The role of L-ascorbic acid recycling in responding to environmental stress and in promoting plant growth

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Abstract

L-Ascorbic acid (Asc) is the most abundant water-soluble antioxidant in plants. It serves as a cofactor for enzymes involved in photosynthesis, hormone biosynthesis, and the regeneration of antioxidants such as α-tocopherol. Once used, Asc can be recycled by several different mechanisms. The short-lived monodehydroascorbate (MDHA) radical, produced following Asc oxidation, can be recycled following reduction by ferredoxin or monodehydroascorbate reductase (MDAR). MDHA can also undergo disproportionation into dehydroascorbate (DHA) and Asc. DHA can be recycled into Asc by dehydroascorbate reductase (DHAR) before it undergoes irrevocable hydrolysis. Through its recycling, Asc content and its redox state are maintained, which is critical under conditions of high demand, for example during high light or other stress conditions that increase reactive oxygen species (ROS) production. This review provides an overview of research in the last decade revealing the role that Asc recycling plays during germination, growth, and reproduction, as well as in response to environmental stress. These findings highlight the importance of DHAR- and MDAR-mediated mechanisms of Asc recycling in maintaining ROS at non-damaging levels while modulating ROS signalling function.

Key words: Ascorbate; L-ascorbic acid, DHAR, MDAR, reactive oxygen species.

Introduction

L-Ascorbic acid (Asc) is the primary water-soluble antioxidant in plants and animals (Levine, 1986; Levine et al., 1995; Sies and Stahl, 1995). In plants, Asc serves as a major redox buffer, as a cofactor for many enzymes, and as a regulator of cell division and growth, as well as in signal transduction (Kerk and Feldman, 1995; Smirnoff and Wheeler, 2000a, b; Noctor et al., 2000; Pignocchi and Foyer, 2003). Given the importance of this vitamin to plant and animal health, increasing Asc content has been the goal of many studies to improve the nutritive value of plants and their stress responses (Chen et al., 2003; Hancock and Viola, 2005; Naqvi et al., 2009).

Although Asc synthesis in animals uses a single pathway, as many as four Asc biosynthetic pathways are present in plants (Smirnoff et al., 2001; Wolucka and Van Montagu, 2003; Lorence et al., 2004; Valpuesta and Botella 2004). Of these, the d-mannose/l-galactose (l-Gal) pathway, also known as the Smirnoff–Wheeler pathway, in which Asc is generated from l-Gal, is responsible for the bulk of Asc synthesis (Wheeler et al., 1998), as suggested by the effect that mutants affecting enzymes in this pathway have on Asc content. In this pathway, l-Gal is generated from mannose-1-phosphate by the conversion of guanosine diphosphate (GDP)-mannose to GDP-l-Gal by GDP-mannose-3',5'-epimerase.
(Wolucka et al., 2001) which is then converted to L-Gal. L-Galactono-1,4-lactone is synthesized from the oxidation of L-Gal by L-galactose dehydrogenase, which is then oxidized to L-ascorbic acid by L-galactono-1,4-lactone dehydrogenase (Siendones et al. 1999; Bartoli et al. 2000).

The vtci mutant of Arabidopsis, deficient in GDP-mannose pyrophosphorylase, contains 25–30% of wild-type Asc levels (Conklin et al., 1999). The vtci2 mutant, lacking GDP-L-galactose phosphorylase (or GDP-L-galactose-hexose-1-phosphate guanyltransferase), contains 10–20% of normal Asc levels (Laing et al., 2007; Linster et al., 2007). VTC5, a parologue of VTC2, is probably responsible for the residual amount of Asc as the vtci2 vtc5 double mutant bleaches and dies within 1 week of transfer to L-Gal-free medium (Dowdle et al., 2007). Because the other identified Asc biosynthetic pathways are unable to compensate for the loss in Asc biosynthetic capacity in the vtci vtc5 mutant, the Smirnoff–Wheeler pathway is likely to be the primary Asc biosynthetic pathway in most organs of the plant.

The role of ascorbate recycling in maintaining the Asc pool size and redox state

Dehydroascorbate (DHA) is generated from the disproportionation of the monodehydroascorbate (MDHA) radical produced following the oxidation of Asc (Fig. 1). Dehydroascorbate reductase (DHAR) catalyses the reduction of DHA to Asc using glutathione (GSH) as the reductant. If DHA is not recycled to Asc, it undergoes irreversible hydrolysis to 2,3-diketogulonic acid. Asc recycling by DHAR, therefore, serves as one means for a plant to recycle DHA into Asc before it is lost from the Asc pool.

Increasing DHAR activity was first achieved by overexpressing a human DHAR gene in tobacco chloroplasts, resulting in a 2-fold increase in the Asc redox state (i.e. the ratio of Asc to DHA) without any alteration in Asc content (Kwon et al., 2001, 2003). These plants also had a lower GSH redox state. However, expression of cytosolic wheat DHAR in tobacco and maize increased both the pool size and redox state of Asc (Chen et al., 2003). Asc content in tobacco leaves increased 2- to 4-fold, with the Asc redox state increasing nearly 3-fold, while the Asc content in maize leaves and developing kernels increased ~2-fold, with a 40% increase in the Asc redox state. The increase in the redox state was due to an increase in Asc and a decrease in DHA, consistent with the function of DHAR. The Asc redox state increased in the apoplastic (which lacks DHAR activity) as well as the symplast, indicating that cytosolic DHAR regulates both. The effect of increasing DHAR expression appeared to be specific to Asc recycling as no increase in Asc biosynthetic activity was observed, although the pool size and redox state of GSH also increased (Chen et al., 2003).

That Asc recycling efficiency can be improved through increases in DHAR expression indicates that the level of DHAR expression is rate limiting, a conclusion supported by studies in other species. For example, expressing an Arabidopsis cytosolic DHAR increased Asc content in tobacco ~2-fold without affecting the Asc redox state (Eltayeb et al., 2006; Yin et al., 2010), whereas expression of an Arabidopsis cytosolic DHAR in Arabidopsis increased foliar Asc content by 2- to 4.25-fold and the redox state by 3- to 16-fold (Wang et al., 2010). A modest increase in Asc content was observed in Arabidopsis expressing a rice cytosolic DHAR (Ushimaru et al., 2006). A similar modest increase in Asc content occurred when rice DHAR was expressed in tobacco chloroplasts, but this was increased further, as was the Asc redox state, when glutathione reductase (GR) was co-expressed (Le Martret et al., 2011). Constitutive overexpression of a cytosolic tomato DHAR resulted in a 1.6-fold increase in Asc in mature green and red ripe fruit of tomato grown under low light, but foliar Asc was unchanged (Haroldsen et al., 2011).

Expression of a sesame DHAR cDNA from the constitutively active Cauliflower mosaic virus (CaMV) 35S promoter increased Asc content 1.5-fold in potato leaves and 1.6-fold in tubers, while expression of the same DHAR cDNA under the control of the patatin promoter, which directs expression specifically in tubers, increased Asc content of tubers 1.1- to 1.3-fold with no increase in leaves (Goo et al., 2008). Asc content was increased 6-fold following expression of rice DHAR under the control of a wheat glutenin promoter in maize endosperm that was also engineered to have increased levels of β-carotene and folate (Naqvi et al., 2009). Therefore, at least in these species, the level of DHAR activity contributes to their Asc pool size and/or redox state.

The presence of nuclear-encoded DHAR isoforms in the cytosol and chloroplast stroma raises the question of which isoform contributes more to the cellular Asc pool size and redox state. In a second study using potato, expression of a cytosolic potato DHAR from the CaMV 35S promoter increased foliar Asc content by >1.6-fold and >1.2-fold in tubers, whereas expression of a chloroplast-localized DHAR from potato increased foliar Asc content up to 1.5-fold with no increase in tubers (Qin et al., 2011). These results indicate that the cytosolic DHAR isoform can regulate Asc in highly different organs, but the ability of the chloroplast-localized DHAR isoform to do so may be limited to photosynthetic tissues.
In its reaction with reactive oxygen species (ROS), for example singlet oxygen (1O₂), superoxide anion (O₂⁻), hydroxyl radical (·OH), and hydrogen peroxide (H₂O₂), or as an enzyme cofactor, Asc is oxidized to the short-lived radical, MDHA, which can rapidly disproportionate non-enzymatically to produce DHA and Asc (Fig. 1). Alternatively, monodehydroascorbate reductase (MDAR) can reduce MDHA to Asc using NADPH as the reductant. Therefore, plants have evolved several mechanisms by which the oxidized forms of Asc can be recycled.

Fewer studies have examined whether MDAR expression is rate limiting. Tobacco overexpressing a cytosolic Arabidopsis MDAR had a slightly higher Asc content and redox state (Yin et al., 2010). Constitutive overexpression of a cytosolic tomato MDAR surprisingly reduced Asc content in mature green tomato fruits, but had no effect on foliar Asc content (Haroldsen et al., 2011). In contrast, constitutive expression of a cytosolic tomato DHAR produced a 1.6-fold increase in Asc in mature green and red ripe tomato fruit grown under low light without altering foliar Asc levels (Haroldsen et al., 2011). Targeting expression of a tomato MDAR to tomato chloroplasts increased foliar Asc content 1.2-fold and, as DHA decreased, the Asc redox state increased 2-fold (Li et al., 2010). Increased MDAR expression improved the chilling tolerance of tomato fruit (Stevens et al., 2008), and increasing MDAR expression in tobacco improved tolerance against salt and osmotic stresses (Eltayeb et al., 2007). From these few studies, it appears that MDAR expression might be rate limiting but perhaps less so than DHAR.

The role of ascorbate recycling in the chloroplast

Because MDHA disproportionates slowly in the chloroplast stroma where the pH is higher during exposure to light, ferredoxin (Fd) and a nuclear-encoded, stromal-localized MDAR are available to reduce MDHA to Asc (Asada, 1999) (Fig. 2). Psac of photosystem I (PSI) reduces Fd, which, in the form of photoreduced Fd (Fdred), donates electrons to NADP⁺ in a reaction catalysed by Fd-NADP⁺ reductase (FNR). Fdred can donate electrons to MDHA, generating Asc at a rate of 10⁷ M⁻¹ s⁻¹ (Miyake and Asada, 1994). Although Fdred reduces both NADP⁺ and MDHA, the rate of MDHA reduction by Fdred is 34-fold greater than by NADP⁺ (Miyake and Asada, 1994); consequently, the reduction of MDHA in the stroma occurs largely through Fd as part of the thylakoidal scavenging system (Fig. 2).

![Fig. 2. Role of ascorbate recycling in photosynthetic-related processes.](https://academic.oup.com/jxb/article-abstract/64/2/433/531153)
The presence of a nuclear-encoded, chloroplast-localized MDAR will recycle any MDHA not reduced by Fd. MDAR is a flavin adenine dinucleotide (FAD) enzyme that uses NADH ($K_m$ 5 µM) or NADPH ($K_m$ 22–200 µM) as the source of the electrons for MDHA reduction (Sano et al., 1995). Asc is also used as part of the Mehler-peroxidase reaction (Fig. 2) to maintain electron flow through PSI in order to prevent the over-reduction of photosystem II (PSII) and photodamage (Asada, 1999). In the Mehler reaction, $O_2^-$ is generated through electron transfer from PSI to oxygen, which superoxide dismutase (SOD) disproportionates to $O_2$ and $H_2O_2$. Ascorbate peroxidase (APX) then catalyses electron transfer from two molecules of Asc to $H_2O_2$ to form water and two molecules of MDHA in the stroma.

Asc can serve as a direct electron donor to PSI and PSII under conditions where the primary electron donor system is impaired, for example under high light (Mano et al., 1997, 2004), with photoreduction of MDHA by Fd (Fig. 2) maintaining electron transport when NADP$^+$ is limiting (Forti and Ehrenheim, 1993; Grace et al., 1995). MDHA is also generated in the thylakoid lumen by violaxanthin de-epoxidase (VDE) for which Asc is a cofactor (Eskling et al., 1997) (Fig. 2). VDE catalyses the conversion of violaxanthin to zeaxanthin in the xanthophyll cycle which functions in the energy-dependent, thermal dissipation of excess absorbed excitation energy. As neither Fd nor MDAR is present in the thylakoid lumen, MDHA cannot be enzymatically recycled to Asc. Consequently, lumenal MDHA disproportionates to Asc and DHA, the latter of which is transported to the stroma for recycling. This disproportionation is facilitated by the low pH of the lumen during light exposure (Asada, 1999; Mano et al., 2004).

**The role of ascorbate recycling in the cytosol**

In addition to photosynthesis, ROS are produced during specific developmental stages. $H_2O_2$ is made in seedling peroxisomes as a by-product of fatty acid $\beta$-oxidation during lipid catabolism (Fig. 3) (Mullen and Trelease, 1996; Graham and Eastmond, 2002). In order to detoxify $H_2O_2$, peroxisomes...
employ catalase (CAT) in the matrix and a membrane-bound APX and MDAR, which detoxify the H$_2$O$_2$ using Asc for this purpose, thus reducing leakage of H$_2$O$_2$ into the cytosol (Yamaguchi *et al*., 1995; Bunkelmann and Trelease, 1996; Mullen and Trelease, 1996; Jiménez *et al*., 1997; Karyotou and Donaldson, 2005). Although apparently not required for growth under normal conditions (Narendra *et al*., 2006), increasing expression from APX3, which is targeted to peroxisomes in *Arabidopsis*, increases tolerance against oxidative stress (Wang *et al*., 1999), suggesting that APX3 detoxifies H$_2$O$_2$ before it damages cellular components.

The importance of peroxisomal APX activity in limiting H$_2$O$_2$-mediated cellular damage was further supported by the finding that peroxisomal-targeted MDAR is necessary to prevent damage by peroxisomal H$_2$O$_2$. The *Arabidopsis* sugar-dependent2 (*sdp2*) mutant is deficient in peroxisomal membrane-associated MDAR4 and is conditionally seedling lethal. *sdp2* seedlings are unable to mobilize storage oil needed to support growth and have higher levels of lipid peroxidation and protein oxidation (Eastmond, 2007) (Fig. 3). The *SDP1*-encoded triacylglycerol (TAG) lipase, largely responsible for the TAG lipase activity associated with oil body membranes (Eastmond, 2006), is inactivated in *sdp2* seedlings, probably through oxidative damage (Eastmond, 2007). These findings suggest that in the absence of MDAR4, some of the peroxisomal H$_2$O$_2$ generated as a consequence of fatty acid β-oxidation escapes and causes oxidative damage to oil bodies, including the inactivation of *SDP1* TAG lipase (Fig. 3). The association of oil bodies with peroxisomes suggests that oil bodies are a target for H$_2$O$_2$ leaking from peroxisomes. Peroxisomes appear less dependent on the APX/MDAR system for protection against H$_2$O$_2$, perhaps because of CAT in the peroxisomal matrix (Eastmond, 2007). Therefore, peroxisomal MDHA-mediated recycling maintains sufficient Asc for APX to reduce H$_2$O$_2$ escaping from peroxisomes to protect the closely associated oil bodies during seedling growth.

### The role of ascorbate recycling in guard cells

Although ROS can be highly damaging, they also function as signalling molecules, for example H$_2$O$_2$ in guard cells, the latter of which controls gas exchange in leaves (Fig. 4). Under normal growth conditions, the regulation of stomatal pores can be quite dynamic, with an increase in H$_2$O$_2$ during the day followed by a decline during the night (Chen and Gallie, 2004). This diurnal change in H$_2$O$_2$ probably results from photosynthetic-related processes such as the Mehler-peroxidase reaction (water–water cycle) and photosynthesis which maintain electron transport through PSII to prevent photooxidation (Asada, 1999). Absorbed light energy exceeding the photosynthetic capacity of the photosystems, for example during peak sunlight in the afternoon, increases H$_2$O$_2$ production, triggering stomatal closure (Schroeder *et al*., 2001). ABA can also elicit H$_2$O$_2$ production as part of the signalling required to promote stomatal closure during

![Fig. 4. The role of DHAR-mediated ascorbate recycling in controlling stomatal behaviour. Under high light, H$_2$O$_2$ is produced from the Mehler reaction or from photosynthesis to maintain electron flow through the photosystems. H$_2$O$_2$ is also produced in guard cells in response to ABA signalling. H$_2$O$_2$ is reduced to H$_2$O by APX using Asc as the reductant. H$_2$O$_2$ rises during the day to a level that triggers stomatal closure. Tobacco plants suppressed for DHAR have increased H$_2$O$_2$ due to impaired recycling of Asc (B) relative to wild-type (WT) plants (A). In contrast, H$_2$O$_2$ is lower in tobacco overexpressing DHAR (C) as a result of enhanced Asc recycling. Representative images of stomata from WT tobacco (A), DHAR-suppressed plants (B), and DHAR-overexpressing plants (C) are shown with an accompanying schematic. The ascorbate–glutathione cycle is shown below each to illustrate how changes in DHAR expression affect stomatal behaviour by regulating the level of H$_2$O$_2$.](https://academic.oup.com/jxb/article-abstract/64/2/433/531153/Recycling-ascorbic-acid-improves-plant-health?redirectedFrom=doi)
conditions of water stress which signals for an increase in cytosolic Ca\(^{2+}\) concentration from H\(_2\)O\(_2\)-activated Ca\(^{2+}\) channels and from intracellular stores (Pei et al., 2000; Schroeder et al., 2001; Zhang et al., 2001). DHA levels increase during the day, a consequence of Asc consumption during H\(_2\)O\(_2\) reduction and a rate-limiting amount of DHAR to recycle DHA efficiently into Asc. The signalling function of H\(_2\)O\(_2\) in guard cells, therefore, is controlled by the rate of its production and the rate of its removal, in which Asc and DHAR play critical roles.

The importance of Asc recycling to guard cell function was illustrated in mutants with altered levels of DHAR expression. Overexpression of DHAR in tobacco resulted in an increase in Asc and a decrease in DHA, thereby increasing the Asc redox state (Chen et al., 2003). Although tobacco overexpressing DHAR grew normally under well-watered conditions, increasing Asc content and its redox state increased transpiration and water loss under normal and water stress conditions (Chen and Gallie, 2004). Increasing DHAR expression in guard cells allowed more efficient regeneration of Asc and scavenging of H\(_2\)O\(_2\), and thus maintained H\(_2\)O\(_2\) below the threshold needed to trigger stomatal closure (Fig. 4), resulting in increased stomatal area, stomatal conductance, transpiration, and water loss while decreasing guard cell responsiveness to ABA and H\(_2\)O\(_2\) signalling.

In contrast, reducing Asc recycling by suppressing DHAR expression caused a greater accumulation of H\(_2\)O\(_2\), which in turn signalled a greater degree of stomatal closure that reduced water loss up to 30% (Chen and Gallie, 2004). Consequently, following a severe water stress, DHAR-suppressed leaves retained turgor, and photochemistry was only slightly reduced relative to well-watered conditions. The importance of Asc recycling in guard cell functioning can thus be understood through the role of Asc as a scavenger of H\(_2\)O\(_2\) whereby the balance between H\(_2\)O\(_2\) production and Asc recycling establishes whether the H\(_2\)O\(_2\) concentration rises to a level that triggers stomatal closure.

The role of ascorbate recycling in response to environmental ROS

Ozone entering a plant rapidly degrades into hydroxyl radicals and other ROS which are first observed in guard cell chloroplasts and membranes but spread to neighbouring cells (Grimes et al., 1983; Mudd, 1997; Joo et al., 2005). Plants limit damage caused by environmental ROS by avoidance, namely reducing entry into leaves, or by tolerance; that is, detoxifying ROS that do enter (Taylor, 1978).

The slower responsiveness of guard cells of DHAR-overexpressing tobacco allows more ozone to diffuse into the leaf interior (Chen and Gallie, 2004, 2005). However, increasing Asc recycling activity increases the apoplastic and symplastic Asc content of all cells and thus increases their ability to detoxify ozone that does enter. This reduces the oxidative load of the leaf (i.e. lower levels of foliar and apoplastic H\(_2\)O\(_2\)) and results in a lower induction of ROS-related enzyme activities, more chlorophyll, and a higher level of photosynthetic activity following an acute exposure to ozone relative to control plants.

Conversely, a reduction in Asc recycling through the suppression of DHAR expression increases guard cell responsiveness to ozone, thereby limiting ozone diffusion into the leaf interior (Chen and Gallie, 2004, 2005). Increasing the responsiveness of guard cells to ozone, however, also limits photosynthetic activity. Moreover, the reduction in Asc recycling reduces the foliar Asc content and redox, state and, consequently, the ability to detoxify any ozone that does diffuse into the leaf interior, resulting in greater damage.

The importance of DHAR-mediated recycling of Asc in abiotic response programmes was validated by subsequent studies. The Arabidopsis AtDHAR3 mutant, which lacks cytosolic DHAR activity and therefore has a lower Asc redox state but not pool size, exhibits increased ozone sensitivity, suggesting that Asc recycling is critical in responding to environmental ROS (Yoshida et al., 2006). AtDHAR3 expression is induced following ozone exposure, further supporting the conclusion that DHAR activity is important for this response programme. Tobacco expressing a cytosolic Arabidopsis DHAR that increased Asc 2-fold exhibited enhanced tolerance to ozone as well as to drought, salt, or polyethylene glycol (Eltayeb et al., 2006).

Increasing the Asc redox state without altering the Asc pool size in tobacco expressing a human DHAR gene in chloroplasts enhanced tolerance to low temperature (15 °C) and 100 mM NaCl, as well as to 5 µM methyl viologen (MV) (which generates O\(_2\)·–), or to 200 mM H\(_2\)O\(_2\) (Kwon et al., 2003). Arabidopsis with increased DHAR expression maintained higher levels of Asc and chlorophyll with reduced levels of membrane damage than did control plants following exposure to high light, high temperature, or following MV treatment (Wang et al., 2010). Despite the small increase in Asc content, Arabidopsis expressing a rice DHAR was more tolerant to salt stress (Ushimaru et al., 2006), suggesting that even small changes in DHAR activity may improve tolerance to some environmental stresses. Expressing a cytosolic Arabidopsis DHAR in tobacco resulted in greater tolerance to aluminium and maintained a higher level of Asc in roots before and after Al treatment (Yin et al., 2010). Roots of these plants also had lower levels of H\(_2\)O\(_2\), lipid peroxidation, and DNA damage, with improved root growth.

Combining an increase in DHAR expression with ROS-detoxifying enzymes has proven to be as good as or better than increasing expression of individual antioxidant enzymes as a means to increase tolerance to abiotic stresses. Co-expression of a chloroplast-localized CuZnSOD and APX enhanced tolerance to MV and salt, which a chloroplast-localized DHAR improved even further when co-expressed (Lee et al., 2007), indicating that the beneficial effect of increasing DHAR expression can be used in a combinatorial approach with other enzymes involved in oxidative stress. Tobacco overexpressing a combination of chloroplast-localized DHAR and GR or glutathione-S-transferase (GST) and GR were more efficient at reducing H\(_2\)O\(_2\) during chilling stress than plants overexpressing DHAR or GST alone. Although expression of each
single transgene failed to confer tolerance to MV-induced oxidative stress, the combinatorial expression of DHAR and GR or GST and GR did (Le Martret et al. 2011), suggesting that increasing chloroplastic DHAR and GR recycling activities increases tolerance to abiotic stress.

Increasing MDAR-mediated Asc recycling can also improve abiotic tolerance. Tomato seedlings overexpressing a chloroplast-targeted tomato MDAR that slightly increased Asc content and doubled the Asc redox state had a lower oxidative load, reduced membrane damage, higher net photosynthetic rate ($P_n$), higher maximal PSII photochemical efficiency ($F_\text{v}/F_\text{m}$), and increased fresh weight when subjected to low or high temperatures (Li et al., 2010). Similar results were obtained following MV treatment. In contrast, antisense transgenic lines exhibiting 54–60% of the wild-type level of MDAR activity with lower Asc content and redox state had a higher oxidative load, greater membrane damage, lower $P_n$, lower $F_\text{v}/F_\text{m}$, and reduced fresh weight under the same stress conditions. These observations suggest that increasing chloroplastic MDAR expression can improve the tolerance of tomato seedlings to certain types of environmental stress, although whether the same protection would be afforded to adult plants remains to be determined.

A salt-tolerant cultivar of pea exhibited greater induction of MDAR expression than did a salt-sensitive cultivar in response to salt stress, although no correlation between DHAR expression and salt tolerance was observed (Hernández et al., 2001), suggesting that MDAR activity may be important during salt stress. This was supported by the observation that overexpression of Arabidopsis MDAR in tobacco increased tolerance to ozone and had reduced $\text{H}_2\text{O}_2$ and increased photosynthetic activity when exposed to salt (Eltabey et al., 2007). An increase in Asc content was observed in tobacco roots in which an Arabidopsis cytosolic MDAR was overexpressed when grown under normal conditions, but not in the presence of Al, whereas DHAR-overexpressing plants had higher Asc content prior to and following Al treatment (Yin et al., 2010). Moreover, no difference in root growth or in the degree of DNA damage was observed between MDAR-overexpressing and wild-type plants, suggesting that overexpression of DHAR, but not MDAR, maintained a higher level of Asc in the presence of Al.

The role of ascorbate recycling during plant growth

The importance of Asc recycling for plant growth was demonstrated in tobacco suppressed for DHAR expression which experienced reduced rates of leaf expansion, slower shoot growth, delayed flowering time, and reduced foliar dry weight (Chen and Gallie, 2006). These phenotypes were accompanied by reduced leaf performance as measured by the preferential loss of chlorophyll $a$, reduced ribulose biphosphate carboxylase/oxygenase, reduced photosynthetic activity in young leaves, as well as the premature senescence of mature leaves. The lower photochemistry was not due to reduced $\text{CO}_2$ diffusion because the level of substomatal $\text{CO}_2$ was elevated over wild-type levels, suggesting that $\text{CO}_2$ was not being used for photochemistry as efficiently. The reduction in photosynthetic activity did correlate with impaired xanthophyll cycle activity and qE [energy dependent non-photochemical quenching (NPQ)] which was accompanied by increased photoinhibition (qI) as indicated by a slower recovery following exposure to high light (Chen and Gallie, 2008). Reductions in the quantum yield of PSII ($\phi\text{PSII}$) and the electron transport rate (ETR) were also observed, while the level of ROS and lipid peroxidation increased, suggesting that reduced Asc recycling probably reduced growth due to a ROS-mediated reduction in leaf function and photosynthetic activity (Chen and Gallie, 2006, 2008). In contrast, increasing DHAR expression did not substantially increase growth relative to wild-type tobacco, although higher levels of RbcL, chlorophyll, and photosynthetic activity were observed in pre-senescent leaves (Chen and Gallie, 2006).

Increasing DHAR expression resulted in increases in xanthophyll pigment and chlorophyll pool sizes, as well as in the ETR and in $\text{CO}_2$ assimilation, particularly at high light intensities, while ROS and qI were reduced (Chen and Gallie, 2008). Thus, DHAR regulates NPQ and contributes to photoprotection during leaf ageing. Because transport of Asc in and out of the chloroplast occurs (Horemans et al., 2000), cytosolic DHAR expression can affect Asc-requiring processes in the chloroplast (Chen and Gallie, 2008), suggesting that DHAR maintains photosynthetic functioning by supporting xanthophyll cycle activity and limiting ROS-mediated damage, which in turn affects the rate of leaf ageing that ultimately affects plant growth.

Asc content is also correlated with seed development and seedling growth. Asc is present in its reduced form during early embryo development but undergoes progressive oxidation so that DHA predominates by seed maturity (Arrigoni et al., 1992; De Gara et al., 1997; Tommasi et al., 2001). During germination, DHA is rapidly recycled into Asc in the absence of Asc biosynthesis (De Gara et al., 1997; Tommasi et al., 2001). Exogenous $\text{H}_2\text{O}_2$ induced MDAR activity in germinating pea seeds (Barba-Espin et al., 2010), suggesting that ROS generated during germination may regulate MDAR expression. Presumably, the changes in the Asc redox state during embryo development and germination are orchestrated by DHAR and MDAR.

The role of ascorbate recycling during reproduction

Early work in onion roots demonstrated that Asc promotes cell division by inducing $G_1$ to $S$ progression of quiescent centre (QC) cells, which lie proximal to the root cap (Liso et al., 1984; Arrigoni et al., 1989; Innocenti et al., 1990; Arrigoni, 1994; Citterio et al., 1994). Maize QC cells contain higher levels of auxin and auxin-induced ascorbate oxidase (AO) activity, with lower levels of Asc than found in adjacent meristematic cells (Kerk and Feldman, 1995). The auxin-mediated induction of AO expression reduces the Asc content of QC cells, thereby maintaining them in $G_1$ (Kerk and Feldman,
High Asc content correlates with rapid tobacco BY-2 cell growth (Kato and Esaka, 1999) and meristematic activity in pea (de Pinto and De Gara, 2004), supporting the notion that Asc promotes mitotic activity.

The effect that DHAR-mediated Asc recycling activity has on cell division can have profound consequences on embryo development as increasing DHAR expression in tobacco induced monozygotic twinning and polycotyly (Chen and Gallie, 2012). The increase in Asc recycling altered cell polarity such that a longitudinal instead of transverse division of the zygote occurred, generating two genetically identical zygotes (Fig. 5). Direct injection of Asc into ovaries of wild-type tobacco within the first 2 d following pollination before the zygote has divided phenocopied the DHAR-induced twinning, confirming that it was the DHAR-mediated increase in Asc that resulted in twinning (Chen and Gallie, 2012). The induction of polycotyly by Asc demonstrated that Asc probably regulates cell division throughout embryo development. The induction of monozygotic twinning and polycotyly by Asc can be understood by its effect on cell polarity and cell division. A change from the normal transverse zygotic division results in the loss of the positional cues needed for proembryo development and instead produces two or more genetically identical zygotes that develop into independent embryos. Similarly, altered cell division during the specification of cotyledon-forming fields can increase cotyledon number. Although increased DHAR-mediated recycling of Asc may affect cell division throughout plant development, such changes would not be as noticeable as changes in embryo or organ number.

**Conclusions**

From these studies, we can conclude that Asc recycling is as important to plant health and development as is Asc biosynthesis itself. Because increases in Asc biosynthetic capacity can take hours or longer (Bartoli et al., 2006), efficient Asc recycling may be critical in responding to sudden increases in light or the imposition of one or more stresses. Regulating Asc recycling activity is essential to maintain ROS at a level that minimizes oxidative damage but permits their important signalling function. For example, the elevated water loss and wilting caused by increased DHAR expression illustrates the importance of maintaining an appropriate balance between avoiding ROS-mediated damage and preserving ROS signalling in guard cells. Asc recycling is equally important to plant development, as the induction of monozygotic twinning and polycotyly caused by increased DHAR expression demonstrates. Consequently, the synthesis and recycling of Asc serve as two integrated means to regulate plant health and development. Given its many functions, it is likely that our appreciation of the importance of Asc recycling to plants will continue to grow in coming years.

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Recycling ascorbic acid improves plant health

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