Chapter 6
Engineering Elevated Vitamin C in Plants to Improve their Nutritional Content, Growth, and Tolerance to Abiotic Stress

Katherine A. Lisko, Siddique I. Aboobucker, Raquel Torres and Argelia Lorence

Abstract Vitamin C (L-ascorbic acid, AsA) is essential for human health; however, despite our dependency on plants as dietary sources of this nutrient, little is known about its metabolism in crops. Ascorbate protects cells and organelles from oxidative damage by scavenging reactive oxygen species that are produced in response to abiotic and biotic insults, and is also a cofactor of many enzymes, controls cell division, affects cell expansion, and is a modulator of plant senescence. Biosynthesis of AsA in plants is carried out by a complex metabolic network involving D-mannose/L-galactose, D-galacturonate, L-gulose, and *myo*-inositol as main precursors. The recent cloning of several genes that regulate AsA synthesis and recycling has facilitated the generation of transgenic plants with enhanced AsA levels, and in some cases as much as sixfold increases in AsA relative to wild-type plants have been achieved. In this review, we provide an overview of research revealing three aspects of the biochemistry of AsA that have not been fully covered elsewhere. First, we discuss the main findings of studies on feeding plant tissues with precursors as a proxy to determine which of the AsA biosynthetic pathways are operational in model and crop plants, and discuss these in the context of the forward and reverse genetic studies that support the operation of each pathway. Next, we critically discuss the consequences of elevating AsA content for plant growth, and finally we explore the effect of AsA content on plant performance under environmental stress.

Keywords Vitamin C · Ascorbic acid · Feeding studies · Abiotic stress · Stress tolerance · Phytoremediation · High throughput phenotyping · Phenomics

A. Lorence (✉)
Department of Chemistry and Physics, Arkansas State University,
P.O. Box 639, Arkansas 72467, USA
e-mail: alorence@astate.edu

K. A. Lisko · S. I. Aboobucker · R. Torres · A. Lorence
Arkansas Biosciences Institute, Arkansas State University,
P.O. Box 639, Arkansas 72467, USA

R. Jetter (ed.), *Phytochemicals—Biosynthesis, Function and Application,*
Recent Advances in Phytochemistry 44, DOI 10.1007/978-3-319-04045-5_6

Abbreviations

L-Gal	= L-Galactose
L-GalL	= L-Galactono-1,4-lactone
D-GalUA	= D-Galacturonic acid
D-Glc	= D-Glucose
D-GlcUA	= D-Glucuronic acid
D-GlucL	= D-Glucuronolactone
L-Gul	= L-Gulose
L-GulL	= L-Gulono-1,4-lactone
D-Man	= D-Mannose
D-Fruc	= D-fructose
MI	= *Myo*-inositol
Suc	= Sucrose
AMR1	= Ascorbic acid mannose pathway regulator 1
AsA	= L-Ascorbic acid, ascorbate, vitamin C
AtERF98	= Arabidopsis ethylene responsive factor 98
AtPAP15	= Purple acid phosphatase 15 from *Arabidopsis thaliana*
DHA	= Dehydroascorbate
DHAR	= Dehydroascorbate reductase
GalDH	= L-Galactose dehydrogenase
GalUR	= D-Galacturonate reductase
GlcUR	= D-Glucuronate reductase
GLDH	= L-Galactono-1,4-lactone dehydrogenase
GLOase	= L-Gulono-1,4-lactone oxidase (a.k.a. GulLO)
GME	= GDP-mannose-3′,5′-epimerase
GNL	= Gluconolactonase
HBK3	= KNOTTED-like homeobox gene from Norway spruce
MIOX	= *myo*-Inositol oxygenase
MDHAR	= Monodehydroascorbate reductase
PMI	= Phosphomannose isomerase
PMM	= Phosphomannose mutase
VTC1	= GDP-mannose pyrophosphorylase
VTC2	= GDP-galactose phosphorylase
VTC4	= L-Galactose-1-phosphate phosphatase

6.1 Introduction

Ascorbate (L-ascorbic acid, AsA, a.k.a. vitamin C) is ubiquitous in plants, and serves a host of different functions. It protects cells and organelles from oxidative damage by scavenging reactive oxygen species (e.g., superoxide and H_2O_2), which are produced by aerobic metabolic processes such as photosynthesis and respiration or by environmental stresses like salt, drought, cold, and excess light. AsA also

participates in the regeneration of vitamin E [17] and acts as a substrate for synthesis of important organic acids (e.g., L-tartaric, L-threonic, L-glyceric, and L-oxalic acids) [24], as well as being a cofactor for enzymes involved in a diverse array of processes including flavonoid and phytohormone biosynthesis and the xanthophyll cycle [23]. There is also growing evidence that AsA participates in the regulation of cell division and elongation [48, 86], modulates flowering time and the onset of senescence [10], acts as a signaling molecule involved in plant response to environmental stresses such as ozone and pathogen attack [17], and regulates cell polarity during embryo development [15]. In short, AsA is crucial to plant health, as illustrated by the fact that no mutant completely devoid of AsA has ever been described. The *vtc2/vtc5* double mutant, a line defective in the expression of two genes in the D-mannose (D-Man)/L-galactose (L-Gal) pathway to AsA that is predicted to entirely lack ascorbate in fact suffers from very early growth arrest and is essentially nonviable [27].

Some animal species, including humans, do not synthesize AsA due to the lack of the enzyme catalyzing the last step of the biosynthetic pathway (L-gulono-1,4-lactone oxidase or GLOase, a.k.a. GulLO), and for them it thus is a vitamin. In addition to its essential roles as redox buffer in key organelles such as the mitochondria and the endoplasmic reticulum [70], vitamin C is an indispensable cofactor in the hydroxylation of proline to lysine, and therefore is essential for collagen synthesis and connective tissue integrity. A consequence of this is that vitamin C deficiency can cause scurvy, a condition characterized by hemorrhages, bleeding gums, and impaired wound healing. Vitamin C is also involved in a wide array of other crucial physiological processes in animals, including the synthesis of cytokines [90], modulation of nitric oxide synthase activity [49], oxidative protein folding and endoplasmic reticulum stress [70, 71], cell proliferation and apoptosis [25], activation of the epithelial cystic fibrosis transmembrane conductance regulator chloride channel [32], maintenance of immune homeostasis [101], promotion of iron absorption and mobilization, and tyrosine, folate and xenobiotic metabolism. In humans, diverse studies indicate that vitamin C may decrease the incidence of various diseases, including dementia [74], cancer [59, 60], stroke [104], heart disease [88], atherosclerosis [76], type 2 diabetes [44], and Charcot–Marie–Tooth disease, a hereditary peripheral neuropathy [83]. According to the 2003–2004 National Health and Nutrition Examination Survey, 7.1 % of the US population was vitamin C-deficient, and smokers, low-income people, and the elderly are among those at increased risk [89]. These data provide a clear rationale for enhancing the vitamin C content of food crops.

Despite the critical importance of AsA to plant health and human nutrition, it is only recently that scientists have succeeded in identifying pathways that lead to AsA synthesis in plants (reviewed in [12, 68]). In contrast to animals, which utilize D-glucuronate (D-GlcUA) as a precursor for AsA synthesis, plants rely on at least four alternative routes for AsA synthesis; these routes utilize myo-inositol (MI) [69], L-gulose (L-Gul) [102], D-Man/L-Gal [100], and D-galacturonate (D-GalUA) [1] as main precursors (Fig. 6.1). The recent cloning of several genes that regulate AsA synthesis and recycling has facilitated the generation of transgenic plants with

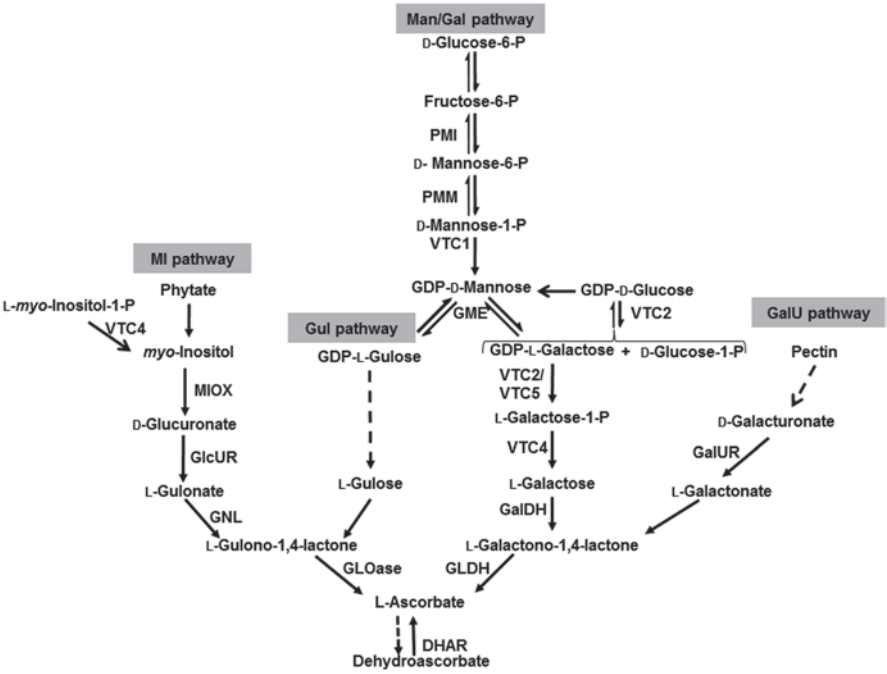

Fig. 6.1 Pathways involved in ascorbate biosynthesis and regeneration in plants: The D-mannose/L-galactose (Man/Gal) route, the L-gulose (Gul) shunt, the D-galacturonate (GalU) pathway, and the myo-inositol (MI) route. A purple acid phosphatase with phytase activity (AtPAP15) has been shown to channel phytate to the MI pathway, while VTC4 has been shown to also use L-myo-inositol-1 phosphate and contribute to both myo-inositol and ascorbate metabolisms. The enzymes participating in the Man/Gal route are: Phosphoglucose isomerase (EC 5.3.1.9); phosphomannose isomerase (PMI, EC 5.1.3.1.8); phosphomannose mutase (PMM, EC 5.4.2.8); GDP-mannose pyrophosphorylase (VTC1, EC 2.7.7.13); GDP-mannose-3′,5′-epimerase (GME, EC 5.1.3.18); GDP-galactose phosphorylase (VTC2, EC 2.7.7.B2); L-galactose-1-phosphate phosphatase (VTC4); L-galactose dehydrogenase (GalDH, EC 1.1.1.48); L-galactono-1,4-lactone dehydrogenase (GLDH, EC 1.3.2.3). The enzymes in the GalU pathway are: D-galacturonate reductase (GalUR) and gluconolactonase (EC 3.1.1.17). The enzymes in the MI pathway are: Inositol phosphate phosphatase (EC 3.1.3.25); myo-inositol oxygenase (MIOX, EC 1.13.99.1); glucuronate reductase (GlcUR, EC 1.1.1.19); gluconolactonase (GNL, EC 3.1.1.17), and L-gulono-1,4-lactone oxidase (GLOase, EC 1.1.3.8). The enzymes involved in AsA recycling are: Monodehydroascorbate reductase (MDHAR, EC. 1.6.5.4) and dehydroascorbate reductase (DHAR, EC 1.8.5.1). Where omitted EC number have not been assigned. (Adapted from [68])

enhanced AsA levels and in some cases as much as sixfold increases in AsA relative to wild-type plants have been achieved (Table 6.1). We and various other groups who discovered these AsA biosynthetic genes have patented their use in metabolic engineering [42].

In 2004, we reported that *myo*-inositol oxygenase (MIOX) overexpression in *Arabidopsis thaliana* leads to plants with 2–3-fold increase in foliar AsA content [69]. During the past 9 years we have worked with multiple generations of these lines and, with the exception of one line where we found gene silencing, we have

Table 6.1 Successful metabolic engineering strategies that have led to high vitamin C plants

Pathway	Plant	Enzyme	Gene source	AsA fold increase	Reference
Myo-inositol	Lettuce and tobacco	GLOase	Rat	2–7	[53]
	Arabidopsis	GLOase	Rat	2–3	[87]
	Arabidopsis	MIOX4	*Arabidopsis*	2–3, N.D., 1.7	[31, 69, 94]
	Tobacco	MIOX4	*Arabidopsis*	2	[78]
	Lettuce	MIOX4	*Arabidopsis*	2–3	[78]
	Arabidopsis	AtPAP15	*Arabidopsis*	2	[105]
	Tomato	MIOX4	*Arabidopsis*	2	[56]
	Arabidopsis	GlcUR	*Arabidopsis*	2–3	Lorence et al. unpublished
D-Mannose/L-Galactose	Tobacco	GMP	Acerola	2	[6, 7]
	Arabidopsis	AMR1	*Arabidopsis*	2–3	[106]
	Tobacco	PMM	Acerola	2	[7]
	Tomato	GME	Tomato	1.6	[107]
	Tomato, potato, and strawberry	VTC2	Kiwifruit	3–6/3/2	[12]
	Arabidopsis	AtERF98	*Arabidopsis*	1.7	[108]
D-Galacturonate	*Arabidopsis*	GalUR	Strawberry	2–3	[1]
	Tomato	GalUR	Strawberry	2	[99]
	Potato	GalUR	Strawberry	2	[47]
Recycling	Tobacco and maize	DHAR	Wheat	2–4	[16]
	Tobacco	DHAR	*Arabidopsis*	2	[28]
	Potato	DHAR	Sesame	1.5	[39]
	Maize	DHAR	Rice	6	[77]
	Arabidopsis	DHAR	*Arabidopsis*	2–4	[98]
	Tomato	DHAR	Tomato	1.6	[45]
	Tomato	MDHAR	Tomato	1.27	[35]
Other pathways	Tomato	MDH	Tomato	6	[79]
	Norway Spruce	HBK3	Norway Spruce	1.6	[11]

Abbreviations of most enzyme names are described in Fig. 6.1; *AMR1* Ascorbic acid mannose pathway regulator 1; *AtERF98 Arabidopsis thaliana* ethylene response factor 98; *HBK3* KNOTTED-like homeobox gene from Norway spruce; *MDHAR* monodehydroascorbate reductase; *MDH* malate dehydrogenase; *N.D.* nondetected

always detected elevated foliar AsA content [56, 78]. In a study published in 2009, Endres and Tenhaken failed to detect differences in AsA content between a wild-type line from the Arabidopsis Stock Center in Europe and our MIOX4 overexpressors, but did detect lower MI content in those lines [31]. In 2011, a group in Hungary provided evidence and independent verification that our MIOX4 overexpressors have elevated AsA (1.7-fold), display enhanced growth (a phenotype noted by the authors and documented with a photograph in the article), and are tolerant to high light stress compared to the wild type background used for transformation [94]. We

further reported that MIOX4 over-expressors continue to have elevated AsA content (1.75-fold) compared to controls not only when grown under normal conditions, but also when subjected to salt, cold, heat, and pyrene stresses [66].

During the past few months, excellent review papers have been published describing in detail novel functions of AsA and its role in plant evolution [33, 36]. Instead of duplicating that effort, in this review we provide an overview of research revealing three aspects of the biochemistry of AsA that have not been fully covered elsewhere. First, we will discuss the main findings of studies on feeding plant tissues with AsA precursors as a proxy to determine which of the AsA biosynthetic pathways are operational in model and crop plants, and discuss these studies in the context of the forward and reverse genetic studies that support the operation of each pathway (Sect. 6.2). Next, we will critically discuss the consequences of elevating AsA content for plant growth (Sect. 6.3). Finally, also the effect of AsA content on plant performance under environmental stress will be reviewed (Sect. 6.4).

6.2 Which Biosynthetic Pathways Leading to Ascorbate Formation are Operational in Plants?

Feeding plant organs, tissues, or cells with AsA precursors has been an invaluable tool that has allowed researchers to gain a better understanding about the plasticity of the metabolic machinery leading to AsA formation in model and crop plants (Table 6.2). This approach has been employed for more than 5 decades in the field. In most cases, the experiment has consisted in the incubation of detached tissues or organs from the plant of interest in an aqueous solution of each of the AsA precursors to be tested. Most feeding experiments have been conducted under continuous light and have lasted between 1 and 24 h. The main goal of these assays has consisted in measuring the *in planta* AsA content resulting from the uptake, transport, and conversion of the substrate in question into AsA. Table 6.2 presents a summary of the studies in which more than one AsA precursor has been tested. The reader should note that we did not include several other published studies in which only one particular precursor has been fed to the plant model of study.

The results of these feeding studies should not be analyzed in isolation but as one of many tools available to understand AsA metabolism. These studies using "cold chemicals" although valuable are considered less sensitive and accurate than those where radioactively labeled precursors have been used.

Table 6.2 illustrates that without exception the AsA pathway that is operational and predominant in tissues of all the plant species analyzed with this approach is the Man/Gal route (a.k.a. Smirnoff–Wheeler pathway, [100]). Of the intermediates in this route, L-Gal is the substrate that has led to the by far highest increases in AsA, followed by L-galactono-1,4-lactone (L-GalL) with the exception of sweet pepper [9] and papaya [9] where L-GalL seems to be preferred over L-Gal. These studies also indicate that this pathway is clearly controlled by substrate availability.

Table 6.2 Feeding studies with AsA precursors in plants

Species	Feeding conditions	Substrate	Percent increase AsA[a]	Reference
Apple (*Malus domestica)*	Flesh peel, flesh, and seed of young (y), and mature (m) fruits, 10 mM, 2 h shaking, 25 °C	Water D-Glc L-Gal L-GalL D-GalUA MI D-GlcUA L-GulL	100 (y), 100 (m) 108 (y), 109 (m) *142* (y), *132* (m) *142* (y), *127* (m) *142* (y), *145* (m) 108 (y), 91 (m) *145* (y), 118 (m) 100 (y), 100 (m)	[61]
Apple (*M. domestica*)	Leaf disks 15 mM, 30 h	Water L-Gal L-GalL L-GulL	100 *300* *300* *150*	[22]
Arabidopsis (*Arabidopsis thaliana*)	Cell suspensions (mid-log phase cells) 15 mM, 30 h 16:8 h photo-period, 24 °C	Water L-Gal L-GalL D-GalUA MI D-GlucL L-GulL	100 *6800* *3180* *3470* 100 *300* *970*	[21]
Bean (*Phaseolus vulgaris*)	Shoots (7–8 day seedlings) 0–2 %, 6–48 h	Water L-GalL D-GulL	100 *364* *157*	[8]
Black currant (*Ribes nigrum* L.)	Flowers 25 mM, 18 h shaking, dark	Water D-Glc L-Gal L-GalL D-GalUA L-Gul MI L-GulL	100 103 *296* *235* 110 108 93 108	[43]
Broccoli (*Brassica oleracea*)	Florets (petiole feeding) 0.5 %, 24 h room temperature, 40 W artificial light	Water D-Man L-Gal L-GalL	100 92 *2087* *233*	[9]
Chestnut rose (*Rosa roxburghii*)	Fruit 25 mM, 24 h room temperature, natural daylight photoperiod	Water Suc D-Fruc L-GalL L-Gul Mannitol D-GlcUA	100 120 125 *200* *140* 136 125	[3]
Green sweet pepper (*Capsicum annuum*)	Whole pepper sliced and immersed to feed 0.5 %, 24 h room temperature, 40 W artificial light	Water D-Man L-Gal L-GalL	100 86 99 *134*	[9]

Table 6.2 (continued)

Species	Feeding conditions	Substrate	Percent increase AsA[a]	Reference
Guava (*Psidium sp.*)	Whole fruit sliced and immersed to feed 0.5 %, 24 h room temperature, 40 W artificial light	Water D-Man L-Gal L-GalL	100 107 *158* *143*	[9]
Kiwi (*Actinidia deliciosa*)	Flesh discs 10 mM, 20 h shaking, 25 °C	Water D-Glc L-Gal L-GalL D-GalUA MI D-GlcUA L-GulL	100 *125* *162* *168* *125* 87 100 *150*	[62]
Papaya (*Carica papaya*)	Whole fruit sliced and immersed to feed 0.5 %, 24 h room temperature, 40 W artificial light	Water D-Man L-Gal L-GalL	100 104 95 *113*	[9]
Pea (*Pisum sativum* L.)	Embryonic axes 25 mM, 8 h dark, 20 °C	Water D-Glc L-GalL L-GulL	100 102 *420* *124*	[82]
Peach (*Prunus persica* L.)	Immature whole fruits (59 days after full bloom) 25–50 mM, 18 h room temperature	Water L-Gal L-GalL D-GalUA L-GulL	100 *350* *200* 100 *125*	[51]
Rice (*Oryza sativa*)	Shoots (s) and roots (r) D-Glc 10–20 mM L-GalL 2–10 mM 72 h natural light, 25–30 °C	Water D-Glc L-GalL	100 *120* (s), *186* (r) *390* (s), *740* (r)	[40]
Strawberry (*Fragaria sp.*)	Fruit 0.5 %, 24 h room temperature, 40 W artificial light	Water D-Man L-Gal L-GalL	100 103 *143* *148*	[9]
Tobacco (*Nicotiana tabacum*)	Young leaves 30 mM, 72 h 22°C, 14:10 h photoperiod light: 40 µmol m^{-2} s^{-1}	Water L-GalL L-GulL	100 *6932* *1068*	[53]
Tobacco (*N. tabacum*)	Leaf discs (young leaves) 5 mM, 6 h light: 400–1,000 µmol m^{-2} s^{-1}	Water D-Man L-Gal	100 100 *200*	[52]

Table 6.2 (continued)

Species	Feeding conditions	Substrate	Percent increase AsA[a]	Reference
Tomato (*Solanum lycopersicum*)	Hairy root cultures 30 mM, 16–264 h 25 ± 2 °C 12 h photoperiod	Water L-Gal D-GalUA	100 *130* *210*	[99]
Tomato (*S. lycopersicum)*	Fruit disks; mature green (m) and red tomatoes (r) 15 mM, 24 h 25 °C, constant light	Water D-Glc D-Man L-Gal L-GalL MI L-GulL	100 (m), 100 (r) 124 (m), 106 (r) *130* (m), *203* (r) *246* (m), *201* (r) *287* (m), *192* (r) 112 (m), *149* (r) *180* (m), *151* (r)	[75]
Wood tobacco (*Nicotiana benthamiana*)	Detached leaves fed via petiole 50 mM, 8 h 25 °C, constant light	Water D-Man L-Gal L-Gul D-GalUA MI L-GulL	100 *132* *338* *184* 104 102 *210*	Aboobucker and Lorence, unpublished

[a] Values were normalized relative to the water control. Values in italics were reported to be significantly different from those of the water controls.
L-Gal L-Galactose; *L-GalL* L-Galactono-1,4-lactone; *D-GalUA* D-Galacturonic acid; *D-Glc* D-Glucose; *D-GlcUA* D-Glucuronic acid; *D-GlucL* D-Glucuronolactone; *L-Gul* L-Gulose; *L-GulL* L-Gulono-1,4-lactonel; *D-Man* D-Mannose; *D-Fruc* D-Fructose; *MI* Myo-inositol; *Suc* Sucrose

In addition to the results of the feeding studies listed in Table 6.2, much biochemical and molecular genetic evidence now exists in support of the Man/Gal route. Genes encoding all the proposed biosynthetic enzymes have been identified in higher plants [19, 20, 27, 34, 50, 58, 65, 72, 81, 102]. Two master regulators of this pathway, *Arabidopsis* AMR1 [106] and AtERF98 [108] have been recently reported as well.

As shown in Table 6.2, there is a significant body of work supporting the operation of alternative routes leading to AsA formation that serve to supplement the synthesis via L-Gal at certain developmental stages and in particular tissues. In the following paragraphs, we discuss the studies supporting the operation of the L-Gul, D-GalUA, and MI pathways.

Only a few of the studies have included L-Gul in the suite of precursors analyzed (Table 6.2). Of those, the one in chestnut rose [3] and the one in wood tobacco (Aboobucker and Lorence, unpublished) provided data supporting the conversion of this substrate into AsA. L-Gul does not seem to be converted into AsA in black currant under the conditions tested [43].

In addition to the feeding studies in which L-Gul has been effectively converted into AsA, molecular evidence supporting the operation of the L-Gul pathway was obtained through the characterization of the *Arabidopsis* GDP-mannose-3′-5′-epimerase (GME) enzyme [102]. GME is able to also synthesize GDP-L-gulose,

and it may be assumed that this substrate can be converted to L-gulono-1,4-lactone (L-GulL) in an analogous way to the conversion of L-Gal to L-GalL and then to AsA. Maruta and collaborators [73] obtained evidence for the operation in tobacco cells of GLOases (a.k.a. GulLOs), enzymes capable of oxidizing L-GulL into AsA. These enzymes also participate in the MI pathway to AsA.

Multiple studies carried out in apples [22], *Arabidopsis* [21], kiwi [62], and tomato fruits [107] report the conversion of D-GalUA into AsA. However, this substrate does not seem to contribute in a significant way to vitamin C synthesis in all fruits, as indicated by the lack of conversion to AsA in black currant [43], peaches [51], and leaves of wood tobacco (Aboobucker and Lorence, unpublished).

Molecular evidence in support of the operation of the GalUA pathway to AsA was provided by the characterization of an enzyme with D-GalUA reductase activity [1] in strawberry. When overexpressed, this enzyme leads to plants with elevated AsA.

Five feeding studies have included MI in the suite of precursors assayed. Of these articles, one provides convincing evidence (statistically significant differences compared to controls fed with water) of the conversion of MI into AsA in red tomatoes [75]. Inositol does not seem to be effectively converted into the final product in apples [61], *Arabidopsis* cells [21], black currant [43], or kiwi fruits [62].

Multiple teams including ours have provided molecular evidence for the operation in higher plants of enzymes capable of using MI and other inositol phosphates including phytate for AsA production [69, 93, 105]. Overexpression of AtMIOX4 led to elevated foliar AsA content in *Arabidopsis*, as demonstrated by us [69] and independently confirmed by Tóth and collaborators [94]. The enzymes that follow MIOX in the proposed MI pathway to AsA are glucuronate reductase (GlcUR), gluconolactonase (GNL), and GLOase (a.k.a. GulLO). Manuscripts with the detailed characterization of at least one member of each of these enzyme families in *Arabidopsis* are in preparation in our group.

A multitude of studies have obtained data showing the effective conversion of L-GulL into AsA in apples [22], *Arabidopsis* [21], beans [8], kiwi [62], peas [82], peaches [51], tobacco [53], tomato [75], and wood tobacco (Aboobucker and Lorence, unpublished). This substrate is structurally quite similar to the immediate AsA precursor in the Man/Gal pathway, L-GalL. However, detailed characterization of L-galactono-1,4-lactone dehydrogenase (GLDH), the terminal enzyme in that pathway, shows that plant GLDHs are highly specific for L-GalL (reviewed in [92]). Therefore, conversion of the alternative substrate L-GulL into AsA is most likely catalyzed by GulLO, the terminal enzyme that connects the L-Gul and MI pathways (Fig. 6.1).

6.3 Effects of Ascorbate Content on Plant Growth

Among the new knowledge that has emerged from the detailed characterization of the function of the various enzymes involved in AsA metabolism in plants are the remarkable negative consequences for growth, morphology, and development of lines that are deficient in this key molecule (Table 6.3). These low-AsA lines have been

Table 6.3 Effects of low ascorbate content on the phenotype, cell structure, and development of plants

Gene mutated	Plant species	Phenotype	References
VTC1	*Arabidopsis*	Reduced growth of aerial tissue Shorter primary root length Accelerated leaf senescence Delayed flowering Cells stop elongation and undergo apoptosis early in development Extensive degradation of grana stacks	[18, 80, 85, 97]
	Potato	Reduced biomass of aerial tissue accelerated leaf senescence	[54]
GME	Tomato	Reduced growth of aerial tissue reduced cell number and size reduced fruit firmness	[37]
GLDH	Rice	Reduced plant height and root length reduced leaf and root weight delayed flowering reduced number of flowers, tillers, and number of grains	[67]
	Tomato	Reduced growth of aerial tissue reduced leaf size reduced fruit diameter and weight reduced cell size	[2]
DHAR	Tobacco	Reduced rate of leaf expansion reduced growth of aerial tissue delayed flowering	[14]

developed either after chemical mutagenesis or via knockout approaches. A common phenotype reported for *Arabidopsis*, potato, rice, tomato, and tobacco low-AsA mutants is a significant reduction of growth and biomass accumulation of both aerial and root tissues. At the cellular level, this reduction in plant size and biomass is linked in some cases with decreased cell size and in others with lower number of cells. Reduced AsA levels also seem to have a negative impact on the number of flowers, number of tillers, the size of the fruits, and seed yield. On the other hand, a question that remains open is whether elevated AsA has positive effects for plant growth and development.

We reported that *Arabidopsis* lines with enhanced AsA content overexpressing enzymes that participate in the inositol pathway, MIOX4 [69] and GLOase [87], accumulate more biomass (measured as dry weight of the aerial tissue) and display a longer inflorescence stem and a wider rosette diameter compared to controls growing in soil under similar conditions. These MIOX4 and GLOase overexpressors also showed enhanced growth of both aerial and root tissues when grown in liquid culture [66, 78]. To our knowledge, this is the first study demonstrating such a marked positive effect on plant growth in lines engineered to have elevated AsA concentrations.

We have recently incorporated the use of a Scanalyzer HTS instrument (LemnaTec, Germany), a powerful tool that allows nondestructive, unbiased, and accurate phenotyping of small plants to the characterization of the high AsA lines (MIOX4 and GLOase). For this experiment, seeds of wild type (untransformed *A.*

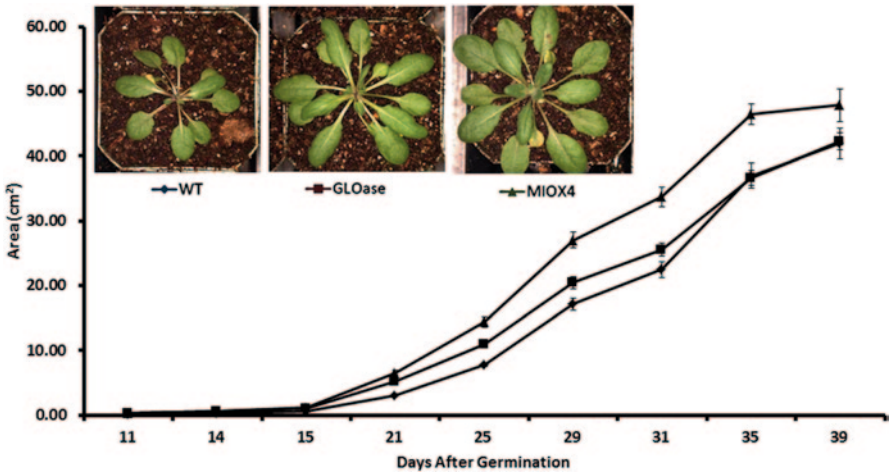

Fig. 6.2 Arabidopsis high vitamin C lines, MIOX4 and GLOase, display enhanced growth rate and biomass accumulation compared to wild-type controls. Seeds were germinated on MS media and seedlings were transferred to soil and grown under controlled conditions (23 °C, 65 % humidity, 14:10 h photoperiod, and 150 µmol m^{-2} s^{-1} light intensity). Images were acquired with a nondestructive high throughput phenotyping system (Scanalyzer HTS, LemnaTec, Germany), and leaf area was measured as previously described [4]. Images shown correspond to plants at the end of the vegetative growth (29 days after germination). Values are means ± standard error ($n = 15$)

thaliana var. Columbia, ABRC stock CS-60000) and homozygous MIOX4 and GLOase lines were cleaned and planted on Murashige and Skoog (MS) plates and vernalized for 3 days at 4 °C. Plates were then incubated in a controlled environmental chamber (Conviron, Pembina, ND) under the following conditions: 23 °C, 65 % humidity, 14:10 h photoperiod, and 150 µmol m^{-2} s^{-1} light intensity. After a week, seedlings were transferred to the soil (Arabidopsis Growing Medium, Lehle Seeds, Round Rock, TX) and grown in QuickPot 15 trays (HerkuPlast, Germany) until maturity under the above conditions. During their entire life cycle, plants were phenotyped with the Scanalyzer HTS. The acquired images were analyzed as previously described [4] to calculate plant growth (measured as foliar area in cm^2). As illustrated in Fig. 6.2, MIOX4 and GLOase overexpressors grew faster and accumulated more biomass than untransformed controls growing under similar conditions.

6.4 Effects of Ascorbate Content on Abiotic Stress Tolerance

Table 6.4 summarizes the results obtained by us and others after detailed characterization of model and crop plants with enhanced AsA content in response to various forms of abiotic stress. The AsA enhancement has been achieved in most cases after constitutive expression of biosynthetic and recycling genes; however, in some cases

Table 6.4 Stress responses of plants with elevated AsA content

Species	Method to elevate AsA	Fold AsA	Plants' performance	Reference
Arabidopsis	OE rice DHAR	1.2	Salt tolerance	[96]
	OE *Arabidopsis* DHAR	2–4.2	Paraquat tolerance Heat tolerance	[16]
	OE *Arabidopsis* MIOX4	1.7	Heat and high-light tolerance	[46]
	OE *Arabidopsis* MIOX4 and rat GLOase	1.48–1.75	Salt tolerance Cold tolerance Heat tolerance Tolerance to pyrene	[46, 66, 78]
Maize	Feeding AsA	1.25	Drought tolerance	[26]
Potato	OE rat GLOase	1.4	paraquat tolerance osmotic stress tolerance salt tolerance	[95]
	OE *Arabidopsis* DHAR	2.2–2.8	Paraquat tolerance osmotic stress tolerance salt tolerance	[30]
Rice	Feeding AsA and AsA precursors	2.9 shoots 6.4 roots	Chilling tolerance drought tolerance Al toxicity tolerance	[52]
Soybean	Feeding AsA	NM	Salt tolerance	[41]
Tobacco	OE human DHAR	No change	Cold tolerance salt tolerance	[57]
	OE wheat DHAR	2–4	ozone tolerance	[13]
	OE *Arabidopsis* DHAR	1.9–2.1	Drought tolerance ozone tolerance salt tolerance osmotic stress tolerance	[28]
	OE *Arabidopsis* MDHAR	2–2.2	Salt tolerance ozone tolerance osmotic stress tolerance	[29]
	OE *Arabidopsis* DHAR	1.3	Tolerance to Al stress	[103]
Tomato	Feeding AsA	NM	Salt tolerance	[91]
	OE tomato MDHAR	1.2	cold tolerance heat tolerance paraquat tolerance	[63]
	OE tomato GME	1.2–1.4 leaves 1.2–1.6 fruits	Paraquat tolerance cold tolerance salt tolerance	[107]
	OE potato DHAR	1.2–1.4 leaves 1.1–1.2 fruits	Paraquat tolerance salt tolerance	[64]
Wheat	Feeding AsA	1.3–1.4	Salt tolerance	[5]

NM not measured; *OE* overexpression

feeding AsA or its precursors has also led to elevated antioxidant content. The big picture that emerges from this summary is that even modest increases in AsA content have led to broad tolerance to common stresses such as salt, cold, ozone, and herbicide treatment. In addition to the overall AsA content, an aspect equally important for the health of the plant tissue is the ratio of reduced to oxidized ascorbate

or AsA redox status. In some of these studies, particularly those where DHAR and MDHAR have been overexpressed, the redox status of the plants changed, helping them overcome the stresses they have been subjected to.

Interestingly, exposure to pyrene, a polycyclic aromatic hydrocarbon and a known inducer of oxidative stress in plants, led to stunted growth of the aerial tissue, reduction in the number of root hairs, and inhibition of leaf expansion in wild-type plants, while these symptoms were less severe in the MIOX4 and GLOase overexpressors [46]. These results indicate the potential of high AsA crops as a tool for phytoremediation applications.

As AsA is intertwined in such a large number of networks (photosynthesis, flowering, ROS signaling, cell growth/division, pathogen response) and elevation of AsA levels has been shown to alter the transcription of many genes [55, 84], there could be unexpected, perhaps negative, consequences to significant elevation of AsA in plants beyond the normal physiological level. Additional research is needed in this regard.

6.5 Conclusions and Perspectives

Feeding studies have revealed that the Man/Gal pathway is operational and predominant in all the plant species analyzed to date. Of the intermediates participating in this route, L-Gal and L-GalL are most effective at increasing AsA content. However, without a doubt, these feeding studies also demonstrate the operation of all the alternative pathways that supplement the one using L-Gal in particular tissues and developmental stages. The research here reviewed indicates the potential of engineering elevated AsA content as an effective strategy to develop crops with enhanced biomass and abiotic stress tolerance. Based on our results and the ones of others, we propose that increases between 1.5- and 6-fold in the total AsA content of plant tissues are necessary to have broad tolerance to abiotic stresses in crops. Future generations of these engineered crops are likely to include the combination of the expression of biosynthetic and recycling genes, and/or the expression of regulatory proteins and transcription factors that have been recently shown to modulate multiple genes at once (e.g., [106, 108]). Although the results obtained by us and others show significant promise for the development of plants with enhanced abiotic stress tolerance, an aspect until now not fully explored is the evaluation of the consequences of elevated AsA content in the ability of plants to interact with insects and other herbivores (reviewed in [38]). This is a crucial aspect that must be evaluated at both greenhouse and field levels before these crops can be deployed.

Acknowledgments This study was supported by funds from the Arkansas Biosciences Institute, the major research component of the Arkansas Tobacco Settlement Proceeds Act of 2000, and a sub-award to AL from the Arkansas INBRE program [National Center for Research Resources (5P20RR016460-11) and the National Institute of General Medical Sciences (8P20GM103429-11) from the National Institutes of Health]. The Scanalyzer HTS was acquired with funds from the

Arkansas Center for Plant-Powered Production (P3) through the RII Arkansas ASSET Initiative (AR EPSCoR), NSF grant # EPS 0701890. KAL and SIA thank the Molecular Biosciences Graduate program at Arkansas State University for a scholarship.

References

1. Agius F, Gonzalez-Lamothe R, Caballero JL, Muñoz-Blanco J, Botella MA, Valpuesta V (2003) Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. Nat Biotechnol 21:177–181
2. 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
3. An HM, Fan WG, Chen LG, Asghar S, Liu QL (2007) Molecular characterization and expression of L-galactono-1,4-lactone dehydrogenase and L-ascorbic acid accumulation during fruit development in *Rosa roxburghii*. J Hortic Sci Biotech 82:627–635
4. Arvidsson S, Perez-Rodriguez P, Mueller-Roeber B (2011) A growth phenotyping pipeline for *Arabidopsis thaliana* integrating image analysis and rosette area modeling for robust quantification of geno-type effects. New Phytologist. doi:10.1111/j.1469-8137.2011.03756.x
5. Athar HR, Khan A, Ashraf M (2008) Exogenously applied ascorbic acid alleviates salt-induced oxidative stress in wheat. Environ Exp Bot 63:224–231
6. 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
7. Badejo AA, Eltelib HA, Fukunaga K, Fujikawa Y, Esaka M (2009) Increase in ascorbate content of transgenic tobacco plants overexpressing the acerola (*Malpighia glabra*) phosphomannomutase gene. Plant Cell Physiol 50:423–428
8. Baig MM, Kelly S, Loewus F (1970) L-Ascorbic acid biosynthesis in higher plants from L-gulono-1,4-lactone and L-galactono-1,4-lactone. Plant Physiol 46:277–280
9. Barata-Soares AD, Gomez MLPA, Henrique deMC, Lajol FM (2004) Ascorbic acid biosynthesis: a precursor study on plants. Braz J Plant Physiol 16:147–154
10. 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
11. Belmonte MF, Stasolla C (2009) Altered HBK3 expression affects glutathione and ascorbate metabolism during the early phases of Norway spruce (*Picea abies*) somatic embryogenesis. Plant Physiol Biochem 47:904–911
12. 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
13. Chen Z, Gallie DR (2005) Increasing tolerance to ozone by elevating foliar ascorbic acid confers greater protection against ozone than increasing avoidance. Plant Physiol 138:1673–1689
14. Chen Z, Gallie DR (2006) Dehydroascorbate reductase affects leaf growth, development, and function. Plant Physiol 142:775–777
15. Chen Z, Gallie DR (2012) Induction of monozygotic twinning by ascorbic acid in tobacco. PLoS ONE 7. doi:10.1371/journal.pone 0039147
16. Chen Z, Young TE, Ling J, Chang S-C, Gallie DR (2003) Increasing vitamin C content of plants through enhanced ascorbate recycling. Proc Natl Acad Sci USA 100:3525–3230
17. 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

18. Conklin PL, Williams EH, Last RL (1996) Environmental stress sensitivity of an ascorbic acid-deficient *Arabidopsis* mutant. Proc Natl Acad Sci USA 93:9970–9974
19. Conklin PL, Norris SN, 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 USA 96:4198–4203
20. 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
21. Davey MW, Gilot C, Persiau G, Østergaard J, Han Y, Bauw GC, Van Montagu MC (1999) Ascorbate biosynthesis in Arabidopsis cell suspension culture. Plant Physiol 121:535–543
22. Davey MW, Franck C, Keulemans J (2004) Distribution, developmental and stress responses of antioxidant metabolism in *Malus*. Plant Cell Environ 27:1309–1320
23. De Tullio MC, Arrigoni O (2004) Hopes, disillusions and more hopes from vitamin C. Cell Molec Life Sci 61:209–219
24. Debolt S, Melino V, Ford CM (2007) Ascorbate as a biosynthetic precursor in plants. Ann Bot 99:3–8
25. Dhar-Mascareño M, Cárcamo JM, Golde DW (2005) Hypoxia-reoxygenation-induced mitochondrial damage and apoptosis in human endothelial cells are inhibited by vitamin C. Free Rad Biol Med 38:1311–1322
26. Dolatabadian A, Modarres-Sanavy SAM, Sharifi M (2009) Alleviation of water deficit stress effects by foliar application of ascorbic acid on *Zea may*s L. J Agron Crop Sci 195:347–355
27. 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
28. Eltayeb AE, Kawano N, Badawi H, Kaminaka H, Sanekata T, Morishima I, Shibahara T, Inanaga S, Tanaka K (2006) Enhanced tolerance to ozone and drought stresses in transgenic tobacco overexpressing dehydroascorbate reductase in cytosol. Physiol Plant 127:57–65
29. Eltayeb AE, Kawano N, Badawi GH, Kaminaka H, Sanekata T, Shibahara T, Inanaga S, Tanaka K (2007) Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt, and polyethylene glycol stresses. Planta 225:1255–1264
30. Eltayeb AE, Yamamoto S, Eltayeb Habora ME, Yin L, Tsujimoto H, Tanaka K (2011) Transgenic potato overexpressing Arabidopsis cytosolic *AtDHAR1* showed higher tolerance to herbicide, drought, and salt stresses. Breeding Sci 61:3–10
31. Endres S, Tenhaken R (2009) *Myo*-inositol oxygenase controls the level of *myo*-inositol in Arabidopsis, but does not increase ascorbic acud. Plant Physiol 149:1042–1049
32. Fischer H, Schwarzer C, Illek B (2004) Vitamin C controls the cystic fibrosis transmembrane conductance regulator chloride channel. Proc Natl Acad Sci USA 101:3691–3696
33. Gallie DR (2013) The role of L-ascorbic acid recycling in responding to environmental stress and in promoting plant growth. J Exp Bot 64:433–443
34. Gatzek S, Wheeler GL, Smirnoff N (2002) Antisense supression of L-galactose dehydrogenase in *Arabidopsis thaliana* provides evidence for its role in ascorbate biosynthesis and reveals light modulated L-galactose synthesis. Plant J 30:541–553
35. Gest N, Garchery C, Gautier H, Jimenez A, Stevens R (2013) Light-dependent regulation of ascorbate in tomato by a monodehydroascorbate reductase localized in peroxisomes and the cytosol. Plant Biotech J 11:344–354
36. 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
37. Gilbert L, Alhagdow M, Nunes-Nesi A, Quemener B, Guillon F, Bouchet B, Faurobert M, Gouble B, Page D, Garcia V, Petit J, Stevens R, Causse M, Fernie AR, Lahaye M, Rothan C, Baldet P (2009) GDP-D-mannose 3,5-epimerase (GME) plays a key role at the intersection of ascorbate and non-cellulosic cell wall biosynthesis in tomato. Plant J 60:499–508
38. Goggin FL, Avila CA, Lorence A (2010) Vitamin C content in plants is modified by insects and influence susceptibility to herbivory. Bioessays 32:777–790

39. Goo YM, Chun HJ, Kim TW, Lee CH, Ahn MJ, Bae SC, Cho KJ, Chun JA, Chung CH, Lee SW (2008) Expressional characterization of dehydroascorbate reductase cDNA in transgenic potato plants. J Plant Biol 51:35–41
40. Guo Z, Tan H, Zhu Z, Lu S, Zhou B (2005) Effect of intermediates on ascorbic acid and oxalate biosynthesis of rice in relation to its stress resistance. Plant Physiol Biochem 43:955–962
41. Hamada AM, Al-Hakimi ABM (2009) Hydroponic treatment with ascorbic acid decreases the effect of salinity injury in two soybean cultivars. Phyton 49:43–62
42. Hancock R (2009) Recent patents on vitamin C: opportunities for crop improvement and single-step biological manufacture. Rec Patents Food Nutr Agric 1:39
43. 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
44. Harding A-H, Wareham NJ, Bingham SA, Khaw K, Luben R, Welch A, Forouhi NG (2008) Plasma vitamin C level, fruit and vegetable consumption, and the risk of new-onset type 2 diabetes mellitus: the european prospective investigation of cancer-Norfolk prospective study. Arch Intern Med 168:1493–1499
45. Haroldsen VM, Chi-Ham CL, Kulkarni S, Lorence A, Bennett AB (2011) Constitutively expressed DHAR and MDHAR influence fruit, but not foliar ascorbate levels in tomato. Plant Physiol Biochem 49:1244–1249
46. Harris RS (2009) Analysis of the protective effects of ascorbic acid on thrichloroethylene and pyrene phytotoxicity. Master's Thesis. Arkansas State University, Jonesboro, AR, USA
47. Hemavathi, Upadhyaya CP, Young KE, Akula N, Kim HS, Heung JJ, Oh OM, Aswath CR, Chun SC, Kim DH, Park SW (2009) Over-expression of strawberry D-galacturonic acid reductase in potato leads to accumulation of vitamin C with enhanced abiotic stress tolerance. Plant Sci 177:659–667
48. Horemans N, Foyer CH, Potters G, Asard H (2000) Ascorbate function and associated transport systems in plants. Plant Physiol Biochem 38:531–540
49. Huang A, Vita JA, Venema RC, Keaney Jr JF (2000) Ascorbic acid enhances endothelial nitric-oxide synthase activity by increasing intracellular tetrahydrobiopterin. J Biol Chem 275:17399–17406
50. Imai T, Karita S, Shiratori G, Hattori M, Numome T, Oba K, Hirai M (1998) L-Galactono-ɣ-lactone dehydrogenase from sweet potato: purification and cDNA sequence analysis. Plant Cell Physiol 39:1350–1358
51. Imai T, Ban Y, Terakami S, Yamamoto T, Moriguchi T (2009) L-ascorbate biosynthesis in peach: cloning of six L-galactose pathway-related genes and their expression during peach fruit development. Physiol Plant 136:139–149
52. Imai T, Ban Y, Yamamoto T (2012) Ectopic overexpression of peach GDP-D-mannose pyrophosphorylase and GDP-D-mannose-3',5'-eperimase in transgenic tobacco. Plant Cell Tiss Organ Cult 111:1–13
53. Jain AK, Nessler CL (2000) Metabolic engineering of an alternative pathway for ascorbic acid biosynthesis in plants. Mol Breeding 6:73–78
54. Keller R, Springer F, Renz A, 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
55. Kiddle G, Pastori GM, Bernard S, Pignocchi C, Antoniw J, Verrier PJ, Foyer CH (2003) Effects of leaf ascorbate content on defense and photosynthesis gene expression in Arabidopsis thaliana. Antiox Redox Signal 5:23–32
56. Kulkarni S (2012) Elevating ascorbate content in tomato and studying the role of jasmonates in modulating ascorbate in Arabidopsis. MS Thesis, Arkansas State University, Jonesboro, AR, USA
57. Kwon SY, Choi SM, Ahn YO, Lee HS, Lee HB, Park YM, Kwak SS (2003) Enhanced stress-tolerance of transgenic tobacco plants expressing a human dehydroascorbate reductase gene. J Plant Physiol 160:347–353

58. 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 USA 104:9534–9539
59. Lee KW, Lee HJ, Surh YJ, Lee CY (2003) Vitamin C and cancer chemo- prevention: reappraisal. Am J Clin Nutr 78:1074–1078
60. Levi F, Pasche C, Lucchini F, La Vecchia C (2001) Dietary intake of selected micro- nutrients and breast-cancer risk. Int J Cancer 91:260–263
61. Li MJ, Ma FW, Zhang M, Pu F (2008) Distribution and metabolism of ascorbic acid in apple fruits (*Malus domestica* Borkh cv. Gala). Plant Sci 174:606–612
62. 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. doi: 10.1371/journal.pone.0014281
63. Li F, Wu QY, Sun YL, Wang LY, Yang XH, Meng QW (2010) Overexpression of chloroplastic monodehydroascorbate reductase enhance tolerance to temperature and methyl viologen-mediated oxidative stress. Physiol Plant 139:421–434
64. Li Q, Li Y, Li C, Yu X (2012) Enhanced ascorbic acid accumulation through overexpression of dehydroascorbate reductase confers tolerance to methyl viologen and salt stresses in tomato. Czech J Genet Plant Breed 48:74–86
65. 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
66. Lisko KA, Torres R, Harris RS, Belisle M, Jullian B, Vaughan MM, Chevone BI, Mendes P, Nessler CL, Lorence A (2013) Elevating vitamin C content via overexpression of myo-inositol oxygenase and L-gulono-1,4-lactone oxidase in Arabidopsis leads to enhanced biomass and tolerance to abiotic stresses. In Vitro Cel Develop Biol Plant49(6):643–655
67. Liu Y, Yu L, Wang R (2011) Level of ascorbic acid in transgenic rice for L-galactono-1,4-lactone dehydrogenase overexpressing or suppressed is associated with plant growth and seed set. Acta Physiol Plant 33:1353–1363
68. Lorence A, Nessler CL (2007) Pathway engineering of the plant vitamin C metabolic network. In: Verpoorte R, Alferman AW, Johnson TS (eds) Applications of plant metabolic engineering. Springer, Dordrecht
69. 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
70. Mandl J, Szarka A, Bánhegyi G (2009) Vitamin C: update on physiology and pharmacology. British J Pharmacol 157:1097–1110
71. Margittai E, Banhegyi G, Kiss A, Nagy G, Mandl J, Schaff Z, Csala M (2005) Scurvy leads to endoplasmic reticulum stress and apoptosis in the liver of guinea pigs. J Nutr 135:2530–2534
72. 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
73. 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
74. Masaki KH, Losonczy KG, Izmirlian G, Foley DJ, Ross GW, Petrovitch H, Havlik R, White LR (2000) Association of vitamin E and C supplement use with cognitive function and dementia in elderly men. Neurology 54:1265–1272
75. Mellidou I, Keulemans J, Kanellis AK, Davey MW (2012) Regulation of fruit ascorbic acid concentrations during ripening in high and low vitamin C tomato cultivars. BMC Plant Biol 12:239
76. Napoli C, Williams-Ignarro S, de Nigris F, Lerman LO, Rossi L, Guarino C, Mansueto G, Di Tuoro F, Pignalosa O, De Rosa G, Sica V, Ignarro LJ (2004) Long-term combined beneficial effects of physical training and metabolic treatment on atherosclerosis in hypercholesterolemic mice. Proc Natl Acad Sci USA 101:8797–8802
77. Naqvi S, Zhu CF, Farre G, Ramessar K, Bassie L, Breitenbach J, Perez Conesa D, Ros G, Sandmann G, Capell T, Christou P (2009) Transgenic multivitamin corn through biofortifica-

tion of endosperm with three vitamins representing three distinct metabolic pathways. Proc Natl Acad Sci USA 106:7762–7767
78. Nessler CL, Lorence A, Chevone B, Mendes P (2007) Increase in plant growth rate, biomass accumulation and stress tolerance in plants over-expressing genes of the ascorbic acid-cell wall biosynthetic network. US Patent Application No 11/908, 551
79. Nunes-Nesi A, Carrari F, Lytovchenko A, Smith AM, Loureiro ME, Ratcliffe AG, Sweetlove LJ, Fernie AR (2005) Enhanced photosynthetic performance and growth as a consequence of decreasing mitochondrial malate dehydrogenase activity in transgenic tomato plants. Plant Physiol 137:611–622
80. Olmos E, Kiddle G, Pellny TK, Kumar S, Foyer CH (2006) Modulation of plant morphology, root architecture, and cell structure by low vitamin C in *Arabidopsis thaliana*. J Exp Bot 57:1645–1655
81. Ostergaard J, Persiau G, Davey MW, Bauw G, van Montagu G (1997) Isolation of a cDNA coding for L-galactono-ɣ-lactone dehydrogenase, an enzyme involved in the biosynthesis of ascorbic acid in plants. J Biol Chem 272:30009–30016
82. Pallanca JE, Smirnoff N (1999) Ascorbic acid metabolism in pea seedlings. A comparison of D-glucosone, L-sorbosone, and L-galactono-1,4-lactone as ascorbate precursors. Plant Physiol 120:453–461
83. Passage E, Norreel JC, Noack-Fraissignes P, Sanguedolce V, Pizant J, Thirion X, Robaglia-Schlupp A, Pellissier JF, Fontés M (2004) Ascorbic acid treatment corrects the phenotype of a mouse model of Charcot-Marie-Tooth disease. Nat Med 10:396–401
84. 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
85. Pavet V, Olmos E, Kiddle G, Mowla S, Kumar S, Antoniw J, Alvarez ME, Foyer CH (2005) Ascorbic acid deficiency activates cell death and disease resistance in *Arabidopsis thaliana*. Plant Physiol 139:1291–1303
86. Potters G, De Gara L, Asard H, Hoemans N (2002) Ascorbate and glutathione: guardians of the cell cycle, partners in crime? Plant Physiol Biochem 40:537–548
87. Radzio JA, Lorence A, Chevone BI, Nessler CL (2003) L-Gulono-1,4-lactone oxidase expression rescues vitamin C-deficient Arabidopsis (*vtc*) mutants. Plant Molec Biol 53:837–844
88. Rinne T, Mutschler E, Wimmer-Greinecker G, Moritz A, Olbrich HG (2000) Vitamins C and E protect isolated cardiomyocytes against oxidative damage. Int J Cardiol 75:275–281
89. Schleicher RL, Carroll MD, Ford ES, Lacher DA (2009) Serum vitamin C and the prevalence of vitamin C deficiency in the United States: 2003–2004 National Health and Nutrition Examination Survey (NHANES). Am J Clin Nutr 90:1252–1263
90. Schwager J, Schulze J (1998) Modulation of interleukin production by ascorbic acid. Vet Immunol Immunopathol 64:45–57
91. Shalata A, Neumann PM (2001) Exogenous ascorbic acid (vitamin C) increases resistance to salt stress and reduced lipid peroxidation. J Exp Bot 52:2207–2211
92. Smirnoff N (2011) Vitamin C: the metabolism anf functions of ascorbic acid in plants. Adv Bot Res 59:107–177
93. Torabinejad J, Donahue JL, Gunesekera BN, Allen-Daniels MJ, Gillaspy GE (2009) *VTC4* is a bifunctional enzyme that affects *myo*-inositol and ascorbate biosynthesis in plants. Plant Physiol 150:951–961
94. Tóth SZ, Nagy V, Puthur JT, Kovacs L, Garab G (2011) The physiological role of ascorbate as photosystem II electron donor: protection against photoinactivation in heat-stressed leaves. Plant Physiol 156:382–392
95. Upadhyaya HCP, Akula N, Young KE, Young KE, Chun SC, Kim DH, Park SW (2010) Enhanced ascorbic acid accumulation in transgenic potato confers tolerance to various abiotic stresses. Biotechnol Lett 32:321–330
96. Ushimaru T, Nakagawa T, Fujioka Y, Daicho K, Naito M, Yamauchi Y, Nonaka H, Amako K, Yamawaki K, Murata N (2006) Transgenic *Arabidopsis* plants expressing the rice dehydroascorbate reductase gene are resistant to salt stress. J Plant Physiol 163:1179–1184

97. Veljovic-Jovanovic SD, Pignocchi C, Noctor G, Foyer CH (2001) Low ascorbic acid in the *vtc*-1 mutant of *Arabidopsis* is associated with decreased growth and intracellular redistribution of the antioxidant system. Plant Physiol 127:426–435
98. Wang Z, Xiao Y, Chen W, Tank K, Zhang L (2010) Increased vitamin C content accompanied by an enhanced recycling pathway confers oxidative stress tolerance in Arabidopsis. JIPB 52:400–409
99. Wevar-Oller AL, Agostini E, Milrad SR, Medina MI (2009) In situ and de novo biosynthesis of vitamin C in wild type and transgenic tomato hairy roots: a precursor feeding study. Plant Sci 177:28–34
100. Wheeler GL, Jones MA, Smirnoff N (1998) The biosynthetic pathway of vitamin C in higher plants. Nature 393:365–369
101. Wintergerst ES, Maggini S, Hornig DH (2006) Immune-enhancing role of vitamin C and zinc and effect on clinical conditions. Ann Nutr Metab 50:85–94
102. Wolucka BA, Van Montagu M (2003) GDP-mannose-3'-5'-epimerase forms GDP-L-gulose, a putative intermediate for the de novo biosyn thesis of vitamin C in plants. J Biol Chem 278:47483–47490
103. Yin L, Wanf S, Eltayeb AE, Uddin MI, Yamamoto Y, Tsuji W, Takeuchi Y, Tanaka K (2010) Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminum stress in transgenic tobacco. Planta 231:609–621
104. Yokoyama T, Date C, Kokubo Y, Yoshiike N, Matsumura Y, Tanaka H (2000) Serum vitamin C concentration was inversely associated with subsequent 20-year incidence of stroke in a japanese rural community: the Shibata study. Stroke 31:2287–2294
105. 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
106. Zhang WY, Lorence A, Gruszewski HA, Chevone B, Nessler CL (2009) *AMR1*, an Arabidopsis gene that coordinately and negatively regulates the D-mannose/L-galactose ascorbic acid biosynthetic pathway. Plant Physiol 150:942–950
107. Zhang C, Liu J, Zhang Y, Cai X, Gong P, Zhang J, Wang T, Li H, Ye Z (2011) Overexpression of SlGMEs leads to ascorbate accumulation with enhanced oxidative stress, cold, and salt tolerance in tomato. Plant Cell Rep 30:389–398
108. Zhang Z, Wang J, Zhang R, Huang R (2012) The ethylene response factor AtERF98 enhances tolerance to salt through the transcriptional activation of ascorbic acid synthesis in Arabidopsis. Plant J 71:273–287