

The Function and Metabolism of Ascorbic Acid in Plants

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Ascorbate is a major metabolite in plants. It is an antioxidant and, in association with other components of the antioxidant system, protects plants against oxidative damage resulting from aerobic metabolism, photosynthesis and a range of pollutants. Recent approaches, using mutants and transgenic plants, are providing evidence for a key role for the ascorbate–glutathione cycle in protecting plants against oxidative stress. Ascorbate is also a cofactor for some hydroxylase enzymes (e.g. prolyl hydroxylase) and violaxanthin de-epoxidase. The latter enzyme links ascorbate to the photoprotective xanthophyll cycle. A role in regulating photosynthetic electron transport has been proposed. The biosynthetic pathway of ascorbate in plants has not been identified and evidence for the proposed pathways is reviewed. Ascorbate occurs in the cell wall where it is a first line of defence against ozone. Cell wall ascorbate and cell wall-localized ascorbate oxidase (AO) have been implicated in control of growth. High AO activity is associated with rapidly expanding cells and a model which links wall ascorbate and ascorbate oxidase to cell wall extensibility is presented. Ascorbate has also been implicated in regulation of cell division by influencing progression from G1 to S phase of the cell cycle. There is a need to increase our understanding of this enigmatic molecule since it could be involved in a wide range of important functions from antioxidant defence and photosynthesis to growth regulation.

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Key words: Ascorbic acid, ascorbate oxidase, cell division, cell wall, growth, oxidative stress, photosynthesis, ozone, vitamin C.

INTRODUCTION

While ascorbic acid (vitamin C) is a familiar molecule because of its dietary significance, most aspects of its metabolism and some aspects of its function in plants are very poorly understood. For example, its biosynthetic pathway has not been firmly established even though it reaches millimolar concentrations in most tissues. Humans and some other animals (including other primates and guinea pigs) depend on ascorbate in their diet because of loss of a functional form of the last enzyme (L-gulonolactone oxidase) of the biosynthesis pathway. Ascorbate is best known for its function as an antioxidant and for its role in collagen synthesis. Collagen deficiency results in the symptoms of scurvy.

The purpose of this review is to outline recent advances and new approaches to the investigation of ascorbate biosynthesis and function. Its role in photosynthesis and photoprotection, in defence against ozone and other oxidative stresses and speculations about its role in cell expansion and cell division will be emphasized. Improved understanding of ascorbate in plants will lead to the possibility of increasing ascorbate concentration in plants by genetic manipulation. This will have benefits for human nutrition and possibly for tolerance of plants to photo-oxidative stresses (Foyer, 1993; Smirnov, 1995).

THE OCCURRENCE AND SUBCELLULAR LOCALIZATION OF ASCORBATE

Ascorbate and ascorbate peroxidase (AP), a peroxidase with specificity for ascorbate as reductant, appear to be universal in photosynthetic eukaryotes including algae and bryophytes (Loewus, 1980; Miyake, Michihata and Asada, 1991). Amongst prokaryotes, AP occurs in some cyanobacteria (Miyake *et al.*, 1991). It is not known if cyanobacteria which lack AP also lack ascorbate. Ascorbate occurs in the cytosol, chloroplasts, vacuoles, mitochondria and cell wall (Anderson, Foyer and Walker, 1983; Rautenkranz *et al.*, 1994). The concentration in chloroplasts can be high (up to 50 mM in spinach) and is probably related to its central role in photosynthesis (Foyer, 1993). Occurrence in the cell wall is not accidental since high affinity carriers for both ascorbate ($K_m = 90 \mu\text{M}$) and dehydroascorbate ($K_m = 20 \mu\text{M}$) occur on the plasma membrane (PM) of barley leaf protoplasts. Dehydroascorbate (DHA), the oxidized form of ascorbate, is taken up more rapidly than ascorbate. The effects of ionophores and channel blockers on ascorbate uptake suggest a requirement for a proton electrochemical gradient across the PM. In contrast, transport into isolated vacuoles is slow and not carrier-mediated (Rautenkranz *et al.*, 1994). Isolated spinach chloroplasts take up ascorbate with a surprisingly low affinity ($K_m = 18\text{--}40 \text{ mM}$) via a saturable carrier (Anderson *et al.*, 1983; Beck, Burkert and Hofman, 1983). Uptake into chloroplasts is inhibited by DHA (Anderson *et al.*, 1983; Beck *et al.*, 1983). High

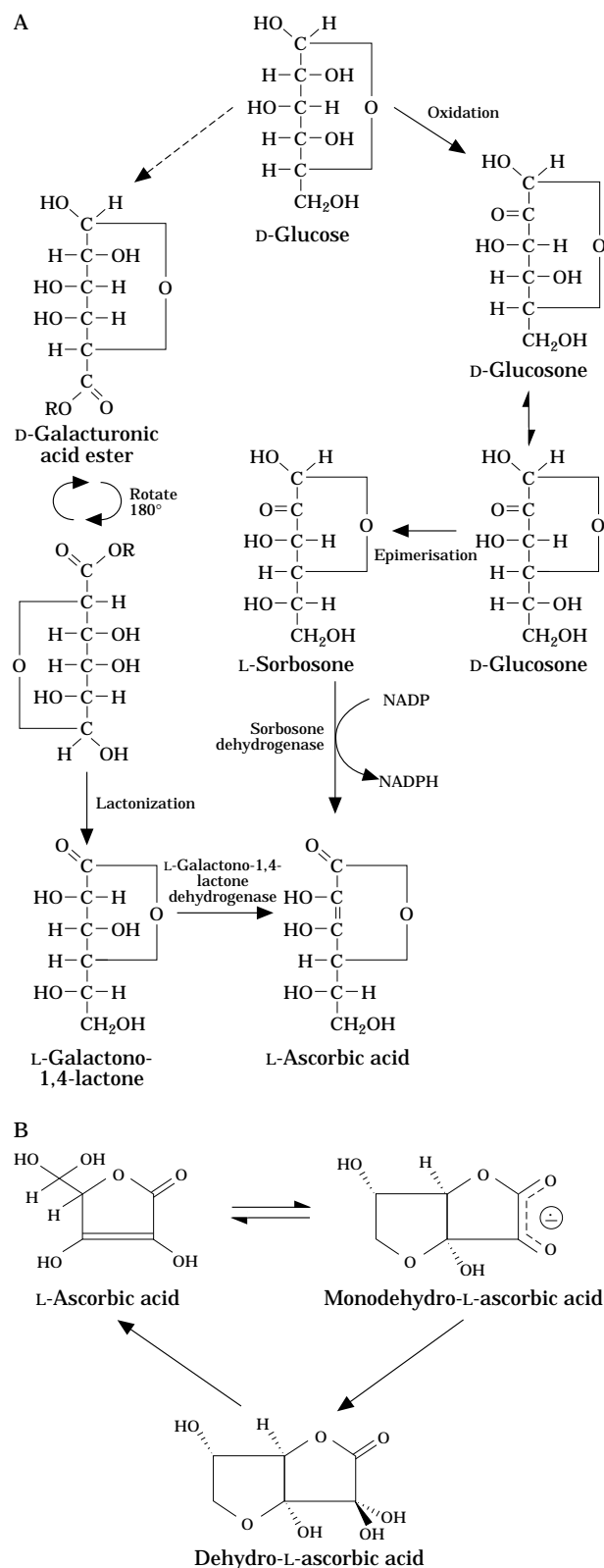


FIG. 1. A, Possible pathways of L-ascorbic acid biosynthesis in plants. Two pathways have been proposed one *via* L-galactono-1,4-lactone which requires inversion of the carbon skeleton, the other *via* the osones D-glucosone and L-sorbose. There is presently no conclusive evidence for either pathway. The possibility that some of the intermediates are sugar phosphates or nucleotides is not shown in the

affinity carriers on the plasma membrane, if generally-occurring, would facilitate movement of ascorbate into the cell wall and allow movement from cell to cell *via* the apoplast.

THE CHEMISTRY AND BIOCHEMISTRY OF ASCORBATE

The reducing properties of ascorbate result from the reactive ene-diol group at C2/3. Acidic properties are provided by ionization of the hydroxyl group at C3 ($pK_a = 4.17$). Oxidation results initially in formation of the monodehydro-ascorbate (MDA) radical. MDA disproportionates to form ascorbate and DHA (Fig. 1 B). DHA is unstable above pH 7 so it is necessary to maintain the total ascorbate pool in a reduced state to prevent rapid loss. The ascorbate pool is about 90% reduced under normal conditions (Foyer, 1993). This is achieved by two enzymes: monodehydroascorbate reductase (MDAR), which uses NAD(P)H as reductant, and dehydroascorbate reductase (DHAR) which uses glutathione as reductant. DHAR links ascorbate to glutathione which is the other major soluble antioxidant in plant cells. These reactions together constitute the ascorbate-glutathione (Halliwell-Foyer-Asada) cycle (Fig. 2; Foyer, 1993). Two enzymes catalyse ascorbate oxidation: ascorbate oxidase (Loewus, 1980; 1988) and ascorbate peroxidase (Asada, 1994).

The biochemical functions of ascorbate can be divided into four categories. (1) Antioxidant. Ascorbate reacts rapidly with superoxide, singlet oxygen, ozone and hydrogen peroxide. It thus participates in removal of these reactive forms of oxygen which are generated during aerobic metabolism and during exposure to some pollutants and herbicides. MDA and DHA are reduced to ascorbate by the ascorbate-glutathione cycle (Fig. 2). Additionally, ascorbate regenerates the lipophilic antioxidant α -tocopherol (vitamin E) from the α -chromanoxyl radical (Asada, 1994). (2) Enzyme cofactor. It is a cofactor for a range of hydroxylase enzymes, for example prolyl and lysyl hydroxylases involved in hydroxyproline (HP) and hydroxylysine synthesis (Davies, Austin and Partridge, 1991). HP-rich glycoproteins, such as extensin, are cell wall structural proteins (Carpita and Gibeau, 1993). Post-translational formation of prolyl residues in these proteins is an important use for intracellular ascorbate in growing cells (Liso *et al.*, 1985). Ascorbate is also required, at least *in vitro*, by ethylene-forming enzyme. This enzyme has sequence homology with other ascorbate-dependent hydroxylases (Smith, Ververidis and John, 1992). (3) Electron transport. Ascorbate is well known as an *in vitro* electron donor for photosynthetic and mitochondrial

scheme. See the text for further details and references. B, Structures and interrelationships between ascorbate and its oxidized forms monodehydroascorbate (MDA) and dehydroascorbate (DHA). MDA and DHA have bicyclic structures in which C3 and C6 form a hemiacetal in addition to the 1,4-lactone ring. MDA is the primary oxidation product of ascorbate and is an exceptionally unreactive free radical. This property is important in the function of ascorbate as a biological antioxidant. MDA disproportionates to ascorbate and DHA. DHA is unstable in aqueous solution and delactonizes to 2,3-diketo-L-gulonic acid and further breakdown products. The enzyme systems which reduce MDA and DHA to ascorbate are shown in Fig. 2.

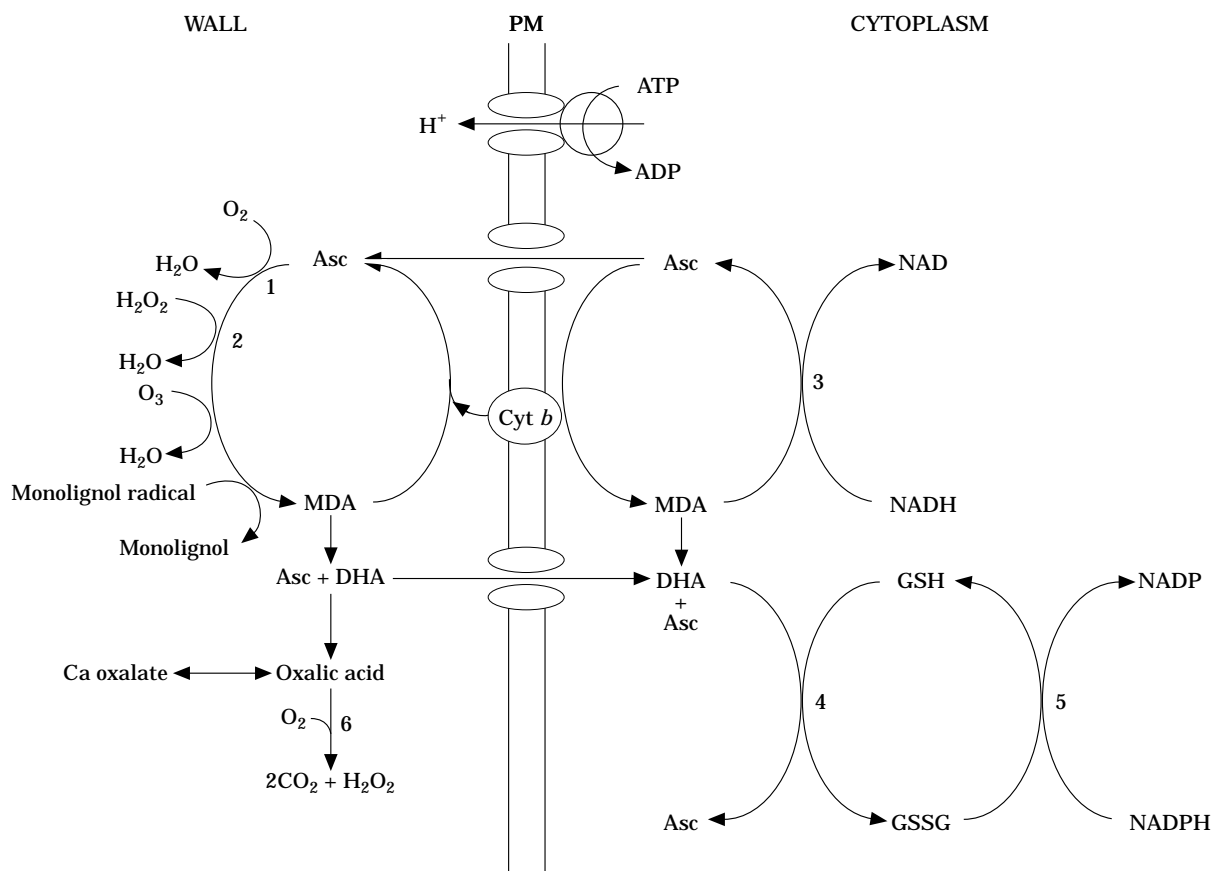


FIG. 2. Proposed relationship between the ascorbate system in the cytoplasm and cell wall and its role in cell wall expansion. Ascorbate is transported *via* a carrier into the cell wall. Ascorbate oxidase (1) catalyses ascorbate oxidation and formation of monodehydroascorbate radical (MDA). MDA accepts electrons from a membrane-bound cytochrome *b*. Cytoplasmic ascorbate is a possible electron donor. Membrane depolarization, caused by transmembrane electron transport, stimulates H^+ -ATPase activity and leads to cell wall loosening. Ascorbate inhibits peroxidative cross-linking of wall polysaccharides and lignin polymerization by scavenging hydrogen peroxide and monolignol radicals. Dehydroascorbate (DHA) could react with amino acid side chains on wall polypeptides and prevent cross linking to matrix polysaccharides. DHA (or ascorbate) gives rise to oxalate which promotes wall loosening by binding calcium. DHA is transported *via* a carrier to the cytoplasm where it is reduced to ascorbate by DHA reductase (4) in the ascorbate–glutathione cycle. Oxalate is removed by oxalate oxidase (6) and the release of hydrogen peroxide and calcium would favour wall tightening. The other pathways for generation of wall hydrogen peroxide are not shown in this scheme. Wall ascorbate also detoxifies ozone. The ascorbate–glutathione cycle and MDA reductase, along with ascorbate peroxidase, also occur in the chloroplasts and components of the cycle have been reported in mitochondria and peroxisomes. See the text for a review of the evidence supporting this scheme. Asc, ascorbate; MDA, monodehydroascorbate; DHA, dehydroascorbate; GSH, glutathione; GSSG, oxidized glutathione; Cyt *b*, cytochrome *b*; PM, plasma membrane. Enzymes: 1, ascorbate oxidase; 2, (ascorbate) peroxidase; 3, monodehydroascorbate reductase; 4, dehydroascorbate reductase; 5, glutathione reductase; 6, oxalate oxidase (germin).

electron transport. Recent evidence suggests that MDA can act as an electron acceptor from PSII *in vivo* (Miyake and Asada, 1992) and that it could act as both electron donor and acceptor in transmembrane electron transport (Asard, Horemans and Caubergs, 1995). The evidence is discussed below. (4) Oxalate and tartrate synthesis. Ascorbate can be cleaved to form oxalate and tartrate (Loewus, 1980; 1988; Saito, 1996). Other pathways could contribute to oxalate synthesis but ascorbate is probably the main source of tartrate in grapes.

HOW DO PLANTS SYNTHESIZE ASCORBATE?

Ascorbate is a major metabolite thus it comes as a surprise that a critical appraisal of the evidence suggests that the

biosynthetic pathway in plants is not known. Two pathways have been suggested (Fig. 1). The first, known as the inversion pathway, suggests that the immediate precursor to ascorbate is L-galactono-1,4-lactone (GAL) (Isherwood and Mapson, 1962). This is analogous to the animal pathway in which L-gulono-1,4-lactone is oxidized to ascorbate by L-gulono-1,4-lactone (GUL) oxidase. This pathway is supported by rapid conversion of exogenous GAL to ascorbate (De Gara *et al.*, 1994) and characterization of a mitochondrial enzyme (GAL dehydrogenase) which catalyses GAL oxidation to ascorbate (Oba *et al.*, 1994; 1995). Further support for this pathway is provided by the action of lycorine. This alkaloid can induce scurvy in animals and decreases ascorbate levels in plants (Arrigoni, Arrigoni-Liso and Calabrese, 1975). De Gara *et al.* (1994) suggest that lycorine inhibits GAL dehydrogenase and GUL oxidase.

However the effect of lycorine has only been tested *in vivo* and its effect on purified GAL dehydrogenase is not known so its specificity must be questioned. Even if GAL is a precursor, it is not known how it is synthesized (Isherwood and Mapson, 1962; Loewus, 1988).

Theoretically, conversion of D-glucose to GAL would require inversion of the carbon skeleton. However, extensive and detailed labelling studies by Loewus and colleagues suggest that the predicted inversion does not occur. Indeed, feeding specifically labelled glucose shows that C1 and C6 of glucose appear predominantly and respectively in C1 and C6 of ascorbate. There is more label randomization in glucose incorporated into polysaccharides which supports lack of inversion and maintenance of the carbon skeleton during ascorbate synthesis from glucose (Loewus, 1980, 1988; Saito, Nick and Loewus, 1990). A non-inversion pathway, involving oxidation of glucose at C2 to produce the unusual osone D-glucosone, has been proposed based on the labelling pattern. D-glucosone is converted to L-sorbosone by epimerization at C5. Oxidation at C3 and lactonization produces L-ascorbate. Apart from the labelling pattern, the only evidence supporting this pathway so far is detection of an enzyme with NADP-dependent sorbosone dehydrogenase activity in spinach and bean leaves (Loewus *et al.*, 1990; Saito, Nick and Loewus, 1990). Glucosone is found in some basidiomycete fungi which produce it by oxidation of glucose by pyranose 2-oxidase. This enzyme generates hydrogen peroxide which may be used by white rot fungi to degrade lignin (Daniel, Volc and Kubatova, 1994). So far no attention has been paid to phosphorylated or UDP derivatives of sugars, sugar acids or lactones as intermediates of ascorbate biosynthesis. The possibility of more than one pathway should not be discounted. In the absence of knowledge of the pathway, little is known about the regulation of ascorbate synthesis. The ascorbate pool is increased in leaves grown at high light intensity and at low temperature. However, unlike glutathione, it does not seem to be strongly responsive to oxidative stress (Smirnoff and Pallanca, 1996). In barley leaves, the ascorbate pool is correlated with photosynthetic capacity and with the supply of soluble carbohydrates (Smirnoff and Pallanca, 1996).

Classical biochemical approaches to ascorbate biosynthesis have not been successful although relatively few laboratories have devoted time to the problem. It is likely that molecular genetics, combined with further biochemical studies, will provide an opening into identification of the pathway. Mutants with altered ascorbate levels could be isolated by mass screening or by selecting plants that are hypersensitive or resistant to oxidative stress. This has recently been achieved in *Arabidopsis thaliana*. *Soz1*, a mutant which was selected for ozone sensitivity has 30% of wild type ascorbate levels (Conklin, Williams and Last, 1996). Labelling studies suggest that *soz1* is defective in ascorbate biosynthesis rather than turnover (Conklin, Pallanca, Smirnoff and Last, unpubl. res.). The role of GAL dehydrogenase could be tested very directly by down-regulating its expression in transgenic plants by using antisense technology. The recent purification of the enzyme (Oba *et al.*, 1995) should make this possible in the near future.

A CENTRAL ROLE FOR ASCORBATE IN PHOTOSYNTHESIS

Ascorbate has a central role in photosynthesis, as the high concentration in chloroplasts would imply. It works in its three biochemical modes. Firstly it acts as an antioxidant by removing hydrogen peroxide (chloroplasts lack catalase) formed by oxygen photoreduction in PSI (Mehler reaction). This is catalysed by ascorbate peroxidase (AP), some of which is bound to thylakoids where it can scavenge hydrogen peroxide as it forms (Miyake and Asada, 1992). This sequence has been termed the Mehler peroxidase reaction (Neubauer and Yamamoto, 1992). Secondly, MDA, formed by AP can act as a direct electron acceptor to PSI (Miyake and Asada, 1992; Foyer and Lelandais, 1993). Thirdly, it is a cofactor for violaxanthin de-epoxidase. In bright light, or when low temperatures and drought limit carbon dioxide fixation, the excess excitation energy is dissipated as heat by zeaxanthin in the light harvesting antennae. Zeaxanthin is formed by successive de-epoxidation of the xanthophyll cycle pigments violaxanthin and antheroxanthin. The de-epoxidase, which is bound to the lumen side of the thylakoid membrane, is dependent on ascorbate as a cofactor (Neubauer and Yamamoto, 1992, 1993). Ascorbate thus contributes to electron flow and to formation of zeaxanthin, which acts as a photoprotectant. The quantitative importance of the Mehler peroxidase reaction *in vivo* (Foyer and Lelandais, 1993) and the extent to which it increases in response to limitation of carbon dioxide fixation by low temperature and drought remain to be determined. Activities of enzymes in the ascorbate glutathione cycle are increased by drought and low temperature suggesting a requirement for increased activity of the cycle under these conditions (Smirnoff and Colombé, 1988; Smirnoff, 1993, 1995). Manipulation of the ascorbate-GSH cycle in chloroplasts by targeted overexpression of AP, GR, MDAR and DHAR should provide further evidence for the role of ascorbate (Foyer, Descourvières and Kunert, 1994) and progress with this is discussed further in the next section in relation to (photo)oxidative stress.

DEFENCE AGAINST OXIDATIVE STRESS

Many stresses, including extreme environments and pollutants, act at least in part by causing oxidative damage (Smirnoff, 1995). Recent approaches using transgenic plants and mutants provide supporting evidence for the role of ascorbate and the ascorbate-glutathione cycle. Expression of bacterial GR in poplar (*Populus tremula* × *P. alba*) chloroplasts increases the ascorbate pool, probably by allowing more rapid reduction of DHA (Foyer *et al.*, 1995). The total GSM pool and its reduction state are also increased in the transformed poplar. In tobacco plants expressing high bacterial GR activity in the cytosol, the ascorbate pool is less oxidized when the leaves are exposed to methyl viologen-induced photo-oxidative stress (Foyer *et al.*, 1991). A number of other experiments with transgenic plants show that increased GR activity increases resistance to (photo)oxidative stress while reduced activity increases susceptibility (Aono *et al.*, 1995).

It has been suggested that cell wall ascorbate provides a first line of defence against ozone and sulphur dioxide (Castillo and Greppin, 1988; Takahama, Veljovic-Ionancovic and Heber, 1992; Luwe, Takahama and Heber, 1993). Ozone fumigation increases ascorbate and ascorbate-glutathione cycle enzyme activity (Castillo and Greppin, 1988). Recently, direct evidence for this has been provided by isolation of *soz1*, an ozone hypersensitive *A. thaliana* mutant which has 30% of wild type ascorbate in its leaves. Ozone sensitivity segregates with ascorbate deficiency (Conklin *et al.*, 1996). Further investigation of the ascorbate-deficient *soz1* *A. thaliana* mutants will provide more information about its role in resistance to oxidative stress as well as the role of ascorbate in photosynthesis and photoprotection. The mutant is sensitive to UV-B and sulphur dioxide as well as ozone indicating further antioxidant roles for ascorbate (Conklin *et al.*, 1996). A glutathione-deficient *A. thaliana* mutant has recently been isolated (Howden *et al.*, 1995) and this should also contribute to understanding of the ascorbate-glutathione cycle.

IS CELL WALL ASCORBATE INVOLVED IN CELL WALL METABOLISM AND CELL EXPANSION?

A role for ascorbate in growth has been suspected for some time. The apoplast contains ascorbate and ascorbate oxidase (AO, E.C. 1.10.3.30). The function of AO has not been understood until recently and there is now strong evidence that wall ascorbate and AO have links with wall metabolism and cell expansion. The characteristics of the ascorbate system in walls will be reviewed and a hypothesis to explain how ascorbate might regulate cell wall expansion is presented.

AO, which oxidizes ascorbate to water and MDA, is a member of the blue copper oxidase family, which also includes laccase and ceruloplasmin (Ohkawa *et al.*, 1989, 1994; Esaka *et al.*, 1990; Messerschmidt and Huber, 1990; O'Malley *et al.*, 1993). It is a glycoprotein and a large proportion of activity is localized in the cell wall (Esaka *et al.*, 1989; Moser and Kanellis, 1994). The K_m for ascorbate (0.05–0.2 mM) and pH optimum (5.5–7.0) (Esaka *et al.*, 1989; Lin and Varner, 1991) suggest that it will be active under wall conditions. Ascorbate and dehydroascorbate (0.1–5 mM) occur in intercellular washing fluid (IWF) obtained by centrifuging tissue which has been vacuum infiltrated with a buffer solution (Castillo and Greppin, 1988; Luwe *et al.*, 1993; Polle *et al.*, 1990; Otter and Polle, 1994; Luwe, 1996). Of the enzymes of the ascorbate-glutathione cycle AP, MDAR and DHAR have not been detected in IWF from *Picea abies* (Polle *et al.*, 1990) and DHAR and GR were not detected in IWF from *Sedum album* leaves (Castillo and Greppin, 1988) although a peroxidase isoform with relatively high affinity for ascorbate was found (Castillo and Greppin, 1986). Very little or no GSH has been detected in the apoplast (Polle *et al.*, 1990; Luwe, 1996). In contrast, MDAR has been detected by immunogold labelling in cell walls of soybean root nodules (Dalton *et al.*, 1993). A scheme for compartmentation of the

ascorbate system between the cytoplasm and wall is shown in Fig. 2.

High AO activity is associated with tissues containing rapidly expanding cells in a wide range of plants. High activity correlates with the beginning of rapid expansion in germinating seeds and can be localized to regions where cells are expanding (Mertz, 1961; Suzuki and Ogiso, 1973). High AO activity is found in rapidly expanding cucurbit fruits. AO activity, protein and mRNA level decrease as the fruit finishes its period of rapid expansion (Lin and Varner, 1991; Esaka *et al.*, 1992). This developmental regulation of AO expression is supported by the effect of light and auxin. In systems in which auxin stimulates growth it also increases AO activity and mRNA levels (Esaka *et al.*, 1992; Takahama, 1994). Continuous far red light, which stimulates expansion of the cotyledons of dark-grown mustard (*Sinapis alba*) seedlings, increases AO activity (Hayashi and Morohashi, 1993). High wall AO activity might be expected to increase wall MDA and DHA. In *Vigna angularis* hypocotyl segments, IAA and darkness, both of which increase the expansion rate of epicotyl segments, result in higher total ascorbate with a greater proportion being DHA. Higher ascorbate and DHA levels would cause higher steady state levels of MDA as predicted from the equilibrium constant of the disproportionation reaction (Takahama, 1994; Takahama and Oniki, 1994). The above observations suggest a close connection between cell expansion, wall ascorbate and AO. The most direct test for a role of AO in growth would be to down-regulate its expression in transgenic plants by antisense technology using the cloned AO. It would be particularly useful if down-regulation were to be targeted to selected tissues (e.g. roots and fruit).

Because of the involvement of AO, any hypothesis to explain the role of wall ascorbate in cell expansion must include MDA or DHA. MDAR and DHAR are not usually present in the wall so there must be another method to maintain reduction of the ascorbate pool. An additional feature of the model in Fig. 2 is a plasma membrane-bound high potential cytochrome *b* which transports electrons from the cytoplasm into the wall. Evidence for this transmembrane electron transport activity has been obtained from studies with highly purified plasma membrane vesicles. PM-bound cytochrome *b* acts as an electron donor to MDA (reducing it to ascorbate) and ferricyanide. Ascorbate is an effective reductant ($K_m = 0.37$ mM). NADH is not an effective reductant so the activity is not a membrane-bound NADH-dependent MDAR (Horemans, Asard and Caubergs, 1994; Asard *et al.*, 1995). The wall therefore has a system to generate MDA via AO activity and a means of reducing MDA to ascorbate. Ascorbate is transported into the apoplast by a carrier. DHA, formed by disproportionation of any MDA which escapes reduction by cytochrome *b*, can be transported by a high affinity carrier into the cytosol (Rautenkranz *et al.*, 1994) where it is reduced to ascorbate by GSH-dependent DHAR. The net result of this system is electron transport across the PM with NAD(P)H as the reductant and extracellular oxygen as the ultimate electron acceptor.

A link can now be made to growth because transmembrane electron transport has been implicated in

stimulation of expansion growth and exogenous MDA also stimulates growth. It has been suggested that electron transport stimulates the plasma membrane H^+ -ATPase (Carrasco-Luna *et al.*, 1995) which, according to the acid growth theory (Rayle and Cleland, 1992), will then lead to increased cell expansion and solute uptake. The possibility that MDA could act as an electron acceptor in the wall and thus stimulate growth is supported by results from experiments on onion (*Allium cepa*) roots. Treatment with MDA (generated by an equimolar mixture of ascorbate and DHA or by copper-catalysed ascorbate oxidation) caused increased growth rate by stimulating cell expansion, vacuolation and solute uptake (Hidalgo, Gonzalez-Reyes and Navas, 1989; Gonzalez-Reyes *et al.*, 1994, 1995). The MDA treatment also causes membrane hyperpolarization which suggests that the treatment increases H^+ -ATPase activity (Gonzalez-Reyes *et al.*, 1995). Control treatments show that the roots did not respond in this way to ascorbate or DHA alone. So far the effect of MDA has only been reported for onion roots and investigations of a range of other systems are required.

This hypothesis explains the relationship between AO and growth but there are a number of other ways in which ascorbate and AO could interact with wall function. Lin and Varner (1991) have suggested that AO generates DHA which reacts with the side chains of lysine and arginine residues in cell walls, thus preventing cross linking of structural proteins with hemicelluloses and polygalacturonate. This would lead to a more extensible wall. A further complication is added to this by the possible formation of oxalate from DHA in the wall. It is known that ascorbate is a precursor of oxalate (Loewus, 1988) but it is not clear if the actual precursor is ascorbate or DHA. Lin and Varner (1991) suggest that wall DHA is converted to oxalate. Oxalate could regulate the level of ionic calcium in the wall by formation of calcium oxalate crystals. Lower calcium increases wall extensibility by reducing cross-linking between polygalacturonate chains. Germin, a cell wall protein, which is expressed in cereal embryos when growth begins (Lane *et al.*, 1992) and in young *Sinapis alba* leaves (Heintzen *et al.*, 1994) has oxalate oxidase activity (Lane *et al.*, 1993). Oxalate oxidase oxidises oxalate to carbon dioxide and hydrogen peroxide. Release of calcium and promotion of peroxidative cross-linking of wall polymers would then lead to wall hardening. Interestingly germin expression is stimulated by salt stress in barley roots (Hurkman, Tao and Tanaka, 1991). Wall oxalate and oxalate oxidase clearly deserve more attention.

The concentration of cell wall ascorbate itself could influence lignification. Lignification is associated with high wall peroxidase (POD) activity and formation of hydrogen peroxide in the wall. POD uses hydrogen peroxide as oxidant to form monolignol radicals from monolignol precursors such as coniferyl alcohol. These then polymerize (Otter and Polle, 1994). This reaction is inhibited by ascorbate which may primarily act by scavenging the monolignol radicals (Fig. 1; Otter and Polle, 1994; Takahama and Oniki, 1994). Ascorbate could also be oxidized by cell wall POD (Otter and Polle, 1994). It is possible that the extent of lignification could be controlled

by the redox state of the wall which will be influenced by the balance between ascorbate and hydrogen peroxide. The wall ascorbate pool is smaller and more oxidized in lignifying spruce needles (38% DHA) than in mature needles (9% DHA). However, the average concentration of ascorbate in the apoplast of lignifying needles (1 mM) is still high enough to inhibit POD. Otter and Polle (1994) therefore suggest that understanding the role of ascorbate requires a method to measure it specifically in lignifying walls. If the rapid formation of superoxide and hydrogen peroxide (oxidative burst) in the walls of cells undergoing the hypersensitive response during pathogen attack (Mehdy, 1994) is to be effective it would have to be associated with oxidation of the wall ascorbate pool and perhaps with reduced ascorbate transport from the cytosol.

In summary, there is strong evidence that apoplastic MDA, generated by AO activity, could have a fundamental role in regulating cell expansion by affecting proton pumping. DHA may also have a role in minimizing interactions between wall proteins and polysaccharides, resulting in looser walls. DHA could generate wall oxalate which might then influence free calcium levels. Oxalate could be removed by oxalate oxidase. Furthermore, in lignifying cells, the balance between ascorbate and hydrogen peroxide in walls could control the rate of polymerization of lignin monomers. So far the possible involvement of ascorbate and ascorbate oxidase has only been investigated in a limited number of systems and more work is required to test these hypotheses. Wall growth is still a poorly-understood phenomenon and the ascorbate system could add another layer of complexity.

CELL DIVISION

Histochemical staining with silver nitrate usually reveals high levels of ascorbate in meristems and ascorbate involvement in cell division has been suggested for both plant and animal cells. In plants, the evidence is based on the increased proportion of cells progressing to from G1 to S phase in onion root meristems and pericycle in response to exogenous ascorbate (Liso *et al.*, 1988; Arrigoni *et al.*, 1989; Innocenti *et al.*, 1990; Arrigoni, 1994; Citterio *et al.*, 1994). In onion roots, this causes a large decrease in the number of cells in the quiescent centre (QC) (Innocenti *et al.*, 1990). Further evidence for the role of ascorbate in controlling the transition from G1 to S phase is provided by studies on maize root QCs. Histochemical detection of ascorbate with silver nitrate and immunolocalization of AO in meristems show that ascorbate is not detectable in the QC while AO levels are high. Isolated QCs have higher levels of AO mRNA than surrounding meristematic cells (Kerk and Feldman, 1995). High AO activity could oxidize any ascorbate transported from cells neighbouring the QC. Arrigoni's group have made use of the alkaloid lycorine to investigate the role of ascorbate. Lycorine decreases the ascorbate content of tissues (Arrigoni, Arrigoni-Liso and Calabrese, 1975). It also inhibits cell division and cell elongation in *Avena* coleoptiles and pea internodes (de Leo *et al.*, 1973) and, in onion roots, it induces the disappearance

of cells in S phase (Arrigoni, 1994). Adding ascorbate reverses the effect of lycorine on cell division (Arrigoni, 1994). While these experiments support the role of ascorbate in cell division and growth, more needs to be known about the specificity of lycorine in inhibiting ascorbate synthesis. There is also evidence that ascorbate or, more likely, MDA stimulates cell proliferation in animal cell cultures by shortening the cell cycle and stimulating entry into S phase (Navas and Gomez-Diaz, 1995). Navas and Gomez-Diaz (1995) implicate transmembrane electron transport supported by extracellular MDA as was discussed for cell expansion above. Another suggestion is that ascorbate increases deoxyribonucleotide reductase activity. This enzyme, which synthesizes deoxyribonucleotides required for DNA replication, requires iron. If iron supply is limited, ascorbate may be required as reductant to release it from the storage protein phytoferritin (Citterio *et al.*, 1994).

CONCLUSIONS

Given the pivotal role of ascorbate in photosynthesis and its possible role in cell division and expansion, it is surprising that so little is known about its metabolism. Further investigation of ascorbate biosynthesis and its role in cell growth are needed. There is now scope for molecular genetics to complement biochemical and physiological approaches. We may then be in a position to increase the vitamin C content of food plants and possibly enhance their tolerance to photo-oxidative stress.

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