

INVITED REVIEW

Recent advances in the role and biosynthesis of ascorbic acid in plants

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ABSTRACT

The past few years have provided many advances in the role and biosynthesis of L-ascorbic acid (AsA) in plants. There is an increasing body of evidence confirming that AsA plays an important role in the detoxification of reactive oxygen species. The role of AsA in photoprotection has been confirmed *in vivo* with the use of *Arabidopsis* mutants. A player in the defence against reactive oxygen species, AsA peroxidase, has been extensively studied at the molecular level, and regulation of this key enzymatic activity appears to occur at several levels. As a cofactor in the hydroxylation of prolyl and lysyl-residues by peptidyl-prolyl and -lysyl hydroxylases, AsA plays a part in cell wall synthesis, defence, and possibly cell division. The maintenance of reduced levels of AsA appears to be highly regulated, involving the interplay of both monodehydroascorbate and dehydroascorbate reductases and possibly auxin. A major breakthrough in plant AsA biosynthesis has been made recently, and strong biochemical and genetic evidence suggest that GDP-mannose and L-galactose are key substrates. In addition, evidence for an alternative AsA biosynthetic pathway(s) exists and awaits additional scrutiny. Finally, newly described *Arabidopsis* mutants deficient in AsA will further increase our understanding of AsA biosynthesis

Key-words: antioxidant; *Arabidopsis*; ascorbate; biosynthesis; cell division; cofactor; hydroxylation; metabolism; mutant.

INTRODUCTION

L-Ascorbic acid (AsA) is a highly abundant metabolite and has important roles in plant stress physiology as well as growth and development. In the detoxification of reactive oxygen species, AsA is a key antioxidant. As an enzyme cofactor, AsA plays significant parts in photoprotection, the wounding response, and insect herbivory as well as cell expansion and division. Finally, AsA is the precursor for

oxalate and tartrate. Given the widespread roles of AsA in plants, it is surprising that only recently has significant progress been made in elucidating the plant AsA biosynthetic pathway. However, new tools have provided us with an increasingly clearer picture of AsA biosynthesis. In the following review, I will detail recent advances in the synthesis and role of AsA in plants.

THE ROLES OF ASCORBIC ACID IN PLANTS

Ascorbic acid as an antioxidant

As an antioxidant, AsA has an important role in protection against oxidative stress. Reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂), superoxide, and the hydroxyl radical cause oxidative stress and are generated by a wide variety of factors in plants. Under normal conditions, ROS are generated during photosynthesis by singlet oxygen formation as well as oxygen photoreduction. Photo-oxidative damage can occur when ROS production exceeds that of the antioxidant capacity. Such conditions occur when high light is combined with other environmental conditions such as drought, temperature extremes, or nutrient deprivation (for a review see Foyer, Lelandais & Kunert 1994). Other abiotic factors in the plant's environment also lead to increased ROS including UV-B, air pollutants (e.g. ozone, sulphur dioxide), redox-active herbicides (e.g. paraquat), and phytotoxic metals (e.g. Zn, Cu, Cd). Plants also generate ROS *in planta* in oxidative bursts that occur during pathogen infection (Lamb & Dixon 1997). H₂O₂ generated during the oxidative burst is thought to play an important role in initiation of the hypersensitive response (Chen, Silva & Klessig 1993; Levine *et al.* 1994), although given the toxicity of ROS such as the hydroxyl radical, ROS levels must be tightly controlled. This control is accomplished through an interconnected network of antioxidants. Within this network, AsA eliminates ROS through multiple mechanisms. AsA has the capacity to directly eliminate several different ROS including singlet oxygen, superoxide, and hydroxyl radicals (Padh 1990). It also maintains the membrane-bound antioxidant α -tocopherol in the reduced state (Packer, Slater & Wilson 1979; Liebler, Kling & Reed 1986) and indirectly eliminates H₂O₂ through the activity of AsA peroxidase (Foyer & Halliwell 1976; Asada & Takahashi 1987; Asada 1992). In addition, AsA has a major

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role in photoprotection as a cofactor in the xanthophyll cycle (reviewed in Eskling, Arvidsson & Åkerlund 1997).

Many studies over the years have investigated the consequences of an oxidative stress on the AsA status in plant cells. Often, the pool of AsA is found to become increasingly oxidized due to a combination of increased oxidation and/or inefficient regeneration. Loss of one electron from AsA results in formation of monodehydroascorbate (MDA). MDA is unstable and unless re-reduced, disproportionates to AsA and dehydroascorbate (DHA). Both MDA and DHA can be reduced back to AsA through the activity of reductases (Foyer & Halliwell 1976; Jablonski & Anderson 1981; Hossain & Asada 1984). In *Triticum durum* seedlings grown at an elevated temperature (~30 °C) for 34 d the AsA : DHA ratio declined significantly (Paolacci *et al.* 1997). Treatment of *Nicotiana plumbaginifolia* with toxic concentrations of either NaCl or CuSO₄ for 120 h resulted in complete oxidation of AsA (Savouré *et al.* 1999). MDA radicals increase in several plant species upon exposure to a variety of ROS-generating stresses including the redox-active herbicide paraquat, cyanide, high light, drought and senescence (Heber *et al.* 1996). The redox status of AsA is at least partially dependent on the size of the AsA pool. In an AsA-deficient mutant of *Arabidopsis* (*vtc1*) grown under non-stress conditions the AsA : DHA ratio is nearly two-times lower than in the wild-type progenitor (Conklin, Williams & Last 1996).

Ascorbate peroxidase

Ascorbate peroxidases (APXs) are a major consumer of AsA, generating MDA in the reduction of hydrogen peroxide to water. APX has a key role in removal of hydrogen peroxide, and APX isoenzymes are located in the chloroplast, cytosol, peroxisome, and mitochondria (Mittler & Zilinskas 1991; Asada 1992; Jimenez *et al.* 1997). The regulation of APX has been the focus of much recent research and appears quite complicated. There is evidence for control at both the level of transcription and/or steady-state mRNA accumulation as well as possibly at the level of translation.

Several groups have demonstrated that increased ROS triggers an increase in the steady-state level of one or more APX mRNAs (Conklin & Last 1995; Kubo *et al.* 1995; Donahue *et al.* 1997; Morita *et al.* 1999). Photooxidative stress caused by exposure to excess light causes rapid elevation of two cytosolic APX mRNAs in *Arabidopsis* (Karpinski *et al.* 1997). Experiments with glutathione and with inhibitors that block reduction or oxidation of the plastoquinone pool have led the Karpinski and Mullineaux groups to speculate that the redox status of glutathione may be involved in transmission of a signal from the excess light-stressed chloroplast to the nucleus (Karpinski *et al.* 1997) that controls APX expression. The authors also make note that AsA could theoretically play a role in this signaling cascade.

APX is also subject to post-transcriptional regulation. Salt and copper stress both cause increases in APX activity whereas the level of the mRNA remains constant

(Lopez *et al.* 1996) or even declines (Savouré *et al.* 1999). Such results could be obtained if APX is under post-transcriptional control. Post-transcriptional control of APX has been shown to occur during induction of programmed cell death in tobacco mosaic virus-infected tobacco (Mittler, Feng & Cohen 1998). Although transcripts encoding a specific cytosolic APX accumulated to high levels, the level of the corresponding APX protein declined. The inhibition of APX expression was shown to occur post-transcriptionally after binding of the APX mRNAs to ribosomes. Furthermore, methyl jasmonate and salicylic acid appear to play a role in this regulation (Mittler *et al.* 1998).

Photoprotection

In addition to its role in protection against photo-oxidative stress through the activity of APX, AsA also has a key role in photoprotection. AsA levels increase in plants grown under high light, and AsA is a cofactor utilized in the xanthophyll cycle (for an extensive review see Eskling *et al.* 1997). This cycle (conversion of violaxanthin to zeaxanthin across the thylakoid membrane) is thought to be involved in non-photochemical quenching of excess light energy in photosystem II (Demmig-Adams & Adams 1990). As early as 1969, AsA was shown to be required *in vitro* as a cofactor for the enzyme violaxanthin de-epoxidase in the conversion of violaxanthin to zeaxanthin (Hager 1969). Recent data obtained with an *in vivo* system support this *in vitro* data. The AsA-deficient *Arabidopsis* mutants (Conklin *et al.* 2000) have lower levels of non-photochemical quenching due to a decrease in this de-epoxidation reaction (P. Mueller and K. Niyogi, personal communication; Smirnov 2000).

Hydroxyproline-rich protein synthesis

AsA is also a cofactor for peptidyl-prolyl and -lysyl hydroxylases, active in the synthesis of hydroxyproline and hydroxylysine (Padh 1990). In plants, the extensively studied and highly abundant hydroxyproline-rich glycoproteins (HRGPs) are key components of the cell wall. These glycoproteins are thought to be involved in structural support of the cell wall and are induced (along with the prolyl hydroxylation reaction) by wounding, ethylene and pathogens (reviewed in Sommer-Knudsen, Bacic & Clarke 1997). Inhibition of peptidyl-prolyl hydroxylase results in major changes in the organization of cellular zones of root development (De Tullio *et al.* 1999). Induction of HRGP synthesis increases the demand for AsA. Indeed, wounding of several different tomato tissues causes a dramatic accumulation of MDA reductase mRNA (Grantz, Brummell & Bennett 1995). Increased levels of the reductase would assist in the maintenance of the level of AsA necessary for efficient hydroxylation. As wounding generates ROS, increased demand for AsA as an antioxidant is no doubt also a factor in the regulation of reductase activity (Grantz *et al.* 1995). There is also evidence that AsA may be involved directly in HRGP induction. Incubation of maize

suspension cells with AsA causes an increase in the accumulation of HRGP mRNA in an ethylene-independent fashion although this induction is not as rapid that seen with ethylene-mediated fungal elicitor (García-Muniz, Martínez-Izquierdo & Puigdomènech 1998). The question of whether AsA plays a part in HRGP regulation during wounding or pathogen attack remains to be answered.

The role of AsA as a cofactor in the synthesis of hydroxyproline may also explain the apparent involvement of AsA in plant cell division.

Cell division

There is some correlative evidence suggesting that AsA is involved in plant cell division. AsA levels are generally found to be high in meristematic tissue and very low in zones with little active cell division such as the maize root quiescent centre (reviewed in Smirnov 1996). In parallel, in non-dividing cells ascorbate oxidase mRNA and activity are high whereas during the transition into active cell division, they are both quite low (Kerk & Feldman 1995; Kato & Esaka 1999). Furthermore, treatment with exogenous AsA enables cell-division competent cells to progress more rapidly from G₁ to S (Citterio *et al.* 1994). Although it appears from these data that AsA is involved in plant cell division, the mechanism of this involvement has not been extensively studied. Furthermore, it is not known if low AsA and high ascorbate oxidase activity in the maize quiescent centre can be generalized to other species or even other cultivars of maize.

Several roles for AsA in cell division have been advanced (Smirnov 1996) but the hypothesis that AsA is required for prolyl-hydroxylation of protein(s) required for progression through the cell cycle is the best supported by published data. Several studies have suggested that HRGPs play a role in progression through the cell cycle (Vera, Lamb & Doerner 1994; Ito *et al.* 1998; De Tullio *et al.* 1999). In pea, AsA does not appear able to induce non-competent cells to divide but rather is necessary for progression of competent cells through the cell cycle (Citterio *et al.* 1994). Inhibition of prolyl hydroxylation inhibits cell division in tobacco protoplasts (Cooper, Heuser & Varner 1994) and causes the majority of onion root cells to arrest at metaphase (De Tullio *et al.* 1999). AsA may be a required component of cell division, however, it seems unlikely that AsA is involved in regulation of cell cycle progression. Cell cycle checkpoint controls are highly conserved across kingdoms and animals contain at least an order of magnitude lower AsA than plants, yet this concentration of AsA appears to be sufficient for progression through the cell cycle. Finally, AsA-deficient *Arabidopsis thaliana* mutants appear to have no gross defect in cell division (P. L. Conklin, unpublished observations).

Cell expansion and ascorbate oxidase

Existing data has been collected and distilled into a hypothesis regarding the role of AsA and ascorbate oxidase in

regulation of cell expansion that was presented by N. Smirnov (Smirnov 1996). Smirnov hypothesized that the cell wall generates MDA via ascorbate oxidase and reduces MDA via a plasma membrane-bound (NAD(P)H-requiring) cytochrome *b* (Horemans, Asard & Caubergs 1994; Asard, Horemans & Caubergs 1995) back to AsA. This AsA is then transported into the apoplastic space. In this cycle, electrons would be transported across the plasma membrane. Electron transport across the plasma membrane is thought to stimulate cell expansion via the acid growth theory (Rayle & Cleland 1992). In addition, the AsA and DHA found in the cell wall most likely influence the cross-linking of cell wall proteins and polysaccharides, lignification, and calcium levels, contributing to control of cell expansion (reviewed in Smirnov 1996). In support of this hypothesis, Kato and Esaka have recently shown that AsA and both the apoplastic enzyme activity of ascorbate oxidase and its mRNA increase in tobacco cells during cellular elongation (Kato & Esaka 1999). Additional details on the role of AsA at the plasma membrane surface can be found in a very recent review (Horemans, Foyer & Asard 2000).

ASCORBIC ACID BIOSYNTHESIS IN HIGHER PLANTS

Early proposal for a 'mammal-like' pathway

Mammals that synthesize AsA do so via the substrates D-glucose, D-glucuronic acid, L-gulonic acid, and L-gulonolactone, which is oxidized to AsA. In this pathway, the carbon skeleton of the primary substrate glucose is inverted in the final product, and this inversion occurs after glucuronate formation (for a review, see Burns 1967). An analogous pathway had been proposed for plants (Isherwood, Chen & Mapson 1954) with D-galacturonate and L-galactono-1,4-lactone as two key intermediates. There is clear evidence that plants possess the ability to convert L-galactono-1,4-lactone to AsA via a galactono-1,4-lactone dehydrogenase (GalLDH; see below). However, when this proposed inversion-type pathway was tested in strawberries using D-glucose labelled with ¹⁴C at either carbon 1, 2 or 6 it became clear that the glucose carbon skeleton is not inverted in plants during the synthesis of AsA. For example, the carbon at position C1 of glucose did not appear at position C6 in the final product, AsA (Loewus, Jang & Seegmiller 1956). Later studies with other plant species confirmed this result and also led to the conclusion that D-galacturonic acid is not a major substrate for plant AsA biosynthesis, reviewed in (Loewus 1999). Although evidence for L-galactono-1,4-lactone as an AsA substrate is strong, the plant pathway does not appear to mirror the inversion pathway of mammals.

D-Glucosone and L-sorbosone as possible intermediates

A non-inversion pathway for the synthesis of AsA in plants with the intermediates D-glucosone and L-sorbosone has

been proposed (Loewus *et al.* 1987). In this pathway, four chemical events are minimally required for the non-inverted conversion of glucose to AsA are present: (1) oxidation of the C1 of glucose; (2) oxidation at C2 or C3; (3) epimerization at C5; and (4) lactonization between C1 and C4 (Loewus 1999). Although a rational proposal for the synthesis of AsA in plants, this pathway does not reconcile the strong evidence that GalLDH activity exists in plants.

Considered together, the experimental data for this pathway as a major mechanism of AsA biosynthesis in higher plants are not highly convincing. In ^{14}C -labelling studies, 4.1% of the label from D- ^{14}C glucosone was incorporated into AsA versus 0.6% from D- ^{14}C glucose over a long (24 h) labelling period, and unlabeled D-glucosone inhibited the conversion of D- ^{14}C glucose to ^{14}C AsA by ~12X (Saito, Nick & Loewus 1990). However, this inhibition should not be used as evidence that glucosone is an intermediate as glucosone has been shown to be moderately toxic to plants (Pallanca & Smirnov 1999). Also, no evidence could be found for the presence of an enzymatic activity in pea seedlings that could effect the direct oxidation of glucose to glucosone (Pallanca & Smirnov 1999). To account for the recovery of ^{14}C AsA from labelled glucosone, it has been hypothesized that this osone is first converted to either fructose or mannose, two substrates in the Smirnov–Wheeler pathway for plant AsA biosynthesis (see below; Pallanca & Smirnov 1999). No difference was found in the incorporation of label into AsA from D- ^{14}C glucose and L- ^{14}C sorbosone, and unlabelled sorbosone only partially inhibited (approximately three-fold) the conversion of D- ^{14}C glucose to ^{14}C AsA (Saito *et al.* 1990). In an independent experiment using pea seedlings, L-sorbosone only slightly inhibited incorporation of label from D- ^{14}C glucose into AsA (Pallanca & Smirnov 1999). An NADP-dependent sorbosone dehydrogenase was partially purified that catalyses the conversion of L-sorbosone to AsA but the affinity of this dehydrogenase for L-sorbosone is quite low (Loewus *et al.* 1990). GalLDH has been shown to oxidize L-sorbosone to AsA with low affinity and this activity may account for the small amount of ^{14}C AsA synthesized from L- ^{14}C sorbosone (Wheeler, Jones & Smirnov 1998). In conclusion, it does not appear that D-glucosone and L-sorbosone are major substrates for AsA biosynthesis.

Biosynthesis via GDP-mannose and L-galactose

Smirnov and colleagues have used biochemical methods to generate evidence for a novel AsA biosynthetic pathway (Fig. 1) that does not predict inversion of the glucose skeleton, with D-mannose-6-P and L-galactose as two key intermediates (Wheeler *et al.* 1998). Supporting their hypothesis that mannose is a key intermediate in the pathway, when Arabidopsis leaves are fed with ^{14}C mannose (which is phosphorylated to mannose-6-phosphate *in vivo*), approximately 10% of the label appears in AsA by the end of a 4 h incubation. This result was also obtained with *Cucurbita pepo* roots (Wheeler *et al.* 1998). Secondly, they showed that ^{14}C L-galactono-1,4-lactone could be formed by supplying a pea embryo extract with ^{14}C GDP-mannose and NAD. They then went on to demonstrate that the ^{14}C L-galactono-1,4-lactone *in vitro*-synthesized from ^{14}C mannose could be converted *in vitro* to ^{14}C AsA with the addition of intact mitochondria (to supply GalLDH) and cytochrome *c* as an electron acceptor. The Smirnov group proposes that the conversion from GDP-mannose to L-galactono-1,4-lactone proceeds via L-galactose. Feeding Arabidopsis leaves and pea seedlings with L-galactose results in an increase in AsA that is similar to that of tissue supplied with L-galactono-1,4-lactone. The role of L-galactose as an AsA substrate was confirmed by Davey and colleagues who reported that Arabidopsis suspension cells rapidly convert exogenously supplied L-galactose to AsA (Table 1; Davey *et al.* 1999). L-galactose can be synthesized from GDP-mannose by a previously described GDP-D-mannose-3,5-epimerase activity (Barber 1971; 1979) that was detected in both pea and Arabidopsis (Wheeler *et al.* 1998). A previously undescribed activity (L-galactose dehydrogenase) also detected in these extracts was partially purified and shown to oxidize L-galactose to L-galactono-1,4-lactone, providing substrate for GalLDH.

Additional support for this newly proposed pathway comes from a study of oxalate synthesis in *Pistia stratiotes*. It has been known for some time that the oxalic acid is produced by cleavage of AsA at the C2/C3 position (Wagner & Loewus 1973). In plants pulse-labelled with L- ^{14}C galactose, significant label was incorporated into oxalic acid. In addition, micrographs showed these labelled cells contained ^{14}C selectively accumulating in calcium oxalate-

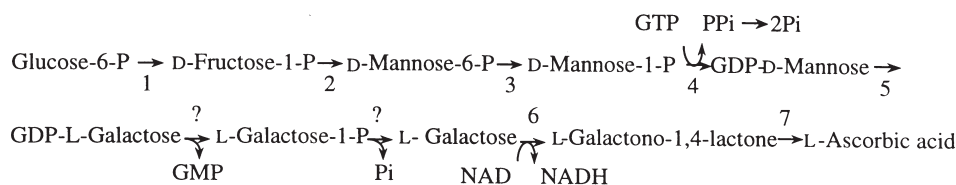


Figure 1. Proposed pathway for L-ascorbic acid biosynthesis in higher plants (Wheeler *et al.* 1998). Enzymes: 1, phosphoglucose isomerase; 2, phosphomannose isomerase; 3, phosphomannomutase; 4, GDP-D-mannose pyrophosphorylase; 5, GDP-D-mannose-3,5-epimerase; 6, L-galactose dehydrogenase; 7, L-galactono-1,4-lactone dehydrogenase.

Substrate	Concentration (mM)	Number of replicates	Relative increase in [AsA] over time ^b
D-glucose	30	1	0.8
Smirnoff–Wheeler pathway:			
D-mannose	30	1	0.3
L-galactose	15	3	68.0
L-galactono-1,4-lactone	15	5	31.8
D-galacturonate (methyl ester)	15	4	34.7
'Mammal-like' pathway:			
D-glucuronolactone	15	3	2.9
D-glucuronic acid (methyl ester)	15	2	8.5
L-gulono-1,4-lactone	15	4	9.7
L-sorbose	15	1	1.2

^aAdapted from [Davey *et al.* 1999, #602], ^brelative increase in comparison to that of control culture over the course of 30 h incubation with substrate.

loaded idioblasts (Keates *et al.* 2000). Therefore, it appears that L-galactose is an effective substrate for the synthesis of oxalic acid and therefore its precursor, AsA.

The rate-limiting step in plant AsA biosynthesis via mannose-1-P and L-galactose is proposed to lie between these two precursors (Wheeler *et al.* 1998; Davey *et al.* 1999). This hypothesis was drawn due to the observation that exogenously supplied D-mannose does not lead to an increased AsA pool (Table 1; Wheeler *et al.* 1998; Davey *et al.* 1999). However, as cells respond rapidly to exogenously supplied L-galactose and L-galactono-1,4-lactone, such that the AsA biosynthetic rate increases by approximately 30- to 70-fold (Davey *et al.* 1999) the rate-limiting step in this pathway must reside upstream of L-galactose. Additional studies with transgenic plants that over-express different biosynthetic enzymes in this pathway are currently being conducted in an attempt to further address this question (N. Smirnoff *et al.* unpublished results; P.L. Conklin, unpublished results).

The Smirnoff–Wheeler pathway resolves the major issues surrounding plant AsA biosynthesis. It retains the four chemical steps minimally required for conversion of glucose to AsA as predicted by Loewus and colleagues (Loewus 1999). In addition, it accommodates both the evidence for a non-inversion pathway and the evidence that L-galactono-1,4-lactone is the final substrate. A fascinating implication of this pathway is that it plays a key role in plant metabolism; in addition to serving as intermediates for AsA biosynthesis, intermediates in this proposed pathway (GDP-mannose, L-galactose) are also utilized in the synthesis of several structural cell wall carbohydrates (the mannans, L-fucose) and protein glycosylation.

Galactono-1,4-lactone dehydrogenase: the final biosynthetic step

Since 1954 when GalLDH activity was first reported to occur in plants (Isherwood *et al.* 1954; Mapson, Isherwood & Chen 1954), much progress has been made in understanding the properties of this mitochondrial enzyme.

Table 1. The effect of proposed substrates on AsA concentration in Arabidopsis cell suspension culture^a

GalLDH activity has been reported in a number of different plant species including kidney bean and strawberry (Baig, Kelly & Loewus 1970), spinach (Hausladen & Kunert 1990), oat (De Gara *et al.* 1992), white potato (Ôba *et al.* 1994), maize (De Gara *et al.* 1994), sweet potato (Ôba *et al.* 1995), and cauliflower (Østergaard *et al.* 1997). Recently, GalLDH was found to be localized specifically to the mitochondrial inner membrane (Siendones *et al.* 1999). GalLDH has been purified to apparent homogeneity from both sweet potato (Ôba *et al.* 1995; Imai *et al.* 1998) and cauliflower (Østergaard *et al.* 1997) mitochondria and is composed of a single polypeptide of 56 kDa. Both groups (Ôba *et al.* 1995; Østergaard *et al.* 1997) concluded from their analyses that GalLDH contains cysteine residues that are important for enzyme activity. Data obtained regarding the kinetics of GalLDH differ somewhat between the cauliflower-derived enzyme and that from sweet potato. The cauliflower GalLDH was found to have an apparent Michaelis constant (k_m) for L-galactono-1,4-lactone of 3.3 mM (Østergaard *et al.* 1997) whereas the sweet potato enzyme has a k_m for the same substrate of only 0.12 mM (Ôba *et al.* 1995). The reason for this rather large discrepancy is not clear. GalLDH is quite specific for L-galactono-1,4-lactone as its substrate (Mapson & Breslow 1958; Ôba *et al.* 1995; Østergaard *et al.* 1997). This is in contrast to related enzymes in both rat (gulono-1,4-lactone oxidase; Nakagawa & Asano 1970) and *Candida albicans* (D-arabino-1,4-lactone oxidase; Huh *et al.* 1994), which can utilize both their primary substrates and L-galactono-1,4-lactone (rat and *Candida*), and L-gulono-1,4-lactone (*Candida*). Lycorine, an alkaloid purified from Amaryllidaceae, was found to be a potent inhibitor of GalLDH activity in partially purified extracts from maize and bean (Arrigoni *et al.* 1997). Oddly, GalLDH purified from sweet potato was found to be inhibited by lycorine (50% inhibition with 68 μ M lycorine; Imai *et al.* 1998) whereas the cauliflower enzyme was not (Østergaard *et al.* 1997). It is difficult to rationalize this disparity but it may reflect differences in the protein purification procedures or the assays for lycorine inhibition.

The mitochondrial locale of GalLDH and its requirement (*in vitro*) for cytochrome *c* as an electron acceptor (Mutsuda *et al.* 1995; Ôba *et al.* 1995; Østergaard *et al.* 1997; Imai *et al.* 1998) prompted Bartoli and colleagues to examine the role of this enzyme in mitochondrial electron transport (Bartoli, Pastori & Foyer 2000). Using mitochondria purified from potato tubers, they found that both the respiration rate and the synthesis of AsA increased significantly when galactono-1,4-lactone was added to the medium. This induction of AsA synthesis was abolished upon addition of the cytochrome *c* oxidase inhibitor, potassium cyanide, suggesting that GalLDH is unable to catalyse the synthesis of AsA when its electron acceptor, cytochrome *c*, is fully reduced (Bartoli *et al.* 2000). These authors propose that GalLDH can act as an alternative source of electrons for the reduction of cytochrome *c*, although as the supply of L-galactono-1,4-lactone may be limited, the impact of this activity on mitochondria respiration is not known (Bartoli *et al.* 2000).

cDNAs have been isolated from GalLDH mRNA in both cauliflower (Østergaard *et al.* 1997) and sweet potato (Imai *et al.* 1998) using peptide sequence data from the purified proteins. A putative mitochondrial transit peptide has been identified (Østergaard *et al.* 1997). From N-terminal amino acid sequence data, GalLDH appears to be first synthesized as a preprotein of 68 kDa (600 amino acids), which is then apparently processed to the mature (calculated) 57.8 kDa of 509 amino acids (Østergaard *et al.* 1997). The predicted amino acid sequences of the cauliflower and sweet potato GalLDH share 77% identity (Imai *et al.* 1998). Both plant-derived polypeptide sequences share domains in common with related enzymes from rat (L-gulonono-1,4-lactone oxidase) and yeast (D-arabino- γ -lactone oxidase), suggesting some commonality of function (Østergaard *et al.* 1997; Imai *et al.* 1998). The identity of the cauliflower GalLDH cDNA clone was confirmed by expression of the cDNA in yeast, which were then shown to have greatly increased levels of GalLDH activity (Østergaard *et al.* 1997).

Evidence suggests that the GalLDH may be a flavoprotein. The absorption spectrum of the purified protein (Ôba *et al.* 1995; Imai *et al.* 1998) and the inhibition of both the sweet potato and cauliflower GalLDH activity by acriflavine (Mapson & Breslow 1958; Ôba *et al.* 1995) suggest that this enzyme is a flavoprotein. However, Østergaard and colleagues did not detect this suggestive absorption spectrum nor was their protein inhibited by flavoprotein inhibitors (Østergaard *et al.* 1997). Furthermore, no sequence identity with known flavin-binding domains was found in the cauliflower GalLDH-predicted amino acid sequence (Østergaard *et al.* 1997). Although it does not completely resolve these differences, it has been suggested that the interaction of the flavin group with GalLDH is non-covalent, unlike the covalent interactions of flavin with related enzymes (Imai *et al.* 1998).

Alternative proposed pathways

Several lines of evidence over the years have led to the suggestion that plants may also harbour alternative pathway(s)

for the synthesis of AsA. Early evidence indicates that D-glucurono-1,4-lactone, D-galacturonic acid, methyl D-galacturonic acid are converted to AsA via an inversion pathway through L-gulonono-1,4-lactone and/or L-galactono-1,4-lactone (Isherwood *et al.* 1954; Loewus, Jang & Seegmiller 1958; Finkle, Kelly, & Loewus 1960; Loewus 1963). This suggestion was recently revisited with the use of Arabidopsis suspension cell cultures. Cells supplied with D-glucuronolactone and the methyl esters of D-glucuronic acid and D-galacturonic acid were shown to have increased concentrations of AsA (Table 1; Davey *et al.* 1999). It is interesting to speculate therefore that these compounds are AsA precursors. However, as direct conversion to AsA was not measured, such results could also be obtained via stimulation of AsA biosynthesis via another route, and/or perturbation of AsA turnover. It was noted that although D-galacturonic acid esters occur naturally in plant cells in the pectin backbone, the esterification does not take place until the backbone is formed in the Golgi, calling into question the availability of this methyl ester for AsA biosynthesis (Davey *et al.* 1999).

Although it is clearly possible that plants possess the capability to convert D-glucuronolactone, D-glucuronic acid (methyl ester) and L-gulonono-1,4-lactone to AsA (Mapson & Isherwood 1956; Loewus 1963; Baig *et al.* 1970), the physiological significance of these activities remains to be tested. However, L-gulonono-1,4-lactone has been detected in plants and current research is underway to characterize and purify the enzyme responsible for reduction of this substrate to AsA (Davey *et al.* 1999). In addition, transgenic tobacco that overexpress a rat L-gulonono-1,4-lactone oxidase have elevated levels of AsA, a further demonstration of the endogenous L-gulonono-1,4-lactone substrate in plants (Jain & Nessler 2000).

Given the evidence that the majority of AsA in plants is synthesized from D-glucose directly via a non-inversion pathway, it has been hypothesized that the uronic acids that can be utilized as AsA substrates could derive from breakdown products of cell wall polysaccharides (Davey *et al.* 1999). Davey and colleagues made the interesting observation that D-glucuronic acid, D-galacturonic acid, D-mannose, and L-galactose are all cell wall polysaccharide precursors, pointing out an interesting possible connection between cell wall polysaccharide turnover and AsA biosynthesis (Davey *et al.* 1999). Additional biochemical, molecular, and genetic work will need to be accomplished to resolve the questions surrounding different possible AsA biosynthetic pathways, their inter-relationships to each other and to other metabolic and catabolic processes within the cell.

ASCORBIC ACID MUTANTS IN ARABIDOPSIS

Mutants have long been used to successfully dissect biochemical pathways, leading to the identification of genes encoding regulators, cofactors, and structural enzymes (Rose, Casselman & Last 1992; Norris, Barrette & Delapenna 1995; Carson *et al.* 1997; Miquel & Browse 1998).

Additionally, mutants can aid in the study of the physiological roles of compounds (Li *et al.* 1993; Pogson *et al.* 1998; Pruitt *et al.* 2000). In a hope to better understand the biosynthesis and role of AsA in plants, *Arabidopsis thaliana* has been used to identify AsA mutants. As described below, one of these mutants lends strong genetic support for the biosynthesis of AsA from GDP-mannose.

Isolation of AsA-deficient mutants

Two large mutant screens in *Arabidopsis* resulted in a collection of AsA mutants. The first screen was for ozone-sensitive mutants and was not a direct screen for AsA mutants (Conklin *et al.* 1996). Following an 8 h exposure to 250 p.p.b. ozone, 2-week-old mutagenized plants were observed for tissue collapse. Over 70 ozone-sensitive mutants were isolated from approximately 100 000 ethyl methanesulfonate (EMS) mutagenized lines. In further tests, two of these ozone-sensitive mutants were found to have reduced AsA levels in their leaves (Conklin *et al.* 1996; 2000). To directly isolate additional AsA mutants, a semi-quantitative assay using nitroblue tetrazolium was developed to rapidly assess the AsA levels in leaf tissue. This simple assay allowed for the direct screening of over 16 000 EMS and Fast-Neutron (FN) mutagenized lines and resulted in the isolation of seven additional AsA-reduced mutants (Conklin *et al.* 2000 and P.L. Conklin, J. Lim *et al.* unpublished results). In summary, approximately 120 000 mutagenized lines were analysed in these two screens resulting in the isolation of nine mutants with reduced AsA levels.

The mutants with reduced levels of leaf AsA consist of nine independent mutants, which fall into four complementation groups: *vtc1*, *vtc2*, *vtc3*, and *vtc4* (*vtc*, vitamin C; P.L. Conklin, unpublished results; Conklin *et al.* 2000). The *vtc* mutants are single gene recessive mutants with the exception of *vtc1* and *vtc2-3*, which both exhibit a gene dosage effect. Each of the *VTC* loci has been placed on the *Arabidopsis* genetic map (Table 2; Conklin *et al.* 1996; 2000). The *VTC1* gene has been identified (see below; Conklin *et al.* 1999) and the other *VTC* genes are currently being map-base cloned. At 2 weeks of age, all of the AsA-

deficient mutants have between 30 and 50% of wild-type levels of AsA in their rosette leaves (Table 2). It is interesting to note that the AsA levels in older leaves of two of the *vtc2* alleles are extremely low (approximately 10% of wild-type). The biochemical/molecular basis for this developmental difference is not yet understood (Conklin *et al.* 2000). In general, the AsA-deficient mutants have no gross visible phenotype under normal growth conditions (Conklin *et al.* 2000) although the *vtc1* rosette can be somewhat smaller under certain growth conditions (P.L. Conklin, unpublished observation).

Free radicals are generated in plant tissue in response to ozone exposure. It is thought that AsA plays a role in detoxifying these free radicals. Surprisingly, the *vtc* mutants vary in their ozone-sensitivity despite the fact that they have similar AsA deficiencies (Table 2; Conklin *et al.* 2000). The *vtc1-1* and the *vtc2-1* mutants were isolated in the ozone-sensitive screen and are quite sensitive to ozone relative to wild-type. The *vtc1-2* mutant is also quite ozone sensitive. These three mutants all have about 30% of wild-type levels of AsA in their leaves. The *vtc2-3*, *vtc3-1* and *vtc4-1* mutants, which accumulate about 40–50% of the wild-type AsA level, are only moderately ozone sensitive. Surprisingly, the *vtc2-2* mutant shows no visible signs of injury as a result of ozone treatment, although it has only 30% of wild-type levels of AsA. The basis for this anomaly remains to be determined.

Identification of the *VTC1* gene

To identify the *VTC1* gene, the *vtc1-1* mutant was initially mapped to a region on chromosome II that had been sequenced by The Institute for Genomic Research (TIGR). TIGR had preliminarily identified an open reading frame, T5I7-7, in this region as a putative mannose-1-phosphate guanyl transferase or GDP-mannose pyrophosphorylase, an enzyme shown in the Smirnoff–Wheeler pathway to be important in AsA biosynthesis (Fig. 1; (Wheeler *et al.* 1998). Four pieces of evidence support the hypothesis that *VTC1* locus encodes the GDP-mannose pyrophosphorylase (Conklin *et al.* 2000). First, the *vtc1-1* mutant is impaired in

Mutant allele	Screening method	Mutagen	Approx. % of wt leaf AsA	Map position (cM) ^a	Ozone sensitivity
<i>vtc1-1</i>	Ozone	EMS	30	Chr II, 74	yes
<i>vtc1-2</i>	NBT	EMS	30	Chr II, 74	yes
<i>vtc2-1</i>	Ozone	EMS	30	Chr IV, 73	yes
<i>vtc2-2</i>	NBT	EMS	30	Chr IV, 73	no
<i>vtc2-3</i>	NBT	EMS	50	Chr IV, 73	moderate
<i>vtc2-4</i>	NBT	FN	50	Chr IV, 73	not tested
<i>vtc3-1</i>	NBT	EMS	40	Chr II, 74	moderate
<i>vtc3-2</i>	NBT	EMS	30	Chr II, 74	not tested
<i>vtc4-1</i>	NBT	EMS	50	Chr III, 5	moderate

Table 2. Summary of AsA-deficient *Arabidopsis* (*vtc*) mutants

^aMap position in centimorgans (cM) is relative to the latest *Arabidopsis* recombinant inbred (RI) genetic map.

its ability to convert both glucose and mannose to AsA. Second, the GDP-mannose pyrophosphorylase activity is reduced in *vtc1-1* leaf extracts when compared with wild-type leaf extracts. Third, in addition to the genetic linkage of the *vtc1-1* mutant and the GDP-mannose pyrophosphorylase gene, *vtc1-1* and *1-2* have identical point mutations that cause a missense mutation, changing a highly conserved proline to serine. Finally, the GDP-mannose pyrophosphorylase gene on BAC T517 complements the *vtc1-1* mutant in transgenic plants, restoring the AsA levels to wild-type. The *vtc1* mutants provide strong genetic evidence to support the Smirnoff–Wheeler AsA pathway, which was postulated primarily on biochemical evidence.

Down-regulation of GDP-mannose pyrophosphorylase in transgenic potato

To further study the roles of GDP-mannose pyrophosphorylase, Keller and coworkers generated transgenic potato plants expressing a gene encoding the potato GDP-mannose pyrophosphorylase in the antisense direction using the cauliflower mosaic virus 35S promoter (Keller *et al.* 1999). Three independent transgenic lines were shown to have significantly reduced levels of GDP-mannose pyrophosphorylase transcript compared with wild-type. The GDP-mannose pyrophosphorylase activity was reduced 41–72% in the transgenic leaves compared with wild-type. Interestingly, the enzyme activity in tubers was not as greatly affected, ranging from 64 to 94% of wild-type.

Keller and colleagues used these transgenic plants to study the effect of reduced GDP-mannose pyrophosphorylase activity on the levels of AsA, cell wall components and protein glycosylation, as well as on plant morphology (Keller *et al.* 1999). The AsA levels in the transgenic plants were significantly reduced relative to wild-type levels. The analysis of cell wall polysaccharides shows a reduction in mannose levels in leaves but interestingly not in tubers. Although GDP-mannose is a substrate for L-fucose biosynthesis (Bonin *et al.* 1997), the levels of this cell wall polysaccharide were unchanged in the transgenic plants. No significant changes could be detected in the protein glycosylation pattern (Keller *et al.* 1999). From these results, it appears that differences may exist in the flux of GDP-mannose through these different biosynthetic pathways. The predicted GDP-mannose deficit in the transgenics results in the reduction of cell wall mannose and AsA content but not fucose and protein glycosylation. Therefore, GDP-mannose may be utilized for protein glycosylation and synthesis of fucose preferentially over AsA biosynthesis and mannose cell wall polysaccharide. A developmental phenotype was apparent in GDP-mannose pyrophosphorylase antisense lines; leaves of the transgenic lines begin to dry out and eventually die, long before senescence occurs in the control lines (Keller *et al.* 1999). As ROS contribute to oxidative damage during senescence, it was hypothesized

that the reduction in AsA may contribute to the early and accelerated senescence observed in the transgenic lines (Keller *et al.* 1999). Additional studies such as this will contribute valuable information on the AsA biosynthetic pathway, and the role of AsA and its precursors in plant growth and development.

THE REGULATION OF ASCORBIC ACID LEVELS

Intuitively, regulation of AsA levels could either be achieved by altering the turnover of AsA or its synthesis. Examples of both mechanisms can be found in the recent literature.

Regulation of AsA levels in root meristems appears to involve turnover through the activity of ascorbate oxidase. The down-regulation of AsA in the non-dividing quiescent centre of the root meristem is thought to involve an interplay between ascorbate oxidase and auxin. Accumulation of auxin in root tips results in increased levels of ascorbate oxidase and decreased AsA (Kerk & Feldman 1994; 1995). Kerk and Feldman proposed that ascorbate oxidase induced by auxin controls the depletion of AsA in the quiescent centre of maize root tips. Recent data sheds some light on the mechanism of this control: ascorbate oxidase was shown to inactivate auxin by oxidative decarboxylation *in vitro*, and auxin is also decarboxylated by intact root tips (a major site of ascorbate oxidase; Kerk, Jiang & Feldman 2000). A model was thus advanced for the interconnected regulation of ascorbate oxidase, auxin, and AsA. In this model, transport of high levels of auxin to the root tip leads to an increase in ascorbate oxidase and a subsequent decrease in AsA, resulting in cell division inhibition. Feedback control of this pathway via decarboxylation of auxin by ascorbate oxidase permits control of root development and therefore regulation of quiescent centre maintenance (Kerk *et al.* 2000).

The activity of GalLDH may play a role in the regulation of AsA through at least partial control of AsA biosynthesis. AsA levels have been shown to decline in leaves as the tissue ages and eventually senesces (Borraccino *et al.* 1994; Bartoli *et al.* 2000). Correlating with AsA levels, GalLDH activity was found to be high in young potato leaves and decrease with age (Bartoli *et al.* 2000). It is interesting to note conversely that sliced/wounded potato tubers have increased amounts of AsA and also GalLDH activity (Öba *et al.* 1994). Recently, the Smirnoff group found that GalLDH activity may be positively affected by light either directly or possibly indirectly via photosynthate supply (see below). A positive correlation was found between AsA levels, light, and GalLDH activity in barley. Furthermore, barley leaves incubated at high light and then transferred to the dark for 24 h were found to have higher GalLDH activity than leaves incubated at a lower fluence prior to dark incubation. In addition, high light-incubated leaves that remained in the light for the 24 h incubation had even higher GalLDH activity. (Smirnoff 2000). Further research

on the effect of light on upstream AsA biosynthetic enzyme activities is needed to clarify the role of GalLDH in controlling the pool size of AsA.

AsA biosynthesis may also be under the control of carbohydrate pools. As discussed above, AsA levels are correlated with the light intensity under which the plant is grown (reviewed in Smirnov & Pallanca 1996; Smirnov 2000). Carbohydrate pools in barley leaves are positively correlated with both light intensity and AsA levels (Smirnov & Pallanca 1996) leading to the suggestion that the AsA biosynthetic apparatus may respond to carbohydrate rather than (or in addition to) light *per se*. In support of this hypothesis, light-grown barley leaf segments incubated in the dark with the addition of glucose or sucrose have increased levels of AsA (Smirnov & Pallanca 1996). However, these increased levels are significantly less than in control leaves incubated in the light with no addition of glucose or sucrose although the carbohydrate pool size was similar in both samples (Smirnov & Pallanca 1996). This suggests that the carbohydrate status alone is not responsible for control of the AsA pool. Clearly, the determinants that control AsA biosynthesis need to be investigated in more detail to tease apart the contributions of such factors as light and carbohydrates.

AsA biosynthesis in plants also appears to be subject to feedback control. An early study provided indirect evidence for feedback control (De Gara *et al.* 1989) and recently, this phenomenon has been investigated in greater detail. Addition of exogenous cold AsA to a [¹⁴C]D-glucose labelling reaction with pea embryos results in a significant decrease in ¹⁴C-AsA, suggestive of feedback control by the end product (AsA) of this biosynthetic pathway (Pallanca & Smirnov 2000). However, the mechanism of this feedback control is not yet understood.

FUTURE PROSPECTS

As the role of AsA in a wide number of important cellular processes is further refined, it is becoming increasingly clear that this small molecule is of crucial importance in plants. From basic operations such as cellular division and expansion to protection from environmental stresses, the influence of AsA on cell function is broad. As AsA is vital for virtually all aerobic eukaryotes, a greater understanding of its role in such processes as cell division and ROS detoxification in plants will further the understanding of these areas in all organisms. On-going research in several laboratories with the goal of increasing AsA biosynthesis in transgenic plants is predicted to result in plants with both increased nutrition and environmental stress tolerance. Future research on AsA biosynthesis and its regulation will benefit greatly from the genomics technologies that are impacting all fields of plant biology. Although AsA research has been ongoing for decades, the work in the last 5 years has led to a vast increase in our understanding of this small and familiar molecule. It is predicted that this trend will continue into the new millennium.

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