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Ascorbate biosynthesis and function in photoprotection

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Ascorbate (vitamin C) can reach very high concentrations in chloroplasts (20–300 mM). The pool size in leaves and chloroplasts increases during acclimation to high light intensity and the highest concentrations recorded are in high alpine plants. Multiple functions for ascorbate in photosynthesis have been proposed, including scavenging of active oxygen species generated by oxygen photoreduction and photorespiration, regeneration of α-tocopherol from α-tocopheryl radicals, cofactor for violaxanthin de-epoxidase and donation of electrons to photosystem II. Hydrogen peroxide scavenging is catalysed by ascorbate peroxidase (Mehler peroxidase reaction) and the subsequent regeneration of ascorbate by reductant derived from photosystem I allows electron flow in addition to that used for CO_2 assimilation. Ascorbate is synthesized from guanosine diphosphate-mannose *via* L-galactose and L-galactono-1,4-lactone. The last step, catalysed by L-galactono-1,4-lactone dehydrogenase, is located on the inner mitochondrial membrane and uses cytochrome *c* as electron acceptor. L-galactono-1,4-lactone oxidation to ascorbate by intact leaves is faster in high-light acclimated leaves and is also enhanced by high light, suggesting that this step contributes to the control of pool size by light. Ascorbate-deficient *Arabidopsis thaliana vtc* mutants are hypersensitive to a number of oxidative stresses including ozone and ultraviolet B radiation. Further investigation of these mutants shows that they have reduced zeaxanthin-dependent non-photochemical quenching, confirming that ascorbate is the cofactor for violaxanthin de-epoxidase and that availability of thylakoid lumen ascorbate could limit this reaction. The *vtc* mutants are also more sensitive to photooxidation imposed by combined high light and salt treatments.

Keywords: *Arabidopsis thaliana vtc* mutants; chlorophyll fluorescence; L-galactono-1,4-lactone dehydrogenase; non-photochemical quenching; vitamin C

1. INTRODUCTION

Oxygen is potentially toxic and even more so when combined with light, pigments and electron transport activity: such conditions are provided in chloroplasts. Photosynthesis releases oxygen, absorbs light and carries out electron transport so the chloroplast therefore needs protection from reactive oxygen species. These include superoxide and hydrogen peroxide formed by oxygen photoreduction, and singlet oxygen formed by transfer of excitation energy to oxygen (Asada 1999). Additionally photorespiration generates hydrogen peroxide from the glycollate oxidase reaction in the peroxisomes. Antioxidants and free radical scavengers are needed to deal with these toxic products of photosynthesis (Noctor & Foyer 1998; Asada 1999; Niyogi 1999). Ascorbate is the most abundant soluble antioxidant in chloroplasts. Interestingly, in mammals, the eye is amongst the tissues containing the highest ascorbate concentration (Halliwell & Gutteridge 1999), which suggests that ascorbate is particularly important in situations where cells contain pigments designed to absorb light effectively.

Given its abundance and the importance of plants as a dietary source of ascorbate (vitamin C) for humans (who are unable to synthesize it), it is surprising that very little is known about ascorbate metabolism in plants. Its biosynthetic pathway is different from mammals and has only recently been established. New information on the role of ascorbate in photosynthesis and photoprotection is beginning to emerge, aided by knowledge of the biosynthetic pathway and the isolation of ascorbate-deficient (*vtc*) mutants of *Arabidopsis thaliana*. This paper reviews the role of ascorbate in photoprotection. New data on the relationship between light and biosynthesis, and the use of ascorbate-deficient *A. thaliana* mutants to investigate photoprotective roles of ascorbate, are presented.

2. THE ROLE OF ASCORBATE IN PHOTOSYNTHESIS

The involvement of ascorbate in photosynthesis has been recognized for some time and has been reviewed relatively recently with emphasis on photoprotection (Smirnoff 2000; Noctor & Foyer 1998; Asada 1999; Niyogi 1999). Possible roles for ascorbate in photosynthesis were suggested by Arnon and co-workers in the early 1950s (Marrè *et al.* 1959; Forti & Jagendorf 1961; Mapson 1962). Initially a role as electron carrier was considered but later as a protectant. Our current knowledge of the roles of ascorbate in photosynthesis was foreshadowed by Marrè *et al.* (1959) who proposed that chloroplasts could both oxidize ascorbate to monodehydroascorbate (MDA) and reduce MDA to ascorbate, these processes being dependent on water splitting and electron flow. They also suggested the involvement of a monodehydroascorbate

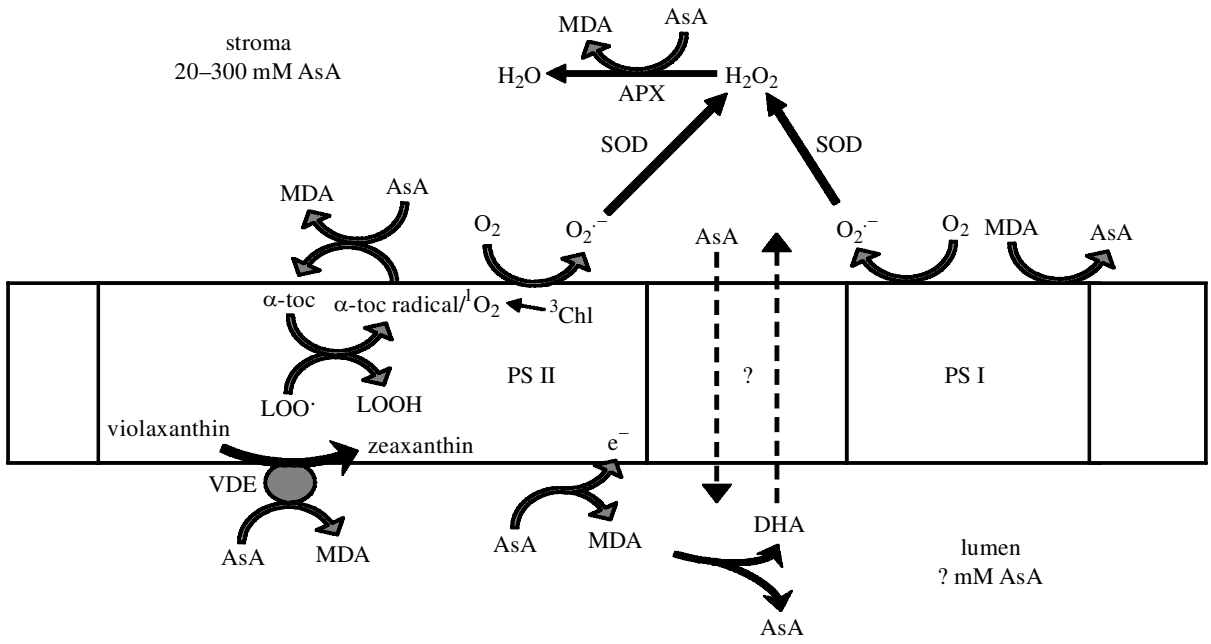

Figure 1. The roles of ascorbate in photosynthesis. The points at which ascorbate acts as an electron donor and monodehydroascorbate acts as an electron acceptor are shown diagrammatically, along with its roles as a cofactor for violaxanthin de-epoxidase, removal of singlet oxygen and regeneration of α-tocopherol from α-tocopheryl radicals. Movement of ascorbate and dehydroascorbate across the thylakoid lumen is shown by dashed lines—a carrier system has not been identified. Reactions that regenerate ascorbate from monodehydroascorbate (NADPH-dependent monodehydroascorbate reductase) and dehydroascorbate (glutathione-dependent dehydroascorbate reductase) are not shown. APX, ascorbate peroxidase; AsA, ascorbic acid/ascorbate; ^{3}Chl, triplet chlorophyll; DHA, dehydroascorbate; LOO·, lipid peroxyl radical; LOOH, lipid hydroperoxide; MDA, monodehydroascorbate radical; $^1O_2$, singlet oxygen; PS I, photosystem I; PS II, photosystem II; SOD, superoxide dismutase; α-toc, α-tocopherol; VDE, violaxanthin de-epoxidase.

reductase enzyme. Forti & Jagendorf (1961) showed that ascorbate stimulates photophosphorylation and allows electron transport by acting catalytically in the Mehler reaction. Mapson (1962) showed that ascorbate is subject to photo-oxidation and that both oxidation and reduction of dehydroascorbate were inhibited by blocking photosynthetic electron transport. Later, when the details of photosynthetic electron transport were more clearly understood, it become clear that ascorbate and glutathione in chloroplasts are oxidized by hydrogen peroxide produced by the Mehler reaction and that reduction of their oxidized forms uses reductant from photosystem I (PS I) (Foyer & Halliwell 1976; Anderson *et al*. 1983; Asada 1999). Current understanding of the roles of ascorbate in photosynthesis can be summarized as follows (figure 1).

(i) Hydrogen peroxide scavenging catalysed by ascorbate peroxidase (APX).
(ii) Direct scavenging of superoxide, hydroxyl radicals and singlet oxygen.
(iii) Regeneration of α-tocopheryl radicals produced when α-tocopherol reduces lipid peroxyl radicals.
(iv) Electron donation to photosystem II (PS II) by lumenal ascorbate.
(v) Cofactor of violaxanthin de-epoxidase (VDE) involved in zeaxanthin-dependent dissipation of excess excitation energy (a component of non-photochemical quenching).

Oxidants formed during photosynthesis (Asada 1999) can be scavenged non-enzymatically while hydrogen peroxide reduction is also catalysed by APX of which there are stromal- and thylakoid-bound forms (Asada 1999). Hydrogen peroxide is formed from dismutation of superoxide that itself is produced by oxygen reduction, either *via* reduced ferredoxin (Mehler reaction) or from PS II (Cleland & Grace 1999). The primary oxidation product of ascorbate is the relatively stable monodehydroascorbate radical (MDA). This can be detected *in vivo* by electron paramagentic resonance spectroscopy in leaves. In some cases it is detected after illumination with bright light but is particularly prominent after imposition of oxidative stress by paraquat, ultraviolet B (UVB) and drought (Heber *et al*. 1996; Hideg *et al*. 1997). It is probable that the importance of ascorbate as a free-radical scavenger depends on the relative stability of the MDA radical: thilyl radicals, formed when thiols such as glutathione scavenge free radicals, are more reactive and are themselves relatively dangerous (Sturgeon *et al*. 1998). The MDA radical disporportionates to ascorbate and dehydroascorbate. Normally the ascorbate pool in leaves and chloroplasts is 90% reduced and this is achieved by systems that reduce MDA and dehydroascorbate (DHA) back to ascorbate. This is important because DHA is unstable, particularly at the pH of illuminated stroma. The ascorbate regeneration systems include direct reduction of MDA by PS I, NADPH-dependent MDA reductase and glutathione-dependent dehydroascorbate reductase. NADPH-dependent glutathione reductase regenerates reduced glutathione (GSH) from oxidized glutathione (GSSG). The net result of the Mehler reaction is that water is the final product of oxygen photoreduction

and that photosynthetically generated reductant is used to regenerate the ascorbate used in this reaction. This allows electron flow in addition to that used for CO_2, NO_3^- and SO_4^{2-} assimilation. The consequences and significance of the so-called Mehler peroxidase or water–water reaction are discussed by Asada (1999, this issue).

3. THE RELATIONSHIP BETWEEN LIGHT AND THE ASCORBATE CONCENTRATION IN LEAVES AND CHLOROPLASTS

The total concentration of ascorbate in leaves is light dependent. Growth at high light intensity produces leaves with higher ascorbate content than at low light intensity (Smirnoff & Pallanca 1996; Grace & Logan 1996; Logan *et al.* 1996). The readjustment of ascorbate concentration is relatively slow, occurring over a period of several days after transfer (Eskling & Åkerlund 1998). Conversely, after transfer to low light, ascorbate content decreases to a new steady state within a few days (Eskling & Åkerlund 1998). The concentration range found in leaves varies from around 2–20 $\mu mol\,g^{-1}$ fresh weight (Wildi & Lutz 1996; Streb *et al.* 1997). The higher concentrations seem to occur more frequently in temperate evergreen and alpine species. Alpine species are also characterized by relatively high concentrations of other antioxidants (glutathione and α-tocopherol) and carotenoids (Wildi & Lutz 1996; Streb *et al.* 1997). GSH, the other major soluble antioxidant, is generally tenfold less concentrated than ascorbate (Noctor & Foyer 1998).

A number of estimates of ascorbate concentration in chloroplasts have been made, generally using chloroplasts isolated in aqueous media. The values quoted range from 10–230 mM (Foyer *et al.* 1983; Rautenkranz *et al.* 1994; Streb *et al.* 1997). Ascorbate is therefore one of the most abundant chloroplast metabolites, even at the most quoted concentration of 20–50 mM, and in alpine plants the concentration is enormous, reaching 300 mM (Streb *et al.* 1997). The pool is generally highly reduced (90% +), unless the chloroplasts are subjected to oxidative stress by paraquat or H_2O_2 treatment (Law *et al.* 1983). The increase in leaf content is also mirrored by equivalent increases in chloroplast ascorbate (Schöner & Krause 1990). Ascorbate is also much more abundant in chloroplasts than GSH, which has a concentration *ca.* 3–4 mM (Foyer & Halliwell 1976; Streb *et al.* 1997). The concentration of ascorbate in the thylakoid lumen is not known for certain and it has been suggested that thylakoids have no ascorbate transport system (Foyer & Lelandais 1996). Assuming there is 50 mM ascorbate in the stroma at pH 8 and that undissociated ascorbic acid (the pK_a of ascorbic acid is 4.2) diffuses across the thylakoid membrane, Eskling *et al.* (1997) calculated that the concentration of ascorbic acid in the thylakoid lumen would be 8 μM in the absence of a carrier. The concentration of ascorbate in the lumen is critical for operation of the xanthophyll cycle, since it is a cofactor of VDE (Eskling *et al.* 1997). VDE prefers undissociated ascorbic acid that predominates over the ascorbate anion at low pH (Bratt *et al.* 1995; Eskling *et al.* 1997). VDE catalyses conversion of antheroxanthin and violaxanthin to zeaxanthin in leaves exposed to high light. Zeaxanthin is involved in non-photochemical quenching by dissipating excitation energy as heat (Niyogi 1999). The predicted concentration of 8 μM is much lower than the measured K_m of 100 μM for VDE. They therefore proposed that there must be thylakoid carriers to transport ascorbate in and DHA out. On the other hand, Mano *et al.* (1997) suggested that 10–20% of chloroplast ascorbate occurs in the thylakoid lumen. Since the thylakoid lumen volume of a typical chloroplast is about 20% of the total chloroplast volume (Lawlor 1993) this measurement implies that lumen and stroma have a similar ascorbate concentration. Further measurements are needed but 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.

The last step of ascorbate biosynthesis occurs in the mitochondria (see §4) and there is little evidence that chloroplasts can synthesize ascorbate. It must therefore be transported from the cytosol. Ascorbate uptake has been measured by isolated chloroplasts and is a facilitated diffusion mechanism with a K_m of 20 mM (Anderson *et al.* 1983; Beck *et al.* 1983; Foyer & Lelandais 1996). The rate of uptake is not sufficient to keep pace with the rate of ascorbate oxidation in the chloroplast (Anderson *et al.* 1983), so ascorbate concentration inside is maintained by the well-known regeneration systems (Asada 1999). The uptake rate must be sufficient to accommodate the relatively slow adjustment in ascorbate pool size that occurs when light intensity changes.

With the exception of inorganic ions such as magnesium, potassium and phosphate (Lawlor 1993), ascorbate is probably the most abundant metabolite in chloroplasts. The question of why so much ascorbate is required in chloroplasts arises. The alpine plants with exceptionally high chloroplast ascorbate may provide a clue—these plants are exposed to a combination of conditions that are conducive to photo-oxidative stress: high light, low temperature and relatively high UVB radiation. Also, given that the rate of ascorbate biosynthesis is relatively sluggish, that transport into the chloroplast is slow and that DHA is unstable, it is possible that a high concentration is needed as an insurance against episodes of increased photo-oxidative stress. It is notable that the ascorbate-deficient *vtc1 A. thaliana* mutant is hypersensitive to UVB radiation, as well as ozone and sulphur dioxide (Conklin *et al.* 1996). Further evidence for the importance of ascorbate in resistance to oxidative stress comes from overexpression and antisense suppression of various APX genes. Antisense suppression of cytosolic APX increases ozone sensitivity (Orvar & Ellis 1997), while overexpression of peroxisomal APX increases hydrogen peroxide resistance (Wang *et al.* 1999). Transcript levels of cytosolic APX increase rapidly when *A. thaliana* leaves are exposed to high light (Karpinski *et al.* 1997, 1999). It seems likely that the cytosolic APX has a role in scavenging photorespiratory hydrogen peroxide that leaks from the peroxisomes as well as any leaking from chloroplasts (Foyer & Noctor 1999). There is evidence that hydrogen peroxide and/or redox signals from Q_B or PQ are the signal inducing APX (Karpinski *et al.* 1999; Morita *et al.* 1999) and that a systemic response is induced in brightly illuminated leaves so that APX transcripts increase in shaded leaves of the same

plant (Karpinski *et al.* 1999). Assuming that the increased cytosolic APX transcript levels translate into increased APX activity, it seems that rapid induction of APX by high light could compensate for the slow response of the ascorbate pool itself.

4. BIOSYNTHESIS PATHWAY

The plant ascorbate biosynthesis pathway differs from that in mammals and a complete scheme has recently been proposed (Wheeler *et al.* 1998; Smirnoff & Wheeler 1999; Conklin *et al.* 1999; Loewus 1999; Smirnoff 2000). The immediate precursor is L-galactose, which is oxidized to L-galactono-1,4-lactone by an NAD-dependent L-galactose dehydrogenase. L-galactono-1,4-lactone is oxidized to ascorbate by L-galactono-1,4-lactone dehydrogenase. This enzyme is located on the inner mitochondrial membrane and donates electrons to cytochrome *c* (Siendones *et al.* 1999; Bartoli *et al.* 2000). L-galactose and L-galactono-1,4-lactone are readily converted to ascorbate when supplied to intact tissue and can elevate the ascorbate pool up to tenfold within a few hours. L-galactose is derived from mannose-1-phosphate *via* guanosine diphosphate (GDP)-mannose and GDP-L-galactose. Evidence for this pathway is derived from ^{14}C-labelling studies (Wheeler *et al.* 1998) and from the ascorbate-deficient *vtc1* mutant of *A. thaliana*. *Vtc1* has *ca.* 30% of wild-type ascorbate and has a lower rate of biosynthesis (Conklin *et al.* 1997). *VTC1* encodes GDP-mannose pyrophosphorylase (Conklin *et al.* 1999). Leaf extracts from mutant plants have about 30% of the activity of GDP-mannose pyrophosphorylase and ascorbate content can be restored by transforming the mutant with the wild-type gene (Conklin *et al.* 1999). Further confirmation of the role of GDP-mannose pyrophosphorylase comes from reduction of its expression by antisense technology in the potato. The resulting plants have lower ascorbate content (Keller *et al.* 1999). A number of details remain to be resolved. The GDP-mannose-3,5-epimerase, proposed to convert GDP-mannose to GDP-L-galactose, has been detected but not characterized in any detail. Conversion of GDP-L-galactose to L-galactose occurs in cell-free extracts but the nature of the reactions is not fully resolved (G. L. Wheeler and N. Smirnoff, unpublished data). It is likely that further ascorbate-deficient *vtc* mutants currently being characterized will be useful for identifying the genes involved in ascorbate biosynthesis (Conklin *et al.* 2000).

L-galactose appears to be a dedicated precursor for ascorbate, while earlier intermediates have other roles. The GDP-sugars are used as cell wall polysaccharide precursors and for protein glycosylation. Antisense potato plants with reduced GDP-mannose pyrophosphorylase activity also have reduced mannose content in their wall polysaccharides (Keller *et al.* 1999). It is currently assumed that the pathway occurs in the cytosol with the exception of the final step in the mitochondria. The integration of L-galactono-1,4-lactone oxidation into the mitochondrial electron transport chain *via* cytochrome *c* could have implications for coordinating ascorbate metabolism with the energy metabolism and redox state of the cell. Currently very little is known about the control of the pathway. Evidence for feedback inhibition or repression of synthesis by ascorbate pool size has been provided (Pallanca & Smirnoff 2000) but nothing is known about the mechanism. Gene probes, antibodies, transgenic plants and quantitative enzyme assays for enzymes involved in the proposed pathway are now becoming available and the next few years should see advances in understanding how ascorbate biosynthesis is controlled.

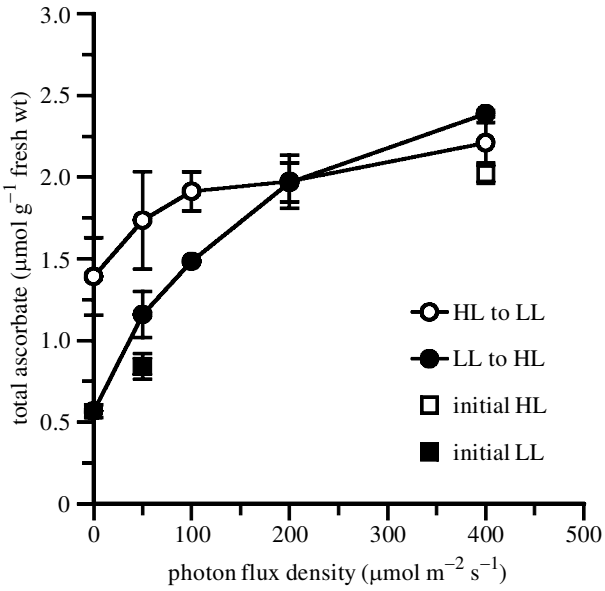

Figure 2. Light dependence of the ascorbate pool in barley leaves (*Hordeum vulgare*). Barley seedlings were grown at photon flux densities of 50 μmol m^{-2} s^{-1} (initial LL) and 200 μmol m^{-2} s^{-1} (initial HL) for seven days. Leaf slices from HL-grown plants (HL to LL) and LL-grown plants (LL to HL) were then floated on water and exposed to a range of light intensities for 24 h before determining total ascorbate concentration. The values are means of three replicates ± standard deviation.

5. MATERIAL AND METHODS

(a) *The effect of light on ascorbate pool size and synthesis*

The primary leaf of seven-day-old barley seedlings was used for investigations of the light dependence of ascorbate synthesis. Plants were grown at 20 °C with a 16 h light period. Portions of leaf 3 cm long were cut from the middle of the primary leaf and sliced transversely into 2 mm segments. The segments (0.2 g per replicate) were floated on water in Petri dishes. L-galactose and L-galactono-1,4-lactone were at concentrations designated for each experiment. The dishes were exposed to continuous light provided by fluorescent tubes at 20 °C. Light intensities are indicated for each experiment. After incubation for the designated time, the leaf segments were extracted and their total ascorbate content (ascorbate + dehydroascorbate) was measured by an ascorbate oxidase assay (Conklin *et al.* 1997).

(b) *Determination of non-photochemical quenching and other chlorophyll fluorescence parameters*

Wild-type *A. thaliana* (Col-0) and *vtc1*, *2*, *3* and *4* mutants (Conklin *et al.* 2000) were grown at 20 °C with a 12 h light

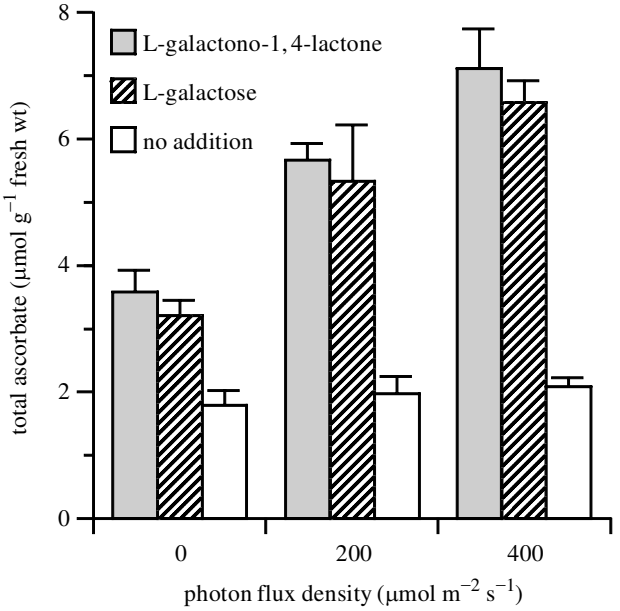

Figure 3. The effect of light on *in vivo* conversion of L-galactose and L-galactono-1,4-lactone to ascorbate. Leaf slices from barley (*Hordeum vulgare*) plants grown at 200 μmol m^{-2} s^{-1} were floated on water with 15 mM L-galactono-1,4-lactone or 15 mM L-galactose. The ascorbate concentration in the leaf slices was determined after incubation for 5.5 h. The values are means of three replicates ± s.d.

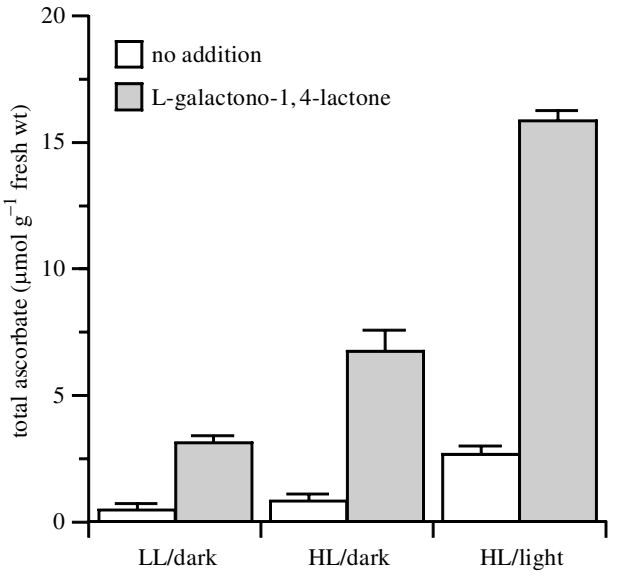

Figure 4. The effect of light on *in vivo* conversion of L-galactono-1,4-lactone to ascorbate. Leaf slices from barley (*Hordeum vulgare*) plants grown at photon flux densities of 80 μmol m^{-2} s^{-1} (LL) and 360 μmol m^{-2} s^{-1} (HL) for three days were floated on water with and without 10 mM L-galactono-1,4-lactone in the dark and light (photon flux density 250 μmol m^{-2} s^{-1}). The ascorbate concentration in the leaf slices was determined after incubation for 24 h. The values are means of three replicates ± s.d.

period at a photon flux density of 200 μmol m^{-2} s^{-1}. Fully expanded leaves from preflowering rosettes were used for chlorophyll fluorescence measurements using a Hansatech FMSl modulated fluorometer (Hansatech Instruments, King's Lynn, UK). The modulated beam was set at a level that had no photochemical effect and the high intensity light pulses (*ca.* 8000 μmol m^{-2} s^{-1}) were saturating. Leaves were dark adapted for 1 h. After an initial dark-adapted measurement of F_v/F_m, a light-response curve for chlorophyll fluorescence parameters was constructed by exposure to a sequence of increasing actinic light levels. After 5 min at each level, F'_o (in far-red light), F_s and F'_m were measured. Chlorophyll fluorescence parameters were calculated as follows: photochemical quenching coefficient [$q_P = (F'_m - F_s)/(F'_m - F'_o)$]; quantum efficiency of PS II [ΦPS II = $(F'_m - F_s)/F'_m$]; non-photochemical quenching coefficient [$q_{NP} = (F_m - F'_m)/(F_m - F'_o)$] and non-photochemical quenching [NPQ = $(F_m - F'_m)/F'_m$] (Schreiber *et al.* 1995). A time-course of development of chlorophyll fluorescence parameters was determined after illumination of dark-adapted leaves with a photon flux density of 1000 μmol m^{-2} s^{-1}.

(c) *Salt-induced photo-oxidation of* Arabidopsis thaliana *seedlings*

Seedlings of wild-type and *vtc1, 2, 3* and *4* mutants (Conklin *et al.* 2000) were grown at 20 °C with a 12 h light period at a photon flux density of 200 μmol m^{-2} s^{-1} in Petri dishes containing MS medium (minus sucrose) with or without 122 mM NaCl (water potential, −0.5 MPa). The dishes were transferred to high light (400 μmol m^{-2} s^{-1}) when indicated. The proportion of seedlings showing complete bleaching of their leaves was recorded at intervals.

6. RESULTS AND DISCUSSION

(a) *The effect of light on ascorbate pool size and synthesis*

The ascorbate pool size in barley leaves is higher when the plants are grown at higher light intensity (Smirnoff 1995; Smirnoff & Pallanca 1996). The dependence of the pool size on photon flux density (PFD) was further investigated by growing barley seedlings at relatively low PFD and then exposing leaf slices to a range of PFDs for 20 h. The ascorbate pool increased to new levels after this time (figure 2). At the highest PFD of 400 μmol m^{-2} s^{-1} it reached the same concentration as intact plants acclimated to this PFD over seven days. Leaf slices from intact plants acclimated to 400 μmol m^{-2} s^{-1} did not show a reduction in the ascorbate pool when incubated at lower light intensities unless kept in the dark. This shows that the readjustment is relatively slow, as has been found in other studies (see § 1) and that the ascorbate pool in the leaf slices behaves in a similar manner to intact leaves on exposure to high light. Therefore barley leaf slices provide a useful system to investigate ascorbate metabolism in relation to light. Exogenous L-galactose and L-galactono-1,4-lactone are readily absorbed and converted to ascorbate by leaves, the reactions being catalysed by L-galactose dehydrogenase and mitochondrial L-galactono-1,4-lactone dehydrogenase (Wheeler *et al.* 1998; Loewus 1999; Bartoli *et al.* 2000). Barley leaf slices supplied with these precursors convert both substrates in a light-stimulated manner over 5.5 h (figure 3). Over 24 h conversion of L-galactono-1,4-lactone to ascorbate is also greater in the

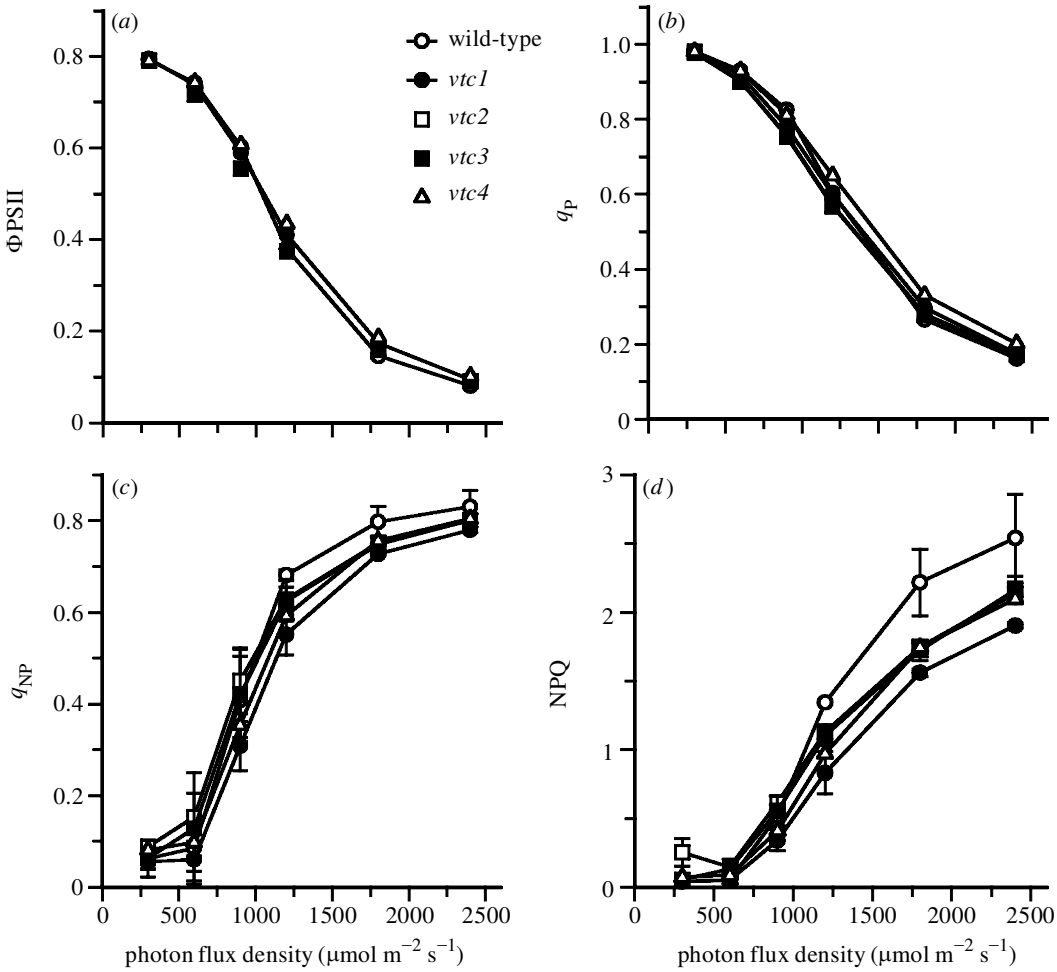

Figure 5. A comparison of the light response of steady-state chlorophyll fluorescence parameters measured in leaves from wild-type and ascorbate-deficient *vtc* mutants of *Arabidopsis thaliana*. (*a*) Quantum efficiency of PS II (ΦPS II). (*b*) Photochemical quenching coefficient (q_P). (*c*) Non-photochemical quenching coefficient (q_{NP}). (*d*) Non-photochemical quenching (NPQ). The values are means of four replicates ± s.e.

light than in the dark (figure 4). Furthermore, when leaves were taken from plants previously acclimated to high or low light, conversion of L-galactono-1,4-lactone to ascorbate in the dark was greater in the previously high-light acclimated leaves (figure 4). These results suggest that L-galactono-1,4-lactone oxidation is enhanced by light. The persistent increase in ascorbate synthesis from L-galactono-1,4-lactone in high light acclimated leaves in the dark suggests that their mitochondria have a larger L-galactono-1,4-lactone dehydrogenase capacity. The further increase in ascorbate synthesis when high-light acclimated leaves were kept in the light suggests that there is also a direct enhancement of L-galactono-1,4-lactone oxidation by light. This is under further investigation to determine if light affects expression of L-galactono-1,4-lactone dehydrogenase and if there is an interaction between light and mitochondrial metabolism that affects L-galactono-1,4-lactone activity. It is surprising that the last step of the pathway, which apparently has a large capacity compared to earlier steps, is affected by light. To understand fully the close coordination between ascorbate pool size and light it will also be necessary to determine the effect of light on earlier steps in the pathway.

(b) *Ascorbate-deficient* Arabidopsis thaliana *mutants have reduced non-photochemical quenching*

Four ascorbate-deficient *A. thaliana* mutants with reduced ascorbate concentration have been isolated (Conklin *et al.* 1996, 2000). They have been named *vtc1*, *2*, *3* and *4*. The young leaves of each mutant have 30–50% of the wild-type ascorbate content. The ascorbate concentration in the rosette leaves of some *vtc2* alleles decreases even further after flowering to 10% of wild-type. The cloning of *VTC1* and its contribution to confirming the proposed ascorbate biosynthesis pathway was described in §1.

Some, but not all, of the *vtc* mutants are ozone hypersensitive while *vtc1* is also more sensitive to sulphur dioxide and UVB (Conklin *et al.* 1996, 2000). Since exogenous ascorbate reverses the ozone hypersensitivity of *vtc1*, the phenotype is unlikely to be caused by pleiotropic effects and this provides strong genetic evidence for an antioxidant role for ascorbate (Conklin *et al.* 1996). Ascorbate is also proposed to play a role in photoprotection by acting as a cofactor for VDE, thus facilitating zeaxanthin synthesis and dissipation of excess excitation energy as heat. The background was reviewed in §1. The bulk of non-photochemical quenching (measured as NPQ) in

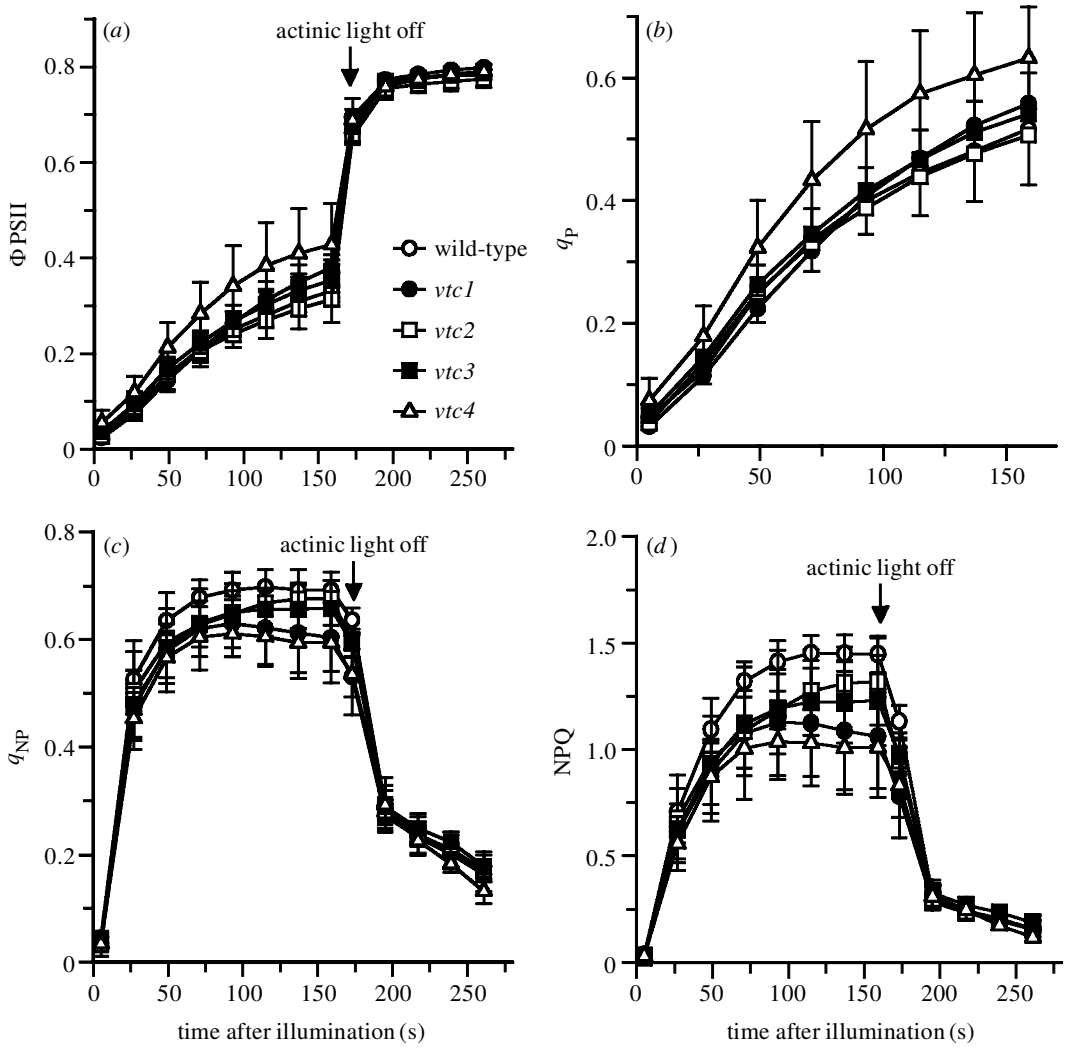

Figure 6. A comparison of the time-course of induction and relaxation (actinic light off) of chlorophyll fluorescence parameters measured in leaves from wild-type and ascorbate-deficient *vtc* mutants of *Arabidopsis thaliana*. Dark-adapted leaves were exposed to actinic light (photon flux density 1000 μmol $m^{-2} s^{-1}$) at zero time. (*a*) Quantum efficiency of PS II (ΦPS II). (*b*) Photochemical quenching coefficient (q_P). (*c*) Non-photochemical quenching coefficient (q_{NP}). (*d*) Non-photochemical quenching (NPQ). The values are means of three replicates ± s.d.

A. thaliana can be attributed to zeaxanthin synthesis (Niyogi *et al.* 1998). Therefore the development of NPQ in wild-type and *vtc* mutants has been assessed by analysis of chlorophyll fluorescence quenching. The plants were grown at low light intensity (200 μmol $m^{-2} s^{-1}$) to avoid photo-oxidative stress and the maximum quantum efficiency (F_v/F_m) was the same in all the strains. Chlorophyll fluorescence measurements were used to compare photochemical quenching (q_P), non-photochemical quenching (calculated as q_{NP} and NPQ) and the quantum efficiency of PS II (ΦPS II) at steady state over a range of light intensities in wild-type and *vtc* mutants (figure 5). There were no significant differences in ΦPS II or q_P, although the latter was very slightly higher in *vtc4*. Non-photochemical quenching measured as q_{NP} was higher in the wild-type leaves, while expression as NPQ showed that all the *vtc* mutants developed lower non-photochemical quenching than the wild-type at irradiances above 1000 μmol $m^{-2} s^{-1}$. A time-course of development of the chlorophyll fluorescence parameters after illuminating dark-adapted leaves was determined and revealed the same differences between wild-type and *vtc* mutants (figure 6). Non-photochemical quenching measured by q_{NP} or NPQ was higher in wild-type leaves by 40 s after illumination. This initial 40 s phase corresponds to zeaxanthin-independent NPQ seen in *npq1*, a VDE-deficient mutant (Niyogi *et al.* 1998). Divergence of the mutants and wild-type after this point suggests that the mutants have reduced zeaxanthin-dependent NPQ that could be attributed to a limitation of VDE activity by ascorbate supply. Because the effect occurs in four non-allelic mutants, it is very likely that ascorbate deficiency is the direct cause of reduced NPQ. The results support previous assumptions that ascorbate supply in the thylakoid lumen is potentially limiting to VDE activity (Neubauer & Yamamoto 1994). It is not known if the reduction in ascorbate concentration in the *vtc* mutants is equal in all subcellular compartments. However, because a relatively small decrease in ascorbate reduces VDE activity, it can be tentatively suggested that the concentration of ascorbic acid in the lumen must be near the K_m of VDE (100 μM; see § 1) and not near the stromal

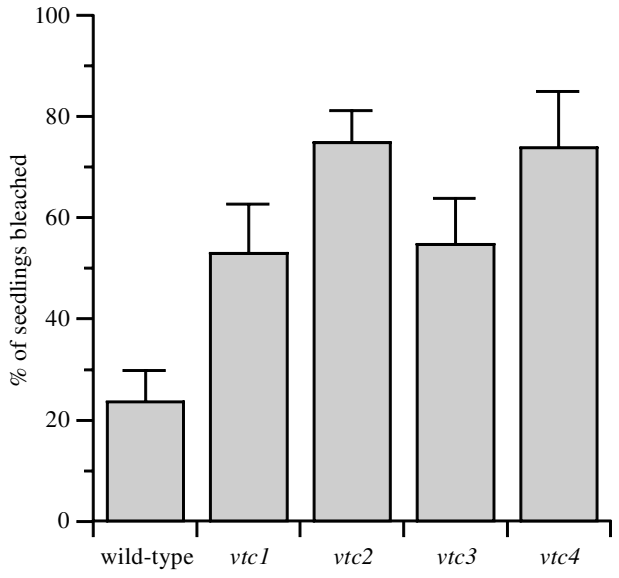

Figure 7. A comparison of the sensitivity of wild-type and ascorbate-deficient *vtc* mutants of *Arabidopsis thaliana* to NaCl-induced photo-oxidation. Ten-day-old seedlings grown with 122 mM NaCl at low light intensity (photon flux density 75 $\mu mol\, m^{-2}\, s^{-1}$) were transferred to higher light intensity (photon flux density 240 $\mu mol\, m^{-2}\, s^{-1}$). After 12 days the proportion of seedlings with all their leaves bleached was determined. The values are means of four replicates ± s.e.

concentration. A very high stromal ascorbate concentration may be required to maintain lumen ascorbate in the apparent absence (Foyer & Lelandais 1996) of a carrier (figure 1). In contrast to the effect of loss of VDE activity in the *npq1* mutant (Niyogi *et al.* 1998) there were no measurable corresponding changes in q_P, ΦPS II and F'_v/F'_m (data not shown) in the *vtc* mutants. This is probably because the reduction in NPQ is smaller than in the VDE-deficient plants.

(c) *The* vtc *mutants are susceptible to salt-induced photo-oxidation*

When young *A. thaliana* seedlings are exposed to a combination of high light intensity and NaCl in the rooting medium, the leaves bleach within a few days (figure 7). NaCl at low light intensity or high light alone does not cause this response, which provides an easily monitored bioassay for photo-oxidation. Under conditions that cause slow bleaching of wild-type seedlings, all the ascorbate-deficient *vtc* mutants bleach more rapidly (figure 7). This provides further evidence for the role of ascorbate in photoprotection. At present the mechanism of protection against salt and light-induced photo-oxidation is not known. It could be related to protection by NPQ, scavenging of superoxide and hydrogen peroxide in chloroplasts and peroxisomes, or regeneration of α-tocopherol. APX and superoxide dismutase activity are induced by this treatment (Tsugane *et al.* 1999), the increase in APX further underlining a role for ascorbate. Tsugane *et al.* (1999) isolated a recessive *A. thaliana* mutant (*pst1*) that is more tolerant to NaCl-induced photo-oxidation and which has higher APX activity. This system and the *vtc* mutants should be useful in assessing the role of ascorbate in photoprotection.

7. CONCLUSIONS

The results presented here identify multiple roles for ascorbate in photosynthesis and photoprotection and show that its synthesis in leaves is controlled by light. However, the question why chloroplasts frequently contain enormous ascorbate concentrations is not yet fully answered. The ascorbate-deficient *vtc* mutants appear to function normally until exposed to oxidative stress, implying that very high ascorbate, as seen in high alpine species, must be maintained to deal with more extreme episodes of photo-oxidative stress. Manipulation of the biosynthetic pathway of ascorbate might now be possible as a number of genes have been cloned and this should provide further insights into its function.

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Discussion

C. Critchley (*Department of Botany, University of Queensland, Australia*). Is there a correlation between PS II activity and ascorbate contents? Chloroplasts and mitochondria are much more closely and intimately associated in the cell than we generally believe.

N. Smirnoff. There appears to be a general correlation between the photosynthetic capacity and leaf ascorbate concentration within a species. For example, in leaves from barley (*Hordeum vulgare*) seedlings, both photosynthetic capacity and ascorbate concentration increase from the leaf base to the tip (N. Smirnoff, unpublished data). Also, as discussed in the paper, acclimation to high light increases ascorbate content perhaps by stimulating biosynthesis. However, there is a tenfold range in leaf ascorbate concentration between different species that is apparently not correlated with PS II activity. As a generalization, it seems that alpine plants and temperate evergreens have the highest concentrations but do not necessarily have a high photosynthetic capacity.

C. Critchley. The close association observed between chloroplast and mitochondria could facilitate the exchange of metabolites and messages involved in the light stimulation of galactonolactone dehydrogenase activity in mitochondria. This enzyme oxidizes galactonolactone to ascorbate.