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Ascorbic Acid in Plants: Biosynthesis and Function

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ABSTRACT: Ascorbic acid (vitamin C) is an abundant component of plants. It reaches a concentration of over 20 m*M* in chloroplasts and occurs in all cell compartments, including the cell wall. It has proposed functions in photosynthesis as an enzyme cofactor (including synthesis of ethylene, gibberellins and anthocyanins) and in control of cell growth. A biosynthetic pathway via GDP-mannose, GDP-L-galactose, L-galactose, and L-galactono-1,4-lactone has been proposed only recently and is supported by molecular genetic evidence from the ascorbate-deficient vtc1 mutant of *Arabidopsis thaliana*. Other pathways via uronic acids could provide minor sources of ascorbate. Ascorbate, at least in some species, is a precursor of tartrate and oxalate. It has a major role in photosynthesis, acting in the Mehler peroxidase reaction with ascorbate peroxidase to regulate the redox state of photosynthetic electron carriers and as a cofactor for violaxanthin de-epoxidase, an enzyme involved in xanthophyll cycle-mediated photoprotection. The hypersensitivity of some of the vtc mutants to ozone and UV-B radiation, the rapid response of ascorbate peroxidase expression to (photo)-oxidative stress, and the properties of transgenic plants with altered ascorbate peroxidase activity all support an important antioxidative role for ascorbate. In relation to cell growth, ascorbate is a cofactor for prolyl hydroxylase that posttranslationally hydroxylates proline residues in cell wall hydroxyproline-rich glycoproteins required for cell division and expansion. Additionally, high ascorbate oxidase activity in the cell wall is correlated with areas of rapid cell expansion. It remains to be determined if this is a causal relationship and, if so, what is the mechanism. Identification of the biosynthetic pathway now opens the way to manipulating ascorbate biosynthesis in plants, and, along with the vtc mutants, this should contribute to a deeper understanding of the proposed functions of this multifacetted molecule.

KEY WORDS: vitamin C, mannose, L-galactose, oxidative stress, antioxidants.

I. INTRODUCTION

L-Ascorbic acid (vitamin C) is apparently a ubiquitous molecule in eukaryotes. It functions as an antioxidant, an enzyme cofactor, and as a precursor for oxalate and tartrate synthesis. It participates in a variety of processes, including photosynthesis, photoprotection, cell wall growth and cell expansion, resistance to environmental stresses and synthesis of ethylene, gibberellins, anthocyanins, and hydroxyproline. Although ascorbate has multiple roles, and plants provide the major source of dietary vitamin C for humans (who are unable to synthesize it), very little is known about the details of its metabolism in plants. At the time of the previous review on ascorbate in this publication (Loewus and Loewus, 1987), the biosynthetic pathway of ascorbate in plants had not been established. Recently, a biosynthetic pathway has been proposed (Wheeler et al., 1998) that opens the way to improving understanding of the metabolism and function of ascorbate in plants. This review focuses on the biosynthetic pathway of ascorbate, considers its catabolism and transport, and evaluates evidence for its various functions. A number of reviews on ascorbate in plants have been published in recent years (Loewus and Loewus, 1987; Noctor and Foyer, 1998; Arrigoni, 1994; Smirnoff, 1996; Smirnoff and Wheeler, 1999; Loewus, 1988, 1999). Therefore, the present review attempts to complement these reviews by concentrating on recent advances.

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II. ASCORBATE BIOSYNTHESIS

A. Historical Perspective

The biosynthetic pathway of ascorbic acid has been a notable gap in our understanding of carbon metabolism in plants. After the first investigations of ascorbate biosynthesis in plants in the 1950s, it was 40 years before a definitive biosynthetic pathway was proposed (Wheeler et al., 1998). The recent elucidation of this pathway will enable rapid advances in our knowledge of ascorbate biosynthesis and its regulation. This section briefly reviews historical evidence leading to the elucidation of the currently proposed pathway. Full details of the early results are given by Loewus (1999).

Early researchers confirmed the conversion of hexose into ascorbate in plants (Ray, 1934; Loewus, 1963). This was also found to be the case in animals, where the biosynthetic pathway was defined in the early 1950s (Burns, 1967). It was shown that animals could convert D-glucuronate into L-gulono-1,4-lactone, which was subsequently oxidized to ascorbate. Significantly, this route of biosynthesis involves an "inversion" of the carbon chain, in which C1 of glucose becomes C6 of ascorbate and C6 becomes C1. The carbon chain inversion was confirmed by feeding specifically labeled glucose and glucuronolactone to rats (Burns, 1967). An analogous pathway was proposed in plants, involving the conversion of D-galacturonic acid to ascorbate via L-galactono-1,4-lactone. Plant extracts were capable of converting D-galacturonic acid derivatives (but not the free acid) and L-galactono-1,4-lactone to ascorbate (Figure 1; Mapson et al., 1954; Isherwood and Mapson, 1962; Mapson and Isherwood, 1956). However, feeding specifically labeled hexoses to plants suggested that no inversion occurred in their conversion to ascorbate (i.e., C1 of glucose became C1 of ascorbate), which is in direct conflict with the proposed biosynthetic pathway (Loewus, 1963). Despite this, the ease and efficiency of L-galactono-1,4-lactone conversion to ascorbate (Pallanca and Smirnoff, 1999; Isherwood et al., 1954) and the eventual purification and cloning of a mitochondrial L-galactono-1,4-lactone dehydrogenase from plants (Ôba et al., 1995; Østergaard et al., 1997; Imai et al., 1998) meant that many researchers found it difficult to dismiss its role in ascorbate biosynthesis.

Other researchers, Loewus and co-workers in particular, were less convinced of the role of L-galactono-1,4-lactone as the physiological ascorbate precursor, and set out to identify an alternative biosynthetic pathway in which the carbon chain of glucose was not inverted (Loewus and Loewus, 1987). An alternative biosynthetic pathway was proposed in which glucose was converted directly to ascorbate via D-glucosone and L-sorbosone (Figure 1; Loewus, 1988). The osones are 2-keto derivatives of the corresponding aldoses. ^{14}C-Labeled glucosone and sorbosone were converted into ascorbate, although the rate of incorporation of label from L-sorbosone into ascorbate was no higher than that of glucose (Saito et al., 1990). Neither substrate was able to increase the ascorbate content of plants when supplied exogenously (Pallanca and Smirnoff, 1999; Conklin et al., 1997; Davey et al., 1999), although a dehydrogenase was detected that could convert L-sorbosone to ascorbate (Loewus et al., 1990). However, the enzyme has a low affinity for its substrate (K_m = 12 m*M*). Basidiomycete fungi contain a pyranose-2-oxidase activity capable of converting glucose to glucosone (Daniel et al., 1994). No such activity capable of oxidizing glucose to glucosone was found in peas, and isotope dilution experiments did not support the notion that the ozones were on the pathway between glucose and ascorbate (Pallanca and Smirnoff, 1999). Therefore, there is no strong evidence to support the existence of this pathway, other than the low rates of labeled D-glucosone and L-sorbosone conversion to ascorbate. Their inability to increase the ascorbate pool of plants suggests that this is not the major route of ascorbate biosynthesis in plants. In contrast, exogenously supplied L-galactono-1,4-lactone can raise the ascorbate pool of plants dramatically (Smirnoff and Pallanca, 1996; Davey et al., 1999), and radiolabeled L-galactono-1,4-lactone is converted very rapidly and completely to ascorbate (Baig et al., 1970). Clearly, L-galactono-1,4-lactone is an extremely effective ascorbate precur-

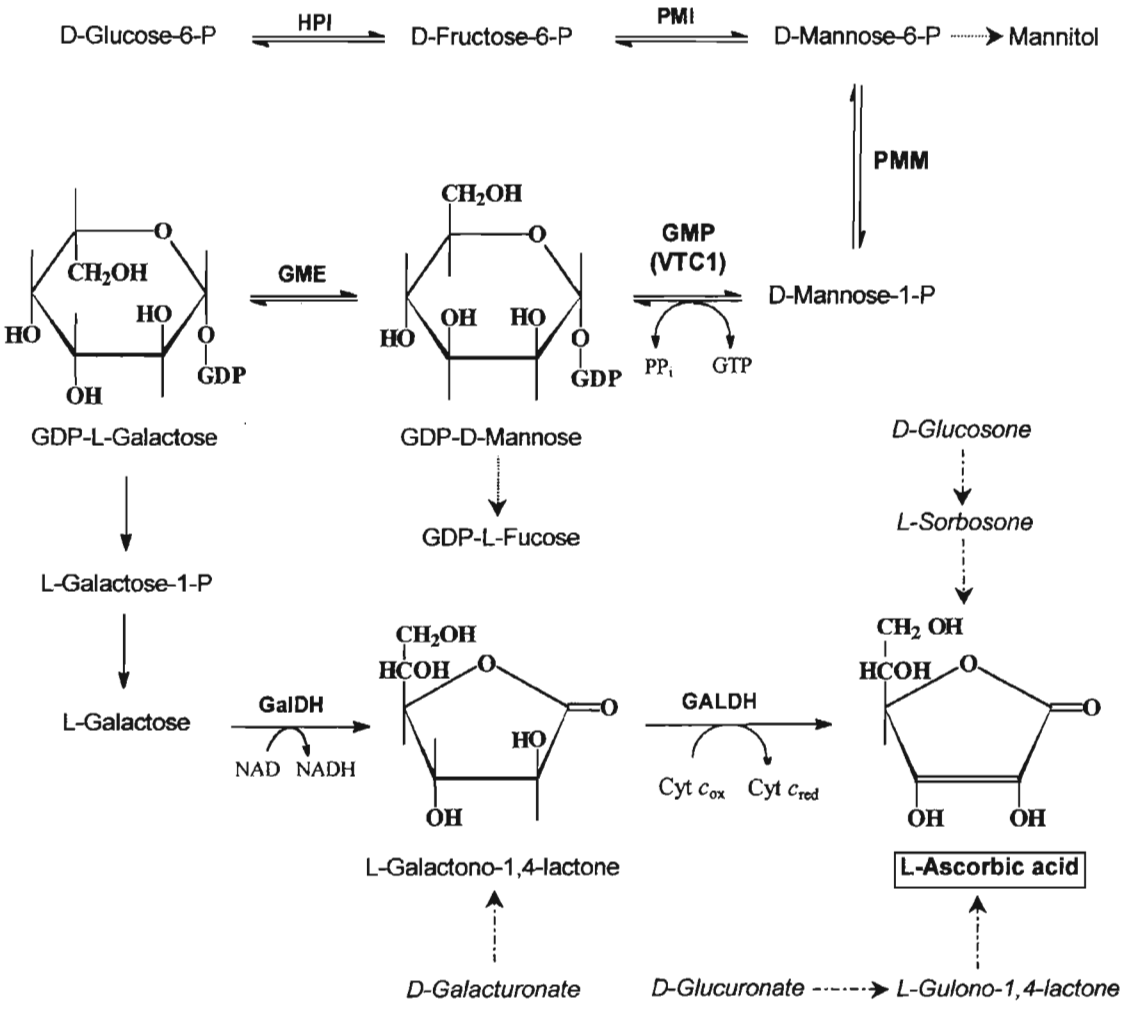

FIGURE 1. Proposed D-mannose/L-galactose biosynthetic pathway of L-ascorbic acid in plants. Branch points and other proposed precursors are also shown. The enzymes are **HPI**, hexose phosphate isomerase; **PMI**, phosphomannose isomerase; **PMM**, phosphomannose mutase; **GMP**, GDP-mannose pyrophosphorylase (product of the *VTC1* gene in *Arabidopsis thaliana*); **GME**, GDP-mannose-3,5-epimerase; **GalDH**, L-galactose dehydrogenase; GALDH, L-galactono-1,4-lactone dehydrogenase. GALDH is located on the outer side of the inner mitochondrial membrane; the other enzymes are probably cytosolic. The enzymes releasing L-galactose from GDP-L-galactose are not fully characterized. GDP-sugar intermediates of the pathway are also used as precursors for synthesis of cell wall polysaccharides and for protein glycosylation. Mannitol is a major end product of mannose metabolism in some plant species. Other possible precursors of ascorbate are shown in italic font. D-galacturonic and glucuronic acids (supplied as methyl derivatives) are incorporated but enzymes have not been characterized. Incorporation of uronic acids into ascorbate would involve "inversion" of the carbon skeleton; however, labeling studies show that only a small proportion of ascorbate could be produced in this way. L-Gulono-1,4-lactone is the precursor of ascorbate in animals, but, in contrast to plants, is oxidized by a microsomal oxidase. There is no evidence that glucosone and sorbosone are synthesized in significant amounts by plants, although the labeled compounds are incorporated into ascorbate.

sor. However, full acceptance of L-galactono-1,4-lactone as the physiological ascorbate precursor was not possible for two reasons. First, there was no evidence that it occurred in plants. Second, no one had proposed a pathway to synthesize this compound without "inversion" of the hexose car-

bon skeleton. As discussed above, carbon skeleton inversion is unlikely to occur in synthesis of ascorbate from glucose.

B. The GDP-Mannose/L-Galactose Pathway

In order to produce ascorbate from glucose without inversion of the carbon chain, three reactions need to occur, although not necessarily in any particular order. The three crucial steps are oxidation at C1 followed by formation of the 1,4-lactone, then oxidation at C2 or C3 to form the *ene*diol group, and finally an epimerization at C5 to change from the D configuration to the L configuration of ascorbate (Loewus, 1988, 1999). Also, the hydroxymethyl group at C6 is conserved during the metabolism of glucose to ascorbate (Loewus, 1999). Recently, a new biosynthetic pathway in plants was proposed, encompassing all the criteria stated above, in which ascorbate is produced from glucose via L-galactono-1,4-lactone but with no inversion of the carbon chain (Wheeler et al., 1998). Therefore, this pathway reconciles two of the established fundamental principles in ascorbate biosynthesis that were previously thought to be contradictory. Evidence for this pathway, shown in Figure 1, is discussed below.

Ascorbate production in plants was proposed to occur via GDP-mannose and L-Galactose. L-Galactose, when supplied to barley leaves, resulted in a dramatic rise in the total ascorbate pool of the leaves similar to that caused by L-galactono-1,4-lactone. Furthermore, it was shown that pea embryonic axes extracts were capable of NAD^+-dependent oxidation of L-galactose at C1 to form L-galactono-1,4-lactone (Wheeler et al., 1998). This novel enzyme was named L-galactose dehydrogenase and its properties are described in Section C.1. Plants are known to synthesize L-galactose because it is a minor component of cell wall polysaccharides (Roberts, 1971), but from an analytical point of view it is masked by larger amounts of D-galactose. Plant enzyme systems had previously been shown to be capable of producing GDP-L-galactose from GDP-D-mannose via an epimerization at C3 and C5 (Barber, 1971, 1979; Feingold, 1982). Since then, this GDP-mannose-3,5-epimerase has not been purified or cloned. The presence of epimerase activity was confirmed in both pea and *A. thaliana* cell-free extracts, but more importantly the production of free L-galactose was also demonstrated by these extracts (Wheeler et al., 1998). Although nonspecific phosphatase, pyrophosphatase, or phosphodiesterase activities could account for appearance of L-galactose, very little free L-mannose is produced from GDP-mannose (Wheeler et al., 1998). A similar observation was made with an enzyme preparation from *Chlorella* (Barber, 1971). Becuase, similar to higher plants, *Chlorella* is able to convert L-galactono-1,4-lactone (Renstrøm et al., 1982-1983) and L-galactose (Marschall and Smirnoff, unpublished results), to ascorbate, these observations provide evidence that there are specific enzymes able to catalyze formation of L-galactose from GDP-L-galactose. However, these enzymes are yet to be purified.

Cell-free extracts from peas were also shown to be capable of producing L-galactono-1,4-lactone and eventually ascorbate from GDP-mannose when supplied with NAD^+ and cytochrome *c*, the cofactors of L-galactose dehydrogenase and L-galactono-1,4-lactone dehydrogenase, respectively (Wheeler et al., 1998). Therefore, it is clear that plants contain enzymes able to convert GDP-mannose to ascorbate. Evidence that this was the route of ascorbate biosynthesis *in vivo* was provided by feeding radiolabeled mannose to *A. thaliana* leaves. Ten percent of the total radioactivity from ^{14}C-mannose was recovered in ascorbate following a 4 h incubation period, compared with 1% when radiolabeled glucose was used as the precursor (Wheeler et al., 1998).

The role of mannose in ascorbate biosynthesis is supported by molecular genetic evidence. An *A. thaliana* mutant (*vtc1*, formerly *soz1*) selected for ozone hypersensitivity (Conklin et al., 1996; Section V) was shown to be deficient in ascorbate biosynthesis (Conklin et al., 1997). The *VTC1* locus has been mapped and the *VTC1* has been cloned. It has homology with GDP-mannose pyrophosphorylase, an enzyme required for synthesis of GDP-mannose from mannose-1-phosphate (Figure 1; Conklin et al., 1999). As a consequence, the *vtc1* mutant displayed only 35% of

this enzyme's activity compared with the wild type and was similarly reduced in the ability of leaves to convert radiolabeled mannose to ascorbate. When the mutant was transformed with the wild-type *VTC1* gene, ascorbate concentration in the transgenic plants was returned to that of wild-type plants (Conklin et al., 1999). This clearly suggests that a reduction in the activity of GDP-mannose pyrophosphorylase is responsible for the deficiency in ascorbate biosynthesis described in the *vtc1* mutant. The mutant *VTC1* had a single point mutation that results in reduced enzyme activity but a similar level of mRNA expression (Conklin et al., 1999). Further evidence supporting the role of this enzyme in ascorbate biosynthesis in plants was provided by potatoes expressing an antisense GDP-mannose pyrophosphorylase construct. Antisense plants displaying a reduction in GDP-mannose pyrophosphorylase activity also have a considerable reduction in their ascorbate concentration (Keller et al., 1999).

We suggest that the D-mannose-L-galactose pathway is currently the foremost candidate for ascorbate biosynthesis in plants. It is supported by the biochemical and molecular genetic evidence discussed above. It also satisfies all the criteria derived from the original radiotracer evidence (Loewus, 1999): oxidation of the hexose precursor at C1 followed by formation of a 1,4-lactone; oxidation at C2 or C3 and epimerization at C5. Therefore, this pathway results in a lack of inversion of the hexose C-skeleton during ascorbate biosynthesis. Other "inversion" type pathways involving uronic acids could contribute to ascorbate synthesis and these are outlined in Section II.E.

C. Ascorbate Biosynthetic Enzymes

1. L-Galactose Dehydrogenase

The identification of L-galactose dehydrogenase in plants was a key step in demonstrating plants possessed the ability to synthesize L-galactono-1,4-lactone (Wheeler et al., 1998). The enzyme oxidizes L-galactose at C1 to produce L-galactono-1,4-lactone with the concurrent reduction of NAD^+. $NADP^+$ was only 10% as effective. This enzyme appears to be specific for L-galactose, with no measurable oxidation of commonly occurring hexoses. Furthermore, no activity was detected with the structural analogues D-arabinose and L-fucose (Wheeler et al., 1998). However, L-galactose dehydrogenase was reported to slowly oxidize L-sorbosone. Wheeler et al. (1998) suggested this could explain the conversion of L-sorbosone into ascorbate (Section II.A). The putative L-sorbosone dehydrogenase described by Loewus et al. (1990) was $NADP^+$-dependant with no measurable activity in the presence of NAD^+, so the relationship between these activities is not clear.

This enzyme appears to be unique in plants in that it oxidizes C1 of a nonphosphorylated hexose. Similar enzymes exist in bacteria (D-galactose dehydrogenase), mammals (L-fucose dehydrogenase) and yeast (D-arabinose dehydrogenase) (Schachter et al., 1969; Maier and Kurtz, 1982; Kim et al., 1996, 1998). Both L-fucose and D-arabinose dehydrogenase have lower substrate specificity than L-galactose dehydrogenase and are capable of oxidizing L-galactose. Interestingly, D-arabinose dehydrogenase is involved in D-erthryoascorbate biosynthesis in yeasts (Kim et al., 1996).

2. L-Galactono-1,4-Lactone Dehydrogenase

By far the most well-characterized step in ascorbate biosynthesis is the conversion of L-galactono-1,4-lactone into ascorbate. L-galactono-1,4-lactone dehydrogenase was first identified more than 40 years ago (Mapson et al., 1954; Mapson and Breslow, 1958). The enzyme has since been purified from a range of plant tissues and recently has been cloned from cauliflower and sweet potato (Østergaard et al., 1997; Imai et al., 1998). It has a subunit and native molecular mass of 56 kDa and a K_m of between 0.2 and 3.0 m*M* for L-galactono-1,4-lactone (Østergaard et al., 1997; Imai et al., 1998; Smirnoff and Wheeler, 1999). L-Galactono-1,4-lactone dehydrogenase is located in mitochondrial membranes (Mutsuda et al., 1995), and the presence of

a mitochondrial targeting sequence in the gene has been suggested (Østergaard et al., 1997). Recently, it has been localized in the inner mitochondrial membrane (Siendones et al., 1999). The enzyme uses only cytochrome *c* as an electron acceptor *in vitro* (Østergaard et al., 1997). Oxidation of L-galactono-1,4-lactone by purified mitochondria and the effect of mitochondrial electron transport inhibitors, that suggest cytochrome *c* is the physiological acceptor and that electrons are fed into the mitochondrial electron transport chain between complexes III and IV (Bartoli et al., 2000). A number of sources have suggested that L-galactono-1,4-lactone dehydrogenase contains a bound flavin group (Mutsuda et al., 1995, Ôba et al., 1995), although this was not the case with the enzyme purified from cauliflower (Østergaard et al., 1997). The analogous enzyme in mammals, L-gulonolactone oxidase, contains a covalently bound flavin group and can also utilize molecular oxygen as electron acceptor (Kiuchi et al., 1982). Inhibition of L-galactono-1,4-lactone dehydrogenase by NEM and PCMB suggest thiol groups are required for activity. It has been suggested that the alkaloid lycorine is a specific inhibitor of this enzyme (Arrigoni et al., 1997), and inhibition of the purified sweet potato enzyme was demonstrated (Imai et al., 1998). Lycorine has also been shown to inhibit ascorbate biosynthesis *in vivo* but also has a number of toxic effects on plant metabolism. Lycorine inhibition was not seen with the purified cauliflower enzyme (Østergaard et al., 1997) and it did not inhibit the conversion of L-galactono-1,4-lactone to ascorbate in *Lilium longiflorum* pollen (Leung and Loewus, 1985) or *A. thaliana* cell cultures (Davey et al., 1999). The reasons for differences in lycorine action observed by different groups remains to be resolved, so it is not clear if lycorine can act as a specific inhibitor of ascorbate biosynthesis.

Plant L-galactono-1,4-lactone dehydrogenase has 28% sequence identity with mammalian L-gulono-1,4-lactone dehydrogenase (Koshizaka et al., 1988) and yeast arabinono-1,4-lactone dehydrogenase (Huh et al., 1994; Nishikimi et al., 1998). L-Gulono-1,4-lactone oxidase from mammals is capable of oxidizing both L-galactono-1,4-lactone and L-gulono-1,4-lactone. Purified L-galactono-1,4-lactone dehydrogenase from cauliflower, spinach, and sweet potato are specific for L-galactono-1,4-lactone. However, the conversion of gulono-1,4-lactone to ascorbate in whole tissue has been demonstrated in cress (Isherwood et al., 1954), strawberry and bean (Baig et al., 1970), and also in partially purified L-galactono-1,4-lactone dehydrogenase from potato (Ôba et al., 1994). Three possibilities exist to explain this discrepancy. First, L-galactono-1,4-lactone dehydrogenase could exist in different isoforms, some of which can oxidize L-gulono-1,4-lactone. Second, L-gulono-1,4-lactone could be oxidized by a separate specific enzyme, and third L-gulono-1,4-lactone could be epimerized at C3 to produce L-galactono-1,4-lactone (Baig et al., 1970). The physiological relevance of this is discussed with reference to uronic acid metabolism in the next section.

D. Relationships between Ascorbate Biosynthesis and Carbohydrate Metabolism

1. D-Mannose Metabolism

The existence of the mannose-L-galactose pathway for ascorbate synthesis provides a major and previously unnoticed fate for mannose. Mannose is known as a major component of polysaccharides that perform structural and storage functions in plants. In addition to this mannose is also involved in protein glycosylation and in the production of glycolipids. The mannose residues found in polysaccharides and glycoproteins are all derived from the sugar nucleotide form of mannose, GDP-mannose. The proposal that GDP-mannose is an intermediate in ascorbate biosynthesis drastically changes our previous conception of the role of this metabolite in plant metabolism.

Mannose-containing polysaccharides are found in a diverse range of plant tissues and species (Herold and Lewis, 1977). Polymers comprising of a mannose backbone and containing less than 5% other glycosyl units are termed mannans. Galactomannans and glucomannans, which contain proportionally higher amounts of

galactose and glucose residues, respectively, are also widely distributed among plants. The endosperm of a number of seeds accumulates mannose containing cell wall polysaccharides (e.g., galactomannans in the Leguminosae) that act as the principal source of carbohydrate for the embryo. Presumably, factors affecting the biosynthesis of these polysaccharides and glycoproteins strongly influence the flux of carbon through to GDP-mannose and its subsequent utilization. Our understanding of these key steps in mannose metabolism will be crucial in understanding the regulation of ascorbate biosynthesis. It is, of course, possible that some of these processes are highly compartmentalized (e.g., production of endosperm reserves) and therefore will not have significant effects on ascorbate biosynthesis. Antisense reduction of GDP-mannose pyrophosphorylase activity in potato reduces the mannose content of cell wall polysaccharides as well as ascorbate accumulation, the effect being more marked in lines with greater suppression. However, the pattern of protein glycosylation was not altered in antisense plants (Keller et al., 1999).

Despite its widespread occurrence in plants, much remains to be learned about mannose metabolism. One particular aspect of mannose metabolism that requires clarification is the role of phosphomannose mutase (PMI) in plants. Exogenous mannose is rapidly phosphorylated to mannose-6-phosphate by hexokinase activities (Schnarrenberger, 1990; Harris et al., 1986). Mannose-6-phosphate can then be incorporated into fructose-6-phosphate (and therefore into mainstream hexose phosphate metabolism) via PMI. The onset of germination in mannan accumulating seeds results in a huge release of free mannose. Consequently, germinating seeds in these species demonstrate appreciable levels of PMI activity (Lee and Matheson, 1984). Additionally, a large number of species (including umbelliferous plants such as celery) synthesize the polyol mannitol for use as a translocated carbohydrate and osmoticum in addition to sucrose (Popp and Smirnoff, 1995). These plants have PMI activity to form mannose-6-phosphate, which is then reduced to mannitol-1-phosphate by mannitol-6-phosphate reductase (Rumpho et al., 1983). In contrast, many other plants are unable to rapidly incorporate mannose-6-P into hexose phosphate metabolism that has been attributed to low activity of PMI or even its absence (Herold and Lewis, 1977). The consequential accumulation of mannose-6-phosphate causes the depletion of cytoplasmic Pi and ATP pools (Loughman et al., 1989; Harris et al., 1986). Large concentrations of exogenous mannose therefore can be toxic to plants. Germination of *A. thaliana* seeds is reversibly inhibited by 7.5 m*M* mannose (Pego et al., 1999). *A. thaliana* plants and maize cell suspension cultures treated with higher D-mannose concentrations (~56 m*M*) show loss of cell viability that has the hallmarks of programmed cell death (apoptosis), including DNA laddering, increased DNAase activity, and release of cytochrome *c* from the mitochondria (Stein and Hansen, 1999). This response was not elicited by D-glucose, L-mannose, or D-galactose. The mechanism of mannose-induced programmed cell death is not clear but inhibition of germination, in contrast to the results of Loughman et al. (1989), is not caused by ATP depletion (Pego et al., 1999). Germination inhibition is reversed by simultaneous supply of glucose or by mannoheptulose, which inhibits hexokinase (Pego et al., 1999). However, tracer levels of mannose are rapidly incorporated into derivatives of fructose 6-phosphate in cultured cells of spinach, rose, and maize (Baydoun and Fry, 1988), although Roberts (1971) concluded that ^{14}C mannose was not metabolized via glycolytic intermediates in maize roots. It is possible that the PMI activity in plants is capable of metabolizing tracer levels of mannose but unable to cope with the huge accumulation of mannose-6-phosphate caused by feeding high concentrations of mannose. Experiments in which large concentrations of mannose are supplied to plants should be interpreted with caution. For example, ^{14}C mannose is rapidly converted to ascorbate in *A. thaliana* leaves (10% in 4 h), although large concentrations of exogenous mannose are unable to increase the ascorbate pool in cultured *A. thaliana* (Davey et al., 1999; Wheeler et al., 1998). Presumably, this is due a sequestration of P_i and a reduction in available GTP for the GDP-

mannose pyrophosphorylase step and/or limited capacity of other enzymes.

It has been demonstrated that there is considerable mannokinase activity in green leaves (Scharrenberger, 1990). Therefore, in the presence of an excess of mannose the sum of activities metabolizing mannose-6-phosphate (i.e., PMI and PMM) would have to be in excess of this kinase activity to prevent an accumulation of mannose-6-phosphate. One must also consider that PMI is a key branch point between mainstream hexose phosphate metabolism and mannose metabolism and is likely to be highly regulated. It would appear that a comprehensive survey of the activity of this enzyme in different species is required before its role in the biosynthesis of GDP-mannose and ultimately ascorbate can be understood.

A possible alternative route of GDP-mannose biosynthesis in plants has been suggested. It has been shown that particulate membrane preparations of pine seedlings were able to produce glucomannans from GDP-^{14}C-mannose in which both the glucose and mannose residues were labeled (Dalessandro, 1986). The same result was seen when GDP-^{14}C-glucose was the precursor. This was interpreted as an indication of the presence of a GDP-glucose-2-epimerase (Dalessandro et al., 1986). Similar results were obtained from *Lilium testaceum* (Wozinecski et al., 1991). The Leguminosae are reported to have appreciable PMI activity, in germinating seeds at least, and therefore would have a lower requirement for an alternative source of GDP-mannose. Interestingly, pea displayed no GDP-glucose-2-epimerase activity (Piro et al., 1993), although this was not the case in mung bean (Elbein, 1969). It must be borne in mind that none of the above studies measured this activity directly and relied on analyzing the monosaccharide content of polysaccharides synthesized from the precursors. Until this enzyme is measured directly, its presence in plants cannot be confirmed. However, it remains a possible source of GDP-mannose in those plants where PMI is deemed to be absent.

Many aspects of mannose metabolism appear to be highly species specific, both in terms of the distribution of mannose polysaccharides and also in the activities of mannose-metabolizing enzymes. The implications of these species differences are of obvious importance in our understanding of ascorbate biosynthesis.

2. L-Galactose Metabolism

L-Galactose was thought to be a relatively rare sugar, occurring in small amounts in cell wall polysaccharides. However, the inability of chromatographic techniques to distinguish between the D and L isomers of this sugar means that small quantities of the L-form are obscured by the more abundant D-isomer unless measures are taken to eliminate one or other from the sample. Up to 25% of the galactose in cell wall polysaccharides of corn root is present as the L-isomer (Roberts and Harrer, 1973), although an estimation of the galactose content in the cell walls of cultured spinach cells suggested a ratio closer to 70:1 in favor of the D-isomer (Baydoun and Fry, 1988). It has been suggested that L-galactose is a widespread constituent of plant cell walls (Roberts and Harrer, 1973). Free L-galactose has never been measured in plants, but its rapid metabolism (Wheeler et al., 1998), combined with the detection problems noted above, suggest that it may well exist in low quantities, which are likely to be obscured by any free D-galactose in the sample. L-Galactose in cell walls is derived from GDP-L-galactose, which is in turn produced from GDP-mannose. No information is available on the relative rates of incorporation of GDP-L-galactose into the cell wall and ascorbate. The details of the conversion of GDP-L-galactose into L-galactose-1-P and on into L-galactose have yet to be defined, but these are the first committed steps in plant ascorbate biosynthesis. These enzymes may prove to be important regulatory steps given the rapid rate of L-galactose and L-galactono-1,4-lactone conversion to ascorbate.

E. The Role of Uronic Acids in Ascorbate Biosynthesis

Current evidence suggests that the major route of ascorbate biosynthesis in plants is via GDP-mannose and L-galactose. However, the need to examine the other possibilities has been stressed

recently (Davey et al., 1999). A brief discussion of alternative pathways is included here, with particular reference to uronic acid metabolism, although it must be made clear that there is at present no convincing evidence that any alternative pathways make a significant contribution to ascorbate biosynthesis *in vivo*. The proposed uronic acid intermediates are shown in Figure 1.

A significant anomaly in our understanding of plant ascorbate biosynthesis is the conversion of uronic acid derivatives to ascorbate. Radiolabel from D-glucuronate and the methyl ester of D-galacturonic acid are incorporated into ascorbate in parsley (Loewus, 1963), and both these substrates effect a substantial rise in the ascorbate pool of cress seedlings (Isherwood et al., 1954) and *A. thaliana* cell cultures (Davey et al., 1999). Feeding specifically labeled uronic acids, indicated that, as predicted, carbon chain inversion occurred in their conversion to aldonic acid lactones. As mentioned previously, studies investigating specifically labeled glucose incorporation showed that the majority of the label recovered from ascorbate was found in the corresponding carbon atom to that labeled in glucose. Although a small percentage of the label could be recovered in the opposite carbon atom (i.e., the carbon atom that would indicate inversion of the carbon chain), a similar percentage was recovered from glucose residues in starch and significantly in the galacturonate residues of pectin. Loewus concluded that this was due to label randomization as a result of triose phosphate metabolism. Although these studies do not rule out uronic acid metabolism as a possible source of galactono-1,4-lactone, they demonstrate that in the various tissues analysed this is certainly not the major route of ascorbate biosynthesis. Davey et al. (1999) suggested a possible role for the conversion of uronic acids to ascorbate in tissues where cell wall breakdown is occurring, for example, rapidly expanding tissue or ripening fruit. Whether this is of physiological significance remains to be seen.

F. Control of Ascorbate Synthesis

Very little is known about the control of ascorbate synthesis or pool size. Now that a biosynthetic pathway has been proposed, and as enzymes are identified and their genes cloned it will become possible to determine the contribution of each part of the pathway to control of synthesis. In embryonic axes of germinating pea seedlings ascorbate synthesis appears to be subject to feedback inhibition or repression (Pallanca and Smirnoff, 2000) as elevating the ascorbate pool by loading with exogenous ascorbate strongly inhibits its synthesis from ^{14}C-glucose. In leaves, ascorbate pool size is light-dependent, probably because of the role of ascorbate in photosynthesis and photoprotection (Section V.A). Leaves acclimated to high light have higher ascorbate concentrations than leaves grown at low light intensity (Smirnoff and Pallanca, 1996; Grace and Logan, 1996; Logan et al., 1996). In some species, such as barley and *A. thaliana* (Smirnoff and Pallanca, 1996; Conklin et al., 1997), ascorbate pool size decreases in the dark, but this can be partially reversed by sucrose or glucose feeding. In contrast, sugar feeding has no effect on ascorbate accumulation in nonphotosynthetic pea embryonic axes (Pallanca and Smirnoff, 1999). The mechanisms behind these effects remain to be discovered.

In squash (*Cucurbita*) roots, boron deficiency strongly reduces root growth rate and ascorbate content (Lukaszewski and Blevins, 1996). Interestingly, root growth could be restored by ascorbate feeding even though the boron deficiency was not alleviated. The biochemical roles of boron in plants are not understood, but the rapid response of ascorbate to boron deprivation could suggest a role for boron in controlling ascorbate biosynthesis. Borates are able to form strong complexes with mannose that could then affect ascorbate biosynthesis. Further work is needed to establish if boron deficiency affects the ascorbate pool by altering its synthesis or turnover.

III. ASCORBATE CATABOLISM: L-TARTRATE AND OXALATE SYNTHESIS

The ascorbate pool turns over at an appreciable rate. For example, in *A. thaliana* leaves the rate of loss is about 2.5% of the pool per hour, estimated from metabolism of ^{14}C-ascorbate and

from the rate of loss of ascorbate in darkened leaf slices (Conklin et al., 1997). Similar measurements on embryonic axes of germinating pea seedlings show a turnover rate of 13% h^{-1} (Pallanca and Smirnoff, 2000). Ascorbate is metabolized to a number of products, including L-tartrate and oxalate. These processes have been demonstrated by Loewus' group and recently have been reviewed comprehensively (Loewus, 1999). The details of tartrate and oxalate formation vary between species. Tartrate forms by a C4/5 cleavage of the ascorbate C-skeleton in the grape family (Vitaceae). In other species, oxalate is formed by C2/C3 cleavage. The other product is L-threonate, which can either be converted to L-tartrate (e.g., *Pelargonium*) or decarboxylated to L-glycerate. A number of plant species accumulate oxalate, often as calcium oxalate crystals deposited in specialized cells (idioblasts). This oxalate may be derived from photorespiratory intermediates such as glycolate (Loewus, 1999; Smirnoff and Wheeler, 1999) but also from ascorbate. The relative importance of these pathways could well differ between species and tissues. Recently, the use of ^{14}C-L-galactose to specifically label the ascorbate pool, in the oxalate-accumulating plant *Pistia stratiotes* has shown that ascorbate is a precursor of oxalate in the calcium oxalate crystals, and is a more effective substrate than glycolate (Keates et al., 2000). These labeling studies have also revealed that the oxalate in the calcium oxalate crystals has appreciable turnover, perhaps by the action of oxalate oxidase (Smirnoff, 1996). Thus, it is possible that in some plants ascorbate-derived oxalate is involved in calcium homeostasis. The mechanism of ascorbate C-skeleton cleavage is not known. $^{18}O_2$ labeling suggests C2/3 cleavage involves oxygenase and hydrolase activity, while C4/5 cleavage is hydrolytic (Saito et al., 1997). It is also possible that cleavage is nonenzymatically mediated by hydrogen peroxide (Loewus, 1999).

IV. SUBCELLULAR LOCALIZATION AND TRANSPORT OF ASCORBATE

Ascorbate probably occurs in all cell types, except in dry seeds (Arrigoni et al., 1991; Pallanca and Smirnoff, 1999). It may be that ascorbate oxidizes during the desiccation phase of seed maturation and cannot be regenerated when water content becomes too low for the operation of the ascorbate-glutathione cycle. In germinating pea seedlings synthesis and accumulation of ascorbate in the embryonic axes starts about 20 h after imbibition (Pallanca and Smirnoff, 1999). Long-distance transport of ascorbate or DHA occurs, probably in the xylem, because ascorbate fed to roots elevates leaf ascorbate concentration and ^{14}C-ascorbate fed to roots appears in leaves (Mozafar and Oertli, 1993). Nothing is known about phloem transport, but ascorbate and DHA would be relatively unstable at the high pH of phloem sap.

Ascorbate is present in all subcellular compartments, including the apoplast (cell wall), chloroplasts, cytosol, vacuoles, mitochondria, and peroxisomes (Rautenkranz et al., 1994; Foyer and Lelandais, 1996; Jimenez et al., 1997). Apoplastic ascorbate may have a role in control of cell growth (Smirnoff, 1996; Section V.D). The high concentration in some fruits (e.g., blackcurrents, *Ribes nigra*) could result from compartmentation and stabilization in acidic vacuoles. The final step of ascorbate biosynthesis is on the inner mitochondrial membrane (Section II.C). Therefore, it must be transported to other organelles. There are likely to be transporters to allow it into mitochondria, chloroplasts, microbodies, vacuole, and apoplast. The best-known ascorbate transport system in plants is on the plasma membrane. It has been characterized in plasma membrane vesicles (Horemans et al., 1996). This has a strong preference for DHA over ascorbate. The K_m for DHA is 24 μM (Horemans et al., 1997, 1998a). DHA uptake is stimulated by preloading the vesicles with ascorbate (Horemans et al., 1998b), suggesting it could act as an ascorbate/DHA exchange mechanism (Figure 2). The K_m for DHA is within the concentration range expected in the apoplast (Smirnoff, 1996). Therefore, the carrier could provide a mechanism to remove DHA from the apoplast and replace it with ascorbate. In contrast to mammalian systems, in which the GLUT1 glucose transporter allows DHA transport by facilitated diffusion (Vera et al., 1993; Welch et al., 1995), this carrier does not transport glucose. In

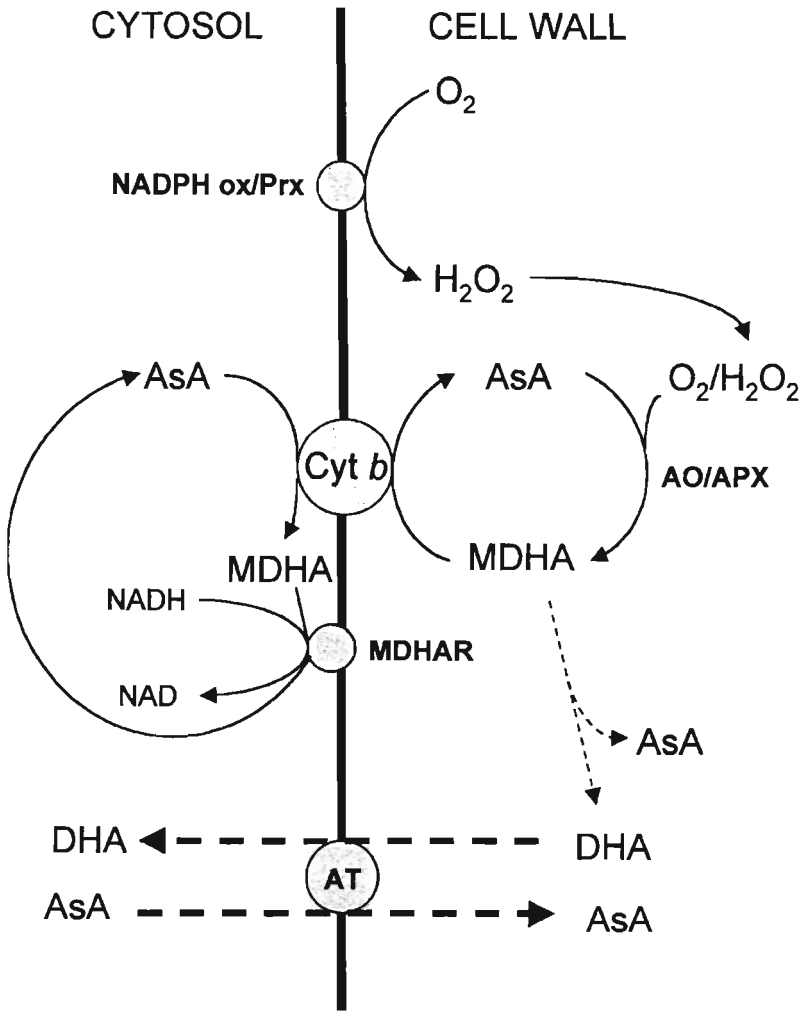

FIGURE 2. Model for proposed ascorbate redox reactions in the cell wall and plasma membrane. Ascorbate is transported into the cell wall by an ascorbate-dehydroascorbate exchanger (AT). In the wall, ascorbate oxidation is catalyzed by ascorbate oxidase (AO), a Cu-containing glycoprotein. High AO activity correlates with rapid cell expansion, but a causal relationship and mechanism are not established. Ascorbate is also oxidized by hydrogen peroxide, this reaction being catalyzed by ascorbate peroxidase (APX) suggested to be localized in the wall. Two molecules of monodehydroascorbate (MDHA), the primary oxidation product of ascorbate, disproportionate to dehydroascorbate (DHA) and ascorbate. The wall apparently lacks enzymes of the ascorbate-glutathione cycle (Figure 3), which can reduce MDHA and DHA back to ascorbate. Instead, wall ascorbate is probably maintained by DHA-ascorbate exchange and a by reduction of MDHA by a membrane-bound cytochrome *b*. The electron donor could be cytosolic ascorbate, the resulting MDHA being reduced to ascorbate by a membrane-bound MDHA reductase. Hydrogen peroxide can be generated by wall-localized oxidases such as oxalate oxidase (Smirnoff, 1996) or by oxygen reduction. The latter occurs during the oxidative burst in response to pathogen attack. The source of hydrogen peroxide is not resolved, a plasma membrane bound NADPH oxidase or a peroxidase (Prx) being possibilities (Bolwell, 1999).

addition to a DHA transporter, mammalian cells have a distinct higher affinity ($K_m = 6\ \mu M$) energy and sodium-dependent ascorbate uptake system (Welch et al., 1995; Rumsey et al., 1999). At high concentration, mammalian cells also exhibit low-affinity sodium-independent ascorbate uptake, and

currently it is not clear if it is a property of the same protein (Rumsey et al., 1999). New ascorbate analogues that do not mimic DHA when oxidized have provided the ability to distinguish the ascorbate-specific transporters from DHA transporters (Rumsey et al., 1999). These analogues could be used in investigations of plant ascorbate transport to establish if ascorbate, as well as DHA, is transported across the plasma membrane.

As well as ascorbate transport across the plasma membrane, a system that facilitates ascorbate-dependent electron transport across the plasma membrane via a *b*-type cytochrome has been identified (Horemans et al., 1994). Apoplastic monodehydroascorbate is reduced by cytochrome *b*. Electrons are donated to cytochrome *b* on the cytosolic side by ascorbate. The resulting cytosolic monodehydroascorbate is reduced to ascorbate by a plasma-membrane bound form of MDHA reductase (Figure 2; Berczi and Moller, 1998). This provides another mechanism, in addition to DHA-ascorbate exchange, to maintain the cell wall ascorbate pool in a reduced state, but it might also contribute to proposed mechanisms of growth regulation by ascorbate (Section V.D). An ascorbate-dependent transmembrane electron transport system may work in mammalian HI-60 cells where reduction of extracellular ferricyanide is dependent on intracellular ascorbate (Van Duijn et al., 1998).

Chloroplastic ascorbate can reach at least 20 m*M*. Surprisingly, although ascorbate is taken up by a saturable carrier, it has a very low affinity (K_m = 20 m*M*) and does not transport glucose (Anderson et al., 1983; Beck et al., 1983; Foyer and Lelandais, 1996). Within the chloroplast, 10 to 20% of the ascorbate occurs in the thylakoid lumen (Mano et al., 1997), where it acts as a co-factor for violaxanthin de-epoxidase (Section V.A). A carrier for ascorbate or DHA on the thylakoid membrane has not been identified.

Much more remains to be learned about ascorbate and DHA transport. Hopefully progress will be made in purifying and cloning the plasma membrane DHA/ascorbate exchanger. This could be most easily achieved, and other transporters identified, by devising screens to identify transport-deficient *A. thaliana* mutants as well as by utilizing ascorbate analogues.

V. FUNCTIONS OF ASCORBATE IN PLANTS

Ascorbate probably has multiple functions, but many of these are very poorly understood (Arrigoni, 1994; Smirnoff, 1996; Noctor and Foyer, 1998). Following from the identification of the ascorbate biosynthesis pathway (Section II.B), it is likely that metabolically engineered plants with increased or decreased ascorbate content will soon be used to probe ascorbate function. The ascorbate-deficient *A. thaliana* mutant *vtc1* (containing 30% of wild-type ascorbate) has already provided strong genetic evidence for the role of ascorbate as an antioxidant in defense against ozone, sulfur dioxide, and UV-B radiation (Conklin et al., 1996). Several other ascorbate-deficient *vtc* mutants are currently being characterized (Conklin et al., 2000). Recent evidence suggests that interpretation of role of ascorbate in ozone resistance is not as straightforward as originally supposed. The evidence concerning apoplastic ascorbate is particularly contradictory (Moldau et al., 1998; Jakob and Heber, 1998). Also, in a range of *A. thaliana* ascorbate-deficient (*vtc*) mutants, not all are ozone hypersensitive despite similar reductions in ascorbate concentration (Conklin et al., 2000). *Vtc1* has no major growth or developmental anomolies, suggesting that, if ascorbate is involved in the growth processes described below, lower concentrations will satisfy these requirements. This observation also implies that *A. thaliana* contains more ascorbate than is needed for antioxidant and photoprotective purposes under "unstressed" laboratory conditions.

A. Antioxidant and Photoprotective Roles of Ascorbate

The antioxidant system of plants differs from that typical of mammalian cells in the relative concentration of ascorbate and glutathione, the two major soluble small molecule antioxidants. Typically, mammalian cells contain 0.1 to 0.8 m*M* ascorbate (0.04 m*M* in plasma) and 2 to 5

m*M* glutathione. In contrast, leaf cells contain 2 to 5 m*M* ascorbate (averaged over all compartments) and < 1 m*M* GSH. Yeast cells also have much higher GSH than erythroascorbate concentrations (Spickett et al., 2000). The significance of this is not known, but it should be noted that ascorbate, compared with glutathione, is a more "effective" free radical scavenger in some respects because its oxidized free radical (MDHA) is much more unreactive, and therefore less damaging, than radicals derived from GSH (Sturgeon et al., 1998). Protection against reactive oxygen species (ROS) and photoprotection have been reviewed recently (Noctor and Foyer, 1998; Asada, 1999; Niyogi, 1999). Here, an "ascorbicentric" view of these processes will be taken. Aerobic metabolism and the interaction of light with pigments generate a number of reactive oxygen species, including superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. These can readily oxidize proteins, unsaturated fatty acids, and DNA, resulting in damage to cell function. Ascorbate reacts with all the above ROS, the product being monodehydroascorbate (MDHA). MDHA can be detected by electron paramagnetic resonance spectroscopy in intact leaves subjected to various stresses (Heber et al., 1996). MDHA disproportionates to form dehydroascorbate (DHA) and ascorbate. DHA is unstable at physiological pH so, to preserve the ascorbate pool, it is necessary to reduce MDHA and DHA back to ascorbate rapidly. Stabilization is achieved by three enzymes comprising the ascorbate-glutathione (GSH) cycle: monodehydroascorbate reductase, GSH-dependent dehydroascorbate reductase, and glutathione reductase. Note that reduction of DHA by GSH also proceeds rapidly at pH 8 (i.e., in the stroma of illuminated chloroplasts) without enzyme catalysis. Evidence for the GSH cycle has been reviewed recently (Smirnoff, 1995; Noctor and Foyer, 1998; Asada, 1999). The enzymes that regenerate ascorbate are covered further in Section V.A.2 and shown in Figure 3.

1. Ascorbate Peroxidase (APX)

Although ascorbate can quite readily reduce hydrogen peroxide nonenzymatically, plants also contain a range of APX isoenzymes (E.C. 1.11.1.11) that catalyze ascorbate-dependent reduction of hydrogen peroxide, producing water and MDHA (Asada, 1992). The utility of APX is illustrated by the effect of altering its expression in transgenic plants. For example, antisense reduction in activity of cytosolic APX increases the ozone sensitivity of tobacco (Orvar and Ellis, 1997) and expression of a cytosolic APX is increased by ozone exposure (Kubo et al., 1995). On the other hand, overexpression of chloroplastic APX, perhaps because chloroplasts are remote from the primary sites of ozone action, has no effect on ozone sensitivity (Torsethaugen et al., 1997). Overexpression of peroxisomal APX3 increases H_2O_2 tolerance (Wang et al., 1999). As pointed out by Loewus (1999), under alkaline conditions H_2O_2 causes cleavage of the ascorbate C-skeleton producing L-threonate and oxalate (see Section III). Perhaps APX minimizes this reaction and encourages MDHA formation; ascorbate is readily regenerated from MDHA (Figure 3; Section V.A.2). APX activity is found in a wide range of cellular locations, including the cytosol, chloroplasts (in the stroma and associated with the thylakoid membranes in the vicinity of PS1), mitochondria, glyoxysomes, and peroxisomes (inside and membrane associated). APX activity has been reported in the cell wall (Vanacker et al., 1998), but the low activities detected make it difficult to be certain that this is not caused by cytosolic contamination. Some of the above compartments, including the peroxisomes, glyoxysomes, and mitochondria, also contain catalase. However, this enzyme has a low affinity for hydrogen peroxide. It seems that the role of APX therefore could be to deal with hydrogen peroxide that leaks from hydrogen peroxide-producing organelles such as the peroxisomes and glyoxysomes. For example, its location on the peroxisome membrane and in the cytosol would enable it to catch escaping hydrogen peroxide. This might be particularly important when photosynthesis and photorespiration rates are high.

A number of plant AXP genes have been sequenced. They are all more closely related to each other and to yeast cytochrome *c* peroxidase than to other peroxidases (Asada, 1992; Ishikawa et al., 1998). APX and cytochrome *c* peroxidase

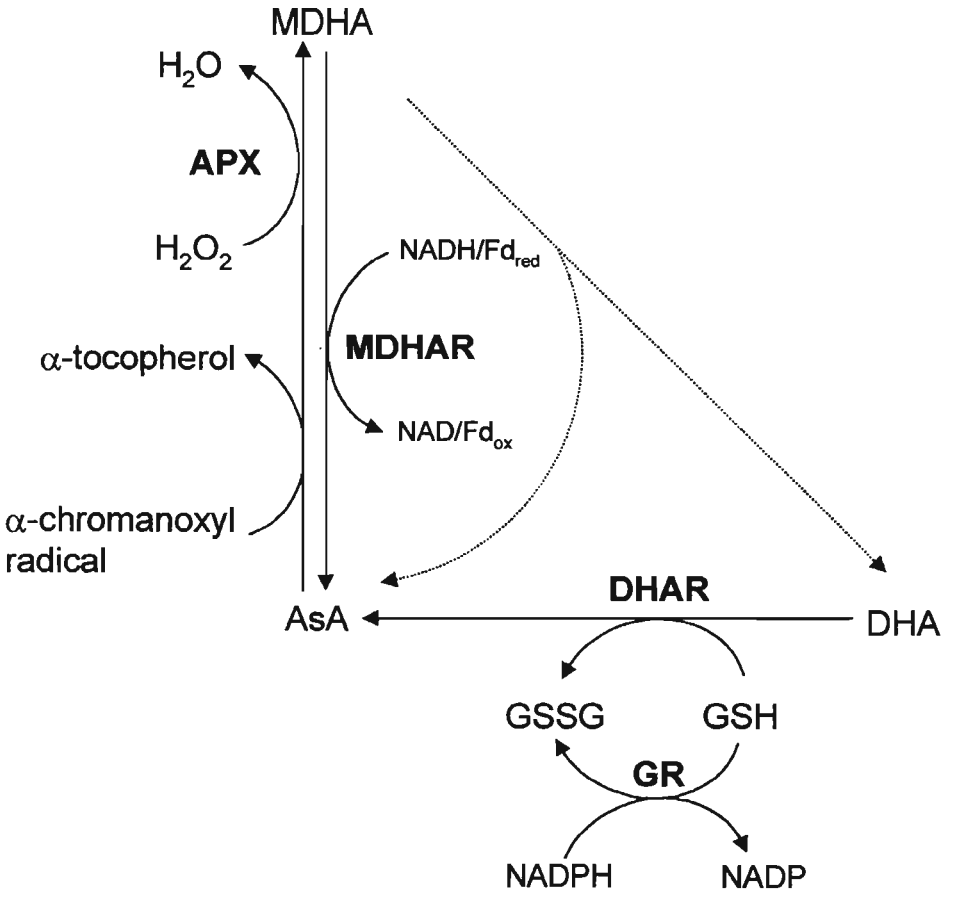

FIGURE 3. Ascorbate oxidation and regeneration from monodehydroascorbate (MDHA) and dehydroascorbate (DHA). Ascorbate scavenges hydrogen peroxide in a reaction catalyzed by ascorbate peroxidase (APX). APX isoforms occur in cytosol, chloroplasts mitochondria, and peroxisomes/glyoxysomes. Membrane-bound APXs occur in thylakoids and peroxisome/glyoxysome membranes. α-Tocopherol (vitamin E) is a membrane-associated antioxidant that scavenges lipid radicals. The product, α-chromanoxyl radical, is reduced to α-tocopherol by ascorbate. Ascorbate is also oxidized in its role as enzyme cofactor (Section V.C). Two molecules of monodehydroascorbate (MDHA), the primary oxidation product of ascorbate, disproportionate to dehydroascorbate (DHA) and ascorbate. MDHA is reduced to ascorbate by NAD(P)H-dependent MDHA reductase (MDHAR) or by reduced ferredoxin (Fd_{red}) in photosystem 1. DHA is reduced to ascorbate by glutathione (GSH)-dependent DHA reductase (DHAR). Oxidized glutathione (GSSG) is reduced to GSH by NADPH-dependent glutathione reductase (GR). The last two reactions comprise the ascorbate-glutathione cycle. The enzymes occur in the cytosol, cholroplast stroma, mitochondrial matrix, and in peroxisomes/glyoxysomes.

have been classed as class 1 peroxidases (Welinder, 1992; Ishikawa et al., 1998). A phylogenetic analysis of amino acid sequences produces a dendrogram that first separates stromal (sAPX) and thylakoid APXs (tAPX) from cytosolic APXs (cAPX) and peroxisomal/glyoxysomal (pAPX, gAPX) forms. Within the latter cluster, cAPX and gAPX form distinct groups (Ishikawa et al., 1998). cAPX consists of a small multigene family in *A. thaliana* (Santos et al., 1996). Spinach tAPX only differs from sAPX in having a hydrophobic C-terminal domain that allows association with the thylakoid membrane (Ishikawa et al., 1996; Yamaguchi et al., 1996). gAPX has a

similar sequence to cAPX but also has a hydrophobic C-terminus that could anchor it to the glyoxysome membrane (Ishikawa et al., 1998). gAPX is on the cytosolic side of the membrane in spinach and pumpkin (Yamaguchi et al., 1995; Ishikawa et al., 1998), but facing the matrix in cotton seed (Bunkelmann and Trelease, 1996). MDHA reductase is also associated with glyoxysome membranes (Bowditch and Donaldson, 1990; Bunkelmann and Trelease, 1996), where it would be well placed to regenerate ascorbate from MDHA.

APX activity is responsive to environmental conditions, particularly those that impose oxidative stress such as excess light, low temperature, and pollutants like ozone (Smirnoff, 1995; Noctor and Foyer, 1998). Also, as noted above, increased or decreased activity of specific APX genes in transgenic plants affects their sensitivity to oxidative stress. In *Arabidopsis thaliana* exposure of leaves to a higher light intensity than that experienced during growth causes a rapid and selective increase in transcripts of *APX1* and *2*, which encode cytosolic APXs (Karpinski et al., 1997). Excess light exposure is suggested to increase hydrogen peroxide formation either through increased oxygen photoreduction by PS1 by the Mehler reaction (Asada, 1999) or perhaps to an even greater extent by increased photorespiration rate and consequent H_2O_2 formation in peroxisomes (Foyer and Noctor, 1999). By using electron transport inhibitors acting before and after plastoquinone (Q_B/PQ) in the photosynthetic electron transport chain, Karpinski et al. (1997) suggested that this induction was associated with increased reduction of Q_B/PQ. The response is also associated with increased hydrogen peroxide, because catalaze infiltation, reduces *APX2* induction, while H_2O_2 feeding increases induction (Karpinski et al., 1997, 1999). Transcription of leaf cytosolic APX therefore could be controlled by a combination of redox signals from Q_B/PQ and from H_2O_2. Similarly, cytosolic APX transcripts are rapidly increased by H_2O_2 in rice embryos. Elevated H_2O_2 was achieved by direct feeding or by inhibition of catalase by aminotriazole (Morita et al., 1999). In transgenic *A. thaliana* expressing a fusion of the *APX2* promotor with a luciferase reporter gene, expression of the reporter can be visualized after excess light or H_2O_2 treatment. This also showed that expression is increased in other leaves on the same plant not directly exposed to excess light (Karpinski et al., 1999). Hydrogen peroxide, which readily diffuses across membranes, is a strong candidate for this mobile signal that can then induce systemic expression of APX in a similar way to systemic acquired resistance to local pathogen attack. This system should allow progress toward understanding the mechanisms behind signaling of oxidative stress. Another approach might be provided by an *A. thaliana* mutant (*pst1*) that has greatly increased tolerance to photooxidative bleaching caused by exposure to combined high NaCl concentration and high light. The mutant seedlings had higher superoxide dismutase activity and much higher APX activity than the wild type (Tsugane et al., 1999). The mutated gene, which is recessive, could be involved in signaling oxidative stress.

Pathogen attack elicits an oxidative burst by generation of hydrogen peroxide in the apoplast of cells near the infection (Bolwell, 1999). This leads to rapid cell death, preventing pathogen spread and may also provide H_2O_2 to act as a signal inducing local programmed cell death (PCD) and systemic resistance in remote tissues (Vanaker et al., 1998). Antisense suppression of catalase in tobacco results in expression of pathogenesis-related (PR) genes (Takahashi et al., 1997). Because H_2O_2 could be key to this mechanism, it is possible that local activity of catalase and APX should be controlled. Cytosolic APX transcripts increase in virus-infected tobacco. However, APX protein decreases as a result of inhibition of the elongation polypeptide on the ribosomes (Mittler et al., 1998, 1999). This illustrates initially the danger of equating increased transcript levels with increased enzyme activity and secondly suggests that suppression of peroxide scavenging by APX is important for pathogen defense. Both the cellular and subcellular localization of APX activity may be important in plant-pathogen interactions. In barley leaves infected with the biotrophic fungus *Blumeria graminis*, a resistant cultivar with a hypersensitive response had a small increase in apoplastic APX activity, while the susceptible host had a large increase

(Vanacker et al., 1998). These results should be viewed with caution because the proportion of total APX activity in the apoplast was about 1% and even though correction for leakage of cytoplasmic marker enzymes was made, this requires confirmation by other means. It has also been proposed that salicylic acid (SA), a signaling molecule involved in pathogen defense, can directly inhibit catalase, although its significance is disputed. However, an SA analogue benzothiadiazole can both induce tobacco mosaic virus resistance better than SA and is a more effective inhibitor of catalase and APX (Wendehenne et al., 1998).

The above evidence all points to APX having a key role in antioxidant and pathogen defense, with specialized and differentially regulated isoforms able to respond to light, oxidative stress, and pathogen attack at the transcriptional and translational levels.

2. Regeneration of Ascorbate from Monodehydroascorbate and Dehydroascorbate

Plants contain enzymes capable of reducing both the MDHA radical and DHA to ascorbate (Figure 3). MDHA reductase is an FAD-containing enzyme with a preference for NADH as reductant. It has isoforms in chloroplasts, cytosol, peroxisomes, and mitochondria (Asada, 1999; Deleonardis et al., 1995) and is also associated with the plasma membrane and glyoxysome membranes (Bowditch and Donaldson, 1990; Berczi and Moller, 1998). Increased activity of MDHA reductase occurs after exposure of plants to a number of oxidative stresses (Smirnoff, 1995). Increased expression of transcripts of a cytosolic MDHA reductase are induced by wounding (Grantz et al., 1995). MDHA is also reduced by a plasma membrane cytochrome *b* system (Section IV) and, in chloroplasts, by reduced ferredoxin (Miyaki and Asada, 1992; Asada, 1999). MDHA, which escapes these reducing systems disproportionates to DHA and ascorbate (Asada, 1999). Thioredoxin reductase is also able to reduce MDHA (May et al., 1998), although the significance is plants is not known.

DHA is reduced to ascorbate by a GSH-dependent DHA reductase. These enzymes have been purified from a number of plant tissues (Asada, 1999; Foyer and Mullineaux, 1998). However, a number of other proteins have DHA reductase activity, including Kunitz type trypsin inhibitors (Trumper et al., 1994), glutaredoxins (Wells et al., 1990), and protein disulfide isomerases (Wells et al., 1990). Therefore, it has been suggested that plants do not have specific GSH-dependent DHA reductase proteins (Morrell et al., 1997). This seems unlikely for the following reasons. GSH-dependent ascorbate reduction occurs in isolated chloroplasts (Smirnoff, 1995). Transgenic plants overexpressing glutathione reductase have higher ascorbate content (Foyer et al., 1995). Finally, a purified rice DHA reductase has a distinct N-terminal amino acid sequence (Kato et al., 1997; Foyer and Mullineaux, 1998). On balance, the evidence favors existence of GSH-dependent DHA reductase. However, this does not exclude a role for the other DHA-reducing enzymes. In mammalian cells, the ability to reduce DHA taken up from the external medium is not inhibited when their GSH content is depleted by treatment with buthionine sulfoximine, an inhibitor of GSH synthesis (Welch et al., 1995; May et al., 1998).

C. Ascorbate as an Enzyme Cofactor

A number of enzymes require ascorbate, at least *in vitro*, as a cofactor. Most of these are 2-oxoglutarate- and Fe(II)-dependent oxygenases in which ascorbate acts as the reductant to maintain the iron as Fe(II) (Prescott and John, 1996). Enzymes of this type in plants include prolyl hydroxylase, which catalyses posttranslational hydroxylation of prolyl residues, notably in the cell wall hydroxyproline-rich glycoproteins (HRGPs) (Sommer-Knudsen et al., 1998). Several enzymes of this type are involved in flavonoid and alkaloid biosynthesis, including anthocyanidin synthase, flavone 3-hydroxylase, flavonol synthase and flavone synthase 1, and alkaloid oxygenases. Two enzymes involved in hormone synthesis are also 2-oxoglutarate-dependent oxygenases: 1-aminocyclopropane-1-carboxylate (ACC) oxidase for ethylene synthesis

and gibberellin 20-oxidase. Ascorbate is also required for myrosinase activity (Prescott and John, 1996).

The HRGPs are predominantly extracellular proteins, including extensins, hydroxyproline-rich glycoproteins, and arabinogalactan-proteins (AGPs), which have as yet poorly defined roles in cell wall structure and growth (Sommer-Knudsen et al., 1998). Strong evidence that ascorbate is required as cofactor for the posttranslational hydroxylation of prolyl residues by prolyl hydroxylase is provided by the effect of 3,4-dehydro-D,L-proline (DP). This inhibits prolyl hydroxylase and results in decreased content of hydroxyproline in pea embryonic axes, carrot root, and potato tuber slices along with a large increase in ascorbate content (De Gara et al., 1991). This implies that the demand for ascorbate for proline hydroxylation is large because the pool is less oxidized when prolyl hydroxylase is inhibited. The effect might be particularly marked in meristems, and it is reported that DP treatment inhibits cell division and causes increased cell size (De Tullio et al., 1999). This could provide an explanation for the reported stimulation of cell division by ascorbate (previously reviewed by Smirnoff, 1996).

The xanthophyll cycle is an important photoprotective system in chloroplasts. When leaves are exposed to high light intensity part of the absorbed energy is reradiated as heat by the carotenoid zeaxanthin in the light-harvesting complex. This reduces photoinhibitory damage to photosystem 2 (Niyogi, 1999). Zeaxanthin is synthesized from the xanthophyll pigments antheraxanthin and violaxanthin by de-epoxidation reactions, catalyzed by violaxanthin de-epoxidase (VDE), which is located in the thylakoid lumen and requires ascorbate as a cofactor (Eskling et al., 1997). It is activated in high light intensity by the decrease in lumen pH that results from rapid photosynthetic electron transport. The xanthophyll cycle is completed by expoxidation of zeaxanthin to reform violaxanthin and antheraxanthin when leaves are returned to dim light. The enzyme requires ascorbate as reductant *in vitro* and prefers the undissociated ascorbic acid that predominates over the ascorbate anion at low pH [pK_a of ascorbic acid = 4.2] (Bratt et al., 1995; Eskling et al., 1997). Ascorbate supply in the chloroplast may limit the rate of zeaxanthin formation *in vivo* (Neubauer and Yamamoto, 1994; Forti et al., 1999). DHA formed by the de-epoxidation in the lumen must move to the stroma to be reduced back to ascorbate. However, no carriers on the thylakoid membrane have been reported (Foyer and Lelandais, 1996). Eskling et al. (1997) calculate that the concentration of ascorbic acid in the thylakoid lumen is 8 μM in the absence of a carrier, assuming 50 mM ascorbate in the stroma at pH 8 and a pK_a of 4.2 for ascorbate. This is much lower than the measured K_m of 100 μM for VDE. They propose that there must be thylakoid carriers to transport ascorbate in and DHA out. VDE has been purified and cloned (Bugos and Yamamoto, 1996; Rockholm and Yamamoto, 1996; Havir et al., 1997). Growth at high light increases both zeaxanthin and ascorbate content of leaves (Smirnoff and Pallanca, 1996; Grace and Logan, 1996; Logan et al., 1996; Eskling and Åkerland, 1998). It is possible that the light responsiveness of the ascorbate pool and the high concentration in the stroma is related to the need to maintain sufficient ascorbate for VDE activity in the thylakoid lumen.

D. Ascorbate Oxidase and Apoplastic Ascorbate: The Role of Ascorbate in Cell Growth

The frequently reported correlation between high ascorbate oxidase (AO) activity and rapid growth, which is particularly marked in, although not confined to, cucurbit fruits and its induction by auxin (Esaka et al., 1992), strongly suggests a role in growth. Ascorbate oxidase activity is very low in embryonic axes of pea seedlings during early germination, and a marked increase in activity just precedes initiation of embryonic axis growth (Pallanca and Smirnoff, 1999). Beyond these correlations, no major advances have been made in determining the role of AO in cell growth because this subject was reviewed several years ago (Smirnoff, 1996). AO is largely apoplastic and is secreted by cells in suspension culture (Esaka et al., 1989). It has been cloned from various cucurbits (Ohkawa et al., 1989; Esaka et al.,

1990; Diallinas et al., 1997; Kisu et al., 1997) and tobacco (Kato and Esaka, 1996). The genes all encode signal sequences specifying secretion. Melon AO is encoded by a multigene family (Diallinas et al., 1997) and the pumpkin gene has an auxin response element (Kisu et al., 1997).

The simplest hypothesis to explain the role of AO in cell growth is that a product of ascorbate oxidation, MDHA or DHA, directly affects cell expansion (Smirnoff, 1996). MDHA stimulates growth and cell expansion in onion roots (Hidalgo et al., 1989; Gonzales-Reyes, 1994, 1995). This is proposed to result from increased transmembrane electron transport using MDHA as electron acceptor via the cytochrome *b* system (Section IV). This depolarizes the plasma membrane and activates the H^+-ATPase resulting in increased ion uptake and/or increased activity of cell wall loosening enzymes. This hypothesis will need further testing, and it is likely that altered expression of AO in transgenic plants will soon reveal more about its function.

Other interactions between ascorbate and the cell wall are possible independently of AO. Ascorbate may inhibit wall peroxidases by scavenging hydrogen peroxide and by direct inhibition. Inhibition of peroxidase activity decreases peroxidative cross-linking of wall polymers, resulting in a more extensible wall (Cordoba-Pedregosa et al., 1996). Fry (1998) has suggested that a combination of ascorbate and traces of transition metals such as Fe and Cu in cell walls generate hydroxyl radicals ($OH^{\cdot}$), which then cause scission of glycosidic bonds of wall polysaccharides. This was shown to occur *in vitro,* but its relevance *in vivo* is not known.

VI. WHERE NEXT?

This review has demonstrated that ascorbate probably has roles in a number of cellular processes from growth to protection against oxidative stress. However, there is still much to learn about all aspects of ascorbate biosynthesis, catabolism, and function. Recent work has finally elucidated a biosynthetic pathway and its implications for the control of ascorbate synthesis, particularly in relation to light and cell wall biosynthesis, can now be addressed. A DHA transporter has been identified and the next stage is to purify and clone it. The significance of transmembrane electron transport via cytochrome *b* using ascorbate and MDHA as electron donor and acceptor needs further investigation. Progress on ascorbate catabolism and on the longstanding mystery of the function of ascorbate oxidase should be made in the next few years. It is becoming clear that glutathione plays an important role in antioxidative defense and redox-based signaling mechanisms, for example, in photoprotection and pathogen defense (Noctor and Foyer, 1998; Karpinski et al., 1999). Therefore, it is likely that ascorbate, whose redox state is often closely linked to glutathione, has a similar role in addition to those roles that are unique to its own chemistry. Ascorbate-deficient mutants are now available (Conklin et al., 2000), and transgenic plants with altered expression of biosynthetic genes will soon be produced. These developments will enable production of plants with reduced or increased ascorbate content, which will be useful to probe the functions of this simple but multifacetted molecule, perhaps even revealing functions that are completely unsuspected.

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