

Mohammad Anwar Hossain  
Sergi Munné-Bosch · David J. Burritt  
Pedro Diaz-Vivancos · Masayuki Fujita  
Argelia Lorence *Editors*

# Ascorbic Acid in Plant Growth, Development and Stress Tolerance

 Springer

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ISBN 978-3-319-74056-0

ISBN 978-3-319-74057-7 (eBook)

<https://doi.org/10.1007/978-3-319-74057-7>

Library of Congress Control Number: 2018933037

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Printed on acid-free paper

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The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

# Preface

L-Ascorbic acid (AsA), vitamin C, is one of the most abundant water-soluble antioxidants in plants and animals. In plants, AsA serves as a major redox buffer and regulates various physiological processes controlling growth, development, signal transduction and stress tolerance. Recent studies on AsA biosynthesis, oxidation, recycling, transport and redox regulation have provided a wealth of information of the mechanisms associated with AsA-mediated plant growth and development, as well as plant stress tolerance. As plant-based foods constitute the principal source of AsA in the human diet, the possibility of increasing the AsA content of plants to improve their nutritional value has also received considerable attention in recent years. The importance of the relationship between reactive oxygen species (ROS) and AsA for regulation of cell signalling and metabolic processes has been well established. In contrast to the single pathway responsible for AsA biosynthesis in animals, plants use multiple pathways to synthesize AsA, and this reflects the metabolic diversity of plants and the importance of AsA for plant health. Any changes in the AsA content of plant cells can result in a diverse range of effects on growth, development and stress tolerance, as AsA is involved in redox signalling, cell cycle regulation, enzyme functioning and the expression of defence and stress-related genes. Being a major component of the Foyer-Halliwell-Asada cycle, AsA helps to modulate oxidative stress tolerance by controlling ROS detoxification, both alone and in cooperation with glutathione (GSH) and other antioxidants. While tremendous progress has been made over the last few years regarding the multiple roles of AsA in improving environmental stress tolerance, the manipulation of the AsA contents in plants as a means to improve stress tolerance and the nutritional value of some crop plants is still an evolving area of research.

In this book, *Ascorbate in Plant Growth Development and Stress Tolerance*, we present a collection of 19 chapters written by leading experts on the importance of AsA metabolism for plant growth, development and stress tolerance and for the production of nutritious plant-based foods. Chapter “Chemistry and Metabolism of Ascorbic Acid in Plants” addresses the chemical properties and redox reactions of AsA and its metabolism, including biosynthesis and degradation. Chapter “The Role of Ascorbate in Plant Growth and Development” describes the involvement of AsA

and its interaction with redox regulatory signalling networks during growth and development, at the cellular, tissue and organ levels, including its participation in cell cycle regulation, seed germination, embryogenesis, root and shoot meristem development and flowering. Chapter “Ascorbate as a Key Player in Plant Abiotic Stress Response and Tolerance” deals with the involvement of AsA metabolism in plants under abiotic stress and how to improve resilience to stressors in transgenic plants with altered enzymes involved in AsA biosynthesis and recycling. Chapter “Ascorbate Peroxidases: Crucial Roles of Antioxidant Enzymes in Plant Stress Responses” describes the physiological roles of ascorbate peroxidase isoforms in plants, by reviewing the findings of key biochemical, physiological and genetic studies. Chapter “Molecular and Functional Characterization of Monodehydroascorbate and Dehydroascorbate Reductases” addresses on the molecular properties, structural and enzymatic, of AsA-regenerating enzymes (monodehydroascorbate reductase, MDHAR and dehydroascorbate reductase, DHAR) and their biochemical functions. Chapter “Regulation of Ascorbic Acid Biosynthesis in Plants” focuses on the regulation of AsA biosynthesis at the transcriptional level by internal and environmental factors affecting the AsA contents of plants. Chapter “Ascorbate-Glutathione Cycle and Abiotic Stress Tolerance in Plants” addresses the role of the ascorbate-glutathione (AsA-GSH) cycle in regulating abiotic stress responses and tolerance. Chapter “Ascorbate-Glutathione Cycle and Biotic Stress Tolerance in Plants” describes the involvement of the AsA-GSH cycle in biotic stress tolerance, the role of compartment-specific changes in the AsA-GSH cycle activity in biotic stress signalling and the AsA- and GSH-dependent mechanisms that enable plants to respond to abiotic and biotic stress combinations. Chapter “Exogenous Ascorbic Acid Mediated Abiotic Stress Tolerance in Plants” gives an overview of physiological, biochemical and molecular effects of exogenous applications of AsA to various plant species under abiotic stress. Chapter “The Role of Ascorbic Acid in Plant-Pathogen Interactions” provides a broad picture of the mechanisms by which AsA interacts with key components of a complex network regulating both basal and induced resistance in different pathosystems. Chapter “Ascorbate Oxidase in Plant Growth, Development, and Stress Tolerance” discusses the roles of ascorbate oxidase in plant growth, development and stress tolerance, based on the current research findings, and examines additional roles the enzyme could play at the cellular level. Chapter “AsA/DHA Redox Pair Influencing Plant Growth and Stress Tolerance” gives an overview of the literature with an emphasis on the role of the AsA/DHA redox pair in plant growth and abiotic and biotic stress tolerance. Chapter “The Role of Plant High-Throughput Phenotyping in the Characterization of the Response of High Ascorbate Plants to Abiotic Stresses” describes the genetic engineering of AsA biosynthetic genes in relation to abiotic stress tolerance and the potential of high-throughput plant phenotyping (phenomics) to accelerate the characterization of phenotypes of model plants and crops with high AsA levels. Chapter “Physiological Role of Ascorbic Acid Recycling Enzymes in Plants” focuses on the primary structure and the expression of genes encoding plant MDHAR and DHAR isozymes, as well as their contributions to the regulation of AsA contents in the leaves and fruits of plants. This chapter also provides information about the roles of MDHAR and

DHAR in chloroplasts, the cytosol and guard cells in relation to plant stress tolerance. Chapter “Ascorbic Acid Biofortification in Crops” provides an overview of our current understanding of AsA metabolism in model species and the ever-expanding molecular genetic toolset that modern molecular breeders can use to improve the AsA contents of crop plants. Chapter “Evolution of the Metabolic Network Leading to Ascorbate Synthesis and Degradation Using *Marchantia polymorpha* as a Model System” explores the evolution of the metabolic network leading to AsA synthesis and degradation using *Marchantia polymorpha* as a model system. Chapter “Ascorbic Acid in Processed Plant-Based Foods” focuses on the use of conventional food processing techniques to stabilize/protect AsA levels in plant-based foods and how novel processing techniques, such as pulsed electric fields and high hydrostatic pressure processing, could improve the retention of biologically active AsA in plant-based foods. Chapter “Ascorbate Metabolism and Nitrogen Fixation in Legumes” deals with AsA metabolism in relation to biological nitrogen fixation, with special reference to the production of ROS and reactive nitrogen species (RNS) in nodules, the benefits of exogenous AsA on N<sub>2</sub> fixation and the metabolic pathways of AsA biosynthesis in plants and nodules. Chapter “Importance of Vitamin C in Human Health and Disease” describes the importance of AsA in human health and disease.

This book is intended to provide comprehensive and in-depth information for advanced students, teachers and plant scientists around the globe, who are working on AsA metabolism in relation to plant growth and development, stress tolerance and quality improvement in plant-based foods. The editors gratefully acknowledge the excellent cooperation of all the experienced and well-versed contributors, who cordially accepted our invitation and contributed to this book. We would also like to extend our thanks to Dr. Kenneth Teng and the editorial staff of Springer, New York, who enabled us to initiate this book project. We believe that the information covered in this book will make a sound contribution to this fascinating area of research.

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# 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.

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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; Smirnov 2000a; Smirnov and Wheeler 2000; Conklin 2001; Smirnov et al. 2001; Smirnov 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 (Smirnov 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

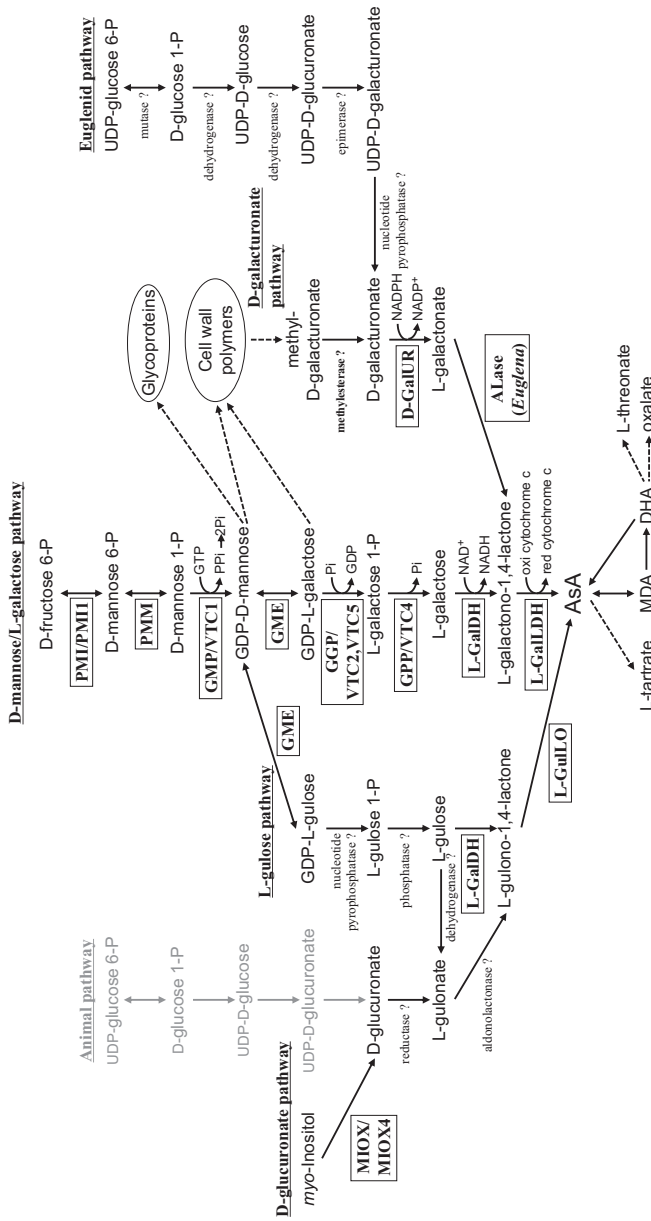
Structurally, AsA is related to hexose sugars and has the conjugated structure of the five-carbon lactone ring, containing an enediol group on C2 and C3. Delocalization of the  $\pi$ -electrons over the C2-C3 enediol group stabilizes the molecule and causes dissociation of the hydrogen of the C3 hydroxyl to become a monovalent anion at physiological pH values since  $pK_{a1}$  is 4.13 ( $pK_{a2} = 11.6$ ). 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, englenid, 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 *Englena*. 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; *PMII* 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* aldololactonase

### 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  $\mu\text{M}$  and 1.89  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein, respectively, whereas those of the recombinant *PMI2* are 372  $\mu\text{M}$  and 22.5  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein, respectively (Maruta et al. 2008). Both *PMI1* and *PMI2* are inhibited by EDTA,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ , 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  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein;  $K_m$ , 29.7  $\mu\text{M}$ ) was more efficient than the latter reaction ( $V_{max}$ , 1.40  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein;  $K_m$ , 65.4  $\mu\text{M}$ ). 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 *VTCl* (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 ( $\text{NH}_4^+$ ), a major nitrogen source for plants. The root growth of *vtc1* mutants was stunted in the presence of  $\text{NH}_4^+$ , whereas they developed roots similar to those of the wild-type plants in the absence of  $\text{NH}_4^+$  (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  $\text{NH}_4^+$  (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 ( $K_m = 376 \pm 24 \mu\text{M}$ ;  $V_{\text{max}} = 1.61 \mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$ ) (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  $\text{NH}_4^+$  (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  $\mu\text{M}$  and 0.31–1.76  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  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-gulonono-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-gulonono-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 colocalized 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  $\mu M$  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  $\mu$ M and 3.7  $\mu$ M, 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  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ . 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 (Alhaghdow 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 aldoketo 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-gulonono-1,4-lactone, enhanced the levels of AsA in immature green fruit. In contrast, L-galactose and D-galacturonate, but not L-gulonono-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).