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Nutrient Uptake Changes in Ascorbate Free Radical-Stimulated Onion Roots[1]

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Long-term treatments with ascorbate free radical-stimulated glucose, fucose, sucrose, and nitrate uptake in *Allium cepa* roots. Glucose and fucose showed saturation kinetics in untreated roots, but after treatment with the ascorbate free radical, uptake was linear with time. Although the rates of nitrate and sucrose uptake increased after treatment with ascorbate free radical, the kinetics were similar to those observed in the controls. Ascorbate and dehydroascorbate inhibited nutrient uptake. The uptake rates for all nutrients increased throughout the 48-h period of pretreatment with ascorbate free radical. During the treatment an increase in the vacuole volume and tonoplast surface area also occurred. These results show the relationship between an increase in vacuolar volume and stimulated nutrient uptake from ascorbate-free radical, resulting in enhanced root elongation. These results suggest that activation of a transplasma membrane redox system by ascorbate-free radical is involved in these responses.

The plant plasma membrane contains one or more redox constituents involved in electron transport from an internal donor to specific acceptors in the apoplast (for reviews, see Crane et al., 1985, 1991). Although NAD(P)H has been identified as a common electron donor for plasma membrane electron transport, the final acceptor is still a matter of controversy. A wide variety of electron acceptors has been applied to test and analyze the transplasmalemma electron transport (Lüthje and Böttger, 1989; Döring et al., 1990). However, except for oxygen and AFR, most of these acceptors are artificial and unlikely to be present in the natural environment of the cell. Furthermore, with the exception of the so-called "turbo system" involved in Fe reduction for Fe uptake (Bienfait, 1985), there is no unequivocal evidence for a specific and relevant role of a plasma membrane redox chain in cell metabolism, despite the suggestion made of a role in the control of cell growth (Crane et al., 1985).

AFR, an intermediate form of the redox pair ASC/DHA, was proposed by Morré et al. (1986, 1988) as a possible natural electron acceptor in plant cells. AFR can be generated from ASC by an ASC oxidase, and both ASC and ASC oxidase have been found in high amounts in the apoplastic space of several species (Mertz, 1964; Castillo et al., 1986; Polle et al., 1990). AFR-reductase activity and endogenous ASC have been found in several plant tissues, including meristems (Arrigoni et al., 1981) and soybean root nodules (Dalton et al., 1992).

Recently, we showed that AFR stimulates root growth in *Allium cepa* by increasing elongation without affecting cell division or proliferation, as a result of an augmented relative volume occupied by vacuoles (cell vacuolization; Hidalgo et al., 1989, 1991). These findings suggest a linkage between the redox chain and processes related to cell growth. Furthermore, we reported that AFR, but not ASC or DHA, caused a continuous hyperpolarization of the plasma membrane of onion root cells (González-Reyes et al., 1992). This energization could then facilitate transport processes across the plasma membrane.

In this paper, we show that the uptake of Glc, Fuc, Suc, and nitrate by onion roots was increased in the presence of AFR, whereas ASC and DHA generally inhibited their uptake. Because vacuole volume and tonoplast surface area also increased significantly during AFR treatment, we conclude that AFR-induced vacuolization and nutrient uptake may be closely related. A possible relationship of these phenomena with the transplasmalemma redox activities is discussed.

MATERIALS AND METHODS

Plant Material and Treatments

Allium cepa L. bulbs were grown in the dark in tap water at a constant temperature (15 ± 0.5°C) and aeration (10–20 mL of air min^{-1}). After 72 h, when most of the roots had reached 3 to 4 cm in length, several bulbs were transferred to aqueous solutions of 1 mM ASC, 1 mM DHA, or AFR for 48 h. Some bulbs were also transferred to distilled water as controls. AFR was obtained by mixing 1 mM ASC and 1 mM DHA in distilled water. Under these conditions, the total amount of AFR formed was calculated as 0.1 μM (Goldenberg et al., 1983). The final pH of the working solutions of ASC, AFR, and DHA was adjusted to 7.0 by adding 1 M imidazole. The mixture of ASC, DHA, and AFR will be referred to as AFR throughout the text. ASC and DHA were purchased from Sigma (Deisenhofen, Germany). All solutions were renewed every 8 h.

[^{3}H]Glc and [^{14}C]Fuc Uptake

After 48 h of treatment with ASC, AFR, or DHA, some bulbs were transferred to fresh solutions of these chemicals

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Abbreviations: AFR ascorbate free radical; ASC, ascorbate; DHA, dehydroascorbate; *Sv*, surface density; *Vv*, volume density of vacuole.

containing 1 mM Glc or Fuc. After 30 min of preincubation, [^{3}H]Glc or [^{14}C]Fuc (Amersham, Amersham, UK; specific activities 21.5 Ci mmol^{-1} and 55 mCi mmol^{-1}, respectively) was added at a final amount of 0.04 or 0.008 μCi mL^{-1}, respectively. Samples were taken every 30 min from the media to monitor the disappearance of isotope. Roots also were collected to determine the amount of isotope incorporated into the tissues. Here, samples consisting of 0.25 g of roots were removed from the bulbs every 30 min, washed in distilled water for 5 min, and immersed in 0.5 M NaOH at 37°C for 24 h. After the roots were homogenized, samples of 100 μL were added to 2 mL of Beckman scintillation cocktail, and dpm were obtained using a Beckman LS 6000TA scintillation liquid counter. Samples of 100 μL from culture media were added to 2 mL of cocktail and measured as above. This assay also was performed during the pretreatment with ASC, AFR, and DHA. After 12, 24, 36, and 48 h of preincubation with these chemicals, [^{3}H]Glc and [^{14}C]Fuc uptake were measured during 4-h periods according to the method described above. The same assay was also performed in control (water-grown) roots.

Nitrate and Suc Uptake

Nitrate and Suc were assayed according to the methods of Cawse (1967) and Dubois et al. (1955), respectively. Their uptake by control and pretreated roots was measured by monitoring the disappearance of each of these components from the culture medium. Thus, potassium nitrate or Suc was added to the culture medium at a final concentration of 0.5 mM at time zero. Samples were taken from the culture medium every 30 min for 4 h for both control and 48-h-treated roots. During the initial treatment with ASC-related compounds, nutrient disappearance was measured as described above but was monitored every 12 h for up to 48 h.

All data concerning nutrient uptake represents the mean value of three different assays. On each assay at least five samples per point were measured. The results included in Figures 1 to 3 showed SE values of less than 10%. Data were analyzed statistically using the Student's t test.

Vacuole and Tonoplast Ultrastructural Quantification

Because the vacuole seems to be the organelle most affected by 48-h AFR treatments, we quantitated the total cell vacuole volume during AFR treatment to establish an additional possible correlation with the nutrient uptake kinetics. For this purpose, eight to 10 roots were fixed at 12-h intervals during a 48-h treatment with AFR and processed for EM. Briefly, roots were fixed in a mixture of 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer for 12 h at 4°C. After the roots were washed in the buffer, they were postfixed in 1% osmium tetroxide for 2 h at 4°C. Samples were dehydrated in ethanol and embedded in Epon 812. Thin sections were obtained from a zone between 1 and 1.5 mm from the root apex and stained with aqueous uranyl acetate and lead citrate. Control experiments with ASC-, DHA-, and water-grown roots were performed as well.

To determine relative volume of vacuoles, 30 to 40 photographic prints were obtained randomly at the above-mentioned intervals at a final magnification of ×13,000. These photographs contained longitudinal sections of whole cortical meristematic cells. A point-counting method using a simple square lattice test system of 6-mm spacing (equivalent to 0.35 μm) was applied to obtain the volume density (Vv) of vacuole. Tonoplast surface density (Sv) was obtained by applying an intersection analysis using a multipurpose test system. For calculations, Weibel's (1979) formula was used:

$$Sv = \frac{4\ Ii}{Pt\ d}$$

where Ii is the number of intersections of the test line segments with tonoplast; Pt is the total number of test points per cell, and d is the test line length (12 mm, equivalent to 0.7 μm).

The stereological study used IMAGO software for image analysis (Sistemas Inteligentes de Visión Artificial Research Group, University of Córdoba). Stereological results were analyzed statistically using the Student's t test or, for nonhomogeneous data, the Kolmogorov-Smirnov U test.

RESULTS

Onion roots growing in the presence of ASC, AFR, or DHA showed time-dependent kinetic patterns for uptake of nitrate and sugars that were different from water-grown roots. However, only AFR had a stimulatory effect on the uptake of all four nutrients, particularly for the uptake of nitrate and Suc (Figs. 1–3).

Nitrate uptake was measured during a 4-h period in both control roots and in roots pretreated with ASC, AFR, or DHA for 48 h. Uptake rates were linear throughout (Fig. 1), but nitrate uptake in AFR-pretreated roots increased significantly compared to water controls. ASC and DHA pretreatments were inhibitory to nitrate uptake.

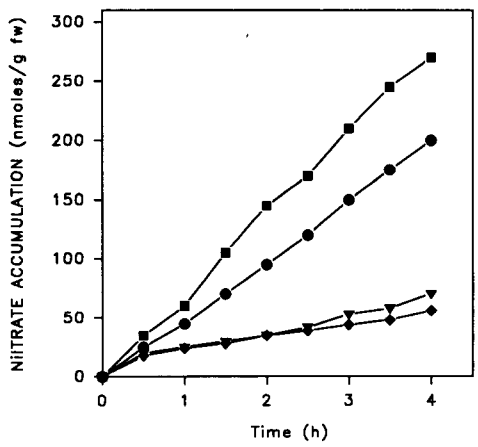

Figure 1. Nitrate accumulation in control roots and in roots pretreated for 48 h with ASC, AFR, or DHA. Two hours after nitrate addition, AFR-pretreated roots accumulated a significantly higher amount of nitrate compared with all other pretreated roots ($P < 0.05$ versus control; $P < 0.01$ versus ASC and DHA). Both ASC and DHA pretreatments markedly inhibited nitrate uptake. ●, Control; ▼, ASC; ■, AFR; ♦, DHA; fw, fresh weight.

Uptake of Suc (Fig. 2) also was increased in AFR-pretreated roots. Here, the time-dependent uptake kinetics were biphasic. There was a phase of rapid uptake followed by a second phase of slower uptake. Significantly higher values of Suc uptake appeared in AFR-pretreated roots 1 h after Suc addition. The uptake kinetics in the ASC experiments were similar to those described for the control but were inhibited slightly 1 h after Suc addition. DHA-pretreated roots showed Suc uptake kinetics very similar to those of the control, but after 1.5 h, uptake was inhibited (Fig. 2).

Glc and Fuc uptake (Fig. 3) exhibited saturation kinetics in control roots but linear kinetics in AFR-pretreated roots. For both sugars, uptake was higher in control roots during the first few hours, but after 3 h of addition, uptake was significantly higher in AFR-treated roots. ASC also stimulated Fuc uptake 3.5 h after its addition but inhibited Glc uptake. On the other hand, DHA inhibited the uptake of both Glc and Fuc (Fig. 3).

We have also evaluated the relative uptake of nitrate and the various sugars during pretreatment with ASC, AFR, and DHA (Figs. 4–6). Nitrate uptake increased significantly during AFR pretreatment and was constant in water-grown roots. However, ASC and DHA inhibited nitrate uptake (Fig. 4). Suc uptake increased only during the preincubation with AFR and was unaltered during pretreatments with ASC and DHA compared with control roots (Fig. 5).

Glc and Fuc increased significantly during the first 24 h of AFR preincubation and then declined (Fig. 6). Although the highest values were obtained after 24 h of pretreatment, at the end of the experiments the uptake rates were also significantly higher than in water-grown roots. Roots exposed to ASC for 24 h exhibited stimulated Glc uptake, but after 24 h Glc uptake declined to control values (Fig. 6A). DHA had no effect on Glc uptake during the first 24 h of exposure, and then a significant decrease was observed for roots exposed to DHA for 36 to 48 h (Fig. 6A). Finally, ASC and DHA

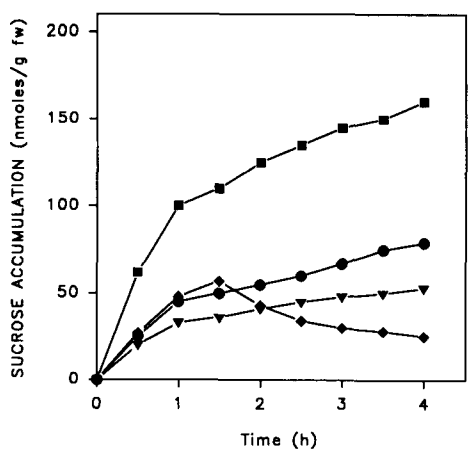

Figure 2. Suc accumulation in control roots and in ASC-, AFR-, or DHA-preincubated roots. One hour after Suc addition, uptake significantly increased in AFR-retreated roots ($P < 0.01$ versus all other conditions). ASC preincubation slightly inhibited Suc uptake, and DHA inhibited uptake 2.5 h after addition. ●, Control; ▼, ASC; ■, AFR; ♦, DHA; fw, fresh weight.

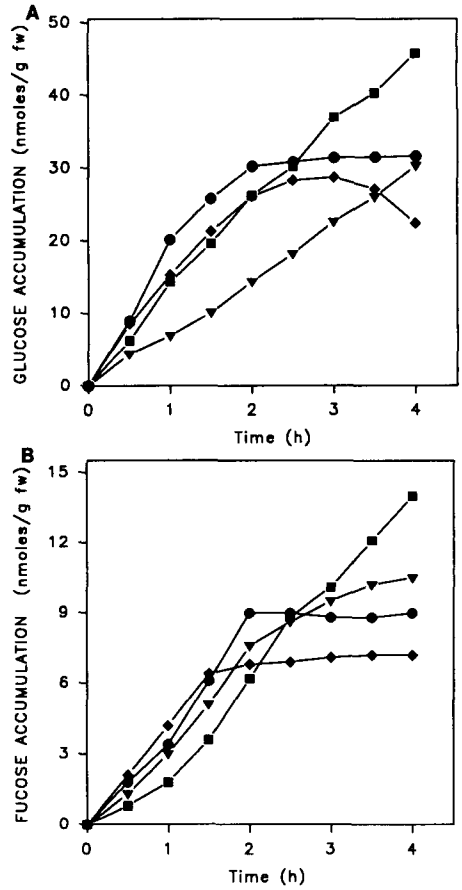

Figure 3. [^{3}H]Glc (A) and [^{14}C]Fuc (B) accumulation in control and in ASC-, AFR-, or DHA-pretreated roots. AFR stimulated uptakes 3.5 h after sugar addition ($P < 0.05$ versus control and DHA). ASC had no significant effect on Fuc uptake and inhibited Glc uptake. DHA inhibited uptake of both sugars. ●, Control; ▼, ASC; ■, AFR; ♦, DHA; fw, fresh weight.

stimulated Fuc uptake after pretreatments of 24 h but subsequently declined (Fig. 6B).

Because vacuole volume and tonoplast surface area grow significantly during 48 h of AFR pretreatment (Hidalgo et al., 1991), we investigated the changes in these vacuolar parameters during the AFR, ASC, and DHA incubations. Both vacuole *Vv* and tonoplast *Sv* increased gradually during the AFR pretreatment, exhibiting kinetics very similar to those observed for nitrate and Suc uptake. The highest values for both stereological parameters were obtained by 36 to 48 h of AFR pretreatment. ASC and DHA did not induce significant changes in the vacuolar parameters compared to water-growing roots (Fig. 7).

DISCUSSION

The results reported in this paper show that previously published data concerning the stimulation of root growth and cell elongation by AFR, but not by ASC or DHA (Hidalgo et al., 1989, 1991), can be correlated with a significant increase in the uptake of nitrate and sugars.

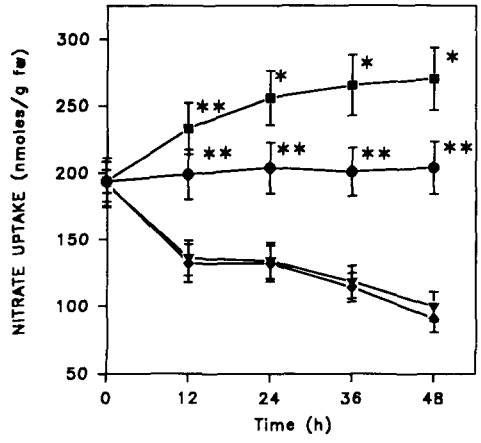

Figure 4. Nitrate uptake in control and during preincubation in the ASC-related compounds. After 12 h of AFR treatment nitrate assimilation was significantly increased (*$P < 0.01$ versus control, ASC, and DHA pretreatments). No significant differences were found among 24-, 36-, and 48-h data. Preincubations with ASC and DHA resulted in a significant inhibition (**$P < 0.01$ versus ASC and DHA). ●, Control; ▼, ASC; ■, AFR; ♦, DHA; fw, fresh weight.

The previous findings demonstrated that long-term (48 h) treatments with AFR led to a more rapid rate of onion root growth (Hidalgo et al., 1991) because of a significant stimulation of cell elongation. There is a higher vacuolization in AFR-stimulated roots (Hidalgo et al., 1989), which constitutes an essential step in the cell enlargement process. The vacuoles provide the turgor pressure required for cell expansion (Cosgrove, 1987). Under these conditions, both a higher rate of nutrient uptake and apparent activation of cell metabolism take place (Navas, 1991). Thus, rates of nitrate and sugar uptake are significantly increased in 48-h AFR-pretreated roots compared with the controls.

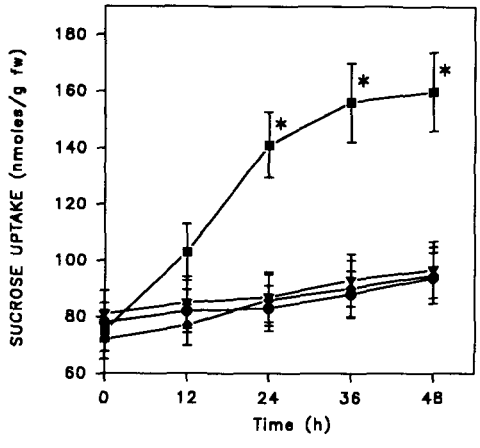

Figure 5. Kinetics of Suc uptake during ASC, AFR, and DHA pretreatments. The uptake rate increased significantly after 24 h of pretreatment ($P < 0.01$ versus 0 and 12 h) and then was maintained (*$P < 0.01$ versus control, ASC, and DHA). ASC and DHA had no effect on Suc uptake rate. ●, Control; ▼, ASC; ■, AFR; ♦, DHA; fw, fresh weight.

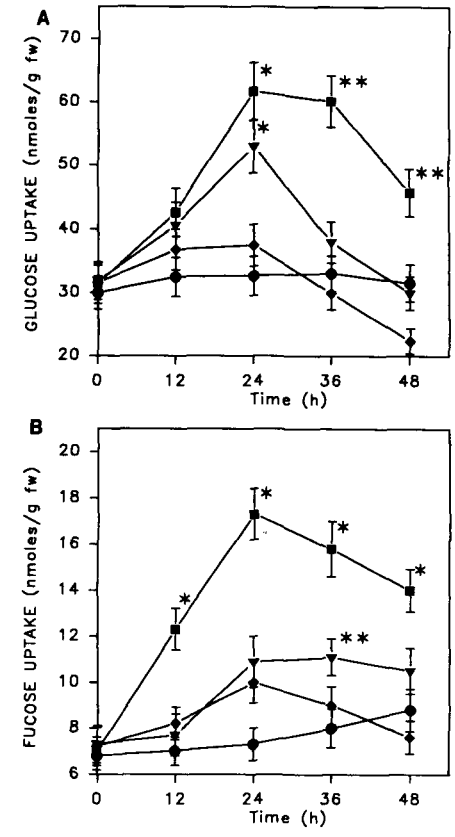

Figure 6. [^{3}H]Glc (A) and [^{14}C]Fuc (B) uptake as a function of pretreatment time with ASC, AFR, or DHA. For both sugars, the highest uptake rates were found after 24 h of AFR treatment ($P < 0.01$ versus 0 and 12 h). Afterward, uptake declined but remained significantly higher compared to all other conditions ($P < 0.01$). ASC and DHA also induced changes in uptake kinetics, but at the end of the preincubations uptake rates were similar to those of the untreated roots (for A, *$P < 0.05$ versus control and DHA; for B, *$P < 0.05$ versus control and **$P < 0.05$ versus control and DHA). ●, Control; ▼, ASC; ■, AFR; ♦, DHA; fw, fresh weight.

Because vacuolization, cell elongation, and nutrient uptake seem to follow the same time-dependent pattern during stimulation of root growth with AFR, a relationship between these phenomena can be inferred. Uptake and assimilation of nitrate by vacuoles have been studied (Beevers and Hageman, 1983; Reinhold and Kaplan, 1984), and it has been proposed that nitrate is transported into the vacuole in response to an interior positive membrane potential set up by the action of the tonoplast H^+-ATPase (Mandala and Taiz, 1985a, 1985b; Kaestner and Sze, 1987). The increase in nitrate assimilation studied here can be correlated with an increased tonoplast ATPase activity after 48 h of treatment with AFR (Hidalgo et al., 1991). Both phenomena appear to be related to increased tonoplast synthesis in AFR-treated roots.

Sugar uptake was significantly increased after 12 h of AFR treatment and reached maximum values at about 36 h. Thereafter rates were maintained or slightly decreased. It has been reported that protons are involved in sugar transport across plant membranes via a H^+/sugar symport in plasma mem-

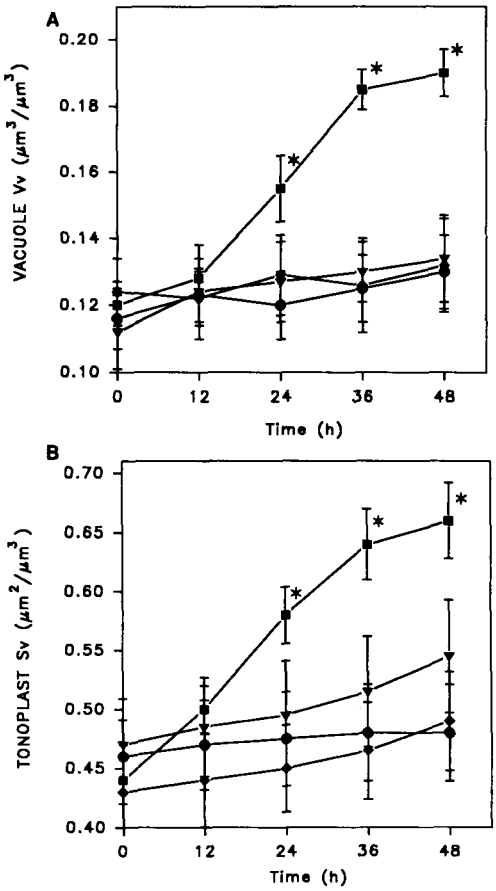

Figure 7. Kinetics of vacuole *Vv* (A) and tonoplast *Sv* (B) during preincubations with ASC, AFR, and DHA. This parameter increased significantly during AFR pretreatment and reached the highest values after 36 to 48 h ($P < 0.01$ versus 0, 12, and 24 h). Vacuole *Vv* is expressed in μm^3 of vacuole μm^{-3} of cell. Tonoplast *Sv* is expressed in μm^2 of membrane surface μm^{-3} of cell (for both parameters, $^*P < 0.01$ versus control, ASC, and DHA). Neither ASC nor DHA induced significant changes in vacuole parameters. ●, Control; ▼, ASC; ■, AFR; ♦, DHA.

brane and a H^+/sugar antiport in tonoplast (Giaquinta, 1983). Thus, an explanation similar to that proposed for nitrate uptake, activation of a H^+-translocating ATPase, may be valid for sugar uptake.

Onion roots growing under constant conditions show steady-state kinetics of growth with an equilibrium between both cell proliferation and elongation (Carmona and Cuadrado, 1986). The presence of AFR in the culture medium, but not ASC or DHA, induces a change in the steady-state kinetics during the first 24 h, increasing cell elongation rate and, as a consequence, increasing final cell length (Hidalgo et al., 1991). The stimulation of nutrient uptake reflects a general stimulation of root metabolism during the accelerated growth. Neither ASC nor DHA was able to increase and/or maintain the uptake of all of the nutrients investigated in this work, despite transient stimulation of Glc and Fuc uptake rates. In fact, both ASC and DHA clearly inhibited nitrate uptake. On the other hand, only AFR had a stimulatory effect on vacuolization. Therefore, a specific effect of AFR on nutrient uptake and in cell vacuolization-elongation is indicated.

The increased vacuolar volume is most likely the result rather than the cause of the more rapid elongation induced by AFR. How AFR enhances elongation and nutrient uptake is unknown. However, it has been shown that AFR stimulates proton extrusion and hyperpolarizes the plasma membrane (González-Reyes et al., 1992), providing a possible mechanism for plasmalemma energization that could facilitate transport processes across the plasma membrane. A specific role for the transplasmalemma redox system in energizing transport mechanisms has been proposed (Misra et al., 1984). Increased accumulation of a number of cations upon the stimulation of the plasma membrane redox system has also been recently reported (Welch et al., 1993).

Nevertheless, AFR action on membrane potential and proton secretion after long-term treatments has not been investigated. Therefore, its role in plasmalemma energization could be proposed only for the initial increased uptake of the nutrients.

In conclusion, long-term treatments with AFR induce a stimulation of root growth and cell enlargement that is accompanied by increased rates of nutrient uptake. Because tonoplast surface and vacuole volume seem to be significantly increased after AFR stimulation, at least a part of these nutrients could be incorporated into these subcellular compartments.

Although the chain of events linking AFR action on onion roots and vacuolization is far from understood, a relationship between a more rapid rate of cell enlargement and a stimulation of nutrient uptake caused by AFR has been shown. The apparent AFR action on the plasma membrane redox system could partially explain these findings.

The existence of an NADH-AFR oxidoreductase activity has been demonstrated in plasma membrane fractions derived from plant tissues (Luster and Buckout, 1989). Preliminary experiments carried out in our laboratory have suggested that onion roots reduce AFR extracellularly, possibly through a transmembrane NADH-AFR reductase. Further characterization of this activity is currently under investigation.

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