Ascorbic acid: metabolism and functions of a multi-facetted molecule

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Ascorbic acid (vitamin C) is the most abundant antioxidant in plants. Its biosynthetic pathway *via* GDP–D-mannose and L-galactose, which was proposed only recently, is now supported by molecular genetic evidence from *Arabidopsis thaliana* and transgenic potato plants. Except for the last step (which is located on the inner mitochondrial membrane) the pathway is cytosolic, sharing GDP–sugar intermediates with cell-wall polysaccharide and glycoprotein synthesis. Ascorbate peroxidase is emerging as a key enzyme in the fine control of H_2O_2 concentration; its expression being controlled by redox signals and H_2O_2. Convincing evidence of the involvement of ascorbate in cell division and growth is also accumulating. Its role as a cofactor in the synthesis of cell wall hydroxyproline-rich glycoproteins is one mechanism for this function.

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Abbreviations

AO	ascorbate oxidase
APX	ascorbate peroxidase
GAL	L-galactono-1,4-lactone
GDP	guanosine diphosphate
GSH	glutathione
GUL	L-gulono-1,4-lactone
HL	high light
HRGP	hydroxyproline-rich glycoprotein
LL	low light
MDHA	monodehydroascorbate
PMI	phosphomannose isomerase
vtc	*vitamin C*

Introduction

Longevity in invertebrates and mice is associated with resistance to oxidative stress, and with mutations that prevent programmed cell death in response to such stress [1]. As a result, there is great public interest in the health-promoting effects of antioxidants, particularly α-tocopherol (vitamin E) [2] and ascorbic acid (vitamin C), which are required in the diet. Plants are the major sources of these vitamins and of other 'nutriceutical' antioxidants such as flavonoids and carotenoids. In plants, antioxidants provide protection against reactive oxygen species created both metabolically and in the environment; although the modular growth pattern of plants (with the exception of seeds) makes the issue of life span less relevant for plants than for animals. It is also becoming apparent that the most prevalent soluble small-molecule antioxidants, ascorbate and glutathione (GSH) [3], are multifunctional and also have roles in photosynthesis, redox signalling, pathogen defence, metal and xenobiotic detoxification, and growth regulation.

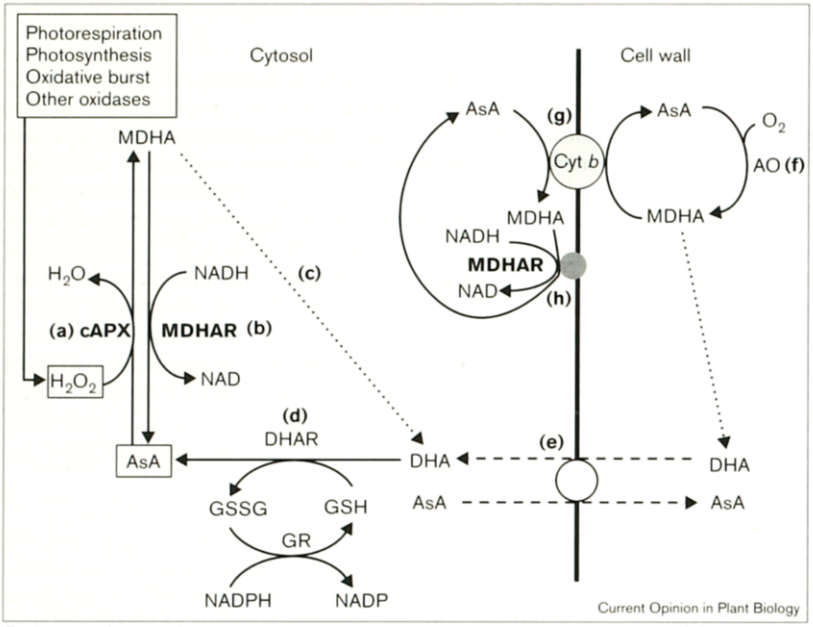
Ascorbate is quantitatively the predominant antioxidant in plant cells, it is found in all subcellular compartments, including the apoplast, and has an average cellular concentration of 2–25 mM or more in the chloroplast stroma. Ascorbate is oxidised by oxygen, superoxide, singlet oxygen and H_2O_2 to the monodehydroascorbate (MDHA) radical [4–6], which disproportionates to form ascorbate and dehydroascorbate. The enzymes and processes involved in ascorbate redox reactions and transport are shown in Figure 1 [7–9].

Interest in ascorbate has increased in recent years and this review covers advances in our understanding of its metabolism and functions. The evidence for a recently-proposed biosynthesis pathway is assessed and its link with cell-wall synthesis is emphasised. Strong evidence is emerging that, in some species, ascorbate is the major precursor of oxalate and could be linked to calcium homeostasis. Ascorbate peroxidase (APX) mediates the scavenging of H_2O_2 by ascorbate. It is apparent that APX has numerous isoforms in various cell compartments that are differentially responsive to redox status and that control H_2O_2 concentration. The role of ascorbate in protecting plants against oxidative stress is also highlighted by the hypersensitivity of the ascorbate-deficient *vtc* (*vitamin C*) mutants of *Arabidopsis thaliana*. Finally, the proposed roles of ascorbate in cell growth and cell division are assessed.

Ascorbate and cell wall polysaccharide synthesis compete for GDP–mannose

A biosynthetic pathway for ascorbate in plants, which is consistent with all of the available evidence, was proposed recently [10,11,12•]. Ascorbate is synthesised from guanosine diphosphate (GDP)–mannose and the pathway shares GDP–sugar intermediates with the synthesis of cell-wall polysaccharides and those glycoproteins that contain D-mannose, L-fucose and L-galactose (Figure 2). The biochemical evidence of the involvement of GDP–mannose pyrophosphorylase is supported by molecular-genetic analysis of the ascorbate-deficient *vtc1 Arabidopsis thaliana* mutant and transgenic potato plants. The *VTC1* gene encodes GDP–mannose pyrophosphorylase [13,14••]. When *vtc1* plants were transformed with the wild-type version of the *VTC1* gene, foliar ascorbate concentration was restored to the wild-type level. In potatoes, antisense suppression of GDP–mannose pyrophosphorylase activity reduced ascorbate content and a close correlation between this activity and ascorbate pool size suggests that this enzyme exerts significant control over pathway flux [15••].

Figure 1



Redox reactions and transport of ascorbate (AsA). For clarity, only reactions in the cytosol and transport across the plasma membrane are shown. **(a)** Hydrogen peroxide reduction by ascorbate is catalysed by cytosolic ascorbate peroxidase (cAPX). **(b)** The oxidation product, monodehyroascorbate radical (MDHA), is reduced to ascorbate by NAD(P)-dependent MDHA reductase (MDHAR). **(c)** Two MDHA molecules can also disproportionate to dehydroascorbate (DHA) and ascorbate (represented non-stoichiometrically by a dotted line). **(d)** DHA is reduced to ascorbate by glutathione (GSH)-dependent glutathione reductase (DHAR), which is, in turn, regenerated from its oxidised form (GSSG) by glutathione reductase (GR). The net result is H_2O_2 removal by the ascorbate–GSH cycle at the expense of NAD(P)H [4,5]. These reactions also occur in the chloroplast stroma, mitochondria and peroxisomes/glyoxysomes. APX is encoded by a gene family with distinct isoforms localised in different organelles; forms with hydrophobic tails are bound to thylakoid and peroxisomal/glyoxysomal membranes [5,28]. A small proportion of APX activity is also reported in the apoplast [39]. There has been controversy over the existence of DHAR; other enzymes (e.g. glutaredoxin, thioredoxin reductase or peroxiredoxin) could reduce DHA but the evidence supports a role for a specific GSH-dependent DHAR [4]. AsA and DHA occur in the cell wall. **(e)** AsA is transported into the wall by facilitated diffusion *via* a plasma membrane transporter in exchange for DHA [8]. **(f)** Ascorbate oxidase (AO) is a secreted glycoprotein that catalyses ascorbate oxidation in the wall. **(g)** The resulting MDHA is probably reduced by a plasma-membrane cytochrome *b* system [7]. It is likely that cytosolic ascorbate is the electron donor and that **(h)** a plasma-membrane-bound MDHAR on the cytosolic side [9] regenerates ascorbate. AO expression correlates with cell expansion although a causal relationship although its mechanism of action has not been established.

In some cases, the antisense suppression resulted in a reduction of mannose and galactose, but not of fucose, residues in wall polysaccharides. Incorporation of these sugar residues into glycoproteins was, however, unaffected ([15••]; GL Wheeler, PL Conklin, N Smirnoff, unpublished data). It has been suggested that most plant species lack phosphomannose mutase (PMI) activity and this accounts for the toxicity of exogenous mannose, which accumulates as mannose-6-phosphate [16,17]. Lack of PMI would necessitate an alternative route to GDP–mannose such as a GDP–glucose-2-epimerase. Nevertheless, the *vtc1* mutation and antisense potato results argue for a role for PMI.

GDP–L-galactose is produced by a double epimerisation of GDP–D-mannose [7]. The enzymes that break GDP–L-galactose down to free L-galactose have been detected ([7]; GL Wheeler, N Smirnoff, unpublished data) but have not yet been characterised in detail. A newly-discovered NAD^+-dependent L-galactose dehydrogenase oxidises L-galactose to L-galactono-1,4-lactone (GAL), the immediate ascorbate precursor [10,11]. This enzyme, which we have purified and cloned (GL Wheeler, S Gatzek, N Smirnoff, unpublished data) is specific for L-galactose and is, as far as we know, the only plant dehydrogenase acting on a non-phosphorylated sugar. The pathway prior to GAL is probably cytosolic but the last step, the oxidation of GAL to ascorbate by GAL dehydrogenase, is mitochondrial. GAL dehydrogenase has been purified and cloned from cauliflower and sweet potato; the genes that encode it in each of these two plant species are nearly identical and include a putative mitochondrial targeting sequence [18,19]. GAL dehydrogenase is bound to the inner membrane of the mitochondria [20•]. GAL donates electrons to cytochrome *c* between complexes III and IV, and the active site probably faces the intermembrane space (CH Foyer, personal communication) so that GAL does not need to be transported into the mitochondrial matrix.

The current evidence suggests that the mannose pathway predominates in the biosynthesis of ascorbate in plants, but we cannot rule out contributions from other pathways. Conversion of radiolabelled (methyl)-D-galacturonate and D-glucuronolactone to ascorbate was noted by Loewus and colleagues some time ago [12•], and the involvement of these compounds has been confirmed by the increased ascorbate pool found after feeding methyl-D-galacturonate, D-glucuronolactone and methyl-D-glucuronate to *A. thaliana* cell cultures [21•]. The physiological significance of these conversions requires further investigation.

L-gulono-1,4-lactone (GUL), the precursor used by mammals in place of GAL, is slowly converted to ascorbate by plants. A separate GUL-oxidising enzyme could exist as purified GAL dehydrogenase is completely specific for GAL. It is now thought that the osone pathway [12•] of ascorbate biosynthesis is not physiologically important [22•].

The recent advances in identifying the ascorbate biosynthetic pathway will allow investigation of its control, most importantly in relation to light intensity. Ascorbate accumulation is increased at high light intensity [23–25], a response which presumably reflects its use in H_2O_2 detoxification (described below), regeneration of oxidised α-tocopherol [6] and as a cofactor for violaxanthin de-epoxidase [25]. Ascorbate concentration decreases in darkened leaves of some species, and this is partially reversed by sucrose or glucose feeding [23]. Synthesis of ascorbate is repressed or feedback-inhibited in pea seedling embryonic axes after their ascorbate content has been increased by feeding with ascorbate [26•].

Oxalate and tartrate are synthesised from ascorbate

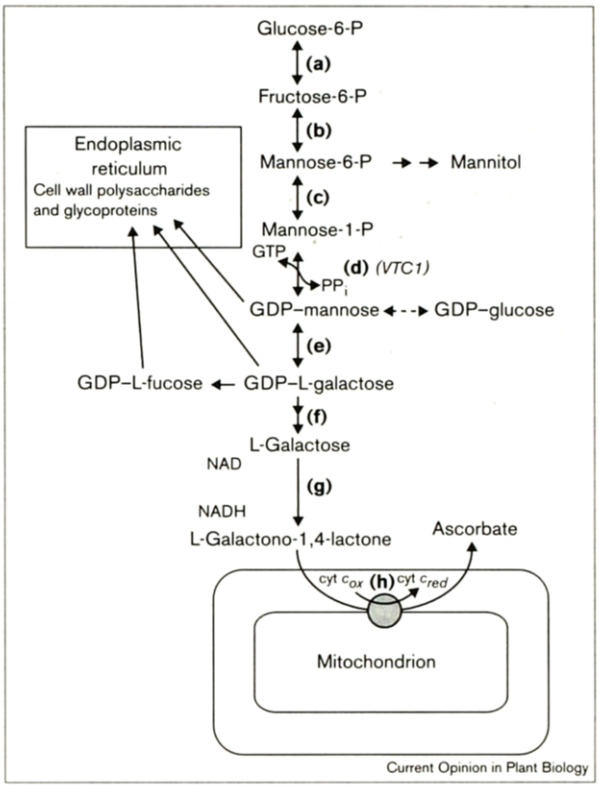
Ascorbate is cleaved at C2/C3 to form oxalate and at either C2/C3 or C4/C5, depending on species, to produce L-tartrate [12•]. Oxalate can also be formed from glycolate. In *Pistia stratiotes*, however, labelling studies show that oxalate is predominantly derived from ascorbate rather than glycolate [27••], and calcium oxalate crystals are accumulated in specialised cells (i.e. idioblasts). Calcium oxalate synthesis could therefore be involved in the regulation of calcium concentration. The enzyme catalysing C2/C3 cleavage has not been identified but idioblast cells would be a good place to start searching for it.

Ascorbate peroxidase is a key enzyme for controlling H_2O_2 concentration

APX catalyses the reduction of H_2O_2 to water and has high specificity and affinity for ascorbate as reductant [5]. Its sequence is distinct from other peroxidases, and different forms of APX occur in the chloroplasts, cytosol, mitochondria, peroxisomes and glyoxysomes. Membrane-bound APXs occur on the peroxisome and thylakoid membranes [5,28]. Hydrogen peroxide is formed by oxygen reduction by the chloroplast and mitochondrial electron transport chains; by certain oxidases, notably peroxisomal glycolate oxidase, during photorespiration; and during the oxidative burst associated with the hypersensitive response to pathogens. Oxygen reduction by Photosystem I, coupled with removal of the resulting H_2O_2 by APX, is termed the Mehler-peroxidase reaction and contributes to the regulation of the redox state of photosynthetic electron carriers [5,29]. Recent work on the response of APX expression to intense light and pathogen attack has highlighted the importance of APX activity in controlling H_2O_2 concentration in signalling.

Photoinhibition and photo-oxidation are caused when low light (LL) acclimated leaves are exposed to high light (HL) intensity. Acclimation involves the induction of antioxidant and photoprotective (e.g. zeaxanthin-dependent non-photochemical quenching) systems [5,24,29,30]. HL causes increased H_2O_2 formation in chloroplasts, either from the Mehler reaction or Photosystem II, and in

Figure 2



The proposed biosynthetic pathway of ascorbate via GDP–mannose and L-galactose [10]. The diagram illustrates links with other pathways, including polysaccharide and glycoprotein synthesis from GDP–sugars. Mannitol is a major carbon translocation compound in some species. GDP–mannose could be formed from GDP–glucose, although lower ascorbate concentration in plants with reduced GDP–mannose pyrophosphorylase activity [14••,15••], suggests that this is not a major route. Reduced GDP–mannose pyrophosphorylase activity also lowers the mannose and L-galactose content of cell-wall polysaccharides. The final oxidation of L-galactono-1,4-lactone to ascorbate on the inner mitochondrial membrane [20•] uses cytochrome *c* as an electron acceptor and is therefore coupled to mitochondrial electron transport. Ascorbate and L-galactono-1,4-lactone presumably enter and leave the intermembrane space freely through porins in the outer membrane. Ascorbate biosynthesis is one of many biosynthetic functions that takes place in plant, but not in animal mitochondria. In contrast, the final step of ascorbate biosynthesis in mammals is catalysed by a microsomal oxidase. Alternative pathways via D-galacturonate and D-glucuronate, or their lactones or esters [21•], are not illustrated and their physiological significance has not yet been established. The enzymes are: **(a)** glucose phosphate isomerase; **(b)** phosphomannose isomerase; **(c)** phosphomannose mutase; **(d)** GDP–mannose pyrophosphorylase; **(e)** GDP–mannose-3,5-epimerase; **(f)** uncharacterised enzymes; **(g)** L-galactose dehydrogenase; and **(h)** L-galactono-1,4-lactone dehydrogenase.

peroxisomes as a result of faster photorespiration. Exposure of *A. thaliana* leaves to HL causes a rapid (i.e. within 30 min) increase in the transcription of *APX1* and *APX2*, which encode two isoforms of cytosolic APX [31]. The promotors for *APX1* and *APX2* have been isolated and fusions with a luciferase reporter gene have been made. The transformants were then used to investigate the pattern of *APX2* expression and the signalling processes that control it [32••]. *APX* induction is correlated to increased reduction-state of quinone B and plastoquinone. Thus, *APX* expression is regulated in same way as has been proposed for light-intensity-dependent expression of other photosynthetic genes [33]. The expression of both antioxidant and photosynthetic genes is therefore co-ordinated through a common redox-signalling system.

Hydrogen peroxide itself is also involved in *APX* induction: exogenous application of H_2O_2 increases *APX* expression in LL, whereas catalase infiltration inhibits the expression of this gene in HL [32••]. Hydrogen peroxide also induces cytosolic APX expression in non-photosynthetic tissue [34••]. Induction of APX by its own substrate may provide a sensitive means of controlling its concentration (assuming that the enzyme is active). This system has also provided evidence of a systemic plant response to HL, possibly mediated by H_2O_2, because leaves remote from the one exposed to HL showed an increase in *APX2::LUCIFERASE* expression and a small increase in H_2O_2 concentration. Because the *APX2* promoter has an extremely sensitive response, it will prove to be a valuable tool for identifying the promotor DNA sequences and DNA binding proteins involved in responses to H_2O_2 or other redox signals. Further progress towards this goal might be made with the use of a recessive *A. thaliana* mutant that has increased tolerance to salinity-induced photo-oxidation [35••]. The mutant has APX and superoxide dismutase activities that are above wild-type levels, particularly after exposure to salt. It is possible that the mutation affects a gene that regulates the expression of antioxidant genes.

Hydrogen peroxide is produced during pathogen attack, and it could provide a direct defence against the pathogen and contribute to programmed cell death in the hypersensitive response. These responses are localised and therefore more difficult to investigate than the leaf responses to HL described above. Antisense suppression of catalase in tobacco results in the expression of pathogenesis-related genes [36]. If H_2O_2 is the key to this mechanism, local activity of catalase and APX should be restricted during pathogen attack. Cytosolic APX transcripts increase in virus-infected tobacco but APX protein concentrations actually decrease as a result of the inhibition of polypeptide elongation on the ribosomes [37,38••]. These findings illustrate the danger of equating increased transcript levels to increased enzyme activity, and suggest that suppression of peroxide scavenging by APX is important for defence against pathogens [39]. The inhibition of catalase and APX by salicylic acid, which is produced in response to infection, might also contribute to local suppression of H_2O_2 scavenging [40].

Vtc mutants and transgenic plants provide evidence of the role of ascorbate in stress resistance

Various stresses, including UV-B, increase the prevalence of MDHA radicals [41]. The *A. thaliana vtc1* mutant has only 30% of wild-type ascorbate [42], which is sufficient for normal growth under non-stressful conditions. A similar reduction in ascorbate concentrations in potatoes expressing antisense GDP–mannose pyrophosphorylase, however, causes visible lesions on leaves and stems, and premature die-back of the shoot [15••]. It is not known if these symptoms are caused by ascorbate deficiency or by defects in the cell wall. *Vtc1* is hypersensitive to ozone, ultraviolet-B light and SO_2, but its ozone sensitivity is reversed by exogenous ascorbate [42]. Plants kept in the dark for a prolonged period are known to be more ozone sensitive, and it has been suggested that this is a result of reduced apoplastic ascorbate [43]. Use of an apoplast-localised dye, whose fluorescence is abolished by oxidation, however, suggests that apoplastic ascorbate is not the major reductant of ozone in the apoplast [44]. Nevertheless, cytosolic ascorbate may be important because antisense reduction of cytosolic APX activity increases the ozone sensitivity of tobacco [45]. Presumably the induction of cytosolic APX (described above) [31,32••] improves the scavenging of H_2O_2 that escapes from the chloroplasts and peroxisomes after evading chloroplast APX and peroxisomal catalase. Furthermore, over-expression of peroxisomal APX3 increases H_2O_2 tolerance [46••]. Ascorbate is probably a limiting factor in the violaxanthin de-epoxidase reaction in the thylakoid lumen [47]; it will therefore be of interest to determine if the reduced ascorbate content of *vtc* mutants affects their zeaxanthin-mediated photoprotection [30].

Ascorbate oxidase and prolyl hydroxylase: roles for ascorbate in growth?

Ascorbate oxidase (AO) is a cell wall localised glycoprotein belonging to the family of blue copper oxidase enzymes. Its role in plants has not been defined but its activity and expression are closely correlated to rapid cell expansion [48•] and it is induced by auxin [49]. A causal relationship that might explain this correlation has not yet been demonstrated and no mechanism of action has yet been established [6]. One possibility is that AO generates MDHA radicals. These radicals are then reduced by transmembrane electron transport (Figure 1) thereby depolarising the plasma membrane and stimulating H^+-ATPase activity. In this model, cell expansion is stimulated by enhanced wall loosening or ion uptake: exogenous MDHA radicals stimulate onion root growth and ion uptake [50,51]. It is likely that transgenic plants with altered AO expression will soon be used to clarify the function of AO. Another role for ascorbate in growth is an involvement in the synthesis of hydroxyproline-rich glycoproteins (HRGPs). Ascorbate is a cofactor for prolyl hydroxylase (as it is for a

range of other oxygenases [6]), which post-translationally hydroxylates proline residues. The HRGPs are cell wall structural proteins and are involved in wall synthesis in dividing cells [52]. Prolyl hydroxylase is inhibited by 3,4-DL-dehydroproline causing increased ascorbate concentration and reduced cell division in onion roots [53,54•]. These results show that HRGP synthesis in meristems is a function of ascorbate and could provide an explanation for the reported effects of ascorbate on cell division.

Conclusions and future developments

Within the past few years knowledge of ascorbate metabolism and function in plants has greatly increased. The identification of the ascorbate biosynthetic pathway will allow us to manipulate the ascorbate content of plants, as has already been achieved with its fellow antioxidants GSH [3,4] and α-tocopherol [2]. This should provide further information on the functions of ascorbate, including those involved in photosynthesis, stress resistance, growth, development and oxidative-stress signalling, that have been identified in my review. Metabolic engineering of ascorbate biosynthesis to produce plants with increased ascorbate concentration will not only provide crops with improved nutritional value, but may well improve their growth and stress resistance. Ascorbate does not act in isolation, so it will be exciting to see the effect of engineering ascorbate biosynthesis along with GSH, α-tocopherol, carotenoids and other antioxidant enzymes; this goal is now within reach.

Update

A recent paper by Conklin *et al.* [55] describes the identification and mapping of three ascorbate-deficient mutants (*vtc2*, *3* and *4*) in addition to the previously characterised *vtc1* [13,14••]. These mutants contain between 20–30% of wild-type ascorbate. Not all of the mutant alleles produce phenotypes that are hypersensitive to ozone, suggesting that the relationship between ascorbate content and ozone resistance [42–45] is not predictable. These mutants should prove useful for identifying further genes involved in ascorbate metabolism and exploring the role of ascorbate in resistance to oxidative stress.

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- •• of outstanding interest

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This paper, along with [15••], provides genetic evidence of the proposed [10] involvement of GDP-mannose in ascorbate biosynthesis. The enzyme catalyses the formation of GDP-mannose from mannose-1-phosphate and GTP. The gene encoding the *vtc1* mutation of *A. thaliana*, which causes ascorbate deficiency, was cloned and found to have homology with GDP-mannose pyrophosphorylase. *Vtc1* leaves had lower GDP-mannose pyrophosphorylase activity than the wild-type, and expression of wild-type *VTC1* in the mutant plants restored ascorbate concentration. Reduced GDP-mannose pyrophosphorylase activity is caused by a point mutation that reduces enzyme-specific activity; transcript levels are unaffected in the mutant.

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This paper, along with [14••], provides genetic evidence of the involvement of GDP-mannose in ascorbate biosynthesis. GDP-mannose pyrophosphorylase was cloned from potato using an *A. thaliana* expressed sequence tag. Antisense expression of this gene in potato resulted in plants with reduced ascorbate concentration. GDP-mannose pyrophosphorylase appears to control ascorbate biosynthesis pathway flux because there is a close correlation between reduction in activity of this enzyme and ascorbate content. The mannose and galactose content of cell wall polysaccharides was also reduced in leaves, as would be expected if GDP-mannose and GDP-L-galactose are precursors of hemicelluloses. The antisense plants also showed necrotic leaf and stem lesions, and accelerated leaf senescence. It is not known if these symptoms are caused by ascorbate deficiency or altered cell wall composition.

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L-galactono-1,4-lactone dehydrogenase is the last enzyme in the ascorbate biosynthesis pathway and its association with the mitochondria has been recognised for some time. This paper uses purified *Phaseolus vulgaris* mitochondria to show that the enzyme is located in the inner mitochondrial membrane. This finding raises the possibility that ascorbate biosynthesis could be controlled through a link to mitochondrial, and therefore chloroplastic, metabolism.

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This paper confirms that L-galactose and L-galactono-1,4-lactone, the proposed immediate precursors of ascorbate [10], are readily converted to ascorbate by *A. thaliana* cell suspensions. In addition, a number of uronic acid derivatives (i.e. methyl-D-galacturonate, D-glucuronolactone and methyl-D-glucuronate) that have previously been shown to be incorporated into ascorbate at radiotracer levels [12•] cause small but detectable increases in ascorbate. The results suggest that alternative pathways involving uronic acid intermediates could make a small contribution to ascorbate synthesis.

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This paper assesses the importance of the osone pathway of ascorbate synthesis [12•] in which D-glucosone and L-sorbosone are proposed as ascorbate precursors. Isotope-dilution experiments show that sorbosone is not involved in ascorbate synthesis. No evidence for glucosone synthesis was found and exogenous glucosone was toxic to pea seedlings. There was a close relationship between the appearance of ascorbate and ascorbate oxidase activity; both are almost absent until 20 h after imbibing the seeds, and the start of embryonic axis growth. These results are further circumstantial evidence of the role of ascorbate and ascorbate oxidase in cell growth.

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The authors show that ascorbate synthesis from ^{14}C-glucose is strongly inhibited by pre-loading pea seedlings with ascorbate. This effect is seen within 3 h of ascorbate loading and provides evidence that ascorbate synthesis is controlled by feedback inhibition. The enzymes subject to feedback regulation must now be identified; this will contribute towards optimising the metabolic engineering of ascorbate biosynthesis. Ascorbate loading increased the rate of ascorbate turnover, determined by metabolism of ^{14}C-ascorbate. The rate of ascorbate turnover is directly proportional to its pool size.

27. •• Keates SE, Tarlyn NM, Loewus FA, Franceschi VR: **L-galactose: source of oxalic acid in calcium oxalate deposition in *Pistia stratiotes*.** *Phytochem* 2000, in press.

Pulse-chase experiments with ^{14}C- ascorbate, L-galactose and glycolate show that ascorbate, not glycolate, is the major source of oxalate in *Pistia stratiotes*. This floating aquatic plant stores calcium oxalate in specialised cells (idioblasts) as a result of stimulation of ascorbate and oxalate synthesis by calcium. Also, contrary to the usual supposition, there is appreciable turnover of ^{14}C-oxalate, showing it is not a dead-end product.

28. Ishikawa T, Yoshimura K, Sakai K, Tamoi M, Takeda T, Shigeoka S: **Molecular characterization and physiological role of a glyoxysome-bound ascorbate peroxidase from spinach.** *Plant Cell Physiol* 1998, **39**:23-34.

29. Foyer CH, Noctor G: **Leaves in the dark see in the light.** *Science* 1999, **284**:599-601.

30. Niyogi KK: **Photoprotection revisited: genetic and molecular approaches.** *Ann Rev Plant Physiol Plant Mol Biol* 1999, **50**:333-359.

31. Karpinski S, Escobar C, Karpinska B, Creissen G, Mullineaux PM: **Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in *Arabidopsis* during excess light.** *Plant Cell* 1997, **9**:627-640.

32. •• Karpinski S, Reynolds H, Karpinska B, Wingsle G, Criessen G, Mullineaux P: **Systemic signalling and acclimation in response to excess excitation energy in *Arabidopsis*.** *Science* 1999, **284**:654-657.

Leaves exposed to high light intensity show rapid increase in expression of cytosolic ascorbate peroxidase as part of a defence against H_2O_2, which is generated by the Mehler reaction and photorespiration [29,31]. The transcripts of one ascorbate peroxidase, *APX2*, are not expressed in low light but increase within 30 min of exposure to high light. *A. thaliana* was transformed with a fusion of the *APX2* promoter and luciferase. This fusion was expressed in the same way as the native gene, allowing luciferase activity to report *APX2* expression in intact leaves exposed to high light. It also provides a sensitive assay for *APX2* expression in leaf extracts. This system was used to show that manipulation of the redox state of the photosystem II electron acceptors quinone B and plastoquinone, using the electron transport inhibitors 3-(3,4-dichlorophenol)-1,1-dimethylurea and 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB), alters *APX2* expression. Increased reduction, as occurs in high light or in the presence of DBMIB, increases *APX2* expression. Antioxidant defences are therefore coupled to photosynthesis and use a similar redox-sensing system as photosynthetic enzymes [33]. *APX2* expression is also increased by its own substrate, ascorbate; the high light response is reduced by infiltrating leaves with catalase. This paper demonstrates a novel systemic response in which exposure of one leaf to high light induces *APX2* expression and reduces photoinhibition in untreated leaves. This phenomenon was named 'systemic acquired acclimation' and H_2O_2 is suggested as a component of the signalling system.

33. Pfannschmidt T, Nilsson A, Allen JF: **Photosynthetic control of chloroplast gene expression.** *Nature* 1999, **397**:625-628.

34. •• Morita S, Kaminaka H, Masumura T, Tanaka K: **Induction of rice cytosolic ascorbate peroxidase mRNA by oxidative stress; the involvement of hydrogen peroxide in oxidative stress signalling.** *Plant Cell Physiol* 1999, **40**:417-422.

Cytosolic APX expression in cultured rice embryos is controlled by H_2O_2. Both H_2O_2 and paraquat are shown to elevate endogenous peroxide and increase APX transcripts within 2 h in a dose-dependent manner. Paraquat generates peroxide via superoxide; inhibition of superoxide dismutase by N,N-diethyldithiocarbamate prevented APX induction showing that superoxide is not involved. Catalase and APX inhibitors (i.e. aminotriazole and hydroxyurea) also induced APX transcripts and increased endogenous H_2O_2. This paper, along with [32••], now provides convincing evidence that cytosolic APX gene expression is controlled by H_2O_2. It should now be possible to uncover the signal transduction systems involved in H_2O_2 signalling. As a cautionary note, this paper and [32••] do not show that increased APX transcript level is associated with more protein or enzyme activity; in some circumstances [37] this is not the case.

35. •• Tsugane K, Kobayashi K, Niwa Y, Ohba Y, Wada K, Kobayshi H: **A recessive *Arabidopsis* mutant that grows photoautotrophically under salt stress shows enhanced active oxygen detoxification.** *Plant Cell* 1999, **11**:1195-1206.

An *A. thaliana* mutant that could be affected in a signalling pathway for oxidative stress is identified. The recessive mutant (*pst1*) is more resistant to salt stress in high light. This treatment causes photo-oxidation, eventually resulting in bleaching of the cotyledons. APX and superoxide dismutase activity are induced by salt treatment. These enzymes have higher activity in the mutant and, notably in the case of APX, the salt-induced increase in activity is greater in the mutant than in wild-type plants. The results, although preliminary, indicate that the gene encoding this mutation could be involved in signalling oxidative stress.

36. Takahashi H, Chen Z, Du H, Liu Y, Klessig DF: **Development of necrosis and activation of disease resistance in tobacco plants with severely reduced catalase levels.** *Plant J* 1997, **11**:993-1005.

37. Mittler R, Feng XQ, Cohen M: **Post-transcriptional suppression of cytosolic ascorbate peroxidase expression during pathogen-induced programmed cell death in tobacco.** *Plant Cell* 1998, **10**:461-473.

38. •• Mittler R, Lam E, Shulaev V, Cohen M: **Signals controlling the expression of cytosolic ascorbate peroxidase during pathogen-induced programmed cell death in tobacco.** *Plant Mol Biol* 1999, **39**:1025-1035.

Cytosolic APX transcripts increase in tobacco leaves showing a hypersensitive programmed cell death response to tobacco mosaic virus. A previous paper [37] had shown that this did not lead to increased APX protein, indeed translation was suppressed, suggesting that H_2O_2, which is required for pathogen defence, is protected. This paper investigates the induction of APX transcripts in detail, and shows that EGTA with a calcium ionophore, paraquat, salicylic acid (see also [40]) and glutathione all induce APX expression. Salicylic acid is not essential to the pathogen response because tobacco expressing salicylate hydroxylase has normal APX expression. Hydrogen peroxide application

did not induce APX expression (in contrast to [32••] and [34••]). This could be because the response to exogenous H_2O_2 is transient [34••]; the treatment period was much longer than that described in other papers. The results indicate that APX expression and activity are controlled in a complex manner when plants are responding to pathogen attack.

39. Vanacker H, Carver TLW, Foyer CH: **Pathogen-induced changes in the antioxidant status of the apoplast in barley leaves.** *Plant Physiol* 1998, **117**:1103-1114.

40. Wendehenne D, Durner J, Chen ZX, Klessig DF: **Benzothiadiazole, an inducer of plant defenses, inhibits catalase and ascorbate peroxidase.** *Phytochem* 1998, **47**:651-657.

41. Hideg E, Mano J, Ohno C, Asada K: **Increased levels of monodehydroascorbate radical in UV-B-irradiated broad bean leaves.** *Plant Cell Physiol* 1997, **38**:684-690.

42. Conklin PL, Williams EH, Last RL: **Environmental stress tolerance of an ascorbic acid-deficient *Arabidopsis* mutant.** *Proc Natl Acad Sci USA* 1996, **93**:9970-9974.

43. Moldau H, Bichele I, Huve K: **Dark-induced ascorbate deficiency in leaf cell walls increases plasmalemma injury under ozone.** *Planta* 1998, **207**:60-66.

44. Jakob B, Heber U: **Apoplastic ascorbate does not prevent the oxidation of fluorescent amphiphilic dyes by ambient and elevated concentrations of ozone in leaves.** *Plant Physiol Biochem* 1998, **36**:313-322.

45. Orvar BL, Ellis BE: **Transgenic tobacco plants expressing antisense RNA for cytosolic ascorbate peroxidase show increased susceptibility to ozone injury.** *Plant J* 1997, **11**:1297-1305.

46. •• Wang J, Zhang H, Allen RD: **Overexpression of an *Arabidopsis* peroxisomal ascorbate peroxidase gene in tobacco increases protection against oxidative stress.** *Plant Cell Physiol* 1999, **40**:725-732.

An *A. thaliana APX3* gene, which encodes a peroxisomal membrane-located ascorbate peroxidase (APX) with a carboxy-terminal extension containing a putative transmembrane domain is over-expressed in tobacco. The enzyme was detected in a membrane fraction of the transgenic plants. The transgenic lines over-expressing *APX3* were more tolerant to aminotriazole as shown by a reduced extent of visible leaf lesions and reduced lipid peroxidation. Aminotriazole is a catalase inhibitor that increases H_2O_2 concentration, particularly in peroxisomes. The results show that membrane-bound peroxisomal APX provides a second line of defence, after catalase, against H_2O_2 leaking from the peroxisome. The third line of defence is cytosolic APX [32••]. The transgenic plants were no more tolerant of paraquat, which largely generates H_2O_2 in the chloroplasts and mitochondria, than wild-type plants.

47. Eskling M, Arvidsson P-O, Åkerlund H-E: **The xanthophyll cycle, its regulation and components.** *Physiol Plant* 1997, **100**:806-816.

48. • Kato N, Esaka M: **Changes in ascorbate oxidase gene expression and ascorbate levels in cell division and cell elongation in tobacco cells.** *Physiol Plant* 1999, **105**:321-329.

AO gene expression and activity increase during the logarithmic growth phase of tobacco BY-2 cells. In cultures treated to induce cell expansion, AO expression and activity, and ascorbate content also increased. In synchronous culture, AO expression was lowest during G_1. These results provide further evidence of a relationship between apoplastic AO and cell expansion, and suggest that AO expression levels vary through the cell cycle.

49. Kisu Y, Harada Y, Goto M, Esaka M: **Cloning of the pumpkin ascorbate oxidase gene and analysis of a *cis*-acting region involved in induction by auxin.** *Plant Cell Physiol* 1997, **38**:631-637.

50. Gonzales-Reyes JA, Hidalgo A, Caler JA, Palos R, Navas P: **Nutrient uptake changes in ascorbate free radical-stimulated roots.** *Plant Physiol* 1994, **104**:271-276.

51. Gonzalez-Reyes JA, Alcain FJ, Caler JA, Serrano A, Cordoba F, Navas P: **Stimulation of onion root elongation by ascorbate and ascorbate free radical in *Allium cepa* L.** *Protoplasma* 1995, **184**:31-35.

52. Sommer-Knudsen J, Bacic A, Clarke AE: **Hydroxyproline-rich plant glycoproteins.** *Phytochem* 1998, **47**:483-497.

53. De Gara L, Tommasi F, Liso R, Arrigoni O: **Ascorbic acid utilization by prolyl hydroxylase *in vivo*.** *Phytochem* 1991, **30**:1397-1399.

54. • De Tullio MC, Paciolla C, Dalla Vechia F, Rascio N, D'Emerico S, De Gara L, Liso R, Arrigoni O: **Changes in onion root development induced by the inhibition of peptidyl-prolyl hydroxylase and influence of the ascorbate system on cell division and elongation.** *Planta* 1999, **209**:424-434.

Ascorbate is a cofactor for a number of enzymes, particularly dioxygenases, in which it maintains the reduced form of the Fe cofactor. These include enzymes involved in ethylene (e.g. ACC [1-aminocyclopropane-1-carboxylic acid] oxidase) and gibberellin synthesis. Prolyl hydroxylase, is an ascorbate-dependent enzyme that hydroxylates prolyl residues that occur in proteins such as the HRGPs. HRGPs are cell-wall structural proteins; they are probably important in assembly of the wall and may provide defence by becoming oxidatively cross-linked in response to wounding or pathogen attack. Cross-linking increases the wall strength and impedes invasion by pathogens. This paper provides evidence of a link between ascorbate and cell division via its role in proline hydroxylation. Treatment of onion roots with 3,4-DL-dehydroproline, a prolyl hydroxylase inhibitor, causes disruption to newly-formed walls and excessive expansion of cells in the elongation zone. At the same time, ascorbate concentration increases, showing that its use by prolyl hydroxylase is a major function in meristems. It is suggested that disruption of wall synthesis results in reduced cell division. It is interesting to recall that scurvy, a disease caused by ascorbate deficiency in mammals, results from reduced synthesis of collagen, an extracellular matrix hydroxy-proline-rich protein.

55. Conklin PL, Saracco SA, Norris SR, Last RL: **Identification of ascorbic acid-deficient *Arabidopsis thaliana* mutants.** *Genetics* 2000, **154**:847-856.