Conklin PL, Williams EH, Last RL (1996) Environmental stress sensitivity of an ascorbic acid-deficient Arabidopsis mutant. Proc Natl Acad Sci U S A 93:9970–9974

Conklin PL, Pallanca JE, Last RL, Smirnoff N (1997) L-ascorbic acid metabolism in the ascorbate-deficient arabidopsis mutant *vtc1*. Plant Physiol 115:1277–1285

Conklin PL, Norris SR, Wheeler GL, Williams EH, Smirnoff N, Last RL (1999) Genetic evidence for the role of GDP-mannose in plant ascorbic acid (vitamin C) biosynthesis. Proc Natl Acad Sci U S A 96:4198–4203

Conklin PL, Saracco SA, Norris SR, Last RL (2000) Identification of ascorbic acid-deficient *Arabidopsis thaliana* mutants. Genetics 154:847–856

Conklin PL (2001) Recent advances in the role and biosynthesis of ascorbic acid in plants. Plant Cell Environ 24:383–394

Conklin PL, Gatzek S, Wheeler GL, Dowdle J, Raymond MJ, Rolinski S, Isupov M, Littlechild JA, Smirnoff N (2006) Arabidopsis thaliana VTC4 encodes L-galactose-1-P phosphatase, a plant ascorbic acid biosynthetic enzyme. J Biol Chem 281:15662–15670

Cruz-Rus E, Amaya I, Sanchez-Sevilla JF, Botella MA, Valpuesta V (2011) Regulation of L-ascorbic acid content in strawberry fruits. J Exp Bot 62:4191–4201

Davey MW, Van Montagu M, Inze D, Sanmartin M, Kanellis A, Smirnoff N, Benzie IJJ, Strain JJ, Favell D, Fletcher J (2000) Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing. J Sci Food Agric 80:825–860

DeBolt S, Hardie J, Tyerman S, Ford CM (2004) Composition and synthesis of raphide crystals and druse crystals in berries of *Vitis vinifera* L. cv. Cabernet sauvignon: ascorbic acid as precursor for both oxalic and tartaric acids as revealed by radiolabelling studies. Aust J Grape Wine Res 10:134–142

DeBolt S, Cook DR, Ford CM (2006) L-tartaric acid synthesis from vitamin C in higher plants. Proc Natl Acad Sci U S A 103:5608–5613

Debolt S, Melino V, Ford CM (2007) Ascorbate as a biosynthetic precursor in plants. Ann Bot 99:3–8

Di Matteo A, Sacco A, Anacleria M, Pezzotti M, Delledonne M, Ferrarini A, Frusciante L, Barone A (2010) The ascorbic acid content of tomato fruits is associated with the expression of genes involved in pectin degradation. BMC Plant Biol 10:163

Dowdle J, Ishikawa T, Gatzek S, Rolinski S, Smirnoff N (2007) Two genes in *Arabidopsis thaliana* encoding GDP-L-galactose phosphorylase are required for ascorbate biosynthesis and seedling viability. Plant J 52:673–689

Foyer CH, Noctor G (2011) Ascorbate and glutathione: the heart of the redox hub. Plant Physiol 155:2–18

Foyer CH, Shigeoka S (2011) Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant Physiol 155:93–100

Foyer C, Rowell J, Walker D (1983) Measurement of the ascorbate content of spinach leaf protoplasts and chloroplasts during illumination. Planta 157:239–244

Franceschi VR, Nakata PA (2005) Calcium oxalate in plants: formation and function. Annu Rev Plant Biol 56:41–71

Fujiki Y, Yoshikawa Y, Sato T, Inada N, Ito M, Nishida I, Watanabe A (2001) Dark-inducible genes from *Arabidopsis thaliana* are associated with leaf senescence and repressed by sugars. Physiol Plant 111:345–352

Gao Y, Nishikawa H, Badejo AA, Shibata H, Sawa Y, Nakagawa T, Maruta T, Shigeoka S, Smirnoff N, Ishikawa T (2011) Expression of aspartyl protease and C3HC4-type RING zinc finger genes are responsive to ascorbic acid in *Arabidopsis thaliana*. J Exp Bot 62:3647–3657

Gatzek S, Wheeler GL, Smirnoff N (2002) Antisense suppression of L-galactose dehydrogenase in *Arabidopsis thaliana* provides evidence for its role in ascorbate synthesis and reveals light modulated L-galactose synthesis. Plant J 30:541–553

Gest N, Gautier H, Stevens R (2013) Ascorbate as seen through plant evolution: the rise of a successful molecule? J Exp Bot 64:33–53

Green MA, Fry SC (2005) Vitamin C degradation in plant cells *via* enzymatic hydrolysis of 4-O-oxalyl-L-threonate. Nature 433:83–87

Grün M, Loewus FA (1984) L-Ascorbic-acid biosynthesis in the euryhaline diatom *Cyclotella cryptica*. Planta 160:6–11

Hancock RD, Viola R (2005a) Improving the nutritional value of crops through enhancement of L-ascorbic acid (vitamin C) content: rationale and biotechnological opportunities. J Agric Food Chem 53:5248–5257

Hancock RD, Viola R (2005b) Biosynthesis and catabolism of L-ascorbic acid in plants. Crit Rev Plant Sci 24:167–188

Hancock RD, Walker PG, Pont SDA, Marquis N, Vivera S, Gordon SL, Brennan RM, Viola R (2007) L-Ascorbic acid accumulation in fruit of *Ribes nigrum* occurs by in situ biosynthesis via the L-galactose pathway. Funct Plant Biol 34:1080–1091

Heazlewood JL, Howell KA, Millar AH (2003) Mitochondrial complex I from Arabidopsis and rice: orthologs of mammalian and fungal components coupled with plant-specific subunits. Biochim Biophys Acta 1604:159–169

Helsper JP, Loewus FA (1982) Metabolism of L-threonic acid in *Rumex x acutus* L. and *Pelargonium crispum* (L.) L'Hér. Plant Physiol 69:1365–1368

Helsper JP, Kagan L, Hilby CL, Maynard TM, Loewus FA (1982) L-Ascorbic acid biosynthesis in *Ochromonas danica*. Plant Physiol 69:465–468

Horner HT, Wagner BL (1995) Calcium oxalate formation in higher plants. In: Khan SR (ed) Calcium oxalate in biological systems. CRC Press, Boca Raton, FL, pp 53–72

Iland PG, Coombe BG (1988) Malate, tartrate, potassium, and sodium in flesh and skin of Shiraz grapes during ripening: concentration and compartmentation. Am J Enol Vitic 39:71–76

Imai T, Karita S, Shiratori G, Hattori M, Nunome T, Oba K, Hirai M (1998) L-galactono-γ-lactone dehydrogenase from sweet potato: purification and cDNA sequence analysis. Plant Cell Physiol 39:1350–1358

Imai T, Ban Y, Terakami S, Yamamoto T, Moriguchi T (2009) L-Ascorbate biosynthesis in peach: cloning of six L-galactose pathwayrelated genes and their expression during peach fruit development. Physiol Planta 136:139–149

Ioannidi E, Kalamaki MS, Engineer C, Pateraki I, Alexandrou D, Mellidou I, Giovannonni J, Kanellis AK (2009) Expression profiling of ascorbic acid-related genes during tomato fruit development and ripening and in response to stress conditions. J Exp Bot 60:663–678

Ishikawa T, Shigeoka S (2008) Recent advances in ascorbate biosynthesis and the physiological significance of ascorbate peroxidase in photosynthesizing organisms. Biosci Biotechnol Biochem 72:1143–1154

Ishikawa T, Dowdle J, Smirnoff N (2006a) Progress in manipulating ascorbic acid biosynthesis and accumulation in plants. Physiol Plant 126:343–355

Ishikawa T, Masumoto I, Iwasa N, Nishikawa H, Sawa Y, Shibata H, Nakamura A, Yabuta Y, Shigeoka S (2006b) Functional characterization of D-galacturonic acid reductase, a key enzyme of the ascorbate biosynthesis pathway, from *Euglena gracilis*. Biosci Biotechnol Biochem 70:2720–2726

Ishikawa T, Nishikawa H, Gao Y, Sawa Y, Shibata H, Yabuta Y, Maruta T, Shigeoka S (2008) The pathway via D-galacturonate/L-galactonate is significant for ascorbate biosynthesis in *Euglena gracilis*: identification and functional characterization of aldonolactonase. J Biol Chem 283:31133–31141

Jain AK, Nessler CL (2000) Metabolic engineering of an alternative pathway for ascorbic acid biosynthesis in plants. Mol Breed 6:73–78

Keller R, Renz FS, Kossmann J (1999) Antisense inhibition of the GDP-mannose pyrophosphorylase reduces the ascorbate content in transgenic plants leading to developmental changes during senescence. Plant J 19:131–141

Kempinski CF, Haffar R, Barth C (2011) Toward the mechanism of NH^{4+} sensitivity mediated by Arabidopsis GDP-mannose pyrophosphorylase. Plant Cell Environ 34:847–858

Kerchev PI, Pellny TK, Vivancos PD, Kiddle G, Hedden P, Driscoll S, Vanacker H, Verrier P, Hancock RD, Foyer CH (2011) The transcription factor ABI4 is required for the ascorbic acid-dependent regulation of growth and regulation of jasmonate-dependent defense signaling pathways in Arabidopsis. Plant Cell 23:3319–3334

Laing WA, Bulley S, Wright M, Cooney J, Jensen D, Barraclough D, MacRae E (2004) A highly specific L-galactose-1-phosphate phosphatase on the path to ascorbate biosynthesis. Proc Natl Acad Sci U S A 101:16976–16981

Laing WA, Wright MA, Cooney J, Bulley SM (2007) The missing step of the L-galactose pathway of ascorbate biosynthesis in plants, an L-galactose guanyltransferase, increases leaf ascorbate content. Proc Natl Acad Sci U S A 104:9534–9539

Laing WA, Martínez-Sánchez M, Wright MA, Bulley SM, Brewster D, Dare AP, Rassam M, Wang D, Storey R, Macknight RC, Hellens RP (2015) An upstream open reading frame is essential for feedback regulation of ascorbate biosynthesis in Arabidopsis. Plant Cell 27:772–786

Leferink NG, van den Berg WA, van Berkel WJ (2008) L-Galactono-γ-lactone dehydrogenase from *Arabidopsis thaliana*, a flavoprotein involved in vitamin C biosynthesis. FEBS J 275:713–726

Li M, Ma F, Shang P, Zhang M, Hou C, Liang D (2009) Influence of light on ascorbate formation and metabolism in apple fruits. Planta 230:39–51

Li M, Ma F, Liang D, Li J, Wang Y (2010) Ascorbate biosynthesis during early fruit development is the main reason for its accumulation in kiwi. PLoS One 5:e14281

Lim B, Smirnoff N, Cobbett CS, Golz JF (2016) Ascorbate-deficient *vtc2* mutants in Arabidopsis do not exhibit decreased growth. Front Plant Sci 13:1025

Linster CL, Clarke SG (2008) L-Ascorbate biosynthesis in higher plants: the role of VTC2. Trends Plant Sci 13:567–573

Linster CL, Gomez TA, Christensen KC, Adler LN, Young BD, Brenner C, Clarke SG (2007) Arabidopsis VTC2 encodes a GDP-L-galactose phosphorylase, the last unknown enzyme in the Smirnoff-Wheeler pathway to ascorbic acid in plants. J Biol Chem 282:18879–18885

Linster CL, Adler LN, Webb K, Christensen KC, Brenner C, Clarke SG (2008) A second GDP-L-galactose phosphorylase in Arabidopsis en route to vitamin C: covalent intermediate and substrate requirements for the conserved reaction. J Biol Chem 283:18483–18492

Loach PA (1976) Physical and chemical data. In: Fasman GD (ed) Handbook of biochemistry and molecular biology, vol 1, 3rd edn. CRC Press, Boca Raton, FL

Loewus FA (1999) Biosynthesis and metabolism of ascorbic acid in plants and of analogs of ascorbic acid in fungi. Phytochemistry 52:193–210

Lorence A, Chevone BI, Mendes P, Nessler CL (2004) *myo*-Inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. Plant Physiol 134:1200–1205

Lukowitz W, Nickle TC, Meinke DW, Last RL, Conklin PL, Somerville CR (2001) Arabidopsis cyt1 mutants are deficient in a mannose-1-phosphate guanylyltransferase and point to a requirement of N-linked glycosylation for cellulose biosynthesis. Proc Natl Acad Sci U S A 98:2262–2267

Maruta T, Yonemitsu M, Yabuta Y, Tamoi M, Ishikawa T, Shigeoka S (2008) Arabidopsis phosphomannose isomerase 1, but not phosphomannose isomerase 2, is essential for ascorbic acid biosynthesis. J Biol Chem 283:28842–28851

Maruta T, Ichikawa Y, Mieda T, Takeda T, Tamoi M, Yabuta Y, Ishikawa T, Shigeoka S (2010) The contribution of Arabidopsis homologs of L-gulono-1,4-lactone oxidase to the biosynthesis of ascorbic acid. Biosci Biotechnol Biochem 74:1494–1497

Matsui T, Kitagawa Y, Okumura M, Shigeta Y, Melino VJ, Soole KL, Ford CM (2015) Accurate standard hydrogen electrode potential and applications to the redox potentials of vitamin C and NAD/NADH. J Phys Chem A 119:369–376

McNair JB (1932) The intersection between substances in plants: essential oils and resins, cyanogen and oxalate. Am J Bot 19:255–271

Melino VJ, Soole KL, Ford CM (2009) Ascorbate metabolism and the developmental demand for tartaric and oxalic acids in ripening grape berries. BMC Plant Biol 9:145

Mieda T, Yabuta Y, Rapolu M, Motoki T, Takeda T, Yoshimura K, Ishikawa T, Shigeoka S (2004) Feedback inhibition of spinach L-galactose dehydrogenase by L-ascorbate. Plant Cell Physiol 45:1271–1279

Müller-Moulé P, (2008) An expression analysis of the ascorbate biosynthesis enzyme VTC2. Plant Mol Biol 68:31–41

Nakata PA (2003) Advances in our understanding of calcium oxalate crystal formation and function in plants. Plant Sci 164:901–909

Niki E (1991) Action of ascorbic acid as a scavenger of active and stable oxygen radicals. Am J Clin Nutr 54:1119S–1124S

Nishikimi M, Yagi K (1996) Biochemistry and molecular biology of ascorbic acid biosynthesis. Subcell Biochem 25:17–39

Noctor G (2006) Metabolic signalling in defence and stress: the central roles of soluble redox couples. Plant Cell Environ 29:409–425

Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 49:249–279

Oba K, Ishikawa S, Nishikawa M, Mizuno H, Yamamoto T (1995) Purification and properties of L-galactono-γ-lactone dehydrogenase, a key enzyme for ascorbic acid biosynthesis, from sweet potato roots. J Biochem 117:120–124

Østergaard J, Persiau G, Davey MW, Bauw G, Van Montagu M (1997) Isolation of a cDNA coding for L-galactono-γ-lactone dehydrogenase, an enzyme involved in the biosynthesis of ascorbic acid in plants. Purification, characterization, cDNA cloning, and expression in yeast. J Biol Chem 272:30009–30016

Pallanca JE, Smirnoff N (2000) The control of ascorbic acid synthesis and turnover in pea seedlings. J Exp Bot 51:669–674

Pastori GM, Kiddle G, Antoniw J, Bernard S, Veljovic-Jovanovic S, Verrier PJ, Noctor G, Foyer CH (2003) Leaf vitamin C contents modulate plant defense transcripts and regulate genes that control development through hormone signaling. Plant Cell 15:939–951

Pineau B, Layoune O, Danon A, De Paepe R (2008) L-Galactono-1,4-lactone dehydrogenase is required for the accumulation of plant respiratory complex I. J Biol Chem 283:32500–32505

Qian W, Yu C, Qin H, Liu X, Zhang A, Johansen IE, Wang D (2007) Molecular and functional analysis of phosphomannomutase (PMM) from higher plants and genetic evidence for the involvement of PMM in ascorbic acid biosynthesis in Arabidopsis and *Nicotiana benthamiana*. Plant J 49:399–413

Qin C, Qian W, Wang W, Wu Y, Yu C, Jiang X, Wang D, Wu P (2008) GDP-mannose pyrophosphorylase is a genetic determinant of ammonium sensitivity in Arabidopsis Thaliana. Proc Natl Acad Sci U S A 105:18308–18313

Rautenkranz A, Li L, Machler F, Martinoia E, Oertli JJ (1994) Transport of ascorbic and dehydroascorbic acids across protoplast and vacuole membranes isolated from barley (*Hordeum vulgare* L. cv Gerbel) leaves. Plant Physiol 106:187–193

Running JA, Burlingame RP, Berry A (2003) The pathway of L-ascorbic acid biosynthesis in the colourless microalga *Prototheca moriformis*. J Exp Bot 54:1841–1849

Saito K, Kasai Z (1969) Tartaric acid synthesis from L-ascorbic acid- 1-^{14}C in grape berries. Phytochemistry 8:2177–2182

Saito K, Kasai Z (1982) Conversion of L-ascorbic acid to L-idonic acid, L-idono-γ-lactone and 2-keto-L-idonic acid in slices of immature grapes. Plant Cell Physiol 23:499–507

Saito K, Kasai Z (1984) Synthesis of L-(+)-tartaric acid from L-ascorbic acid *via* 5-Keto-D-gluconic acid in grapes. Plant Physiol 76:170–174

Saito K, Ohmoto J, Kuriha N (1997) Incorporation of ^{18}O into oxalic, L-threonic and L-tartaric acids during cleavage of L-ascorbic and 5-keto-D-gluconic acids in plants. Phytochemistry 44:805–809

Sakamoto S, Fujikawa Y, Tanaka N, Esaka M (2012) Molecular cloning and characterization of L-galactose-1-phosphate phosphatase from tobacco (*Nicotiana tabacum*). Biosci Biotechnol Biochem 76:1155–1162

Schertl P, Sunderhaus S, Klodmann J, Grozeff GE, Bartoli CG, Braun HP (2012) L-Galactono-1,4-lactone dehydrogenase (GLDH) forms part of three subcomplexes of mitochondrial complex I in *Arabidopsis thaliana*. J Biol Chem 287:14412–14419

Schimmeyer J, Bock R, Meyer EH (2016) L-Galactono-1,4-lactone dehydrogenase is an assembly factor of the membrane arm of mitochondrial complex I in Arabidopsis. Plant Mol Biol 90:117–126

Sharples SC, Fry SC (2007) Radioisotope ratios discriminate between competing pathways of cell wall polysaccharide and RNA biosynthesis in living plant cells. Plant J 52:252–262

Shigeoka S, Maruta T (2014) Cellular redox regulation, signaling, and stress response in plants. Biosci Biotechnol Biochem 78:1457–1470

Shigeoka S, Nakano Y, Kitaoka S (1979a) The biosynthetic pathway of L-ascorbic acid in *Euglena gracilis* z. J Nutr Sci Vitaminol 25:299–307

Shigeoka S, Nakano Y, Kitaoka S (1979b) Some properties and subcellular localization of L-gulono-γ-lactone dehydrogenase in Euglena gracilis z. Agric Biol Chem 43:2187–2188

Siendones E, Gonzalez-Reyes JA, Santos-Ocana C, Navas P, C rdoba F (1999) Biosynthesis of ascorbic acid in kidney bean. L-galactono-γ-lactone dehydrogenase is an intrinsic protein located at the mitochondrial inner membrane. Plant Physiol 120:907–912

Smirnoff N (2000a) Ascorbate biosynthesis and function in photoprotection. Philos Trans R Soc Lond Ser B Biol Sci 355:1455–1464

Smirnoff N (2000b) Ascorbic acid: metabolism and functions of a multifaceted molecule. Curr Opin Plant Biol 3:229–235

Smirnoff N, Wheeler GL (2000) Ascorbic acid in plants: biosynthesis and function. Crit Rev Biochem Mol Biol 35:291–314

Smirnoff N, Conklin PL, Loewus FA (2001) Biosynthesis of ascorbic acid in plants: a renaissance. Annu Rev Plant Physiol Plant Mol Biol 52:437–467

Smirnoff N, Gatzek S (2004) Ascorbate biosynthesis: a diversity of pathways. In: Asard H, May JM, Smirnoff N (eds) Vitamin C: functions and biochemistry in animals and plants. BIOS Scientific Publishers Ltd, Oxford, pp 7–29

Stafford HA (1959) Distribution of tartaric acid in the leaves of certain angiosperms. Am J Bot 46:347–352

Sturgeon BE, Sipe HJ Jr, Barr DP, Corbett JT, Martinez JG, Mason RP (1998) The fate of the oxidizing tyrosyl radical in the presence of glutathione and ascorbate. Implications for the radical sink hypothesis. J Biol Chem 273:30116–30121

Tanaka H, Maruta T, Ogawa T, Tanabe N, Tamoi M, Yoshimura K, Shigeoka S (2015) Identification and characterization of AtNUDX9 as a GDP-D-mannose pyrophosphohydrolase: its involvement in root growth inhibition in response to ammonium. J Exp Bot 66:5797–5808

Torabinejad J, Donahue JL, Gunesekera BN, Allen-Daniels MJ, Gillaspy GE (2009) VTC4 is a bifunctional enzyme that affects myoinositol and ascorbate biosynthesis in plants. Plant Physiol 150:951–961

Tsuchiya J, Yamada T, Niki E, Kamiya Y (1985) Interaction of galvinoxyl radical with ascorbic acid, cysteine and glutathione in homogeneous solution and in aqueous dispersions. Bull Chem Soc Jpn 58:326–330

Urzica EI, Adler LN, Page MD, Linster CL, Arbing MA, Casero D, Pellegrini M, Merchant SS, Clarke SG (2012) Impact of oxidative stress on ascorbate biosynthesis in Chlamydomonas via regulation of the VTC2 gene encoding a GDP-L-galactose phosphorylase. J Biol Chem 287:14234–14245

Valpuesta V, Botella MA (2004) Biosynthesis of L-ascorbic acid in plants: new pathways for an old antioxidant. Trends Plant Sci 9:573–577

Wagner G, Loewus F (1973) The biosynthesis of (+)-tartaric acid in *Pelargonium crispum*. Plant Physiol 52:651–654

Wagner C, Sefkow M, Kopka J (2003) Construction and application of a mass spectral and retention time index database generated from plant GC/EI-TOF-MS metabolite profile. Phytochemistry 62:887–900

Wang J, Yu Y, Zhang Z, Quan R, Zhang H, Ma L, Deng XW, Huang R (2013) Arabidopsis CSN5B interacts with VTC1 and modulates ascorbic acid synthesis. Plant Cell 25:625–636

Wheeler GL, Jones MA, Smirnoff N (1998) The biosynthetic pathway of vitamin C in higher plants. Nature 393:365–369

Wheeler G, Ishikawa T, Pornsaksit V, Smirnoff N (2015) Evolution of alternative biosynthetic pathways for vitamin C following plastid acquisition in photosynthetic eukaryotes. elife 4:e06369

Wolucka BA, Van Montagu M (2003) GDP-mannose 3′,5′-epimerase forms GDP-L-gulose, a putative intermediate for the *de novo* biosynthesis of vitamin C in plants. J Biol Chem 278:47483–47490

Wolucka BA, Van Montagu M (2007) The VTC2 cycle and the *de novo* biosynthesis pathways for vitamin C in plants: an opinion. Phytochemistry 68:2602–2613

Wolucka BA, Persiau G, Van Doorsselaere J, Davey MW, Demol H, Vandekerckhove J, Van Montagu M, Zabeau M, Boerjan W (2001) Partial purification and identification of GDP-mannose 3′,5′-epimerase of *Arabidopsis thaliana*, a key enzyme of the plant vitamin C pathway. Proc Natl Acad Sci U S A 98:14843–14848

Yabuta Y, Yoshimura K, Takeda T, Shigeoka S (2000) Molecular characterization of tobacco mitochondrial L-galactono-γ-lactone dehydrogenase and its expression in *Escherichia coli*. Plant Cell Physiol 41:666–675

Yabuta Y, Mieda T, Rapolu M, Nakamura A, Motoki T, Maruta T, Yoshimura K, Ishikawa T, Shigeoka S (2007) Light regulation of ascorbate biosynthesis is dependent on the photosynthetic electron transport chain but independent of sugars in Arabidopsis. J Exp Bot 58:2661–2671

Yang JC, Loewus FA (1975) Metabolic conversion of L-ascorbic-acid to oxalic-acid in oxalateaccumulating plants. Plant Physiol 56:283–285

Yang XY, Xie JX, Wang FF, Zhong J, Liu YZ, Li GH, Peng SA (2011) Comparison of ascorbate metabolism in fruits of two citrus species with obvious difference in ascorbate content in pulp. J Plant Physiol 68:2196–2205

Yoshimura K, Nakane T, Kume S, Shiomi Y, Maruta T, Ishikawa T, Shigeoka S (2014) Transient expression analysis revealed the importance of expression level in light/dark regulation of ascorbate biosynthesis in Arabidopsis. Biosci Biotechnol Biochem 78:60–66

Zechmann B, Stumpe M, Mauch F (2011) Immunocytochemical determination of the subcellular distribution of ascorbate in plants. Planta 233:1–12

Zhang L, Dong S (2004) The electrocatalytic oxidation of ascorbic acid on polyaniline film synthesized in the presence of camphorsulfonic acid. J Electroanal Chem 568:189–194

Zhang W, Gruszewski HA, Chevone BI, Nessler CL (2008) An Arabidopsis purple acid phosphatase with phytase activity increases foliar ascorbate. Plant Physiol 146:431–440

Zhao S, Liu L (2016) Expression and crystallographic studies of the *Arabidopsis thaliana* GDP-D-mannose pyrophosphorylase VTC1. Acta Crystallogr F Struct Biol Commun 72:795–798