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Ascorbate and Plant Cell Growth

Francisco Córdoba[1] and José A. González-Reyes[2]

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Ascorbate and related enzymes are involved in the control of several plant growth processes. Ascorbate modulates cell growth by controlling (i) the biosynthesis of hydroxyproline-rich proteins required for the progression of G1 and G2 phases of the cell cycle, (ii) the cross-linking of cell wall glycoproteins and other polymers, and (iii) redox reactions at the plasma membrane involved in elongation mechanisms. The effect of ascorbate on onion root elongation is reviewed here. The ascorbate free radical induces a high vacuolization responsible for elongation. This effect may be dependent on the activity of the redox system linked to the plasma membrane. Current data are discussed on the basis of the modulation of the plasma membrane energetic state derived from the ascorbate-induced hyperpolarization and the activity of an intrinsic transplasmalemma ascorbate-regenerating enzyme.

KEY WORDS: Ascorbate; cell wall; onion root elongation.

SYNTHESIS, TRANSPORT, AND FUNCTIONS

In higher plants, ascorbic acid is generated from glucose via oxidation at C-1, epimerization at C-5, and a new oxidation at C-2 or C-3. Although all the intermediates involved in these pathways have not been identified yet, D-glucosone and L-sorbosone have been putatively proposed (Loewus, 1988; Loewus *et al.*, 1990; Saito *et al.*, 1990). The following is a summary of the possible ascorbate biosynthesis pathways in plant cells:

D-glucose → D-glucosone → L-sorbosone
→ L-ascorbic acid

In plant, ascorbate levels depend on the age of the organism. Dry seeds do not contain ascorbic acid, but after germination a rapid synthesis of ascorbate can be measured. The concentration of this compound in the cell decreases again with senescence or after depletion of carbohydrate reserves (Penel and Castillo, 1991). *Vicia faba* seeds acquire the capability of synthesizing ascorbic acid shortly before the onset of seed desiccation; thus, the plant reaches sufficient ascorbic levels to reestablish the metabolic activity when germination starts (Arrigoni *et al.*, 1992). Mature pollen grains of *Dasypyrum villosum* are endowed with the ascorbate biosynthetic pathway and actively synthesize ascorbic acid (De Gara *et al.*, 1993). In another report, Cakmak *et al.* (1993) found that germination of wheat seeds is associated with an enhancement of ascorbate peroxidase (an H_2O_2-scavenging enzyme) and monodehydroascorbate-reductase, an ascorbate-regenerating enzyme.

Exogenous ascorbate can be also incorporated in animal cells by transport and uptake mechanisms, but in plants little is known about this process. It has been reported recently (Mozafar and Oertli, 1993) that ascorbic acid is absorbed from the culture media by soybean roots and leaves by an active process that requires metabolic energy, and the kinetics fit with a typical saturation-curve pattern. However, the increase of ascorbic levels after external application is very temporary, so a rapid disappearance, especially in roots, can be observed. The authors interpreted these data as suggesting that ascorbate transport and its metabolism are closely related to the intracellular carbohydrate pool, but more experiments

[1] Departamento de Ciencias Agroforestales, Universidad de Huelva, Spain.
[2] Departamento de Biología Celular, Universidad de Córdoba, Spain.

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are needed to ascertain the physiological contribution of transport mechanisms to the general metabolism of this compound in plants.

Ascorbate synthesis seems to be restricted to the cytosol (Loewus, 1988) and it is subsequently transported to chloroplast and apoplast, two plant cell compartments especially enriched in ascorbate, by mechanisms which are still unclear. Beck *et al.* (1983) reported that intact spinach chloroplasts took up ascorbate by a facilitated diffusion transport mechanism, competitively inhibited by dehydroascorbate (see also Foyer *et al.*, 1991). Transport of ascorbate from cytoplasm to apoplast has been observed by Castillo and Greppin (1988) in leaves of *Sedum album* and by Luwe *et al.* (1993) in spinach leaves subjected to ozone exposure.

Under physiological conditions, the newly synthesized ascorbic acid is mainly used as a reductant in a variety of reactions. It is essential in photosynthesis by removing active oxygen species. Ascorbate can also regenerate some membrane-bound radical quenchers such as α-tocopherol and zeaxanthin (Foyer *et al.*, 1991). The ascorbate function is extended to the removal of free radical oxygen species produced by air pollutants, certain herbicides, and other cytotoxic compounds derived from lipid peroxidation (for a review, see Penel and Castillo, 1991). Most of these detoxification processes are functional in chloroplasts and apoplast. In this last location as well as in the plasma membrane, ascorbate and related enzymes can be of particular importance in regulating growth and development in plants.

ASCORBATE, PEROXIDASES AND CROSS-LINKING PROCESSES AT THE CELL WALL

Until now, works concerning ascorbate control of plants cell growth have had a double focus. First, ascorbate together with peroxidases seems to regulate the synthesis of several cell wall components. A very well known function of plant peroxidases concerns the cross-linking of cell wall components and the polymerization of lignin and suberin monomers at the expenses of hydrogen peroxide, together with an important role in detoxification processes (Penel and Castillo, 1991). On the other hand, ascorbate and its free radical have been involved in the control of plasma membrane redox state as well as in the modulation of nutrient uptake and other plant growth events, such as elongation (see below).

In animals, an essential role of ascorbic acid in the hydroxylation of proline during biosynthesis of collagen has been firmly established (see, e.g., Padh, 1990). In plants, growth depends on the biosynthesis of hydroxyproline-rich proteins as well. Arrigoni *et al.* (1977) demonstrated that lycorine, a specific inhibitor of ascorbate biosynthesis, strongly blocked the biosynthesis of hydroxyproline-rich proteins during aging of stored tuber slices. The addition of ascorbate almost completely prevented the lycorine inhibition. In another report, the same group (Liso *et al.*, 1984) showed that the presence of lycorine inhibited cell division in *Vicia faba* and *Allium cepa* roots. Once again, the addition of ascorbate prevented or reversed the inhibitory effect of lycorine. These results were explained by an ascorbate-mediated control of the biosynthesis of hydroxyproline-rich proteins required for the progression of G1 and G2 phases of the cell cycle. More recently, the involvement of ascorbate in the control of the biosynthesis of hydroxyproline-rich proteins was supported by the observation that the ascorbate content in several plant materials was enhanced after inhibition of prolyl-hydroxylase by dehydroproline (De Gara *et al.*, 1991).

Ascorbate may also regulate lignification and/or the formation of cross-linking by peroxidative reactions at the cell wall. Extensin, which may be the major structural protein in the cell wall of many species, is an insoluble hydroxyproline-rich protein (Cooper *et al.*, 1984; Cassab and Varner, 1988). An inverse relationship between the rate of elongation and the concentration of hydroxyproline-rich wall proteins has been reported, which suggests that extensin stiffens the wall during growth, thus reducing the rate of elongation (see Cassab and Varner, 1988). Extensin is secreted as single soluble monomeric hydroxyproline forms that are slowly insolubilized in the cell wall via the oxidative formation of isodityrosine cross-links (Cooper and Varner, 1983; Fry, 1986a). An *in vivo* assay of covalent wall binding of newly synthesized (pulse-labeled) extensin was partially prevented by inhibitors of peroxidase such as dithiothreitol, ascorbate, or cyanide (Fry, 1986a). Similarly, low pH or the addition of ascorbate to root slices of *Daucus carota* inhibited the cross-linking of soluble extensin by blocking the formation of isodityrosine (Cooper and Varner, 1984). Thus, it is probable that ascorbate itself and/or ascorbate-regulated peroxidase system in the cell wall regulates expansion growth by controlling the cross-linking of wall glycoproteins.

In another experimental model, it was shown that

the blue-light calcium-dependent inhibition of stem elongation in *Cucumber* could be abolished by ascorbic acid treatment (Shrinkle and Jones, 1988). In parallel, ascorbate or low pH inhibited the peroxidase isolated from the cell-wall free space. These results were interpreted according to an inverse correlation between growth rate and cell-wall peroxidase activity. In this sense, it has been reported that in epicotyls of *Cicer arietinum*, a particular fraction of cell wall peroxidases named Px-2 decreased its enzymatic activity when cell wall loosening was induced by an acid pH (Valero *et al.*, 1991). More recently, Zheng and van Huystee (1992) have reported a direct inverse relationship between peroxidase and the elongation of peanut hypocotyls. The above results strongly support the idea that peroxidase-driven reactions at the cell wall decrease its extensibility by increasing cross-linkage of several polymers and proteins (Fry, 1986b). A question to be elucidated is whether ascorbate inhibition of peroxidase is really a physiological mechanism involved in the modulation of growth in plants.

Ascorbate is a common compound in the apoplastic space (Castillo and Greppin, 1988; Polle *et al.*, 1990; Takahama and Oniki, 1992; Luwe *et al.*, 1993). The apoplastic compartment also contains peroxidases, phenolics, and other compounds that are substrates for the cross-linking reactions in cell walls, catalyzed by peroxidases (Castillo and Grepping, 1986; Fry, 1986a, 1986b; Bolwell, 1988; Polle *et al.*, 1990; Penel and Castillo, 1991). H_2O_2 is the other substrate required for peroxidase reaction, and it is probably generated from redox reactions at the cell wall (Elstner and Heupel, 1976) or plasma membrane (Askerlund *et al.*, 1987; Rubinstein and Luster, 1993). It has been reported that ascorbate inhibits the oxidation of phenolics by the peroxidase–H_2O_2 system (Takahama, 1992), and that H_2O_2-dependent oxidation of ascorbate in apoplast was stimulated by phenolics (Takahama and Oniki, 1992). Thus, the above data taken together support the idea that ascorbate regulates lignification, suberization, or the formation of cross-linking in cell walls. If so, an inverse relation between ascorbate levels and peroxidase-dependent cross-linking in the cell wall should occur, as has been suggested by Takahama and Oniki (1992).

ASCORBATE AND ELONGATION IN ONION ROOTS

Ascorbate and ascorbate free radical are known to be involved in processes related to plant growth, and concretely in onion root elongation. It was reported first that long-term treatments (about 24–48 h) with ascorbate free radical resulted in an increased cell size. Ultrastructural examination of root meristems showed a strong vacuolization of the cells: both mean size and mean number of vacuoles significantly increased after 48-h incubation with ascorbate free radical. Neither ascorbate nor dehydroascorbate induced these changes (Hidalgo *et al.*, 1989). The kinetics of the vacuome in meristematic cells during ascorbate free radical incubation was exhaustively studied as well (Navas, 1991).

Further analysis showed that ascorbate free radical was able to stimulate cell growth and vacuolization at any region of the root (meristematic, differentiation, and elongation zones), and it was also confirmed that ascorbate and dehydroascorbate had no significant effect on root elongation. On the other hand, the mitotic pattern remained unaltered during the incubation with ascorbate free radical. Vacuolization was accompanied by an increase in acid phosphatase and nitrate-sensitive ATPase activity (Hidalgo *et al.*, 1991). The authors concluded that the ascorbate free radical treatment affects only the elongation component of root growth (see Carmona and Cuadrado, 1988) rather than the production of new cells at the meristems.

The results were explained on the basis of redox reactions at the plasma membrane. Redox processes at the plasmalemma have been observed in all eukaryotic cells (Crane *et al.*, 1985), and in plants they have been attributed different functions: ferric chelate reduction, reduction of dioxygen, superoxide anion generation, etc. (see, for example, Crane *et al.*, 1991; Rubinstein and Luster, 1993). Each one of these functions seems to be mediated by different redox systems, and their presence may depend on the different plant species. For instance, the so-called "turbo-system" has a specific role related to iron uptake in dicots and nongrass monocots (Bienfait, 1985). However, all the plants investigated so far have a "standard system" likely involved in plasma membrane energization (Böttger *et al.*, 1985; Böttger and Lüthen, 1986), cell elongation, and proliferation (Crane *et al.*, 1985).

Redox systems detected in plant plasmalemma use internal NAD(P)H as a common source for electrons (Qiu *et al.*, 1985; Buckhout and Hrubec, 1986; Luster and Buckhout, 1989), although ascorbate itself was able to reduce the intramembrane cyt *b*

while NAD(P)H was a poor reductant (Asard *et al.*, 1992). However, each redox system may have one or more specific acceptors which in the case of the "standard system" have not yet been determined. Artificial acceptors have classically been applied to investigate the plasmalemma redox system in plants (Lüthje and Böttger, 1989; Döring *et al.*, 1990). In spite of the usefulness of these electron acceptors in the study of the physiology of the transmembrane redox system, natural molecules have to be used in order to elucidate the role of the system in the natural environment of the cell. Therefore, possible natural electron acceptors were studied.

The ascorbate free radical had been proposed as a possible electron acceptor (Morré *et al.*, 1986), and the results summarized above were interpreted on the basis of ascorbate free radical as an electron acceptor of the "standard system." There are several facts that support this possibility.

(i) Plant cells can produce and secrete ascorbate (see above). In some cases, relatively large amounts of ascorbate have been found in the apoplastic space (Mertz, 1964; Castillo and Greppin, 1988; Polle *et al.*, 1990; Takahama and Oniki, 1992; Luwe *et al.*, 1993).

(ii) The presence of an ascorbate oxidase and ascorbate-dependent peroxidases has also been found in this plant compartment; these enzymes catalyse the partial oxidation of ascorbate to produce ascorbate free radical (Mertz, 1964; Arrigoni *et al.*, 1981; Penel and Castillo, 1991). Therefore, natural ascrobate free radical generation at the cell wall compartment can be assumed.

(iii) More recently, Horemans *et al.* (1994) have shown that ascorbate free radical may be used as electron acceptor for the cyt *b*-mediated transplasmalemma electron transport in bean hypocotyls.

Hidalgo *et al.* (1989, 1991) suggested that ascorbate free radical could act as electron acceptor for the transplasma redox system, which could be activated by the presence of relatively large amounts of the free radical, and that this redox activity resulted in a stimulation of cell growth. To test this hypothesis, more experiments were developed.

González-Reyes *et al.* (1992) reported that ascorbate free radical induces a very rapid hyperpolarization of plasmalemma in *Allium cepa* roots. Ascorbate and dehydroascorbate only induced a short transient (2–3 min) hyperpolarization, while the effects of ascorbate free radical were observed in periods longer than 30 min. Moreover, all the three redox forms of ascorbate induced a rapid stimulation of H^+ efflux. However, the effect of both ascorbate and dehydroascorbate was also transient and followed by a period of influx, while the stimulation induced by ascorbate free radical was stable for hours. These results do not explain the stimulation of root elongation since the time scales were different: long-term treatments (24–48 h) for root growth stimulation (Hidalgo *et al.*, 1989, 1991) and short-term treatments for H^+ secretion and membrane potential experiments (González-Reyes *et al.*, 1992). However, the latest results seem to indicate changes in the energetic state of the plasmalemma that could result in the activation of mechanisms related to cell elongation. Changes in the energetic state of plasma membrane have also been found using artificial electron acceptors (Döring, 1990).

There are more data showing a correlation between ascorbate free radical treatments and cell growth. For instance, it has recently been reported that long-term treatments with ascorbate free radical result in an increased uptake of several nutrients (sugars and nitrate) in parallel with cell vacuolization. The suggestion was made that the stimulation of root growth by ascorbate free radical also results in an increase in the nutrient uptake pattern (González-Reyes *et al.*, 1994a).

More recently (González-Reyes *et al.*, 1994b), it has been shown that ascorbate can also stimulate root elongation when culture conditions allow ascorbate to be oxidized: at 15°C in the presence of Cu^{+2}, or simply at 25°C. Under both conditions the ascorbate oxidation rate is high and produces ascorbate free radical as a first step in the oxidation process (Creutz, 1981; Njus *et al.*, 1990). On the contrary, inhibition of ascorbate oxidation leads to an inhibition of ascorbate-mediated acceleration of root elongation. These results suggest that ascorbate free radical formed from ascorbate oxidation can act in the activation of growth-related processes.

During the last years there have been several attempts to explain the action of ascorbate free radical in both animal and plant cells (Alcaín *et al.*, 1991; Rodríguez-Aguilera *et al.*, 1993). Although there are more data concerning animal cells, we propose a similar mechanism for plant cells which is summarized in the attached scheme.

External ascorbate free radical may be formed at the cell wall by ascorbate (auto) oxidation in the presence of copper ions, or by the addition of an

ascorbate/dehydroascorbate mixture that yields a measurable amount of ascorbate free radical. In plants, the presence of several enzymes (oxidases/peroxidases) at the apoplast contribute to the supply of ascorbate free radical. Ascorbate free radical can then accept electrons from the plasma membrane redox system to be reduced to ascorbate. This ascorbate regeneration has previously been reported in animal cells, and explained partially on the basis of the existence of a transmembrane NADH-ascorbate free radical oxidoreductase (Alcaín *et al.*, 1991). Data obtained in our laboratory show that plasma membrane isolated from onion roots oxidize NADH at 20 nmol mg^{-1} protein min^{-1} when ascorbate free radical is present in the reaction mixture (Serrano *et al.*, 1994). A similar value has been reported in corn root plasma membrane (Luster and Buckhout, 1988).

Very recently, Horemans *et al.* (1994) have shown that extracellular ascorbate can be regenerated by a mechanism using intracellular ascorbate as electron donor, cyt *b* as intraplasma membrane electron transporter, and ascorbate free radical as extracellular electron acceptor. This reaction is different from that using NADH, since this nucleotide is a very poor electron donor to cyt *b*. Other mechanisms to stabilizate ascorbate are possible as well: for instance, dehydroascorbate derived from ascorbate and/or from the ascorbate free radical can enter into the cells, and be rereduced to ascorbate by a glutathione-dependent oxidoreductase (Rose and Bode, 1992; Penel and Castillo, 1992), or by a cytosolic NADH-ascorbate free radical oxidoreductase (Arrigoni *et al.*, 1981; Hossain *et al.*, 1984; Borracino *et al.*, 1986). An exhaustive analysis of ascorbate regeneration via a transplasma membrane ascorbate free radical reductase in animal cells is included in another article of this series.

In onion roots, the first morphological effect of ascorbate free radical is vacuolization, which can be observed 12 h after its application. The increase in number and volume of vacuoles is accompanied by an increase in the tonoplast surface as well (Hidalgo *et al.*, 1989, 1991; González-Reyes *et al.*, 1994a). Although how plasmalemma hyperpolarization induces vacuole increase is still unknown, it has been established that vacuoles could provide a turgor pressure for cell expansion (Cleland, 1971; Schnepf and Deichgräber, 1979), and under these circumstances, the increased cell elongation could be a consequence of the vacuole growth. Moreover, it has been observed that roots growing in the presence of ascorbate free radical incorporate nitrate and several sugars at higher rates than untreated roots and the kinetics of the nutrient incorporation seems to run in parallel with vacuolization. Neither ascorbate or dehydroascorbate have an effect on vacuolization or nutrient uptake (Hidalgo *et al.*, 1989, 1991; González-Reyes *et al.*, 1994a).

These results seem to indicate a relationship between ascorbate free radical, root growth, and redox processes at the plasma membrane. However, there are still a great number of questions to answer. For example, little is known about proteins or other components related to redox processes at the plasma membrane and whether some of these components are physiologically related to the ascorbate system. Finally, a physiological regulation of the involved redox components should exist. For instance, the response to growth hormones probably depends on membrane redox status. If this is confirmed, a physical link between hormone receptors and redox components is expected to occur. On the other hand, possible connections between the redox system and transport phenomena is also an important matter to be investigated.

CONCLUDING REMARKS

In plants, ascorbate has been involved in several processes, many of them related to cell growth. The specific role of ascorbate in these events seems to depend on its relationship with peroxidases, hydroxyproline-rich glycoproteins, and plasma membrane redox reactions. However, there are many aspects still to be investigated in order to clarify and give a complete picture of ascorbate functions in plant cell metabolism. Some of the most relevant aspects are shown in Fig. 1.

Since several peroxidases use ascorbate as a natural substrate, it would be of interest to ascertain which ascorbate-peroxidases are actually related to growth control, compared to others involved in detoxification mechanisms. Also, ascorbate seems to play an important role in the control of cell proliferation, most likely by modulating hydroxyproline-rich proteins synthesis. On the other hand, ascorbate also has been shown to inhibit the cross-linking of soluble extensin, thus stimulating elongation. If both processes occur naturally, they are probably correlated and a mechanism of common regulation is expected to occur.

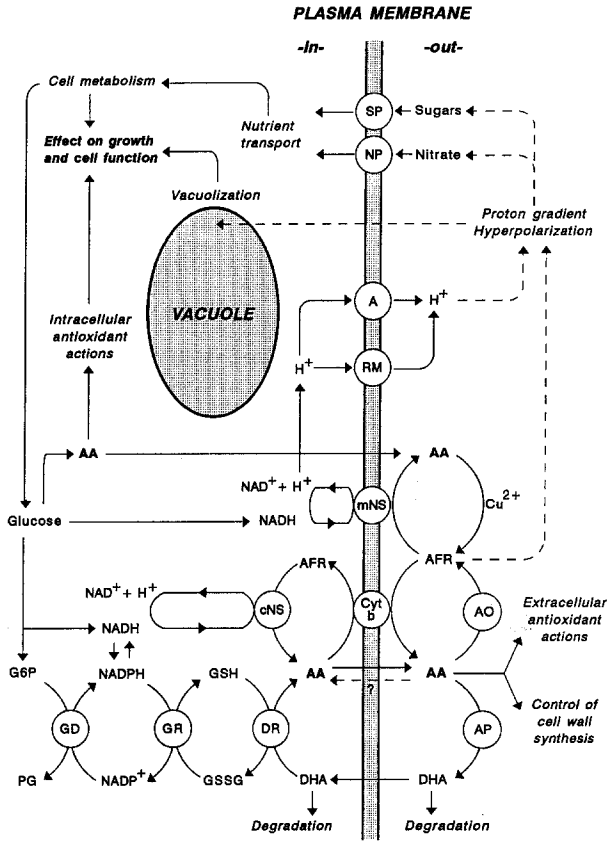

Fig. 1. Ascorbate (AA) is synthesized from glucose in the cytosol and acts as an antioxidant in a variety of cell processes, including those related to the synthesis of hydroxyproline-rich proteins involved in cell division. Intracellular ascorbate can transfer electrons through an intramembrane cyt *b* to the extracellular space (apoplast). On the other hand, AA may be transported to the outer cell space. In this location, AA itself and/or together with peroxidases participate in the control of the synthesis of cell-wall components. The oxidation of AA by ascorbate oxidase (AO) and/or AA peroxidase (AP), or by the catalytic action of copper ion, produces ascorbate free radical (AFR) that can trigger a hyperpolarizing effect leading to vacuolization and other growth-associated events as increased nutrient transport through carriers of sugars (SP) or nitrate (NP). AFR is also a substrate to regenerate AA in the apoplast via the intramembrane cyt *b* or via an integral NADH-AFR oxidoreductase (mNS). The oxidation of cytosolic NADH produces H^+ that passes out of the cell through the H^+-ATPase (A) and/or through a membrane redox mediator (RM). The acidification of the extracellular space would stimulate cell growth too. Finally, the apoplastic AA and AFR are oxidized to dehydroascorbate (DHA) that can degrade or move back into the cell. A series of consecutive enzyme-catalyzed reactions can contribute to the regeneration of ascorbate. The involved enzymes are glucose-6-phosphate dehydrogenase (GD), glutathione reductase (GR), and DHA-reductase (DR). A cytosolic NADH-AFR oxidoreductase (cNS) can also contribute to the regeneration of oxidized AA.

Redox reactions at the plasma membrane seem to play a significant role in elongation. Ascorbate itself has been proposed as an electron donor while ascorbate free radical acts as electron acceptor for this system. In spite of the suggested role of electron transfer from ascorbate to ascorbate free radical in modulating plasma membrane redox potential, the events leading to vacuolization and/or cell growth as well as the molecular mechanisms involved are unknown. Moreover, ascorbate can reduce other external compounds; whether one or more of these molecules are involved in redox processes occurring at the cell wall is also unknown at present.

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