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Characterization of the last step of lignin biosynthesis in *Zinnia elegans* suspension cell cultures

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Abstract **The last step of lignin biosynthesis in *Zinnia elegans* suspension cell cultures (SCCs) catalyzed by peroxidase (ZePrx) has been characterized. The k_3 values shown by ZePrx for the three monolignols revealed that sinapyl alcohol was the best substrate, and were proportional to their oxido/reduction potentials, signifying that these reactions are driven exclusively by redox thermodynamic forces. Feeding experiments demonstrate that cell wall lignification in SCCs is controlled by the rate of supply of H_2O_2. The results also showed that sites for monolignol β-*O*-4 cross-coupling in cell walls may be saturated, suggesting that the growth of the lineal lignin macromolecule is not infinite.**

Keywords: Lignin; Monolignols; Peroxidase; Suspension cell cultures; *Zinnia*

1. Introduction

Lignins are plant cell wall-located phenolic heteropolymers which result from the oxidative coupling of three monolignols, *p*-coumaryl, coniferyl and sinapyl alcohols, in a reaction mediated by class III plant peroxidases [1]. The cross-coupling reaction produces an optically inactive hydrophobic heteropolymer composed of H (*p*-hydroxyphenyl), G (guaiacyl) and S (syringyl) units, respectively [2]. The spatial and temporal control of lignin biosynthesis is extremely important since lignification is a metabolically costly process that requires large quantities of carbon skeletons and reducing equivalents [3]. Plants do not possess a mechanism to degrade lignins [4], so any carbon invested in lignin biosynthesis is not recoverable. Consequently, lignified cells represent a significant carbon sink and, as such, plants must carefully balance the synthesis of lignin polymers against the availability of resources.

The metabolic flux (carbon allocation) in the phenylpropanoid pathway is controlled at multiple enzymatic levels [5]. Studies using lignifying cell cultures of *Pinus taeda*, a gymnosperm, have established [6] that both the carbon allocation to the pathway and its differential distribution into the two monolignols, *p*-coumaryl and coniferyl alcohol, is controlled by the rate of phenylalanine supply and the differential modulation of cinnamate-4-hydroxylase (C4H) and *p*-coumarate-3-hydroxylase (C3H), respectively. A similar role, as rate limiting step, has recently been proposed for the polymerization of monolignols catalyzed by class III plant peroxidases [7].

Zinnia elegans is a flowering plant commonly used as a model for studying xylem differentiation [8]. This is in part due to the simplicity and duality of the lignification pattern shown by stems and hypocotyls but also to the nature of the peroxidase isoenzyme complement, which is almost completely restricted to the presence of a basic peroxidase isoenzyme [9]. The lignification pattern of *Z. elegans* seedlings is unique in that, at a certain developmental stage, they offer simultaneously two models of lignification that closely resemble those occurring in gymnosperms and angiosperms. Thus, in 25/30-d old plants, hypocotyl lignins are mainly composed of G/S units in a 42/58 ratio, while stem lignins contain significant amounts of H units in a H/G/S ratio of 22/56/22 [10]. That is, S units predominate in the hypocotyl, while G units predominate in the stem. In this regards, *Z. elegans* hypocotyl lignins are typical of angiosperms, while the lignins of the young stem partially resemble those which occur in gymnosperms, since (H+G) alone constitute 78% of the lignin building blocks.

The *Z. elegans* peroxidase (ZePrx) has been cloned and its primary structure determined [11]. Four full-length cDNAs encoding the ZePrx have been isolated (AJ880392-5). They contain an identical 966-bp ORF encoding 321 amino acids but differ in the 5′-untranslated region (5′-UTR). Although little is known about the precise function of the 5′-UTRs, it is accepted [12] that these regions contain sequences that can form secondary structures and interact with proteins which regulate mRNA transport, stability and translation. The immature polypeptide of the ZePrx contains a signal peptide (N-terminal pro-peptide) of 30 amino acids, which directs the polypeptide chain to the ER membrane. Since the polypeptide has no C-terminal propeptide extension to target the proteins for vacuolar transport and does not contain any of the ER motifs for retrograde transport (the KDEL, HDEL and DEL tails), it can be affirmed that the protein follows the default pathway, which ends in the plant cell wall [11]. This cell wall localization of the protein is corroborated by the recovery of the enzyme in the spent medium fraction of *Z. elegans* suspension cell cultures (SCCs) [11].

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Abbreviations: DHPs, dehydropolymers; SCCs, suspension cell cultures; ZePrx, *Zinnia elegans* peroxidase

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The four genes codifying the ZePrx consist of three exons and two introns [11], one of type 2 and the other of type 3, according to Tognolli et al. [12] nomenclature. Both the nature and position of these introns suggest that the *ZePrxs* belong to the peroxidase gene subgroup of class *a*, already described in *Oryza* and *Arabidopsis* [12]. The homologies found between introns [11] put forward that duplications of the *ZePrxs* occurred by recombination within introns, as has been described for two horseradish basic peroxidase genes coded in tandem [13]. Thus, the ZePrx is encoded by a complex multigene family in the genome of *Z. elegans*, as might be expected from its crucial role in lignin biosynthesis.

In this report, the major basic peroxidase (ZePrx) from lignifying *Z. elegans* SCCs has been purified and the enzymatic step in SCCs catalyzed by this peroxidase has been characterized. The results show that ZePrx constitutes a particular and novel subgroup within class III plant peroxidases, for which the name of *syringyl peroxidase* is proposed.

2. Materials and methods

2.1. Cell cultures

SCCs of *Z. elegans* (cv. Envy, Chiltern Seeds, Cumbria, England) were established from friable calluses in 250 mL flasks as described [11]. Cultures were grown on a rotator shaker (100 rpm) at 25 °C and in darkness in 90 mL of culture medium, and sub-cultured every 8 d by diluting 1:1 (v/v) in fresh medium, until they reached 68.70 (±10.99)% packed cell volume, which corresponds to a cell density of 1.37 (±0.45) × 10^8 cells mL^{-1}. Feeding experiments were performed by adding H_2O_2 and/or sinapyl alcohol to exponential-growing SCCs (4-d old) at a rate of 0.1 μM s^{-1} for 10 h.

2.2. Purification of ZePrxs

To purify ZePrxs, the spent medium fraction from SCCs was salted out with $(NH_4)_2SO_4$ up to 95% saturation, and the total protein was recovered by centrifugation. The ZePrxs were purified in a four-step protocol, which included chromatography on Phenyl-Sepharose™ 6 Fast Flow (Amersham Biosciences), Superdex™ 75 (Amersham Biosciences), SP Sepharose Fast Flow (Amersham Biosciences), and affinity chromatography on Concanavalin A-Sepharose® 4B (Sigma, Madrid, Spain) [11]. This last chromatography yielded two MALDI-TOF-homogeneous peroxidase fractions (ZePrx33.44 and ZePrx34.70, named according to their respective M_r values determined by MALDI-TOF) [11]. M_r differences between ZePrxs were due to the glycan moieties, as deduced from the total similarity of the N-terminal sequences (LSTTFYDTT) and by the 99.9% similarity of the tryptic fragment fingerprints obtained by RP-nano LC [11].

2.3. Determination of kinetic properties

The oxidation of the lignin precursors, *p*-coumaryl alcohol ($\Delta\varepsilon_{259}$ = 14 756 M^{-1} cm^{-1}), *p*-coumaryl aldehyde ($\Delta\varepsilon_{324}$ = 20 732 M^{-1} cm^{-1}), coniferyl alcohol ($\Delta\varepsilon_{262}$ = 9750 M^{-1} cm^{-1}), coniferyl aldehyde ($\Delta\varepsilon_{341}$ = 13 400 M^{-1} cm^{-1}), sinapyl alcohol ($\Delta\varepsilon_{271}$ = 4140 M^{-1} cm^{-1}), and sinapyl aldehyde ($\Delta\varepsilon_{340}$ = 11 900 M^{-1} cm^{-1}), by ZePrxs in the presence of H_2O_2 was assayed spectrophotometrically at 25 °C in a reaction medium containing 50 mM Tris-acetate buffer, pH 5.0. Phenolic concentrations ranged from 6.25 to 100 μM, while H_2O_2 concentrations ranged from 2.5 to 50 μM. The k_1 and k_3 values for the six lignin precursors were determined as described [14].

2.4. End-wise polymerization of sinapyl alcohol by ZePrxs and HPLC identification of the polymerization products

The end-wise polymerization of sinapyl alcohol by either ZePrx33.44 or ZePrx34.70 was performed in a 50 mM Na-phosphate buffer, pH 5.0, which contained 5 mg sinapyl alcohol (dissolved in 50 μL acetone and added at a rate of 5 μL h^{-1} to a 5 mL reaction medium), and equimolar amounts of H_2O_2. HPLC analyses of dehydrogenation polymers (DHPs) of sinapyl alcohol were performed using a Waters system (Millipore Corp., Waters Chromatography, Milford, MA) comprising a Model 600 controller, Model 600 pump, Rheodyne 7725i manual injector and Waters 996 Photodiode Array detector. The data were processed with the Waters Millenium® 2010 LC version 2.10 software. Gel permeation (GP) HPLC of the DHPs was carried out at room temperature on a 30 cm × 7.8 mm I.D. TSK-Gel G2500HR column (TosoHaas, TOSOH Corp., Montgomeryville, PA) using DMF as eluent at a flow rate of 1 mL min^{-1} [15]. Due to the different λ_{max} of the different DHPs, chromatographic profiles were recorded at the maximum wavelength (λ_{max} chromatogram). M_r calibration in the GP column was performed using poly(styrene) standards (Aldrich, Madrid, Spain) and hydrolytic lignin (Aldrich).

2.5. Isolation of cell walls and thioacidolysis analyses

The thioacidolysis of lyophilized cells and DHPs of sinapyl alcohol was performed as described [16]. Gas chromatography–mass spectrometry (GC–MS) analyses were performed using a Hewlett Packard 5890 Series II gas chromatograph, an HP 5972 mass spectrometer and an HP5 (30 m × 0.25 mm I.D.) column [16]. Mass spectra were recorded at 70 eV.

2.6. Chemicals

Coniferyl alcohol, coniferyl aldehyde, sinapyl alcohol and sinapyl aldehyde were purchased from Sigma. The rest of the chemicals were obtained from various suppliers and were of the highest purity available. *p*-Coumaryl alcohol and *p*-coumaryl aldehyde were generously provided by Hoon Kim and John Ralph (US Dairy Forage Research Center, Madison, WI).

3. Results and discussion

3.1. Lignins in SCCs of Z. elegans

The predominant inter-unit linkages in lignins from plant cell walls are the aryl-glycerol-β-aryl ether (β-*O*-4) bonds, the frequencies of which reportedly vary from 50% in gymnosperms to 80% in angiosperms [2], and are the targets of thiocidolysis [17]. In angiosperms, such as *Z. elegans* [10], the β-*O*-4 linear core is mainly composed of intercalated G,S units. Thioacidolysis coupled to GC–MS analyses of *Z. elegans* SCCs reveals the presence of the thioethylated monomers (*erythro* and *threo* isomers) arising from the aryl-glycerol-β-aryl ether structures derived from coniferyl and sinapyl alcohols (Fig. 1). Quantitative analysis revealed that the lignification pattern of *Z. elegans* SCCs closely resembles that occurring in hypocotyls, the plant organ from which SCCs are derived. Thus, SCCs lignins are composed of G/S units in a 38/62 ratio, which matches the 42/58 (G/S) ratio found for hypocotyl lignins [10]. From these results, it becomes apparent that the metabolic flux of monolignols and their further incorporation into lignins is similar in *Z. elegans* SCCs as in hypocotyls. These results lend weight to the use of SCCs as a model system for characterizing the enzyme responsible for the oxidative coupling of monolignols into lignins.

3.2. Biochemical characterization of ZePrx

To characterize the enzyme responsible for the oxidative coupling of monolignols into lignins in *Z. elegans*, the ZePrx was purified to homogeneity from the spent medium fraction of SCCs [11]. The purification protocol yielded two MALDI-TOF-homogeneous peroxidase fractions (ZePrx33.44 and ZePrx34.70, named according to their respective M_r values determined by MALDI-TOF) [11]: one (ZePrx34.70), fully glycosylated, and weakly retained by the column, and the other (ZePrx33.44), partially glycosylated, and strongly retained by the column [11]. The two purified proteins showed absorption

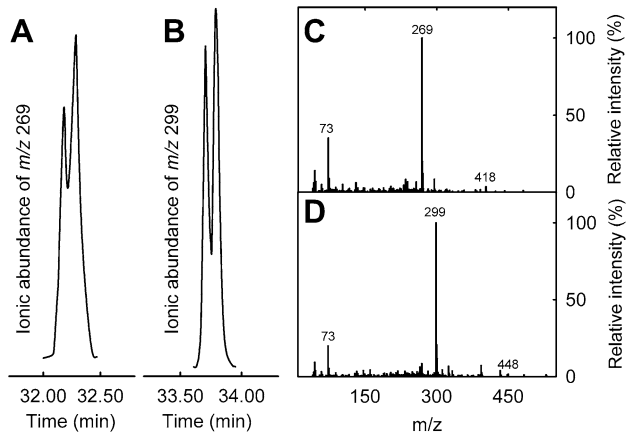

Fig. 1. GC profiles (A and B) and mass spectra (C and D) of the thioethylated monomers arising from aryl-glycerol-β-aryl ether structures derived from coniferyl (A and C) and sinapyl (B and D) alcohol in *Z. elegans* SCC cell walls.

maxima in the visible region at 403 (Soret band), and at 500 and 640 nm, which are typical and unequivocal for hemo-containing high-spin ferric secretory (class III) peroxidases [18].

The oxidation of cinnamyl alcohols and aldehydes by the ZePrxs yields hyperbolic curves, which fits well with the irreversible ping-pong mechanism shown by peroxidases involved in lignin biosynthesis [19]. This mechanism is characterized by a three-step cycle involving a two-electron enzyme oxidation: $FeIII + H_2O_2 \rightarrow CoI + H_2O$ (k_1); followed by two one-electron reductions: (i) $CoI + RH \rightarrow CoII + R^{\cdot}$ (k_2) and (ii) $CoII + RH \rightarrow FeIII + R^{\cdot}$ (k_3), where FeIII, CoI, CoII and RH are the native enzyme, compound I, compound II, and the monolignol, respectively.

The oxidation of the three monolignols, *p*-coumaryl, coniferyl and sinapyl alcohols, and their corresponding aldehydes, by the two ZePrxs was studied. The k_1 (CoI formation constant – which monitors the reactivity of the enzyme with H_2O_2) and k_3 (CoII reduction constant – which monitors the ability of the oxidized form of the enzyme, CoII, to oxidize phenolics) values for the six phenolics are reported in Table 1. From Table 1, it may be concluded that sinapyl alcohol was, by far, the best substrate for both enzymes. To judge from the magnitude of the k_3 values, the reactivity of ZePrxs towards sinapyl alcohol was at least twice higher than that shown by prototypical peroxidases, such as the *Arabidopsis* ATP2 and horseradish A2 peroxidases, for coniferyl alcohol [20], while the reactivity of ZePrxs towards coniferyl alcohol was 1/5–1/10 lower than that shown by prototypical peroxidases for coniferyl alcohol [20]. Prototypical peroxidases, such as the *Arabidopsis* ATP2 and horseradish A2 peroxidases, only show trace activity against sinapyl alcohol [20].

Although no clear tendency was observed for the effect of glycosylation on the k_1 in the case of *p*-coumaryl aldehyde, the full glycosylation which occurred in ZePrx34.70 increased the k_1 of the enzyme when *p*-coumaryl alcohol and coniferyl alcohol were used as substrates, while the k_1 was reduced when the substrates were coniferyl aldehyde, sinapyl alcohol and sinapyl aldehyde. Likewise, the full glycosylation which occurs in ZePrx34.70 reduces the reactivity of the oxidized form of the enzyme, CoII (k_3), with coniferyl alcohol, but increases the reactivity with sinapyl alcohol. No clear tendency was found for the effect of glycosylation on the k_3 values in the case of *p*-coumaryl alcohol, *p*-coumaryl aldehyde, coniferyl aldehyde or sinapyl aldehyde (Table 1). These results agree with the observation that glycans modulate the catalytic activity of peroxidases through a mechanism in which glycans might assist in guiding, and Phe141 in catching, electron donors at the substrate binding site of the enzyme [20].

Most class III plant peroxidases previously described as being involved in lignin biosynthesis, such as the *Arabidopsis* ATP2 and the horseradish A2 peroxidases [20,21], with some exceptions [22], are usually capable of oxidizing coniferyl alcohol, but lack the ability to oxidize sinapyl alcohol, despite the fact that sinapyl alcohol is more prone to oxidation than either coniferyl alcohol or *p*-coumaryl alcohol [23]. These results suggest that, although peroxidase-catalyzed reactions are driven by redox thermodynamic forces [24], substrate accommodation in (exclusion from) the catalytic centre of the enzyme may determine the real role played by each peroxidase isoenzyme in lignin biosynthesis [21]. In fact, the oxidation of sinapyl alcohol by prototypical peroxidases is sterically hindered due to unfavourable 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 [21].

Unlike that which occurs with the prototypical peroxidases, *Arabidopsis* ATP2 and the horseradish A2 peroxidases [20], the k_3 values shown by the two ZePrxs for the three monolignols were linearly proportional to the oxido/reduction potentials of the phenolate species (Fig. 2). The results shown in Fig. 2 led us to conclude that ZePrxs, unlike *Arabidopsis* ATP2 and horseradish A2 peroxidases [20,21], have no steric hindrance in the hemo crevice for actively discriminating between the three monolignols, and suggest that the ZePrx-catalyzed reactions are driven exclusively by redox thermodynamic forces, where substrate hindrance only plays a minor role during catalysis. ZePrxs thus constitute a particular and novel subgroup within class III plant peroxidases, for which the name

Table 1
Compound I formation constant (k_1) and Compound II reduction constant (k_3) of ZePrxs for phenolics utilized in lignin biosynthesis

Substrate	k_1 ($\mu M^{-1}\ s^{-1}$)		k_3 ($\mu M^{-1}\ s^{-1}$)	
	ZePrx34.70	ZePrx33.44	ZePrx34.70	ZePrx33.44
p-Coumaryl alcohol	2.9 ± 0.2^{a}	1.5 ± 0.1^{b}	0.07 ± 0.01	0.07 ± 0.0
p-Coumaryl aldehyde	1.7 ± 0.3	1.8 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
Coniferyl alcohol	8.1 ± 0.6^{a}	6.6 ± 0.4^{b}	0.6 ± 0.1^{a}	0.9 ± 0.1^{b}
Coniferyl aldehyde	4.7 ± 0.1^{a}	6.5 ± 0.1^{b}	5.5 ± 0.1	5.5 ± 0.3
Sinapyl alcohol	14.9 ± 0.1^{a}	18.0 ± 0.1^{b}	13.0 ± 0.9^{a}	7.9 ± 0.2^{b}
Sinapyl aldehyde	4.9 ± 0.1^{a}	9.3 ± 0.2^{b}	5.7 ± 0.3	5.4 ± 0.1

Values are means ± S.E. Values followed by different super indexes are statistically different at $P = 0.05$.

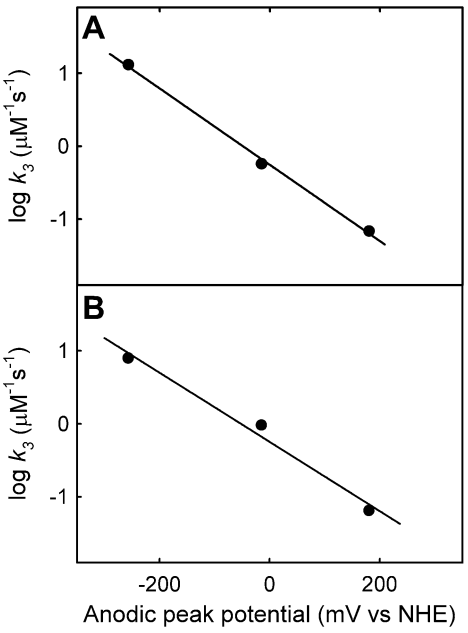

Fig. 2. Plot of the logk_3 (Compound II reduction constant) vs the oxido/reduction potentials for *p*-coumaryl, coniferyl and sinapyl alcohols, during their oxidations by ZePrx34.70 (A) and ZePrx33.44 (B). Values for the anodic peak potential in mV vs NHE were taken from [23].

of *syringyl peroxidases* is proposed. This observation is supported by the fact that ZePrxs show only 45.70% homology at amino acid level with typical class III (guaiacyl) peroxidases, such as the *Arabidopsis* ATP2 peroxidase.

3.3. ZePrx-mediated formation of lignin-like sinapyl alcohol dehydropolymers

Since sinapyl alcohol is the best substrate for both ZePrxs, the oligomeric nature of the oxidation products of sinapyl alcohol oxidation by both ZePrxs was studied. End-wise polymerization showed that both ZePrxs are able to oxidize sinapyl alcohol to yield highly polymerized lignins, which were resolved by GP-HPLC (Table 2) in a polymer of $M_r \geqslant 18200$ (polymerization degree, $n \geqslant 87$), and in oligomers with mean M_r values of 4050 ($n \approx 19$), 1270 ($n \approx 6$) and 670 ($n \approx 3$). No differences were found in the relative abundance of the polymers and oligomers of sinapyl alcohol during its oxidation by both ZePrxs. Sinapyl alcohol DHPs of $M_r \geqslant 18200$ formed during the ZePrxs-catalyzed oxidations show M_r values greater than those of Kraft lignins ($M_r = 9900$) [25], and were of the same order as those of milled wood and enzyme lignins (MWEL) ($16000 < M_r < 36000$) [26], which are the solubilized lignin samples that most closely resemble lignins *in vivo*.

The most characteristic lignin absorption band in the UV region is the B-band which absorbs at 268–287 nm [27], and which is due to aromatic structures with saturated side chains such as that formed by repetitive aryl-glycerol-β-aryl ether (β-*O*-4) bonds. Others bonds present in minor amounts in lignins give rise to conjugated systems, such as those represented by stilbenes, isoeugenol, α-carbonyl and biphenyl compounds, which cause bathochromic shifts of the basic guaiacyl B bands by as much as 10–30 nm [27]. In this respect, it is necessary to mention that the sinapyl alcohol DHPs ($M_r \geqslant 18200$, $n \geqslant 87$, Fig. 3A) and oligomers ($M_r = 4050$, $n \approx 19$, Fig. 3B) formed during the ZePrx-catalyzed oxidations show B-absorb-

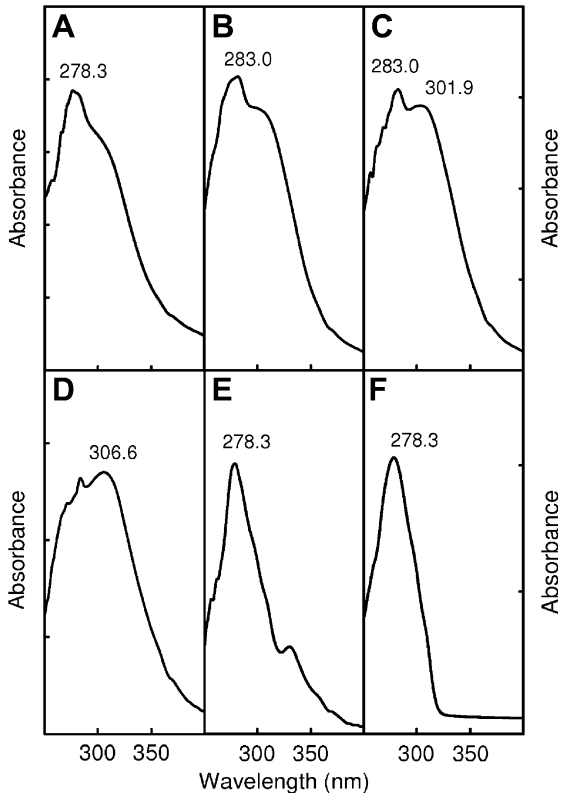

Fig. 3. UV spectra of the products of sinapyl alcohol polymerization by ZePrx34.70 using the end-wise protocol, and resolved by GP-HPLC into a polymer of $M_r \geqslant 18226$ (A, polymerization degree, $n \geqslant 87$), and into oligomers with mean M_r values of 4052 (B, $n \approx 19$), 1274 (C, $n \approx 6$), and 667 (D, $n \approx 3$). Spectrum E possibly corresponds to the quinone-methide of sinapyl alcohol, while spectrum F is from unreacted sinapyl alcohol.

Table 2
Quantitative determination by GP-HPLC of the products of sinapyl alcohol polymerization by ZePrx34.70 and ZePrx33.44

Peak	R_t	M_r	*n*	Area (mV s) (%)	
				ZePrx34.70	ZePrx33.44
1	5.2	⩾ 18200	⩾ 87	4060 ± 60 (10 ± 1)	1650 ± 280 (9 ± 0)
2	7.5	4050	19	31700 ± 630 (72 ± 3)	16700 ± 2370 (73 ± 23)
3	8.3	1270	6	4260 ± 760 (10 ± 1)	2500 ± 550 (11 ± 1)
4	8.8	670	3	120 ± 40 (4 ± 0)	1050 ± 220 (4 ± 0)
5	9.3	270	1	1160 ± 220 (4 ± 0)	570 ± 260 (3 ± 1)

Peak 5 was possibly the quinone-methide of sinapyl alcohol. *n*, polymerization degree.

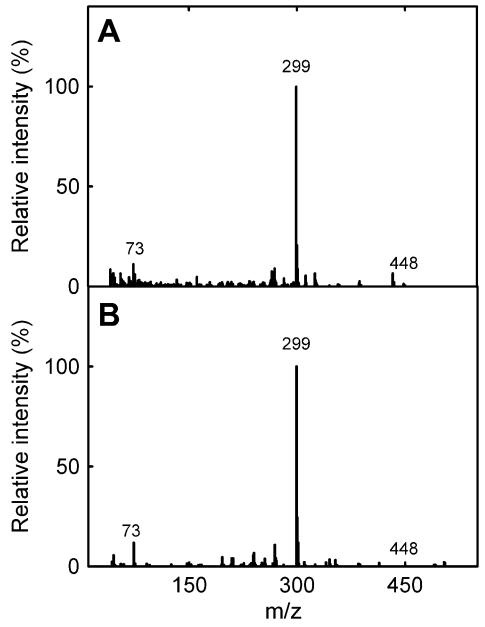

Fig. 4. Mass spectra of the thioethylated monomers arising from aryl-glycerol-β-aryl ether structures derived from sinapyl alcohol in sinapyl alcohol DHPs formed during the ZePrx34.70 (A) and ZePrx33.44 (B)-catalyzed reactions.

tion bands in the 268–287 nm range, suggesting that these polymers and oligomers are rich in β-*O*-4 bonds. To confirm this, thioacidolysis coupled to GC–MS analyses of DHPs derived from sinapyl alcohol was performed. The results allowed to identify the presence of the thioethylated monomers (*erythro* and *threo* isomers) arising from the aryl-glycerol-β-aryl ether structures derived from sinapyl alcohol (Fig. 4). Taken together, the results obtained suggest that the sinapyl alcohol DHPs formed during the ZePrx-catalyzed reaction closely resemble native lignins as regards their M_r, their UV-spectroscopic properties and the unequivocal presence of β-*O*-4 bonds.

3.4. Rate limiting factors during the last step of lignin biosynthesis in Z. elegans SCCs

From the results described above, it may be concluded that *Z. elegans* SCCs are able to lignify and that a peroxidase which fulfils all the requirements to be involved in lignification is present in SCCs. To study which factors control the step catalyzed by ZePrxs in *Z. elegans* SCCs, feeding experiments with H_2O_2 and H_2O_2 + sinapyl alcohol were performed. The results (Table 3) showed that feeding with H_2O_2 at a rate of 0.1 μM s^{-1} increased the β-*O*-4-linked monomers derived from both coniferyl and sinapyl alcohol and incorporated into lignins by 780% and 250%, respectively. These results point to the presence of a pool of monolignols in the culture medium, and suggest that the rate-limiting step of the lignification in SCCs is the availability of H_2O_2. These results agree with previous observations, using other model systems [28,29], that identify the rate of supply of H_2O_2 as the true rate-limiting step of the oxidative coupling of monolignols into lignins.

Surprisingly, when fed with H_2O_2 + sinapyl alcohol at a rate of 0.1 μM s^{-1}, the total amount of the β-*O*-4-linked monomers derived from coniferyl alcohol fell to values below the limit of detection (Table 3), while the total amount of the β-*O*-4-linked monomers derived from sinapyl alcohol increased by 570%. These results suggest that feeding with sinapyl alcohol blocks the incorporation of coniferyl alcohol into SCC lignins, as it may be expected from the ratio of the k_3 values for sinapyl/coniferyl alcohol shown by ZePrxs, which is 21 for ZePrx34.70 and ~9 for ZePrx33.44 (Table 1). But perhaps the most exciting results were obtained when the total amount of β-*O*-4-linked monomers of coniferyl and sinapyl alcohols incorporated in *Z. elegans* SCC lignins were compared. Feeding with H_2O_2 increased the total amount of β-*O*-4-linked monomers by 455%, whereas the addition of H_2O_2 + sinapyl alcohol did not further increase the total amount of β-*O*-4-linked monomers, since they only reached 351% (Table 3). These results suggest that cell wall lignification in *Z. elegans* SCCs is not only controlled by the rate of supply of H_2O_2, but also that cell wall sites for monolignol β-*O*-4 cross-coupling may become saturated, so that the lineal growth of the lignin macromolecule resulting from the β-*O*-4 coupling of monolignols is far from being infinite.

Table 3
Quantitative determination of the thioethylated monomers (*erythro* and *threo* isomers) arising from the aryl-glycerol-β-aryl ether structures derived from coniferyl (*m/z* 269) and sinapyl (*m/z* 299) alcohol in cell walls after feeding SCCs with H_2O_2 and with H_2O_2 + synapyl alcohol

Treatment	Total ionic current (TIC × 10^{-8}) mg^{-1}		
	m/z 269 (%)	*m/z* 299 (%)	*m/z* 269 + *m/z* 299 (%)
None	2.3[a] (100)	3.7[a] (100)	6.0 (100)
H_2O_2	17.9[b] (778)	9.4[b] (254)	27.3 (455)
H_2O_2 + Synapyl alcohol	nd[c] (0)	21.1[c] (570)	21.1 (351)

Values followed by different super indexes are statistically different at $P = 0.05$. nd, not detected.

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References

[1] Ros Barceló, A. (1997) Lignification in plant cell walls. Int. Rev. Cytol. 176, 87–132.
[2] Ralph, J., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P.F., Marita, J.M., Hatfield, R.D., Christensen, J.H. and Boerjan, W. (2004) Lignins: natural polymers from oxidative coupling of 4-hydroxyphenyl-propanoids. Phytochem. Rev. 3, 29–60.
[3] Amthor, J.S. (2003) Efficiency of lignin biosynthesis: a quantitative analysis. Ann. Bot. 91, 673–695.
[4] Lewis, N.G. and Yamamoto, E. (1990) Lignin: occurrence, biogenesis and biodegradation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 455–496.
[5] Boerjan, W., Ralph, J. and Baucher, M. (2003) Lignin biosynthesis. Annu. Rev. Plant Biol. 54, 519–546.
[6] Anterola, A.M., Jeon, J., Davin, L.B. and Lewis, N.G. (2002) Transcriptional control of monolignol biosynthesis in *Pinus taeda*. J. Biol. Chem. 227, 18272–18280.
[7] Blee, K.A., Choi, J.W., O'Connell, A.P., Schuch, W., Lewis, N.G. and Bolwell, G.P. (2003) A lignin-specific peroxidase in tobacco whose antisense suppression leads to vascular tissue modification. Phytochemistry 64, 163–176.
[8] Fukuda, H. (1996) Xylogenesis: Initiation, progression and cell death. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 299–325.

[9] López-Serrano, M., Fernández, M.D., Pomar, F., Pedreño, M.A. and Ros Barceló, A. (2004) *Zinnia elegans* uses the same peroxidase isoenzyme complement for cell wall lignification in both single-cell tracheary elements and xylem vessels. J. Exp. Bot. 55, 423–431.
[10] Ros Barceló, A., Gómez Ros, L., Gabaldón, C., López-Serrano, M., Pomar, F., Carrión, J.S. and Pedreño, M.A. (2004) Basic peroxidases: the gateway for lignin evolution. Phytochem. Rev. 3, 61–78.
[11] Gabaldón, C., López-Serrano, M., Pedreño, M.A. and Ros Barceló, A. (2005) Cloning and molecular characterization of the basic peroxidase isoenzyme from *Zinnia elegans*, an enzyme involved in lignin biosynthesis. Plant Physiol. 139, 1138–1154.
[12] Tognolli, M., Penel, C., Greppin, H. and Simon, P. (2002) Analysis and expression of the class III peroxidase large gene family in *Arabidopsis thaliana*. Gene 288, 129–138.
[13] Fujiyama, K., Takemura, H., Shibayama, S., Kobayashi, K., Choi, J.K., Shinmyo, A., Takano, M., Yamada, Y. and Okada, H. (1988) Structure of the horseradish peroxidase isoenzyme C genes. Eur. J. Biochem. 173, 681–687.
[14] Morales, M. and Ros Barceló, A. (1997) A basic peroxidase from vacuoles and cell walls from *Vitis vinifera*. Phytochemistry 45, 229–232.
[15] López-Serrano, M. and Ros Barceló, A. (2001) Reversed-phased and size-exclusion chromatography as useful tools in the resolution of peroxidase-mediated (+)-catechin oxidation products. J. Chromatogr. 919, 267–273.
[16] Pomar, F., Caballero, N., Pedreño, M. and Ros Barceló, A. (2002) H_2O_2 generation during the auto-oxidation of coniferyl alcohol drives the oxidase activity of a highly conserved class III peroxidase involved in lignin biosynthesis. FEBS Lett. 529, 198–202.
[17] Lapierre, C., Pollet, B. and Rolando, C. (1995) New insights into the molecular architecture of hardwood lignins by chemical degradative methods. Res. Chem. Intermed. 21, 397–412.
[18] Yamazaki, I. and Yokota, K.N. (1973) Oxidation states of peroxidase. Mol. Cell. Biochem. 2, 39–52.
[19] Ros Barceló, A. and Pomar, F. (2001) Oxidation of cinnamyl alcohol and aldehydes by a basic peroxidase from lignifying *Z. elegans* hypocotyls. Phytochemistry 57, 1105–1113.
[20] Nielsen, K.L., Indiani, C., Henriksen, A., Feis, A., Becucci, M., Gajhede, M., Smulevich, G. and Welinder, K.G. (2001) Differential activity and structure of highly similar peroxidases. Spectroscopic, crystallographic and enzymatic analyses of lignifying *Arabidopsis thaliana* peroxidase A2 and horseradish peroxidase A2. Biochemistry 40, 11013–11021.
[21] Østergaard, L., Teilum, K., Mirza, O., Mattsson, O., Petersen, M., Welinder, K.G., Mundy, J., Gajhede, M. and Henriksen, A. (2000) *Arabidopsis* ATP A2 peroxidase. Expression and high-resolution structure of a plant peroxidase with implications for lignification. Plant Mol. Biol. 44, 231–243.
[22] Sasaki, S., Nishida, T., Tsutsumi, Y. and Kondo, R. (2004) Lignin dehydrogenative polymerization mechanism: a poplar cell wall peroxidase directly oxidizes polymer lignin and produces in vitro dehydrogenative polymer rich in β-O-4 linkage. FEBS Lett. 562, 197–201.
[23] Kobayashi, T., Taguchi, H., Shigematsu, M. and Tanahashi, M. (2005) Substituent effects of 3,5-disubstituted *p*-coumaryl alcohols on their oxidation using horseradish peroxidase-H_2O_2 as the oxidant. J. Wood Sci. 51, 607–614.
[24] Folkes, L.K. and Candeias, L.P. (1997) Interpretation of the reactivity of peroxidase compound I and II with phenols by the Marcus equation. FEBS Lett. 412, 305–308.
[25] Ringena, O., Lebioda, S., Lehnen, R. and Saake, B. (2006) Size-exclusion chromatography of technical lignins in dimethyl sulfoxide/water and dimethylacetamide. J. Chromatogr. 1102, 154–163.
[26] Baumberger, S., Dole, P. and Lapierre, C. (2002) Using transgenic poplars to elucidate the relationship between the structure and the thermal properties of lignins. J. Agr. Food Chem. 50, 2450–2453.
[27] Lin, S.H. (1982) Derivative ultraviolet spectroscopy of lignin and lignin model compounds – a new analytical technique. Sven. Papperstidn., R162–R171.
[28] Nose, M., Bernards, M.A., Furlan, M., Zajicek, J., Eberhardt, T.L. and Lewis, N.G. (1995) Towards the specification of consecutive steps in macro-molecular lignin assembly. Phytochemistry 39, 71–79.
[29] Ferrer, M.A., Pedreño, M.A., Muñoz, R. and Ros Barceló, A. (1990) Oxidation of coniferyl alcohol by cell wall peroxidases at the expense of indole-3-acetic acid and O_2. A model for the lignification in the absence of H_2O_2. FEBS Lett. 276, 127–130.