JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Structural Motifs of Syringyl Peroxidases Are Conserved during Angiosperm Evolution

LAURA V. GÓMEZ ROS,† GINÉS J. AZNAR-ASENSIO,† JOSE A. HERNÁNDEZ,‡ MARIA A. BERNAL,§ MARÍA J. L. NÚÑEZ-FLORES,† JUAN CUELLO,† AND ALFONSO ROS BARCELÓ*,†

Department of Plant Biology, University of Murcia, E-30100 Murcia, Spain; Department of Plant Breeding and Physiology, CEBAS-CSIC, E-30100 Murcia, Spain; and Department of Plant Biology, University of La Coruña, E-15071 La Coruña, Spain

The most distinctive variation in the monomer composition of lignins in vascular land plants is that between the two main groups of seed plants. Thus, whereas gymnosperm (softwood) lignins are typically composed of guaiacyl (G) units, angiosperm (hardwood) lignins are largely composed of similar levels of G and syringyl (S) units. However, there are some studies that suggest that certain angiosperm peroxidases are unable to oxidize sinapyl alcohol, and a coniferyl alcohol shuttle has been proposed for oxidizing S units during the biosynthesis of lignins. With this in mind, a screening of the presence of S peroxidases in angiosperms (including woody species and forages) was performed. Contrarily to what might be expected, the intercellular washing fluids from lignifying tissues of 25 woody, herbaceous, and shrub species, belonging to both monocots and dicotyledons, all showed both S peroxidase activities and basic peroxidase isoenzymes analogous, with regard the isoelectric point, to the *Zinnia elegans* basic peroxidase isoenzyme, the only S peroxidase that has been fully characterized. These results led to the protein database in the search for homologies between angiosperm peroxidases and a true eudicot S peroxidase, the *Z. elegans* peroxidase. The findings showed that certain structural motifs of S peroxidases are conserved within the first 15 million years of angiosperm history, because they are found in peroxidases from the two major lineages of flowering plants, eumagnoliids and eudicotyledons, of note being the presence of these peroxidases in *Amborella* and Nymphaeales, which represent the first stages of angiosperm evolution. These phylogenetic studies also suggest that guaiacyl peroxidases apparently constitute the most "evolved state" of the plant peroxidase family evolution.

KEYWORDS: Basic peroxidases; evolution; lignin heterogeneity; phylogeny; syringyl moieties; *Zinnia elegans*

INTRODUCTION

Lignins are three-dimensional heteropolymers that result from the oxidative coupling of three *p*-hydroxycinnamyl alcohols, *p*-coumaryl, coniferyl, and sinapyl alcohols, in a reaction mediated by both laccases and class III plant peroxidases (*1*), leading to an optically inactive hydrophobic heteropolymer (*2*), composed of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, derived from *p*-coumaryl, coniferyl, and sinapyl alcohols, respectively. The most distinctive variation in lignin monomer composition in vascular plants is that found between the two main groups of seed plants. Thus, lignins are typically composed of G units in gymnosperms, with a minor proportion of H units, whereas in angiosperms lignins are mainly composed of similar levels of G and S units (*2*). In grasses (*3*), lignins are even more complex, because they also contain significant amounts of ester-bound *p*-coumaric acid. In such a scenario, it is generally assumed that the chemical complexity of lignins has increased during the course of plant evolution, from ancient pteridophytes and gymnosperms to the most evolved grasses.

Lignins represent the most abundant organic compound on the Earth's surface after cellulose, accounting for about 25% of plant biomass (*4*). They are found specifically in vascular plants (tracheophyta) and occur in greatest quantity in the secondary cell walls of xylem vessels and tracheids, as well as fibers and sclereids (*1*). Lignins have been identified in pteridophytes (ferns, lycophytes, and horsetails), widely considered to be the earliest-divergent living vascular plants, and are likely to have played a key role in the colonization of the terrestrial landscape by plants during the Ordovician to Silurian transition, 400−450 million years ago (*4*).

* Author to whom correspondence should be addressed (e-mail rosbarce@um.es; fax + 34 968 363 963; telephone + 34 968 364 945).
† University of Murcia.
‡ CEBAS-CSIC.
§ University of La Coruña.

10.1021/jf063164t CCC: $37.00
Published on Web 04/18/2007

Peroxidases (class III plant peroxidases, EC 1.11.1.7) are the main enzymes involved in the process of monolignol assembly that leads to lignin biosynthesis. Peroxidases are usually classified into acidic (isoelectric point below 7.0) and basic (isoelectric point above 7.0) isoenzymes. Both types of peroxidases are capable of oxidizing *p*-coumaryl and coniferyl alcohol. However, whereas basic peroxidases are also able to oxidize sinapyl alcohol, typical acidic peroxidases, with some exceptions (*5*, *6*), are generally regarded as poor catalysts with this substrate (*7*). Because sinapyl alcohol is more prone to oxidation than either coniferyl alcohol or *p*-coumaryl alcohol (*8*), this observation suggests that substrate accommodation in (exclusion from) the catalytic center of the enzyme determines the real role played by each peroxidase isoenzyme in lignin biosynthesis.

In fact, the oxidation of sinapyl alcohol by certain G acidic peroxidases is sterically hindered due to unfavorable hydrophobic interactions between the sinapyl alcohol methoxy atoms and the conserved I-138 and P-139 residues at the substrate binding site of the enzyme (*9*), although other factors, such as substrate hydrophilicity, should also be considered (*8*). This is not apparently the case for most S basic peroxidases, which have no apparent steric restrictions for oxidizing sinapyl moieties (*10*−*13*). This observation would explain why antisense suppression of basic S peroxidases in transgenic plants produces decreased levels of both G and S lignins (*14*), whereas antisense suppression of acidic peroxidases produces only decreased levels of G lignins (*15*).

In accordance with their key role in lignin biosynthesis, cationic (basic) S peroxidases are differentially expressed during the transdifferentiation of *Zinnia elegans* mesophyll cell cultures, where they act as molecular markers of xylogenesis (*12*, *16*). *Z. elegans* is a eudicot, but peroxidases capable of oxidizing sinapyl alcohol have also been described in gymnosperms (*17*), which paradoxically are reported to lack S-type lignins. In fact, the observation that certain structural motifs (certain amino acid sequences and certain β-sheet secondary structures) of peroxidases capable of oxidizing sinapyl alcohol are also present in peroxidases from gymnosperms which lack S-type lignins (*17*), suggests that the evolutionary gain of the monolignol branch leading to the biosynthesis of S lignins was made possible because the enzymes responsible for its polymerization evolved very early during the evolution of land plants. These results also suggest that these peroxidases were present in an ancestral plant stock, prior to the radiation of tracheophytes, a suggestion confirmed by the presence of the same structural motifs in peroxidases from mosses and liverworts (*17*).

Our previous phylogenetic studies on S peroxidases (*17*) have shown that the genes codifying these enzymes could predate the appearance of vascular plants themselves, and might even be contemporaneous with the acquisition of the most primitive short-distance water and nutrient transport systems that coevolved with mosses and liverworts (*18*). This observation would agree with the fact that an ancestral phenylpropanoid pathway is still present in bryophytes, where peroxidases of a versatile nature have been involved in the metabolism of cell wall located *p*-hydroxycinnamic acid derivatives (*19*). Because the most characteristic property of S peroxidases is the absence of steric restrictions at the substrate binding site for oxidizing *p*-hydroxyphenylpropanoid metabolites (*13*), this observation agrees with the existence of an ancestral basic model of cell wall architecture and function, which would have evolved before the evolutionary divergence of bryophytes, ferns, and seed plants and which have been finely tuned in response to specific evolutionary pressures.

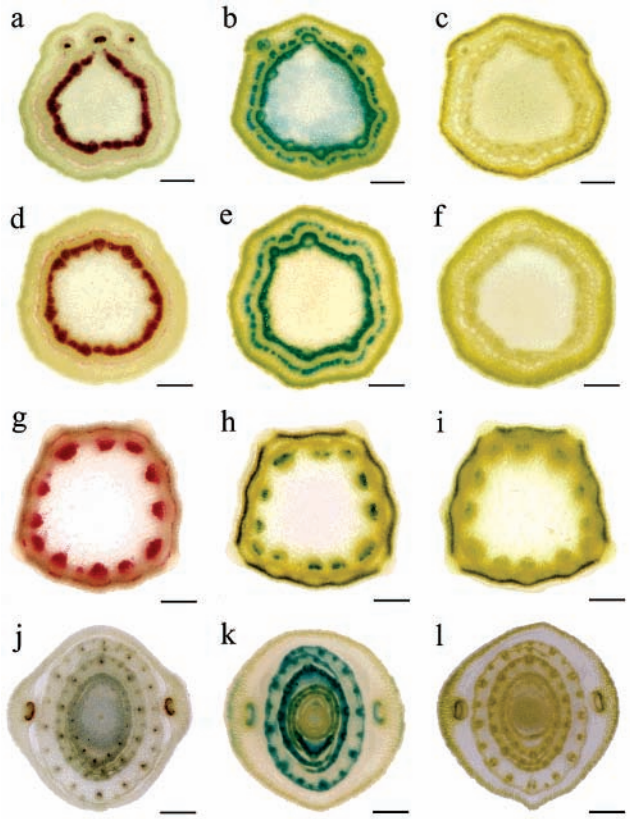

Figure 1. Micrographs of transversal stem sections of the node (**a**–**c**) and the internode (**d**–**f**) of *P. dulcis*, *M. sativa* (**g**–**i**), and *Zea mays* (**j**–**l**), after staining with phloroglucinol (**a**, **d**, **g**, and **j**) to reveal *p*-hydroxycinnamaldehyde-rich lignins and after staining with TMB (**b**, **e**, **h**, and **k**) to reveal sites of peroxidase/H_2O_2 localization. Controls in the presence of 1.0 mM ferulic acid (**c**, **f**, **i**, and **l**) support the peroxidase/H_2O_2-dependent nature of the staining reaction described in panels **b**, **e**, **h**, and **k**). Bars = 300 μm.

Most crops utilized as woods, forages, and human foods belong to angiosperms. In this paper, we search for homologies between angiosperm peroxidases and the *Z. elegans* basic peroxidase, the only S peroxidase that has been characterized and cloned (*12*, *13*). For this, we studied the colocalization of lignin and peroxidase, the ability of peroxidase for oxidizing S moieties, and the peroxidase isoenzyme patterns in a set of differentially evolved angiosperms. The results showed that certain structural motifs of S peroxidases are conserved within the first 15 million years of angiosperm history because they are found in peroxidases from the two major lineages of flowering plants—eumagnoliids and eudicotyledons—with the remarkable presence of these peroxidases in *Amborella* and Nymphaeales, which represent the first stages of angiosperm evolution (*20*). Our phylogenetic studies also suggest that G peroxidases, which show sterically derived constraints for the catalysis, constitute the most "evolved state" of the plant peroxidase family evolution.

MATERIALS AND METHODS

Plant Species and Growth Conditions. Young branch apices (1−3 cm long) of *Populus alba* (poplar), *Magnolia grandiflora*, *Betula pendula* (birch), *Washingtonia filifera*, and *Citrus aurantium* (bitter orange tree) were harvested in April and May from trees at least 10 years old, which were growing on the campus of the University of Murcia. Seedlings of *Zinnia elegans* L (cv. Envy), *Annona cherimola*, *Malus domestica* (apple tree), *Prunus dulcis* (almond tree), *Quercus*

Table 1. Peroxidase Activity Measured with 4-Methoxy-α-naphthol (MN), TMB, Syringaldazine (SYR), and Coniferyl Alcohol (CA) in IWF of Angiosperms[a]

plant species	peroxidase activity[b]					
	MN	TMB	SYR	CA	III ASC	I ASC
Magnolia grandiflora	56.0 ± 8.5	nd	nd	nd	nd	nd
Populus alba	11.5 ± 0.3	18.7 ± 2.6	nd	11.1 ± 1.3	0.5 ± 0.1	0.8 ± 0.1
Citrus aurantium	9.1 ± 5.1	38.3 ± 3.0	nd	10.2 ± 0.7	7.0 ± 0.5	0.0
Betula pendula	1.4 ± 0.1	nd	nd	nd	nd	nd
Annona cherimola	3.2 ± 0.1	16.9 ± 0.3	nd	7.3 ± 0.7	0.2 ± 0.0	0.1 ± 0.0
Malus domestica	2.8 ± 0.1	6.1 ± 1.2	nd	10.5 ± 1.3	nd	nd
Prunus dulcis	2.3 ± 0.1	7.8 ± 0.5	nd	7.4 ± 0.2	nd	nd
Quercus ilex	0.9 ± 0.1	3.0 ± 0.2	nd	2.8 ± 0.1	0.00	0.00
Zinnia elegans	0.4 ± 0.0	1.7 ± 0.0	0.9 ± 0.0	2.4 ± 0.0	0.00	0.00
Cataranthus roseus	1.2 ± 0.0	nd	nd	nd	nd	nd
Medicago sativa	5.1 ± 1.9	nd	nd	nd	nd	nd
Trifolium repens	4.4 ± 0.3	17.4 ± 2.6	2.2 ± 0.2	16.0 ± 2.0	nd	nd
Coleus blumei	0.2 ± 0.0	nd	nd	nd	nd	nd
Capsicum annuum	nd	13.5 ± 0.2	3.2 ± 0.1	6.7 ± 0.0	nd	nd
Secale cereale	3.6 ± 0.2	0.7 ± 0.0	1.8 ± 0.9	4.3 ± 0.9	nd	nd
Avena sativa	14.8 ± 3.2	29.4 ± 3.0	0.8 ± 0.1	27.8 ± 3.3	nd	nd
Zea mays	6.4 ± 0.8	11.4 ± 0.1	nd	4.8 ± 0.0	0.4 ± 0.0	3.1 ± 0.1
Areca lutescens	3.1 ± 1.0	nd	nd	nd	nd	nd
Narcissus tazetta	0.4 ± 0.1	nd	nd	nd	nd	nd
Phalaenopsis amabilis	2.0 ± 0.1	nd	nd	nd	nd	nd
Juncus maritimus	16.4 ± 1.3	nd	nd	nd	nd	nd
Asparagus setaceus	0.1 ± 0.0	nd	nd	nd	nd	nd
Phoenix dactylifera	23.3 ± 1.3	49.6 ± 3.3	nd	19.4 ± 0.6	0.0 ± 0.0	0.2 ± 0.0
Dracaena drago	0.5 ± 0.1	nd	nd	nd	nd	nd
Washingtonia filifera	41.9 ± 3.4	nd	nd	nd	nd	nd

[a] *p*CMB-insensitive (III ASC) and *p*CMB-sensitive (I ASC) peroxidase activities against ascorbic acid are also shown. [b] Peroxidase activity is given in nkat g^{-1} of FW. Values are means ± SE ($n = 3$). nd, not determined.

illex, *Catharanthus roseus*, *Medicago sativa* (alfalfa), *Trifolium repens* (clover), *Coleus blumei*, *Capsicum annuum* (pepper), *Secale cereale* (rye), *Avena sativa* (oats), *Zea mays* (corn), *Phoenix dactylifera* (palm), and *Dracaena drago* were grown until they reached 15 cm in a greenhouse. *Narcissus tazetta* (narcissus), *Phalaenopsis amabilis* (orchid), *Juncus maritimus* (rush), and *Asparagus setaceous* were purchased from a local nursery. Transdifferentiating *Z. elegans* mesophyll cell cultures were established from true leaves from 14-day-old seedlings, which were surface-sterilized in 10% (v/v) commercial NaOCl and rinsed in sterile distilled water. Single cells were isolated and cultured for 3 days in a differentiating medium as described (*16*).

Histochemical Stains for Monitoring Lignins, H_2O_2, and Peroxidase. 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 10−15 min (*21*). Peroxidase/H_2O_2 localization was monitored using the 3,5,3′,5′-tetramethylbenzidine (TMB) endogenous H_2O_2-dependent method (*22*). 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 (*22*), a competitive inhibitor of peroxidase, the oxidation of which 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-filtered for 10 periods of 30 s at 1.0 kPa and 4 °C with 50 mM sodium acetate buffer (pH 5.0) containing 1 M KCl and 50 mM $CaCl_2$. Later, the sections were quickly dried and subsequently centrifuged in a 25 mL syringe barrel placed within a centrifuge tube at 900*g* for 10 min at 4 °C. IWF samples were desalted and concentrated using the Amicon Ultra-15 system (Millipore, Carrigtwohill, Ireland).

Contamination by cytoplasmic constituents, as monitored by the activity of glucose-6-phosphate dehydrogenase (*16*), was always <0.1% relative 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 (*16*). Using the same method, the recovery of IWFs was extremely high because apoplast-specific proteins were absent from symplastic (IWF-extracted tissue) fractions (*16*).

In the case of *Z. elegans* tracheary elements, cells were separated from the culture medium by centrifugation at 100*g* for 1 min at 4 °C, the supernatant constituting the apoplastic protein fraction. This protein fraction was desalted by chromatography on PD-10 Sephadex G-25 (Amersham Bioscience) columns equilibrated in 50 mM sodium acetate buffer, pH 5.0, containing the protease inhibitors, 1.0 mM phenylmethanesulfonyl fluoride (PMSF) and 1.0 mM benzamidine, and concentrated using Ultrafree-0.5 (Millipore).

Determination of Peroxidase Activity in IWFs. Peroxidase activities were determined in IWFs in assay media containing 50 mM sodium acetate buffer (pH 5.0) and 500 μM H_2O_2, using as electron donors 4-methoxy-α-naphthol, TMB, and syringaldazine (*23*). Peroxidase activity in IWFs was also measured with the lignin precursors, coniferyl alcohol, sinapyl alcohol, coniferyl aldehyde, and sinapyl aldehyde, as described (*10*). Finally, peroxidase activities in IWFs from monocots were also measured using *p*-coumaric acid, ferulic acid, and sinapic acid according to the method of Takahama et al. (*24*).

Ascorbate peroxidase (APX) activities were measured in a reaction medium containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, and 0.5 mM ascorbic acid (*22*), by monitoring the decrease in absorbance at 290 nm ($\epsilon_{290} = 2.8\ mM^{-1}\ cm^{-1}$). Class I APX activity was distinguished from class III APX activity by preincubation of IWF samples for 10 min with 50 μM sodium *p*-chloromercuribenzoate (*p*CMB), a specific suicide inhibitor of class I APXs (*22*).

Isoelectric Focusing (IEF) and Visualization of Enzymatic Activities. Disc-IEF and slab-IEF were performed as described by Ros Barceló et al. (*22*) and Pomar et al. (*25*), respectively. IEF under nonequilibrium conditions (NEIEF) was performed as described by López-Serrano et al. (*16*). Protein migration was followed at 4 °C using cytochrome *c* as a migration marker, and peroxidase isoenzymes were stained with 4-methoxy-α-naphthol (*16*).

Sequence Homology Analyses of Peroxidases. Sequence homology analysis was carried out using algorithms of CLUSTALW (http://www.ebi.ac.uk), PROSITE (http://www.expasy.org/prosite), and the integrated software Molecular Evolutionary Genetics Analysis (version 3.1, Masatoshi Nei, http://www.megasoftware.net) (*26*). Protein se-

Table 2. Peroxidase Activity Measured with Coniferyl Alcohol, Coniferyl Aldehyde, Sinapyl Alcohol, and Sinapyl Aldehyde in IWF of Angiosperms

	peroxidase activity[a]			
plant species	coniferyl alcohol	coniferyl aldehyde	sinapyl alcohol	sinapyl aldehyde
Zinnia elegans	2.4 ± 0.0	5.9 ± 0.2	7.8 ± 0.1	6.8 ± 0.2
Trifolium repens	16.0 ± 2.0	5.7 ± 2.0	1.5 ± 0.3	0.8 ± 0.2
Capsicum annuum	6.7 ± 0.0	5.3 ± 0.1	2.5 ± 0.0	2.4 ± 0.0
Secale cereale	4.3 ± 1.0	3.3 ± 0.2	0.1 ± 0.0	0.3 ± 0.0
Avena sativa	27.8 ± 3.3	19.2 ± 2.1	1.9 ± 0.1	0.4 ± 0.1

[a] Peroxidase activity is given in nkat g^{-1} of FW. Values are means ±SE ($n = 3$).

Table 3. Peroxidase Activity Measured with *p*-Coumaric Acid, Ferulic Acid, and Sinapic Acid in IWF of Monocots

	peroxidase activity[a]		
plant species	*p*-coumaric acid	ferulic acid	sinapic acid
Zea mays	3.7 ± 0.1	26.1 ± 1.4	1.3 ± 0.1
Areca lutescens	7.0 ± 0.2	9.6 ± 0.5	0.4 ± 0.0
Dracaena drago	0.5 ± 0.0	4.2 ± 0.6	0.1 ± 0.0
Juncus maritimus	4.9 ± 0.1	10.0 ± 1.1	0.3 ± 0.0
Narcissus tazetta	1.0 ± 0.0	2.3 ± 0.1	0.1 ± 0.0
Phalaenopsis amabilis	0.9 ± 0.2	7.8 ± 0.5	0.5 ± 0.0
Washingtonia filifera	28.3 ± 3.0	53.2 ± 6.8	7.8 ± 0.8
Asparagus setaceus	0.1 ± 0.0	0.5 ± 0.0	0.3 ± 0.0

[a] Peroxidase activity is given in nkat g^{-1} of FW. Values are means ±SE ($n = 3$).

quences were withdrawn from the NCBI (http://www.ncbi.nlm.nih.gov/) and the PeroxiBase (http://peroxidase.isb-sib.ch), a class III plant peroxidase database (*27*). Cluster analyses were performed by using the "Minimum Evolution" method, a distance method whereby evolutionary distances are computed for all pairs of analyzed sequences forming a matrix of pairwise distances, the phylogenetic tree being constructed by analysis of the relationships among these distance values (*28*).

Chemicals. TMB−HCl, 4-methoxy-α-naphthol, syringaldazine, *p*-coumaric acid, ferulic acid, sinapic acid, coniferyl alcohol, sinapyl alcohol, coniferyl aldehyde, and sinapyl aldehyde were purchased from Sigma-Aldrich (Madrid, Spain). The other chemicals were of the highest purity available.

RESULTS AND DISCUSSION

Histochemical Localization of H_2O_2/Peroxidase in the Angiosperm Xylem. To study whether H_2O_2 production and peroxidase activity are colocalized in the same tissues where lignins are synthesized, stem sections of differentially evolved angiosperms were stained by means of the Wiesner reaction, because it is known that angiosperm lignins are rich in 4-*O*-linked coniferyl and sinapyl aldehyde end groups, the targets of the phloroglucinol reaction (*21*). Phloroglucinol staining of differentially evolved angiosperms revealed that lignification in the stem was mainly restricted to the xylem, whereas phloem tissues were only very weakly stained (**Figure 1a**,**d**,**g**,**j**). Stain was independent of the angiosperm lineage, because similar results were found in angiosperms belonging to both Eudicotyledons (**Figure 1a**,**d**,**g**) and Eumagnoliids (**Figure 1j**). Illustrated examples from Eudicotyledons were the woody species, *Prunus dulcis* (**Figure 1a**,**d**), and the herbaceous species, *Medicago sativa* (**Figure 1g**), and an illustrated example from Eumagnoliids was *Zea mays* (**Figure 1j**).

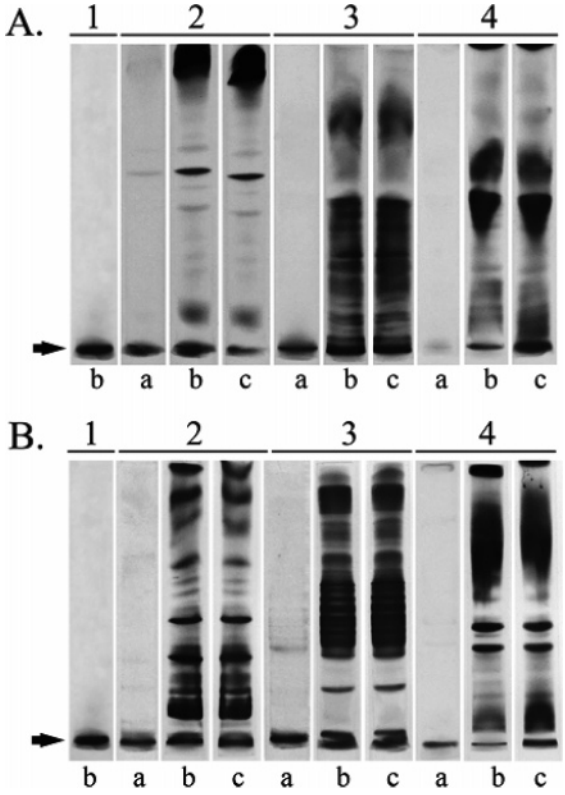

Figure 2. (**A**) Peroxidase isoenzyme patterns obtained by disc-IEF of the apoplastic protein from tracheary elements of *Z. elegans* (1), *Prunus dulcis* (2), *Malus domestica* (3), and *Annona cherimola* (4). (**B**) Peroxidase isoenzyme patterns obtained by disc-IEF of the apoplastic protein from tracheary elements of *Z. elegans* (1), *Zea mays* (2), *Avena sativa* (3), and *Phoenix dactylifera* (4). Peroxidase isoenzymes were stained with 4-methoxy-α-naphthol in the absence of H_2O_2 (lanes a), in the presence of H_2O_2 (lanes b), and in the presence of H_2O_2 and *p*CMB (lanes c). Arrows indicate the position of peroxidases homologous to the *Z. elegans* basic peroxidase.

The independence of the pattern of lignin deposition from the angiosperm lineage was confirmed by the localization of peroxidase/H_2O_2. In this case, stain was observed in woody species both in phloem and in xylem (**Figure 1b** shows the node and **Figure 1e** the internode of *P. dulcis*) after staining with TMB. In the herbaceous species, *M. sativa* (Eudicotyledon, **Figure 1h**) and *Z. mays* (Eumagnoliid, **Figure 1k**), the stain was mainly observed in the xylem. Controls in the presence of ferulic acid (**Figure 1c**,**f**,**i**,**l**) support the peroxidase-dependent nature of the staining reaction shown in **Figure 1b**,**c**,**h**,**k**. From these results, it may be concluded that H_2O_2 production and peroxidase activity are colocalized in the tissue area where lignins are being synthesized.

Syringyl Peroxidases in Angiosperms. It might be expected that if angiosperm lignins are rich in S units (*2*), the presence of a peroxidase capable of oxidizing both coniferyl and sinapyl alcohol would be of great advantage for the expensive process of lignin assembly. To test this hypothesis, peroxidase activity was measured in the IWFs from several plant species with syringaldazine, the prototype substrate of S peroxidases (*23*), and with coniferyl alcohol, for comparative purposes. The results showed that, in all of the species where these peroxidases were assayed, IWF peroxidases were capable of oxidizing not only syringaldazine (**Table 1**) but also sinapyl alcohol and sinapyl aldehyde (**Table 2**). Peroxidases from the IWFs of these species apparently have no steric restrictions during catalysis because they were also able to oxidize both nonphysiological substrates

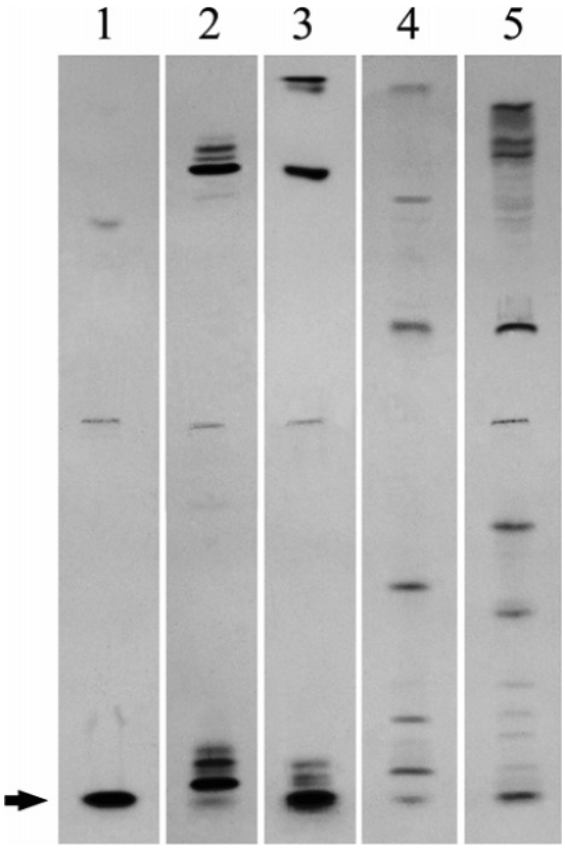

Figure 3. Peroxidase isoenzyme patterns obtained by slab IEF of the apoplastic protein from tracheary elements of *Z. elegans* (1), *Medicago sativa* (2), *Capsicum annuum* (3), *Trifolium repens* (4), and *Secale cereale* (5). Peroxidase isoenzymes were stained with 4-methoxy-α-naphthol and H_2O_2. Arrow indicates the position of peroxidases homologous to the *Z. elegans* basic peroxidase.

such as 4-methoxy-α-naphthol and TMB (**Table 1**) and physiological substrates such as coniferyl alcohol and ascorbic acid (**Table 1**). The oxidation of ascorbic acid by these apoplastic peroxidases deserves special attention because IWFs also contain true (class I) ascorbate peroxidases (**Table 1**), which are the major apoplastic enzymes responsible for H_2O_2 detoxification (*22*).

In the monocotyledon lineage belonging to Eumagnoliids, ester-linked *p*-hydroxycinnamic acids have been described as nucleation points for lignin anchorage and growth (*3*, *29*), underscoring their decisive role during the first stages of cell wall lignification. It should be noted that the IWF peroxidases from all monocotyledons assayed were able to oxidize not only *p*-coumaric and ferulic acids but also sinapic acid (**Table 3**). Taken together, these results showed that the ample presence of S peroxidases in IWFs from angiosperms makes unnecessary the presence of a coniferyl alcohol shuttle to oxidize S units (*24*).

The best-characterized S peroxidase is the *Z. elegans* peroxidase because this has been purified and cloned (*12*), and the *β-O*-4 polymers resulting from the oxidation of sinapyl alcohol catalyzed by this peroxidase have been characterized (*13*). With this in mind, a rapid method was developed for screening peroxidase isoenzymes homologous to the *Z. elegans* peroxidase in the apoplast of basal and evolved angiosperms. Isoenzymes were analyzed by IEF, because the isoelectric point is determined by the balance between the acidic and basic amino acids of the protein and thus reflects similarities at the amino acid level of the isoenzymes.

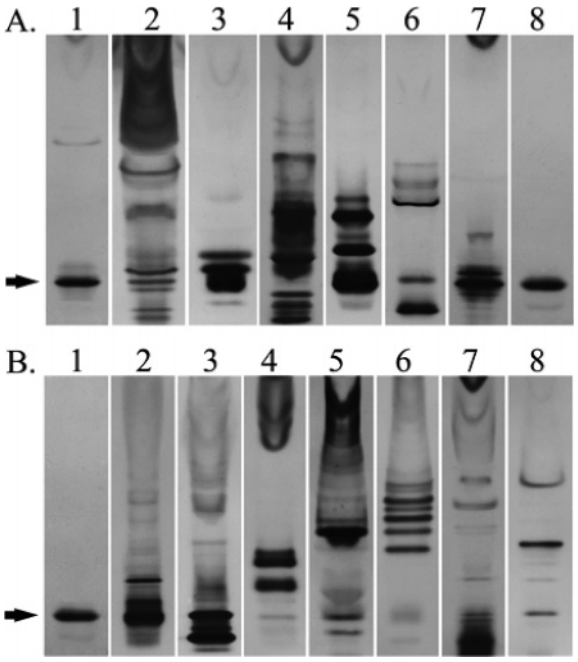

Figure 4. (**A**) Peroxidase isoenzyme patterns obtained by nonequilibrium isoelectric focusing (NEIEF) of the apoplastic protein of tracheary elements of *Z. elegans* (1), *Magnolia grandiflora* (2), *Betula pendula* (3), *Populus alba* (4), *Citrus aurantium* (5), *Catharanthus roseus* (6), *Medicago sativa* (7), and stems from *Z. elegans* (8). (**B**) Peroxidase isoenzyme patterns obtained by NEIEF of the apoplastic protein of tracheary elements of *Z. elegans* (1), *Areca lutescens* (2), *Juncus maritimus* (3), *Dracaena drago* (4), *Narcissus tazetta* (5), *Phalaenopsis amabilis* (6), *Washingtonia filifera* (7), and *Asparagus setaceus* (8). Peroxidase isoenzymes were stained with 4-methoxy-α-naphthol and H_2O_2. Arrows indicate the position of peroxidases homologous to the *Z. elegans* basic peroxidase.

Table 4. Accession Number (AN), Number of Amino Acids (NAA), Theoretical M_r (TM), and Theoretical Isoelectric Point (TIP) of the Peroxidase Sequences Studied

taxon	species	AN	NAA	TM	TIP
ANITA	*Amborella trichopoda*	CV002678	303	33309	8.14
ANITA	*Nuphar advena*	354084 PGN[a]	243		
Eumagnoliids	*Secale cereale*	BE587237[a]	152		
Eumagnoliids	*Zea mays*	AX546792	303	32795	9.05
Eumagnoliids	*Avena sativa*	AF078872	291	30161	8.44
Eumagnoliids	*Asparagus officinalis*	AB042103	303	33412	8.85
Eudicotyledons	*Populus alba*	BAE16616	301	32228	8.46
Eudicotyledons	*Malus domestica*	CN995809	302	34717	8.69
Eudicotyledons	*Prunus dulcis*	BU575219[a]	154		
Eudicotyledons	*Arabidopsis thaliana*	X99952	305	31936	4.82
Eudicotyledons	*Armoracia rusticana*	P80679	305	31899	4.72
Eudicotyledons	*Medicago sativa*	CO511956	305	34636	8.88
Eudicotyledons	*Trifolium repens*	AJ011939	303	32964	9.07
Eudicotyledons	*Capsicum annuum*	AAL35364	300	32318	7.70
Eudicotyledons	*Catharanthus roseus*	AY769111[a]	131		
Eudicotyledons	*Lycopersicun esculentum*	L13653	304	32942	8.91
Eudicotyledons	*Zinnia elegans*	AJ880395	291	30863	8.47

[a] The sequences deposited in the data banks are incomplete and lack the C-ends.

Peroxidase isoenzyme patterns in these species obtained by disc-IEF (**Figure 2**), slab-IEF (**Figure 3**) and NEIEF (**Figure 4**) showed the common presence not only of analogues (isoenzymes of similar mobility in the IEF gels) but also of homologues (isoenzymes of identical mobility in IEF gels) of the basic peroxidase isoenzyme isolated from differentiating *Z. elegans* tracheary elements. Thus, peroxidases homologous to the *Z. elegans* basic isoenzyme were found by disc-IEF in the

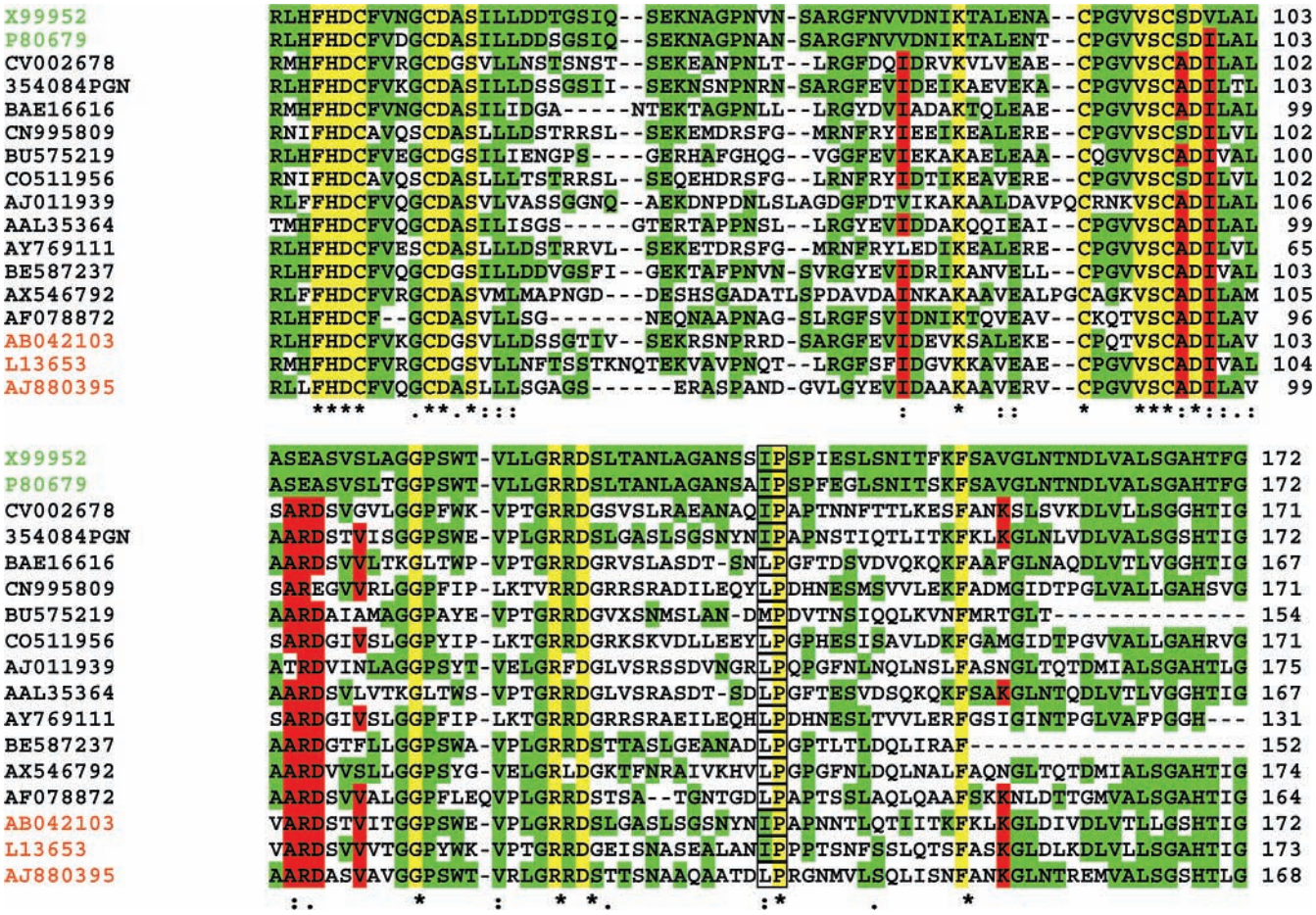

Figure 5. Amino acid sequence alignment of eudicot G and S peroxidases with angiosperm peroxidases. Structural motif characteristics of S peroxidases were determined by alignment of the *Z. elegans* basic isoenzyme (AJ880395) with the tomato (*Lycopersicon esculentum*) basic S peroxidase (L13653) and with the asparagus (*Asparagus officinalis*) basic S peroxidase (AB042103), a set of peroxidases having a well-known capacity for oxidizing S moieties. Structural determinants of S peroxidases (shaded in red) were deduced by comparing amino acid sequence motifs common to these three eudicot peroxidases with the amino acid sequence motifs of two typical G peroxidases, the *Arabidopsis thaliana* A2 peroxidase (X99952) and the *Armoracia rusticana* A2 peroxidase (P80679). Sequence homology analyses were restricted to the region between the distal (H41 in ATP A2) and the proximal (H169 in ATP A2) histidines that determine the conformation of the catalytic center of the enzymes. This region also comprises the amino acids I138, P139, and S140 (numbered according to ATP A2, black arrowheads), which determine the conformation and hydrophobicity of the substrate-binding site (*9*). Alignments of this zone for the peroxidases selected revealed highly conserved amino acid residues common to all peroxidases (shaded in yellow) and partially conserved amino acid residues of ATP A2 and HRP A2 present also in some of the resting peroxidases (shaded in green). Database accession numbers are as in **Table 4**. Consensus symbols: "∗" means that the amino acid residues in the column are identical in all sequences of the alignment; ":" means that conserved substitutions have been observed; "." means that semiconserved substitutions are observed.

Eudicotyledons, *Prunus dulcis* (**Figure 2A**, lane 2b), *Malus domestica* (**Figure 2A**, lane 3b), and *Anona cherimola* (**Figure 2A**, lane 4b), and in the Eumagnoliids, *Zea mays* (**Figure 2B**, lane 2b), *Avena sativa* (**Figure 2B**, lane 3b), and *Phoenix dactylifera* (**Figure 2B**, lane 4b). Slab-IEF (**Figure 3**), in which isoelectrofocusing of proteins involves migration on the gel surface, gave similar results because peroxidases homologous to the *Z. elegans* basic isoenzyme were found in all of the species assayed, among them the Eudicotyledons, *Medicago sativa* (lane 2), *Capsicum annuum* (lane 3), and *Trifolium repens* (lane 4), and the Eumagnoliid, *Secale cereale* (lane 5). Finally, NEIEF (**Figure 4A**,**B**), in which identical mobility is determined not only from identical p*I* but also from an identical mass, showed the presence of peroxidases homologous to the *Z. elegans* basic isoenzyme in all of the Eudicotyledons (**Figure 4A**) and in all the Eumagnoliids (**Figure 4B**) studied. Homologous isoenzymes were even found in the IWFs of the basal angiosperm, *Magnolia grandiflora* (**Figure 4A**, lane 2).

The results described above demonstrate the wide presence of peroxidases homologous to the *Z. elegans* basic isoenzyme in angiosperms on the basis of molecular properties, such as the p*I* and the molecular mass. However, this homology was even closer because similarities were also seen in some catalytic properties. In fact, a key property of the *Z. elegans* basic peroxidase isoenzyme is its ability to show a certain oxidase activity in the absence of H_2O_2 (*30*), which is accompanied by its insensibility to thiol reagents, such as *p*CMB; this last property is typical of all class III peroxidases (*22*). Homology was also observed at this level, because peroxidases homologous to the *Z. elegans* basic isoenzyme showed both oxidase activity in the absence of H_2O_2 (**Figure 2**, lanes a) and a total insensitivity to the thiol reagent, *p*CMB (**Figure 2**, lanes c). These results led us to search for the protein sequence database of the NCBI and the PeroxiBase to verify the presence in angiosperms of peroxidases homologous at the amino acid sequence level to the *Z. elegans* basic isoenzyme.

Structural Motifs of the Primary Structure of the *Z. elegans* S Peroxidase Are Strongly Conserved during Angiosperm Evolution. Structural motifs of angiosperm S peroxidases were determined by alignment of the *Z. elegans* basic

isoenzyme (AJ880395) with a tomato (*Lycopersicon esculentum*) S peroxidase of basic nature (L13653) (*5*) and with a basic peroxidase from asparagus (*Asparagus officinalis*) (AB042103) (*11*) (**Table 4**), the capacity of which for oxidizing S moieties is well-known (*5*, *11*−*13*). Structural determinants of S peroxidases were deduced by comparing amino acid sequence motifs common to these three eudicot peroxidases with the amino acid sequence motifs of two typical G peroxidases, the *Arabidopsis thaliana* (ATP) A2 peroxidase (X99952) (*9*) and the *Armoracia rusticana* (horseradish) A2 peroxidase (HRP A2, P80679) (*7*) (**Table 4**). All of these peroxidases show an N-terminal propeptide but lack a C-terminal extension, supporting the view that they are cell wall located enzymes, with a putative role in lignification. Sequence homology analyses were restricted to the region between the distal (H41 in ATP A2) and the proximal (H169 in ATP A2) histidines that either surround or determine the conformation (relaxation capacity) of the catalytic center of the enzymes. This region also comprises the amino acids P69, I138, P139, S140, and R175 (numbered according to ATP A2), which determine the conformation and hydrophobicity of the substrate-binding site (*9*). The region considered is extremely distinctive of class III plant peroxidases because it is codified by exon 2 in most peroxidase genes (*12*) and delimited by type 2 and type 3 introns (two introns actively involved in recombinations between plant peroxidase genes) (*12*). Finally, this region has been shown to be one of the most highly conserved in class III plant peroxidases during land plant evolution (*31*).

As may be expected, alignments of this zone (**Figure 5**) for the peroxidases selected revealed highly conserved amino acid residues common to all peroxidases (shaded in yellow) and partially conserved amino acid residues of ATP A2 and HRP A2 in some of the resting peroxidases (shaded in green). Structural motifs differentially present in all eudicot S peroxidases and absent from the G peroxidases, ATP A2 and HRP A2, are shaded in red (**Figure 5**). These motifs are I76 (in *Z. elegans* peroxidase) (V80 in ATP A2), A94 (in *Z. elegans* peroxidase) (S98 in ATP A2), 101-ARD (in *Z. elegans* peroxidase) (105-SEA in ATP A2), and K151 (in *Z. elegans* peroxidase) (V155 in ATP A2). Some of these amino acids, such as the A94 (in *Z. elegans* peroxidase) (S98 in ATP A2), form part of the structural motif VSC**A**D in S peroxidases, which contrasts with that found in G peroxidases, VSC**S**D (**Figure 5**).

Structural motifs common to these three eudicot S peroxidases were found in peroxidases from woody eudicotyledons, such as *Populus alba* (BAE16616), *Malus domestica* (CN995809), and *Prunus dulcis* (BU575219); in peroxidases from herbaceous eudicotyledons, such as *Medicago sativa* (CO511956), *Trifolium repens* (AJ011939), *Capsicum annuum* (AAL35364), and *Catharanthus roseus* (AY769111); and in peroxidases from Eumagnoliids, such as *Secale cereale* (BE587237), *Zea mays* (AX546792), and *Avena sativa* (AF078872). Structural motifs common to these three eudicot S peroxidases were even found in peroxidases from basal angiosperms belonging to the ancient ANITA lineage, such as *Amborella trichopoda* (CV002678) and *Nuphar advena* (354084PGN). In the case of *Nuphar*, *Prunus*, *Secale*, and *Catharanthus* peroxidases, the cell wall localization could not be confirmed because the sequences deposited in the databanks are incomplete and lack the C-ends (see **Table 4**). The reliability of the molecular approach followed in this study is confirmed by the fact that the *P. alba* peroxidase (BAE16616), which shows all of the structural motifs common to S peroxidases (**Figure 5**), shows a differential capacity for oxidizing sinapyl alcohol (*32*), the prototype substrate for S peroxidases.

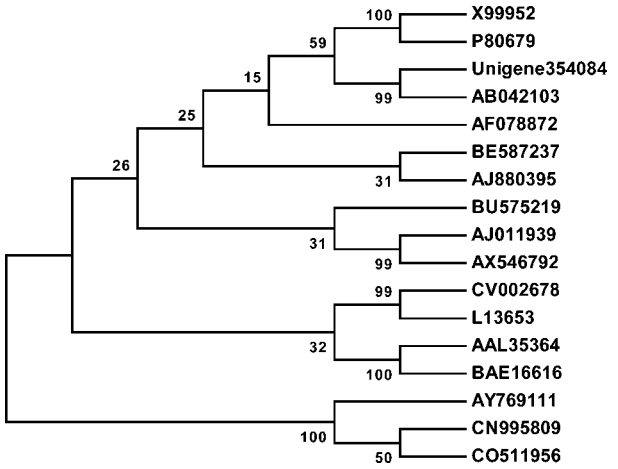

Figure 6. Phylogenetic tree of eudicot S peroxidases using the minimum evolution method. Database accession numbers are as in **Table 4**. In this cluster analysis, both the *Arabidopsis thaliana* (X99952) and the *Armoracia rusticana* (P80679) G peroxidases appeared as a more evolved branch within angiosperm peroxidase evolution.

Phylogenetic Tree of Angiosperm Syringyl Peroxidases. Cluster analysis by the Minimum Evolution method (**Figure 6**) suggests that all of these S peroxidases constitute an ancestral branch, the origins of which go back to ancestral (nonvascular) plant lineages within the monophyletic origin of land plants (*20*). In this cluster analysis, both the *Arabidopsis thaliana* (X99952) and the *Armoracia rusticana* (P80679) G peroxidases (Cruciferae/Brassicaceae) (both located in the top) appeared as a more evolved branch within class III plant peroxidase evolution. The results described above are not surprising because cell wall located class III plant peroxidases are found in most vascular plant lineages (*31*), molecular clocks suggesting that angiosperms are monophyletic (*20*). In this scenario, it is not surprising that S peroxidases, the main enzymes responsible for lignin building construction, appeared early in the evolution of land plants and that these enzymes, as occurs for all highly expressed proteins which evolved slowly, have been largely conserved during angiosperm evolution.

Conclusion. After screening for the presence of S peroxidases in IWFs from lignifying tissues of 25 woody, herbaceous, and shrub species, belonging to both monocots and dicotyledons, it was found that all of these species show not only S peroxidase activities but also basic peroxidase isoenzymes analogous, with regard to the isoelectric point, to the *Zinnia elegans* basic peroxidase isoenzyme, the only S peroxidase that has been fully characterized. A search in the protein database for homologies between angiosperm peroxidases and true eudicot S peroxidases showed that certain structural motifs of S peroxidases are conserved within the first 15 million years of angiosperm history, because they are found in peroxidases from the two major lineages of flowering plants—eumagnoliids and eudicotyledons—with the remarkable presence of these peroxidases in *Amborella* and Nymphaeales, which represent the first stages of angiosperm evolution. Finally, our phylogenetic studies also suggest that guaiacyl peroxidases constitute the most "evolved state" of the plant peroxidase family evolution.

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Received for review November 3, 2006. Revised manuscript received March 8, 2007. Accepted March 16, 2007. This work was supported by grants from the Fundación Séneca (Project 00545/PI/04), MCYT (BOS2002-03550), and MEC (BFU2006-11577/BFI)-FEDER. L.V.G.R. holds a fellowship (FPI) from the MCYT.

JF063164T