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Research article

Looking for *Arabidopsis thaliana* peroxidases involved in lignin biosynthesis

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ABSTRACT

Monolignol polymerization into lignin is catalyzed by peroxidases or laccases. Recently, a *Zinnia elegans* peroxidase (ZePrx) that is considered responsible for monolignol polymerization in this plant has been molecularly and functionally characterized. Nevertheless, *Arabidopsis thaliana* has become an alternative model plant for studies of lignification, filling the gaps that may occur with *Z. elegans*. The arabidopsis genome offers the possibility of performing bioinformatic analyses and data mining that are not yet feasible with other plant species, in order to obtain preliminary evidence on the role of genes and proteins.

In our search for arabidopsis homologs to the ZePrx, we performed an exhaustive *in silico* characterization of everything from the protein to the transcript of *Arabidopsis thaliana* peroxidases (AtPrxs) homologous to ZePrx, with the aim of identifying one or more peroxidases that may be involved in monolignol polymerization.

Nine peroxidases (AtPrx 4, 5, 52, 68, 67, 36, 14, 49 and 72) with an E-value greater than 1e-80 with ZePrx were selected for this study. The results demonstrate that a high level of 1D, 2D and 3D homology between these AtPrxs and ZePrx are not always accompanied by the presence of the same electrostatic and mRNA properties that indicate a peroxidase is involved in lignin biosynthesis.

In summary, we can confirm that the peroxidases involved in lignification are among AtPrx 4, 52, 49 and 72. Their structural and mRNA features indicate that exert their action in the cell wall similar to ZePrx.

1. Introduction

The xylem constitutes the longest pathway for water transport in vascular plants. It is a simple, low resistance pathway which enables water to be transported in large quantities with great efficacy, especially from the roots to the leaves [1]. Most terminally differentiated cells fulfill specialized functions until they die, but in the case of the xylem, its function does not really begin until after cell death [2]. Thus, functional water-conducting cells have no membranes or organelles, and the remaining thick lignified cell walls form hollow tubes through which water can flow with relatively little resistance [1]. Terminal xylem elements (water-conducting cells) are internally coated with lignins, which confer resistance against tensile forces of the water columns, provide structural support and flexural stiffness to the aerial organs and impart water impermeability. Lignins can also provide protection against the microbial degradation of cell walls [3]. In this context, plant cell wall lignification is one of the main restrictive factors in the use and recycling of plant biomass [4].

Lignins constitute the most abundant organic compound on the surface of the Earth after cellulose, accounting for 25% of plant biomass [5]. They are found specifically in vascular plants (Tracheophyta) [5], and occur selectively in the secondary cell walls of xylem vessels and tracheids but also in fibers and sclereids. Lignins have been identified in pteridophytes (ferns, lycophytes and horsetails), the first vascular plants, and probably played a key role in the colonization of the terrestrial landscape by plants during the Ordovician–Silurian transition, 400–450 million years ago [5].

Lignins are three-dimensional, amorphous heteropolymers that are the result of oxidative coupling of three p-hydroxycinnamyl alcohols: p-coumaryl, coniferyl and sinapyl alcohols, in a reaction mediated both by laccases and class III plant peroxidases [5], although it remains unclear whether laccases participate in constitutive lignification [6,7]. This cross-coupling reaction produces an optically inactive hydrophobic heteropolymer [8] composed of

Abbreviations: AREs, AU-rich regions; AtPrx, *Arabidopsis thaliana* peroxidase; DST, downstream sequence; ORF, open reading frame; UTR, untranslated region; ZePrx, basic peroxidase isoenzyme from *Zinnia elegans*.

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p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively. Lignins are therefore ill-defined phenolic heteropolymers, whose monomeric composition varies greatly and which are covalently associated with certain polysaccharides, probably of a pectin nature [9], in the plant cell wall.

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 [10]. Plants do not possess a mechanism to degrade lignins [11], 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.

One of the most important and critical stages in lignin biosynthesis is the process that occurs through the oxidation of various monomer precursors and their subsequent assembly in the cell wall.

From a metabolic (and probably functional) point of view, this is the only specific step in the biosynthesis pathway of lignins. This step, which also renders metabolic flow through the route irreversible, is catalyzed by peroxidase.

Peroxidases (class III plant peroxidases, EC 1.11.1.7) are the main enzymes involved in the process of monolignol assembly that leads to lignin biosynthesis. They are heme-containing enzymes which catalyze the one-electron oxidation of the three monolignols (RH) at the expense of H_2O_2, yielding phenoxy radicals (R•) and water [12]:

$$2RH + H_2O_2 \rightarrow 2R^{\bullet} + 2H_2O$$

Class III plant peroxidases are usually classified into acidic (isoelectric point below 7.0) and basic (isoelectric point above 7.0) peroxidases. Both p-coumaryl alcohol and coniferyl alcohol (in the case of most acidic peroxidases), and p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (in the case of most basic peroxidases), are thus dimerized and polymerized in xylem cell walls in a reaction dependent on H_2O_2 [13] delivered by an NADPH-oxidase-like enzyme [14].

In addition, class III plant peroxidases belong to a multigene family, whose evolution seems to be correlated with the increasing complexity of plant cell wall architecture [15,16], and it is known that a high duplication rate in some plants has led to large multigene families, as suggested by the presence of 138 peroxidase-encoding genes in *Oryza* [16], and 73 peroxidase-encoding genes in Arabidopsis [17–19]. Either subfunctionalization or neofunctionalization could explain the conservation of these duplicate genes and the presence of such large multigene families in which each paralog could become specialized for a determined task.

Participation of class III plant peroxidases in lignin synthesis has been studied in many plant species and tissue culture systems [20–23]. Most of these studies have been based on finding peroxidases with suitable catalytic properties, i.e., the ability to oxidize monolignol substrates and the proper localization of proteins or gene expression in lignifying xylem and, more specifically, within the lignifying cell walls [24].

Although many studies on anionic peroxidases have been reported, e.g. anionic AtPrx53 (ATPA2) [21,22], evidence of cationic peroxidases involved in lignification has also been reported, e.g. a cationic peroxidase of tomato with pI 9.6 [24,25], and more importantly, the cationic peroxidase from *Zinnia elegans* (ZePrx) has been shown to be involved in lignification [26].

In this latter case, the ZePrx is the only one to have recently been molecularly and functionally characterized [26–28], and it is considered responsible for the process of lignification in Zinnia plants [28].

This basic peroxidase is highly conserved, probably due to the fact that lignification has been an important process during plant evolution, has a high capacity to oxidize both coniferyl and sinapyl alcohols and has been located in lignifying secondary cell walls in zinnia hypocotyls and cell suspension cultures [26,27,29]. *Z. elegans* is an annual flowering plant belonging to the Asteraceae family which is frequently used as a model for lignification studies [30]. The cell wall of lignifying *Z. elegans* hypocotyls and stems contain a basic peroxidase (EC 1.11.1.7) of pI ~ 10.2, which shows coniferyl alcohol oxidase activity [31,32].

The lignification pattern of *Z. elegans* seedlings is unique in that, at a specific developmental stage, they simultaneously offer two models of lignification that closely resemble those occurring in gymnosperms and angiosperms. Furthermore, *Z. elegans* [33] and, recently, arabidopsis (*Arabidopsis thaliana*) [34] offer the unique possibility of working with cell cultures that resemble differentiating xylem cells and constitute exceptionally useful models for monitoring the expression of enzymes from the lignin biosynthetic pathway, especially the segment that is concerned with the phenylpropanoid backbones [35,36].

The *Z. elegans* peroxidase (ZePrx) has been cloned and its primary structure determined [26]. 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) [26,37,38].

All these constraints strongly suggest that this peroxidase is one of the enzymes responsible for cell wall lignification, a consideration which is supported by its localization in lignifying xylem vessels [13,39].

However, *Z. elegans* is recalcitrant to transformation processes using the technique of T-DNA with *Agrobacterium tumefaciens*.

In contrast, arabidopsis has become a model plant in studies of xylem development [40]. In arabidopsis, secondary growth can occur in the root, hypocotyl and inflorescence stem, and the architecture of these secondary tissues is very similar to that in trees (e.g. *Populus* sp.) [41]. Therefore, *Arabidopsis* is a perfect plant model for studies of lignification, filling the gaps that may occur with *Z. elegans*.

Here, we report our search for *Arabidopsis thaliana* homologs to the ZePrx implicated in lignification, for which we performed an exhaustive bioinformatic characterization of everything from the protein to the transcript of *Arabidopsis thaliana* peroxidases homologous to ZePrx, with the aim of identifying one or more proteins that could shed light on the last step of lignin biosynthesis. Homology studies at 1D, 2D and 3D together with studies of stability and longevity/half-life of mRNA were carried out with the aim of identifying the structural characteristics that a peroxidase involved in lignification must fulfill.

The results show that a high structural homology is not sufficient to indicate the function of a protein, and other parameters such as surface charge, post-translational modifications, amino acid positions and stability and longevity/half life of mRNA are necessary to obtain a good approximation.

2. Results and discussion

2.1. Blast analyses and phylogenetic tree

The *Arabidopsis thaliana* genome encodes 73 peroxidases. We searched for those peroxidases which presented a higher homology with the basic peroxidase from *Z. elegans* (ZePrx), an enzyme involved in lignification [26]. BLAST analysis (http://www.arabidopsis.org) was conducted to verify the homology of ZePrx with the entire *A. thaliana* genome, and an alignment between the sequence of interest and the closest homolog peroxidases was performed.

The results of the analysis are shown in Table 1. Ten peroxidases with an E-value greater than 1e-80 with ZePrx were selected for