J Plant Growth Regul (1996) 15:53–56

Journal of
Plant
Growth
Regulation

Rooting Hastened in Onions by Ascorbate and Ascorbate Free Radical

R. C. de Cabo,[1] J. A. González-Reyes,[1] F. Córdoba,[2] and P. Navas[1,*]

[1]Departamento de Biología Celular, Facultad de Ciencias, Universidad de Córdoba, 14004 Córdoba; and [2]Departamento de Ciencias Agroforestales, Universidad de Huelva, 21819 Huelva, Spain

Received August 14, 1995; accepted March 18, 1996

Abstract. Treatment of onion bulbs with ascorbate or its free radical hastened root emergence on the basal plate in relation to treatments with water or dehydroascorbate. This stimulation was accompanied by a significant increase of DNA synthesis per primordium. After a 24-h imbibition, ascorbate and ascorbate free radical also increased cell length. Ascorbate and ascorbate free radical apparently activated the onset of cell proliferation in root primordia, resulting in a shortening in G_1–S transition. The possible action of the ascorbate system at the plasma membrane level is discussed.

Key Words. Ascorbate—Ascorbate free radical—Root elongation

Ascorbic acid (ASC) is involved in the control of plant cell growth and development. For instance, ASC regulates the biosynthesis of hydroxyproline-rich proteins required for the progression of G_1 and G_2 phases of the cell cycle in several plant roots (Arrigoni et al. 1977, De Gara et al. 1991, Liso et al. 1984). In other experimental models, ASC seems to regulate cell expansion by controlling the cross-linking of wall polymers (Fry 1986, Penel and Castillo 1991).

Another way to explain the effect of ASC on plant growth is derived from studies of the ASC-dependent redox activities in plasma membrane from onion roots. Hidalgo et al. (1989, 1991) showed that long treatments with the ascorbate free radical (AFR), an intermediate form of the redox pair ASC/dehydroascorbate (DHA), stimulate cell vacuolization and elongation as well as root growth without affecting the cell proliferation pattern. Short term treatments of onion roots with AFR stimulated proton extrusion and energized plasma membrane by hyperpolarization (González-Reyes et al. 1992). Morré et al. (1986) have postulated the existence of an NADH-AFR-regenerating enzyme linked to the plasma membrane from plant sources. According to this hypothesis AFR produced in the apoplast by chemical or enzymatic oxidation of ASC would modify the energization state of the plasma membrane by regulating the plasmalemma redox chain (see Córdoba and González-Reyes 1994).

The aim of this paper is to test whether ASC and its derivatives affect the onset of cell proliferation at the onion basal plate primorida. Our results show that AFR and ASC, but not DHA, accelerates the transition between G_1 and S phases of the cell cycle, resulting in a hastening of root development.

Materials and Methods

One or two layers of *Allium cepa* L. bulbs (20–30 g) were removed and discarded and the remainder washed in tap water for 10 min. Bulbs were placed in cylindric glass receptacles of about 80-mL capacity. Only the bases of the bulbs remained immersed in the incubation medium which was aerated by a continuous bubbling at the rate of 10–20 mL of air min^{-1}. Incubations were in the dark at 15 ± 0.5°C. The working solutions were: distilled water as control, 1 mM glucose, mM ASC, 1 mM DHA and AFR. Ascorbate free radical was prepared as described previously (González-Reyes et al. 1992). The final concentration of AFR was 0.1 μM. The pH of all solutions was adjusted to 6.5.

At the indicated times, samples consisting of at least 30 root primordia were removed after each treatment from each bulb, quickly washed in distilled water, and pulse labeled with [^{3}H]thymidine (7.9×10^5 Bq $mmol^{-1}$; Amersham International, Amersham, UK). Pulse duration was 10 min, and the final [^{3}H]thymidine concentration was 3.77×10^{11} Bq m^{-3}. After exhaustive washing with distilled water, [^{3}H]thymidine incorporation was determined by the method of Tsao et al. (1987), which expresses incorporation as cpm/A_{260}. In our assays, 0.1 mL of sample from individual homogenized primordia was added to 2 mL of Beckman scintillation mixtures and cpm were determined using an LS-6000TA Scintillation Liquid-Counter (Beckman).

Alternatively, some primordia were used for autoradiography. After the pulse, primordia were fixed in a mixture of ethanol:acetic acid (3:1) for 24 h and processed as described elsewhere (González-Fernández et al. 1992). An Ilford nuclear emulsion (type K.5) was used, and the exposure time was 20 days at −30°C. Labeled and unlabeled nuclei were counted and then used to calculate the labeling index (percentage

Abbreviations: ASC, ascorbic acid; AFR, ascorbate free radical; DHA, dehydroascorbate.

* Author for correspondence.

Table 1. No. of roots sprouted from onion bulb basal plates (per cm^2 of basal plate surface) after 48 and 72 h imbibition.[a]

Addition	Concentration	Roots/cm^2 48 h	72 h
None (control)		6 ± 3	15 ± 5
ASC	1 mM	14 ± 6[b]	18 ± 6
AFR	0.1 μM	14 ± 4[b]	19 ± 6
DHA	1 mM	7 ± 3	12 ± 3
Glucose	1 mM	8 ± 3	16 ± 4

[a] Data are expressed in mean value ± S.D. Scoring was performed in at least nine bulbs/treatment in three separate assays.
[b] $p < 0.01$ vs control, DHA and glucose additions.

of labeled nuclei) as a test of nuclei reaching the S phase of the cell cycle.

Finally several primordia were fixed in a mixture of 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7, at 4°C for 12 h, then transferred to 1% osmium tetroxide in the same buffer for 1 h at the same temperature, washed in buffer, dehydrated in an ethanol series, and embedded in Epon 812 resin. Longitudinal sections (0.5–1 μm thick) of whole primordia were stained with toluidine blue. Cell lengths were measured using a semiautomatic image analyzer IBAS (Kontron, Germany).

The results included in this paper were obtained from at least three separate experiments. Statistical comparisons of mean values were performed using the Student's *t* test.

Results

Root development was hastened by both ASC and AFR as deduced from the increased number of visible radicles after 48 h of imbibition with either chemical (Table 1). Glucose and DHA did not affect root development, so that stimulation could not have resulted from nutrient effect. No differences were significant after 72 h.

The time course of [^{3}H]thymidine incorporation indicates maximum DNA synthesis 28 h after initiation of imbibition for control and ASC- and DHA-treated primordia (Fig. 1). However, with AFR this maximum occurred earlier and was higher. Therefore, most of the subsequent experiments were performed at these times.

Labeling index gradually increased between 24 and 28 h of incubation and was higher for both ASC and AFR compared with control and DHA treatments (Fig. 2). Moreover, for AFR the highest values were found 26 h after treatment, whereas equivalent values appeared only after a 28-h incubation in ASC. In control (water) and DHA treatments, labeling indices remained significantly lower.

Cell lengths were measured in primordia treated with the different solutions for 26 h (AFR) or 28 h (ASC, DHA, or water) (Fig. 3). Control and DHA-treated primordia contained many small cells (up to 17-μm length) and few large or very large cells (up to 27 or 37 μm, respectively), whereas AFR- and ASC-treated tissues

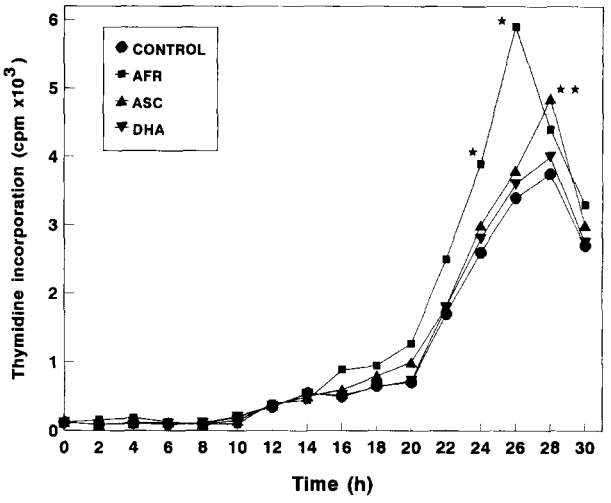

Fig. 1. Kinetics of [^{3}H]thymidine incorporation in root primordia. Onion bulbs were incubated in different solutions and at the indicated times, primordia were removed and submitted to the autoradiographic technique. * $p < 0.01$ vs ASC, DHA, and control. ** $p < 0.01$ vs DHA and control.

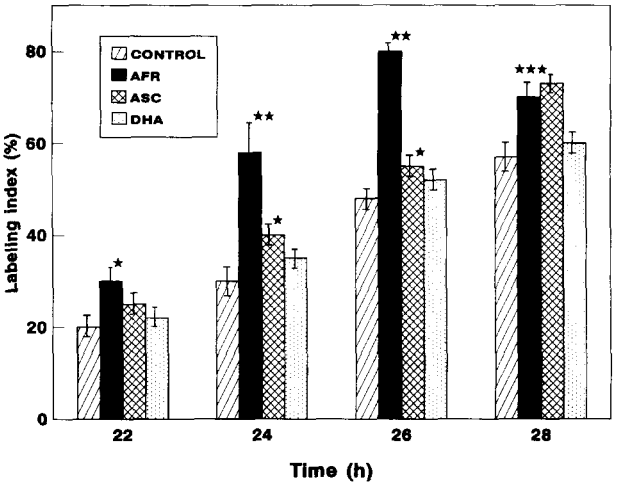

Fig. 2. Labeling indices in onion root primordia 22–28 h after treatment with ascorbic acid redox forms or water (control). * $p < 0.05$ vs control. ** $p < 0.01$ vs ASC, DHA, and control. *** $p < 0.01$ vs DHA and control.

contained a higher proportion of large and very large cells (see Fig. 3).

Discussion

Our experimental data show a stimulation of root elongation in onion bulbs treated with either ASC or AFR. The stimulation seems to result from both an increase in the number of cells entering the S phase of the cell cycle

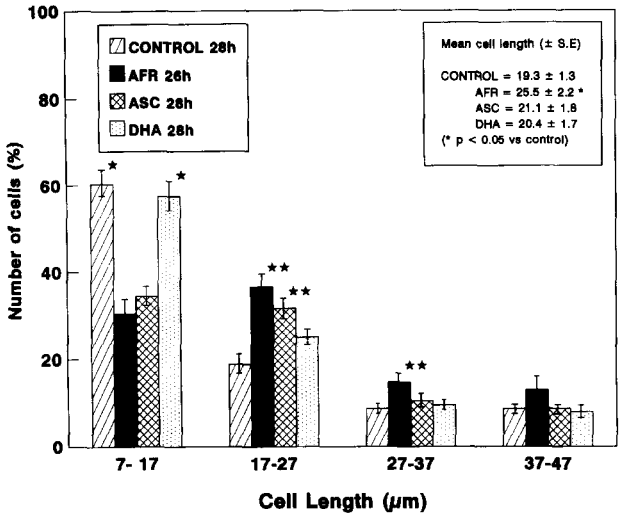

Fig. 3. Distribution of cell sizes in onion root primordia incubated for 26 h in AFR or for 28 h in ASC, DHA, or water. Measurements were restricted to a 500-μm-long region after discarding the apical 500 μm of the primordia. Mean values for cell length in every treatment are also included in the figure (n = 300 cells/treatment). * $p < 0.01$ vs AFR and ASC. ** $p < 0.01$ vs control and DHA.

and an increase in cell length. Both mechanisms are involved in the regulation of cell cycle progression and are interdependent (Carmona and Cuadrado 1986, Navarrete et al. 1983). In our experiments, ASC and AFR induced a shorter G_0/G_1 period and consequently allowed a higher number of cells to enter S phase compared with primordia stimulated to grow in water or DHA.

Currently, it is well known that ASC inhibits apoplastic and cell wall-bound peroxidases, which seem to be involved in cell wall stiffening during plant growth (Fry 1986, Takahama 1994). Therefore, ASC may stimulate elongation by inhibiting cross-linking reactions at the cell wall. On the other hand, AFR has been shown to stimulate onion root elongation, although this effect was explained on the basis of plasmalemma hyperpolarization (Córdoba and González-Reyes 1994, González-Reyes et al. 1992).

Accelerated cell expansion, via inhibition of peroxidase activity or via plasmalemma energization, may be responsible for the shortening of the G_0/G_1 to S transition and the consequent increase in the number of cells entering S phase. However, the reduced form, ASC, does not seem to stimulate cell elongation in onion roots (Hidalgo et al. 1991). Thus, a mechanism including the action of both ASC and AFR must be proposed.

We have reported that ASC may stimulate root elongation if culture conditions are favorable to a high oxidation rate of ASC to AFR, the latter being the only form that stimulates elongation in onion roots (González-Reyes et al. 1994). Therefore, the stimulation of root sprouting by ASC seems to depend on the ability of this chemical to be oxidized during the incubation period. Since ASC stimulates neither elongation nor DNA synthesis in detached primordia (de Cabo et al. 1993), ASC oxidation may depend on the environment within the basal plate rather than within the primordium itself. However, this possibility has to be investigated further.

Very recently, Citterio et al. (1994) reported a stimulation of the onset of cell proliferation in pea roots. They suggest that ASC accelerates the beginning of S phase by speeding up the transfer of Fe^{2+} from ferritin to deoxyribonucleotide reductase. Although this possibility is feasible, more data are needed to ascertain whether external ASC and/or AFR is transported from the culture medium to the cell. DHA inhibits ASC transport in chloroplasts (Beck et al. 1983) and in protoplasts isolated from barley (Rautenkranz et al. 1994). In our work, a mixture of ASC and DHA was used as the source of AFR; ASC probably could not be taken up by the cells under these conditions.

In cultured animal cells, serum seems to be an essential requirement for the transition from quiescence to proliferation. In root primordia only imbibition in water is required to induce proliferation. Our results may indicate that water, probably at the plasma membrane level (González-Reyes et al. 1992), accelerates the cascade of events leading to the onset of proliferation in the primordia contained in dormant onion bulbs.

Acknowledgments. We are grateful to Dr. C. de la Torre (CIB-CSIC, Madrid) and Dr. F. J. Alcaín (University of Córdoba) for comments and suggestions. This work was partially supported by the Spanish DGICYT (project PB92-0714).

References

Arrigoni O, Arrigoni-Liso R, Calabrese G (1977) Ascorbic acid requirements for biosynthesis of hydroxyproline-containing proteins in plants. FEBS Lett 85:135–138

Beck E, Burkert A, Hofmann M (1993) Uptake of L-ascorbate by intact spinach chloroplasts. Plant Physiol 73:41–45

Carmona MJ, Cuadrado A (1986) Analysis of growth components in *Allium* roots. Planta 86:183–189

Citterio S, Sgorbati S, Scippa S, Sparvoli E (1994) Ascorbic acid effect on the onset of cell proliferation in pea root. Physiol Plant 92:601–607

Córdoba F, González-Reyes JA (1994) Ascorbate and plant cell growth. J Bioenerg Biomembr 26:399–405

de Cabo R, González-Reyes JA, Navas P (1993) The onset of cell proliferation is stimulated by ascorbate free radical in onion root primordia. Biol Cell 77:231–233

De Gara L, Tommasi F, Liso R, Arrigoni O (1991) Ascorbic acid utilization by prolyl hydroxylase in vivo. Phytochemistry 30: 1397–1399

Fry SC (1986) Cross-linking of matrix polymers in the growing cell walls of angiosperms. Annu Rev Plant Physiol 37:165–180

González-Fernández A, Aller P, Sans J, De la Torre C (1992) Early and late replicating DNA involved in the G_1 to S transition in *Allium cepa* L. meristematic cells. Biol Cell 74:243–247

González-Reyes JA, Alcaín FJ, Caler JA, Serrano A, Córdoba F, Navas P (1994) Relationship between apoplastic ascorbate regeneration and the stimulation of root growth in *Allium cepa* L. Plant Sci 100:23–29

González-Reyes JA, Döring O, Navas P, Obst G, Böttger M (1992) The effect of ascorbate free radical on the energy state of the plasma membrane of onion (*Allium cepa* L.) root cells: Alteration of K^+ efflux by ascorbate? Biochim Biophys Acta 1098:177–183

Hidalgo A, García-Herdugo G, González-Reyes JA, Morré DJ, Navas P (1991) Ascorbate free radical stimulates onion root growth by increasing cell elongation. Bot Gaz 152:282–288

Hidalgo A, González-Reyes JA, Navas P (1989) Ascorbate free radical enhances vacuolization in onion root meristems. Plant Cell Environ 12:455–460

Liso R, Calabrese G, Bitonti MB, Arrigoni O (1984) Relationship between ascorbic acid and cell division. Exp Cell Res 150:314–320

Morré DJ, Navas P, Penel C, Castillo FJ (1986) Auxin-stimulated NADH oxidase (semidehydroascorbate reductase) of soybean plasma membrane: Role in acidification of cytoplasm? Protoplasma 133:196–197

Navarrete MH, Cuadrado A, Cánovas JL (1983) Partial elimination of G_1 and G_2 periods in higher plant cells by increasing the S period. Exp Cell Res 148:273–280

Penel C, Castillo FJ (1991) Peroxidases of plant plasma membranes, apoplastic ascorbate, and relation of redox activities to plant pathology. In: Crane FL, Morré DJ, Löw HE (eds) Oxidoreduction at the plasma membrane: Relation to growth and transport. Vol 2. Plants. CRC Press, Boca Raton, FL, pp 121–147

Rautenkranz AAF, Li L, Mächler F, Märtinoia E, Oertli JJ (1994) Transport of ascorbic and dehydroascorbic acids across protoplast and vacuole membranes isolated from barley (*Hordeum vulgare* L. cv Gerbel) leaves. Plant Physiol 106:187–193

Takahama U (1994) Redox state of ascorbic acid in the apoplast of stems of *Kalanchoe daigremontiana*. Physiol Plant 89:791–798

Tsao MS, Sanders GHS, Grisham JW (1987) Regulation of growth of cultured hepatic epithelial cells by transferrin. Exp Cell Res 171:52–62