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Review

Roles of cell wall peroxidases in plant development

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This paper forms part of a special issue of *Phytochemistry* dedicated to the memory and legacy of Professor (Godfrey) Paul Bolwell, MA DSc (Oxon). (1946–2012), internationally-recognised plant biochemist and Regional Editor of *Phytochemistry* (2004–2012). He is much missed by his friends.

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ABSTRACT

Class III peroxidases (CIII Prxs) are plant specific proteins. Based on *in silico* prediction and experimental evidence, they are mainly considered as cell wall localized proteins. Thanks to their dual hydroxylic and peroxidative cycles, they can produce ROS as well as oxidize cell wall aromatic compounds within proteins and phenolics that are either free or linked to polysaccharides. Thus, they are tightly associated to cell wall loosening and stiffening. They are members of large multigenic families, mostly due to an elevated rate of gene duplication in higher plants, resulting in a high risk of functional redundancy between them. However, proteomic and (micro)transcriptomic analyses have shown that CIII Prx expression profiles are highly specific. Based on these omic analyses, several reverse genetic studies have demonstrated the importance of the spatio-temporal regulation of their expression and ability to interact with cell wall microdomains in order to achieve specific activity *in vivo*. Each CIII Prx isoform could have specific functions *in muro* and this could explain the conservation of a high number of genes in plant genomes.

Contents

1. Introduction

Plant primary cell walls (CWs) mainly contain carbohydrate polymers and proteins. The polysaccharide part is composed of cellulose microfibrils interlaced with hemicellulose cross-linking glycans. This network is embedded within a gel matrix of pectic polysaccharides (Carpita and Gibeaut, 1993). The arabinoxylan content of primary cell walls of commelinid monocots is relatively high when compared with dicots and non-commelinid monocots

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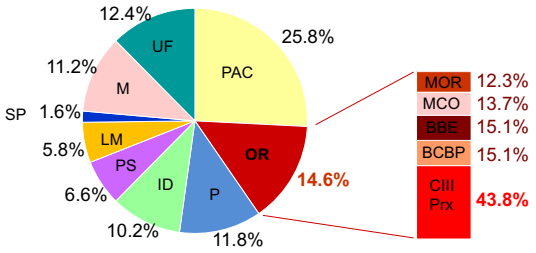

Fig. 1. Distribution of the 500 *A. thaliana* CWPs within nine functional classes. PAC: proteins acting on carbohydrates; OR: oxido-reductases; P: proteases; ID: proteins with interacting domains; PS: proteins possibly involved in signaling; LM: proteins related to lipid metabolism; SP: structural proteins; M: miscellaneous proteins; UF: proteins of yet unknown function. ORs constitute the second most enriched functional class and are subdivided as follows: MOR: miscellaneous oxido-reductases (among which only 1 laccase); MCO: multicopper oxido-reductases; MCO: multicopper oxido-reductases; BBE: berberine bridge oxido-reductases; BCBP: blue copper binding proteins; CIII Prxs: class III peroxidases. Note that CIII Prxs represent about one half of the identified ORs.

and also these polymers can be cross-linked through phenolic substitutions on the polysaccharides. In commelinid monocots, arabinoxylan-linked ferulic and *p*-coumaric acids replace xyloglucans and extensins as cross linkers. Plant CWs can be thickened in specialized cell types to form secondary walls strengthened with additional hydrophobic polymers (e.g., lignins in xylem or suberin and lignins in endodermis Casparian strips). This extracellular matrix is a dynamic structure that plays numerous roles in the physical control of growth, the establishment of cell shape and the maintenance of structural integrity of the plant body in response to environmental cues. Cell wall proteins (CWPs) also play an important role in all these processes. More than 20 proteomics studies have been performed on various *Arabidopsis thaliana* CW-enriched fractions. Around 500 different CWPs have been identified and distributed in nine functional classes (Fig. 1, Albenne et al., 2013; Jamet et al., 2008). The most abundant category contains proteins modifying carbohydrates (25.8% of the identified CWPs). Among other things, the proteins belonging to this class may contribute to CW polysaccharide disassembly and re-assembly processes. The second most abundant category of CWPs is that of oxido-reductases (OR) (14.6%) with about one half of these ORs belonging to the green plant-specific heme class III peroxidases (CIII Prxs).

Together with other CWPs, CIII Prxs are assumed to be involved in CW dynamics, (Cosgrove, 2005; Fry, 2004), but they are also supposed to play roles in other biological processes (Passardi et al., 2005). They belong to large multigenic families (e.g., 73 members in *A. thaliana*, 181 in *Eucalyptus grandis*, 138 in *Oryza sativa* and 143 in *Brachypodium distachyon*) (Fawal et al., 2013). The expansion of CIII Prx gene families (large number of genes) and gene duplication events (gene clusters specific to each species) appears to be chaotic (Dunand et al., 2011). Although there is no evidence to date, relationships between land plant emergence and plant CW evolution may exist (Passardi et al., 2004).

CIII Prxs are characterized by conserved key amino acid residues necessary for heme binding, electron transfer or 3D-structure stability (Welinder, 1992). They also contain variable domains (access channel) in relation to their substrate diversity. CIII Prxs are mainly considered as secreted/apoplastic/CW proteins (Welinder, 1992), but vacuolar isoforms also exist (Carter et al., 2004). The complex roles of CIII Prxs could be explained both by the diversity of their substrates and their spatio-temporal regulation of expression. Thus, their functional analysis remains challenging.

During plant growth, cell expansion is tightly associated to CW loosening and stiffening. The balance between these two processes can be precisely controlled by the antagonistic activities of CIII Prxs (Fig. 2). Indeed, they are able to either (i) build a rigid wall by forming bonds by oxidizing aromatic CW compounds (monolig-

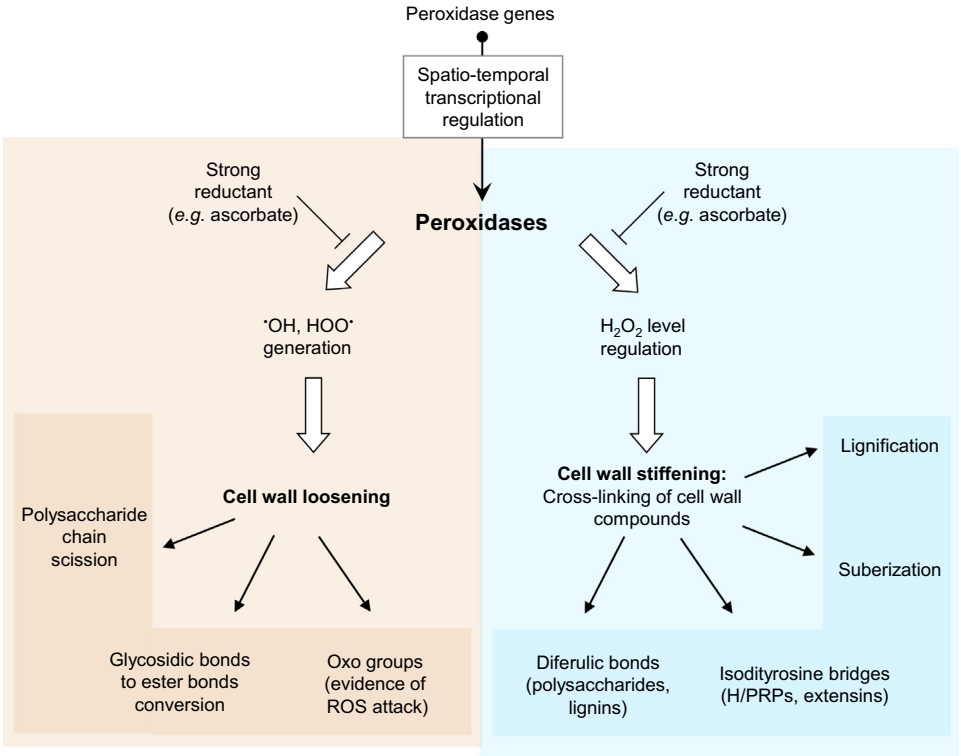

Fig. 2. Dual activity of CW peroxidases. CIII Prxs are capable of generating reactive oxygen species (ROS) such as ·OH and HOO·, but can also regulate the level of hydrogen peroxide (H_2O_2). Therefore, they play a pivotal role in cellular growth by controlling the subtle balance between CW loosening (left part of the figure) and *de novo* cell wall synthesis/cell wall strengthening (right part of the figure). H/PRPs = Hydroxyproline/Proline-Rich Proteins.

nols, cinnamic acids, aromatic amino acids...) in the presence of H_2O_2, or (ii) loosen CWs by regulating the local concentration of H_2O_2 or generating radical oxygen species (ROS) such as hydroxyl radical ($OH^{\cdot}$) which break covalent bonds in cell wall polymers (Schopfer, 2001). CIII Prxs can also control cell elongation through their auxin oxidase activity (Cosio et al., 2009). Thus, the entire expansion process could be controlled by different CIII Prx isoforms that are in turn regulated by fine transcriptional and post-transcriptional tunings.

The purpose of this review is to update the knowledge on the involvement of CIII Prx in cell wall remodelling during development, mainly focusing on *A. thaliana*. The large sets of available transcriptomic and proteomic data argue towards the drawbacks of functional redundancy. However, a few elegant reverse genetic studies have pointed to the importance of the precise *in muro* localization of some CIII Prxs with the observation of clear (micro)-phenotypes in mutants.

2. Pharmacological and biochemical approaches

The involvement of enzyme-mediated electron transfer from O_2 leading to the CIII Prx dual action mode has been studied using various pharmacological approaches (Fig. 3). Several chromogenic/fluorogenic generic substrates for CIII Prxs (such as 4-chloronaphthol, diaminobenzidine, guaiacol, *O*-dianisidine or 3′-(*p*-hydroxyphenyl) fluorescein) are available to detect H_2O_2 (in the presence of exogenous CIII Prx) and to measure CIII Prx activity (in the presence of exogenous H_2O_2) *in vitro* or *in situ*. Other aromatic substrates, such as monolignols or extensins, can also be used to obtain more information about CIII Prx activities (Brownleader et al., 2000; Magliano and Casal, 1998; Marjamaa et al., 2006). However, although purified CIII Prxs can show distinct substrate specificities *in vitro*, it does not necessarily mean that these differences are relevant *in vivo* (de Marco et al., 1999).

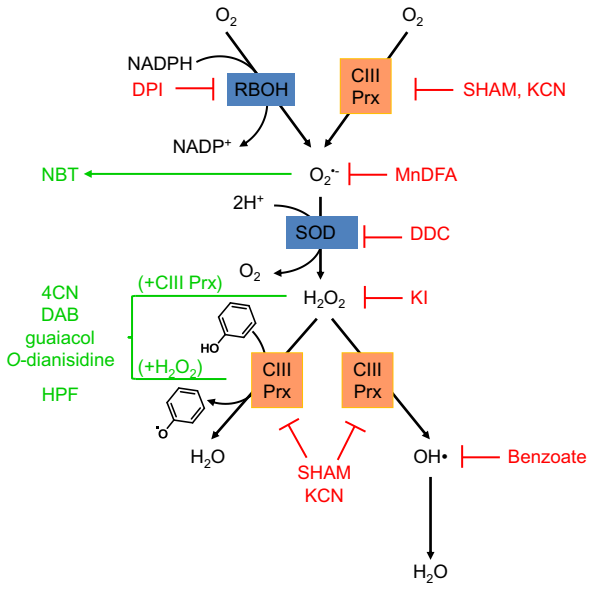

Fig. 3. CIII Prx-mediated redox electron transfer and positioning of pharmacological inhibitors and chromogenic/fluorogenic probes and substrates. The different steps of the enzyme-mediated redox electron transfer from O_2 allowing aromatic ring oxidation (peroxidative cycle on the left) and $OH^{\cdot}$ production (hydroxylic cycle on the right) by CIII Prxs are represented in black. NADPH oxidase (RBOH), superoxide dismutase (SOD) and CIII Prxs are positioned and underlined in blue and orange, respectively. Pharmacological inhibitors (in red) are positioned from the top to the bottom as follows. DPI: diphenyleneiodonium, NADPH oxidase (RBOH) inhibitor; SHAM: salicylhydroxamic acid, CIII Prx inhibitor; KCN: potassium cyanide, CIII Prx inhibitor; MnDFA: manganese desferal, $O_2^{\cdot-}$ scavenger; DDC: diethyldithiocarbamic acid, superoxide dismutase (SOD) inhibitor; KI: potassium iodide, H_2O_2 scavenger; Benzoate, $OH^{\cdot}$ scavenger; Note that KCN and SHAM inhibit all CIII Prx isoforms. Chromogenic/fluorogenic substrates and probes (in green) are positioned from the top to the bottom as follows. NBT: nitroblue tetrazolium, chromogenic probe for $O_2^{\cdot-}$; 4 CN: 4-chloronaphtol; DAB: diaminobenzidine; guaiacol and *O*-dianisidine are chromogenic probes for H_2O_2 and/or substrates for CIII Prxs depending on the addition of exogenous CIII Prx and/or H_2O_2, respectively. HPF: 3′-(*p*-hydroxyphenyl) fluorescein is a fluorogenic probe for H_2O_2 and/or substrate for CIII Prxs depending on the addition of exogenous CIII Prx and/or H_2O_2, respectively. Note that chromogenic/fluorogenic CIII Prx substrates are substrates for all CIII Prx isoforms.

The appearance or disappearance of specific CIII Prx activities during a particular biological process or at a specific localization has been reported *in vivo* (CIII Prx activity staining of tissues at the macroscopic and/or microscopic level) and *in vitro* (protein extraction, separation and Prx activity staining) (Allison and Schultz, 2004; Loukili et al., 1999). However, it is difficult to associate a band observed on a zymogram with a particular CIII Prx isoform. Indeed, protein purification from gel is not straightforward and the enzymatic activity detected is not representative of the amount of protein and of the number of isoforms present in one band. In addition, the use of generic chromogenic CIII Prx substrates does not allow the discrimination between isoforms or to determine if all the isoforms have been detected.

A whole set of enzyme inhibitors and intermediate scavengers is available (Fig. 3). The link between the production of ROS in the apoplast/CW and their role during many developmental and physiological processes is well established (Dunand et al., 2007; Lariguet et al., 2013; Lee et al., 2013). Among ROS, hydroxyl radicals ($OH^{\cdot}$) are extremely reactive, and can split covalent bonds in all kinds of organic molecules close to its production area. Initiation of $OH^{\cdot}$ production occurs from O_2 in the presence of a reductant such as NAD(P)H, but also following a reaction catalyzed by CIII Prxs (Haber–Weiss reaction) (Chen and Schopfer, 1999) (Fig. 3). Non-enzymatic cleavage by $OH^{\cdot}$ of CW polysaccharides such as pectins and xyloglucans, has been demonstrated *in vitro* with an implication in cell elongation (Fry, 1998; Schopfer, 2001).

Together with *in vivo* localization of CIII Prxs, the use of ROS probes and scavengers suggests that CIII Prx play important roles especially during germination. Indeed, ROS and CIII Prx activity are co-localized in the CW of the endosperm prior to its rupture (Lariguet et al., 2013). The involvement of CIII Prxs during testa and endosperm rupture has been suggested but no specific isoform has been identified so far. Besides, ROS released by germinating seeds may protect the emerging embryo against pathogens (Schopfer et al., 2001).

Altogether, pharmacological and *in vitro* approaches suggest that (i) one CIII Prx can oxidize different substrates (e.g., any of the generic chromogenic/fluorogenic substrates), (ii) different CIII Prxs can oxidize the same substrate (e.g., horseradish CIII Prx (HRP) and any other tested CIII Prx can be used to polymerize monolignols *in vitro*) (Demont-Caulet et al., 2010) and (iii) a single CIII Prx can be found in different tissues with different potential protein partners. These approaches give interesting clues regarding the roles of CIII Prx, but they do not reveal their specific function. As discussed in the following paragraphs, proteomic and transcriptomic analyses together with reverse genetic studies contribute to bypass this limitation.

3. Spatio-temporal regulation of gene expression and protein distribution

CIII Prxs share structural features (Welinder, 1992) suggesting similar mechanisms of action confirmed by the oxidation of the same generic substrates *in vitro*. The function of each CIII Prx may be related to spatio-temporal regulation of gene expression

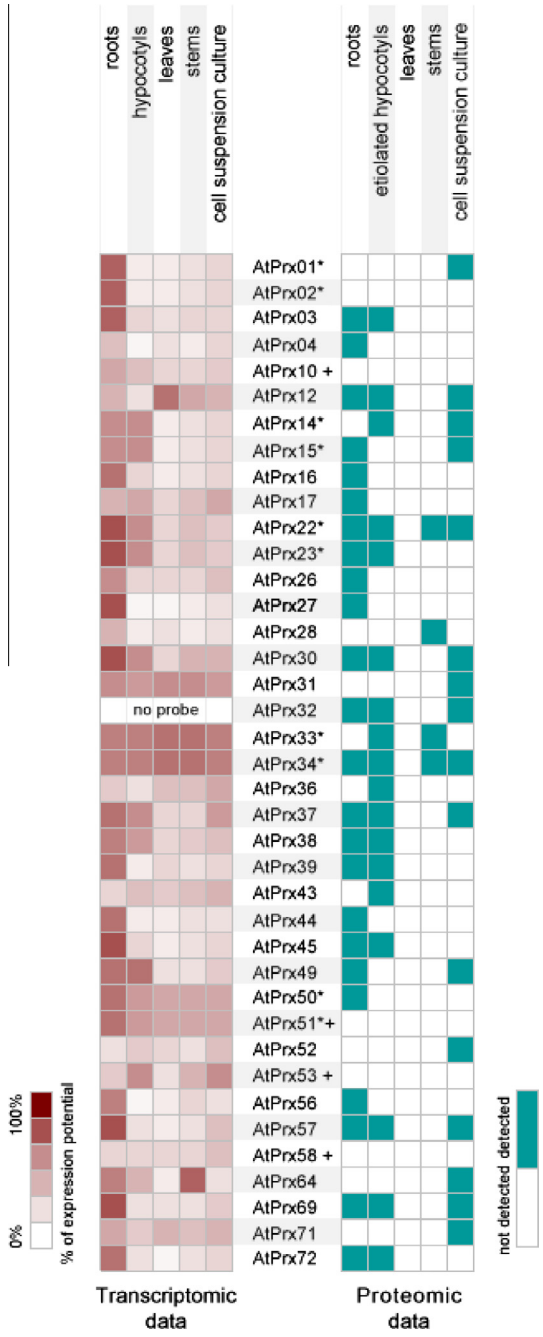

Fig. 4. Comparison of transcriptomic and CW proteomic data of *A. thaliana* roots, hypocotyls, leaves, stems and suspension cultures. Transcriptomic data was obtained from the public database Genevestigator (https://www.genevestigator.com/) and color-coded to reflect the expression level of each gene in the selected organs/tissues. Proteomic data correspond to CIII Prxs identified in several CW proteome studies (WallProtDB, www.polebio.lrsv.ups-tlse.fr/WallProtDB/, unpublished data). * Denote genes whose microarray data should be taken cautiously since the corresponding probe is probably not specific. + Indicate peroxidases detected in a least one proteome not reported here.

and protein distribution. Indeed, promoter sequences of *A. thaliana* CIII Prxs are highly diversified, suggesting different regulatory pathways (Cosio and Dunand, 2009). New sets of data are available to assist functional analysis including (i) organ-specific transcript and protein profiling (Fig. 4), (ii) sub-cellular localization (Tanz et al., 2013) and (iii) micro-transcriptomic studies giving access to time-controlled cell-specific expression profiles (Birnbaum et al., 2003).

3.1. Proteomics complements transcriptomics

Prx enzymatic activity can be detected in all organs and almost all tissues, with a particularly high level in roots. The distribution of CIII Prxs in plant organs is illustrated in various transcriptomic/cell wall proteomic studies performed on roots, hypocotyls, leaves, stems and cell suspension cultures (Fig. 4). The reason for the preferential accumulation of CIII Prxs in roots has not been elucidated yet, but could be related to gain of functions during the land emergence process (Delaux et al., 2012). In some instance, the lack of a probe (*AtPrx32*) or the presence of a single probe per tandem genes (*AtPrx01*/*02*, *AtPrx14*/*15*, *AtPrx33*/*34*, *AtPrx50*/*51*) does not allow accurate detection of transcripts (Fig. 4). On the contrary, proteomics helps precisely identify CIII Prx isoforms through proteotypic peptides as illustrated by the specific profiles observed for proteins-couples encoded by the tandem genes mentioned above. Four CIII Prxs (AtPrx10/51/53/58) have been described as being transcribed in one of the five organs analyzed, but their specific peptides have only been found in samples not shown in Fig. 4 (http://www.polebio.lrsv.ups-tlse.fr/WallProtDB/). Altogether, both approaches appear complementary to analyze CIII Prx transcript and protein distribution at the organ level.

3.2. Cell wall vs vacuolar CIII Prxs

Based on the presence of a predicted signal peptide and the absence of known sorting/retention signals, CIII Prxs are considered as secreted extracellular proteins. Proteomic studies show that CIII Prxs are mainly found in apoplastic/CW fractions (Fig. 1 and Supplementary Table 1). Among the 73 *A. thaliana* CIII Prxs, 32 were found in extracellular fractions, 17 in plasma membrane (PM)-enriched fractions and 7 in vacuole-enriched fractions. This result is consistent with the presence of a predicted signal peptide in 71 CIII Prxs. No correlation has been found between the presence of CIII Prx in PM fractions and the bioinformatic prediction of trans-membrane domains or glycosylphosphatidylinositol (GPI) anchors (Supplementary Table 1). Alternatively, CIII Prxs could be trapped in PM fractions due to interactions with transmembrane proteins, similar to AtPrx64 interacting with CASP1 in Casparian strips (Lee et al., 2013; discussed in more detail below). In the case of the 7 CIII Prxs identified in vacuolar fractions, a C-terminus extension (CTPP) predicted to allow vacuolar targeting (Carter et al., 2004) is always found (Supplementary Table 1). Only AtPrx22 and AtPrx23 display such a CTPP without having been identified in vacuolar fractions so far. Moreover, green fluorescent protein (GFP) imaging data allowed the visualization of 8 CIII Prx-fluorescent protein fusions in CW (Supplementary Table 1). However, in the case of predicted vacuolar CIII Prxs, this type of fusion may hinder the vacuolar targeting because of the inaccessibility of the CTPP vacuolar sorting sequence (Costa et al., 2008; Matsui et al., 2011). Finally, CIII Prxs probably have to be considered as extracellular or vacuolar proteins according to the absence or presence of a CTPP, respectively.

3.3. Microtranscriptomics: the example of roots

In recent years, transcriptomics studies have reached such resolution, as to select specific cells or tissues. As an example, the pioneer cell sorting of root tissues expressing cell-specific GFP constructs using proptoplasts allowed the isolation of five root tissue samples at three developmental stages: stele, endodermis, cortex and endodermis, epidermis, lateral root cap (Birnbaum et al., 2003). CIII Prx gene expression values above the detection limit have been found for 44 CIII Prxs. They have been organized in clusters of different sizes, from single to several CIII Prxs (Supplementary Table 2). Interestingly, these data were recently used

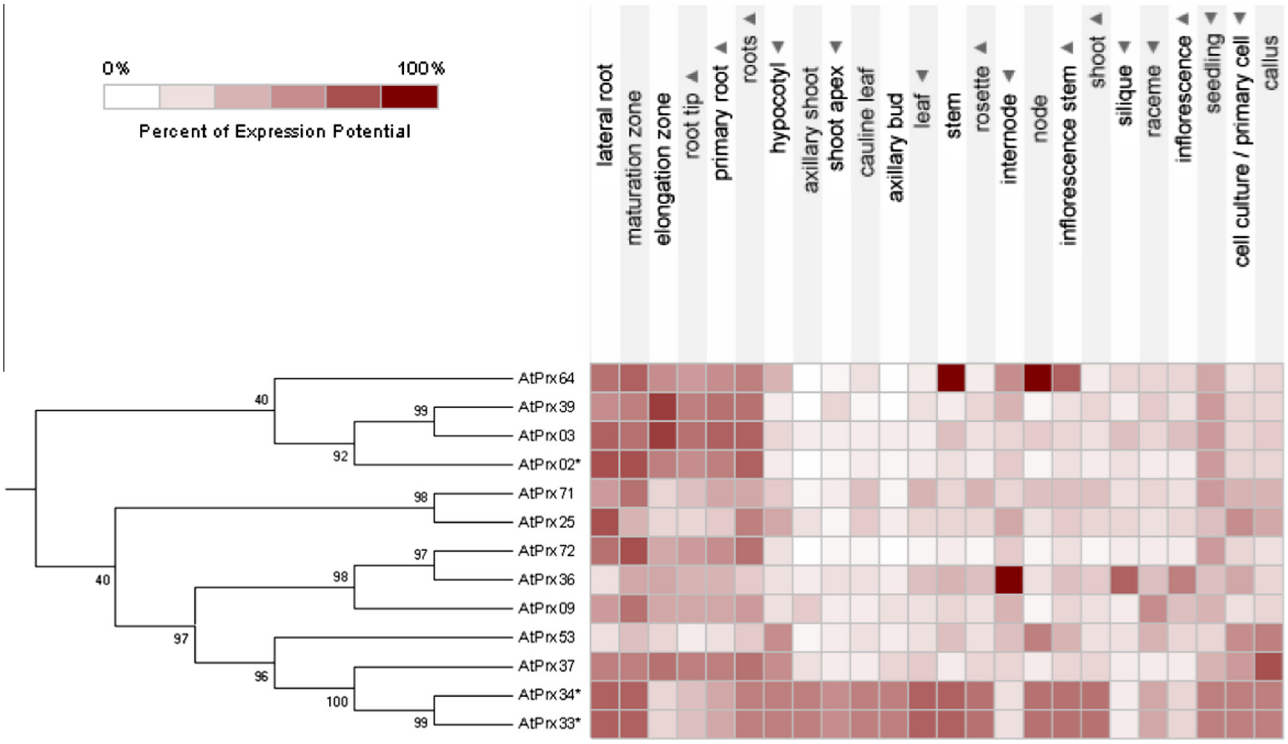

Fig. 5. Transcriptomic data of thirteen selected CIII Prx genes. Affymetrix microarray data were retrieved from Genevestigator (Zimmermann et al., 2004) and color-coded to reflect the expression level of each gene in the selected organs/tissues. Genes with available reverse genetic analysis have been selected. They are listed in Supplementary Table 3.

to select five CIII Prx genes highly expressed in endodermis (*AtPrx03/09/39/64/72*) regardless of their expression in other tissues, which led to the functional characterization of AtPrx64 that will be discussed below (Lee et al., 2013).

4. Reverse genetic studies: recent discovery of CIII Prx functions

Pharmacological approaches, global transcriptomic and proteomic analyses do not allow the assignment of a function to a given gene, especially if it belongs to a multigene family such as CIII Prxs. For example, organ-specific transcriptomics do not necessarily show specific expression profiles for a sub-set of CIII Prx genes for which reverse genetic data has been published (Fig. 5 and Supplementary Table 3). For example, although several genes show root-specific expression, only *atprx64* has an endodermis phenotype (see below). Together with these global analyses, the study of T-DNA insertion mutants has recently enabled the functional characterization of a few CIII Prxs (Supplementary Table 3).

Based on their confirmed or putative functions, CIII Prxs can be classified as having CW stiffening roles, CW loosening roles, other roles and no attributed role. AtPrx64 (Lee et al., 2013), AtPrx02/25/71 (Shigeto et al., 2013), AtPrx37 (Pedreira et al., 2011) and AtPrx72 (Herrero et al., 2013) have been attributed a role in cell wall stiffening, mostly through lignin polymerization in cell walls of various cell types. On the other hand, AtPrx36 (Kunieda et al., 2013), AtPrx33/34 (Passardi et al., 2006) and AtPrx53 (Jin et al., 2011) have been suggested to play roles in wall loosening based on the phenotype of the corresponding mutants. Their CW targets have not been identified, but CW loosening could be due to the breaking of polysaccharides bonds. Two elegant studies demonstrating the roles of AtPrx64 in cell wall stiffening (Lee et al., 2013) and of AtPrx36 in cell wall loosening (Kunieda et al., 2013) have illustrated the importance of precise *in muro* localization of these CIII Prxs in order to perform their function.

4.1. Role of AtPrx64 and satellite proteins in lignification of Casparian strips in root endodermis

A series of papers has recently illustrated how a given CIII Prx can be accurately targeted to CW micro-domains together with its protein partners to perform localized cell wall modification. Trans-membrane proteins were recently discovered and named CAsparian Strip membrane domain Proteins (CASPs) (Roppolo et al., 2011). CASP1, 2, 3, 4, 5 belong to a unique clade within the *A. thaliana* uncharacterized protein family UPF0497 (38 members) and localize to the electron dense Casparian Strip membrane Domain (CSD) in root endodermal cells. The 5 CASPs strongly interact together. The correct deposition of lignin along the CSD to form functional Casparian strips depends on the coordinated action of several proteins: CASP1, a specific NADPH oxidase (Rbohf), a dirigent-domain containing protein (ESB1) and AtPrx64 (Hosmani et al., 2013; Lee et al., 2013; Naseer et al., 2012). Together, they form a scaffold of interacting proteins. As mentioned above, *AtPrx64* was chosen as a candidate in Casparian strip lignification together with *AtPrx03/09/39/72* on the basis of transcript enrichment in the endodermis (Supplementary Table 2; Lee et al., 2013). However, the *per03/09/39/72* quadruple mutant did not display any lignin phenotype, whereas inducible tissue-specific *AtPrx64* amiRNA lines exposed the lignifying role of AtPrx64 (Lee et al., 2013). Given that each member of this scaffold belongs to a multigenic family, it is speculated that such a mechanism could be widespread in ensuring *in muro* specificity of the action of ROS within CW micro-domains.

The localization of CIII Prxs in CW micro-domains could also be due to interactions with CW polysaccharides as shown for a CIII Prx from zucchini (Carpin et al., 2001). This CIII Prx contains a cluster of three basic amino acids (Arg-262, Arg-268, Arg-271) involved in the binding of Ca^{2+}-polygalacturonate *in vitro*. CIII Prxs belonging to the *AtPrx32/33/34/37/38* cluster of *A. thaliana* contain similar

patterns of three basic amino acids and AtPrx33 and AtPrx34 have been shown to interact *in vitro* and *in vivo* with Ca^{2+}-polygalacturonate (Passardi et al., 2006; Shah et al., 2004). The importance of this binding for CIII Prx functions in plant CW needs to be investigated further.

4.2. The role of AtPrx36 in the extrusion of seed coat mucilage in A. thaliana

AtPrx36 has been demonstrated to be a target of two redundant transcription factors (NARS1 and NARS2) that affect seed development and seed mucilage extrusion following seed imbibition (Kunieda et al., 2008). More precisely, AtPrx36 was shown to play a role in mucilage extrusion (Kunieda et al., 2013). Spatio-temporal expression of *AtPrx36* in the outermost integument second cell layer (oi2) of seeds for a few days around the embryo torpedo stage conducted to the precise protein localization in micro-domains of oi2 outer CW. Oi2 is also named mucilage secretory cells (MSCs) and the AtPrx36 deposition zones corresponds to the CW micro-domains that break following seed imbibition. Accordingly, *atprx36* shows a severe phenotype of mucilage extrusion delay corresponding to the failure of polarized CW rupture. This suggests that AtPrx36 enables the modification of oi2 outer CW micro-domains leading to the organized breaking of the CW following seed imbibition. It has been suggested that the observed phenotype could result from a default in CW loosening. However, the substrates of AtPrx36 remain to be identified.

5. Prospects

CIII Prxs are members of large multigenic families with numerous recent duplication events with enhanced possibilities of functional redundancy. At present, only a low number of CIII Prxs from *A. thaliana* and other plants have been assigned a function *in planta*. Biochemical, pharmacological, transcriptomic, proteomic, cell biology and genetic approaches need to be used in concert to unravel their functions. Multiple mutant and RNAi strategies might be necessary in the case of highly homologous genes, such as those resulting from tandem duplication. Micro-transcriptomic analyses such as those performed on roots demonstrated the spatio-temporal regulation of CIII Prx gene expression. Unfortunately, the lack of some probes together with the low specificity of others reduces the quality of the data. RNA sequencing technologies and *in situ* hybridization will help overcome these drawbacks.

Recent examples of functional characterization of some CIII Prxs have pointed out the importance of their precise localization in CWs to fulfill their function *in muro*. This is supported by the detection within, but also the interaction of those CIII Prxs with particular CW micro-domains such as Casparian strips, outer CW subdomains of seed MSCs or Ca^{2+}-pectate domains. The identification of CIII Prx binding sites (proteins or CW micro-domains) and of their substrates *in muro* are now crucial points for the understanding of the function of all the CIII Prxs. Finally, since the CIII Prx copy number exploded after land plant emergence, the analysis of a basal green organism, with much less CIII Prx isoforms will help us understand their original function and the diversification of their roles in plant CWs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.07.020.

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