Planta (1999) 209: 424–434

Planta

Changes in onion root development induced by the inhibition of peptidyl-prolyl hydroxylase and influence of the ascorbate system on cell division and elongation

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Received: 14 October 1998 / Accepted: 31 May 1999

Abstract. Post-translational hydroxylation of peptide-bound proline residues, catalyzed by peptidyl-prolyl-4 hydroxylase (EC 1.14.11.2) using ascorbate as co-substrate, is a key event in the maturation of a number of cell wall-associated hydroxyproline-rich glycoproteins (HRGPs), including extensins and arabinogalactan-proteins, which are involved in the processes of wall stiffening, signalling and cell proliferation. *Allium cepa* L. roots treated with 3,4-DL-dehydroproline (DP), a specific inhibitor of peptidyl-prolyl hydroxylase, showed a 56% decrease in the hydroxyproline content of HRGP. Administration of DP strongly affected the organization of specialized zones of root development, with a marked reduction of the post-mitotic isodiametric growth zone, early extension of cells leaving the meristematic zone and a huge increase in cell size. Electron-microscopy analysis showed dramatic alterations both to the organization of newly formed cell walls and to the adhesion of the plasma membranes to the cell walls. Moreover, DP administration inhibited cell cycle progression. Root tips grown in the presence of DP also showed an increase both in ascorbate content (+53%) and ascorbate-specific peroxidase activity in the cytosol (+72%), and a decrease in extracellular "secretory" peroxidase activity (−73%). The possible interaction between HRGPs and the ascorbate system in the regulation of both cell division and extension is discussed.

Key words: *Allium* (root development) – Ascorbate – Cell division – Cell expansion – Hydroxyproline-containing protein – Root development

Introduction

Both cell division and extension contribute to root growth. The shape and length of cells are developmentally regulated by their extension rates and by the polarity of growth (Fowler and Quatrano 1997). Cell extension and polarized growth result from a complex balance of strictly interconnected physical and biochemical factors. As water enters the cell, the protoplasm gradually increases in volume and the different cell wall components (polysaccharides, pectins and structural proteins) must change their composition and aggregation to allow cell wall relaxation and extension (Cosgrove 1993). Conversely, cessation of elongation occurs as a consequence of molecular events leading to stiffening of the wall texture. It is conceivable that both functions (relaxation and stiffening) are in a dynamic equilibrium.

Ascorbate involvement in the processes of cell division (Liso et al. 1984, 1988) and extension (Arrigoni 1994; Cordoba and Gonzalez-Reyes 1994) is a well-established phenomenon. Moreover, a positive correlation has been clearly observed between experimentally induced changes in ascorbate content and growth rate of *Lupinus albus* seedlings (Arrigoni et al. 1997a). Nevertheless, because the complex roles of ascorbate in cell metabolism are not yet completely understood, the mechanism(s) of ascorbate action on growth processes has not been fully explained. Navas and co-workers thoroughly investigated the possible role of the ascorbate system (particularly the ascorbate free radical, AFR) as an inducer of cell elongation by means of the activation of ion transport leading to vacuole enlargement (Gonzalez-Reyes et al. 1994). However, although the turgor pressure represents the driving force of elongation, it must be accompanied by relaxation of the cell wall structural components to yield cell enlargement (Cosgrove 1993).

Structural glycoproteins characterized by the presence of hydroxyproline residues are relatively abundant in the extracellular matrix of plants. The expression 'hydroxyproline-rich glycoproteins' (HRGPs) is used to

Abbreviations: AFR = ascorbate free radical; APX = ascorbate peroxidase; DP = 3,4-DL-dehydroproline; HRGP = hydroxyproline-rich glycoprotein; PIG = postmitotic isodiametric growth

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identify the four major groups of such proteins: extensins, arabinogalactan proteins, proline/hydroxyproline-rich glycoproteins and solanaceous lectins (Sommer-Knudsen et al. 1997). Extensins are thought to form cross-links with wall pectic substances (Qi et al. 1995) and between extensin chains themselves, the latter catalyzed by extensin peroxidase (Schnabelrauch et al. 1996). It is well known that the hydroxylation of specific proline residues is necessary for the glycosylation of extensins (Kieliszewski et al. 1994). It is reasonable to assume that the interactions of HRGPs with other wall components could at least in part regulate cell wall assembly and the degree of its extensibility.

The synthesis of HRGPs is a multi-step process involving translation of mRNAs, hydroxylation of peptide-bound proline residues in the endoplasmic reticulum, processing in the Golgi apparatus and eventually their transport and assembly in the cell wall. The hydroxylation step requires as co-substrates peptidyl-proline, ascorbate, Fe^{2+} and 2-oxoglutarate, and is operated by peptidyl-prolyl-4 hydroxylase (Hedden 1992). Ascorbate is a highly specific electron donor for prolyl hydroxylase and is necessary for the hydroxylation reaction to occur (Arrigoni et al. 1977; De Gara et al. 1991; Tschank et al. 1994). This link between HRGPs synthesis and the ascorbate system targets the hydroxylation step as a key event of metabolic regulation of cell growth.

In order to test whether ascorbate could regulate plant growth by affecting HRGP hydroxylation, we analyzed the effects of 3,4-DL-dehydroproline (DP), a proline analogue specifically inhibiting peptidyl-prolyl hydroxylation (Cooper and Varner 1983; De Gara et al. 1991; Cooper et al. 1994), on growth and on the activities of the enzymes of the ascorbate system in *Allium cepa* roots.

In the present paper, we report experimental evidence that DP administration markedly affects root growth with two different and apparently contrasting mechanisms: (i) by inducing a dramatic early extension of cells in the post-mitotic growth zone, thus causing production of larger cells and altering the organization of root specialized developmental zones; (ii) by causing anomalies to the cell cycle and inhibiting cell division. In parallel, a general model of the multiple action of the ascorbate system on root growth is presented.

Materials and methods

Growth conditions and treatments. Onion (*Allium cepa* L.) bulbs were locally purchased and grown hydroponically so that only their basal plates were immersed in water. After 72 h in dark at 20 °C the bulbs were washed and subjected to different treatments as indicated. Inhibition of peptidyl-prolyl hydroxylase activity was obtained using DP according to Cooper and Varner (1983). In order to test the efficacy and selectivity of DP, either 3,4-L-dehydroproline or *cis*-hydroxyproline were used in control experiments. The changes in root growth, ascorbate and hydroxyproline contents and cell-cycle progression which were observed using the mixed isomers could be detected using one-half concentrations of 3,4-L-dehydroproline, whereas roots treated with *cis*-hydroxyproline in the range 250–750 μM showed values comparable to samples grown in water (data not shown). Root growth was measured using a flexible ruler. Apical root segments of 10 mm length were used for both protein extraction and ascorbate determination.

Light and electron microscopy. Tip segments from onion roots incubated for 20 h in distilled water (control) or in 500 μM DP were fixed in 6% glutaraldehyde and processed for light and trasmission electron microscopy as previously described (Zanchin et al. 1993).

For light microscopy, thin sections (1 μm thick) were cut with an Ultracut Reichert-Jung ultramicrotome, stained with 1% toluidine blue and 1% tetraborate (1:1, v/v), and observed and photographed under a Leitz ortholux microscope.

For electron microscopy, ultrathin sections (60 nm) were stained with lead citrate and examined with a Hitachi HS9 electron microscope operating at 75 kV.

For the estimation of cell divisions, the roots were fixed in a 3:1 ethanol:acetic acid (v/v), hydrolyzed in 1 N HCl at 60 °C for 5 min and stained with Schiff's reagent for 2 h. Segments of 1 mm starting from the tip were dissected and squashed in a drop of 45% acetic acid. For each treatment, five squash preparations were made; for each slide, 1000 cells were scored, giving a total of 5000 cells scored. The mitotic index was calculated as percentage of the ratio between dividing cells and number of cells scored.

Measurement of peptide-bound hydroxyproline content. Cell-wall fractions for the assay of hydroxyproline content were isolated according to Kieliszewski et al. (1990). Root tips (10 mm) were frozen in liquid N_2, ground to a fine powder with a mortar and pestle and suspended in 1 M NaCl. The suspension was sonicated with Sonifier 250 (Branson) for 7 min with 20-s pulses at 0–4 °C, then centrifuged at 1000 *g* for 5 min. The resulting pellet was washed and re-centrifuged. The procedure was repeated five times. The obtained pellet was freeze-dried and subjected to hydrolysis in 6 N HCl for 18 h at 110 °C. Protein-bound hydroxyproline content was estimated according to Arrigoni et al. (1977) using Ehrlich's reagent.

Determination of ascorbic acid and dehydroascorbic acid. Samples were ground in 5% metaphosphoric acid. The homogenate was centrifuged for 15 min at 12 000 *g* and the supernatant used for determinations according to Zhang and Kirkham (1996).

Enzyme analysis. Immediately after cutting, root tips were washed with water and homogenized in a chilled mortar with 50 mM Tris-HCl buffer (pH 7.5; buffer A) containing 0.25 M sucrose and 1 mM magnesium acetate. The homogenate was filtered through two layers of gauze and centrifuged at 1000 *g* for 5 min. The supernatant was re-centrifuged at 20 000 *g* for 15 min and the resulting supernatant assayed as the cytosolic fraction. The pellet resulting from the first centrifugation step was resuspended in buffer A plus 1% Triton X 100 and re-centrifuged. The pellet was washed three times in buffer A and re-centrifuged. The fraction obtained (cell wall preparation) did not show glucose-6-phosphate dehydrogenase activity, as assayed according to Löhr and Waller (1974).

Spectrophotometric assays of ascorbate peroxidase (APX), AFR reductase and dehydroascorbate reductase activity were performed according to Arrigoni et al. (1997a). Total peroxidase activity was measured according to Ferrer et al. (1990) using 4-methoxy-α-naphthol as substrate. Proteins were measured according to Bradford (1976) using bovine serum albumin as a standard. Native-PAGE was performed using a stacking gel containing 4.3% acrylamide and a running gel containing 7.3% acrylamide with a running buffer composed of 4 mM Tris-HCl (pH 8.3) and 38 mM glycine. After the run at 2 °C, the gel was washed with distilled water.

Isoenzymes of APX were visualized after incubation of the gel for 15 min at room temperature in 0.1 M Na-phosphate buffer (pH 6.4) containing 4 mM ascorbate and 4 mM H_2O_2. The gel washed in water was stained in the dark with a solution of 0.125 N HCl

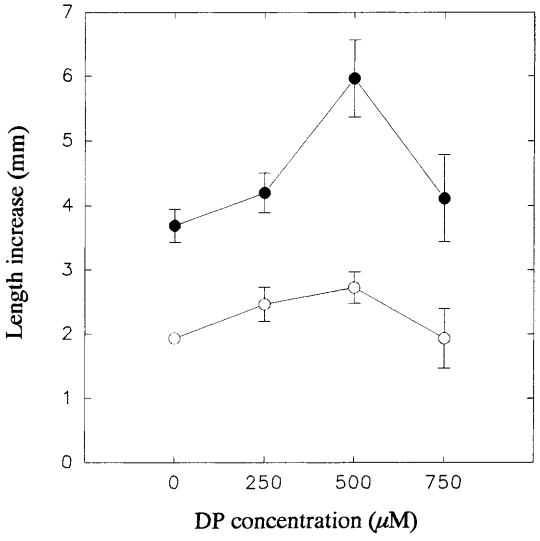

Fig. 1. Increase in length of roots after short-term DP incubation. Onion roots were incubated for 6 (*open symbols*) and 20 h (*filled symbols*) with different DP concentrations. Mean values ± SE of three independent experiments

containing 0.1% ferricyanide and 0.1% ferrichloride (w/v) for 10 min. The APX isoenzymes were located as achromatic bands on a Prussian blue background, as a result of the reaction of ferrichloride and ferrocyanide, the latter having been produced by the reduction of ferricyanide with unreacted ascorbate. Specific inhibition of APX activity by *p*-chloromercuribenzoic acid (pCMB; Amako et al. 1994) was obtained pre-incubating gels for 10 min in 0.1 M Na-phosphate buffer (pH 6.4) containing 500 μM pCMB. Gels were then washed with distilled water, incubated with the substrates and stained as above.

In-vivo staining of total peroxidase activity. The method described by de Pinto and Ros-Barcelo (1997) was used. After incubation either in distilled water (controls) or in 500 μM DP solution, roots were washed with distilled water and bulbs suspended over beakers filled with 50 mL of Tris-acetate buffer (pH 5) containing 1 mM 4-methoxy-α-naphthol and 0.33 mM H_2O_2, so that roots were completely submerged for 15 min. Peroxidase activity could be observed by the deposition of blue stain.

Analysis by SDS-PAGE and immunoblotting. Proteins electrophoresed under denaturing conditions according to Schägger and von Jagow (1987) were either silver-stained or transferred for immunoblot. Western blots were performed according to Harlow and Lane (1988). Proteins were transferred to Immobilon polyvinylidenedifluoride membranes (Bio-Rad). Rabbit polyclonal antibodies against tomato extensin were a generous gift from Dr. Mike Brownleader (University of Westminster, London, UK). The membrane was blotted with peroxidase-conjugated goat anti-rabbit IgG and diaminobenzidine was used for detection.

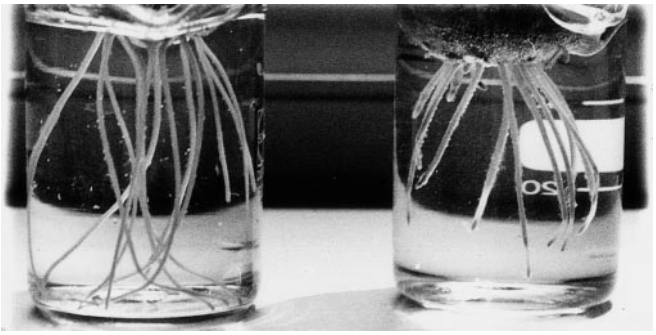

Fig. 2. Effect of DP application on root growth. Two onion bulbs with roots of similar length (4–5 cm) were immersed for 40 h either in distilled water (*left*) or 500 μM DP (*right*). The inhibitory effect of DP treatment on root growth is clear

Results

Treatment with DP affects root growth. Application of DP influences *Allium cepa* root growth with a complex and apparently contradictory kinetic (Figs. 1, 2). In 'short-term' incubations (Fig. 1), after 6 h of treatment 250 and 500 μM DP induced a 27% and a 41% increase in root length, respectively. A higher DP concentration (750 μM) induced neither stimulation nor inhibition as compared to control samples. After 20 h of treatment the same trend was observed. As a consequence of applying 750 μM DP, however, some of the treated roots appeared to be damaged. Somewhat surprisingly, the longer incubation time totally reversed the apparent stimulatory effect of DP administration and a dramatic inhibitory effect could be clearly observed after 40 h of treatment with 500 μM DP (Fig. 2). The inhibitory effect of DP on growth of onion roots was largely due to the arrest of the cell cycle: the mitotic index fell nearly to zero in the root meristem after 40 h of DP treatment (Table 1). Incubation for 20 h in 500 μM DP caused little change in the mitotic index, but most of the dividing cells were apparently blocked in metaphase (Table 1). This remarkable effect is also shown in Fig. 3 for two representative examples of control and treated root tip preparations. In *Pisum sativum* the inhibitory effect of DP on both root growth and cell division is

Table 1. Mitotic index (MI) and percentage of mitotic phases in the root meristem of *Allium cepa*. Each determination is based on five squash preparations. For each, 1000 cells were scored. Standard errors are indicated. Only sporadic divisions could be observed after 40 h of incubation

Treatment	MI	Mitotic phases (%)			
		Prophases	Metaphases	Anaphases	Telophases
Control	10.4 ± 0.7	60	17.7	21	1.15
500 μM DP, 20 h	8.31 ± 0.5	21.35	75.45	3.2	0
500 μM DP, 40 h	0.5 ± 0.1	–	–	–	–

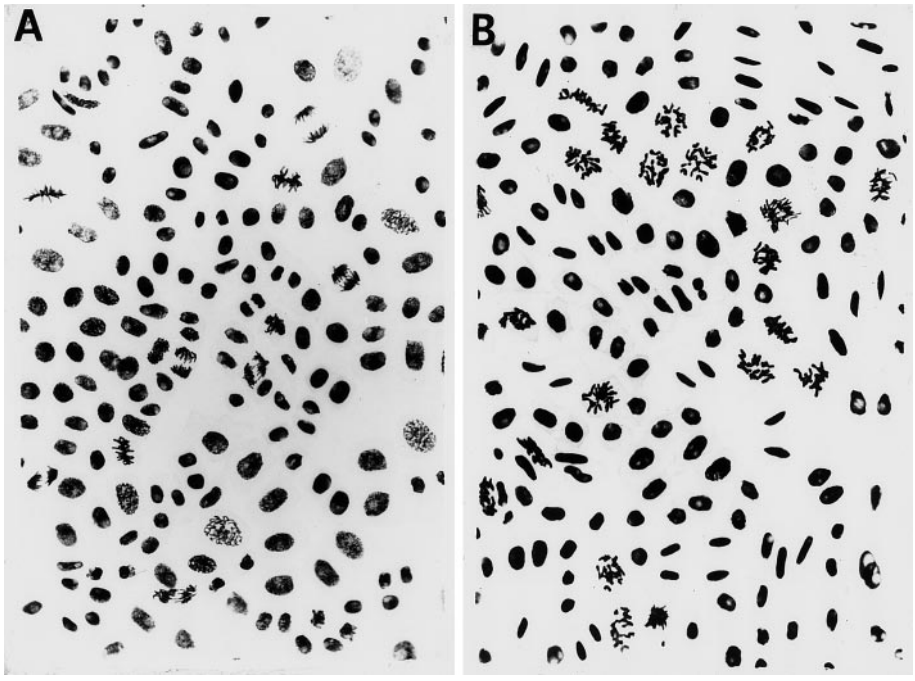

Fig. 3A,B. Effect of DP on cell division. Squash preparations of tips (1 mm) from onion roots incubated for 20 h either in distilled water (**A**) or in 500 μM DP (**B**) were stained with Schiff's reagent. Treated cells are apparently blocked in metaphase

more precocious and is already evident after 7.5 h (data not shown).

Administration of DP causes changes in root cell organization into specialized zones. Light microscopy of DP-treated roots showed that root organization is strongly affected by treatment. Cells leaving the meristematic zone were clearly more extended than their control counterparts (Fig. 4). In the zone between 600 and 800 μm from the root tip, the average cell length (± SE) of four different cortical cell files was 18.25 ± 1.8 and 36.75 ± 4.7 μm in control and treated samples, respectively. This apparently 'anticipated' anisodiametric growth strongly reduced the postmitotic isodiametric growth (PIG) zone described by Baluška et al. (1996). Treated cells also had larger vacuoles, whereas controls had numerous small vacuoles scattered in the cytoplasm.

Changes in cell wall-plasma membrane adhesion and cell wall deposition induced by DP. Electron-microscopy analysis showed that DP treatment strongly affected the ultrastructural organization of the cell wall. Transverse sections of controls (Fig. 5A,B) showed well-structured walls and spatial contiguity between the plasma membrane and the wall. In contrast, treated cells had highly irregular walls that were not homogeneously electron dense. The plasma membrane did not adhere to the cell wall, leaving open spaces which resembled a 'plasmolytic effect' (Fig. 5C,D). Longitudinal sections showed that the treatment had striking effects on the newly forming transverse walls (Fig. 6). In controls, deposition of vesicles containing materials regularly occurred, forming an ordered cell plate (Fig. 6A) from which a well-structured and defined cell wall was built up (Fig. 6B). In contrast, clusters of vesicles were oddly distributed in treated samples (Fig. 6C), leading sometimes to the formation of an irregularly organized cell wall, showing large masses protruding into the cell (Fig. 6D).

Changes in the contents of protein-bound hydroxyproline and ascorbate induced by DP. Incubation for 20 h in 500 μM DP caused a 56% decrease in cell-wall-associated peptide-bound hydroxyproline [mean value of three experiments ± SE was 2.9 ± 0.3 mg (g DW)$^{-1}$ in controls and 1.27 ± 0.1 in treated samples, respectively). The SDS-PAGE and silver staining of cell wall fractions showed two additional protein bands in DP-treated samples (Fig. 7A). It is interesting that these bands cross-reacted with a polyclonal antibody against tomato extensin (Fig. 7B).

As mentioned above, ascorbate is a specific co-substrate for prolyl hydroxylase. It has been previously demonstrated that the inhibition of prolyl-hydroxylase activity induces an increase in ascorbate content in carrot, potato slices and pea embryos (De Gara et al. 1991). In growing *Allium cepa* roots a 53% increase in ascorbate content was observed after 20 h of DP treatment (Fig. 8A). A small amount of dehydroascorbate, the oxidized form of ascorbate, could be detected in both control and treated roots; DP, however, seemed to lower the dehydroascorbate content slightly (Fig. 8B).

Treatment with DP affects the activities of APX and extracellular peroxidases. The activities of cytosolic redox enzymes of the ascorbate system are reported in Table 2. No significant difference was observed between control and treated samples in the activity of both AFR reductase and dehydroascorbate-reducing proteins, the enzymes devoted to ascorbate regeneration from its oxidized forms. Treatment with DP induced a 72%

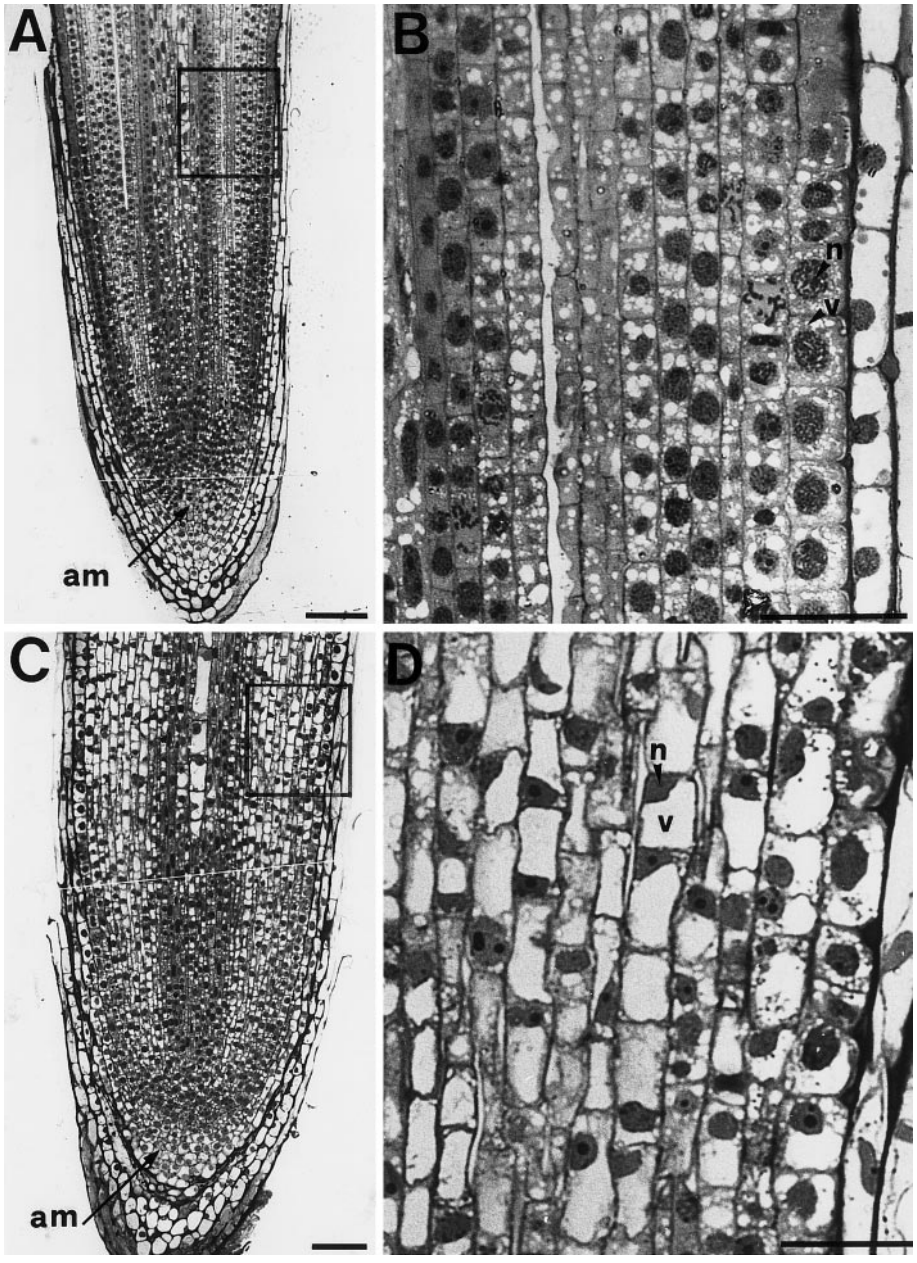

Fig. 4A–D. Light micrographs of longitudinally sectioned onion root tips. In the control root (**A**) an extended PIG zone, with cells maintaining their isodiametric shape, can be seen throughout the apical meristem. **B** Higher magnification of the area framed in A, 600–800 μm from the apical meristem. The cells show very juvenile characteristics, with large nuclei and numerous small vacuoles scattered in the cytoplasm. Most of them have an isodiametric shape and several are still dividing. **C** Root treated for 20 h with 500 μM DP. Note the wider root diameter and the larger cell sizes. The PIG zone is reduced in length and the cells acquire an elongated shape closer to the apical meristem. **D** Higher magnification of the area framed in C, 600–800 μm far from the apical meristem. Most cells are elongated, with a large central vacuole. Dividing cells are not visible. *am*, apical meristem; *n*, nucleus; *v*, vacuole. Bars = 100 μm (**A,C**), 50 μm (**B,D**)

increase in APX activity. Figure 9 shows the electrophoretic profile of APX isoforms. The same electrophoretic pattern of bands, all sensitive to inhibition by *p*-chloromercuribenzoic acid (data not shown), was detected in both control and treated samples. In accordance with spectrophotometric measurements, higher APX activity could be observed after DP treatment (Fig. 9). Oxidation of ascorbate without addition of H_2O_2 occurred to the same extent in both control and treated samples. This oxidation, however, seemed to be due to non-enzymatic reactions, rather than to ascorbate oxidase activity, since no band could be detected by activity staining of polyacrylamide gels (data not shown).

Total peroxidase activity in the cytosolic fraction, measured using the non-physiological substrate 4-methoxy-α-naphthol which acts as an electron donor for both APX and the other peroxidases, was substantially unchanged; conversely, cell-wall-associated (extracellular) peroxidases showed much lower activity in DP-treated samples (Table 2). In-vivo staining for total peroxidase activity showed that in treated roots the blue stain (indicative of activity) was mainly confined to the distal part (approx. 0.5 cm), whereas controls were stained throughout (Fig. 10).

Discussion

As yet, the main difficulty in trying to identify the complex events regulating root growth is to put together the scattered information obtained investigating separately the morphological, cellular and molecular aspects of root development.

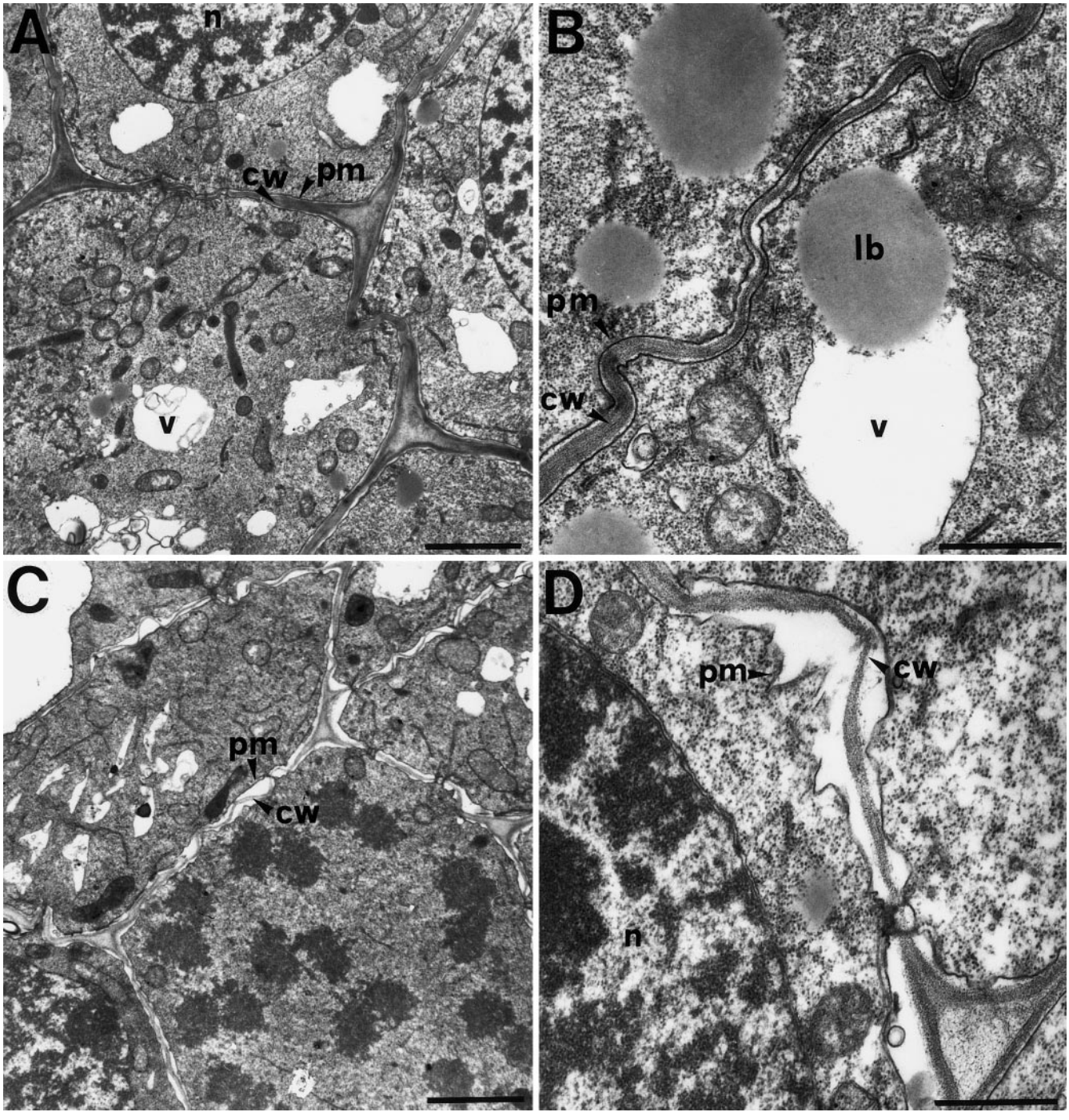

Fig. 5A–D. Electron micrographs of transverse sections of the apical meristematic zone. **A,B** Meristematic cells of a control root. **A** Note the thin, well-structured cell walls. **B** Higher magnification showing a compact and homogeneously electron-dense cell wall, regularly bordered by the plasma membrane. **C,D** Meristematic cells of a root incubated for 20 h with 500 μM DP. **C** The cell walls look distorted, with a non-adherent plasma membrane. **D** Higher magnification showing a misshaped cell wall and large open spaces between wall and plasma membrane. *cw*, cell wall; *lb*, lipidic body; *n*, nucleus; *pm*, plasma membrane; *v*, vacuole. Bars = 2.5 μm **(A,C)**, 1 μm **(B,D)**

A wide range of extracellular hydroxyproline-containing proteins has been described and characterized. Ascorbate-dependent hydroxylation of proline residues is essential for HRGPs to play their different roles in cell organization. Our experimental approach using DP as a specific inhibitor of peptidyl-prolyl hydroxylase activity allowed us to obtain a general view of the effects of HRGP underhydroxylation on root growth, cell organization and the ascorbate system.

Our data strongly indicate that the synthesis of hydroxyproline-containing proteins is necessary for cell division, thus further substantiating data reported by Cooper et al. (1994) about dramatic changes in cell division and wall regeneration patterns in DP-treated tobacco protoplasts. In the present study, we observed few differences in the mitotic index up to 20 h of

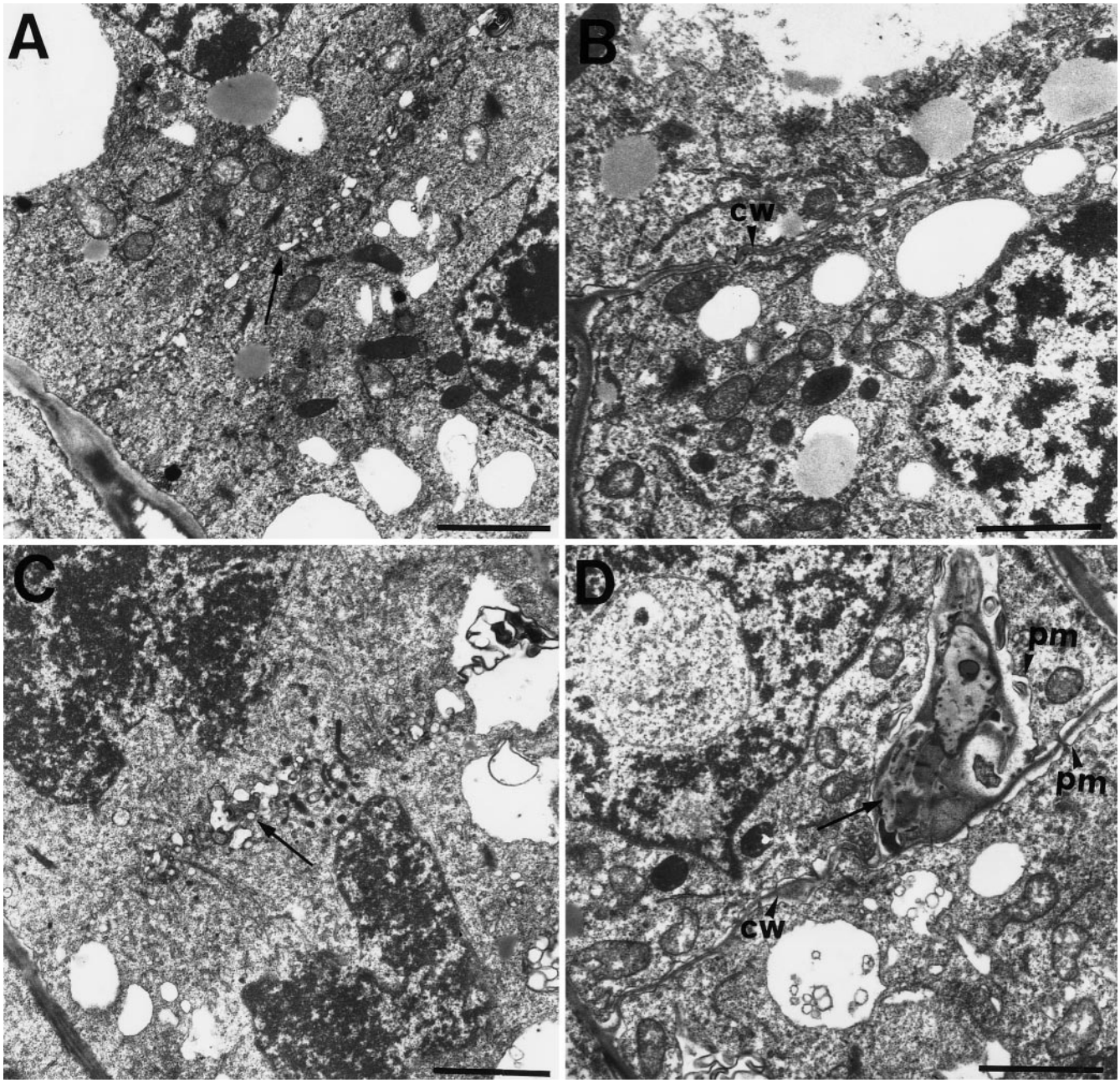

Fig. 6A–D. Electron micrographs of longitudinal sections of the apical meristematic zone. **A,B** Meristematic cells of a control root. **A** A forming cell plate with small and well-lined-up vesicles (*arrow*). **B** A young, very thin transverse cell wall showing a regular organization. **C,D** Meristematic cells of a root incubated for 20 h with 500 μM DP. **C** Oddly distributed vesicles (*arrow*) along a forming cell plate. **D** A misshaped cell wall with a mass of material (*arrow*) protruding into the cell. The plasma membrane is away from the cell wall. *cw*, cell wall; *pm*, plasma membrane. Bars = 2 μm

treatment, and this is not surprising, since the cell cycle of onion meristematic cells requires approximately 17–18 h (Liso et al. 1988).

We have observed that longer DP incubation (up to 40 h) totally blocks both cell division and length increase in treated roots. The interpretation of these data is, however, difficult as there could be a more pervasive and less specific DP effect on protein properties in general. In fact, in the long run, DP incorporation into proteins, and particularly into proline-rich proteins, is likely to affect the function of these proteins and the binding of proteins which recognize such sequences, as in the case of profilin (Gibbon et al. 1998).

The relevant DP-induced increase in the percentage of metaphases and corresponding decrease in the number of anaphasic, telophasic and prophasic cells, and the dramatic reduction in the mitotic index (Table 1) clearly indicate the involvement of hydroxyproline-containing protein(s) in different points of the cell-cycle progression. To our knowledge, such a protein(s) has not been identified yet. Nevertheless, the expression of a gene correlated with mitosis whose transcript shows high homology with HRGPs has been recently reported

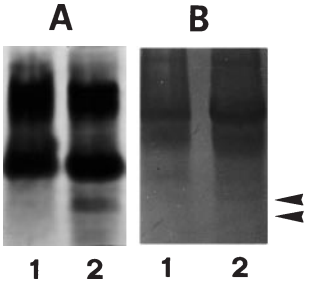

Fig. 7A,B. SDS-PAGE and Western blot with an anti-extensin antiserum. Cell wall fractions extracted from control roots (*lanes 1*) and from roots treated for 20 h with 500 μM DP (*lanes 2*) were electrophoresed in SDS-PAGE. Half of each gel was silver-stained (**A**) and the other half was blotted with an antibody against tomato extensin (**B**). *Arrow heads* indicate two additional protein bands detected in DP-treated samples. For each lane, 30 μg of total protein was loaded

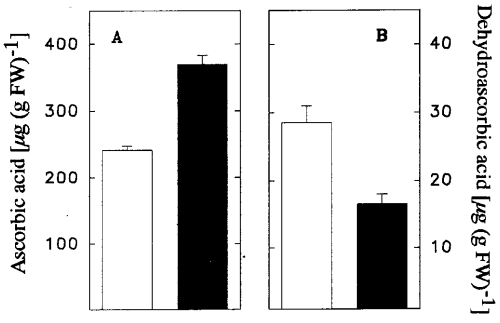

Fig. 8A,B. Increase in ascorbic acid content induced by DP treatment. Ascorbic acid (**A**) and dehydroascorbic acid (**B**) were extracted from roots immersed for 20 h either in distilled water (*open bars*) or in 500 μM DP (*filled bars*). Mean values ± SE of three independent experiments are shown

(Woo and Hawes 1997). It is tempting to speculate that the molecular mechanism of ascorbate requirement for cell division could be the utilization of ascorbate for the hydroxylation of proline residues of this or other analogue proteins. It is noteworthy that DP administration up to 1 mM did not cause any change in cell-cycle progression either in human fibroblasts or in hybrid human-hamster cell lines (M. Rocchi, personal communication), strongly suggesting that the results observed in our experiments could be a direct consequence of the interaction between different cell structures and compo-

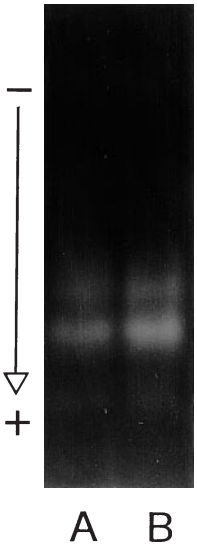

Fig. 9. Native-PAGE and activity staining of APX. Protein extracts from roots immersed for 20 h either in distilled water (*lane A*) or in 500 μM DP (*lane B*) were loaded onto gel (300 μg of total proteins per lane) and stained for APX activity

nents of the extracellular matrix of the plant cell (Fowler and Quatrano 1997). It has been observed that specialized regions of the cell wall are involved in the regulation of microtubule orientation and organization of the cortical domain. A possible involvement of HRGPs in microtubule stabilization also derives from the observation that DP treatment affects vescicle deposition and cell plate formation (another cytoskeleton-driven process). Moreover, Akashi and Shibaoka (1991) indicated the possibility that extensin could interact with plasma-membrane components which in turn can regulate the organization of cortical microtubules. Cytoskeleton organization and orientation of cortical microtubules have been observed to undergo dramatic changes in the transition from a slow, near-isotropic mode of cell growth observed in the PIG zone, to the rapid anisotropic growth characteristic of the zone of elongation (Baluška et al. 1996). Indeed we observed that DP administration strongly reduces the PIG zone and induces early extension.

Electron microscopy analysis clearly shows that DP treatment affects the adhesion of the plasma membrane

Table 2. Enzyme activities. Protein extracts from roots incubated for 20 h in distilled water (control) or 500 μM DP were assayed with the different substrates. Mean of three independent experiments ± SE. ASC, ascorbate; DRP, dehydroascorbate-reducing proteins; MN, 4-methoxy-α-naphthol; POD, total peroxidase activity. Significance of data was analyzed with the Student's *t*-test

Enzyme assayed	Treatment		*t*-test
	Control	500 μM DP	
AFR red [nmol NADH oxidized min^{-1} (mg protein)$^{-1}$]	329 ± 95	364 ± 22	n.s.
DRP [nmol ASC oxidized min^{-1} (mg protein)$^{-1}$]	174 ± 18	148 ± 24	n.s.
APX [nmol ASC oxidized min^{-1} (mg protein)$^{-1}$]	88 ± 16	150 ± 15	$P < 0.05$
Extracellular POD [nmol MN oxidized min^{-1} (mg protein)$^{-1}$]	640 ± 63	174 ± 12	$P < 0.05$
Cytoplasmic POD [nmol MN oxidized min^{-1} (mg protein)$^{-1}$]	233 ± 12	253 ± 30	n.s.

n.s. Not significant

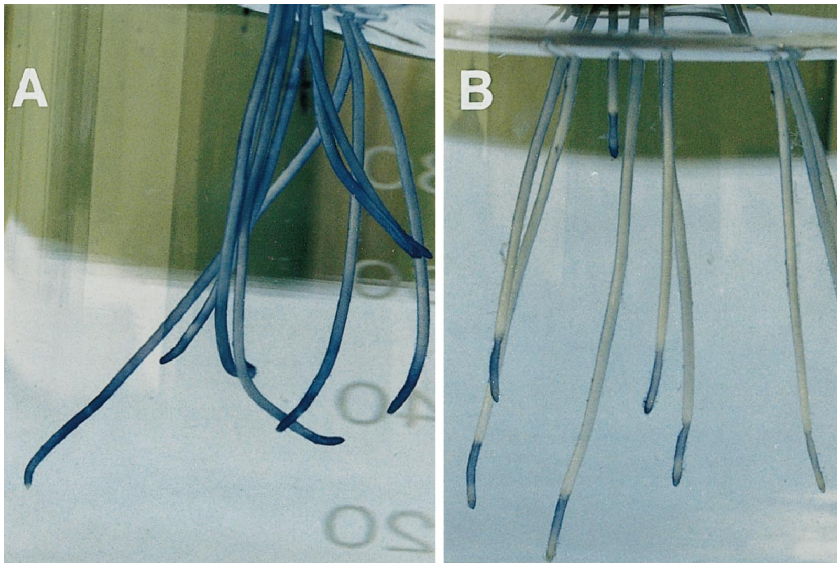

Fig. 10A,B. In-vivo staining of total peroxidase activity. Onion roots immersed for 20 h either in distilled water (**A**) or in 500 μM DP (**B**) were stained for total peroxidase activity using 4-methoxy-α-naphthol as an electron donor. The blue stain is indicative of activity

to the cell wall, thus indicating that HRGP hydroxylation is required also in this case. This is in agreement with previous observations on the presence of punctate fluorescence on the surface of protoplasts derived from onion epidermal strips stained with an anti-extensin antiserum (Pont-Lezica et al. 1993). Immunological studies also indicate that in the rice root apex four glycoproteins located both in the cell walls and plasma membrane share a common epitope recognized by monoclonal antibodies against a homologous HRGP (Smallwood et al. 1995). According to some authors, arabinogalactan proteins could also be involved in the cell wall-plasma membrane adhesion (Pennell et al. 1989). A role for arabinogalactan proteins in the processes of cell division and expansion has also been proposed (Serpe and Nothnagel 1994). Moreover, it has been recently reported that two arabinogalactan proteins from *Nicotiana alata* and from *Pyrus communis* are attached by a glycosylphosphatidylinositol membrane anchor (Youl et al. 1998). The presence of a proline-rich linker protein (Goodwin et al. 1996) has also been observed, although no information is available about the possible presence of hydroxylated proline residues in this or other proteins whose sequence has been deduced from cDNAs. We hypothesize that underhydroxylation of one or more HRGP(s) putatively involved in the cell wall-plasma membrane adhesion could cause the observed malfunction.

Inhibition of the hydroxylation of peptide-bound proline residues in onion roots caused a clear stimulation of cell expansion (Figs. 1, 4). In a widely accepted view, extensins could regulate the degree of cell wall extensibility by connecting different structures via both covalent and non-covalent cross-links (Cassab and Varner 1988; Qi et al. 1995; Schnabelrauch et al. 1996). According to our data, cell enlargement induced by 500 μM DP treatment up to 20 h could be due to changes in this extensin network, which in turn would induce cell wall loosening. It should also be considered that extensin glycosylation occurs by arabinosylation of hydroxyproline residues, and is necessary for the structural role of the protein (Sommer-Knudsen et al. 1997). It is noteworthy that western blotting with a tomato polyclonal anti-extensin antibody showed additional cross-reactive bands in DP-treated samples (Fig. 7B). These bands could be tentatively interpreted as deglycosylation products and/or could be the consequence of a different aggregation of extensin molecules, although more detailed analysis is required to elucidate this point.

Our results also suggest that the induction of cell expansion by DP is not only linked to hydroxylation processes, but is due to the consequences that the inhibition of prolyl hydroxylase has on the ascorbate system: DP treatment, while inhibiting the peptidyl-proline hydroxylation, determines a rise in the ascorbate content (since the prolyl-hydroxylase is the most important ascorbate consumer enzyme in the cell), a net increase in the APX activity and concomitantly a remarkable decrease in extracellular peroxidase activity.

The observed increase in APX activity apparently correlates with increased ascorbate availability, as previously and repeatedly observed (Arrigoni et al. 1997b; Conklin et al. 1997), although the mechanism underlying this activation is still far from being elucidated. It is, however, well known that APX is regulated at the post-transcriptional level, since mRNA accumulation apparently does not correlate with enzyme activity (Lopez et al. 1996; Mittler et al. 1998).

The removal of H_2O_2 by APX could be an important step in the regulation of cell wall cross-links. It is generally assumed that H_2O_2 can freely circulate in different cell compartments (Mittler et al. 1998). Moreover, H_2O_2 could form transient adducts with different molecules (Schubert and Wilmer 1991). Hydrogen peroxide is used as a substrate by an array of peroxidases, including extensin peroxidase (Schnabelrauch et al. 1996); in addition, plasma membrane-associated arabinogalactan proteins are oxidatively cross-linked in the presence of H_2O_2 (Kjellbom et al. 1997). Ascorbate

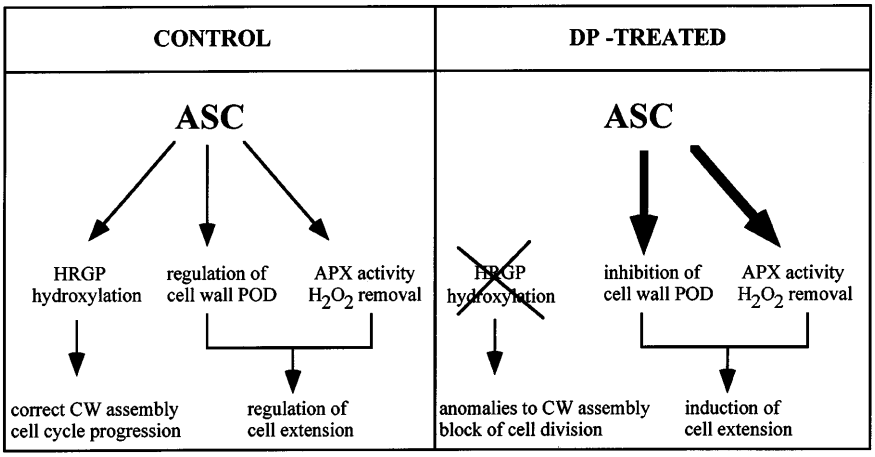

Fig. 11. Possible effects of ascorbate on plant growth. The proposed scheme summarizes anomalies observed as a consequence of DP treatment and presents a general model of ascorbate (*ASC*) action on root growth. *CW*, cell wall; *POD*, extracellular peroxidases

peroxidase is generally considered the most effective scavenger of H_2O_2 in nearly all cell compartments (Arrigoni 1994). Since APX has an affinity for H_2O_2 higher than that of the other peroxidases, it could reasonably be argued that when APX activity rises, the amount of H_2O_2 available for the so-called secretory peroxidases decreases and with it their activities.

However, the decrease of peroxidase occurring in DP-treated roots could also be due to the increased ascorbate content in the cells: there are several indications that ascorbate inhibits cell wall peroxidases (Takahama and Oniki 1992; Cordoba-Pedregosa et al. 1996). Increased ascorbate availability resulting from the inhibition of prolyl-hydroxylase could therefore act both by up-regulating APX (thus removing H_2O_2) and directly inhibiting the activity of the secretory peroxidase(s). A third possible role has recently been suggested by Fry (1998), who observed an ascorbate-dependent formation of hydroxyl radicals involved in the scission of cell wall polysaccharides.

Due to the fact that for our biochemical investigations, we used apical root segments of 10 mm, the values and activities measured represent an average of possible different conditions occurring in the different zones of root development. This means that differences observed could be underestimated. It is noteworthy that in-vivo staining of total peroxidase activity shows that peroxidases are apparently confined to the distal zone in DP-treated roots, whereas proximal to this zone the activity is substantially lacking; in control roots the stain is also clearly detectable proximal to the root apex. In trying to understand these data, it is necessary to make some assumptions. Since it has been ascertained that in dividing cells the activity of APX is very high, whereas that of the secretory peroxidases is negligible (De Gara et al. 1996), the stain in the root tips is due essentially to APX activity. During cell differentiation, while APX activity gradually decreases, a rise in activity of the other peroxidases occurs (De Gara et al. 1996); accordingly the blue stain above the root apex in the control is mainly due to their activities. The lack of stain proximal to the root apex in treated roots can be attributed to a dramatic decrease in secretory peroxidases, as indicated from data in Table 2, probably due to the increase in the ascorbate content which directly inhibits their activities.

The results of our observation are summarized in the scheme shown in Fig. 11, in which a central role of ascorbate in growth regulation is proposed. According to this model, ascorbate would regulate cell division and plasma membrane-cell wall adhesion via hydroxylation of proline residues of specific proteins, whereas cell elongation would be a consequence of both direct inhibition of cell-wall-associated peroxidase activity and APX-mediated removal of H_2O_2, which is used as a substrate by peroxidases.

Further experiments have been planned to identify specific hydroxyproline-containing protein(s) involved in the processes of cell division and elongation and of plasma membrane adhesion to the cell wall.

The authors thank Dr. Mike Brownleader (Westminster University, London UK) for kindly supplying tomato anti-extensin antibodies. The authors also thank Prof. M. Rocchi (Institute of Genetics University of Bari, Italy) for sharing unpublished results and two anonymous referees for useful comments on the manuscript.

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