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RESEARCH PAPER

Changes in the ascorbate metabolism of apoplastic and symplastic spaces are associated with cell differentiation

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Abstract

Ascorbate levels and redox state, as well as the activities of the ascorbate related enzymes, have been analysed both in the apoplastic and symplastic spaces of etiolated pea (*Pisum sativum* L.) shoots during cellular differentiation. The ascorbate pool and the ascorbate oxidizing enzymes, namely ascorbate oxidase and ascorbate peroxidase, were present in both pea apoplast and symplast, whereas ascorbate free radical reductase and dehydroascorbate reductase were only present in the symplastic fractions. During cell differentiation the ascorbate redox enzymes changed in different ways, since a decrease in ascorbate levels, ascorbate peroxidase and ascorbate free radical reductase occurred from meristematic to differentiated cells, whereas ascorbate oxidase and dehydroascorbate reductase increased. The activity of secretory peroxidases has also been followed in the apoplast of meristematic and differentiating cells. These peroxidases increased their activity during differentiation. This behaviour was accompanied by changes in their isoenzymatic profiles. The analysis of the kinetic characteristics of the different peroxidases present in the apoplast suggests that the presence of ascorbate and ascorbate peroxidase in the cell wall could play a critical role in regulating the wall stiffening process during cell differentiation by interfering with the activity of secretory peroxidases.

Key words: Ascorbate, cell wall, class III peroxidases, dehydroascorbate, galactono-γ-lactone dehydrogenase, plant cell differentiation, reactive oxygen species.

Introduction

The regulation of the cellular redox state is a crucial point for plant development and responsiveness to environmental stimuli. Several redox metabolites are involved in maintaining the ideal redox balance in plant tissues, among which ascorbate (ASC) plays a pivotal role by acting as a redox buffer as well as a sensor of metabolic changes involving redox reactions (de Pinto *et al*., 1999; Pastori and Foyer, 2002; Foyer and Noctor, 2003). In recent years, much attention has been paid to the roles of ASC metabolism in stress responses (Kubo *et al*., 1995; Noctor and Foyer, 1998; Asada, 1999; Smirnoff, 2000; Schützendübel and Polle, 2002; Agrawal *et al*., 2002). However, it is widely accepted that ASC is also involved in plant development (Arrigoni, 1994; Cordoba and Gonzáles-Reyes, 1994; De Gara, 2003). The levels and redox balance of ASC are often indicative of certain developmental stages or conditions. Young tissues, characterized by an active metabolism, often have higher ASC levels and cytosolic ASC peroxidase (APX) activity than mature tissues, whereas, a decrease in ASC content and in APX activity occurs in ageing tissues (De Gara *et al*., 1991, 1996; Borraccino *et al*., 1994). Consistently, treatments, which delay the senescence-related decrease in ASC and APX,

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Abbreviations: AFR, ascorbate free radical; AFRR, ascorbate free radical reductase; AOX, ascorbate oxidase; APX, ascorbate peroxidase; ASC, ascorbate; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; IEF, isoelectric focusing; IWF, intercellular washing fluid; PAGE, polyacrylamide gel electrophoresis; *p*CMB, *p*-chloromercuribenzoate; POD, class III peroxidases; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate.

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also delay leaf senescence (Borraccino *et al*., 1994). Seed maturation is another example in which changes in ASC metabolism are representative of different physiological conditions. The resting phase, reached at the end of orthodox seed dehydration, is preceded by a strong decrease in ASC content and APX activity, the levels of which become undetectable in the majority of mature orthodox seeds (Arrigoni *et al*., 1992; De Gara *et al*., 2003). On the other hand, when the metabolic processes are recovered during germination, the ascorbate pool rises, as do the activities of the ASC-utilizing enzymes (De Gara *et al*., 1997; Tommasi *et al*., 2001). The seedlings of the heterotic F_1 maize hybrid B73×Mo17, having a higher growth rate than the parental lines, also have higher activities of the ASC redox enzymes as well as of L-galactono-γ-lactone dehydrogenase (GLDH), the last enzyme in the ASC biosynthetic pathway (De Gara *et al*., 2000). The involvement of ASC in the regulation of plant growth has been further confirmed by studies of the *Arabidopsis* mutant *vtc-1*. This mutant, which has an ASC level almost 70% lower than that of the wild type, is smaller and has delayed flowering and accelerated senescence (Veljovic-Jovanovic *et al*., 2001). It is widely accepted that ASC is required for the normal progression of the cell cycle; therefore it could affect plant development by influencing cell proliferation (for a recent review see Potters *et al*., 2002). More recently, the involvement of ASC in cell elongation has been confirmed (Arrigoni *et al*., 1997; De Tullio *et al*., 1999; De Gara, 2003). Tobacco Bright Yellow 2 cells, over-expressing a pumpkin ASC oxidase (AOX), undergo more rapid elongation than untransformed controls (Kato and Esaka, 2000). Consistently, the phenotype of tobacco plants is affected by their transformation with AOX cDNA, in both a sense and an anti-sense orientations (Pignocchi *et al*., 2003). Interestingly, AOX is an apoplastic enzyme, which supports the idea that changes in apoplastic ASC metabolism are crucial, not only as a front line of defence against environmental perturbations, but even for plant development (De Tullio *et al*., 1999; Pignocchi and Foyer, 2003).

It has been reported that the presence of ASC in the cell wall promotes hydroxyl radical production and the consequent oxidative scission of structural polysaccharides, an event promoting cell wall loosening (Fry, 1998). Moreover, ASC is known to affect the activity of secretory peroxidases (class III peroxidases; POD) involved in wall stiffening, because it reduces the phenolic radicals produced as intermediates of the catalytic POD reaction, thus making POD activity fruitless, at least as far as substrate oxidation is concerned (Takahama, 1993; De Gara, 2004).

In the present study the ASC level, redox state and its related enzymes, as well as POD isoenzymes, were analysed in the apoplastic and symplastic compartments of different segments of pea (*Pisum sativum* L.) shoots which were indicative of different differentiation levels. Data obtained show opposite gradients in the ASC system and PODs during cell differentiation. In particular, the changes in ASC metabolism occurring in the cell wall and the analysis of the expression patterns and the kinetic characteristics of the APX and POD isoenzymes located in this compartment suggest that ASC and APX activity could have a regulatory effect on POD-dependent wall stiffening.

Materials and methods

Plant material

Pea seeds (*Pisum sativum* L.) were imbibed for 12 h and then germinated on moist vermiculite at 22 °C in the dark. After a week of germination pea shoots had their sub-apical portion bent into a hook and three internodes were present in most of the seedlings. Homogeneous seedlings (8–10 cm in height) were chosen and different shoot segments were collected. The zone above the hook deprived of the external more differentiated leaves was indicated as segment 1, the hook zone as segment 2, and two segments of 1 cm of length of the second internode as segments 3 and 4 (Fig. 1).

Mitotic index and cell size

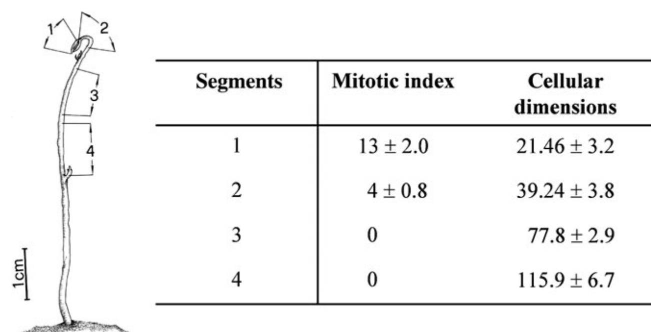
For the estimation of mitotic index the segments were fixed in a 3:1 ethanol/acetic acid (v/v), hydrolysed in 1 N HCl at 60 °C for 5 min and stained with Schiff's reagent for 2 h. Each segment was squashed in a drop of 45% acetic acid. For each segment, five squash preparations were made, and for each slide, 1000 cells were scored. The mitotic index was calculated as a percentage of the ratio between dividing cells and the number of cells scored.

Cell size was determined from optical microscopy images (×250). The pea segments were fixed in formalin:acetic acid:ethanol (1:1:0.5, by vol.), dehydrated in an ethanol series, and embedded in paraffin. Then the segments were longitudinally sectioned at 7–10 μm and stained with safranin-fast green.

Optical microscopy images were analysed with UTHSCSA Image Tool software. For each segment the length measurements were taken of 1000 cells.

Cellular compartmentalization

Pea segments were homogenized with a medium consisting of 0.3 M mannitol, 1 mM EDTA, 30 mM HEPES (pH 7.5), 4 mM cysteine, and 0.1% BSA (lysis buffer) in a ratio of 1:5 (v:v). The homogenate



Segments	Mitotic index	Cellular dimensions
1	13 ± 2.0	21.46 ± 3.2
2	4 ± 0.8	39.24 ± 3.8
3	0	77.8 ± 2.9
4	0	115.9 ± 6.7

Fig. 1. Changes in mitotic index and cell size in different segments of etiolated pea shoots. Four segments having different stages of differentiation were collected as described in the Materials and methods. Both the mitotic index and cellular size analyses were performed by scoring 5000 cells for each segment. The reported values are the means of five independent experiments ±SE.

was filtered and then centrifuged at 1500 g for 10 min to remove cell debris and nuclei. Mitochondria were precipitated at 14 000 g for 15 min and gently washed three times with a solution containing 0.3 mM mannitol, 1 mM EDTA, 10 mM HEPES (pH 7.4), and 0.1% BSA (washing medium), reprecipitating at 14 000 g for 15 min, and finally resuspended in 0.5–1 ml of washing medium. The supernatant after the first 14 000 g centrifugation was again centrifuged at 30 000 g for 20 min and the supernatant so obtained was used as the cytosolic fraction.

IWFs were extracted by a vacuum infiltration/centrifugation technique. Shoot segments were washed in deionized water for 5 min, and then vacuum-infiltrated for 10 min at 0.9 kPa with 50 mM $CaCl_2$ in 50 mM TRIS-HCl buffer at pH 6.5. The sections were quickly dried and centrifuged at 1000 g for 10 min at 4 °C in a 10 ml syringe barrel placed within a centrifuge tube (IWF_1).

In order to test whether the non-covalently-bound apoplastic enzymes were completely extracted, a second extraction with higher ionic strength buffer was performed according to Lin and Kao (2001). The same segments used for the IWF_1 fraction were homogenized on ice in 30 mM phosphate buffer (pH 7.0) with a buffer-to-tissues ratio of 0.5 ml g^{-1} fresh weight. The homogenates were centrifuged at 3000 g for 10 min and the pellet was washed with the extraction buffer by centrifuging at 3000 g for 10 min three times. The supernatants were discarded. In order to release the enzymes, the 3000 g pellet was dispersed in extraction buffer containing 1 M KCl, shaken gently for 1 h, and then centrifuged at 3000 g for 10 min. This supernatant was used after being dialysed overnight against the extraction buffer (IWF_2).

Contamination by cytoplasmic enzymes of the two IWF fractions, as monitored by the activity of glucose-6-phosphate dehydrogenase (Ros Barceló, 1998), was always less than 1.5% and 0.5% for IWF_1 and IWF_2, respectively.

Enzyme assays

Spectrophotometric determination of enzymes was carried out using a Beckman (Fullerton, CA) DU 7000 spectrophotometer.

AOX (L-ascorbate: oxygen oxidoreductase, EC 1.10.3.3), was tested by measuring the oxidation rate of ASC at 265 nm in a reaction mixture consisting of 50 μM ASC, 50–100 μg protein, and 0.1 M phosphate buffer, pH 6.4.

APX (L-ascorbate: hydrogen peroxide oxidoreductase, EC 1.11.1.11), DHAR (glutathione: dehydroascorbate oxidoreductase, EC 1.8.5.1) and AFRR (NADH: ascorbate free radical oxidoreductase, EC 1.6.5.4) activities were tested as described in de Pinto *et al.* (2003).

Class III peroxidases (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) activity was estimated at 25 °C using 4-methoxy-α-naphthol as substrate (Ferrer *et al.*, 1990).

GLDH (L-galactono-1,4-lactone: ferricytochrome-*c* oxidoreductase, EC 1.3.2.3) was assayed in the mitochondrial fraction following the reduction of cytochrome *c* at 550 nm (Ôba *et al.*, 1995) in a reaction medium that contained 50 mM TRIS-HCl (pH 8), 60 μM cytochrome *c*, 1 mM sodium azide, 1 mM L-galactono-1,4-lactone (GL), 1 μM FAD, and 10–15 μg proteins and utilizing an extinction coefficient of 27 mM^{-1} cm^{-1}. Sodium azide was added to inhibit the cytochrome oxidase activity and to avoid an underestimation of GLDH. The variation in absorbance not dependent on GL was subtracted.

Protein measurement was performed according to Bradford (1976) using bovine serum albumin as a standard.

Gel electrophoresis and protein purification

Native polyacrylamide gel electrophoresis (PAGE) and the stain of the gels for APX and DHAR were performed as previously reported (De Gara *et al.*, 1997). The gels for PODs were stained by incubation for 15 min at 25 °C with 1 mM 4-methoxy-α-naphthol and 0.3 mM H_2O_2 in TRIS-acetate pH 5.0 as described in Ferrer *et al.* (1990).

P1 and P3 were purified from the IWF_1 obtained from segments 3 and 4, whereas APX was purified from the IWF_1 obtained from segments 1 and 2. The three proteins were eluted from the gels as described in De Gara *et al.* (1997). Briefly, after the electrophoretic run, a lane was cut from each gel and dyed to identify the position of each band. The areas containing the isoenzymes of interest were then electro-eluted with a GE 200 SixPac Gel Eluter (Hoefer Scientific Instruments, San Francisco, CA) following the experimental conditions recommended by the supplier. The values of K_m for APX, P1, and P2 were obtained by Michaelis-Menten analysis using Sigma Plot software (SPSS Inc. Chicago, Illinois).

Sodium dodecyl sulphate (SDS)-PAGE was carried out according to the method of Laemmli (1970), using a separating gel of 12.5% acrylamide. The proteins of SDS-PAGE were silver-stained as described by Oakley *et al.* (1980). The molecular mass of the proteins was estimated by SDS-PAGE using molecular mass standards (Bio-Rad Laboratories, Richmond, CA).

Isoelectric focusing (IEF) was performed on 5% polyacrylamide gels in 3.5–10.5 pH gradients using a MiniProtean II (Bio-Rad Laboratories, Richmond, CA) electrophoresis kit. Protein migration was allowed at 200 V for 90 min at 4 °C using cytochrome *c* as a migration marker. The isoelectric point of proteins was determined by measuring the local pH along the IEF gel.

Western blot analysis

After SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane as described in de Pinto *et al.* (2002). Immunoblotting was carried out using anti-APX monoclonal antibody (AP6 from Saji *et al.*, 1990) and with goat anti-mouse immunoglobulin G conjugated with alkaline phosphatase (Promega Madison, WI) as previously reported by de Pinto *et al.* (2002).

RNA extraction and Northern blot analysis

Frozen segments were ground to a fine powder in liquid N_2 using a sterile mortar. Total RNA was extracted from pea segments using the RNeasy plant minikit (Quiagen S.p.A., Milan Italy) according to the supplier's recommendation. Residual DNA was removed from the RNA samples using a DNA-*free* kit (Ambion, Inc. Austin, TX). Equal amounts of RNA for each sample (15 μg) were separated in 1.5% agarose gels and visualized with ethidium bromide to confirm the correct spectrophotometric quantification and to assess RNA integrity. Total RNA (5 μg) was separated on 1.5% (w/v) agarose gels after formaldehyde/formamide denaturation in MOPS buffer, and transferred to nylon membranes (Boehringer Mannheim) according to Sambrook *et al.* (1989). Membranes were prehybridized and hybridized at 65 °C in 7% sodium dodecyl sulphate (SDS), 1% bovine serum albumin (BSA), and 1 mM ethylenediaminetetraacetic acid (EDTA) in 0·5 M sodium phosphate buffer (pH 7.2), containing 50 mg ml^{-1} salmon sperm DNA. A cDNA of the cytosolic ascorbate peroxidase from tobacco, kindly supply by Dr Christiane Gatz (University of Gottingen, Germany), was used as a probe. A ^{32}P-labelled cDNA cytosolic APX probe was prepared by the method of Feinberg and Vogelstein (1983). Prehybridization was carried out for 4–6 h and hybridization was carried out overnight. Membranes were washed once in 2× SSC, 0.1% SDS at room temperature for 15 min and twice in 1× SSC, 1% SDS at 65 °C for 15 min. Filters were autoradiographed using X-ray film (Biomax Kodak; Kodak, Rochester, NY, USA) with an intensifying screen at −80 °C.

Determination of ascorbate content

For cytosolic extraction of ascorbate, plant tissues (0.5–3 g) were homogenized with 8 volumes of cold 5% (w/v) *m*-phosphoric acid at 4 °C in a porcelain mortar. The homogenate was centrifuged at 20 000 g

for 15 min at 4 °C, and the supernatant was collected for the analysis. For the apoplastic extraction of ascorbate, the IWF was collected in a tube containing 50 µl of cold 10% (w/v) *m*-phosphoric acid to stabilize the AA in the IWF. ASC and dehydroascorbate (DHA) were measured according to de Pinto *et al*. (1999).

Results

Mitotic index and cell elongation along pea shoot

Different segments along etiolated pea shoots were analysed for their mitotic index and cell size. The zone above the hook deprived of the external differentiating leaves (segment 1) only contained meristematic cells that continuously undergo division, in fact, the highest value of the mitotic index was found in this zone, whereas the cell length was the smallest (Fig. 1). The hook zone (segment 2) mainly consisted of cells that had stopped dividing and were in the elongation phase, with only a few cells, the procambial ones, still dividing. The cells of the second internode (segments 3 and 4) had nearly completed the distension phase having almost reached their final size (Fig. 1). Indeed, the cells of the basal internode had the same dimensions as segment 4 (data not shown).

Symplastic ascorbate metabolism during cell elongation

The highest value of the cytosolic ascorbate pool (ASC+DHA) was found in the meristematic cells (segment 1); it decreased progressively during cell elongation, reaching levels almost three times lower in the cells of segments 3 and 4 (Fig. 2A). The cytosolic component of the ascorbate pool was always strongly shifted toward the reduced form, with a redox state remaining around values of 0.9 (between 0.94 in segment 1 and 0.90 in segment 4). The ASC biosynthetic capability also decreased from segment 1 to 4 as indicated by the activity of GLDH. This enzyme had an activity four times higher in segment 1 than in segment 2. In the cells of the second internode (segments 3 and 4) the GLDH was almost ten times lower than that of the meristem (Fig. 2B). The cytosolic fraction of the pea shoot cells also had a moderate AOX activity, that seemed to be higher in segments 3 and 4 than in segment 1 (Fig. 2C).

The cytosolic APX activity also decreased from the zone of dividing cells to that of the elongated ones (Table 1). The decrease in APX activity from the meristematic zone to the second internode was confirmed by native PAGE assays, which showed that only one band with decreasing APX activity was present in the pea cytosolic fraction (Fig. 3A). The analysis of western and northern blotting indicated that the decrease in APX activity was parallel with the decrease in the amount of the protein, as well as in the expression of its gene occurring from segments 1 to 4 (Fig. 3B, C).

The activity of the cytosolic ascorbate free radical reductase (AFRR) also decreased, even if less and more gradually than APX (Table 1). On the other hand, DHAR had almost the same activity in segments 1 and 2, but significantly increased in segments 3 and 4 (Table 1). Segments 1 and 2 also seemed to have a population of DHA

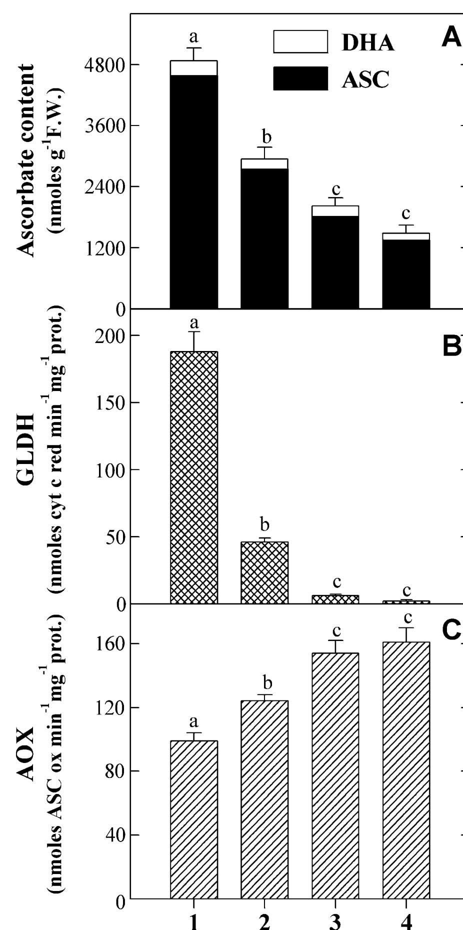


Fig. 2. Symplastic ascorbate pool (A) and activities of GLDH (B) and cytosolic AOX (C) in the different segments of pea shoots. The changes in the ASC and DHA contents, cytosolic AOX, and mitochondrial GLDH activities were followed from the meristematic zone (segment 1) to differentiated ones (segments 3 and 4). The reported values are the means of five independent experiments ±SE. Different letters indicate values the differences of which were statistically significant (Student's *t*-test, *P* at least <0.05).

Table 1. *Changes in the cytosolic ASC redox enzymes during differentiation of pea shoots*

Four segments characterized by different levels of cellular differentiation were utilized for the extraction of the cytosolic fraction and the determination of APX, AFRR, and DHAR activities. The values reported in the table are the means of four independent experiments ±SE. Statistically significant differences using Student's *t*-test, ($P<0.05$) are indicated by different letters.

Segments	APX (nmol ASC oxidized min^{-1} mg^{-1} protein)	AFRR (nmol NADH oxidized min^{-1} mg^{-1} protein)	DHAR (nmol DHA reduced min^{-1} mg^{-1} protein)
1	315±13 a	1148±104 a	210±18 a
2	251±19 a	910±81 a	230±14 a
3	199±15 b	811±66 a, b	330±23 b
4	137±6 c	740±79 b	300±15 b

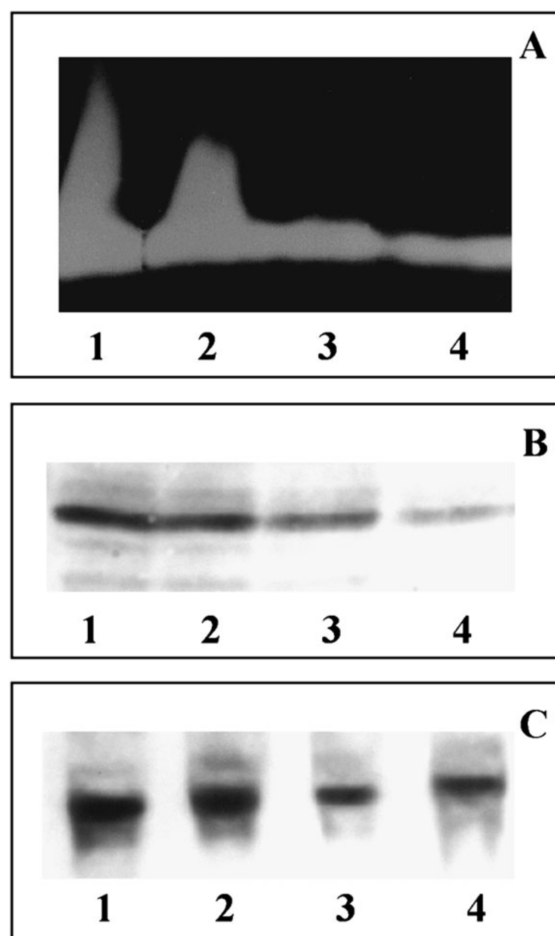


Fig. 3. APX enzymatic profile and expression during differentiation. The analyses of the isoenzymatic profile, gene expression, and protein amount of cytosolic APX were performed in the four segments characterized by different levels of cellular differentiation. (A) Representative native PAGE of cytosolic APX. 300 μg of protein extracts were loaded in each line. (B) Representative immunoblotting analysis performed using a specific APX antibody (see Materials and methods for details). Protein extracts (15 μg) were loaded in each line. (C) Representative northern blotting analysis performed using a specific APX cDNA (see Materials and methods). 5 μg of total RNA were loaded in each line.

reducing proteins partly different from segments 3 and 4 (Fig. 4).

Apoplastic ascorbate metabolism

The IWF of etiolated pea seedlings did not contain either AFRR or DHAR, since their activities were often completely undetectable or, at most, in the percentage range of glucose 6-phosphate dehydrogenase, which was used as a marker of cytosolic contamination (see Materials and methods for details). On the other hand, extracellular fluids had both AOX and APX activities (Fig. 5A, B). In the IWF_1 from each segment, 30–35% of the total AOX activity of the whole homogenate was present. This enzyme had the lowest activity in segments 1 and 2, and the highest in segment 3 (Fig. 5A). On the other hand, APX activity

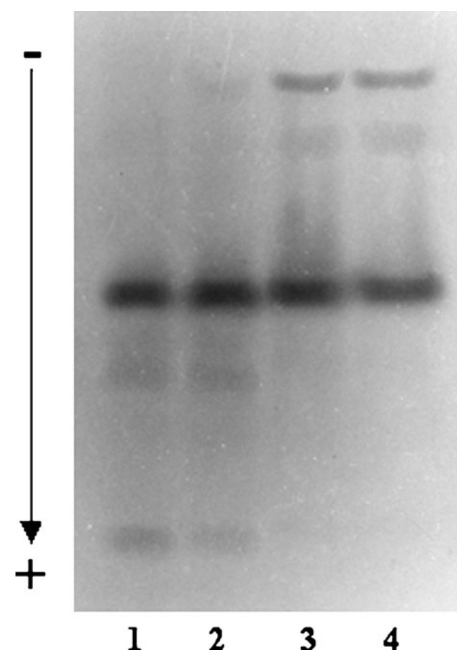


Fig. 4. Electrophoretic pattern of cytosolic DHA reducing proteins during cellular differentiation. Representative native PAGE of cytosolic DHAR in the four segments having different levels of differentiation. 200 μg of protein extracts were loaded in each line.

decreased from segment 1 to segment 4 (Fig. 5B). The percentage of the extracellular APX activity with respect to total APX activity also decreased from values of about 10% in segment 1 to 3% in segment 4, but it was higher in all the segments than the values indicative of cytosolic contamination. Native PAGE showed the presence in the apoplastic fluids of a single protein with APX activity, with a similar migration rate to the cytosolic isoenzyme (data not shown). ASC and DHA were also present in the apoplastic space (Fig. 5C). The ascorbate pool extracted with the extracellular fluids was much higher in segment 1 than in the others, however, a progressive further decrease was also evident from segment 2 to segment 4. The ascorbate redox state in the apoplast was always very low (about 0.1 in the first segment and 0.05 in the other segments), thus indicating that most of the vitamin C in the apoplastic space was present in its oxidized form.

The activities of the ASC oxido-reduction enzymes were also assayed in the IWF obtained after a second extraction with a higher ionic strength buffer (IWF_2, see Materials and methods for details). In the IWF_2 fraction, AOX was the only detectable ASC-redox enzyme, and its activity was again higher in the second internode than in segments 1 and 2, as shown by both specific activity and electrophoretic profile analyses (Fig. 6).

Apoplastic class III peroxidases

When the peroxidase activity of the IWF_1 was assayed by using 4-methoxy-α-naphthol as the electron donor, an

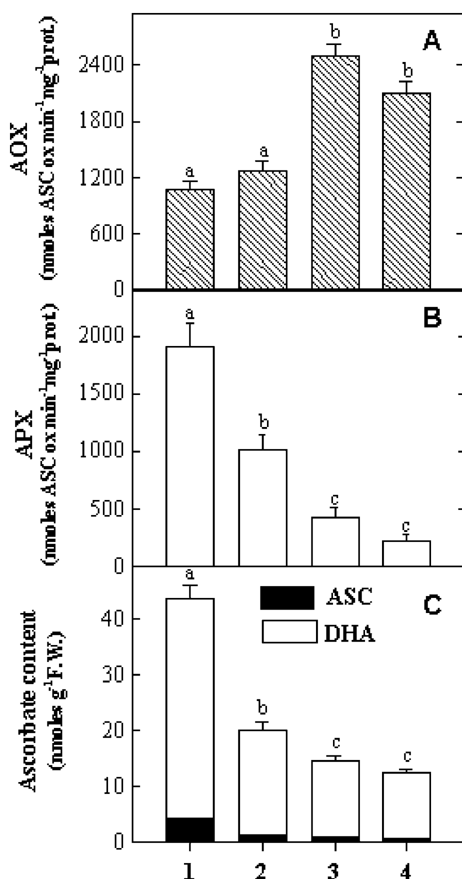


Fig. 5. Changes in the activities of AOX (A), APX (B), and in the total ascorbate content (C) in the IWF_1 of pea shoots. The IWF_1 was extracted from the four segments having different levels of cellular differentiation, as indicated in the Materials and methods. The reported values are the means of five independent experiments ±SE. Different letters indicate values which statistically differed (Student's *t*-test, P <0.01).

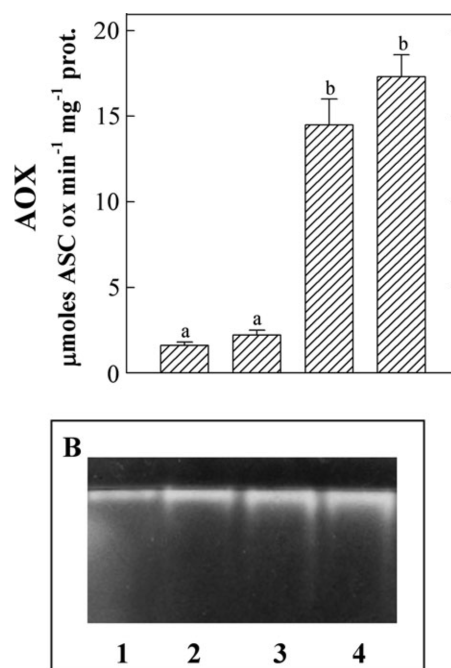


Fig. 6. Changes in the AOX of IWF2 during differentiation. IWF2s were obtained from the four different segments as described in the Materials and methods, and used for both the measurement of specific activity and the presence of isoenzymatic forms. (A) Specific activity of AOX. The reported values are the means of five independent experiments ±SE. Different letters indicate values which statistically differed (Student's *t*-test (paired tests); P <0.01). (B) Representative electrophoretic pattern of AOX from IWF2. 200 μg of protein extracts were loaded in each line.

unspecific substrate that can be used both by APX and PODs, a significant increase was detected from segment 1 to segment 4. In particular, the IWF_1 of the second internode had four to six times higher activity than that of the meristematic and hook zones (Fig. 7A). The native PAGE stained with 4-methoxy-α-naphthol showed that different populations of peroxidases were present in the different segments (Fig. 7B), in particular, a protein numbered as P2 in the figure was evident only in the meristematic zone, whereas P3 only appeared in the elongated cells of the second internode. In spite of native PAGE not giving quantitative information on the enzyme activity, the remarkable increase in the intensity of the proteins with the lowest migration rate (P1) from segments 1–2 to segments 3–4 suggested that this POD isoenzyme had a higher activity in elongated cells than in dividing ones. The proteins with the highest migration rate (P6) that showed decreasing intensity from segment 1 to 4 had the same migration rate as APX. In order to verify whether this band actually corresponded to the APX or was a class III peroxidase, the gel was incubated for 10 min with *p*-chloromercuribenzoate (*p*CMB), an APX inhibitor that does not affect POD activity (Amako *et al.*, 1994), prior to the addition of 4-methoxy-α-naphthol and hydrogen peroxide. The only protein, the activity of which was remarkably inhibited by the *p*CMB pretreatment, was P6, thus confirming that it was an APX isoenzyme (data not shown).

The IWF_2 also showed POD activity. In this fraction, a gradual increase was evident during differentiation, since POD-specific activities reached values four times higher in segment 4 than those measured in segment 1 (Table 2). Native PAGE of IWF_2 showed the presence of a single POD isoenzyme, the intensity of which also increased from segments 1 to 4 (data not shown).

Purification and characterization of apoplastic APX and PODs

With the aim of obtaining further information on the physiological role of apoplastic APX, a partial kinetic characterization of the enzyme was performed. Two POD isoenzymes (P1, the activity of which progressively increased along the shoot and P3, an isoenzyme appearing only in the second internode) were purified and partially characterized. Since the conventional protein purification procedures were made difficult by the low amount of the

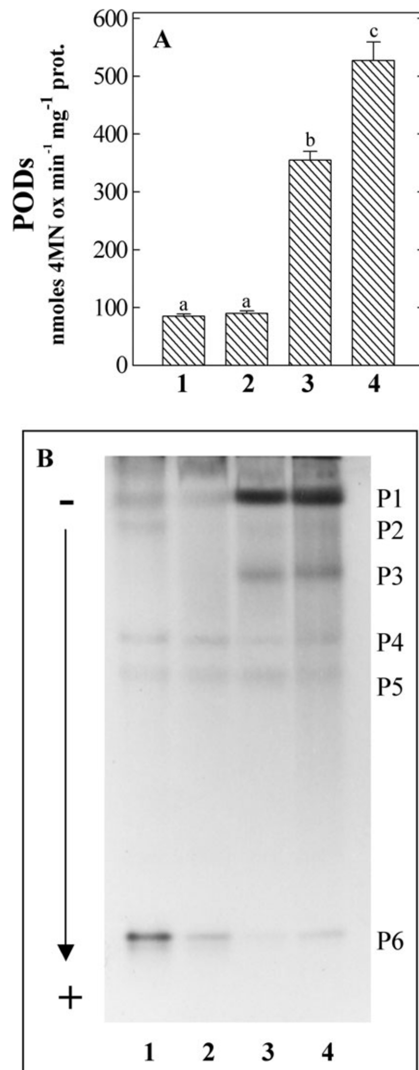


Fig. 7. Changes in the activity of PODs in the IWF_1 of different segments of pea shoots. IWF_1s were obtained from the four segments as described in the Materials and methods and used for the analyses of specific activity and isoenzymatic profiles. (A) Specific activity of PODs. The values are the means of five independent experiments ±SE. Different letters indicate values which statistically differed (Student's *t*-test (paired tests); P <0.01). (B) Representative electrophoretic pattern of PODs in the IWF_1. 100 μg of protein extracts were loaded in each line.

proteins obtained in the IWF, the purification of the chosen proteins was attempted by eluting them from native PAGE (see Materials and methods for details). The elution of APX gave 20-fold protein purification with a recovery of 42%. When analysed by SDS-PAGE, APX appeared not to be completely purified, since two bands were present after silver staining, even if one protein was only very faintly dyed (data not shown). On the other hand, the elution of P1 and P3 from native PAGE was sufficient for complete purification of the two isoenzymes, which proved to have the same molecular masses of 85 kDa (Table 3).

The two purified PODs had different isoelectric points (9.5 and 7.5 for P1 and P3, respectively). The isoelectric point of the apoplastic APX was about 5.5. Moreover, the three enzymes had different affinity for the common substrate, i.e. hydrogen peroxide. Indeed, the K_m for H_2O_2 of APX was 116 and 260 times lower than those of P1 and P3, respectively (Table 3).

Table 2. *Changes in the activity of PODs in the IWF_2 fractions during differentiation of pea shoots*

The IWF_2 fractions were obtained from the four pea shoot segments as described in the Materials and methods. The specific activity of POD was estimated by using 4-methoxy-α-naphthol (4MN) as electron donor. The reported values are the means of four independent experiments ±SE. Statistically significant differences using the Student's *t*-test, (P <0.05) are indicated by different letters.

Segments	PODs activity (nmol 4MN oxidized min^{-1} mg^{-1} protein)
1	314±41 a
2	595±63 b
3	933±55 c
4	1362±90 d

Table 3. *Characterization of the purified apoplastic peroxidases*

APX, and two different POD isoenzymes (P1 and P3) were purified from IWF_1 by eluting the bands from a native PAGE gel (see Materials and methods). The purified proteins were then analysed by SDS-PAGE and IEF for the characterization of their molecular weight (MW) and isoelectric point (IP). The Michaelis-Menten analysis of the three isoenzymes were also performed. The reported values are the means of four independent experiments ±SE for K_m and the means of three independent experiments for MW and IP.

Proteins	MW (kDa)	IP	K_m (μM)
APX	27	5.5	1.48±0.9
P1	85	9.5	173±21
P3	85	7.5	390±53

Discussion and conclusions

Cytosolic changes in the ascorbate metabolism

The data reported here clearly indicate that gradual changes in ascorbate metabolism occur along the etiolated pea shoot, from the meristem to the differentiated zones. The meristematic tissues having the highest level of ASC are also characterized by the highest activity of GLDH, the last enzyme of the main ASC biosynthetic pathway in higher plants (Smirnoff and Wheeler, 2000). These results are compatible with the requirement of a large amount of ASC for the normal progression of the cell cycle, an event characterizing meristematic tissues (Liso *et al.*, 1984; Kerk and Feldman, 1995). Consistently, in cultured tobacco cells the highest amount of ASC is found in the exponential growth phase, when the mitotic index also has the highest value, while in the phases with a lower mitotic index the ASC content is also lower (Kato and Esaka, 1999; de Pinto *et al.*, 2000). In pea meristematic tissues cytosolic APX also

has the highest activity, suggesting that reactive oxygen species (ROS) levels must be maintained at minimal levels in the dividing cells. It has been reported that under conditions overproducing ROS, as in the presence of toxic metals, the cell cycle is strongly prolonged and, as a consequence, cell proliferation reduced (Francis, 1998). Depending on its intensity, oxidative stress can even completely block the cell cycle (Reichheld *et al*., 1999). Western and northern blotting analyses indicate that the decrease in cytosolic APX during cell differentiation seems to be mainly regulated at the level of gene expression.

In spite of being an apoplastic enzyme, AOX activity is also present in the cytosolic fractions, even if it is not clear whether this AOX activity is indicative of the presence of the enzyme in the cellular compartment or is due to the contamination of the Golgi vesicles containing active AOX on route to the cell wall. The AOX detected in the cytosolic fraction increases from segments 1 to 4, similarly to that which occurs in the cell wall, even if in the symplast the differences between segments are less marked.

It is worth noting that, in the cytosolic fraction, the ASC pool is always strongly shifted towards the reduced form, its redox state remaining almost unchanged in all the analysed segments. The activity of the two ASC recycling enzymes, AFRR and DHAR, strongly contribute to maintaining the high ASC redox state. These two enzymes are part of the so-called ascorbate–glutathione cycle, a series of co-ordinated reactions in which the ascorbate required for APX-dependent H_2O_2 removal is continuously regenerated by draining reducing equivalents from pyridine nucleotides (Noctor and Foyer, 1998). Ascorbate free radical (AFR, also known as monodehydroascorbate or ascorbyl), the first product of ASC oxidation, spontaneously undergoes dismutation, a reaction yielding one molecule of ASC and one of DHA from two molecules of AFR. Therefore the activity of AFRR is critical for regulating DHA production, because it avoids AFR dismutation by directly reducing the hemiquinone radical to ASC. Interestingly, the decrease in AFRR occurring from segment 1 to segment 4 is balanced by an increase in DHAR. The presence of different DHAR bands in segments 1 and 2 versus segments 3 and 4, as shown by native PAGE analysis, suggests that, during cell differentiation, various proteins with DHA reducing activity are expressed to different degrees. However, the different pattern of DHARs could also be the result of tissue specificity, apart from their developmental regulation.

Apoplastic changes in the ascorbate metabolism

ASC is considered the most abundant low-molecular-weight antioxidant present in the apoplast, where it acts as both a redox buffer and a redox sensing metabolite (Pignocchi and Foyer, 2003). In etiolated pea shoots the apoplast contains a certain amount of the ASC pool (ASC plus DHA) that decreases from the meristematic zone to differentiated ones. In contrast to the situation in the cytosol, most of the pool is present as DHA, the ASC redox state being 0.1 in segment 1 and about 0.05 in the other segments. A predominance of DHA has also been reported in the apoplast of other tissues (Vanacker *et al*., 1998; Córdoba-Pedregosa *et al*., 2003*a*, *b*), and, apart from being a consequence of ASC utilization in this cellular compartment, it could also be due to the lack of ASC recycling enzymes. Indeed, neither AFRR nor DHAR have been detected in the pea seedling apoplast, although the absence of the ASC recycling enzymes might not be common to all plant species or organs, since moderate activities of the two enzymes have been detected in the IWF of oat leaves and onion roots (Vanacker *et al*., 1998; Córdoba-Pedregosa *et al*., 2003*a*). However, the possibility that the oxidized forms of ASC could be reduced in the cell wall by these recycling enzymes is also questioned by the fact that the presence of pyridine nucleotides and glutathione, the electron donors of AFRR and DHAR, respectively, is rather uncertain in this cellular compartment (Luwe, 1996; Pignocchi and Foyer, 2003). Other pathways have been proposed to supply ASC more efficiently in the cell wall. An ASC/DHA exchange system, localized in the plant plasma membrane, provides ASC to the apoplast and drives DHA within cells where it is reduced to ASC (Horemans *et al*., 2000). Interestingly, this transport seems to be modulated during differentiation, having the highest rate in the active proliferating cells (Horemans *et al*., 2003). This evidence is in agreement with the presence of higher ASC levels in the apoplast of the meristematic zone than in the differentiated ones. As far as AFR reduction is concerned, a plasma membrane *b* type cytochrome, shuttling electrons from symplastic ASC to apoplastic AFR, has been characterized in several plant cells (Verelst and Asard, 2003). However, whatever the pathways for ASC regeneration active in the cell wall, the strong predominance of DHA over ASC underlines that they are insufficient to cope with the oxidative load of this cellular compartment. In the cell wall, ASC can be non-enzymatically oxidized by ROS and other reactive chemical species generated here. Moreover, at least two ASC oxidizing enzymes are present in the etiolated pea seedling apoplast, AOX and APX. In spite of the physiological function of AOX not having been clearly identified, evidence suggests that it regulates the ASC levels in this cellular compartment, since transgenic tobacco plants over- or under-expressing the AOX gene have remarkably lower or higher ASC levels in their apoplast, respectively (Pignocchi *et al*., 2003). An increasing body of data underlines that a positive relationship exists between AOX activity and cell expansion. Tobacco protoplasts over-expressing a pumpkin AOX cDNA have a higher elongation rate than untransformed control protoplasts (Kato and Esaka, 2000). Moreover, transformed tobacco plants expressing AOX in the sense and anti-sense orientation have altered phenotypes: plants having higher apoplastic AOX are significantly taller than wild types,

whereas, plants transformed by anti-sense technologies are smaller (Pignocchi *et al.*, 2003). It has been suggested that AOX increases the rate of cell elongation by generating AFR and DHA. Indeed, AFR could stimulate such a process by inducing membrane hyperpolarization, with a consequent increase in ion uptake and vacuole enlargement (Hindalgo *et al.*, 1989; Córdoba and Gonzales-Reyes, 1994), whereas, DHA promotes cell wall plasticity by hindering the cross-links between structural proteins and hemicellulose. Oxalic acid, which is produced by DHA degradation, further increases wall loosening by reducing the number of calcium bridges between pectin chains (Lin and Varner, 1991). Interestingly, a remarkable increase in AOX activity occurs in segment 3, where cells undergo the major elongation, by almost doubling their average size with respect to cells of segment 2.

Apopastic peroxidases

Peroxidases of both class I (APX) and III (POD) are present in the etiolated pea seedling apoplast. The opposite behaviour of the peroxidases belonging to the two classes (decrease in APX during differentiation and increase in POD, both in the activity and in the number of isoenzymes expressed) is compatible with their different physiological roles. APX is considered an antioxidant enzyme, its physiological role being confined to that of a ROS scavenger, whereas, PODs mainly utilize H_2O_2 for oxidizing their reducing substrates. Through their peroxidative reaction, apoplastic PODs catalyse the cross-links between matrix components and the polymerization of lignin, thus increasing the stiffening and reducing the extensibility of the cell wall (Iiyama *et al.*, 1994; Pomar *et al.*, 2002). Indeed, a negative correlation between peroxidase activity and cell elongation is widely accepted (Zheng and Van Huystee, 1992; Zarra *et al.*, 1999). Since both APX and POD utilize H_2O_2 as an oxidizing substrate, it is expected that they compete for it, when they have the same cellular localization, as in the case of the IWF enzymes. In particular, in the meristematic tissues, where H_2O_2 production is much lower than in differentiating ones (Schopfer, 1994), APX could hinder the activity of POD because its activity is higher than in other segments and it has an affinity for H_2O_2 much higher than that of PODs (the K_m for H_2O_2 of APX being about 100 and 300 times lower than those of P1 and P3, respectively). The APX decrease from segment 1 to segment 4 is consistent with the increasing requirement of POD activity for the processes involved in cell wall differentiation. Interestingly, an inverse correlation between APX and PODs has also been reported in the cell wall of onion roots (Córdoba-Pedregosa *et al.*, 1996, 2003*a*, *b*). It has been reported that APX is not present in the IWF from mature pea leaves (Hernández *et al.*, 2001) suggesting that this enzyme could only be transiently present in the apoplast of meristematic or not yet completely differentiated cells. As for the relationship existing in the cell wall between ASC metabolism and POD, it must be mentioned that ASC itself negatively affects the POD catalytic reaction, because the phenolic radicals, produced as an intermediate in the POD reaction, can be reduced to their initial form by ASC. Indeed, the oxidation of the POD reducing substrates (which is important for wall stiffening and, in general, for wall differentiation) can be negatively affected depending on ASC concentration (Takahama, 1993).

In conclusion, these data underline that the redox pairs ASC/AFR and ASC/DHA and their redox enzymes are important for cell differentiation. The results reported here clearly show that ASC levels change remarkably during cell differentiation, and that the ASC related enzymes seem to be developmentally regulated. Therefore, not only does the ASC availability *per se* alter plant growth, as in the case of *vtc-1 Arabidopsis* mutant or transgenic plants in which the physiological ASC levels have been impaired by genome transformation (Veljovic-Jovanovic *et al.*, 2001; Pignocchi *et al.*, 2003), but in addition all the ASC redox enzymes are involved in plant development. Moreover, the capability of ASC to connect and co-ordinate apoplastic and symplastic metabolism, besides its redox properties and its possible action as a redox sensor of environmental changes, further support the importance of the ASC system as a key component in plant development.

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