DARWIN REVIEW

From Zinnia to Arabidopsis: approaching the involvement of peroxidases in lignification

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Abstract

Zinnia elegans constitutes one of the most useful model systems for studying xylem differentiation, which simultaneously involves secondary cell wall synthesis, cell wall lignification, and programmed cell death. Likewise, the in vitro culture system of Z. elegans has been the best characterized as the differentiation of mesophyll cells into tracheary elements allows study of the biochemistry and physiology of xylogenesis free from the complexity that heterogeneous plant tissues impose. Moreover, Z. elegans has emerged as an excellent plant model to study the involvement of peroxidases in cell wall lignification. This is due to the simplicity and duality of the lignification pattern shown by the stems and hypocotyls, and to the basic nature of the peroxidase isoenzyme. This protein is expressed not only in hypocotyls and stems but also in mesophyll cells transdifferentiating into tracheary elements. Therefore, not only does this peroxidase fulfill all the catalytic requirements to be involved in lignification overcoming all restrictions imposed by the polymerization step, but also its expression is inherent in lignification. In fact, its basic nature is not exceptional since basic peroxidases are differentially expressed during lignification in other model systems, showing unusual and unique biochemical properties such as oxidation of syringyl moieties. This review focuses on the experiments which led to a better understanding of the lignification process in Zinnia, starting with the basic knowledge about the lignin pattern in this plant, how lignification takes place, and how a sole basic peroxidase with unusual catalytic properties is involved and regulated by hormones, H2O2, and nitric oxide.

Key words: Lignification, peroxidase, syringyl, tracheary element, xylem differentiation, Zinnia elegans.

Introduction

The xylem constitutes the longest pathway for water transport in vascular plants. It is a simple pathway of low resistance, which enables water to be transported in large quantities and with great efficiency from the roots to the leaves. The xylem is composed of non-conducting cells including parenchyma and fibres, and conducting cells, or tracheary elements (TEs; i.e. vessel elements and tracheids), which are essential for transport. Xylem formation has been the subject of numerous studies of differentiation in higher plants, not only because its function is essential to the existence of vascular plants, but also because xylem formation is considered one of the most distinctive differentiation processes which occur in land plants.

In the primary xylem, tracheids are differentiated from procambial cells, while in the secondary xylem these cells are derived from vascular cambium. The tracheids can be induced in vitro from multiple cell types, such as parenchyma, cortex, and leaf mesophyll (Roberts et al., 1988; Fukuda, 1992).
Cell walls of mature xylem elements are impregnated with lignins (Boudet et al., 1995), which are the most abundant organic compound on the surface of the Earth after cellulose, accounting for 25% of plant biomass (Higuchi, 1990). Lignins impart water impermeability and resistance against tensile forces of the water columns, and confer structural support and flexural stiffness to the aerial organs. Lignification also takes place in response to wounding and pathogen attack, creating a barrier to pathogen penetration (Ros Barceló, 1997).

Lignins are three-dimensional heteropolymers resulting from the oxidative coupling of three p-hydroxyphenyl (p-coumaryl, coniferyl, and sinapyl) alcohols (i.e. monolignols). The cross-coupling reaction of monolignol radicals produces a highly hydrophobic heteropolymer composed of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units (Vanholme et al., 2010).

Lignins are deposited mainly in TEs, fibres, and sclereids (Ros Barceló, 1997). However, lignin composition varies among species, phylogenetic groups, cell types, development stage, and even seasonal growth. Different stresses provoke a change in lignin composition, and this shift depends on the stress which caused it (Moura et al., 2010).

The biosynthesis of lignins proceeds through a long sequence of reactions that involve (i) the shikimate pathway, which provides l-phenylalanine and l-tyrosine; (ii) the common phenylpropanoid pathway from l-phenylalanine (and/or l-tyrosine) to the p-hydroxyphenyl-CoAs; and (iii) the lignin-specific pathway, which channels the p-hydroxyphenyl-CoAs toward the synthesis of monolignols, and their later polymerization in the cell walls (Boerjan et al., 2003).

According to the literature (Lewis and Yamamoto, 1990; Ros Barceló, 1997; Gavnholt and Larsen, 2002), an enzyme that plays a role in monolignol oxidation must fulfill the following criteria: (i) spatial and temporal correlation of the enzyme with active lignification; (ii) apoplast localization; (iii) altered lignification in plants with modified levels of the enzyme; (iv) substrate specificity; (v) wide distribution among different plant phylogenetic groups; and (vi) high affinity for cinnamyl alcohols. Two candidate enzymes have been proposed as responsible for monolignol oxidation: laccases and peroxidases. Nowadays, the participation of peroxidases in the lignification of plant cell walls is beyond doubt, though it is also accepted that laccases are involved in lignin formation (for a recent review, see Berthet et al., 2012). It appears laccases are able to catalyse the synthesis of G units, but the formation of S units is restricted to a peroxidase-mediated oxidation (Berthet et al., 2011). Although much research has been performed to assess the role of class III peroxidases in lignification, only a few isoenzymes have actually been correlated with lignin formation. As a matter of fact, the basic peroxidase of Zinnia elegans (ZePrx) seems to be the only peroxidase isoenzyme that has been fully characterized and unequivocally linked to lignification (Gabaldón et al., 2005a, 2006).

As described above, the biosynthesis of lignin is developmentally activated in specific tissues and cell types, such as xylem elements during vascular development, but this process can also be activated in cells that normally do not accumulate lignins, such as leaf mesophyll cells, in response to wounding and hormonal factors (Fukuda, 1997). Thus, lignin genes which are transcriptionally silenced in certain cell types may be activated in response to hormonal factors, so that the use of these cell types constitutes a special and valuable system for studying the nature and time-course of lignin gene transcription (Demura et al., 2002). In addition, studies on TE differentiation in plant cell cultures have provided strong support for xylem differentiation from cambial derivatives, since mesophyll cultured cells can be induced by a variety of external stimuli to proceed through temporally controlled metabolic and developmental programmes to the formation of single-cell-derived dead TEs (Fukuda, 1997; Demura et al., 2002). The best characterized cell culture system used for these studies is that derived from isolated Z. elegans mesophyll cells. Zinnia mesophyll cells can be induced to differentiate into cells resembling TEs in a medium containing suitable levels of auxins and cytokinins (Fukuda, 1997), and have been considered as a suitable model for studying the sequence of events which take place during xylem differentiation, including programmed cell death (PCD) and secondary cell wall synthesis (Fukuda, 1997; Milioni et al., 2002). This makes it possible to study the biochemistry and physiology of xylogenesis without the complexity which heterogeneous plant tissues impose. TE differentiation from mesophyll cells thus represents a unique and interesting model system, in which secondary cell wall deposition, lignin biosynthesis, and PCD may be studied, and where the inter-relationship among these unique cellular events may be explored.

On the other hand, Z. elegans is one of the most useful plants for studying cell wall lignification. This is due to the simplicity and duality of the lignification pattern shown by the stems and hypocotyls, and to the nature of the peroxidase isoenzyme complement, which is almost completely restricted to the presence of a basic peroxidase isoenzyme (Ros Barceló et al., 2004). Moreover, Zinnia suspension cultured cells (SCCs) have emerged as a good source of peroxidase, since this protein, being located in cell walls, is rapidly secreted into the culture medium, allowing its extraction and purification (Gabaldón et al., 2005a). Taking into account all of the above, this review sheds light on a better understanding of the lignification process in Zinnia and how lignin formation is achieved. In this review, we would like to embrace Professor Ros Barceló’s contributions regarding lignification in Zinnia, starting with the basic knowledge about the pattern of lignins in this plant, how lignification takes place, and how a sole basic peroxidase with unusual catalytic properties is involved. We will also discuss the involvement of nitric oxide (NO) and H₂O₂ in xylogenesis of both single cell cultures and differentiating xylem. Likewise, as the basic peroxidase that is expressed in the transdifferentiating process is the same peroxidase complement in both lignifying xylem and Zinnia hypocotyl-derived cell cultures, we will draw attention to this basic peroxidase, which has been purified and further characterized, biochemically, structurally, and molecularly. In summary, we would like to highlight how Professor Ros Barceló contributed to shed light not only on the close relationship between peroxidases and lignification but also on how these basic peroxidases are regulated.
Lignification in *Z. elegans*

Molecular mechanisms underlying monolignol transport across cell membrane

While monolignols are synthesized in the cytosol, monolignol polymerization takes place in the cell wall. The exact mechanism whereby this occurs is unclear, and none of the proposed systems has been completely ruled out or accepted (Liu, 2012). Several pathways have been suggested: (i) translocation through the 4-O-glcunosylated forms; (ii) exocytosis via the enoplasmatic reticulum (ER–Golgi); (iii) passive diffusion via hydrophobic reactions through the plasma membrane; and (iv) active transport via different transporters.

The glucosylated forms of monolignols (p-coumaryl alcohol glucoside, coniferin, and syringin) have been found in gymnosperms and some angiosperms. It was suggested that the glucosylated forms may be a pathway existing in gymnosperms (Liu et al., 2011), but recent evidence in *Arabidopsis* suggests that monolignol glucosides are storage forms of monolignols but not the direct precursors of lignin (Chapelle et al., 2012).

Currently, there is no evidence that supports the transport via Golgi vesicles. Kaneda et al. (2008) used [^3H]phenylalanine feeding in the developing xylem to observe that the Golgi vesicles did not load any phenylpropanoid-derived compounds. However, the passive diffusion is supported by the fact that other non-canonical phenolics are incorporated into the lignin polymer, such as p-hydroxyphenyl aldehydes and p-hydroxycinnamic acids (Boerjan et al., 2003). This variety of monomers would require non-selective transport, such as passive diffusion through the plasma membrane and hydrophobic–hydrophilic interactions (Boija and Johansson, 2006).

Several lines of evidence suggest the implication of ATP-binding cassette (ABC) transporters in translocating monolignols into the cell wall (Miao and Liu, 2010): (i) their expression was correlated with lignification in *Arabidopsis* (Ehlting et al., 2005) and *Zinnia* (Pesquet et al., 2005); (ii) monolignol transport depends on the presence of ATP; (iii) specific ABC transporter inhibitors reduce the uptake of monolignols by membrane vesicles, even in the presence of ATP; (iv) this uptake displays typical protein–ligand binding kinetics; and (v) this uptake of lignin precursors by membrane vesicles is a selective process (Liu, 2012). However, the precise identification of which specific ABC transporters are involved in lignification remains unclear because *Arabidopsis* mutants, deficient in ABC transporters whose expression had a good correlation with phenylpropanoid–lignin biosynthesis, did not present any reduction of lignification in the xylem (Kaneda et al., 2011). Instead, some of these mutants impaired auxin polar transport in *Arabidopsis* stem, indicating that the ABC transporters found in vascular tissues and correlated with their lignification may be involved in other biological processes. Therefore, research at the current time is aimed at the use of multiple gene-deficient mutant lines for the related ABC transporters with the aim of finding a shortage of lignification in a tissue or specific xylem cell (Liu, 2012).

Lignin composition is an environment- and development-regulated process

Lignin monomer composition is different among several groups of plants. In the past, S lignins have been generally assumed to be characteristic of angiosperms, but recent studies confirmed that S lignins are also present in gymnosperms, lycophytes, and ferns, and even in non-vascular plants such as liverworts and red algae (Fig. 1A; for reviews, see Li and Chapple, 2010; Vanholme et al., 2010; Weng and Chapple, 2010; Novo-Uzal et al., 2012). However, the genotype is not the only factor determining differences in lignin. First, lignin abundance and composition are affected by environmental factors (Fig. 1B), both biotic and abiotic (Moura et al., 2010). Secondly, lignification is also a process modulated by development (Fig. 1B), and varies among organs, tissues, and developmental stage of the secondary cell wall (Schuetz et al., 2013). Regarding development, the particular lignin composition of *Z. elegans* seedlings is unique because at a certain developmental stage (25- to 30-day-old plants), the same plant shows simultaneously two different modes of lignification in hypocotyls and epicotyls (Fig. 1C). Thus, the hypocotyl composition resembles that occurring in angiosperms, whereas the epicotyl composition partially resembles that found in gymnosperms, since H+G units alone constitute 78% of the lignin building blocks (Ros Barceló et al., 2004). Moreover, not only were residues from the three monolignols detected, but also some other unusual monomers. Obviously, lignin monomer composition is affected by the regulation of different enzymes involved in monolignol biosynthesis such as O-methyl transferases, ferulate 5-hydroxylase, and peroxidases, which catalyse the last step of lignin formation, oxidizing the monolignols. How the *Z. elegans* peroxidase complement is able to oxidize different monomers? How is such a process regulated?

Hydrogen peroxide is required to regulate xylem lignification

H$_2$O$_2$ is a molecule that may play several roles in lignification and is required for the reaction driven by peroxidase to oxidize monolignols. Moreover, it is now well known that H$_2$O$_2$ is also a potent signalling molecule, involved not only in the regulation of cell wall strengthening, but also in a plethora of physiological processes such as acquiring resistance, senescence, phytoalexin production, photosynthesis, stomatal opening, and the cell cycle (Dimitrov Petrov and van Breusegem, 2012). However, H$_2$O$_2$ is also a toxic compound, which can lead to damage to a variety of biological molecules. Therefore, H$_2$O$_2$ has multiple functions in the plant, but causes damage at elevated concentrations, so that a very strict control of H$_2$O$_2$ concentration in plant cells is compulsory (Dimitrov Petrov and van Breusegem, 2012).

In such a way, H$_2$O$_2$ production in xylem cells of *Z. elegans* is not an exception since it represents a strongly regulated developmental process, showing a peak at the moment at which cell wall lignification in the xylem begins (Ros Barceló et al., 2002a). At such a moment, when only
differentiating xylem vessels have begun to lignify, H$_2$O$_2$ is mainly observed at the outer face of the plasma membrane of both xylem (non-lignifying) parenchyma cells and adjacent living (differentiating) thin-walled xylem cells (Ros Barceló, 2005). H$_2$O$_2$ production is a property not only of *Z. elegans* lignifying xylem, but also of such xylem of different vascular plants, gymnosperms, and angiosperms (including mono- and dicotyledons) (Ros Barceló, 1998b). From studies of the hypersensitive response, it is generally recognized that the main sources of H$_2$O$_2$ are NADPH-dependent oxidases such as respiratory burst oxidase homologue (rboh) and cell wall-associated peroxidases, but other minor sources of H$_2$O$_2$ also exist (Dimitrov Petrov and van Breusegem, 2012). In the case of *Z. elegans* xylem cells, the experiments carried out by Ros Barceló (1998a, 1999) suggest that they generate extracellular H$_2$O$_2$ via a plasma membrane-localized NADPH-dependent oxidase (Fig. 2). In these studies, he took advantage of different inhibitors of NADPH-dependent oxidase, peroxidase, phospholipase C, protein kinase, and protein phosphatase. H$_2$O$_2$ production in the xylem of *Z. elegans* was sensitive to diphenylene iodonium (DPI), pyridine, imidazole, and quinacrine (Ros Barceló, 1998a, 1999), well known inhibitors of NADPH oxidase. DPI also inhibits H$_2$O$_2$ production in spruce SCCs during lignin formation (Kärkönen et al., 2009). It has been reported that DPI is able to inhibit both peroxidase-mediated generation of H$_2$O$_2$ and NADPH-oxidase-mediated generation of the O$_2^-$ anion (Bolwell et al., 1998). However, other authors found that DPI has an effect on NADPH oxidase, but has no effect on generation of the O$_2^-$ anion (and subsequent H$_2$O$_2$ production) by peroxidases (Ros Barceló and Ferrer, 1999). The plethora of NADPH oxidase inhibitors tested in *Z. elegans* support its role in H$_2$O$_2$ generation in the xylem. Moreover, a Rac small GTPase, a component of the NADPH oxidase complex, is located in xylem parenchyma cells and precursor of TEs (Nakanomyo et al., 2002), and

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**Fig. 1.** Lignification is modulated by the genotype, development, and the environment. (A) Phylogenetic tree showing the lignin monomer composition among major plant lineages (modified from Li X, Chapple C. 2010. Understanding lignification: challenges beyond monolignol biosynthesis. Plant Physiology 154, 449–452. www.plantphysiol.org. Copyright American Society of Plant Biologists). (B) Model summarizing the effect of genotype, development, and environment on both lignin abundance and monomer composition through class III peroxidases and other enzymes involved in lignin biosynthesis. (C) Different lignin monomer composition between epicotyls and hypocotyls in *Zinnia elegans* (developmental regulation; data from Ros Barceló et al., 2004). *Ginkgo wood; **Ginkgo cell cultures.

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**Fig. 2.** Model showing the two sources of H$_2$O$_2$ in the lignification process of *Zinnia elegans*.
an mRNA coding for a putative rboh (NADPH oxidase) is differentially expressed during the transdifferentiation of Z. elegans mesophyll cells into TEs (Demura et al., 2002). Such a putative NADPH oxidase is up-regulated by auxins (a plant hormone involved in vascular differentiation) during the early stages of TE differentiation (Yoshida et al., 2009). Despite the role of NADPH oxidase in H$_2$O$_2$ production, this molecule can also be generated during the auto-oxidation of coniferyl alcohol by the Z. elegans basic peroxidase in a buffered solution at pH 5.0, in a process that resembles which occurs in the apoplast (Pomar et al., 2002). Thus, H$_2$O$_2$ could be generated by peroxidases even when the concentration generated by the NADPH oxidase is not sufficient. Indeed, a study with an inhibitor of peroxidases (salicylhydroxamic acid) suggests the presence of a H$_2$O$_2$-generating basic peroxidase in Zinnia (Karlsson et al., 2005). A dual-source model for H$_2$O$_2$ generation in Z. elegans is the emerging picture (Fig. 2).

**Nitric oxide regulates peroxidase in the lignifying xylem**

Besides H$_2$O$_2$, another interesting point about the regulation of lignification in Z. elegans is the role of NO. NO is an important molecule that acts in many tissues, participating in the regulation of several physiological processes in plants and other organisms (Palavan-Unsal and Arisan, 2009). It is synthesized by a wide variety of mechanisms in plants, and its role in signalling, regarding plant growth and development, and responses to stress is well known. Indeed, as occurs with H$_2$O$_2$, NO is produced by Zinnia xylem cells (Gabaldón et al., 2005b). By means of two NO-donor compounds [sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP)], NO itself, and the NO-scavenger compound 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), NO was shown to be a direct effector of the activity of Z. elegans peroxidase in the lignifying xylem. The first three compounds (SNP, SNAP, and NO) were able to inhibit the activity of Z. elegans peroxidase in vitro and in vivo (Ferrer and Ros Barceló, 1999). As an additional proof of the specificity of such inhibition, PTIO was able to revert such inhibition in vitro as it did with tetramethylbenzidine oxidase activity (belonging to the peroxidase enzymes) in vivo (Ros Barceló et al., 2002b). However, H$_2$O$_2$ production detected by histochemical staining with starch/KI reagent was not affected by the NO donor SNP, so the enzymatic system for H$_2$O$_2$ release in the xylem is still active even if peroxidase is inhibited. This suggests that NO may regulate xylem lignification by its direct action on peroxidase activity, without affecting H$_2$O$_2$ production by the same enzyme. However, this does not preclude other regulatory functions of NO in the lignification of Z. elegans, as we will see later in this review. The way in which NO causes the direct kinetic effect on Zinnia peroxidase is not known, but sensing NO by plant enzymes leads to metal nitrosylation, S-nitrosoylation, and tyrosine nitration (Gómez-Ros et al., 2012). Moreover, it should be noted that NO has been shown to react with the higher oxidation states of horseradish peroxidase (HRP), being a substrate for the enzyme (Glover et al., 1999).

**Regulation of lignification in Z. elegans**

**Zinnia elegans transdifferentiating cells help to understand how xylogenesis occurs**

Fukuda and Komamine (1980), based on the work of Kohlenbach and Schmidt (1975), established an in vitro experimental system in which isolated mesophyll cells from Z. elegans leaves were directly differentiated into TEs in a culture medium containing auxins and cytokinins. This experimental system depends on various factors including plant age and the conditions in which the Z. elegans plant is grown, and also the specific culture conditions (Fukuda, 1992; Roberts et al., 1992). In this way, the Zinnia system is particularly useful for xylogenesis studies, specifically to analyse the sequence of different steps which occur during TE differentiation. In fact, the differentiation is an inducible process, triggered by the establishment of the culture itself, and the addition of hormones, wherein the cells differentiate to a semi-synchronous high frequency (McCann et al., 2000). Furthermore, due to their morphological characteristics, these cells can be easily identified. This property, together with the fact that the induction of in vitro TE differentiation is a relatively simple process to reproduce, makes this system very useful in studies of cell differentiation.

**Stages of TE differentiation from Z. elegans mesophyll cells**

The Zinnia experimental system has been very useful for the elucidation of the different steps involved in TE differentiation. The cell differentiation process is divided into three stages (Fig. 3) (Fukuda, 1997).

**Stage I**

The induction of differentiation occurs as a result of both the establishment of the culture and the addition of a combination of cytokinins and auxins, although Oda et al. (2005) highlighted the importance of brassinosteroids (BRs) as the factors that induce TE differentiation in Arabidopsis SCCs, the levels of these compounds being higher in the final stages of Zinnia TE differentiation (Yamamoto et al., 2001). During this stage, which corresponds to the dedifferentiation process, isolated mesophyll cells lose their ability to photosynthesize. This dedifferentiation involves the expression of induced genes by the establishment of the culture (wounding), and the acquisition by the cells of the ability to grow and differentiate again in a new environment.

**Stage II**

The undifferentiated cells restrict their development potential (which is limited to the ability to differentiate only into TEs), and an early expression of genes involved in TE differentiation occurs. In fact, during stage II, the calcium levels increase transiently (Fukuda, 1997) and the expression of several proteins is induced.

**Stage III**

This differentiation process involves the formation of the secondary cell wall, protoplast autolysis, and PCD. Specifically,
the PCD, by which individual cells degrade their contents autonomously, takes place at the end of stage III, and it is strongly associated with secondary cell wall formation (Fukuda, 1997). Therefore, the transition from stage II to stage III is a point of no return in the differentiation.

Most visible changes produced during TE differentiation occur on the cell surface, but changes in the cell wall architecture and its influence on the differentiation process have not been analysed in detail, except for the process of lignification, which occurs specifically on the secondary cell walls (McCann et al., 2000). In fact, the production of a thick secondary wall formed by polysaccharides, structural proteins, and lignins is essential for the TE-specific development. Thus, in differentiating TEs, the phenoxyl radicals derived from the p-hydroxycinnamyl alcohols are coupled to form a growing lignin polymer on cell walls. Specifically, materials that form the secondary cell wall begin to be deposited within 48 h of culture initiation, while lignin deposition is observed later.

During protoplast autolysis, hydrolytic enzymes such as cysteine and serine proteases, RNases, S1 nuclease-type, acid phosphatases and lipases are expressed for catalysing the degradation of macromolecules (Fukuda, 2004). These enzymes are accumulated in the vacuole until this organelle is degraded, so that the maintenance of vacuolar compartmentalization is necessary for cells which are differentiating into TEs (Fukuda et al., 1998). Then, the vacuole collapses and hydrolytic enzymes attack different organelles, degrading the cellular content, including the plasma membrane and part of the primary cell walls (Obara et al., 2001). Specifically, the degeneration of all organelles, including the nucleus, begins when the tonoplast is degraded, a process which defines the initial stage of PCD, and that occurs several hours after thickenings of the secondary cell wall become visible. Finally, in the last stage, the rupture of the vacuole and the release of digestive enzymes lead cells to lose all their cellular content and form hollow structures, reinforced by secondary cell walls (Kuriyama and Fukuda, 2000).

**Hydrogen peroxide production and localization in both the vascular bundles and the lignifying cell cultures of Z. elegans**

Taking into account the different events that take place in transdifferentiating Z. elegans mesophyll cell cultures undergoing TE differentiation, and in order to identify the sites of H$_2$O$_2$ production in this system, Ros Barceló’s group described four different cell developmental stages (Gómez-Ros et al., 2006): (i) undifferentiated mesophyll cells which have acquired the competence to differentiate; (ii) thin-walled transdifferentiating mesophyll cells; (iii) thick-walled transdifferentiating (secondary cell wall-forming) TEs; and (iv) thick-walled differentiated (dead) TEs. Thereby, using CeCl$_3$ staining and electron microscopy, during the first three cell stages, electron-dense deposits, indicative of H$_2$O$_2$
production and localization, were mainly observed on the cell surface in areas comprising the cell wall and the membrane, while in the last developmental stage, electron-dense deposits were clearly seen in both the primary and secondary cell wall, uniformly distributed throughout the ornamental structure (Fig. 4; Gómez-Ros et al., 2006). That is, transdifferentiating Z. elegans mesophyll cells did not show an oxidative burst but live in a strongly oxidative state during the entire culture period. In fact, when H₂O₂ levels in the culture medium were measured, they were in the order of 1–3 mM. These values were consistent with those found in the lignifying xylem of Z. elegans, where H₂O₂ levels during the bulk of lignification were established to be in the millimolar range (Ros Barceló et al., 2002a). In order to check the local H₂O₂ production, Gómez-Ros et al. (2006) also monitored these four cell types involved in TE differentiation by confocal laser scanning microscopy using 2',7'-dichlorofluorescein-diacetate as fluorescent probe. Green fluorescence was mainly shown (Fig. 5A, B) by undifferentiated (parenchyma-like) thin-walled cell elements (i.e. undifferentiated competent mesophyll cells and thin-walled transdifferentiating mesophyll cells), while differentiating and differentiated thick-walled TEs showed lower fluorescence levels. These results confirmed the zones of H₂O₂ production monitored by electron microscopy, and that all cell types involved in TE differentiation were able to produce H₂O₂. A similar pattern of fluorescence was also observed in Z. elegans vascular bundles stained with the H₂O₂ probe since thin-walled green fluorescent cells (Fig. 5C) surround thick-walled xylem vessels whose secondary thickenings shone brightly when observed by polarized light microscopy (Fig. 5D; Gómez-Ros et al., 2006). The analogy between both systems became even more evident when transdifferentiating Z. elegans mesophyll cells were stained with phloroglucinol to reveal lignins since the only lignifying cells were the thick-walled transdifferentiating (secondary cell wall-forming) TEs, whereas undifferentiated (parenchyma-like) thin-walled cell elements did not begin to lignify (Fig. 5E; Gómez-Ros et al., 2006). Therefore, undifferentiated and non-lignifying thin-walled cell elements supplied H₂O₂ to differentiated and lignifying TEs, suggesting

Fig. 4. Distribution of electron-dense deposits indicative of H₂O₂ production and localization in transdifferentiating Zinnia elegans mesophyll cells stained with CeCl₃ at two different developmental stages: (A, B) thick-walled transdifferentiating tracheary elements and (C, D) thick-walled differentiated tracheary elements, as viewed by electron microscopy. Bar=5 µm. Taken from Protoplasma, Vol. 227, 2006, 175–183. Two distinct cell sources of H₂O₂ in the lignifying Zinnia elegans cell culture system. Gómez-Ros LV, Paradiso A, Gabaldón C, Pedreño MA, de Gara L, Ros Barceló A, with kind permission from Springer Science and Business Media.
that a degree of cell–cell cooperation exists in relation to H$_2$O$_2$ which is required for lignifying TEs. In fact, Schuetz et al. (2013) described in their review some studies using the Zinnia cell culture system, in which lignification of TEs proceeds even after PCD. Thus, when dead TEs were moved from TE induction medium to a medium containing added monolignols, the TEs were able to use these monolignols to continue lignification post-mortem (Schuetz et al., 2013, and references therein). Because Zinnia cell cultures contain many cells that remain in a state similar to xylem parenchyma cells (McCann et al., 2001), it has been hypothesized that these parenchyma-like cells may act as good neighbours for the differentiating TEs and provide them with an exogenous supply of monolignols. Such a model comes from the observation that some lignin biosynthesis genes are expressed not only in lignifying TEs and fibres of Arabidopsis, tobacco and popular, but also within xylem parenchyma cells situated adjacent to dead TEs (Schuetz et al., 2013, and references therein).

It has also been suggested that, in addition to the release of hydrolytic enzymes during the vacuolar collapse, monolignols stored in that compartment could be released, and that lignification is therefore primarily a post-mortem process (Pesquet et al., 2010). In this model, monolignols would be synthesized prior to cell death, and small amounts might be deposited and polymerized in the cell wall. However, the bulk of monolignols would be stored in the vacuole, and only when the vacuole collapses would the monolignols diffuse rapidly into the cell wall to be polymerized, and most lignin polymerization would therefore occur after PCD of TEs. Monolignol localization using microautoradiography suggests that TEs in Arabidopsis are still living while the cell wall is being lignified (Kaneda et al., 2008), but this observation does not preclude lignification continuing to proceed following PCD, through either the vacuolar release of monolignols or their acquisition from neighbouring non-lignifying cells.

Nitric oxide production and localization in both the vascular bundles and the lignifying cell cultures of Z. elegans

Although the metabolic cascade leading to cell death in xylem cells has been partially described, little is known about the diffusible signalling molecules that switch this unavoidable process off. One of these possible signalling molecules is NO, involved in plant cell differentiation and other types of PCD such as pathogen-induced PCD that occur during the hypersensitive response (Neill et al., 2003; Wendehenne et al., 2004). Therefore, as Neill stated (Neill, 2005), another important contribution of Ros-Barceló’s group was the first report that demonstrated an essential signalling role for NO in regulating PCD and lignification during xylem formation (Gabaldón et al., 2005b). Gabaldón et al. (2005b), using Z. elegans as a model species, worked with two complementary experimental systems to study xyleogenesis: developing vascular bundles in Zinnia stems and the in vitro Zinnia cell cultures. In the first system, confocal laser scanning microscopy of stem sections incubated with an NO-sensitive fluorescent dye...
(4,5-diaminofluorescein-2 diacetate) revealed that NO production was largely confined to xylem cells. Moreover, a spatial gradient of NO production inversely related to the degree of lignification was observed, with the highest NO fluorescence being seen in just-differentiating xylem cells, and the lowest in differentiated and lignified xylem cells (Fig. 6A, B).

These observations in planta were mirrored by those made using the transdifferentiating Zinnia mesophyll cell cultures undergoing TE differentiation. In this second system, Gabaldón et al. (2005b) found that there was a temporal gradient of NO production, with low NO fluorescence in the undifferentiated mesophyll cells, the highest NO fluorescence in both thin-walled transdifferentiating mesophyll cells and thick-walled transdifferentiating (secondary cell wall-forming) TEs, and then low NO fluorescence again in thick-walled differentiated TEs (Fig. 6C). These results suggested that a burst of NO was produced when transdifferentiating mesophyll cells acquired the competence for cell death, constituting the point of no return, and NO production was sustained while secondary cell wall synthesis and cell autolysis were in progress. These results also explained why just-differentiating thin-walled xylem cells in vascular bundles also exhibited an NO burst (Fig. 6B). That NO is essential for both differentiation and PCD was demonstrated by the effect of the NO scavenger PTIO, which very effectively removed NO from cells both in planta and in vitro cultures. Removal of NO from the cultured cells with PTIO resulted in dramatic reductions both in PCD and in the formation of TEs.

Although absolute verification requires genetic and transgenic analyses, these data strongly indicated that NO was a key factor mediating PCD and lignification during xylogenesis. In addition, NO might well directly affect the activity of some of the enzymes of lignin biosynthesis as well as increase the transcription of their genes (Gabaldón et al., 2005b). This suggestion emerged from the observation that all the branching enzymes as well as rate-limiting enzymes of the lignin biosynthetic pathway are haem proteins and therefore possible targets of NO action. This fact is especially important since any possible metabolic control of these enzymes by NO synthesized by the developing xylem would enable it to regulate not only the global p-hydroxy- cinnamyl alcohol pools in lignifying plant cells and the H/G/S ratio for carbon partitioning, but also their rates of polymerization. In addition, although many genes induced or repressed during xylogenesis have been identified from transcriptional profiles of transdifferentiating Zinnia cells (Demura et al., 2002), the analysis of these profiles in the presence of PTIO could be an excellent way to determine which genes are regulated directly by NO.

Xylem maturation and TE differentiation are processes tightly regulated mainly by plant growth regulators and transcriptional factors

As described above, during TE differentiation, deposition of the secondary cell wall, which in turn is reinforced by lignins, and cell death results in the formation of a functional cell corpse devoid of cytoplasm. Auxins and cytokinins are needed for Zinnia TE differentiation, but their function is only related to the early events of the transdifferentiating process. However, BRs are believed to play a role during the late events of Zinnia TE differentiation since inhibition of BR synthesis in these in vitro cultures prevents cells from maturing and undergoing cell death.

Fig. 6. (A) Temporal inverse relationship between nitric oxide production and the lignin content in the Zinnia elegans xylem. (B) Nitric oxide production by a young Z. elegans vascular bundle from stems, after staining with 4,5-diaminofluorescein-diacetate as viewed by confocal laser scanning microscopy. (1, asterisk), thin-walled xylem cells; (2, arrowheads), differentiating thick-walled xylem cells; and (3, arrows), differentiated thick-walled xylem cells. P, phloem fibres; X, xylem vessel. Bar=40 μm. (C) Nitric oxide production by transdifferentiating Z. elegans mesophyll cells at different developmental stages. Bright-field (1, 3, 5, 7) and fluorescent (2, 4, 6, 8) images of undifferentiated mesophyll cells (1, 2), thin-walled transdifferentiating mesophyll cells (3, 4), thick-walled transdifferentiating tracheary elements (5, 6), and thick-walled differentiated tracheary elements (7, 8), stained with 4,5-diaminofluorescein-diacetate, as viewed by confocal laser scanning microscopy. Bar=15 μm.
On the other hand, the signals related specifically to xylem cell death are poorly understood due to difficulties found in identifying signalling which is only directed towards cell death without affecting secondary cell wall formation. Most pharmacological agents that block xylem cell death also block secondary cell wall formation, suggesting that these two processes are tightly co-regulated (Bollhöner et al., 2012). However, it is clear that even though the different phases of xylem maturation are jointly regulated, it is likely that the individual processes have separate controls as well. It has been recently shown that differentiated Zinnia TEs accumulate ethylene (Pesquet and Tuominen, 2011) so that the addition of silver thiosulphate allowed development of secondary cell walls, but TEs do not lignify or die, which means that silver thiosulphate-mediated arrest of TE differentiation is probably due to blocking cell death, which, in turn, blocks lignification. Therefore, ethylene seems to interfere in these processes in the Zinnia in vitro system. However, this conclusion is not supported by Arabidopsis mutant plants where no developmental defects were observed in any dominant ethylene receptor or downstream signalling mutants, although complete removal of ethylene biosynthesis is lethal (Tsuchisaka et al., 2009).

Nevertheless, it is possible that the Zinnia system actually reveals some basic regulatory aspects of xylem differentiation that are masked or compensated in intact vascular tissues (Bollhöner et al., 2012). Interestingly, ACAULIS5 (ACL5), which encodes the enzyme involved in the biosynthesis of thermospermine, controls xylem specification through its action on the duration of xylem vessel differentiation (Muñiz et al., 2008). In fact, the shorter lifetime of the xylem vessels in the Arabidopsis acl5 mutant resulted in the development of small spiral-type xylem vessels (instead of the normally predominant pitted vessels observed in the wild type), absence of xylem fibres, and early cell death in vessels, suggesting that ACL5 prevents premature xylem maturation and cell death. Likewise, the addition of thermospermine blocked TE differentiation almost completely in the Zinnia in vitro system (Kakehi et al., 2010), which could be due to accentuated protection against premature TE differentiation.

In that scenario, SAC51 [a basic helix–loop–helix (bHLH) transcription factor SUPPRESSOR OF ACAULIS51], which is a putative target of ACL5, acts as a negative regulator of the expression of genes which control xylem vessel maturation, so it is proposed that ACL5 (or thermospermine) blocks xylem maturation by activating translation of SAC51 (Vera-Sirera et al., 2010). In this way, the translational control of SAC51 by thermospermine ensures an appropriate level of the SAC51 bHLH transcription factor that acts as a negative regulator of cell death-related genes (e.g. those encoding nucleases and cysteine proteases). According to this model, the lack of thermospermine would decrease the production of the bHLH transcription factor, which would result in derepression of cell death genes. Currently, there is no experimental evidence for the regulation of translation efficiency by thermospermine, or for the direct regulation of cell death genes by SAC51 (Vera-Sirera et al., 2010).

On the other hand, an inhibitory role in TE cell death has also been assigned to a NAC transcription factor, XYLEM NAC DOMAIN1 (XND1). A loss-of-function mutation in XND1 resulted in Arabidopsis cell size and rate differentiation similar to those observed in the acl5 mutant, suggesting that XND1 and ACL5 function in the same way. Therefore, it is possible that ACL5 and its putative target SAC51 act upstream of XND1 to control the rate of TE differentiation (Bollhöner et al., 2012).

In the light of genetic and pharmacological experiments, TE cell death seems to be co-regulated with secondary cell wall formation, and it has been impossible to separate these two tightly linked processes in the Zinnia in vitro culture system. However, the occurrence of TE cell death without any secondary wall formation has been reported in at least two Arabidopsis mutants (Cools et al., 2011). Therefore, it seems probable that secondary cell wall formation and cell death are induced through a common signalling cascade, diverting at some point to control the two processes separately (Bollhöner et al., 2012).

Evidence for this cascade came from the analysis of the NAC domain transcription factors VASCULAR-RELATED NAC-DOMAIN6 and 7 (VND6 and VND7) and SECONDARY WALL-ASSOCIATED NAC-DOMAIN PROTEIN1 (SND1), amongst others. VND6, VND7, and SND1 are expressed in TEs, and their overexpression induced ectopic secondary wall formation with a pattern that was reminiscent of TEs in Arabidopsis (Zhong et al., 2006; Yamaguchi et al., 2008). Also, dominant repression of each of these factors led to inhibition of secondary wall formation in Arabidopsis specific cell types (Zhong et al., 2007), so these transcription factors work as master switches of secondary cell wall formation. Surprisingly, post-translational activation of both VND6 and VND7 resulted in transcriptional activation of genes involved in not only secondary wall formation but also cell death. This activation was mediated by the binding of VND6 and VND7 to promoter regions carrying the TE-regulating cis-element (TERE) (Ohashi-Ito et al., 2010; Yamaguchi et al., 2011), suggesting that these NAC master switches, VND6 and VND7, regulate the expression of both cell death- and secondary cell wall-related genes, and TE cell death was an integral part of TE differentiation.

Experiments in the Zinnia in vitro system and Arabidopsis and Zinnia plants provided evidence for a non-cell-autonomous post-mortem lignification

Secondary cell walls are reinforced by lignins during xylem maturation and TE differentiation. It has been speculated that TE lignification occurs at least partially after cell death, during TE differentiation, but no efforts were taken to track the dynamics of lignin deposition. It has been observed that the addition of lignin precursors to already lignified cells induced through a common signalling cascade, diverting at some point to control the two processes separately (Bollhöner et al., 2012).
(2011) showed that lignin deposition occurs post-mortem (i.e. after TE cell death) using live-cell imaging of differentiating Arabidopsis TEs, although the inter-relationship between these processes was not tested. Post-mortem lignification implies that TE lignification occurs by the release of monolignols from dying TEs and/or in a non-cell-autonomous manner in which other cells are required to achieve full TE lignification after their death. Recently, Pesquet et al. (2013) demonstrated that Zinnia TEs can fully lignify after their death if the appropriate lignin monomers are supplied to the culture medium. Moreover, the fact that secondary cell walls can lignify post-mortem implies that lignin-oxidizing enzymes (including peroxidases) would be present and remain active in TE secondary walls even after cell death. Also these authors checked that lignification in xylem vessels continued post-mortem in Zinnia plants, when they analysed protoxylem and metaxylem vessels in the different internodes by Fourier transform-infrared (FT-IR) microspectroscopy, and used phloroglucinol-HCl staining on transverse sections obtained from the different internodes. In this way, the increasing trend of lignification in proto- and metaxylem vessels with the ageing of the cells supported TE post-mortem lignification in planta. Likewise, Pesquet et al. (2013) also analysed the expression and localization of CCR and CAD by multiplex in situ reverse transcription–PCR (RT–PCR), and confocal microscopy in both differentiating cells and stem sections of Z. elegans demonstrated that both TEs and non-TE cells in cultures and stems expressed these genes although only TEs lignified. Additionally, non-TE cells were able to produce both ROS and phenolic compounds required for TE post-mortem lignification, suggesting that non-TE (parenchymal) cells that surround dead TEs participate in the TE lignification process. Moreover, they used knockout Arabidopsis plants in some non-TE-expressed genes, observing that the proportion of H-, G-, and S-type lignins of xylem tissues was affected, and therefore proposed that parenchymal cells of the xylem were able to express specific genes from lignin biosynthesis acting as nurse cells to enable post-mortem TE lignification. Although the identity of the signals that triggered lignin polymerization remain unclear, it is evident that TE lignification relies on interactions with other cells of the xylem, and that ROS signalling is involved (Gómez-Ros et al., 2006; Pesquet et al., 2013). Considering that TEs lignify after their cell death, it can be assumed that a large set of genes/transcription factors must be involved in the regulation of TE lignification in a non-cell-autonomous manner, although further studies are required to show whether their function is directly related to post-mortem TE lignification (Pesquet et al., 2013).

Involvement of a basic peroxidase in TE differentiation

The last step in lignin biosynthesis, which involves the oxidation of p-hydroxycinnamyl alcohols to form the different monomers, is catalysed by both laccases and, especially, peroxidases (Ros Barceló, 1997). Peroxidases have been widely reported to be expressed during differentiation of TEs. In the early 1980s, Fukuda and Komamine (1982) reported that peroxidase activity was correlated to TE formation. They described a peak of peroxidase activity, especially in the ionically cell wall-bound fraction, just before lignification, and during active lignin biosynthesis. Hence, peroxidase was considered as a marker of the transdifferentiation of mesophyll cells. However, they were not able to associate one specific isoenzyme with this process. Church and Galston (1988) identified one cationic peroxidase as a marker in the transdifferentiation process, describing the appearance of this isoenzyme not only before lignification, but even before TEs become visible. Sato et al. (1995a) correlated one cationic peroxidase to lignin formation, by means of the substrate affinity and abundance of this isoenzyme during TE formation. Later, this peroxidase was purified from Zinnia roots and its amino acid sequence was partially resolved (Sato et al., 1995b). Further evidence of the participation of peroxidases in differentiating Zinnia TEs, in this case achieved by gene expression methodology, came from the experiments of Demura et al. (2002), who performed microarray analysis with a cDNA library prepared from Zinnia cultured cells transdifferentiating into TEs. They reported the specific expression of a sole peroxidase during the stage where lignification of the secondary cell wall takes place. However, they did not characterize it.

One of the main questions that researchers have been dealing with, concerning TE differentiation, lies in confirming that the transdifferentiation process occurring in mesophyll cells, including the whole array of networks, also takes place in the differentiation into xylem cells in plants. As a matter of fact, secondary cell wall thickenings differ in lignifying xylem and tracheary single-cell cultures, suggesting the existence of different networks of signals controlling cell wall thickenings in the two systems. López-Serrano et al. (2004) identified a strongly cationic peroxidase in hypocotyls, stems, and leaves of Zinnia, which happened to be located in the cell wall. A strongly cationic peroxidase was also found to be specifically expressed during the transdifferentiation of Z. elegans mesophyll cells. This peroxidase was absent from mesophyll cells and the culture medium of cells that do not undergo transdifferentiation. Moreover, its expression was correlated with other proteins known to be markers of TE differentiation in cell cultures, such as an RNase (Stacey et al., 1995; Fukuda, 1997). Based on the isoenzyme pattern obtained by isoelectric focusing (IEF), the strongly basic peroxidase expressed during differentiation of Zinnia TEs is the same one that was expressed by the stems and the hypocotyls. López-Serrano et al. (2004) concluded that the basic peroxidase expressed during TE formation was a marker of this process and, even more, Z. elegans used one sole peroxidase for xylem lignification, both in TEs and in lignifying xylem, suggesting the existence of only one lignification programme regardless of the different ontogenesis pathways.

The fact that only one peroxidase was expressed during TE differentiation, as well as during xylem lignification, led to further molecular cloning and characterization of this basic peroxidase (Gabaldón et al., 2005a). These authors developed specific antibodies against this purified peroxidase, which specifically labelled one strongly cationic peroxidase expressed in hypocotyls, stems, and TEs. These data confirmed the
previous assumption that the peroxidase expressed in xylem cells and TEs was the same (Gabaldón et al., 2005a).

In 2006, Sato et al. reported the molecular cloning of a cationic peroxidase that was specifically expressed during the transdifferentiation of Zinnia mesophyll cells into TEs and also in immature xylem vessels in Zinnia seedlings. This peroxidase, named ZPO-C, is particularly interesting since it was able to oxidize sinapyl alcohol and coniferyl alcohol. ZPO-C was found to be ionically bound to the cell wall.

**Characterization and regulation of Z. elegans basic peroxidase**

**Biochemical and molecular characterization of Zinnia basic peroxidase**

Peroxidase activity has been found in many lignifying tissues or cell cultures, where these enzymes are usually present in a large number of isoforms, which can also be generated by post-transcriptional and post-translational modifications (Welinder et al., 2002). This feature, along with the low substrate specificity, makes specific functions of individual peroxidases difficult to assign (Hiraga et al., 2001). Regarding peroxidases that may participate in lignification of cell walls, the assignment of the putative function is based on their catalytic properties and the correlation of peroxidase activity or gene expression with active lignification. Unfortunately, the isolation of peroxidases with a known role in lignification has not been achieved so frequently. However, several peroxidases involved in lignification have been fully sequenced and characterized in gymnosperms and angiosperms (Christensen et al., 1998; Østergaard et al., 2000; Quiroga et al., 2000; Koutaniemi et al., 2005; Gabaldón et al., 2005a; Sato et al., 2006). Most of these characterized peroxidases show specific catalytic properties, and are able to oxidize the three p-hydroxyxinnamyl alcohols.

In Z. elegans, two peroxidases have been cloned and characterized to date, ZePrx (Gabaldón et al., 2005a) and ZPO-C (Sato et al., 2006). While mRNA encoding ZePrx was isolated from hypocotyls, ZPO-C mRNA was isolated from mesophyll cells that had been cultured for 48 h in the appropriate medium to undergo TE differentiation. Despite the fact that the literature describes a main peroxidase isoenzyme in hypocotyls and TEs, with the same PI and Mx (López-Serrano et al., 2004), these two peroxidase isoenzymes differ in nucleotide sequence. It should be mentioned that none of these sequences matches with the tryptic fragments previously described by Sato et al. (1995b) for a peroxidase purified from Zinnia roots.

The ZPO-C mRNA was specifically expressed in differentiating TEs, with active secondary cell wall thickening, and no gene expression was detected when mesophyll cells were not differentiating (Sato et al., 2006). ZePrx was also detected in active differentiating TEs, and in roots, hypocotyls, stems, and SCCs, but not in cotyledons or leaves (Gabaldón et al., 2005a). Both proteins were purified and their kinetic properties characterized. Whereas Sato et al. (2006) purified a recombinant protein (ZPO-C:6×His), ZePrx was purified from the spent medium of SCCs, which according to previous assays was demonstrated to be a valuable source of the enzyme (Gabaldón et al., 2005a).

As Ros Barceló and Pomar (2001) reported previously for a protein extract of Zinnia, the best substrate for ZePrx was the sinapyl alcohol (Gabaldón et al., 2005a). The preference for sinapyl over coniferyl alcohol or its aldehyde is an exceptional feature shown by this Zinnia peroxidase and not so commonly found in other lignification-related peroxidases (de Marco et al., 1999; Martínez-Cortés et al., 2012). Even many peroxidases with a recognized role in lignification, such as Arabidopsis ATP A2, are unable to use sinapyl alcohol as substrate (Østergaard et al., 1996, 2000). In contrast, the recombinant ZPO-C:6×His preferred coniferyl alcohol over sinapyl alcohol as substrate, with an enzymatic activity ~5-fold higher for coniferyl alcohol (Sato et al., 2006).

This differential capacity for oxidizing S moieties has defined a new type of peroxidases, named syringyl peroxidases (Ros Barceló et al., 2007), which will be reviewed later. Peroxidase isoenzymes capable of oxidizing S moieties have been described in several angiosperms such as tomato, poplar, Zinnia, and sugarcane (Brownleader et al., 1995; Christensen et al., 1998; Gabaldón et al., 2005a; Cesarino et al., 2013), as well as gymnosperms, ferns, liverworts, and mosses (Gómez-Ros et al., 2007a; Espiñeira et al., 2011), whose lignins are not usually composed of S units (Vanholme et al., 2010).

**Zinnia basic peroxidase is involved in the oxidation of monolignols**

Cell cultures have become a useful tool to study lignification as well as enzymes participating in monolignol oxidation, since the culture medium can be considered as the apoplastic fluid which is easily isolated (Kärkönen and Koutaniemi, 2010). However, the lignin induced after stress (including shaking of SCCs) often differs from developmental lignin and contains more H units (Kärkönen and Koutaniemi, 2010). Nevertheless, the lignification pattern of Z. elegans SCCs closely resembles that occurring in hypocotyls, the plant organ from which SCCs were derived. SCC lignins are composed of G/S units in a 38/62 ratio, while the G/S ratio found in hypocotyl lignins was 42/58 (Gabaldón et al., 2006), indicating that Zinnia SCCs are a model system for characterizing the enzyme responsible for the oxidative coupling of monolignols into lignins.

**Regulation of the basic peroxidase of Zinnia by hormones, hydrogen peroxide, and nitric oxide**

Though several peroxidase genes have been cloned, there is an important lack of data regarding regulation of peroxidase gene expression. Recently, the promoter of ZePrx has been cloned and sequenced (Gutiérrez et al., 2009), and the responsive elements described (Gutiérrez et al., 2009; López Nuñez-Flores et al., 2010; Gómez-Ros et al., 2012). Peroxidases have multiple functions and are able to oxidize several substrates, so it is expected that gene expression of these enzymes is regulated by a broad plethora of stimuli. The promoter of ZePrx
has been shown to have elements responsive to hormones involved in xylem differentiation, such as auxins, cytokinins, and BRs (Gutiérrez et al., 2009), as well as gibberellic acid (López Núñez Flores et al., 2010), and H₂O₂ and NO (Gómez-Ros et al., 2012). Gutiérrez et al. (2009) described that ZePrx activity was enhanced when Zinnia plants were treated with auxins and cytokinins, but was repressed with the addition of BRs. When auxins, cytokinins, and BRs were added in combination, the inhibitory effects of BRs predominate over the activating effects of auxins and cytokinins. These results mirrored the induction or repression of secondary growth caused by the addition of the above-mentioned hormones (Gutiérrez et al., 2009). The essential and necessary role of an auxin flow in xylem cell differentiation and TE formation is well known (Fukuda, 1997; Ohashi-Ito and Fukuda, 2010), as is the importance of cytokinins and BRs in procambial cell formation and vascular cell elongation (Ye, 2002). Moreover, BRs regulate vascular differentiation, promoting xylem formation (Fukuda, 2004). Recently the in vitro induction of TEs in Populus was reported (Yamagishi et al., 2013), which, unlike most described systems, is achieved in an auxin-free medium containing BRs as the sole hormone. Thus, auxins and cytokinins, together with BRs appear to be the main hormones controlling vascular differentiation. Hence, the presence of putative cis-elements known to confer regulation by plant hormones in the ZePrx promoter is not surprising.

It is worth noting that ZPO-C was not expressed in a culture medium with different concentrations and combinations of auxins and cytokinins, suggesting that, unlike ZePrx, the expression of ZPO-C mRNA is not induced by auxin or cytokinin, but is linked to TE differentiation requiring both auxins and cytokinins (Sato et al., 2006).

The promoter of ZePrx contains several cis-elements where different transcriptional factors, such as MYB, NAC, AP2, and MADS, bind. Several MYB proteins have been shown to play an important role in regulating the lignin biosynthesis pathway, in a complex regulation cascade that has not been completely unravelled yet (Zhong and Ye, 2009). Nevertheless, these MYB transcriptional factors bind to AC elements placed in the promoters of the genes involved in lignin biosynthesis, except in the ferulate 5-hydroxylase gene (Zhou et al., 2009; Zhao and Dixon, 2011). These AC elements have also been found in the promoter of ZePrx, suggesting a coordinate regulation of lignin biosynthesis. Therefore, auxins, cytokinins, and BRs directly regulate ZePrx, and the ZePrx promoter may be the target of a set of transcription factors (NAC, MYB, AP2, MADS, and class I and III HD Zips) which are up-regulated by these hormones during secondary growth, supporting a role for ZePrx in xylem differentiation/lignification (Gutiérrez et al., 2009).

As mentioned before, H₂O₂ and NO are important biological molecules in plants, formed during xylem differentiation in Z. elegans, and apparently play important roles during xylogenesis (Gabaldón et al., 2005b; Gómez-Ros et al., 2006). It has been shown that treatments with H₂O₂ and an NO donor (S-nitroso glutathione) provoke the induction of ZePrx activity in Zinnia seedlings, as well as the time-dependent induction of ZePrx transcripts as determined by quantitative PCR, suggesting that H₂O₂ and NO modulate the expression of ZePrx (Gómez-Ros et al., 2012).

**Syringyl peroxidases pre-date the angiosperm–gymnosperm divergence**

At this point, two peroxidases had been described as responsible for the last step of lignification in the transdifferentiation of Zinnia mesophyll cells into TEs (López-Serrano et al., 2004; Gabaldón et al., 2005a; Sato et al., 2006). However, judging from their partial amino acid sequences, these two peroxidases were two different proteins, but, interestingly, both peroxidases were highly basic proteins. The role of basic or acidic peroxidases responsible for cell wall lignification has always been controversial. In general, both acidic and basic peroxidases are able to oxidize coniferyl alcohol, but acidic peroxidases are reported as poor catalysts of sinapyl alcohol, which is surprising since sinapyl alcohol is more prone to oxidation than coniferyl alcohol. These results suggest that substrate accommodation in the catalytic centre of the enzyme determines the real role played by each peroxidase isoenzyme in lignin biosynthesis (Ros Barceló et al., 2004). Studies on ATP A2 of Arabidopsis have concluded that the overlapping of I138 and P139 from the substrate-binding site with the methoxy group in sinapyl alcohol hinders the docking of the substrate (Østergaard et al., 2000). This overlap apparently does not occur at the substrate-binding site in most peroxidases, where the I138 residue is substituted by an L138 (Ros Barceló et al., 2004).

Previous studies have demonstrated that the peroxidase complement of Z. elegans, unlike many peroxidases, had high affinity for sinapyl alcohol, which undoubtedly supports the role of this peroxidase in lignin formation. It might be expected that a peroxidase capable of oxidizing both coniferyl and sinapyl alcohols should be highly conserved during the evolution of vascular plants, since its presence would confer a great economy to the expensive process of lignin assembly. Ros Barceló and Aznar Asensio (2002) performed IEF analysis that revealed the presence of strongly basic peroxidases homologous to those of Zinnia in several species of the Asteraceae family. Moreover, the ionically cell wall-bound fraction showed high peroxidase activity when syringaldazine, a chemical analogue of sinapyl alcohol, was used.

Taking into account that the peroxidase isolated from Z. elegans was present in other Asteraceae members and was responsible for lignification in both xylem cells and TEs, one question arose: was this peroxidase involved in lignification conserved during plant evolution? IEF analysis revealed, in different woody and herbaceous angiosperms (Ros Barceló et al., 2003; Gómez-Ros et al., 2007b), the presence of peroxidases homologous and analogous to the strongly basic peroxidase from Z. elegans. Moreover, these species had peroxidase isoenzymes able to use sinapyl alcohol or syringaldazine as substrates. The capacity of these homologous peroxidases to oxidize sinapyl alcohol highly is important since angiosperm lignins are composed of G and S moieties. However, these strongly basic peroxidases were also able to oxidize coniferyl...
alcohol, ultimately to form G moieties, and may be present in other plant species. Again, the IEF technique was chosen to identify homologous peroxidases in non-angiosperm species. Interestingly, peroxidases with a similar pI to those of *Zinnia* were found in gymnosperms and other basal land plants, such as ferns, lycophytes, and even liverworts, regardless of the presence of S groups in their lignins (Ros Barceló et al., 2004; Gómez Ros et al., 2007a; Espíñeira et al., 2011). These results suggest that basic peroxidases should be present in ancestral plant species, prior to the radiation of seed plants, and that these preserved isoenzymes might have an important specific role in the lignification of cell walls.

The unique catalytic properties of ZePrx have led to the consideration of a particular and novel subgroup within class III plant peroxidases, for which the name S peroxidases has been proposed (Gabaldón et al., 2006). These peroxidases are characterized by the capacity to oxidize sinapyl alcohol or syringaldazine with high affinity (Ros Barceló et al., 2007). Ros Barceló et al. (2007) identified the structural motifs that determine the S peroxidases, based on comparing the amino acid sequence of ZePrx with the amino acid sequences of a set of peroxidases whose capacity for oxidizing S moieties was well known, and the sequence of two typical G peroxidases, the ATP A2 and the HRP A2 (Fig. 7). The structural motifs, differentially present in all of the S peroxidases and absent from the G peroxidases, are necessary and sufficient to determine the S-oxidizing activity of peroxidases (Ros Barceló et al., 2007). Moreover, the determinants of S peroxidases have been conserved since the first 15 million years of angiosperm history because they are found in peroxidases from the two major lineages of flowering plants (Fig. 7, Gómez-Ros et al., 2007a).

![Fig. 8A](https://example.com/fig8a.png)  ![Fig. 8B](https://example.com/fig8b.png)

Taking into account that the structures of only a few peroxidases have been resolved, in order to shed light on the ZePrx tertiary structure, theoretical comparative 3D models of ZePrx and ATP A2 were constructed (Fig. 8) from the primary structure of the corresponding mature proteins to explain the differential catalytic properties between G and S peroxidases (Ros Barceló et al., 2007). Some significant differences emerged between the ZePrx and ATP A2 tertiary structure. For example, in comparison with the G peroxidase ATP A2 (Fig. 8A), helix D' was absent from the *Z. elegans* S peroxidase (Fig. 8B). This observation might be important because, in ATP A2 peroxidase, helix D' relies on the haem prosthetic group (Fig. 8A, C). The absence of helix D' in S peroxidases represents a relaxation factor for the haem crevice, enabling the docking of S moieties because helix D' fixes the IPS motif in ATP A2, which determines the conformation and hydrophobicity of the substrate-binding site (Østergaard et al., 2000) (Fig. 8A, C).

Similarly to the helix D', the β-strand I (R173–R175) in ATP A2 was absent from all of the S peroxidases, in which novel β-strands were found instead (Gómez-Ros et al., 2007b). Special attention should be paid to the novel β-strand in ZePrx, which is upstream of the proximal histidine (Fig. 8B, D), and which therefore influences the catalytic centre of the enzymes. All of these factors are likely to condition the substrate specificity of S peroxidases, determining the unique catalytic properties, although their real importance in catalysis should be confirmed by crystallographic data and site-directed mutagenesis.

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<th>Arabidopsis</th>
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Fig. 7. Structural alignment of G and S peroxidases. Structural motif descriptions of S peroxidases were determined by alignment of the *Zinnia elegans* peroxidase (AJ880395) with *Picea* (AJ809340), *Populus* (BAE16616), *Asparagus* (ABO42103), *Lycopersicon* (L136635), *Pinus* (AAG02215), and *Ceratoptersis* (BE643121) peroxidases, a set of peroxidases whose capacity for oxidizing S moieties is well known (Quiroga et al., 2000; Gabaldón et al., 2005a; Koutaniemi et al., 2005). Structural determinants of S peroxidases (in red) were deduced by comparing amino acid sequence motifs common to these seven peroxidases with the amino acid sequence motifs of two typical G peroxidases, the *Arabidopsis* peroxidase, ATP A2 (X999952), and the horseradish peroxidase, HRP A2 (P80679). Alignments for the peroxidases selected revealed highly conserved amino acid residues common to all peroxidases (in yellow), and partially conserved amino acid residues of ATP A2 and HRP A2 present in some of the other peroxidases (in green). The amino acid residues I138, P139, and S140 (asterisks) determine the conformation and hydrophobicity of the substrate-binding site in ATP A2 (Østergaard et al., 2000).
Current knowledge of the involvement of peroxidases in oxidative dehydrogenation

Current approaches to determination of the function of specific peroxidase isoenzymes are focused on studying the suppression or overexpression of the genes encoding peroxidases. Regarding the identification of a peroxidase isoenzyme involved in monolignol oxidation, several works were carried out in the last 20 years, with different outcomes. El-Mansouri et al. (1999) overexpressed a basic peroxidase from tomato, resulting in an increment of lignin content in transgenic plants, and the antisense suppression of a basic peroxidase in tobacco caused a reduction of lignin levels (Talas-Ogras et al., 2001). In transgenic lines of aspen, lignin content was reduced up to 20% with the down-regulation of PrxA3a, an acidic peroxidase (Li et al., 2003). Nevertheless, the down-regulation of an acidic isoenzyme of tobacco did not produce a decrease in lignification (Lagrimini et al., 1997). These results suggest it is not an individual peroxidase that is responsible for lignification, but some isoenzymes would participate in lignin formation.

Other attempts were made to identify lignin-specific peroxidases by purification from French bean. In fact, the subsequent cloning of the cDNA from a purified French bean cell wall cationic peroxidase made it possible to screen a cDNA library derived from lignifying tobacco stems. Amongst the clones obtained, TP60 showed a regulated high expression during xylogenesis in a differentiating tissue culture system. The functional analysis of TP60 was investigated by antisense expression, but the transformed plants only showed few phenotypic changes without any apparent significant effect on growth. However, the major physiological change produced by down-regulating TP60 was on overall lignin deposition which decreased up to 50% together with a reduction of the levels of G and S units (Blee et al., 2003). More recently, Kavousi et al. (2010) analysed a tobacco transgenic line (1074) screened from plants expressing the antisense TP60. Morphologically 1074 plants had underdeveloped xylem, with both fibres and vessels having thin cell walls and limited secondary wall thickening. Despite these changes, the mutant plants grew and were viable, indicating that the lignification pattern can be manipulated considerably without causing any serious deleterious effects on their overall growth, which means that other specific cell wall peroxidases may overcome limitations of those which were down-regulated. Therefore, the down-regulation of specific lignin peroxidases using antisense strategies cannot identify the specific peroxidases involved in the oxidative dehydrogenation of lignin precursors, although it is a valid strategy for modifying the lignin content in herbaceous and woody species.

Recently, Shigeto et al. (2013) have found in Arabidopsis three cationic cell wall-bound peroxidase homologues to a cationic cell wall-bound peroxidase (CWPO-C) from Populus alba (Sasaki et al., 2004), which was previously reported to be involved in lignification. The analysis of the Arabidopsis knock-out phenotypes provided in vivo evidence that these three CWPO-C homologues (AtPrx2, AtPrx25, and AtPrx71) were involved in the lignification of the stems. In a similar way, Herrera et al. (2013) reported the involvement of AtPrx72, a peroxidase homologue to ZePrx of Zinnia, in stem lignification of Arabidopsis. The knock-out mutants showed a lower amount of lignin as well as a decrease in syringyl units, mainly in the interfascicular fibres, while the xylem lignins showed no particular alteration.

These types of studies may help to explain the elusive mechanism of lignin polymerization by plant peroxidases and to identify which are the peroxidases responsible for lignification in vascular tissues.

Acknowledgements

We would like to dedicate this review to the memory of Professor Alfonso Ros Barceló, who passed away in January 2012. His absence is felt as mentor, man of science, and friend. Being able to continue with his work is an honour. This work was supported by the Ministerio de Ciencia e Innovación (MICINN, Spain), the European Commission FEDER (BFU2009-08151), and the Fundación Séneca, Agencia de Ciencia y Tecnología de la Región de Murcia en el marco de II PCTRM 2007-10 (08610/PI/08). FF-P and JH hold a FPU fellowship, and EN-U holds a JdC grant from MICINN (Spain).

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