

Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence

P. L. CONKLIN¹ & C. BARTH²

¹Department of Biological Sciences, Bowers Hall, State University of New York College at Cortland, Cortland, NY 13045, USA and ²Boyce Thompson Institute for Plant Research, Tower Road., Ithaca, NY 14853, USA

ABSTRACT

Ascorbic acid is a well-known antioxidant and cellular reductant with an intimate and complex role in the response of plants to ozone. It is clear from a number of studies that sensitivity to ozone is correlated with total ascorbic acid levels, and that a first line of defence against the reactive oxygen species generated in the apoplastic space by ozone is ascorbic acid. For activity, ascorbic acid must be in the fully reduced state. Therefore, both the rate of ascorbic acid synthesis and recycling via dehydroascorbate and monodehydroascorbate reductases are critical in the maintenance of a high ascorbic acid redox state. Active transport of ascorbic acid across the plasma membrane is necessary to achieve reduction of oxidized ascorbic acid by cytoplasm-localized reductases. It has been known for some time that the chlorotic lesions produced by exposure to ozone are not unlike lesions produced by the hypersensitive response to avirulent pathogen attack. Surprisingly, activation of a defence gene-signalling network by both ozone and pathogens is influenced by the level of ascorbic acid. Indeed, in addition to acting simply as an antioxidant in the apoplastic space, ascorbic acid appears to be involved in a complex phytohormone-mediated signalling network that ties together ozone and pathogen responses and influences the onset of senescence.

Key-words : antioxidant; apoplast; Arabidopsis; ascorbate; ascorbic acid; cofactor; hydroxylation; metabolism; mutant; ozone; pathogens; senescence; transport.

Abbreviations : AA, L-ascorbic acid; ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylate; APX, ascorbate peroxidase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GMPase, GDP-mannose pyrophosphorylase; GSH, reduced glutathione; GSSG, oxidized glutathione; HR, hypersensitive response; JA, jasmonic acid; MDA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; NAT, Na⁺-dependent L-ascorbic acid transporter; NCED, nine-*cis*-epoxycarotenoid dioxy-

genase; PAL, phenylalanine ammonium lyase; PCD, programmed cell death; PM, plasma membrane; ROS, reactive oxygen species; PR, pathogenesis-related; SA, salicylic acid; SAG, senescence associated gene; SAR, systemic acquired resistance.

INTRODUCTION

For many years, it has been clear that the well-known antioxidant and cellular reductant, L-ascorbic acid (AA) has a significant role in prevention of the cellular damage imposed by ozone. As early as 1960, foliar application of AA was demonstrated to act as an ozone protectant (Freebairn 1960). It is the facile oxidation of AA to the primary oxidation product, monodehydroascorbate radical (MDA) that defines the biological activity of this small molecule. MDA that is not reduced by the reductase activity detailed below disproportionate to the labile dehydroascorbate (DHA) and AA. If DHA is not rapidly reduced enzymatically back to AA, it is lost from the cell. Despite the high turnover of AA (Pallanca & Smirnoff 2000) intracellular levels of AA are in the millimolar range. A combination of *de novo* synthesis and efficient AA recycling via reductases (see below for details) are obviously important in maintenance of this high AA concentration. Realization of the importance of this *de novo* synthesis led to the recent elucidation of the major AA biosynthetic pathway in plants (Wheeler, Jones & Smirnoff 1998). This pathway includes the intermediates GDP-mannose and L-galactose and has been supported in part by the identification of the *Arabidopsis thaliana* *VTC1* gene, which encodes a GDP-mannose pyrophosphorylase (Conklin *et al.* 1999). Several additional genes encoding the biosynthetic enzymes of this major pathway have been identified including L-galactose dehydrogenase (Gatzek, Wheeler & Smirnoff 2002), GDP-mannose 3,5-epimerase (Wolucka *et al.* 2001), and L-galactono-1,4-lactone dehydrogenase (Imai *et al.* 1998). The latter enzyme catalyzes the final step in the AA biosynthetic pathway, the conversion of L-galactono-1,4-lactone into AA. However, some studies suggest that there is an alternative AA biosynthetic pathway, which occurs through D-galacturonic acid that is converted into L-galactonic acid by D-galacturonic acid reductase (Isherwood & Mapson

Correspondence: Patricia L. Conklin. Fax: 607-753-2927; e-mail: conklinp@cortland.edu

Table 1. Known or predicted ascorbate peroxidase (APX) loci in *Arabidopsis thaliana*

Gene Designation	Locus	Localization ^a
APX1	At1g07890	cytoplasm
APX1b	At3g09640	cytoplasm
APX, putative	At4g35970	transmembrane
tAPX	At1g77490	thylakoid
APX, putative	At4g09010	thylakoid lumen
APX3	At4g35000	peroxisome

^aBased on annotations from The Institute for Genomic Research (TIGR), The Arabidopsis Information Resource (TAIR) and the Munich Information Center for Protein Sequences (MIPS).

1956; Loewus & Kelly 1961). L-galactonic acid can then be converted into L-galactono-1,4-lactone, the direct precursor of AA. Recently, Agius and coworkers demonstrated that over-expression of D-galacturonic acid reductase in ripe strawberry fruit increases AA levels (Agius *et al.* 2003).

Although it is tempting to categorize AA function simply based on its obvious role as a small molecular antioxidant, it has become increasingly clear that AA function is intertwined in a complex network that meshes the plant's responses to ozone, pathogens, and the onset of senescence. Below, we attempt to summarize the role of AA in detoxification of the reactive oxygen species (ROS) generated by ozone, and furthermore to clarify the more global role of AA in the response of a plant to the environment.

ASCORBIC ACID AND OZONE TOLERANCE

The role of ascorbate peroxidase

Ascorbate peroxidase (APX) isoenzymes are present in most subcellular compartments within the plant cell (see Table 1 for a list of *Arabidopsis* APX loci and the localization of the encoded protein products) and promote the dismutation of hydrogen peroxide to water with the concurrent oxidation of AA to MDA (Shigeoka *et al.* 2002). Hydrogen peroxide has been shown to be a major player in the regulation of cytosolic ascorbate peroxidase gene expression (Shigeoka *et al.* 2002). In response to ozone exposure, cytosolic APX mRNA levels are elevated (Willekens *et al.* 1994; Conklin & Last 1995; Kubo *et al.* 1995; Orvar, McPherson & Ellis 1997), suggesting that increased levels of this isoenzyme have a functional role in detoxification of the elevated levels of ROS generated by ozone. Indeed, tobacco plants that express an antisense version of cytosolic APX and have clearly decreased cytosolic APX activity are more sensitive to ozone exposure (Orvar & Ellis 1997). One might therefore predict that elevation of APX activity via a transgenic approach may increase ozone resistance. However, transgenic over-expression of cytosolic APX in an ozone-sensitive tobacco line failed to alter its sensitivity (Orvar & Ellis 1997). Similarly, over-expression of chloroplastic APX also does not provide protection against either chronic or acute ozone exposure (Torsethau-

gen *et al.* 1997). Therefore, it is apparent that APX activity is an essential component of ozone tolerance but greatly elevating APX alone cannot alleviate ozone-generated ROS. This may be due at in part to limitations on the availability of the reduced AA in the cytosol that is utilized as a substrate by APX (see Fig. 1). It is interesting to note that (in spinach) chloroplastic APX transcript levels are unresponsive to stresses imposed by a variety of treatments such as methyl viologen, drought, ABA, and salinity, suggesting that the genes encoding the chloroplastic APX isoenzymes are constitutively expressed (Yoshimura *et al.* 2000).

Total ascorbic acid levels correlate with ozone tolerance

Ascorbic acid is an integral weapon in the defence against ROS generated by ozone. It is therefore not surprising that the levels of total AA correlate with ozone resistance across a wide variety of plant species. In a recent study, the AA pool was approximately two times higher in the ozone-resistant Bel B tobacco relative to the ozone-sensitive Bel W3, in both control and ozone-treated (150 p.p.b. ozone, 5 h) leaves (Pasqualini *et al.* 2002). Furthermore, soybean leaflets of an ozone-tolerant cultivar Essex maintained higher total AA levels than the ozone sensitive-Forrest cv. under both ozone-treatment and control conditions (Robinson & Britz 2000). The AA-deficient *Arabidopsis thaliana* mutant *vtc1* contains approximately one-third of the wild-type level of AA and was isolated by virtue of its sensitivity to ozone. Restoration of ozone-resistance in this mutant was achieved by artificial elevation of AA by pre-treatment with the AA biosynthetic precursor, L-galactono-1,4-lactone (Conklin *et al.* 1997). Similarly, elevation of total AA in radish with this biosynthetic precursor (Maddison *et al.* 2002) and in barley by direct AA feeding (Machler *et al.*

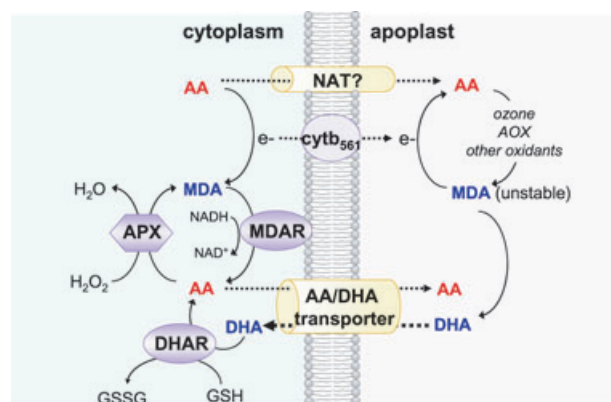


Figure 1. Oxidation-reduction reactions of ascorbic acid (AA) in the cytoplasm and apoplast along with known or proposed AA/DHA transport mechanisms across the plasma membrane. Monodehydroascorbate radical (MDA), monodehydroascorbate reductase (MDAR), ascorbate oxidase (AOX), dehydroascorbate (DHA), dehydroascorbate reductase (DHAR), Na⁺-dependent L-ascorbic acid transporter (NAT), and ascorbate peroxidase (APX).

1995) was found to alleviate ozone-induced injury. These studies confirm and extend the early results of H.T. Freebairn who, in 1960 clearly demonstrated that pre-treatment of Pinto beans with AA resulted in partial to complete ozone resistance (Freebairn 1960).

Apoplastic ascorbic acid – the front line of ozone defence

Within the apoplast, the interception and detoxification of ozone and/or the reactive oxygen intermediates that result from its dissolution is one of the main defensive mechanisms thought to govern resistance of plants to ozone. It has been estimated that approximately 10% of the AA pool is localized within the apoplastic space (Fig. 1; Noctor & Foyer 1998) or less than 2 mM if one assumes that 10% of fresh weight is attributable to the apoplast (Burkey, Eason & Fiscus 2003). Several older studies suggest that the pool of apoplastic AA is an important component of the defence against ozone injury (Castillo & Greppin 1988; Luwe, Takahama & Heber 1993; Polle, Wieser & Havranek 1995). For example, in one study, younger leaves, containing the highest level of apoplastic AA, were also the most resistant to ozone exposure (Luwe *et al.* 1993). In the ozone-tolerant radish in which elevation of intracellular AA was achieved via pre-treatment with L-galactono-1,4-lactone, apoplastic AA was also elevated (Maddison *et al.* 2002). In contrast, total AA was found to be decreased to 23% of wild type in the ozone-sensitive AA-deficient *vtc1* mutant (Veljovic-Jovanovic *et al.* 2001).

Although tolerance to ozone has been shown to correlate with an elevated level of AA in the apoplast, this relationship does not hold in all circumstances. In snap bean (*Phaseolus vulgaris* L.) genotypes grown under low concentrations of ozone, apoplastic AA was significantly higher (six to eight times) in the resistant relative to the sensitive genotypes. Exposure to higher levels of ozone over an 8 d period revealed no such global correlation between ozone tolerance and apoplastic AA levels across several genotypes with a wide range of ozone sensitivities (Burkey & Eason 2002). However, if one focuses on a comparison between the most ozone-sensitive (Provider) and resistant snap bean genotypes (S156), the concentration of total AA in the apoplast was consistently higher (two times) in Provider relative to S156 regardless of the ozone treatment (Burkey *et al.* 2003). Mathematical modelling suggests that, in general, the concentration of apoplastic AA should be sufficient to directly detoxify the majority of ozone absorbed into the leaf (Plochl *et al.* 2000). However, as the authors of this study point out, the variation in ozone sensitivities between different species clarifies the fact that other detoxification mechanisms are also involved.

Ascorbic acid redox state in the apoplast

Apoplastic AA is most likely an important first line of defence against ozone, but it is essential that this antioxidant is maintained in a reduced state to effect this defensive

action. Therefore, the redox status of AA in the apoplast cannot be overlooked. The previously mentioned ozone-resistant snap bean genotype 'Provider' maintained a strikingly higher apoplastic AA:(AA + DHA) ratio relative to the sensitive genotype '156' upon exposure to ozone (Burkey *et al.* 2003).

That the redox state of AA is an important factor in protection against ozone-induced oxidative injury is clearly demonstrated in transgenic plants that over-express ascorbate oxidase in the apoplast. Ascorbate oxidase oxidizes AA to MDA, which rapidly disproportionates to DHA and AA (Fig. 1). In these transgenic tobacco lines, the pool of apoplastic AA is largely oxidized. In one study, the reduced AA in the apoplast fell from 40% of the total in wild type to only 3% in the over-expression line (Pignocchi *et al.* 2003). In a second independent study, the pool of reduced apoplastic AA (which was 30% in the wild-type line) fell to undetectable levels in the transgenic line, and resulted in greatly enhanced foliar injury upon chronic ozone exposure (Sanmartin *et al.* 2003). Tobacco expressing ascorbate oxidase in the antisense orientation maintain a higher percentage of reduced AA (66 versus 40% in the wild type; Pignocchi *et al.* 2003) but the effect of this altered AA redox status on ozone sensitivity has not been reported. In both studies, over-expression of ascorbate oxidase in the apoplast did not dramatically affect total AA levels nor result in a large change in the total AA redox status (Pignocchi *et al.* 2003; Sanmartin *et al.* 2003).

A number of studies have shown that the proportion of apoplastic AA in the reduced state is much lower than that of the intracellular AA (Noctor & Foyer 1998). This fact suggests that reduction of apoplastic MDA and DHA and/or transport of (fully reduced) AA into the apoplast as a limiting factor in maintenance of a high apoplastic redox status. Reduction of apoplastic AA appears to be intimately linked to transport across the plasma membrane.

Recycling of MDA and DHA to ascorbic acid

An ascorbate-reducible transmembrane b-type cytochrome *c* (plant PM cyt b_{561}) has been implicated for the reduction of MDA back to AA within the apoplastic compartment (Fig. 1). Three Arabidopsis genes that encode putative plant PM cyt b_{561} proteins have been found (Asard *et al.* 2001) and plant PM cyt b_{561} proteins that are fully ascorbate-reducible have been purified from bean, corn and Arabidopsis (Trost *et al.* 2000; Berczi, Luthje & Asard 2001; Berczi, Caubergs & Asard 2003). Using AA-loaded 'right-side-out' vesicles (vesicles with the apoplastic face exposed), plant PM cyt b_{561} could be shown to transport electrons from the internal AA across the plasma membrane to external electron acceptors such as MDA (Asard, Horemans & Caubergs 1992). The functional significance of this plant PM cyt b_{561} has not yet been demonstrated in intact plants. It would be of considerable interest to alter the concentration of this protein in the plasma membrane via insertional inactivation, RNA interference, or over-expression and monitor the affect on

apoplastic AA redox status and associated sensitivity to ozone stress.

NADH-dependent monodehydroascorbate reductases that reduce MDA back to AA have been localized to several subcellular compartments including the plastids, mitochondria, microbodies, and cytoplasm (Arrigoni & De Tullio 2002 for a review). Indeed, as seen in Table 2, at least five predicted MDAR genes are present in the *Arabidopsis* genome.

A monodehydroascorbate reductase (MDAR) has been found to be associated with the inner (cytoplasmic) side of the plasma membrane in spinach (Fig. 1). This enzyme has been shown to preferentially utilize NADH as an electron donor (Berczi & Moller 1998). Models have been proposed in which this MDAR is involved in recycling AA that is oxidized to MDA on the cytoplasmic side of the plasma membrane by the action of the above mentioned plant PM cyt b_{561} protein (Pignocchi & Foyer 2003). We are aware of no evidence indicating that MDAR activity in general is regulated in response to the elevated ROS generated by ozone. In one study, it was noted that fumigation of *Arabidopsis thaliana* with 0.1–0.15 p.p.m. ozone for up to a week had no significant effect on total MDHAR activity (Kubo *et al.* 1995). However, at least in one species, it is clear that cytosolic MDHAR expression is not merely constitutive but can be varied at the level of mRNA. The abundance of a putative cytosolic MDAR mRNA from tomato (*Lycopersicon esculentum* Mill.) was shown to be inversely proportional to total AA and greatly elevated in response to wounding (Grantz, Brummell & Bennett 1995).

Transport of ascorbic acid/DHA across the plasma membrane

Similar to MDAR, glutathione-dependent dehydroascorbate reductases (DHAR) that reduce DHA back to AA, have also been localized to several subcellular compartments and at least five DHAR loci have been found in the *Arabidopsis* genome (Table 2).

In addition to the probable reduction of MDA in the apoplast via cyt b_{561} and a plasma membrane associated MDAR, there is much evidence suggesting that the apoplastic DHA that disproportionates from MDA is actively transported across the plasma membrane and reduced by a DHAR in the cytosol (Fig. 1; for a recent review see Horemans, Foyer & Asard 2000). Although the probable existence of an AA carrier in the plasma membrane had been suspected for some time (Mozafar & Oertli 1993), it was not until 1997 that Horemans and colleagues demonstrated that this carrier had preferential high affinity for DHA (Horemans, Asard & Caubergs 1996). This transporter of DHA across isolated plasma membranes and intact leaves relies on a proton gradient across the membrane for activity (Rautenkranz *et al.* 1994; Horemans, Asard & Caubergs 1998; Kollist, *et al.* 2001). In AA-loaded plasma membrane vesicles, DHA transport into the vesicles is enhanced, which led to the suggestion that transport of DHA into the

Table 2. *Arabidopsis thaliana* loci predicted to encode dehydroascorbate reductases (DHAR) and monodehydroascorbate reductases (MDAR)^a

Gene Product	Locus	Localization ^a
DHAR	At1g19550	unknown
	At1g19570	cytoplasm
	At1g75270	cytoplasm/membrane
	At5g16710	plastids
	At5g36270	cytoplasm/membrane
MDAR	At1g63940	plastids, mitochondria ^b
	At3g09940	cytoplasm
	At3g27820	mitochondria
	At3g52880	cytoplasm
	At5g03630	cytoplasm

^aBased on annotations from The Institute for Genomic Research (TIGR), The Arabidopsis Information Resource (TAIR) and the Munich Information Center for Protein Sequences (MIPS). ^bDual-targeted to both plastids and mitochondria (Obara, Sumi & Fukuda 2002).

cytosol is concurrent with export of AA out of the cell (Horemans *et al.* 1998). The activity of this transporter changes at different times in the cell cycle, indicating that DHA/AA transport across the plasma membrane is a regulated process (Horemans *et al.* 2003). However, as protoplasts with ten to twenty times elevated levels of AA did not take up DHA at significantly higher rates than control protoplasts, it has been speculated that the millimolar concentration of AA normally present in the cytosol is not limiting this transport system (Horemans *et al.* 2003). Transport of DHA across the plasma membrane of the AA-deficient *vtc1* cells is currently being quantified to further investigate the possible role of internal AA concentrations on the rate of DHA/AA transport (Horemans personal comm.). It may indeed be that it is not the rate of transport of DHA/AA that is limiting but rather the cytosolic recycling of DHA back to AA, as detailed in the next section of this review.

In the plant plasma membrane, other AA transporters may exist and perhaps several different carriers are present (for a review see Horemans *et al.* 2000). In animals, the major carriers of AA across the plasma membrane are nucleobase Na⁺-dependent L-ascorbic acid transporters (NATs; Tsukaguchi *et al.* 1999). Although a number of plant NATs (Fig. 1) have been identified, none to-date have been shown to be involved in active AA transport (Argyrou *et al.* 2001; <http://www.uni-frankfurt.de/fb15/botanik/mcb/AFGN/fluegge.htm>).

It is not known whether DHA/AA transport across the plasma membrane is altered in response to ozone. However, early observations that AA accumulates in the apoplast upon exposure to ozone led to the speculation that indeed, this transport may be regulated (Horemans *et al.* 2000).

Recycling of ascorbic acid in the apoplast and cytosol

Reduction of DHA back to AA may be a limiting factor in the resistance to ozone. The strong positive relationship between ozone resistance and the total pool size of AA was discussed earlier. It is logical to hypothesize that this total AA pool is dependent on both the rate of synthesis, and the rate of reduction of MDA and the more labile DHA. The rate of AA synthesis is regulated to a large degree by light intensity (Smirnoff & Pallanca 1996). Turnover of AA via loss through DHA decay is not insignificant. In pea seedlings, approximately 40% of the total AA pool was metabolized over a 22 h period (Pallanca & Smirnoff 2000). This turnover may be due in large part to limiting DHAR activity. In a recent study, transgenic maize and tobacco that over-express a wheat DHAR had significantly elevated total AA (approximately two to four times) and the ratio of reduced AA to total AA (the AA redox status) rose from 1.5 to more than 4 in the tobacco transgenics (Chen *et al.* 2003). If these transgenics have elevated apoplastic AA, one might predict that they may be more resistant to ozone and other ROS-generating environmental stresses. In a similar study, a human DHAR gene was over-expressed in transgenic tobacco and targeted to the chloroplasts. Although these plants did not have elevated total AA relative to non-transgenic controls, the AA:(DHA + AA) ratio increased to approximately twice. The DHAR-overproducing transgenics were more tolerant to methyl viologen (which primarily generates superoxide in the chloroplasts), hydrogen peroxide, low temperature, and salt-induced stresses (Kwon *et al.* 2003), suggestive of a limitation in the ability of non-transgenic plants to recycle DHA produced in response to ROS.

Ozone is a major air pollutant that may negatively affect plant growth, development and thus productivity, if the plant antioxidant capacity is not sufficient (Heagle, Miller & Pursley 2003). High levels of AA are a prerequisite to efficiently detoxify ROS in order to reduce ozone-induced tissue damage. The ozone response, on the other hand, mimics pathogen-induced responses and can activate premature senescence (Miller, Arteca & Pell 1999). Therefore, ozone is a useful tool to study plant-signalling pathways triggered upon pathogen and ozone exposure as well as during senescence. Recent studies reveal evidence that AA plays an important role in both plant–pathogen interactions and senescence.

OZONE, PATHOGENS, AND SENESCENCE – CONNECTIONS WITH ASCORBIC ACID

Ozone response resembles the hypersensitive response in incompatible plant–pathogen interactions

Ozone has become widely used in studies of the physiological responses of plants to oxidative stress. Acute exposures

to ozone (150–300 p.p.b) for a short time period (4–6 h) cause necrotic lesions on leaves and induce plant reactions that resemble the hypersensitive response (HR), suggesting similarities between ozone- and pathogen-induced responses (Kangasjärvi *et al.* 1994; Sharma & Davis 1994; Sandermann *et al.* 1998; Rao, Koch & Davis 2000a).

HR is a form of programmed cell death (PCD) in plants. HR is considered part of a complex defence response to microbial pathogens in which death of host cells at the site of avirulent pathogen entry occurs within a few hours of pathogen attack (incompatible interaction, i.e. plant is resistant to the invading pathogen) (Crute *et al.* 1994; Katagiri, Thilmony & He 2002).

The similarity between ozone responses and the HR-like PCD in plants is related to the occurrence of ROS in the apoplast and the induction of defence responses. ROS that derive from ozone exposure are thought to trigger an oxidative burst in affected cells, resulting in a variety of biochemical changes (Schraudner *et al.* 1998; Rao & Davis 1999; Pellinen, Palva & Kangasjarvi 1999). Similarly, associated with pathogen-induced HR is a rapid oxidative burst at the site of microbial infection. ROS production during HR (and recognition of the invading pathogen) and upon ozone exposure is presumably mediated by a NAD(P)H oxidase localized in the plasma membrane (Levine *et al.* 1994; Rao & Davis 2001). ROS-induced signal-transduction networks appear to involve a Map(K) signalling cascade pathway(s) (Vranova, Inze & Van Breusegem 2002).

Generally, ROS formed during ozone exposure (Rao & Davis 2001) and HR (Leon, Lawton & Raskin 1995; Ryals *et al.* 1996) activate ethylene, salicylic acid (SA) and jasmonic acid (JA) signalling pathways. These pathways serve to induce defence-gene expression to counteract the invading pathogen and to minimize lesion formation in plants exposed to ozone. Ethylene synthesis and emission increase in plants exposed to ozone (Overmyer *et al.* 2000). Ethylene triggers PCD in the accelerated cell death mutant (*acd1*) (Greenberg & Ausubel 1993) and is involved in regulating PCD in plant–pathogen interactions (Bent *et al.* 1992). SA signalling is required for ozone-induced cell death responses (Overmyer, Brosche & Kangasjarvi 2003; Vahala *et al.* 2003), induction of pathogenesis-related proteins (PR) (Greenberg 1996; Dong 1998; Dempsey, Shah & Klessig 1999), and systemic acquired resistance (SAR – increased systemic acquired resistance to subsequent infection by a broad range of pathogens) (for reviews see Ryals *et al.* 1996; Shah & Klessig 1999). JA biosynthesis is induced in ozone-treated Arabidopsis and hybrid poplar plants within several hours of treatment (Koch *et al.* 2000; Rao *et al.* 2000b). Treatment with JA has been shown to reduce the extent of cell death in tobacco (Orvar *et al.* 1997). Arabidopsis mutants constitutively expressing JA, such as *cev1*, are more resistant to powdery mildew (Ellis & Turner 2001). These plant hormones do not act independently in response to ozone and/or pathogens, but rather in a complex signalling network (Dong 1998; Rao & Davis 2001).

The ascorbate-deficient mutant *vtc1* and the role of ascorbic acid in this signalling network

AA is a major cellular antioxidant involved in ozone-induced ROS detoxification as detailed above. As ROS are responsible for the triggering of a phytohormone-regulated signal transduction pathway in response to pathogens and ozone, the role of AA in this response was investigated. The AA-deficient *Arabidopsis* mutant *vtc1* (vitamin C-deficient) was employed in several studies to address this question. The *vtc1* mutant was isolated by its sensitivity to ozone, but was also found to exhibit increased sensitivity to other abiotic stress factors, such as sulphur dioxide, UV-B radiation and freezing (Conklin, Williams & Last 1996).

In a recent study (Pastori *et al.* 2003), *vtc1* was subjected to a large-scale microarray analysis to identify genes that are differentially expressed in the AA-deficient mutant relative to the wild type in the absence of added ROS. One hundred and seventy-one transcripts that were either increased or decreased in *vtc1* versus wild type were identified. Specifically, the most dramatic changes in transcript abundance were observed in genes involved in plant defence responses against biotic stress. PR proteins, such as PR-1, PR-2 and PR-5, and lytic enzymes, such as β -glucanases and chitinases, were significantly increased in *vtc1* compared to wild type. In contrast, major antioxidant enzymes, such as catalase and APX were largely unaffected in *vtc1*. Interestingly, when the AA content was artificially elevated by feeding with 10 mM ascorbate resulting in AA levels similar to wild type treated equally, the transcript abundance was reversed in *vtc1*, indicating that low AA induces defence responses and high ascorbate suppresses the induction of defence genes.

One would predict that constitutively induced defence-gene expression in *vtc1* might correlate with resistance to pathogens. Barth *et al.* (2004) tested this hypothesis with *vtc1* and wild type, using the virulent bacterium *Pseudomonas syringae* pv. *maculicola* ES4326 and the virulent fungus *Peronospora parasitica* pv. *Noco*, the cause of downy mildew. The bacterial growth of *P. syringae* was significantly lower in *vtc1* than in wild type. In addition, hyphal development and fungal conidiophore production was significantly reduced in *vtc1* and also *vtc2*, a second non-allelic AA-deficient mutant. PR-1 and PR-5 proteins were of higher abundance in *vtc1* than in wild type, particularly within the first 24 h post-inoculation with *P. syringae*, as revealed by Western blot analysis. This supports and extends the observation by Pastori *et al.* (2003) of elevated PR transcript accumulation in this mutant.

Induction of the PR genes in *vtc1* may be due to an SA-dependent pathway as the total SA content was found to be approximately six-fold higher in *vtc1* than in wild type. Virulent pathogens have been reported to induce non-specific resistance responses via the induction of SA synthesis and PR proteins (Glazebrook *et al.* 1997; Rogers & Ausubel 1997). However, the defence responses elicited by virulent pathogens are either activated more slowly and/or

they are activated to lower levels than the defence response induced by avirulent pathogens. An oxidative burst elicited by virulent pathogens is usually very small or absent (Crute *et al.* 1994 and references therein). Counter to our evidence suggesting a role for SA in induction of PR genes in *vtc1*, increased transcript levels of phenylalanine ammonium lyase (PAL), an enzyme that is required for the synthesis of SA, were not observed. Pastori *et al.* therefore concluded that the constitutive induction of PR proteins in *vtc1* occurs through an SA-independent pathway.

PR gene induction in the AA-deficient mutant may occur via altered SA-dependent signalling. However, there is also evidence for the possible involvement of other phytohormone signalling pathways (Pastori *et al.* 2003). Many recent studies suggest the specific requirement of AA as a cofactor for the activity of 2-oxoacid-dependent dioxygenases, a class of enzymes that includes those regulating the synthesis of hydroxyproline-containing proteins and hormones in plants (and animals) (Arrigoni & De Tullio 2000, 2002). Indeed, we review evidence below that suggests an involvement of AA as a cofactor in the synthesis of ABA, gibberellin (GA), and ethylene.

AA-dependent dioxygenases are involved in ABA biosynthesis. Specifically, NCED, a dioxygenase catalyzing the formation of xanthoxin, the precursor of ABA, can be activated by the addition of both AA and Fe³⁺ (Schwartz *et al.* 1997). Pastori *et al.* (2003) observed that transcripts encoding NCED are up-regulated in *vtc1* compared to wild type, suggesting that low AA in *vtc1* decreases the flux through the dioxygenase reaction. They hypothesized that the observed elevation of NCED transcript in the mutant compensates for the decreased cofactor (AA) availability, and results in increased ABA biosynthesis (Pastori *et al.* 2003). ABA has been demonstrated to induce PR genes in several other plant species, such as in rice (Agrawal *et al.* 2001) and in *lithospermum* (Yu *et al.* 1999).

AA is also strictly required by some enzymes that are involved in GA biosynthesis (Arrigoni & De Tullio 2000). Pastori *et al.* also showed that in *vtc1*, two 2-oxoglutarate-dependent dehydrogenases, which may be involved in GA biosynthesis, are expressed at higher levels in *vtc1* versus wild type. A role of GA in pathogen defence has been suggested, for example, in tomato (van den Heuvel *et al.* 2001) and in arbuscular mycorrhizal plants of *Linum usitatissimum* when infected by fungal pathogens (Dugassa, vonAlten & Schonbeck 1996).

Ethylene, in addition to SA and ABA, also plays a role in the pathogen response (as detailed above) and specifically in the induction of PR genes (Knoester *et al.* 1995; Grimmig *et al.* 2003). Although not tested to-date, it is possible that altered ethylene biosynthesis could also play a role in the pathogen resistance and the ozone sensitivity of *vtc1*. In ethylene biosynthesis, AA is required for 1-aminocyclopropane-1-carboxylate (ACC) oxidase that forms ethylene (Dong, Fernandez-Maculet & Yang 1992). The microarray analysis of *vtc1* revealed the up-regulation of an ethylene-responsive transcription factor when the endogenous AA content in *vtc1* is artificially elevated (Pastori *et al.*

2003; supplemental data), suggesting that AA availability may regulate ethylene biosynthesis/signalling.

Taken together, analyses of the *vtc1* mutant suggest that AA affects cell signalling. Presumably, the availability and/or the redox status of AA regulate enzyme activity directly or modulate redox-sensitive proteins which trigger signalling cascades (Pignocchi & Foyer 2003). Thereby, AA modulates the content of several signalling molecules, such as SA, ABA, ethylene and GA, thus regulating plant defence responses as well as developmental processes. SA may act in concert with ABA and/or ethylene to induce defence-related proteins and other antimicrobial compounds in response to pathogen attack, resulting in increased pathogen resistance.

Pathogen resistance and ozone sensitivity – a connection

Given that pre-treatment with ozone generally leads to increased pathogen resistance (Plazek *et al.* 2001; Sandermann *et al.* 1998), it may seem somewhat counterintuitive that a mutant sensitive to ozone would be resistant to pathogens. How does the pathogen-resistance phenotype of *vtc1* connect to the increased sensitivity to ozone? Upon ozone exposure, the extent of an oxidative burst and the resulting accumulation of SA in wild-type plants are presumably low, but sufficient to induce defence-gene expression conferring ozone resistance, but not high enough to trigger PCD. JA apparently mediates this response by attenuating SA biosynthesis in wild type, as *jar1*, a JA-insensitive mutant, exhibits higher levels of H₂O₂ and SA after ozone exposure, resulting in increased sensitivity to ozone compared with the wild type (Rao *et al.* 2000b). In contrast, in *vtc1*, ozone fumigation may cause higher than wild-type levels of SA to accumulate (given that the baseline concentration is elevated), resulting in the activation of PCD and ozone sensitivity visualized in the form of chlorotic lesions and tissue collapse. Upon virulent pathogen infection, the somewhat elevated levels of SA, other phytohormone alterations and downstream defence gene activation in this mutant may paradoxically serve to generate resistance. However, as the ozone exposure and pathogen experiments as well as the microarray analysis on wild type and *vtc1* were performed with plants at two different developmental stages, it is difficult to directly compare the ozone fumigation and pathogen studies.

Exposure of plants to anthropogenic sources of ROS has only occurred very recently in evolutionary time due to the relatively short coexistence of land plants with modern man. However, the interaction of plants with pathogens has occurred over a much longer span of time. Unfortunately, the signal transduction pathway that has evolved to so efficiently limit pathogen spread inadvertently causes global tissue destruction when triggered by the ROS generated by ozone. Therefore, if one alters this signalling pathway (by for example, altering AA levels), responses to pathogens and anthropogenic sources of ROS are both altered.

Role of ascorbic acid in the triggering of senescence by ozone

Chronic ozone exposures (100 p.p.b) over a long time period (days to months) can induce symptoms of chlorosis and premature senescence (Pell, Schlagnhauser & Arteca 1997; Miller *et al.* 1999). Such ozone exposures result in the induction of several *senescence associated genes* (*SAGs*) that are induced during natural senescence, whereas transcript levels of photosynthetic genes (*rbcS*, *cab*) decrease (Miller *et al.* 1999). Genes identified as *SAGs* encode proteins involved in protein metabolism, such as cysteine proteases, glutamine synthase, aspartic protease, RNases, but also enzymes involved in response to pathogens and oxidative stress, such as PR proteins, glutathione-S-transferase, nitrilases, cinnamyl alcohol dehydrogenases and catalase (for a review see Lim, Woo & Nam 2003).

AA deficiency in *vtc1* results in the induction of some *SAGs*, such as *SAG13*, *SAG15*, *SAG27*, whereas transcription levels of others, such as *SAG29* and *SAG29*, are not elevated (Barth *et al.* 2004). These results suggest that *vtc1* enters at least some stages of senescence prematurely. This is supported by the fact that incubation of wild-type and *vtc1* leaves in the dark caused faster senescence of *vtc1* leaves than of wild-type leaves, as indicated by the enhanced chlorophyll loss and higher transcript levels of *SAG13* (dark-induced senescence, Fig. 2a). Furthermore, in the presence of exogenous AA, expression levels of *SAG13* and *PR1* are decreased to wild-type levels both in the dark and in the light (Barth *et al.* 2004), indicating that low AA promotes senescence, whereas high AA delays senescence (Navabpour *et al.* 2003). In addition to this premature senescence phenotype, we have found that an early flowering phenotype also co-segregates with the mutant *vtc1* allele (Fig. 2b). However, contrary to this early flowering phenotype of *vtc1*, the Foyer laboratory reported delayed development and flowering of *vtc1* versus wild type (Veljovic-Jovanovic *et al.* 2001; Pastori *et al.* 2003; Kiddle *et al.* 2003). One explanation for the reported variations in the *vtc1* phenotype may be the different growth conditions used in the studies (i.e. 8-h photoperiod in the Foyer laboratory versus 16-h photoperiod in the Conklin laboratory).

A faster senescence phenotype has been reported previously in transgenic potato plants expressing GMPase (the enzyme defective in *vtc1*) in antisense (Keller *et al.* 1999). In comparison with controls, transgenic lines exhibited decreased levels of AA and senesced earlier. The authors hypothesized that imbalance between antioxidant deficiency and ROS results in the premature senescence phenotype in these transgenic plants. Oxidative stress has long been associated with senescence (Prochazkova *et al.* 2001; Navabpour *et al.* 2003). However, it is not known whether these antisense GMPase plants indeed contain increased levels of ROS. In fact, the *vtc1* mutant does not appear to suffer from oxidative stress under optimal growth conditions despite its AA deficiency. A general increase in antioxidant enzymes was not observed (Conklin *et al.* 1997; Veljovic-Jovanovic *et al.* 2001) and elevated levels of hydro-

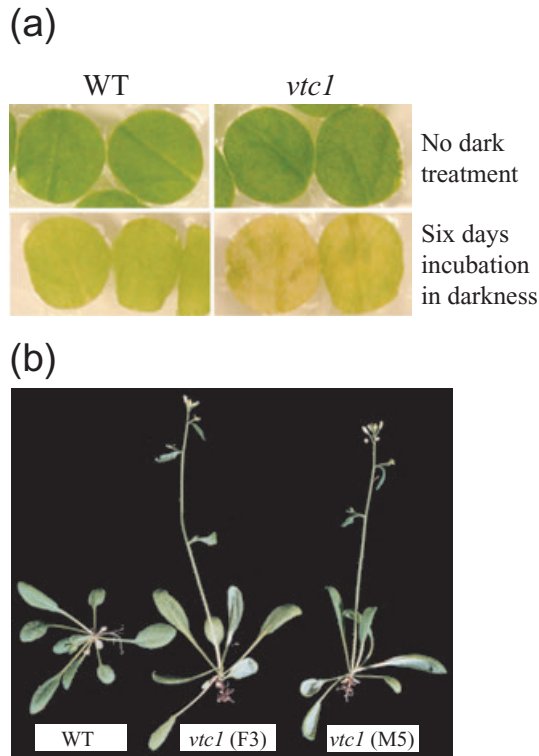


Figure 2. (a) The phenotype of 5-week-old, wild-type and *vtc1* mutant plants before and after a 6 d treatment in darkness. Note that dark incubation accelerates senescence in *vtc1* leaf discs, which is evident in the pronounced chlorophyll loss compared to wild type. (b) Early flowering phenotype of *vtc1* versus wild type. Shown are a wild-type plant, a representative F₃ *vtc1* mutant individual descended from a F₂ (parental cross: Col-0 × *vtc1*) ozone-sensitive (*vtc1/vtc1*) plant and an M5 *vtc1* plant. In contrast, F₃ individuals from single F₂ ozone-resistant (*VTC1/VTC1*) plants do not have this early flowering phenotype (data not shown). F₃ individuals from five *VTC1/VTC1* and five *vtc1/vtc1* F₂ plants were included in this analysis.

gen peroxide (Veljovic-Jovanovic *et al.* 2001) and super oxide anion radicals (Barth and Conklin, unpublished results) could not be detected in *vtc1*. Therefore, it appears that entrance into at least some stages of senescence prematurely (and the induction of defence-related genes) in *vtc1* is independent of ROS, but instead is due to an alteration in the relative levels of certain phytohormones, specifically SA, ABA, and ethylene (as detailed above).

A number of plant hormones that are induced upon ozone exposure are also involved in promotion of senescence (SA and ABA; Weaver *et al.* 1998; Morris *et al.* 2000) or modulation of the timing of senescence (ethylene; Nakashima *et al.* 1997; Weaver *et al.* 1998). Numerous studies have shown that SAGs are differentially regulated by SA, ethylene, ABA, cytokinin and methyl jasmonate, suggesting that multiple signalling pathways are activated during the senescence process (Becker & Apel 1993; Oh *et al.* 1996; Chung *et al.* 1997; Park *et al.* 1998; Weaver *et al.* 1998).

Several genes, such as *SAG13*, *SAG15* are induced by

ethylene and ABA treatment (Nakashima *et al.* 1997; Weaver *et al.* 1998). This suggests that different signalling pathways control responses to ozone, pathogen attack and senescence and that there is cross talk between those pathways. However, gene expression during natural senescence is distinct from premature senescence induced by ozone or AA deficiency. For example, *SAG12* is not induced by ozone or other ROS generators such as silver nitrate, but only during natural senescence (*SAG12* = natural senescence marker) (Morris *et al.* 2000; Navabpour *et al.* 2003). In 5-week-old wild type and *vtc1*, *SAG12* expression could not be detected (Barth and Conklin unpublished results).

Based on the recent findings in *vtc1* we propose a model that attempts to tie together the ozone-sensitive, the pathogen-resistant and the premature senescence phenotypes caused by AA deficiency (Fig. 3). Possibly, premature senescence of *vtc1* contributes to pathogen-resistance. This age-related resistance phenomenon, which has been reported previously in many plant species, is dependent on the accumulation of SA (Kus *et al.* 2002). However, other SA-independent signalling pathways presumably contribute to pathogen resistance as well.

CONCLUSIONS

There has been much progress towards a better understanding of AA biosynthesis, transport, its antioxidant function in response to ozone and the role of AA in response to pathogens and senescence. It has become clear that apoplastic as well as cytosolic AA is important for detoxifica-

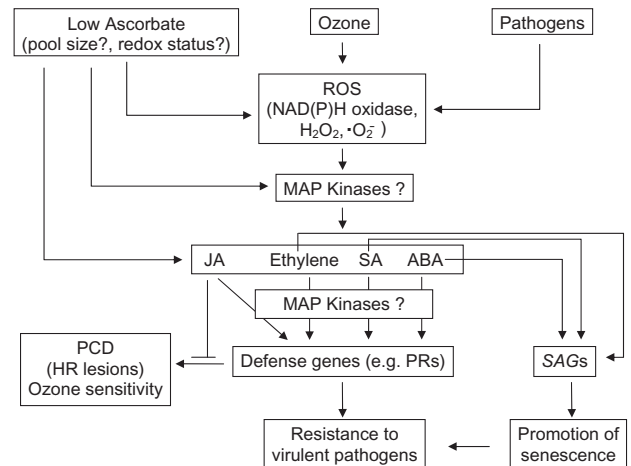


Figure 3. A hypothetical model illustrating that low AA, ozone and pathogens can activate similar plant defence pathways. Presumably, low levels of AA result in heightened levels of ROS, through which MAP kinases may be activated, thereby inducing certain phytohormones that trigger defence responses. However, the redox status and/or the abundance of AA may also directly result in the induction of plant hormones resulting in the expression of defence-related genes and senescence associated genes, which may contribute to resistance to virulent pathogens. On the other hand, SA, ethylene and ABA initiate HR and lesion formation, a response that is attenuated by JA, resulting in ozone sensitivity.

tion of ROS derived from ozone. However, the role of AA goes beyond that of simply an antioxidant given its apparent involvement in a complex signalling pathway that mediates responses to ozone. An intriguing question in this respect is how AA influences gene expression. AA may modulate a MAP kinase pathway, such as through SIPK, an SA-induced protein kinase that can be induced by ozone (Jonak *et al.* 1996; Samuel, Miles & Ellis 2000). In mammalian cells a JNK kinase involved in ROS signal transduction is inhibited by AA (Chuang & Yang 1998). Pathogen exposure of plants with low amounts of AA could cause increased ROS signal transduction, resulting in pronounced HR-like lesions and increased resistance to virulent pathogens. However, direct evidence for such signalling is lacking. Knowledge about regulatory genes that control interactions of several signalling molecules in this network will provide valuable information on how plants respond to both biotic and abiotic stress.

ACKNOWLEDGMENTS

We apologize to the many colleagues whose articles we were not able to cite due to space limitations. Research in the author's laboratory was supported by grant 98-35100-7000 from the Plant Responses to the Environment Program of the National Research Initiative Competitive Grants Program, US Department of Agriculture and the German Academic Exchange Service.

REFERENCES

- Agius F., Gonzalez-Lamothe R., Caballero J.L., Munoz-Blanco J., Botella M.A. & Valpuesta V. (2003) Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. *Nature Biotechnology* **21**, 177–181.
- Agrawal G.K., Rakwal R., Jwa N.S. & Agrawal V.P. (2001) Signaling molecules and blast pathogen attack activates rice *OsPR1a* and *OsPR1b* genes: a model illustrating components participating during defense/stress response. *Plant Physiology and Biochemistry* **39**, 1095–1103.
- Argyrou E., Sophianopoulou V., Schultes N. & Diallinas G. (2001) Functional characterization of a maize purine transporter by expression in *Aspergillus nidulans*. *Plant Cell* **13**, 953–964.
- Arrigoni O. & De Tullio M.C. (2000) The role of ascorbic acid in cell metabolism: between gene-directed functions and unpredictable chemical reactions. *Journal of Plant Physiology* **157**, 481–488.
- Arrigoni O. & De Tullio M.C. (2002) Ascorbic acid: much more than just an antioxidant. *Biochimica et Biophysica Acta* **1569**, 1–9.
- Asard H., Horemans N. & Caubergs R.J. (1992) Transmembrane electron transport in ascorbate-loaded plasma membrane vesicles from higher plants involves a b-type cytochrome. *FEBS Letters* **306**, 143–146.
- Asard H., Kapila J., Verelst W. & Berczi A. (2001) Higher-plant plasma membrane cytochrome b561: a protein in search of a function. *Protoplasma* **217**, 77–93.
- Barth C., Moeder W., Klessig D.F. & Conklin P.L. (2004) The timing of senescence and response to pathogens is altered in the ascorbate-deficient Arabidopsis mutant vitamin c-1. *Plant Physiology* **134**, 1784–1792.
- Becker W. & Apel K. (1993) Differences in gene-expression between natural and artificially induced leaf senescence. *Planta* **189**, 74–79.
- Bent A.F., Innes R.W., Ecker J.R. & Staskawicz B.J. (1992) Disease development in ethylene-insensitive *Arabidopsis thaliana* infected with virulent and avirulent *Pseudomonas* and *Xanthomonas* pathogens. *Molecular Plant Microbe Interactions* **5**, 372–378.
- Berczi A. & Moller I.M. (1998) NADH-monodehydroascorbate oxidoreductase is one of the redox enzymes in spinach leaf plasma membranes. *Plant Physiology* **116**, 1029–1036.
- Berczi A., Caubergs R.J. & Asard H. (2003) Partial purification and characterization of an ascorbate-reducible b-type cytochrome from the plasma membrane of *Arabidopsis thaliana* leaves. *Protoplasma* **221**, 47–56.
- Berczi A., Luthje S. & Asard H. (2001) b-type cytochromes in plasma membranes of *Phaseolus vulgaris* hypocotyls, *Arabidopsis thaliana* leaves, and *Zea mays* roots. *Protoplasma* **217**, 50–55.
- Burkey K.O. & Eason G. (2002) Ozone tolerance in snap bean is associated with elevated ascorbic acid in the leaf apoplast. *Physiologia Plantarum* **114**, 387–394.
- Burkey K.O., Eason G. & Fiscus E.L. (2003) Factors that affect leaf extracellular ascorbic acid content and redox status. *Physiologia Plantarum* **117**, 51–57.
- Castillo F.J. & Greppin H. (1988) Extracellular ascorbic acid and enzyme activities related to ascorbic acid metabolism in *Sedum album* L. leaves after ozone exposure. *Environmental and Experimental Botany* **28**, 231–238.
- Chen Z., Young T.E., Ling J., Chang S.C. & Gallie D.R. (2003) Increasing vitamin C content of plants through enhanced ascorbate recycling. *Proceedings of the National Academy of Sciences of the USA* **100**, 3525–3530.
- Chuang S.M. & Yang J.L. (1998) Roles of reactive oxygen species in the activation of JNK, p38, and ERK by chromium (VI) in non-small-cell lung carcinoma cells. *Cancer Detection and Prevention* <http://www.cancerprev.org/Journal/Issues/22/101/36/2360>.
- Chung B.C., Lee S.Y., Oh S.A., Rhew T.H., Nam H.G. & Lee C.H. (1997) The promoter activity of *sen1*, a senescence-associated gene of Arabidopsis, is repressed by sugars. *Journal of Plant Physiology* **151**, 339–345.
- Conklin P.L. & Last R.L. (1995) Differential accumulation of antioxidant mRNAs in *Arabidopsis thaliana* exposed to ozone. *Plant Physiology* **109**, 203–212.
- Conklin P.L., Norris S.R., Wheeler G.L., Williams E.H., Smirnov N. & Last R.L. (1999) Genetic evidence for the role of GDP-mannose in plant ascorbic acid (vitamin C) biosynthesis. *Proceedings of the National Academy of Sciences of the USA* **96**, 4198–4203.
- Conklin P.L., Pallanca J.E., Last R.L. & Smirnov N. (1997) 1-ascorbic acid metabolism in the ascorbate-deficient Arabidopsis mutant *vtc1*. *Plant Physiology* **115**, 1277–1285.
- Conklin P.L., Williams E.H. & Last R.L. (1996) Environmental stress sensitivity of an ascorbic acid-deficient Arabidopsis mutant. *Proceedings of the National Academy of Sciences of the USA* **93**, 9970–9974.
- Crute I., Beynon J., Dangel J.L., Holub E.B., Mauch-Mani B., Slusarenko A., Staskawicz B. & Ausubel F.M. (1994) Microbial pathogenesis of *Arabidopsis*. In *Arabidopsis* (eds E.M. Meyerowitz & C.R. Somerville), pp. 705–747. Cold Spring Harbor Laboratory Press, Plainview, NY.
- Dempsey D.A., Shah J. & Klessig D.F. (1999) Salicylic acid and disease resistance in plants. *Critical Reviews in Plant Sciences* **18**, 547–575.
- Dong X. (1998) SA, JA, ethylene, and disease resistance in plants. *Current Opinion in Plant Biology* **1**, 316–323.

- Dong J.G., Fernandez-Maculet J.C. & Yang S.F. (1992) Purification and characterization of 1-aminocyclopropane-1-carboxylate oxidase from apple fruit. *Proceedings of the National Academy of Sciences of the USA* **89**, 9789–9793.
- Dugassa G.D., von Alten H. & Schonbeck F. (1996) Effects of arbuscular mycorrhiza (AM) on health of *Linum usitatissimum* L. infected by fungal pathogens. *Plant and Soil* **185**, 173–182.
- Ellis C. & Turner J.G. (2001) The Arabidopsis mutant *cev1* has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. *Plant Cell* **13**, 1025–1033.
- Freebairn H.T. (1960) The prevention of air pollution damage to plants by the use of Vitamin C sprays. *Journal of Air Pollution Control Association* **10**, 314–317.
- Gatzek S., Wheeler G.L. & Smirnov N. (2002) Antisense suppression of 1-galactose dehydrogenase in *Arabidopsis thaliana* provides evidence for its role in ascorbate synthesis and reveals light modulated 1-galactose synthesis. *Plant Journal* **30**, 541–553.
- Glazebrook J., Zook M., Mert F., Kagan I., Rogers E.E., Crute I.R., Holub E.B., Hammerschmidt R. & Ausubel F.M. (1997) Phytoalexin-deficient mutants of Arabidopsis reveal that *PAD4* encodes a regulatory factor and that four *PAD* genes contribute to downy mildew resistance. *Genetics* **146**, 381–392.
- Grantz A., Brummell D.A. & Bennett A.B. (1995) Ascorbate free-radical reductase messenger-RNA levels are induced by wounding. *Plant Physiology* **108**, 411–418.
- Greenberg J.T. (1996) Programmed cell death: a way of life for plants. *Proceedings of the National Academy of Sciences of the USA* **93**, 12094–12097.
- Greenberg J.T. & Ausubel F.M. (1993) *Arabidopsis* mutants compromised for the control of cellular damage during pathogenesis and aging. *Plant Journal* **4**, 327–341.
- Grimmig B., Gonzalez-Perez M.N., Leubner-Metzger G., et al. (2003) Ozone-induced gene expression occurs via ethylene-dependent and -independent signalling. *Plant Molecular Biology* **51**, 599–607.
- Heagle A.S., Miller J.E. & Pursley W.A. (2003) Growth and yield responses of potato to mixtures of carbon dioxide and ozone. *Journal of Environmental Quality* **32**, 1603–1610.
- van den Heuvel K.J.P.T., Hulzink J.M.R., Barendse G.W.M. & Wullems G.J. (2001) The expression of *tgas118*, encoding a defensin in *Lycopersicon esculentum*, is regulated by gibberellin. *Journal of Experimental Botany* **52**, 1427–1436.
- Horemans N., Asard H. & Caubergs R.J. (1996) Further characterisation of a vitamin C carrier in the plasma membrane of beans (*Phaseolus vulgaris*). *Archives of Physiology and Biochemistry* **104**, 790–790.
- Horemans N., Asard H. & Caubergs R.J. (1998) Carrier mediated uptake of dehydroascorbate into higher plant plasma membrane vesicles shows trans-stimulation. *FEBS Letters* **421**, 41–44.
- Horemans N., Asard H., Van Gestelen P. & Caubergs R.J. (1998) Facilitated diffusion drives transport of oxidised ascorbate molecules into purified plasma membrane vesicles of *Phaseolus vulgaris*. *Physiologia Plantarum* **104**, 783–789.
- Horemans N., Foyer C.H. & Asard H. (2000) Transport and action of ascorbate at the plant plasma membrane. *Trends in Plant Science* **5**, 263–267.
- Horemans N., Potters G., De Wilde L. & Caubergs R.J. (2003) Dehydroascorbate uptake activity correlates with cell growth and cell division of tobacco bright yellow-2 cell cultures. *Plant Physiology* **133**, 361–367.
- Imai T., Karita S., Shiratori G., Hattori M., Nunome T., Ôba K. & Hirai M. (1998) 1-galactono- γ -lactone dehydrogenase from sweet potato: purification and cDNA sequence analysis. *Plant Cell Physiology* **39**, 1350–1358.
- Isherwood F.A. & Mapson L.W. (1956) Biological synthesis of ascorbic acid: the conversion of derivatives of D-galacturonic acid into 1-ascorbic acid by plant extracts. *Biochemical Journal* **64**, 13–22.
- Jonak C., Kiegerl S., Ligterink W., Barker P.J., Huskisson N.S. & Hirt H. (1996) Stress signaling in plants: a mitogen-activated protein kinase pathway is activated by cold and drought. *Proceedings of the National Academy of Sciences of the USA* **93**, 11274–11279.
- Kangasjärvi J., Talvinen J., Utriainen M. & Karjalainen R. (1994) Plant defence systems induced by ozone. *Plant Cell and Environment* **17**, 783–794.
- Katagiri F., Thilmony R. & He S.Y. (2002) The *Arabidopsis thaliana*-*Pseudomonas syringae* interaction. In *The Arabidopsis Book* (eds C.R. Somerville & E.M. Meyerowitz), pp. 1–39. American Society of Plant Biologists, Rockville, MD, USA.
- Keller R., Springer F., Renz A. & Kossmann J. (1999) Antisense inhibition of the GDP-mannose pyrophosphorylase reduces the ascorbate content in transgenic plants leading to developmental changes during senescence. *Plant Journal* **19**, 131–141.
- Kiddle G., Pastori G.M., Bernard S., Pignocchi C., Antoniow J., Verrier P.J. & Foyer C.H. (2003) Effects of leaf ascorbate content on defense and photosynthesis gene expression in *Arabidopsis thaliana*. *Antioxidants and Redox Signaling* **5**, 23–32.
- Knoester M., Bol J.F., Vanloon L.C. & Linthorst H.J.M. (1995) Virus-induced gene-expression for enzymes of ethylene biosynthesis in hypersensitively reacting tobacco. *Molecular Plant-Microbe Interactions* **8**, 177–180.
- Koch J.R., Creelman R.A., Eshita S.M., Seskar M., Mullet J.E. & Davis K.R. (2000) Ozone sensitivity in hybrid poplar correlates with insensitivity to both salicylic acid and jasmonic acid. The role of programmed cell death in lesion formation. *Plant Physiology* **123**, 487–496.
- Kollist H., Moldau H., Oksanen E. & Vapaavuori E. (2001) Ascorbate transport from the apoplast to the symplast in intact leaves. *Physiologia Plantarum* **113**, 377–383.
- Kubo A., Saji H., Tanaka K. & Kondo N. (1995) Expression of Arabidopsis cytosolic ascorbate peroxidase in response to ozone or sulfur dioxide. *Plant Molecular Biology* **29**, 479–489.
- Kus J.V., Zaton K., Sarkar R. & Cameron R.K. (2002) Age-related resistance in Arabidopsis is a developmentally regulated defense response to *Pseudomonas syringae*. *Plant Cell* **14**, 479–490.
- Kwon S.Y., Choi S.M., Ahn Y.O., Lee H.S., Lee H.B., Park Y.M. & Kwak S.S. (2003) Enhanced stress-tolerance of transgenic tobacco plants expressing a human dehydroascorbate reductase gene. *Journal of Plant Physiology* **160**, 347–353.
- Leon J., Lawton M. & Raskin I. (1995) Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco. *Plant Physiology* **108**, 1673–1678.
- Levine A., Tenhaken R., Dixon R. & Lamb C. (1994) H₂O₂ from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* **79**, 583–593.
- Lim P.O., Woo H.R. & Nam H.G. (2003) Molecular genetics of leaf senescence in Arabidopsis. *Trends in Plant Science* **8**, 272–278.
- Loewus F.A. & Kelly S. (1961) The metabolism of p-galacturonic acid and its methyl ester in the detached ripening strawberry. *Archives of Biochemistry and Biophysics* **95**, 483–493.
- Luwe M.W.F., Takahama U. & Heber U. (1993) Role of ascorbate in detoxifying ozone in the apoplast of spinach (*Spinacia oleracea* L.) leaves. *Plant Physiology* **101**, 969–976.
- Machler F., Wasescha M.R., Krieger F. & Oertli J.J. (1995) Damage by ozone and protection by ascorbic acid in barley leaves. *Journal of Plant Physiology* **147**, 469–473.
- Maddison J., Lyons T., Plochl M. & Barnes J. (2002) Hydroponically cultivated radish fed 1-galactono-1,4-lactone exhibit increased tolerance to ozone. *Planta* **214**, 383–391.

- Miller J.D., Arteca R.N. & Pell E.J. (1999) Senescence-associated gene expression during ozone-induced leaf senescence in *Arabidopsis*. *Plant Physiology* **120**, 1015–1024.
- Morris K., Mackerness S.A.H., Page T., John C.F., Murphy A.M., Carr J.P. & Buchanan-Wollaston V. (2000) Salicylic acid has a role in regulating gene expression during leaf senescence. *Plant Journal* **23**, 677–685.
- Mozafar A. & Oertli J.J. (1993) Vitamin-C (ascorbic-acid) – uptake and metabolism by soybean. *Journal of Plant Physiology* **141**, 316–321.
- Nakashima K., Kiyosue T., Yamaguchi-Shinozaki K. & Shinozaki K. (1997) A nuclear gene, *erd1*, encoding a chloroplast-targeted Clp protease regulatory subunit homolog is not only induced by water stress but also developmentally up-regulated during senescence in *Arabidopsis thaliana*. *Plant Journal* **12**, 851–861.
- Navabpour S., Morris K., Allen R., Harrison E., S.A.H.-M. & Buchanan-Wollaston V. (2003) Expression of senescence-enhanced genes in response to oxidative stress. *Journal of Experimental Botany* **54**, 2285–2292.
- Noctor G. & Foyer C.H. (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**, 249–279.
- Obara K., Sumi K. & Fukuda H. (2002) The use of multiple transcription starts causes the dual targeting of *Arabidopsis* putative monodehydroascorbate reductase to both mitochondria and chloroplasts. *Plant and Cell Physiology* **43**, 697–705.
- Oh S.A., Lee S.Y., Chung I.K., Lee C.H. & Nam H.G. (1996) A senescence-associated gene of *Arabidopsis thaliana* is distinctively regulated during natural and artificially induced leaf senescence. *Plant Molecular Biology* **30**, 739–754.
- Orvar B.L. & Ellis B.E. (1997) Transgenic tobacco plants expressing antisense RNA for cytosolic ascorbate peroxidase show increased susceptibility to ozone injury. *Plant Journal* **11**, 1297–1305.
- Orvar B.L., McPherson J. & Ellis B.E. (1997) Pre-activating wounding response in tobacco prior to high-level ozone exposure prevents necrotic injury. *Plant Journal* **11**, 203–212.
- Overmyer K., Brosche M. & Kangasjarvi J. (2003) Reactive oxygen species and hormonal control of cell death. *Trends in Plant Science* **8**, 335–342.
- Overmyer K., Tuominen H., Kettunen R., Betz C., Langebartels C., Sandermann H. Jr & Kangasjarvi J. (2000) Ozone-sensitive *Arabidopsis rcd1* mutant reveals opposite roles for ethylene and jasmonate signaling pathways in regulating superoxide-dependent cell death. *Plant Cell* **12**, 1849–1862.
- Pallanca J.E. & Smirnov N. (2000) The control of ascorbic acid synthesis and turnover in pea seedlings. *Journal of Experimental Botany* **51**, 669–674.
- Park J.H., Oh S.A., Kim Y.H., Woo H.R. & Nam H.G. (1998) Differential expression of senescence-associated mRNAs during leaf senescence induced by different senescence-inducing factors in *Arabidopsis*. *Plant Molecular Biology* **37**, 445–454.
- Pasqualini S., Della Torre G., Ferranti F., Ederli L., Piccioni C., Reale L. & Antonielli M. (2002) Salicylic acid modulates ozone-induced hypersensitive cell death in tobacco plants. *Physiologia Plantarum* **115**, 204–212.
- Pastori G.M., Kiddle G., Antoniw J., Bernard S., Veljovic-Jovanovic S., Verrier P.J., Noctor G. & Foyer C.H. (2003) Leaf vitamin C contents modulate plant defense transcripts and regulate genes that control development through hormone signaling. *Plant Cell* **15**, 939–951.
- Pell E.J., Schlagnhauser C.D. & Arteca R.N. (1997) Ozone-induced oxidative stress: mechanisms of action and reaction. *Physiologia Plantarum* **100**, 264–273.
- Pellinen R., Palva T. & Kangasjarvi J. (1999) Short communication: subcellular localization of ozone-induced hydrogen peroxide production in birch (*Betula pendula*) leaf cells. *Plant Journal* **20**, 349–356.
- Pignocchi C. & Foyer C.H. (2003) Apoplastic ascorbate metabolism and its role in the regulation of cell signalling. *Current Opinion in Plant Biology* **6**, 379–389.
- Pignocchi C., Fletcher J.M., Wilkinson J.E., Barnes J.D. & Foyer C.H. (2003) The function of ascorbate oxidase in tobacco. *Plant Physiology* **132**, 1631–1641.
- Plazek A., Hura K., Rapacz H. & Zur I. (2001) The influence of ozone fumigation on metabolic efficiency and plant resistance to fungal pathogens. *Journal of Applied Botany-Angewandte Botanik* **75**, 8–13.
- Plochl M., Lyons T., Ollerenshaw J. & Barnes J. (2000) Simulating ozone detoxification in the leaf apoplast through the direct reaction with ascorbate. *Planta* **210**, 454–467.
- Polle A., Wieser G. & Havranek W.M. (1995) Quantification of ozone influx and apoplastic ascorbate content in needles of Norway spruce trees (*Picea abies* L., Karst) at high altitudes. *Plant, Cell and Environment* **18**, 681–688.
- Prochazkova D., Sairam R.K., Srivastava G.C. & Singh D.V. (2001) Oxidative stress and antioxidant activity as the basis of senescence in maize leaves. *Plant Science* **161**, 765–771.
- Rao M.V. & Davis K.R. (1999) Ozone-induced cell death occurs via two distinct mechanisms in *Arabidopsis*: the role of salicylic acid. *Plant Journal* **17**, 603–614.
- Rao M.V. & Davis K.R. (2001) The physiology of ozone induced cell death. *Planta* **213**, 682–690.
- Rao M.V., Koch J.R. & Davis K.R. (2000a) Ozone: a tool for probing programmed cell death in plants. *Plant Molecular Biology* **44**, 345–358.
- Rao M.V., Lee H., Creelman R.A., Mullet J.E. & Davis K.R. (2000b) Jasmonic acid signaling modulates ozone-induced hypersensitive cell death. *Plant Cell* **12**, 1633–1646.
- Rautenkranz A.A.F., Li L.J., Machler F., Martinoia E. & Oertli J.J. (1994) Transport of ascorbic and dehydroascorbic acids across protoplast and vacuole membranes isolated from barley (*Hordeum-vulgare* L cv Gerbel) Leaves. *Plant Physiology* **106**, 187–193.
- Robinson J.M. & Britz S.J. (2000) Tolerance of a field grown soybean cultivar to elevated ozone level is concurrent with higher leaflet ascorbic acid level, higher ascorbate-dehydroascorbate redox status, and long term photosynthetic productivity. *Photosynthesis Research* **64**, 77–87.
- Rogers E.E. & Ausubel F.M. (1997) *Arabidopsis* enhanced disease susceptibility mutants exhibit enhanced susceptibility to several bacterial pathogens and alterations in PR-1 gene expression. *Plant Cell* **9**, 305–316.
- Ryals J., Neuenschwander U., Willits M., Molina A., Steiner H. & Hunt M. (1996) Systemic acquired resistance. *Plant Cell* **8**, 1809–1819.
- Samuel M.A., Miles G.P. & Ellis B.E. (2000) Ozone treatment rapidly activates MAP kinase signalling in plants. *Plant Journal* **22**, 367–376.
- Sandermann H., Ernst D., Heller W. & Langebartels C. (1998) Ozone: an abiotic elicitor of plant defence reactions. *Trends in Plant Science* **3**, 47–50.
- Sanmartin M., Drogoudi P.A., Lyons T., Pateraki I., Barnes J. & Kanellis A.A. (2003) Over-expression of ascorbate oxidase in the apoplast of transgenic tobacco results in altered ascorbate and glutathione redox states and increased sensitivity to ozone. *Planta* **216**, 918–928.
- Schraudner M., Moeder W., Wiese C., Van Camp W., Inzé D., Langebartels C. & Sandermann H. Jr (1998) Ozone-induced oxidative burst in the ozone biomonitor plant, tobacco Bel W3. *Plant Journal* **16**, 235–245.

- Schwartz S.H., Tan B.C., Gage D.A., Zeevaart J.A. & McCarty D.R. (1997) Specific oxidative cleavage of carotenoids by VP14 of maize. *Science* **276**, 1872–1874.
- Shah J. & Klessig D.F. (1999) Salicylic acid: signal perception and transduction. In *Biochemistry and Molecular Biology of Plant Hormones* (eds P.P.J. Hooykaas, M.A. Hall & K.R. Libbenga), pp. 513–541. Elsevier Science B, V., Amsterdam, The Netherlands.
- Sharma Y.K. & Davis K.R. (1994) Ozone-induced expression of stress-related genes in *Arabidopsis thaliana*. *Plant Physiology* **105**, 1089–1096.
- Shigeoka S., Ishikawa T., Tamoi M., Miyagawa Y., Takeda T., Yabuta Y. & Yoshimura K. (2002) Regulation and function of ascorbate peroxidase isoenzymes. *Journal of Experimental Botany* **53**, 1305–1319.
- Smirnoff N. & Pallanca J.E. (1996) Ascorbate metabolism in relation to oxidative stress. *Biochemical Society Transactions* **24**, 472–478.
- Torsethaugen G., Pitcher L.H., Zilinskas B.A. & Pell E.J. (1997) Overproduction of ascorbate peroxidase in the tobacco chloroplast does not provide protection against ozone. *Plant Physiology* **114**, 529–537.
- Trost P., Berczi A., Sparla F., Sponza G., Marzadori B., Asard H. & Pupillo P. (2000) Purification of cytochrome b-561 from bean hypocotyls plasma membrane. Evidence for the presence of two heme centers. *Biochimica et Biophysica Acta* **1468**, 1–5.
- Tsukaguchi H., Tokui T., Mackenzie B., Berger U.V., Chen X.Z., Wang Y.X., Brubaker R.F. & Hediger M.A. (1999) A family of mammalian Na⁺-dependent 1-ascorbic acid transporters. *Nature* **399**, 70–75.
- Vahala J., Keinänen M., Schutzendubel A., Polle A. & Kangasjarvi J. (2003) Differential effects of elevated ozone on two hybrid aspen genotypes predisposed to chronic ozone fumigation. Role of ethylene and salicylic acid. *Plant Physiology* **132**, 196–205.
- Veljovic-Jovanovic S.D., Pignocchi C., Noctor G. & Foyer C.H. (2001) Low ascorbic acid in the *vtc-1* mutant of *Arabidopsis* is associated with decreased growth and intracellular redistribution of the antioxidant system. *Plant Physiology* **127**, 426–435.
- Vranova E., Inze D. & Van Breusegem F. (2002) Signal transduction during oxidative stress. *Journal of Experimental Botany* **53**, 1227–1236.
- Weaver L.M., Gan S., Quirino B. & Amasino R.M. (1998) A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. *Plant Molecular Biology* **37**, 455–469.
- Wheeler G.L., Jones M.A. & Smirnoff N. (1998) The biosynthetic pathway of vitamin C in higher plants. *Nature* **393**, 365–369.
- Willekens H., Van Camp W., Van Montagu M., Inze D., Langebartels C. & Sandermann H. Jr (1994) Ozone, sulfur dioxide, and ultraviolet B have similar effects on mRNA accumulation of antioxidant genes in *Nicotiana plumbaginifolia* L. *Plant Physiology* **106**, 1007–1014.
- Wolucka B.A., Persiau G., Doorselaere J.V., Davey M.W., Demol H., Vandekerckhove J., Van Montagu M., Zabeau M. & Boerjan W. (2001) Partial purification and identification of GDP-mannose 3',5'-epimerase of *Arabidopsis thaliana*, a key enzyme of the plant vitamin C pathway. *Proceedings of the National Academy of Science of the USA* **98**, 14843–14848.
- Yoshimura K., Yabuta Y., Ishikawa T. & Shigeoka S. (2000) Expression of spinach ascorbate peroxidase isoenzymes in response to oxidative stresses. *Plant Physiology* **123**, 223–234.
- Yu H.J., Mun J.H., Kwon Y.M., Lee J.S. & Kim S.G. (1999) Two cDNAs encoding pathogenesis-related proteins of *Lithospermum erythrorhizon* display different expression patterns in suspension cultures. *Journal of Plant Physiology* **155**, 364–370.

Received 21 January 2004; received in revised form 15 March 2004; accepted for publication 22 March 2004