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The apoplastic antioxidant enzymatic system in the wood-forming tissues of trees

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Abstract. The complete apoplastic enzymatic antioxidant system, composed by class I ascorbate peroxidases (class I APXs), class III ascorbate peroxidases (class III APXs), ascorbate oxidases (AAOs), and other class III peroxidases (PRX), of wood-forming tissues has been studied in *Populus alba*, *Citrus aurantium* and *Eucalyptus camaldulensis*. The aim was to ascertain whether these enzymatic systems may regulate directly (in the case of APXs), or indirectly (in the case of AAOs), apoplastic H_2O_2 levels in lignifying tissues, whose capacity to produce and to accumulate H_2O_2 is demonstrated here. Although class I APXs are particularly found in the apoplastic fraction of *P. alba* (poplar), and class III APXs are particularly found in the apoplastic fraction of *C. aurantium* (bitter orange tree), the results showed that the universal presence of AAO in the extracellular cell wall matrix of these woody species provokes the partial or total dysfunction of apoplastic class I and class III APXs, and of the whole plethora of non-enzymatic redox shuttles in which ascorbic acid (ASC) is involved, by the competitive and effective removal of ASC. In fact, the redox state (ASC/ASC+DHA) in IWFs of these woody species was zero, and thus strongly shifted towards DHA (dehydroascorbate), the oxidized product of ASC. This imbalance of the apoplastic antioxidant enzymatic system apparently results in the accumulation of H_2O_2 in the apoplast of secondary wood-forming tissues, as can be experimentally observed. Furthermore, it is hypothesized that since AAO uses O_2 to remove ASC, it could regulate O_2 availability in the lignifying xylem and, thorough this mechanism, AAO could also control the activity of NADPH oxidase (the enzyme responsible for H_2O_2 production in lignifying tissues) at substrate level, by controlling the tension of O_2. That is, the presence of AAO in the extracellular cell wall matrix appears to be essential for finely tuning the oxidative performance of secondary wood-forming tissues.

Keywords: apoplast · ascorbate oxidase · ascorbate peroxidase · ascorbic acid · H_2O_2 · lignification · peroxidase

Introduction

The tree life form imposes several different physiological and morphological constraints compared with those pertaining to herbaceous plants. Many of the processes that distinguish trees from herbs take years to fully develop and express themselves, as it is the case of wood formation. Trees and shrubs form hard, long-lasting structures that are distinct from the soft stems and branches of herbs, especially annuals. The lignocellulosic cell walls of trees and shrubs are critical for their survival, stature, competitive ability and provision of habitat; they have a dramatic influence on ecosystem cycles (Boehle et al. 1996). Trees and shrubs are found intermixed with herbaceous plants in many phylogenetic groups within the angiosperms (Groover 2005), showing that the tree growth habit has been lost or acquired many times during evolution (Boehle et al. 1996). The herbaceous life form is thus considered to be the derived state, evolving numerous times from tree-like ancestors.

If there is something that characterises the tree life form it is secondary growth and wood formation (Mellerowicz et al. 2001). Wood formation is initiated in the vascular cambium, where cambial derivatives develop into xylem cells through the process of division, expansion, secondary cell wall formation, lignification and, finally, programmed cell death (Mellerowicz et al. 2001). Lignified cells thus constitute most wood, the importance of lignification being underlined by the fact that lignins account for about 25% of plant biomass (Ros Barceló 1997).

Lignin is a complex hydrophobic network of phenylpropanoid units derived from *p*-hydroxycinnamyl alcohols, which are cross-linked by xylem class III peroxidases (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) (Christensen et al. 1998), in a reaction strictly dependent on H_2O_2 (Nose et al. 1995; Ros Barceló and Pomar 2001; Pomar et al. 2002a). This need for H_2O_2 before cell wall lignification can start explains why lignifying xylem tissues are universally able to sustain H_2O_2 production (Czaninski et al. 1993; Olson and Varner 1993; Schopfer 1994; Ros Barceló 1998a, 1998b and 2005). However, the ability of the lignifying xylem to produce H_2O_2 is not confined to this vascular tissue. Indeed, most plant tissues are able to produce H_2O_2, either constitutively or under stress situations, and the result is not always net H_2O_2 accumulation (Hernández et al. 2001; Mittler et al. 2004). The reason for this last observation is that plant cells also contain a whole battery of enzymatic antioxidant systems capable of removing H_2O_2 from the apoplast (Vanacker et al. 1998; Pignocchi and Foyer 2003). Class I APX (L-ascorbate: H_2O_2 oxidoreductase, EC 1.11.1.11) is one of such enzymatic systems, and its substrate, ascorbic acid, is by far the most abundant low-molecular-weight antioxidant in the apoplast (Pignocchi and Foyer 2003).

Class I APXs are involved in the removal of H_2O_2 in a reaction strictly dependent on ASC (de Gara 2004). Class I APXs are widely distributed in plant cells, where they are present in the cytosol, chloroplasts, microbodies, mitochondria and the cell wall, in different isoenzymatic forms which, in turn, are diversely regulated (Nakano and Asada 1981; de Gara and Tommasi 1999; Jiménez et al. 1998; de Leonardis et al. 2000; Córdoba-Pedregosa et al. 2003; Hernández et al. 2004). Class I APXs are different from class III peroxidases in several aspects. The latter are the classical secretory plant peroxidases, and are responsible for cell wall

93 lignification and other cell wall stiffening processes which conclude in the
94 maturation of the cell wall (Passardi et al. 2004). Both types of peroxidase are not
95 easily distinguishable since it is known that some class I APXs are able to oxidize
96 phenolic compounds (Chen and Asada 1989; Jiménez et al. 1998), whereas some
97 class III PRXs (those that should be renamed as class III APXs to avoid confusion)
98 show great specificity for ASC (Vianello et al. 1997; de Gara 2004). Most of the
99 knowledge on this type of APX have been obtained in tea, where a class III APX
100 (TcPOD I) has been purified and characterized (Kvaratshelia et al. 1997). TcPOD I
101 is a glycosylated protein which oxidizes phenols and ASC to the same extent, and is
102 probably an extracellular enzyme secreted through the conventional default pathway
103 (Kvaratshelia et al. 1997). Nevertheless, both types of peroxidases can be
104 distinguished by the use of thiol reagents, such as *p*-chloromercuribenzoate (*p*-
105 CMB), which specifically inhibits class I APXs (Chen and Asada 1989; Jiménez et
106 al. 1998).

107 The developmental regulation of the activity/expression of class I APXs
108 and class III PRXs seems to be different (de Pinto and de Gara 2004; Córdoba-
109 Pedregosa et al. 2004 and 2005). Class I APXs are generally associated with
110 meristematic and actively growing young tissues, whereas class III PRXs are
111 generally expressed in non-growing tissues, where cell wall stiffening processes are
112 emerging. This situation is further complicated by the fact that differentiating
113 xylem cells are able to produce nitric oxide (NO), which is a potent endogenous
114 regulator of the activity of these hemeproteins (Gabaldón et al. 2005, Neil 2005).

115 Both class I APXs and class III PRXs use H_2O_2 as their electron
116 acceptor, and therefore compete for the same substrate when they are present in the
117 same cellular compartment. Class I APXs could be responsible for H_2O_2

detoxification, preventing its accumulation in the apoplastic space when it is produced in excess (de Gara 2004). For the efficient removal of H_2O_2 by class I APXs, ASC levels in the apoplastic space should be sustainable. ASC sustainability in cell walls is regulated by different mechanisms: for example, apoplastic ASC pools may be regenerated by a plasma membrane cytochrome *b* type protein (Horemans et al. 2000), through which electrons shuttle from cytoplasmic ASC to apoplastic ascorbic free radical (AFR), the first product of the oxidation of ASC by APXs. On the other hand, ASC and DHA (the product of the dismutation of AFR) may also be transported by specific plasma membrane carriers, which transport ASC from the cytosol to the cell wall, and DHA from the cell wall to the cytosol, where the DHA is promptly reduced to ASC (Horemans et al. 2000).

However, to complete the picture of the antioxidant enzymatic system in cell walls, it is necessary to mention the main enzyme which consumes ASC in the apoplastic space, AAO (L-ascorbate: O_2 oxidoreductase, EC 1.10.3.3). This is a cell wall localized glycoprotein belonging to the family of blue copper oxidases (de Tullio et al. 2004), which catalyzes the aerobic oxidation of ASC to AFR which, in turn, rapidly disproportionates to ASC and DHA. Although its biological function in the cell wall is not totally understood (de Tullio et al. 2004), it has been demonstrated that AAO regulates the apoplastic ascorbate pool and therefore the redox state (ASC/ASC+DHA), reducing apoplastic ASC levels to values which, in certain cases, are below the detection threshold (Sanmartin et al. 2003; Pignocchi et al. 2003). That is, the presence of AAO in cell walls seems to provoke the partial or total dysfunction of apoplastic class I and class III APXs, and all the plethora of non-enzymatic redox shuttles in which ASC is involved (Takahama 1993), by the competitive and effective removal of ASC.

To date, most of the data concerning apoplastic APXs and AAOs have been obtained from leaves, etiolated (poorly lignifying) hypocotyls, and roots, from several herbs (Vanacker et al. 1998; Hernández et al. 2001; Pignocchi and Foyer 2003; de Pinto and de Gara 2004; Liso et al. 2004; Córdoba-Pedregosa et al. 2005), but there are no data available on APX and AAO levels in the apoplast of wood-forming tissues. In this report, we study APX and AAO levels in the apoplast from young branches of trees to ascertain whether these enzymatic systems regulate directly (in the case of APX), or indirectly (in the case of AAO), apoplastic H_2O_2 levels in secondary wood-forming tissues. This is especially important to describe the oxidative performance of secondary wood-forming lignifying xylem cells, since the presence of APXs in the lignifying xylem would keep H_2O_2 levels to minimum values, while the presence of AAOs in the lignifying xylem would protect H_2O_2 levels, since it is known (Sanmartin et al. 2003; Pignocchi et al. 2003) that the apoplastic ASC pool is partially or totally removed by the presence of this enzyme. For this task, we chose as a model tree, poplar (*Populus alba*), since this is one of the few trees that may be genetically transformed and regenerated (Brunner et al. 2004). Studies were extended to two other trees (*Citrus aurantium* and *Eucalyptus camaldulensis*), and to an annual herb (*Zinnia elegans*).

Materials and methods

Plant material

Young branches of *Populus alba* (poplar), *Eucalyptus camaldulensis* (eucalyptus) and *Citrus aurantium* (bitter orange tree) were harvested in April and May from trees at least 10 years old which were growing in the University Campus. Seedlings of *Zinnia elegans* L (cv. Envy) were grown for 30 days in a greenhouse under

daylight conditions at 25 °C on Humus King™ (type 3) (Impra S.L., El Ejido, Almería, Spain) containing 30 % organic C, 0.5 % organic N, and 52 % total organic material, pH 5.5-6.0. Fertilizers present in the humus were 120-160 mg/L N, 100-130 mg/L P_2O_5 and 150-200 mg/L K_2O.

Histochemical stains for monitoring lignins and H_2O_2 localization/production

Lignins were detected using the Wiesner test by soaking 0.5 mm-thick sections in 1.0 (w/v) phloroglucinol in 25:75 (v/v) HCl-ethanol for 10 to 15 min (Pomar et al. 2002b). H_2O_2 localization/accumulation was monitored by staining 250-500 μm thick sections with the KI/starch reagent, composed of 4 % (w/v) starch and 0.10 M KI (Olson and Varner, 1993; Ros Barceló 1998a), adjusted to pH 5.0 with KOH. Areas of H_2O_2 localization/accumulation were monitored by observing the development of a dark stain on the cut surface over a period of 1 to 10 hr. Controls were performed in the presence of 200 U/ml catalase.

For monitoring H_2O_2 production we used the 3,5,3',5'-tetramethylbenzidine (TMB) endogenous peroxidase-dependent method (Ros Barceló 1998b). For this, sections were directly incubated for 10 min at 25 °C in a staining solution composed of 0.1 mg/ml TMB-HCl in 50 mM Tris-acetate buffer (pH 5.0). Controls were performed in the presence of 0.1 mM ferulic acid (Ros Barceló et al. 2000), a competitive inhibitor of peroxidase, whose oxidation is strictly dependent on H_2O_2.

Isolation of intercellular washing fluids (IWFs)

To obtain IWFs, 5 mm-thick sections were washed three times with deionized water, and subsequently vacuum-infiltrated for 10 periods of 30 s at 1.0 kPa and 4 ºC with

50 mM Na-acetate buffer (pH 5.0) containing 1 M KCl, 50 mM $CaCl_2$ and 1 mM ASC. Later, the sections were quickly dried and subsequently centrifuged in a 25-ml syringe barrel placed within a centrifuge tube at 900 g for 5 min at 4 ºC. IWF samples were desalted and concentrated using the Centricon 10^{TM} system (Amicon Inc., Beverly, MA, USA).

Contamination by cytoplasmic constituents, as monitored by the activity of glucose-6-phosphate dehydrogenase (Ros Barceló 1998a), was always less than 0.1 % with regard to that found in the cytosolic fraction. Further confirmation of the absence of noticeable symplastic contamination in this apoplastic fraction was obtained by protein fingerprint analysis. SDS-PAGE analyses of the major proteins in the symplastic fractions showed the presence of specific proteins, which were almost totally absent from the respective apoplastic fractions (López-Serrano et al. 2004). Using the same method, the recovery of IWFs was extremely high since apoplast specific proteins were absent from symplastic (IWF-extracted tissue) fractions (López Serrano et al. 2004).

Determination of the (ASC/ASC+DHA) ratio in IWFs

To obtain IWFs for ASC/DHA analysis, sections were vacuum-infiltrated for 10 periods of 30 s at 1.0 kPa and 4 ºC with 2% metaphosphoric acid (w/v). The rest of the protocol was as described above for the extraction of enzymes. ASC and DHA contents in the IWFs were directly determined by HPLC according to Hernández et al. (2001).

Determination of peroxidase, ascorbate peroxidase and ascorbate oxidase activies in IWFs

PRX activities were determined in IWFs in assay media containing 50 mM Na-

219 acetate buffer (pH 5.0) and 500 μM H_2O_2, using as electron donors 1.0 mM 4-
220 methoxy-α-naphthol (ε_{595} = 21,600 $M^{-1}cm^{-1}$), 0.1 mM coniferyl alcohol (ε_{262} = 9,600
221 M^{-1} cm^{-1}) or 320 μM TMB-HCl (ε_{652} = 39,000 M^{-1} cm^{-1}). Controls were carried out
222 in the absence of H_2O_2.

223 APX activities were measured in a reaction medium containing 50 mM
224 K-phosphate buffer (pH 7.0), 0.1 mM H_2O_2 and 0.5 mM ASC (Amako et al. 1994),
225 by monitoring the decrease in absorbance at 290 nm (ε_{290} = 2.8 mM^{-1} cm^{-1}). Class I
226 APX activity was distinguished from class III APX activity by pre-incubation of
227 plant extracts for 10 min with 50 μM of Na-*p*CMB, a specific suicide inhibitor of
228 class I APXs (Amako et al. 1994).

229 AAO activities were determined (Moser and Kanellis 1994) in a reaction
230 medium containing 35 mM K-phosphate buffer (pH 5.3), 0.002% (w/v)
231 metaphosphoric acid, and 0.15 mM ASC, by monitoring the changes in absorbance
232 at 265 nm (ε_{265} = 9246 M^{-1} cm^{-1}). Controls were performed in the presence of 0.1
233 mM sodium azide.

234

235 *Extraction of symplastic enzymes*

236 After removal the IWFs, sections were frozen in liquid nitrogen, and homogenized
237 in 100 mM K-phosphate buffer (pH 7.8), containing 2% polyvinylpolypyrrolidone,
238 0.5% Triton X-100, and 5 mM ASC (Polle et al. 1996). The homogenate was
239 centrifuged at 48000 g for 20 min, and the supernatants were desalted using a
240 Sephadex G-25 (PD-10 columns, Pharmacia, Germany) column equilibrated with 50
241 mM K-phosphate buffer (pH 7.0), containing 1 mM ASC.

242

243 *Isolation of cell walls and assay for ASC oxidation*

244 Cell walls were prepared and purified through a Triton X-100 washing procedure, as
245 described by Ros Barceló (1998b). To monitor ASC oxidation by isolated cell walls,
246 an amount of purified cell walls, equivalent to 1.0 g FW, was incubated with stirring
247 for 1 h at 25°C in an assay medium (10.0 mL) containing 35 mM K-phosphate
248 buffer (pH 5.3), 0.002% (w/v) metaphosphoric acid, and 0.15 mM ASC. Controls
249 were performed in the presence of 0.1 mM sodium azide, in the presence of a heat
250 (100°C)-denatured cell wall fraction, and in the presence of catalase (200 U/mL).
251 After this time, the cell walls were centrifuged at 1100 g for 5 min at 5ºC, the
252 amount of ASC being determined by the decrease in absorbance at 290 nm (ε_{290} =
253 2.8 mM^{-1} cm^{-1}).
254
255 *Isoelectric focusing (IEF), polyacrylamide gel electrophoresis (PAGE) and staining*
256 *of enzymatic activities*
257 IEF and staining of peroxidase isoenzymes with 4-methoxy-α-naphthol was
258 performed as described (Ferrer et al. 1990). Controls were carried out either in the
259 absence of H_2O_2, or in the presence of 0.5 mM Na-*p*-CMB, an inhibitor of class I
260 APXs (Chen and Asada, 1989). In the last case, a 20 min pre-incubation of the gels
261 in the presence of the inhibitor was performed before peroxidase staining.
262 Proteins were subjected to discontinuous PAGE under non-denaturing
263 and non-reducing conditions essentially as described (Pomar et al. 2002a), except
264 that the gels were supported with 10% (w/v) glycerol (Mittler and Zilinskas 1993).
265 In the case of APX and AAO activities, polyacrylamide gels were pre-run for 30
266 min in the carrier buffer containing 2 mM ASC, prior to the application of the
267 protein samples (Mittler and Zilinskas 1993).
268 Staining for APX activity was performed in successive steps. First, gels

were pre-equilibrated for 30 min the gels in 50 mM Na-posphate buffer (pH 7.0), containing 2 mM ASC. Then, the gels were incubated for 20 min in 50 mM Na-phosphate buffer (pH 7.0), containing 4 mM ASC and 2 mM H_2O_2. Finally, the gels were washed with buffer for 1 min, and stained with 50 mM Na-phosphate buffer (pH 7.8), containing 28 mM N,N,N',N'-tetramethylethylenediamine (TEMED) and 2.45 mM nitrotetrazolium blue chloride (NBT). APX activities were observed as achromatic bands on a purple-blue background. Controls for class I APXs were performed in the presence of 0.5 mM Na-*p*-CMB.

Staining for AAO activity was performed as reported for APX activities, but using 0.1 M Na-phosphate buffer (pH 5.3), containing 4 mM ASC, and in the absence of H_2O_2. After washing, the gels were stained in the same staining solution described above containing TEMED and NBT. AAO activities were observed as achromatic bands on a purple-blue background. Controls were performed in the presence of 1 mM sodium azide.

Chemicals

TMB-HCl, KI, ferulic acid, horseradish peroxidase C, *Cucurbita sp.* AAO, NBT, TEMED, and catalase (from bovine liver, EC 1.11.1.6) were purchased from Sigma (Madrid, Spain). Ascorbic acid and sodium azide were from Merck (Barcelona, Spain). The other chemicals were of the highest purity available.

Results

Histochemical localization of H_2O_2 production/accumulation in the secondary xylem

Re-initiation of sustainable secondary growth is one of the characteristic of trees during spring, when the axillary buds elongate and develop into lateral branches.

Secondary growth of young branches during spring is easily monitored in poplar and bitter orange tree by light microscopy. In these cases, secondary growth arises from the periclinal (tangential plane) division of elongated fusiform cambial initial cells, which generate the wood elements, vessels, parenchyma cells and fibres, on the inner side, and phloem cells (sieve tubes, parenchyma cells and fibres) on the outer side.

During spring, the xylem and phloem from young branches of poplar begin to lignify (Fig. 1a). Such tissue specificity in the case of lignification is also showed for H_2O_2 production, since cytochemical probes with the TMB reagent revealed that only lignifying tissues show a strong stain reaction (Fig. 1b). Nevertheless, H_2O_2 is only accumulated in the secondary xylem, as is revealed by the KI-starch reagent (Fig. 1c) and not in the phloem. Controls in the presence of ferulic acid (Fig. 1d) support the peroxidase/H_2O_2-dependent nature of the staining reaction described in Figure 1b.

Similar patterns for H_2O_2 production were also found in bitter orange tree (Figs. 2a-d) and eucalyptus (Figs. 2e-g), where H_2O_2 production was restricted to a few cell layers of the differentiating (lignifying) secondary xylem, as was the case in bitter orange tree (Fig. 2b, arrowheads), or to a large number of cells layers of the differentiating (lignifying) secondary xylem, as was the case in eucalyptus (Fig. 2f). In bitter orange tree, the pattern found for H_2O_2 production (Fig. 2b) was similar to that obtained for H_2O_2 accumulation (Fig. 2c). Interestingly, no H_2O_2 production was observed in the phloem of bitter orange tree or eucalyptus, despite the lignifying nature of these vascular tissues (Figs. 2a,e). Controls in the presence of ferulic acid (Fig. 2d,g) lent weight to the peroxidase/H_2O_2-dependent nature of the staining reaction described in Figures 2 b,f.

Since the TMB reagent is able to reveal H_2O_2-production at short times (Ros Barceló 1998b and 2005), while the KI-starch reagent is able to reveal H_2O_2 accumulation at longer times (Olson and Varner 1993, Ros Barceló 2005), it seems that H_2O_2 is not only produced by the secondary xylem (Figs. 1b, 2b and 2f), but is also accumulated in the secondary xylem (Figs. 1c, 2c), at the very same time and place that lignification is beginning.

Nature of the peroxidase isoenzyme complement in the secondary xylem

The results shown in Figures 1b, 2b and 2f, obtained with the TMB-endogenous peroxidase dependent probe, revealed that H_2O_2 is produced by the secondary xylem of trees. It has been shown (Ros Barceló 1998b) that for this staining to be successful, peroxidase must be localized in the same secondary tissues. To confirm this tissue distribution of peroxidase, exogenous H_2O_2 (0.1 mM) was added to the TMB staining medium. However, addition of H_2O_2 does not reveal further staining in other tissues, the only difference being that the reaction of the secondary xylem was faster and, with time, stronger. These results clearly indicate that peroxidase is only localized in H_2O_2-producing vascular tissues. For this reason, the nature of peroxidase activity and the nature of the peroxidase isoenzyme complement were studied in IWFs from these species, the research being extended to the herb, *Zinnia*.

Results show that the IWFs of all these species contained PRX activities capable of oxidizing 4-methoxy-α-naphthol, TMB and coniferyl alcohol (Table 1), the last a substrate for lignin biosynthesis. In the case of citrus orange tree, eucalyptus and *Zinnia*, oxidation rates of 4-methoxy-α-naphthol and coniferyl alcohol by IWFs were measured both in the absence and in the presence of exogenous H_2O_2 (Table 1), the rates in its absence never exceeding 10 % of the rates

345 measured in its presence. However, in the case of poplar, the IWFs show an
346 appreciable substrate oxidation rate in the absence of H_2O_2, particularly when
347 assayed with 4-methoxy-α-naphthol (Table 1), suggesting that an oxidase activity
348 independent of H_2O_2 is also present in this species.

349 This observation was confirmed by IEF analysis of the peroxidase
350 isoenzymes present in this apoplastic fraction. In fact, IEF analyses of IWFs from
351 poplar revealed the presence of a protein with oxidase activity, whose activity was
352 not stimulated by H_2O_2 (Fig. 3a, lanes 1 and 2, white arrow). Such enzymatic
353 activities have been described in poplar previously (Ranocha et al. 1999), but were
354 not found in IWFs of bitter orange tree (Fig. 3b), eucalyptus (Fig. 3c) or *Zinnia* (Fig.
355 3d), species in which all the bands showing phenol-oxidizing activity were most
356 intense in the presence of H_2O_2 (lanes 1 and 2). This was particularly the case with
357 the most basic PRX isoenzymes (Fig. 3, arrowheads). In poplar, IWFs also showed
358 peroxidase isoenzymes, whose activity was inhibited by the inhibitor of class I
359 peroxidases, *p*CMB (Fig. 3a, lanes 2 and 3, arrows). This result clearly demonstrates
360 the presence of class I peroxidases in the IWFs of this species.
361
362 *Determination of APXs in IWFs*
363 The presence of class I peroxidases in IWFs from poplar was confirmed by
364 measuring *p*CMB-sensitive APX activities, since ASC is the prototype natural
365 substrate of these enzymes. The results (Table 2) showed that APX activities
366 measured in IWFs from poplar were inhibited by *p*CMB. In bitter orange tree IWFs,
367 APX activities were not inhibited by *p*CMB, whereas both eucalyptus and *Zinnia*
368 showed no APX activity in IWFs. For comparative purposes, Table 2 also shows
369 APX activities in the symplastic fraction of these species, in which most of

symplastic APX activities were sensitive to *p*CMB. These results suggest that IWFs from poplar contained class I (*p*CMB-sensitive) APXs, while IWFs from bitter orange tree contained class III (*p*CMB-insensitive) APXs. The IWFs from eucalyptus and *Zinnia* did not contain either type of APX. These results also illustrate the variability found in the apoplastic antioxidant enzyme pattern between woody species.

The presence of class I APXs in IWFs from poplar was confirmed by anionic PAGE, which showed the presence of one highly mobile APX isoform (Fig. 4a, lane 1, arrow), whose activity was inhibited by *p*CMB (Fig 4a, lane 2, arrow). By anionic PAGE, this *p*CMB-sensitive APX isoform showed the same mobility as a peroxidase isoform stained with 4-methoxy-α-naphthol (Fig. 4a, lane 3, arrow), supporting the results previously obtained by IEF (Fig. 3a), which showed the existence in poplar of 4-methoxy-α-naphthol peroxidases sensitive to *p*CMB. In the case of bitter orange tree, the only APX isoform detected (Fig. 4b, lanes 1 and 2, arrow) was not sensitive to *p*CMB (Fig. 4b, lanes 3 and 4, arrow), supporting the previous observation that only class III APX activities are presents in the IWFs of this species (Table 2). This is not surprising since the prototype class III peroxidase, horseradish peroxidase C, also shows APX activity after migration by anionic PAGE (Fig. 4c, lanes 1 and 2). In the case of eucalyptus (Fig. 4d) and *Zinnia* (Fig 4e), no APX (either class I or class III) activities were detected by anionic PAGE.

Determination of AAOs in IWFs

As in the case of APX activities, AAO activities were detected in the apoplastic fraction of poplar and bitter orange tree, but not in eucalyptus or *Zinnia* (Table 3). In the species where AAO was measured, levels of AAO activity were of the same

order as found for APX activities (Table 2), suggesting that both enzymatic activities contribute equally to the apoplastic enzymatic antioxidant system. In the case of eucalyptus and *Zinnia*, although it was not possible to determine AAO activities in the IWF fraction, cell walls isolated from both species were able to aerobically oxidize ASC. In fact, amounts of cell walls equivalent to 1 g FW were able to oxidize 15 μmol ASC in less than 1 h in the presence of catalase. This oxidation was exclusively due to AAO activities since the addition of H_2O_2, in the absence of catalase, does not stimulate the ASC oxidation rate

Analyses by anionic PAGE of AAO isoforms in the IWFs from poplar showed three azide-sensitive achromatic bands, one of low mobility and two of high mobility (Fig. 5a, lanes 1 and 2, arrows). In bitter orange tree, three azide-sensitive achromatic bands of medium mobility were observed (Fig. 5b, lanes 1 and 2, arrows). The low mobility of the azide-sensitive achromatic band in poplar is not surprising, since AAO from *Cucurbita* spp shows a similar low mobility by anionic PAGE (Fig. 5a and 5b, lanes 3). Again, no AAO activity was detected by anionic PAGE in IWFs from eucalyptus (Fig. 5c) or *Zinnia* (Fig 5d).

Determination of the ascorbic acid redox state (ASC/ASC+DHA) in IWFs

Analysis by HPLC of ascorbate species (ASC and DHA) in IWFs from poplar, bitter orange tree, eucalyptus and *Zinnia* showed that ASC levels were, in all the cases, below the threshold for detection, and only DHA could be detected. The redox state (ASC/ASC+DHA) was thus zero, and therefore strongly shifted towards DHA, the oxidized product of ASC.

Discussion

The secondary xylem of poplar, bitter orange tree and eucalyptus are not only able to produce H_2O_2, but also to accumulate during the phase of active lignification which follows the re-initiation of secondary growth during spring it (Figs. 1 and 2). Such H_2O_2 production and accumulation has previously been observed in *Zinnia* (Ros Barceló 1998a, 2005), where evidence suggests that H_2O_2 is produced by an NADPH oxidase-like enzyme, broadly similar to the phagocytic NADPH oxidase of mammalian cells (Ros Barceló 1998a). The evidence obtained in *Zinnia* also suggests (Ros Barceló 2005) that this H_2O_2 is produced on the outer-face of the plasma membrane of both differentiating thin-walled xylem cells and non-lignifying xylem parenchyma cells, from where it diffuses, through the continuous cell wall space, to differentiated lignifying xylem vessels**.**

However, there is a gap in our knowledge of why lignifying xylem tissues are able to accumulate H_2O_2, since some plant tissues, which are able to produce H_2O_2 constitutively, do not shown net H_2O_2 accumulation (Hernández et al. 2001; Mittler et al. 2004). The results obtained here suggest that the reason for such H_2O_2 accumulation is an imbalance of the apoplastic antioxidant enzymatic system of the secondary xylem provoked by the presence of AAO, which triggers the apoplastic redox state (ASC/ASC+DHA) until zero or nearly zero values.

It is now accepted (de Gara 2004) the presence in the apoplast of three H_2O_2 detoxifying systems which use ASC as a source of reducing equivalents: class I APXs, class III APXs, and class III peroxidases, which are unable to oxidize ASC directly, but which use a redox phenol shuttle to carry out this task. The use of ASC as reducing substrate for removing H_2O_2 by all these enzymatic systems offers several advantages for the plant cell, since the ASC oxidation does not produce toxic or reactive molecules. In fact, AFR, the first product of ASC oxidation by these

445 enzymes, shows insignificant reactivity with O_2 (de Gara 2004), unlike phenolic
446 radicals (Pomar et al. 2002a), and, therefore, does not trigger O_2^- production, thus
447 avoiding the emergence of a possible $O_2^-/H_2O_2/OH^\bullet$ cascade in the apoplast (Mittler
448 et al. 2004; de Gara 2004). Moreover, both AFR and DHA, the final product of ASC
449 oxidation in the apoplastic space, may be continuously reduced to ASC, using the
450 cytoplasmic pyridine nucleotide and glutathione pools (de Gara and Tommasi
451 1999).

452 The first of these ASC-dependent antioxidant systems is constituted by
453 class I APXs, which are specific for ASC. Although some class I APX genes are
454 expressed in vascular tissues (Fryer et al. 2002), class I APXs are generally
455 associated with meristematic and actively growing young tissues (de Pinto and de
456 Gara 2004; Córdoba-Pedregosa et al. 2005), and this could explain their absence
457 from lignifying tissues in three of the four species studied (Table 2). In fact, class I
458 APXs were only detected in poplar (Fig. 4a, Table 2), and are generally absent from
459 the apoplast of most mature plant tissues (Polle et al. 1990; Hernández et al. 2001).
460 For this reason, it is hard to imagine that class I APXs constitute the central pivot of
461 the H_2O_2 detoxifying system in the apoplast of lignifying tissues.

462 The second ASC-dependent antioxidant system in the apoplast is
463 constituted by class III APXs, which are only moderately specific for ASC. Class III
464 APXs are generally expressed in non-growing tissues (de Pinto and de Gara 2004),
465 where the cell wall stiffening process is emerging. Class III APXs were only
466 detected in the apoplastic fractions of bitter orange tree (Table 2 and Fig. 4), and
467 therefore neither appears as a possible candidate for constituting the core of the
468 H_2O_2 detoxifying system in the apoplast of lignifying tissues.

469 The third ASC-dependent antioxidant enzymatic system in the apoplast is

constituted by class III peroxidases, which, although unable to oxidize ASC directly, can perform this task when they are coupled by means a phenolic/phenolic radical shuttle (Takahama 1993; Otter and Polle 1994). In this mechanism, phenolic radicals ($R^{\bullet}$), which have been generated by a H_2O_2-dependent peroxidase-catalyzed reaction (2 RH + H_2O_2 $\rightarrow$ 2 $R^{\bullet}$ + H_2O), may act as diffusible oxidants to oxidize a secondary substrate, such as ASC (2 $R^{\bullet}$ + ASC $\rightarrow$ 2 RH + DHA). This type of reaction has been described for the oxidation of ASC mediated by the coniferyl alcohol radical ($R^{\bullet}$) (Takahama 1993; Otter and Polle 1994), and may have important physiological connotations in the detoxification of H_2O_2 in the apoplast of lignifying tissues, since peroxidases unable to oxidize ASC, such as those present in the apoplast of eucalyptus and *Zinnia* (Table 2) could oxidize this substrate using coniferyl alcohol as redox shuttle. The only condition that these peroxidases must fulfil is an intrinsic capacity for oxidizing coniferyl alcohol, and this condition is completely fulfilled by the peroxidases present in the apoplast of eucalyptus and *Zinnia*, and even by the peroxidases present in the apoplast from bitter orange tree and poplar (Table 1).

In fact, the thermodynamic driving force for the two-electron transfer reaction, which constitutes the basis of this shuttle, is given by the difference between the oxidation/reduction potentials of coniferyl alcohol/coniferyl alcohol radical ($R^{\bullet},H^{+}$ /RH, E = + 0.810 V) and ASC/DHA (E = + 0.06 V), ΔE = E ($R^{\bullet},H^{+}$/RH) - E (DHA/ASC) = + 0.804 V, which gives a change of free energy for the reaction, ΔG [ΔG = -n F ΔE] of - 155.17 kJ mol^{-1}, and which is responsible for the spontaneous nature of the reaction. This theoretical consideration suggests that class III peroxidases, which paradoxically do not show APX activity, may be the better candidates for constituting the core of the ASC-dependent H_2O_2 detoxifying

system in the apoplast of lignifying tissues, since they are largely able to oxidize coniferyl alcohol (Table 1), the redox mediator, which is present in μM amounts in lignifying tissues (Hosokawa et al. 2001).

However, all these apoplastic H_2O_2 detoxifying systems, which use ASC as source of reducing equivalents, may either be partially non-functional or dysfunctional in the lignifying cell wall in the presence of AAO. AAO oxidizes ASC at the expense of O_2, and contrarily to class I APXs, is exclusively localized in the cell wall of plant tissues and, more specifically, in the cell wall of vascular tissues (Liso et al. 2004). AAOs are present in IWFs from poplar and bitter orange tree (Table 3 and Fig. 5) and, although they are not present in IWFs from both eucalyptus and *Zinnia*, it is known that blue copper oxidases mRNAs are differentially expressed in the secondary xylem of poplar (Mellerowicz et al. 2001), eucalyptus (Paux et al. 2004) and *Zinnia* (Demura et al. 2002). The reason why AAOs are not detected in the IWFs of eucalyptus and *Zinnia* probably resides in the fact that these enzymes are covalently-bound to the cell wall, a situation already described for the laccase-type blue copper oxidases in *Zinnia* (Liu et al. 1994), since isolated cell wall fractions from eucalyptus and *Zinnia* are able to oxidize ASC aerobically.

Although the biological function of AAOs in the cell wall remains to be totally understood (de Tullio et al. 2004), it has been demonstrated that AAO regulates the apoplastic ascorbate pool and therefore the redox state (ASC/ASC+DHA), reducing apoplastic ASC levels to values below the detection threshold (Sanmartin et al. 2003; Pignocchi et al. 2003). Under these circumstances, the apoplastic redox state is zero, or near to zero, apparently due to the fact that when apoplastic AAO activity is present, the rate of ASC oxidation far exceeds the

520 capacity of DHA transport to the cytosol (Pignocchi and Foyer 2003).

521 In fact, the effectiveness of this ASC removal system compared with
522 class I APXs is such that the presence of AAO in the apoplastic space of non-
523 growing tissues provokes the total shift of the redox state (ASC/ASC+DHA)
524 towards DHA (de Pinto and de Gara 2004), the oxidized product of ASC. This does
525 not occur in the apoplast of young tissues, where class I APXs predominate (de
526 Pinto and de Gara 2004). Zero values were found for the redox state of ascorbic acid
527 in IWFs from poplar, bitter orange tree, eucalyptus and *Zinnia*, and similar results
528 (with values of zero or close to zero) have been reported in the xylem of *Larix*
529 during the early stages of lignification (Antonova et al. 2005). In other words, it is
530 conceivable that the presence of AAO in cell walls provokes the partial or total
531 dysfunction of apoplastic class I and class III APXs, and of all the plethora of non-
532 enzymatic redox shuttles in which ASC is involved (Takahama 1993), through the
533 competitive and effective removal of ASC, allowing the accumulation of H_2O_2 in
534 the apoplast of lignifying plant tissues, as can be observed experimentally (Figs. 1
535 and 2).

536 Furthermore, since AAO uses O_2 to remove ASC, it could regulate O_2
537 availability in the lignifying xylem (Tullio et al. 2004). Thorough this mechanism,
538 AAO could also control the activity of NADPH oxidase (the enzyme responsible for
539 H_2O_2 production in lignifying tissues) (Ogawa et al. 1997; Ros Barceló 1998a) at
540 substrate level, by controlling the tension of O_2. That is, AAOs, through a double
541 mechanism, appear to be essential for the fine control of the oxidative performance
542 of lignifying secondary wood-forming tissues.

543

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709

710

711 **Fig. 1 a**) Histochemical localization of lignins in poplar using the phloroglucinol/HCl
712 reagent. **b**) Histochemical localization of H_2O_2 production in poplar using the TMB
713 reagent. **c**) Histochemical localization of H_2O_2 accumulation in poplar using the
714 KI/starch reagent. **d**) Control for the histochemical localization of H_2O_2 production in
715 poplar using the TMB reagent, performed in the presence of 0.1 mM ferulic acid. Bar =
716 300 μm.

717

718 **Fig. 2** Histochemical localization of lignins in **a**) bitter orange tree and **e**) eucalyptus
719 using the phloroglucinol/HCl reagent. Histochemical localization of H_2O_2 production in
720 **b**) bitter orange tree and **f**) eucalyptus using the TMB reagent. Histochemical
721 localization of H_2O_2 accumulation in **c**) bitter orange tree using the KI/starch reagent.
722 Controls for the histochemical localization of H_2O_2 production in **d**) bitter orange tree
723 and **g**) eucalyptus using the TMB reagent, performed in the presence of 0.1 mM ferulic
724 acid. Bar = 600 μm.

725

726 **Fig. 3** Isoenzyme pattern obtained by isoelectric focusing in 3.5-10.5 pH gradients (top,
727 acidic; bottom, basic) of IWF proteins from **a**) poplar, **b**) bitter orange tree, **c**)
728 eucalyptus and **d**) *Zinnia*, stained with 4-methoxy-α-naphthol in the absence (1) and in
729 the presence (2) of H_2O_2, and in the presence of H_2O_2 after pre-incubation with *p*CMB
730 (3). The black arrows mark class I APXs, the white arrows mark H_2O_2-independent
731 oxidases, and the black arrowheads mark the strongly basic H_2O_2-dependent
732 peroxidases.

733

734 **Fig 4 a)** Isoenzyme pattern obtained by native PAGE of IWF proteins from poplar after

735 staining for APX activity in the absence (1) and in the presence (2) of *p*CMB, and after
736 staining with 4-methoxy-α-naphthol/H_2O_2 (3). **b)** Isoenzyme pattern obtained by native
737 PAGE of IWF proteins from bitter orange tree after staining for APX activity in the
738 absence (1) and in the presence (3) of *p*CMB, and after staining with 4-methoxy-α-
739 naphthol/H_2O_2 in the absence (2) and in the presence (4) of *p*CMB. **c)** Isoenzyme pattern
740 obtained by native PAGE of horseradish peroxidase c after staining for APX activity
741 (1), and after staining with 4-methoxy-α-naphthol/H_2O_2 (2). **d)** Isoenzyme pattern
742 obtained by native PAGE of IWF proteins from eucalyptus after staining for APX
743 activity (1). **e)** Isoenzyme pattern obtained by native PAGE of IWF proteins from
744 *Zinnia* after staining for APX activity (1), and after staining with 4-methoxy-α-
745 naphthol/ H_2O_2 (2)
746
747 **Fig 5 a)** Isoenzyme pattern obtained by native PAGE of IWF proteins from poplar after
748 staining for AAO activity in the absence (1) and in the presence (2) of azide. **b)**
749 Isoenzyme pattern obtained by native PAGE of IWF proteins from bitter orange tree
750 after staining for AAO activity in the absence (1) and in the presence (2) of azide. **c)**
751 Isoenzyme pattern obtained by native PAGE of IWF proteins from eucalyptus after
752 staining for AAO activity (1). **d)** Isoenzyme pattern obtained by native PAGE of IWF
753 proteins from *Zinnia* after staining for AAO activity (1). Lanes 3 in **a**) and **b**) is the
754 isoenzyme pattern obtained by native PAGE of *Cucurbita* AAO after staining for AAO
755 activity.
756

Table 1. Peroxidase activities in IWFs of young branches (*C. aurantium, P. alba* and *E. camaldulensis*) and stems (*Z. elegans*) from different plant species, in the absence and in the presence (0.1 mM) of H_2O_2. Values are the mean ± SE (n). n, number of determinations.

Species	**Peroxidase activity (nmol S oxidized min^{-1} g^{-1} FW)**					
	4-Methoxy-α-naphthol		**3,3',5,5'-Tetramethylbenzidine**		**Coniferyl alcohol**	
	- H_2O_2	**+ H_2O_2**	**- H_2O_2**	**+ H_2O_2**	**- H_2O_2**	**+ H_2O_2**
P. alba	15.74 ± 0.04 (3)	125.2 ± 11.63 (3)	0 ± 0 (3)	1124 ± 158 (3)	2.53 ± 0.96 (3)	668 ± 79 (3)
C. aurantium	18.80 ± 0.03 (6)	320.3 ± 18.45 (6)	0 ± 0 (6)	2300 ± 180.0 (6)	0.360 ± 0.015 (6	616 ± 45 (6)
E. camaldulensis	0.143 ± 0.034 (3)	23.18 ± 5.76 (3)	0 ± 0 (3)	79.86 ± 12.65 (3)	0.01 ± 0.00 (3)	98.2 ± 9.54 (3)
Z. elegans	0.56 ± 0.00 (4)	6.23 ± 0.85 (5)	0 ± 0 (4)	104.2 ± 1.94 (4)	0.59 ± 0.20 (4)	53.73 ± 6.13 (4)

Table 2. APX activities in IWFs and symplastic fractions of young branches (*C. aurantium, P. alba* and *E. camaldulensis*) and stems (*Z. elegans*) from different plant species, in the absence and in the presence (50 μM) of *p*CMB. Values are the mean ± SE (n). n, number of determinations. [a]Differences were significant at P <0.01 according to Duncan´s Multiple Range Test

Species	**APX activity (μmol ascorbic acid oxidized min^{-1} g^{-1} FW)**					
	IWF			**Symplast**		
	- *p*CMB	**+ *p*CMB**	**Δ *p*CMB**	**- *p*CMB**	**+ *p*CMB**	**Δ *p*CMB**
P. alba	0.081 ± 0.021 (14)[a]	0.033 ± 0.009 (10)[a]	0.048	3.26 ± 0.36 (8)[a]	0.70 ± 0.22 (5)[a]	2.56
C. aurantium	0.405 ± 0.026 (15)	0.422 ± 0.029 (10)	0	9.77 ± 0.70 (6)[a]	4.22 ± 0.35 (5)[a]	5.55
E. camaldulensis	0 ± 0 (11)	0 ± 0 (9)	0	1.91 ± 0.09 (10)[a]	0 ± 0 (10)[a]	1.91
Z. elegans	0 ± 0 (10)	0 ± 0 (10)	0	0.70 ± 0.02 (10)[a]	0.22 ± 0.01 (10)[a]	0.48

Table 3. AAO activities in IWFs of young branches (*C. aurantium, P. alba* and *E. camaldulensis*) and stems (*Z. elegans*) from different plant species, both in the absence and in the presence (0.1 mM) of azide. Values are the mean ± SE (n). n, number of determinations. [a]Differences were significant at P <0.01 according to Duncan´s Multiple Range Test

Species	**AAO activity (nmol ascorbic acid oxidized min^{-1} g^{-1} FW)**	
	- Azide	+ Azide
P. alba	56 ± 20 (10)[a]	7 ± 5 (6)[a]
C. aurantium	866 ± 78 (10)[a]	15 ± 5 (5)[a]
E. camaldulensis	0 ± 0 (9)	0 ± 0 (5)
Z. elegans	0 ± 0 (6)	0 ± 0 (5)

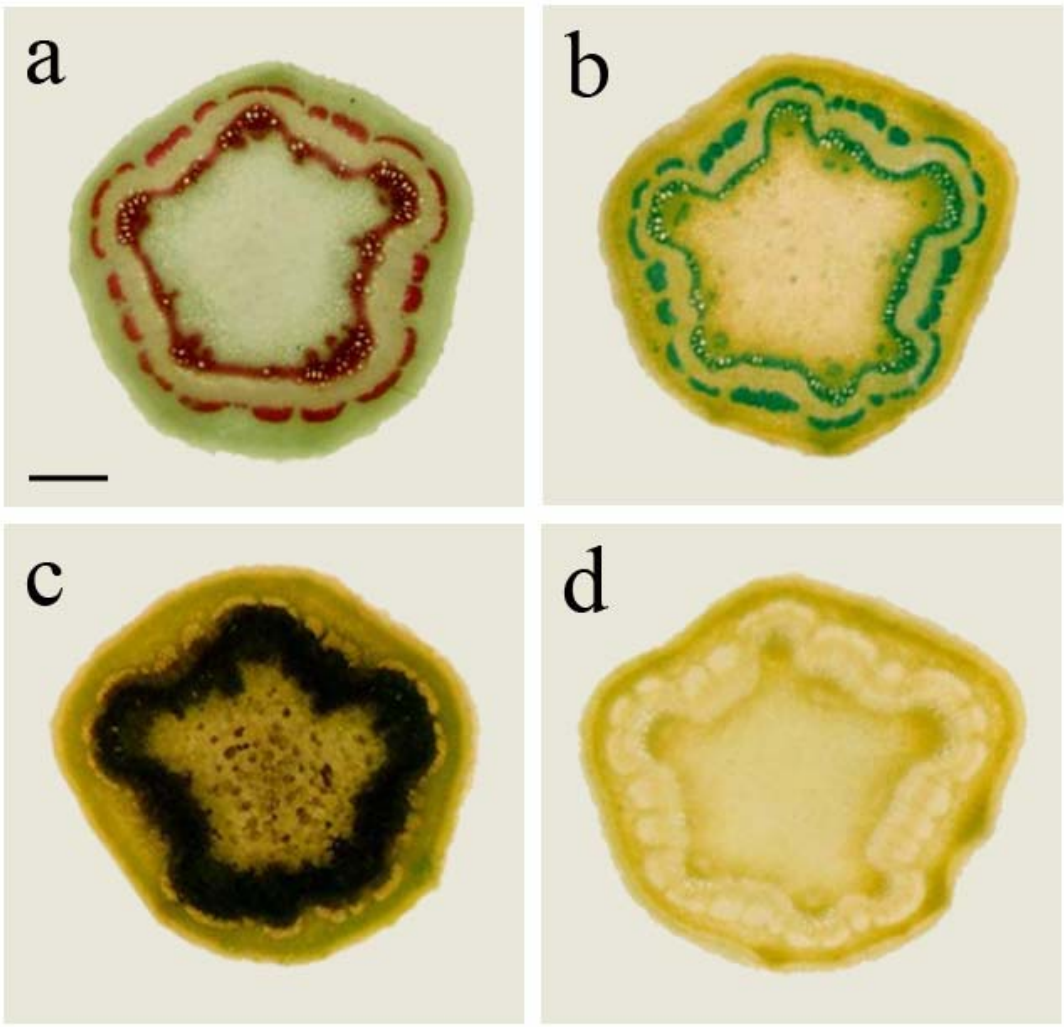

Figure 1

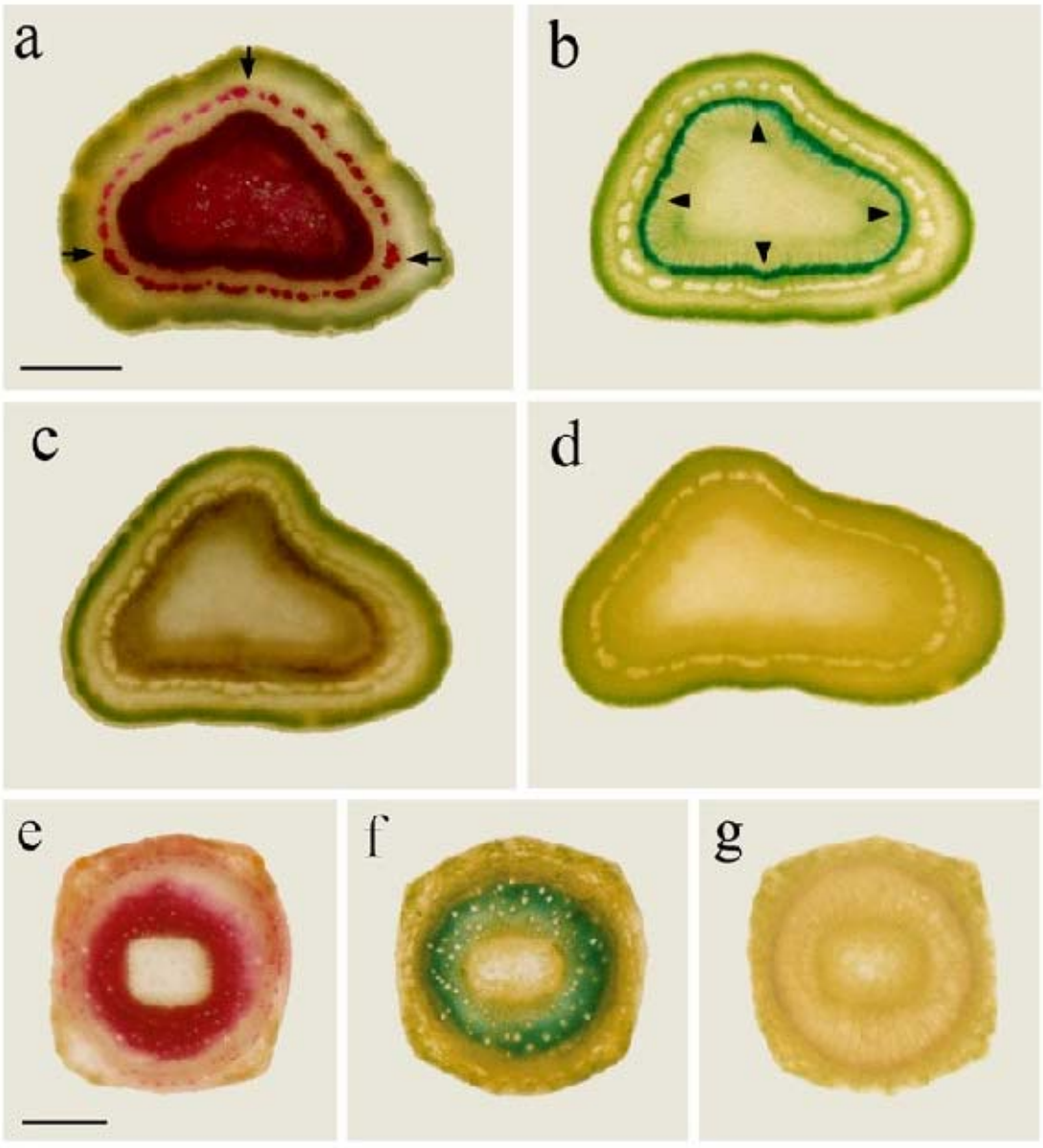

Figure 2

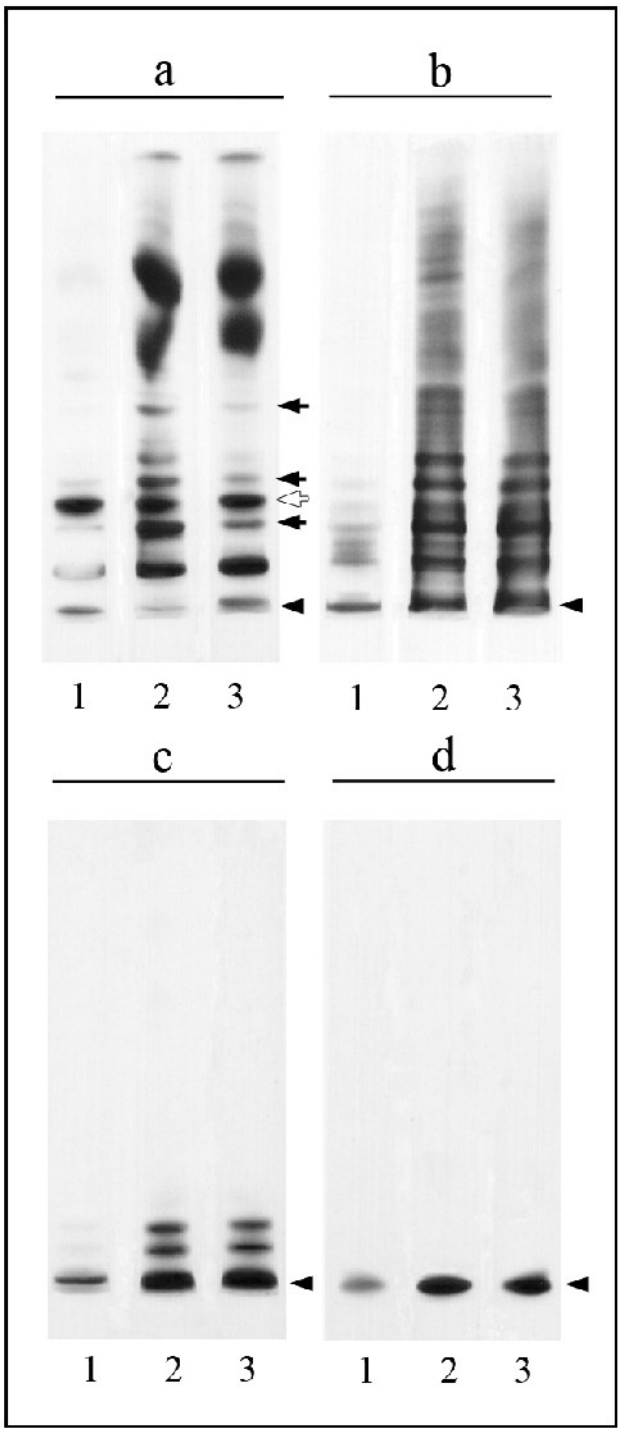

Figure 3

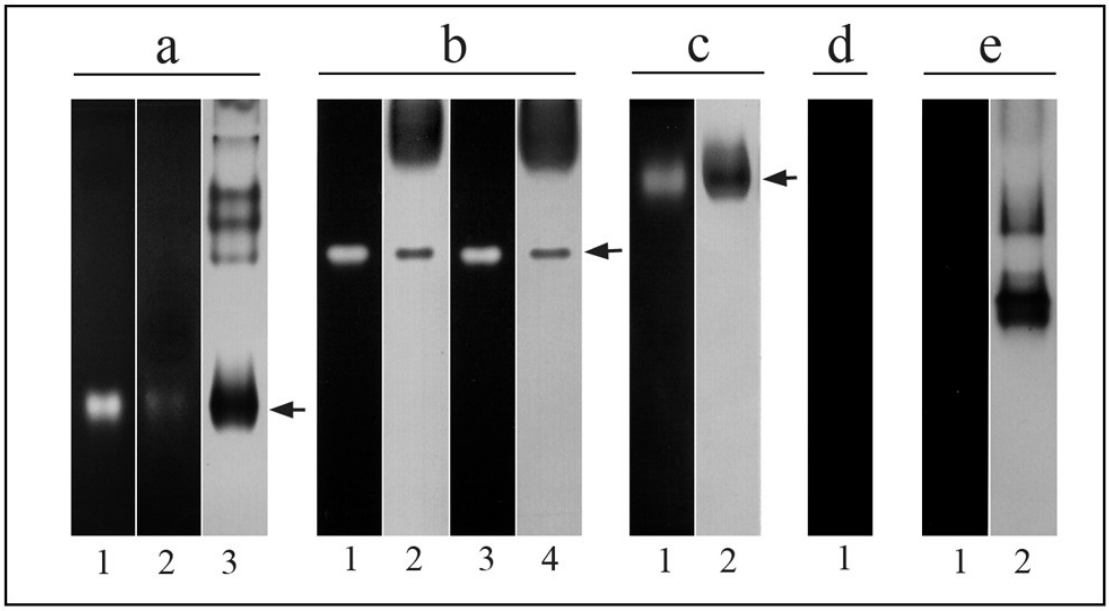

Figure 4

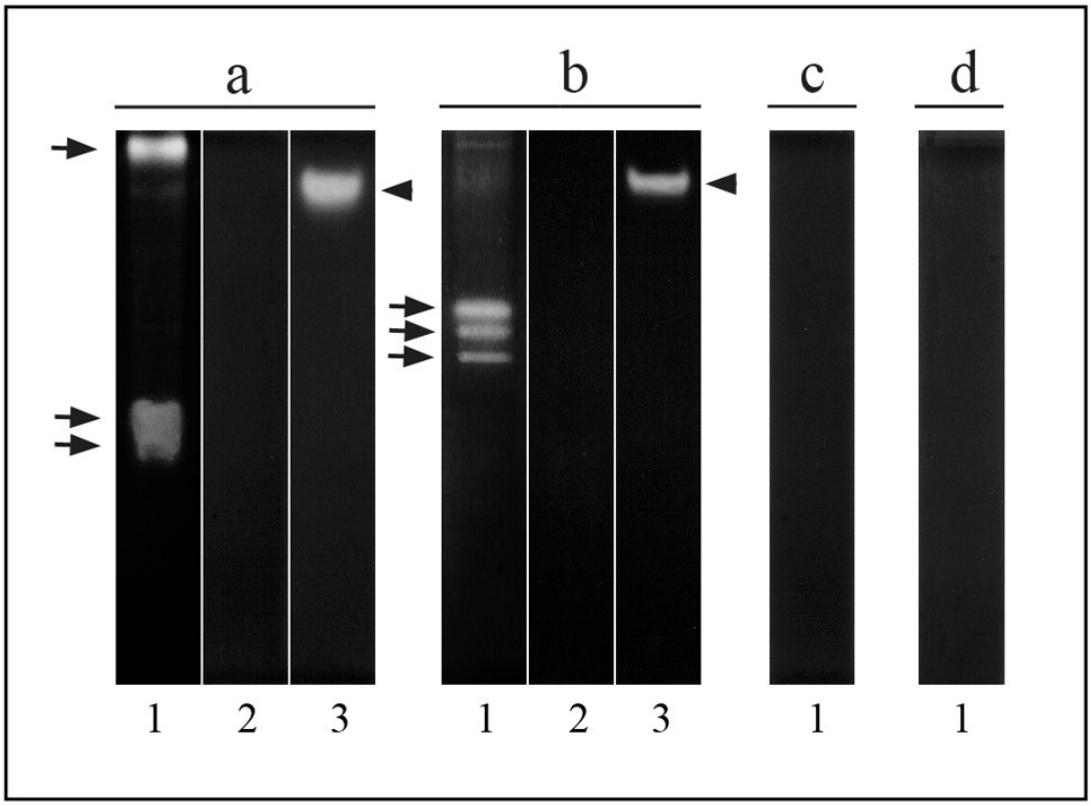

Figure 5