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FOCUS REVIEW PAPER

Specific functions of individual class III peroxidase genes

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Abstract

In higher plants, class III peroxidases exist as large multigene families (e.g. 73 genes in *Arabidopsis thaliana*). The diversity of processes catalysed by peroxidases as well as the large number of their genes suggests the possibility of a functional specialization of each isoform. In addition, the fact that peroxidase promoter sequences are very divergent and that protein sequences contain both highly conserved domains and variable regions supports this hypothesis. However, two difficulties are associated with the study of the function of specific peroxidase genes: (i) the modification of the expression of a single peroxidase gene often results in no visible mutant phenotype, because it is compensated by redundant genes; and (ii) peroxidases show low substrate specificity *in vitro* resulting in an unreliable indication of peroxidase specific activity unless complementary data are available. The generalization of molecular biology approaches such as whole transcriptome analysis and recombinant DNA combined with biochemical approaches provide unprecedented tools for overcoming these difficulties. This review highlights progress made with these new techniques for identifying the specific function of individual class III peroxidase genes taking as an example the model plant *A. thaliana*, as well as discussing some other plants.

Key words: Auxin catabolism, defences, genes, isoforms, lignification, proteomic, regulation pathways, stresses, transcriptome analysis, transgenic plants.

Introduction

Secreted class III plant peroxidases (EC 1.11.1.7) belong to a superfamily that contains three different classes of peroxidases (Welinder, 1992). The three classes have in common a haem formed by protoporphyrin IX and Fe(III), and share a very similar three-dimensional structure (Fig. 1; Welinder, 1992, Edwards *et al*., 1993, Smulevich *et al*., 2006). However, the three classes of peroxidases show low identity in their primary amino acid sequences and have distinct functions and reaction mechanisms. The intracellular class I can be found in most living organisms, except animals. Its widespread distribution—in particular its presence in prokaryotes—suggests that class I peroxidases are probably at the origin of the two other classes (Passardi *et al*., 2007). Their main function in the cell is detoxification of excess H_2O_2 (Skulachev, 1998; Erman and Vitello, 2002; Shigeoka *et al*., 2002). Class I peroxidases are split into three distinct groups: ascorbate peroxidases (APxs; EC 1.11.1.11), cytochrome *c* peroxidases (CcPs; EC 1.11.1.5), and catalase–peroxidases (CPs; EC 1.11.1.6). APxs have a very high affinity for ascorbate and are found in photosynthetic organisms. In higher plants, they are subdivided according to their subcellular localization: chloroplastic (stroma and thylakoid-bound), peroxisomal, and cytoplasmic (Teixeira *et al*., 2004). CcPs are located in the mitochondrial intermembrane space and use cytochrome *c* as electron donor. CPs can function as a regular peroxidase but they can also oxidize another molecule of H_2O_2 (catalase activity) and many other substrates (Obinger *et al*., 1999). Class II, encoded exclusively by fungi, are divided into manganese peroxidases (EC 1.11.1.13), lignin peroxidases (EC 1.11.1.14), and versatile peroxidases (EC 1.11.1.16; Ruiz-Dueñas *et al*., 2001). Class II peroxidases have a major role in degradation of soil debris (Piontek *et al*., 2001; Martinez *et al*., 2005), since no other haem peroxidase is able to degrade lignin.

Class III peroxidases—on which this review is centred—are present as large multigene families in all land plants (Tognolli *et al*., 2002; Duroux and Welinder, 2003;

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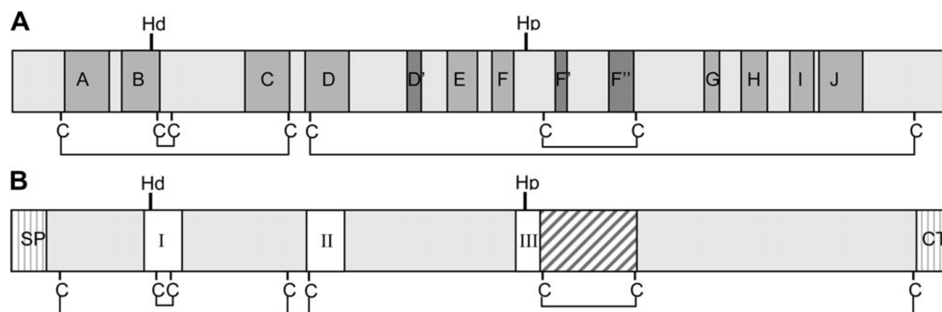

Fig. 1. Schematic structures of class III peroxidases. (A) Secondary structure. The helix composition and position is represented using cysteine (C) conserved residues and distal and proximal histidine (Hd and Hp) as reference. Helices in dark grey are specific for class III peroxidases. Other helices are conserved among class I, II, and III peroxidases. (B) Primary structure. The highly conserved domains I, II, and III correspond to the distal haem-binding domain, an unknown domain, and the proximal haem-binding domain. The hatched region corresponds to a putative variable domain responsible for the catalytic specificity. The signal peptide (SP) and C-terminal extension (CT) are highly variable in length and amino acid composition.

Passardi *et al*., 2004*a*). They have possibly played a critical role during land colonization by plants, either by allowing formation of rigid plant structures or by adapting the organism to a more oxygenated environment (Duroux and Welinder, 2003; Passardi *et al*., 2004*a*). Class III peroxidases are generally secreted into the cell wall or the surrounding medium and the vacuole (Neuhaus, 1996; Welinder *et al*., 2002; Matsui *et al*., 2003). In their regular peroxidative cycle, class III plant peroxidases catalyse the reduction of H_2O_2 by taking electrons to various donor molecules such as phenolic compounds, lignin precursors, auxin, or secondary metabolites (Hiraga *et al*., 2001; Passardi *et al*., 2004*b*). Recently, a separate hydroxylic cycle, which leads to the formation of various reactive oxygen species (ROS), has been described (Liszkay *et al*., 2003; Passardi *et al*., 2004*a*), thus initiating a host of implications for the class III peroxidases.

The diverse functions of class III peroxidases

In striking contrast to the first two peroxidase classes, assigning a function to a class III peroxidase is a rather complex task. Probably as a consequence of the large number of genes and the two possible catalytic cycles, class III plant peroxidases are involved in a broad range of physiological processes (Fig. 2) throughout the plant life cycle (for a review, see Passardi *et al*., 2005). They are, for example, involved in cell wall metabolism (Barcelo and Pomar, 2001; Passardi *et al*., 2004*b*), wound healing (Bernards *et al*., 1999; Allison and Schultz, 2004), and auxin catabolism (Gazaryan *et al*., 1996). They are also believed to be involved in removal of H_2O_2, oxidation of toxic reductants, defence against pathogen or insect attack, as well as symbiosis and normal cell growth. In addition class III peroxidases can generate highly reactive ROS (Liszkay *et al*., 2003; Passardi *et al*., 2004*a*) which can possess an intrinsic activity during different environmental responses and developmental processes, including the oxidative burst, the hypersensitive response (HR), or cell elongation (Bolwell *et al*., 2002; Schopfer *et al*., 2002; Delannoy *et al*., 2003; Liszkay *et al*., 2004; Bindschedler *et al*., 2006). Alternatively, ROS can also act as part of signal transduction pathways (Laloi *et al*., 2004) during specific mechanisms, including biotic and abiotic stress responses, allelopathic plant–plant interactions, cell division/elongation, and programmed cell death (Bethke and Jones, 2001; Bais *et al*., 2003; Foreman *et al*., 2003; Apel and Hirt, 2004; Foyer and Noctor, 2005).

The diversity of the processes catalysed by peroxidases as well as the great number of their genes suggests the existence of functional specialization of the members of this protein family. The fact that all plant peroxidase sequences contain both conserved regions and variable parts (Fig. 1) further supports this hypothesis. Many authors have reported the appearance or disappearance of specific peroxidase isoforms during a particular process or in a particular localization (e.g. Loukili *et al*., 1999; Allison and Schultz, 2004). Nevertheless it is generally difficult to associate the band observed on an isoelectric focusing (IEF) gel with a particular protein mainly because protein purification is not straightforward. It is also difficult to link a band on an IEF gel with a particular gene because no obvious quantitative relationship exists between the transcript expression level and the protein activity (Dunand *et al*., 2003). Beside, because of different post-translational modifications, often more than one protein form (isoform) originates from a particular gene (e.g. Gabaldon *et al*., 2007; Laugesen *et al*., 2007).

In other attempts to identify the function of specific class III peroxidases, several authors reported the generation of transgenic plants related to different peroxidase genes. Although some of these studies provided interesting data (e.g. Ostergaard *et al*., 2000; Heggie *et al*., 2005; Bindschedler *et al*., 2006), many of these approaches have produced disappointing, inconclusive results (e.g. Sherf *et al*., 1993; McIntyre *et al*., 1996; Kristensen *et al*., 1997; Ray *et al*., 1998; Schlimme *et al*., 2002), and the *in planta* role of most peroxidases remains elusive. Two difficulties

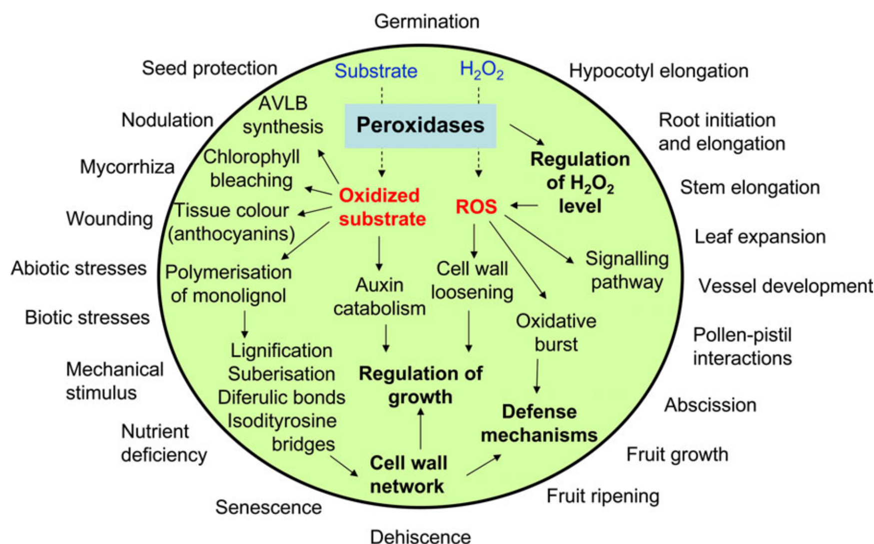

Fig. 2. The diverse functions and roles of class III peroxidases (AVLB=α-3′,4′-anhydrovinblastine).

are inherent in peroxidases: (i) the modification of the expression of a single gene often results in no visible mutant phenotype, because it is compensated by redundant peroxidase genes; and (ii) peroxidases have the ability to react with numerous plant compounds *in vitro*, although it is uncertain which of these compounds are *in planta* substrates. *In vitro* activity cannot therefore offer precise information on the specific activity of different isoforms. Nevertheless, the generalization of molecular biology approaches such as microarray analysis offers a tool of unprecedented power to overcome these difficulties. Here, the recent progress made in identifying the specific function of individual class III peroxidase isoforms is discussed. The review is centred on *A. thaliana* because it is the plant in which the greatest number of class III peroxidase genes have been related to a function. Limited data concerning other plant species illustrating the recent findings concerning class III peroxidase function are also included and discussed. An apology is offered to all authors whose work is not cited.

Peroxidase genes in *Arabidopsis thaliana*

Arabidopsis thaliana is currently a very popular organism in plant biology. Its short life cycle, small genome size, and availability of large T-DNA insertion mutant collections have rendered it one of the principal model systems. Moreover, the advent of molecular biology and the completion of the *Arabidopsis* genome sequence have contributed to helping researchers discover a large variety of gene functions. In *A. thaliana*, 73 sequences encoding class III peroxidase genes have been identified (Tognolli *et al*., 2002; Welinder *et al*., 2002) of which at least 66 are expressed in 6-week-old plants (Valério *et al*., 2004). The remaining genes may be expressed following environmental stress or in other developmental stages. Microarray analysis as a high-throughput technology is the approach that has putatively identified the highest number (36) of individual isoforms in specific mechanisms. In comparison, proteomics and transgenic approaches have allowed the identification of 12 and nine genes, respectively. Other techniques are scarcely represented. In total, 44 out of 73 class III peroxidase genes have been reported to be putatively involved in a specific mechanism (Table 1).

It should be noted that this review is based only on papers that have mentioned class III peroxidases in their published results. A higher number of putative class III peroxidase gene functions would certainly be identified if raw microarray data or a microarray database such as GENEVESTIGATOR (Zimmermann *et al*., 2004) were exhaustively exploited. Although microarrays are an interesting and productive approach, it is important that data be confirmed by more reliable methods, such as real-time quantitative PCR (RT-qPCR) or reverse genetic in order to be validated. Among the microarray data reported in this review only six peroxidase genes (out of the 36 identified) have been also monitored by RT-qPCR (Kumari *et al*., 2008). Therefore, microarray data are in most cases purely indicative and further work would be necessary to confirm the specific function of the gene of interest. In addition, global transcriptome approaches such as microarray analysis should be cautiously evaluated because they can identify both directly and indirectly regulated genes. Moreover, they are based on the transcript level and are not necessarily linked to protein activity (e.g. Dunand *et al*., 2003). In contrast, proteomic approaches working directly on proteins are difficult methods, particularly concerning purification and removal of background proteins (Andersen and Mann, 2006). Consequently, many published proteomes contain

Table 1. *List of* Arabidopsis thaliana *class III peroxidase genes putatively involved in a specific mechanism*

Blot, northern blot; DD, differential display; ish, *in situ* hybridization; qPCR, real-time-qPCR; RT, semi-quantitative RT-PCR; SAGE, serial analysis of gene expression; SSH, suppression substractive hybridization; TNT, trinitrotoluene.

Protein name	Method	Organ	Mechanism of interest of the study	References
AtPrx02	Microarrays	Roots	Low oxygen response	Klok *et al*. (2002)
	Microarrays	Stem	Monolignol polymerization	Ehlting *et al*. (2005)
	Microarrays	Whole plant	Arsenic stress	Abercrombie *et al*. (2008)
	Microarrays/qPCR	Roots	Aluminium stress	Kumari *et al*. (2008)
AtPrx03	Transgenic plants	Roots	Cold-inducible tolerance	Llorente *et al*. (2002)
	Microarrays	Stamen	Stamen abscission	Cai and Lashbrook (2008)
AtPrx07	Microarrays	Seedlings	TNT treatment	Mentewab *et al*. (2005)
AtPrx09	Microarrays	Stem	Monolignol polymerization	Ehlting *et al*. (2005)
AtPrx11	Microarrays	Shoots	Cuticle metabolism regulation in response to abiotic stress	Cominelli *et al*. (2008)
AtPrx12	Microarrays	Roots	Low oxygen response	Klok *et al*. (2002)
	Proteomic	Hypocotyls	Cell elongation	Irshad *et al*. (2008)
AtPrx13	Macroarrays/RT	Siliques	Pod shatter	Cosio *et al*. (2008*b*)
AtPrx17	Microarrays	Stem	Monolignol polymerization	Ehlting *et al*. (2005)
	Microarrays	Stamen	Stamen abscission	Cai and Lashbrook (2008)
	Transgenic plants	Siliques	Lignification in pod shatter	Cosio *et al*. (2008*b*)
AtPrx21	Microarrays	Seedlings	Wound stress	Cheong *et al*. (2002)
	Microarrays	Leaves	Non-host resistance to fungus	Narusaka *et al*. (2005)
	Microarrays	Leaves	Infection by *Pseudomonas syringae*	Mohr and Cahill (2007)
	Transgenic plants	Leaves	Fungus defence	Chassot *et al*. (2007)
	Microarrays	Stamen	Stamen abscission	Cai and Lashbrook (2008)
	Microarrays	Roots	Aluminium stress	Kumari *et al*. (2008)
AtPrx22	Proteomics	Seedlings	Potassium deficiency	Kang *et al*. (2004)
AtPrx27	Microarrays	Seedlings	TNT treatment	Mentewab *et al*. (2005)
	Microarrays/qPCR	Roots	Aluminium stress	Kumari *et al*. (2008)
AtPrx28	Microarrays/SSH	Stigma	Stigma-specific gene	Swanson *et al*. (2005)
	Microarrays/blot	Pistil	Pollen–pistil interactions	Tung *et al*. (2005)
AtPrx30	Microarrays	Stem	Monolignol polymerization	Ehlting *et al*. (2005)
	Microarrays	Stamen	Stamen abscission	Cai and Lashbrook (2008)
	Macroarrays/RT	Siliques	Pod shatter	Cosio *et al*. (2008*b*)
	Proteomics	Hypocotyls	Cell elongation	Irshad *et al*. (2008)
AtPrx31	Microarrays	Stamen	Stamen abscission	Cai and Lashbrook (2008)
AtPrx32	Proteomics	Hypocotyls	Cell elongation	Irshad *et al*. (2008)
AtPrx33	Transgenic plants	Leaves	Oxidative burst	Bindschedler *et al*. (2006)
	Transgenic plants	Roots	Root length	Passardi *et al*. (2006)
	Microarrays	Stamen	Stamen abscission	Cai and Lashbrook (2008)
	Proteomics	Hypocotyls	Cell elongation	Irshad *et al*. (2008)
AtPrx34	cDNA library/blot	Roots	Aluminium stress	Richards *et al*., 1998)
	Microarrays	Shoots	Phosphate starvation	Hammond *et al*. (2003)
	Microarrays	Leaves	Ozone stress	Ludwikow *et al*. (2004)
	Transgenic plants	Leaves	Oxidative burst	Bindschedler *et al*. (2006)
	Transgenic plants	Roots	Root length	Passardi *et al*. (2006)
	Microarrays	Leaves	Infection by *Pseudomonas syringae*	Mohr and Cahill (2007)
	Microarrays	Stamen	Stamen abscission	Cai and Lashbrook (2008)
	Proteomics	Hypocotyls	Cell elongation	Irshad *et al*. (2008)
AtPrx36	Proteomics	Hypocotyls	Cell elongation	Irshad *et al*. (2008)
AtPrx37	SSH/blot	Roots	Orobanche defence	Vieira Dos Santos *et al*. (2003)
	Microarrays	Leaves	Ozone stress	Ludwikow *et al*. (2004)
	Microarrays	Stem	Monolignol polymerization	Ehlting *et al*. (2005)
	Microarrays	Leaves	Infection by *Pseudomonas syringae*	Mohr and Cahill (2007)
AtPrx39	Microarrays/SSH	Stigma	Stigma-specific gene	Swanson *et al*. (2005)
	Microarrays	Pistil	Pollen–pistil interactions	Tung *et al*. (2005)
AtPrx42	Microarrays	Stem	Xylem secondary cell wall formation	Yokoyama and Nishitani (2006)
	Microarrays	Stamen	Stamen abscission	Cai and Lashbrook (2008)
	Microarrays	Roots	Aluminium stress	Kumari *et al*. (2008)

Table 1. *Continued*

Protein name	Method	Organ	Mechanism of interest of the study	References
AtPrx43	Proteomics	Hypocotyls	Cell elongation	Irshad *et al*. (2008)
AtPrx45	SAGE	Roots	TNT stress	Ekman *et al*. (2003)
	Microarrays	Stamen	Stamen abscission	Cai and Lashbrook (2008)
	Proteomics	hypocotyls	Cell elongation	Irshad *et al*. (2008)
	Microarrays	Roots	Aluminium stress	Kumari *et al*. (2008)
AtPrx49	DD/ish/blot	Root galls	Compatible interaction with nematode	Vercauteren *et al*. (2001)
	Microarrays/qPCR	Roots	Aluminium stress	Kumari *et al*. (2008)
AtPrx50	Microarrays	Roots	Low oxygen response	Klok *et al*. (2002)
	Microarrays	Shoots	Phosphate starvation	Hammond *et al*. (2003)
	Microarrays	Stamen	Stamen abscission	Cai and Lashbrook (2008)
AtPrx51	Microarrays	Stamen	Stamen abscission	Cai and Lashbrook (2008)
AtPrx52	Microarrays	Leaves	Ozone stress	Ludwikow *et al*. (2004)
	Microarrays	Leaves	Insect defence	Little *et al*. (2007)
	Microarrays	Leaves	Infection by *Pseudomonas syringae*	Mohr and Cahill (2007)
	Microarrays	Stamen	Stamen abscission	Cai and Lashbrook (2008)
AtPrx53	Transgenic plants	Whole plant	Lignification of vascular bundles	Ostergaard *et al*. (2000)
	Microarrays	Stamen	Stamen abscission	Cai and Lashbrook (2008)
	Macroarrays/RT			Cosio *et al*. (2008*b*)
AtPrx55	Siliques	Pod shatter		
AtPrx56	Microarrays/qPCR	Roots	Aluminium stress	Kumari *et al*. (2008)
AtPrx57	Microarrays	Seedlings	TNT treatment	Mentewab *et al*. (2005)
	Microarrays	Whole plant	Arsenic stress	Abercrombie *et al*. (2008)
	Proteomics	Hypocotyls	Cell elongation	Irshad *et al*. (2008
AtPrx58	Microarrays/ish/blot	Pistil	Pollen–pistil interactions	Tung *et al*. (2005)
	Microarrays/SSH	Stigma	Stigma-specific gene	Swanson *et al*. (2005)
AtPrx59	Microarrays	Seedlings	Mechanical stimulus	Moseyko *et al*. (2002)
	Microarrays	Leaves	Infection by *Pseudomonas syringae*	Mohr and Cahill (2007)
	Microarrays	Roots	Aluminium stress	Kumari *et al*. (2008)
AtPrx60	Microarrays	Roots	Low oxygen response	Klok *et al*. (2002)
AtPrx62	Microarrays	Seedlings	Wound stress	Cheong *et al*. (2002)
	Microarrays	Shoots	Phosphate starvation	Hammond *et al*. (2003)
	Microarrays	Roots	Cadmium stress	Weber *et al*. (2006)
	Transgenic plants	Leaves	Fungus defence	Chassot *et al*. (2007)
	Microarrays/qPCR	Roots	Aluminium stress	Kumari *et al*. (2008)
AtPrx64	Microarrays	Stem	Xylem secondary cell wall formation	Yokoyama and Nishitani (2006)
	Microarrays	Roots	Aluminium stress	Kumari *et al*. (2008)
AtPrx66	Transgenic plants	Roots	Lignification of vascular bundles	Sato *et al*. (2006)
AtPrx67	Microarrays	Stamen	Stamen abscission	Cai and Lashbrook (2008)
	Microarrays	Roots	Aluminium stress	Kumari *et al*. (2008)
AtPrx68	Microarrays	Shoots	Cuticle metabolism regulation in response to abiotic stress	Cominelli *et al*. (2008)
AtPrx69	Microarrays	Shoots	Phosphate starvation	Hammond *et al*. (2003)
	Microarray	Seedlings	Sulphur deficiency	Nikiforova *et al*. (2003)
	Microarrays	Leaves	Infection by *Pseudomonas syringae*	Mohr and Cahill (2007)
	Microarrays	Whole plant	Arsenic stress	Abercrombie *et al*. (2008)
	Proteomics	Hypocotyls	Cell elongation	Irshad *et al*. (2008)
	Microarrays/qPCR	Roots	Aluminium stress	Kumari *et al*. (2008)
AtPrx70	Microarrays/RT	Shoots	Ozone treatment	Miyazaki *et al*. (2004)
AtPrx71	Microarrays	Seedlings	Wound stress	Cheong *et al*. (2002)
	Microarrays	Roots	Low oxygen response	Klok *et al*. (2002)
	Microarrays	Shoots	Phosphate starvation	Hammond *et al*. (2003)
	Protein purification	Cell culture	Hypo-osmolarity response	Rouet *et al*. (2006)
	Microarrays	Stem	Xylem secondary cell wall formation	Yokoyama and Nishitani (2006)
	Transgenic plants	Leaves	Fungus defence	Chassot *et al*. (2007)
	Microarays	Leaves	Insect defence	Little *et al*. (2007)
	Microarrays	Leaves	Infection by *Pseudomonas syringae*	Mohr and Cahill (2007)
	Microarrays	Stamen	Stamen abscission	Cai and Lashbrook (2008)
AtPrx72	Proteomics	Hypocotyls	Cell elongation	Irshad *et al*. (2008)
AtPrx73	Microarrays	Roots	Aluminium stress	Kumari *et al*. (2008)

significant numbers of 'false positives', although the constant progress of mass spectrometry-based proteomics will probably soon remedy these inaccuracies. However, to date, transgenic approaches are in principle more reliable, although ectopic overexpression of a protein can create artefacts, and silencing is not totally specific since it might result in silencing of more than a single gene. In conclusion, it was decided to highlight a limited number of representative microarray data and focus on more conclusive approaches in this review.

Defence and stress response mechanisms appear widely among the factors regulating class III peroxidase genes: metals (Richards *et al*., 1998; Weber *et al*., 2006; Abercrombie *et al*., 2008; Kumari *et al*., 2008), pathogens (Vieira Dos Santos *et al*., 2003; Narusaka *et al*., 2005; Chassot *et al*., 2007; Little *et al*., 2007; Mohr and Cahill, 2007), ozone stress (Ludwikow *et al*., 2004; Miyazaki *et al*., 2004), cold (Llorente *et al*., 2002), anoxia (Klok *et al*., 2002), phosphate starvation (Hammond *et al*., 2003), sulphur depletion (Nikiforova *et al*., 2003), and potassium deficiency (Kang *et al*., 2004) have been reported to affect expression of peroxidases gene. Few studies concern developmental aspects (Swanson *et al*., 2005; Tung *et al*., 2005; Cai and Lashbrook, 2008; Cosio *et al*., 2008*b*; Irshad *et al*., 2008). Surprisingly, no gene has been related to auxin catabolism, although this is a classical function attributed to class III peroxidases. However, a heterologous study with a zucchini peroxidase indicated that endogenous *A. thaliana* genes were also related to auxin oxidase activity (Cosio *et al*., 2008*a*). The main data and representative results are briefly presented and discussed according to their putative mechanisms.

Stresses, defence and tolerance

Several roles have been attributed to plant peroxidases in response to biotic and abiotic stresses. During plant defence mechanisms they can have a cell wall cross-linking activity (formation of lignin, extensin cross-links, dityrosine bonds; Chen *et al*., 2002) and create a highly toxic environment by producing vast amounts of ROS (oxidative burst, hypersensitive response; Delannoy *et al*., 2003; Bindschedler *et al*., 2006), which results in adverse growth conditions for microorganisms. Alternatively, ROS can act as part of signal transduction pathways involved in defence mechanisms (Laloi *et al*., 2004). In the case of wounding, peroxidase expression is probably triggered in order to repair the damaged tissue but also as a preventive defence mechanism against pathogen attacks (El Mansouri *et al*., 1999).

Gene alterations have been studied in an amazing variety of stress conditions. Fourteen class III peroxidase genes in total are related to metal stresses. Thirteen of these genes are associated with aluminium stress in roots (Richards *et al*., 1998; Kumari *et al*., 2008). One gene is found in arsenic stress only (Abercrombie *et al*., 2008). Two genes are related to both arsenic and aluminium (Abercrombie *et al*., 2008; Kumari *et al*., 2008). One gene is related to both aluminium and cadmium stress in roots (Weber *et al*., 2006; Kumari *et al*., 2008). Metals are known to drive oxidative stress in plants (Landberg and Greger, 2002). It is generally thought that antioxidation activity related to class III peroxidases could contribute in part to metal tolerance (Chiang *et al*., 2006). Trinitrotoluene (TNT) treatment that also results in oxidative stress induced one specific gene in common with aluminium treatment and another one in common with arsenic treatment (Mentewab *et al*., 2005), possibly indicating a role for these peroxidases in antioxidation activity.

Various pathogen stresses also affect peroxidase gene expression. Seven genes were identified by microarrays during infection by *Pseudomonas* spp. (Mohr and Cahill, 2007). Three genes have been identified in leaves attacked by fungus (Chassot *et al*., 2007), and two genes after oviposition by pierid butterflies (Little *et al*., 2007). Three genes (*AtPrx21*, *AtPrx62*, and *AtPrx71*) were induced after both fungus (Chassot *et al*., 2007) and wound stress (Cheong *et al*., 2002), but also other stresses, possibly indicating the action of these three proteins in general defence mechanisms. *AtPrx21* was detected after wounding (Cheong *et al*., 2002), fungus (Chassot *et al*., 2007), *Pseudomonas* spp. (Mohr and Cahill, 2007), and aluminium (Kumari *et al*., 2008) stresses. Similarly *AtPrx62* was detected after wounding (Cheong *et al*., 2002), fungus (Chassot *et al*., 2007), cadmium (Weber *et al*., 2006), and aluminium (Kumari *et al*., 2008) stresses. *AtPrx71* was also detected after wounding (Cheong *et al*., 2002), fungus (Chassot *et al*., 2007), *Pseudomonas* spp. (Mohr and Cahill, 2007), and insect (Little *et al*., 2007) stresses. Induction of these three genes by such a number of different stresses suggests that the corresponding proteins are essential for defence. Not surprisingly, constitutive expression of these genes in wild-type plants significantly increased resistance to *Botrytis cinerea* (Chassot *et al*., 2007). Whereas wild-type plants are close to 0% resistant, overexpression of the *AtPrx62* gene resulted in >80% resistant plants. Overexpression of *AtPrx21* and *AtPrx71* genes, respectively, resulted in ~30% resistant plants. Interestingly other treatments and stresses also induced these genes. In the case of *AtPrx21*, stamen abscission enhanced expression (Cai and Lashbrook, 2008), suggesting that AtPrx21 has a protective role against pathogen attack during the cell separation process. *AtPrx62* was also induced by phosphate starvation (Hammond *et al*., 2003). In the case of *AtPrx71*, phosphate starvation (Hammond *et al*., 2003), as for *AtPrx62*, but also low oxygen (Klok *et al*., 2002), xylem secondary cell wall formation (Yokoyama and Nishitani, 2006), and stamen abscission (Cai and Lashbrook, 2008) induced this gene, possibly indicating an involvement in signalling rather

than directly in defence mechanisms. Indeed, the peroxidase hydroxylic cycle (Liszkay *et al*., 2003; Passardi *et al*., 2004*a*) leads to the formation of various ROS which can act as part of signal transduction pathways (Laloi *et al*., 2004). In addition, the fact that AtPrx71 protein has also been purified in relation to H_2O_2 production (oxidative burst) induced by hypo-osmolarity in cell cultures (Rouet *et al*., 2006) supports this hypothesis. Unfortunately, the lack of physiological characterization of the transgenic plants hinders the further identification of the specific functions of these three genes. It would be interesting to compare, for example, lignin content and H_2O_2 levels in leaves of overexpressing mutants with those in the wild type and loss-of-function mutants if they are available.

A remarkable study concerned the isoform AtPrx03 that was identified in cold response (Llorente *et al*., 2002). *AtPrx03* transcripts were exclusively detected in etiolated seedlings and roots of adult plants. Plants overexpressing *AtPrx03* also exhibited an increase in dehydration and salt tolerance, while antisense suppression of *AtPrx03* expression produced dehydration- and salt-sensitive phenotypes. Studies on *AtPrx03* expression showed that the gene was induced in etiolated seedlings, but not in roots, exposed to dehydration, salt stress, or abscisic acid (ABA). These results again suggest the existence of common mechanisms of defence/tolerance to different stresses, but also the existence of a complex regulation through different signalling pathways of an individual peroxidase gene.

Some unclear results are also reported. The identification of AtPrx22 in potassium deficiency (Kang *et al*., 2004) remains to be confirmed. Indeed, in the text, the authors mentioned At2g38380 (AtPrx22) as the protein produced after K^+ starvation, but curiously they annotated it as a peroxiredoxin. Some contradictory results also appeared in two different studies concerning aluminium stress in roots (Richards *et al*., 1998; Kumari *et al*., 2008), and ozone stress in shoots (Ludwikow *et al*., 2004; Miyazaki *et al*., 2004). In both cases, the two studies resulted in the identification of different peroxidase genes. These discrepancies can be attributed to biological variations, growth conditions, experimental variations, and use of different detection criteria, but also illustrate the limits of global approaches and the need for complementary analysis to be conclusive. Another surprising result concerns *AtPrx49* that was induced in aluminium stress (Kumari *et al*., 2008) but was also reported in the compatible interaction with nematodes (Vercauteren *et al*., 2001). This result could indicate some type of common defence mechanism that is overcome by the nematode in this particular case.

Lignification

Lignification is one of the functions classically attributed to class III peroxidases. Lignification occurs during normal growth but also during defence responses (El Mansouri *et al*., 1999; Cosio *et al*., 2008*b*). *AtPrx42*, *AtPrx64*, and *AtPrx71* were identified by microarrays during xylem secondary cell wall formation (Yokoyama and Nishitani, 2006). Similarly, AtPrx53 was shown to be expressed in vascular bundles by fusion of the regulatory sequence with β-glucuronidase (GUS; Ostergaard *et al*., 2000). The corresponding proteins are therefore putatively involved in lignification, although definitive proof would require further investigation, especially since *AtPrx71* is induced by many different conditions, as mentioned earlier. Some of the genes identified during hypocotyl cell elongation could also be involved in lignification (Irshad *et al*., 2008). Class III peroxidases restrict cell growth by forming phenolic linkages in the wall (Pedreno *et al*., 1995).

Two other well-documented studies concern the two peroxidases AtPrx66 and AtPrx17 that are putatively involved in lignification. *AtPrx66* showed a high degree of homology to *ZePrx01* (*ZPO-C*), a peroxidase gene of *Zinnia elegans* that is expressed specifically in differentiating tracheary elements and catalyses lignin formation *in vitro* (Sato *et al*., 2006). *AtPrx66* was specifically expressed in root vessels (Sato *et al*., 2006), which suggests a putative function for this gene in lignification.

Recently, AtPrx17 function in the lignification of siliques has been demonstrated. In *A. thaliana*, the dehiscence zone (DZ) and the endocarp b (en*b*) are composed of highly specialized cells essentially involved in the pod shatter mechanism (Roeder and Yanofsky, 2006). Lignification of the en*b* layer happens at stage 17 of silique development (Roeder and Yanofsky, 2006). This lignification step is necessary for a proper shatter mechanism (Spence *et al*., 1996). Plants treated with SHAM, a peroxidase inhibitor, showed a delay of silique shatter (Cosio *et al*., 2008*b*). A first screen to study isoform expression during flower and silique development was performed with macroarrays composed of short highly specific sequences (90–400 bp) corresponding to the 73 peroxidase genes (Valério *et al*., 2004). Several putative candidates for peroxidases involved in flower and silique development have been identified. Lines of T-DNA insertion mutated for these individual peroxidases were monitored for peroxidase protein activity and gene expression level in flowers and siliques. AtPrx17 was identified and shown through analysis in diverse mutant plants to be involved in lignification of the en*b* of the silique (Cosio *et al*., 2008*b*). A careful analysis of the *AtPrx17* regulation pathway was also conducted. Key regulators of *AtPrx17* expression were AGL15/18 transcription factors that are known to be involved in age-dependent mechanisms (Fernandez *et al*., 2000; Adamczyk *et al*., 2007). Gibberellins (GAs) also had an effect on expression levels of the AtPrx17 gene and protein. In this study,

three other genes (*AtPrx13*, *AtPrx30*, and *AtPrx55*) were identified as being mainly expressed in flowers and regulated by the transcription factors SHP1 and SHP2 (Cosio *et al*., 2008*b*). These three genes might therefore have a putative role in DZ lignification as this mechanism is known to be regulated by SHP1 and SHP2 (Liljegren *et al*., 2004). Nevertheless, T-DNA mutants of these genes did not show any particular phenotype. Therefore, the precise role of these three peroxidase genes is not yet clear. However, the success of this study illustrates the interest in combining expression data with reverse genetics to identify rapidly and efficiently the genes required for complex molecular processes.

Interestingly *AtPrx17* and *AtPrx30* have been identified amongst other peroxidase genes by microarrays in the stamen abscission zone (AZ; Cai and Lashbrook, 2008) and in monolignol polymerization (Ehlting *et al*., 2005), suggesting their involvement in both silique DZ and stamen AZ lignification. There is accumulating evidence that common mechanisms exist between the different cell separation process events of a plant life (Sander *et al*., 2001; Roberts *et al*., 2002; Stenvik *et al*., 2006). It is also known that at the proximal side of the separation zone, cell walls become heavily impregnated with both lignin and suberin, and this may be related to peroxidase activity (vanDoorn and Stead, 1997).

Auxin catabolism, a heterologous study

Heterologous studies of peroxidase isoforms from other plant species expressed in *A. thaliana* also helped to assess peroxidases function. This kind of approach is obviously particularly useful when studying unsequenced and/or difficult to transform plants species but they also give information on endogenous *A. thaliana* genes. For example, in previous studies, the identification and isolation of an anionic peroxidase CpPrx01 (APRX) from the apoplast of zucchini was reported (Carpin *et al*., 1999, 2001). More investigations were needed to determine the function and regulation of CpPrx01 *in planta*. The CpPrx01 protein sequence is largely conserved among Cucurbitaceae, but absent from the other Eudicotyledons. The closest homologue in *A. thaliana* only shows 53% identity and displays significant structural differences (Cosio *et al*., 2008*a*). Therefore, the localization and effect of heterologous CpPrx01 were analysed in transgenic *A. thaliana* (Cosio *et al*., 2008*a*). The protein was localized near the cell wall, mainly produced in the elongation area of the hypocotyls, and responds to exogenous auxin, confirming data previously obtained in zucchini (Dunand *et al*., 2002). In addition, the ectopic overexpression of CpPrx01 induced changes in growth pattern and a significant reduction of endogenous indole-3-acetic acid (IAA) level. In contrast, silencing of CpPrx01 resulted in an increase of the endogenous IAA level in transgenic *A. thaliana*, certainly due to the silencing of endogenous peroxidases also involved in auxin catabolism. Since CpPrx01 is a heterologous protein, its suppression should not result in any phenotype. The observed phenotype can only result from affected endogenous proteins. The antisense strategy is known to be unspecific. For example, *A. thaliana* plants expressing the heterologous French bean peroxidase type 1 (FBP1) resulted in the silencing of at least two endogenous genes in leaves (Bindschedler *et al*., 2006). It is highly probable that the silencing of *CpPrx01* in *A. thaliana* also affected the transcription of *Arabidopsis* peroxidases involved in auxin catabolism and therefore result in a higher endogenous auxin content in silenced *CpPrx01* seedlings. Moreover, bands were missing on IEF gels of silenced *CpPrx01* (Cosio *et al*., 2008*a*). Nevertheless, further work will be necessary to identify the genes concerned and to assess their function.

Discrepancies: the case of AtPrx33 and AtPrx34

Although several studies, such as those concerning stigma-specific peroxidase, give concordant results (Swanson *et al*., 2005; Tung *et al*., 2005), there obviously exist some discrepancies difficult to explain with the current level of knowledge. AtPrx33 and AtPrx34 provide a useful example.

An early defence response to pathogen attacks is the oxidative burst leading to the production of ROS including H_2O_2 (Doke *et al*., 1996). *Arabidopsis thaliana* plants expressing the heterologous FBP1 exhibited an impaired oxidative burst and enhanced susceptibility to pathogens, but showed a normal morphology (Bindschedler *et al*., 2006). Analysis of the expression of the mutant gene by microarray and RT-qPCR showed that *AtPrx33* and *AtPrx34*—two close homologues—were silenced in leaves.

Other authors also studied *AtPrx33* and *AtPrx34* (Passardi *et al*., 2006). They showed that genes were mainly expressed in the cell wall of roots. Their role in the cell wall was investigated using (i) insertion mutants that have suppressed or reduced expression of *AtPrx33* or *AtPrx34* genes, respectively; (ii) the corresponding double mutant; and (iii) a mutant overexpressing *AtPrx34*. The major phenotypic consequences of these genetic manipulations were observed at the level of seedling root lengths. Seedlings lacking *AtPrx33* transcripts had shorter roots than the wild-type controls, and roots were even shorter in the double mutant. Seedlings overexpressing *AtPrx34* exhibited significantly longer roots. These modifications of root length were accompanied by corresponding changes of cell length. The results suggested that AtPrx33 and Atprx34 are involved in the reactions that promote cell elongation in roots and that this most probably occurs within cell walls.

What explanation could there be for the fact that a peroxidase gene would be at the same time responsible for

root cell length and leaf oxidative burst? Moreover, *AtPrx34* was also identified in microarray studies concerning infection by *Pseudomonas* spp. (Mohr and Cahill, 2007), aluminium stress (Richards *et al*., 1998), ozone stress (Ludwikow *et al*., 2004), phosphate starvation (Hammond *et al*., 2003), cell elongation (Irshad *et al*., 2008), and stamen abscission (Cai and Lashbrook, 2008). Taken together, there are data supporting the involvement of AtPx34 in defence mechanisms as well as developmental processes. Both proteins were also identified in proteome analysis of leaf vacuoles (Carter *et al*., 2004). Since it is doubtful that an individual peroxidase protein can have several different functions *in planta*, further experiments are needed to explain these discrepancies. It would be interesting to test, for example, susceptibility to pathogens, phosphate starvation, and tolerance to aluminium and ozone of the loss-of-function and overexpressing mutants of *AtPrx33* and *AtPrx34*. Monitoring the root length of the FBP1 mutant also seems necessary. Nevertheless, in this particular study,overexpressing and loss-of-function single and double mutants clearly showed opposite phenotypes (Passardi *et al*., 2006), providing strong evidence for the involvement of AtPrx33 and AtPrx34 in cell wall elongation. As mentioned earlier, transgenic plant approaches have proven to be more conclusive than other approaches.

Relationship between structure and function in class III peroxidases

The protein structure of the class III peroxidases (Fig. 1), as well as key amino acid residues and protein size, are highly conserved between orthologues and paralogues even in evolutionarily distant plant families (Ostergaard *et al*., 2000; Nielsen *et al*., 2001; Welinder *et al*., 2002). Several key features are reported [horseradish peroxidase-(HRP) C numbering] notably the proximal (Thr171, Asp222, Thr225, Ile228) and distal (Asp43, Gly48, Asp50, Ser52) Ca^{2+}-binding sites, the central active site residues Arg38, Phe41, His42, Asn70, and His170, and the carbonyl of Pro139 that accepts a hydrogen bond from reducing substrates and determines peroxidase substrate specificity (for a review, see Veitch, 2004). Residues 44–75 in the BC loop are also important for activity in plant peroxidases as Asn70 in this loop is hydrogen-bonded to the active site distal His42, thereby orienting the hydrogen-bonding network in the distal cavity and regulating the p*K*a value of this histidine. A conserved glutamic acid (Glu64) participates in the same hydrogen-bonding network, which also involves the distal Ca^{2+}.

Because of the protein structure conservation, there is no simple correlation between the amino acid sequence similarity and the function, as illustrated by, for example, the study of AtPrx62 and AtPrx71 that have been identified together as responding to stresses and development in different studies (see above and Table 1). The two proteins have similar amino acid sequences (Tognolli *et al*., 2002; Welinder *et al*., 2002). Theoretical comparative models of AtPrx62, and AtPrx71 but also AtPrx25, AtPrx69, Udp1, and cotton and pepper peroxidases were constructed from the primary structure of the corresponding mature proteins (Douroupi *et al*., 2005). This modelling approach dictated that the predicted mature proteins contain the important and characteristic residues of the plant peroxidase superfamily. No particular structural feature was found in AtPrx62 and AtPrx71, except an uneven distribution of charges on the surface of AtPrx62 and AtPrx71 (Douroupi *et al*., 2005). Positively charged surface residues were clustered in a remote area, away from the putative channel for substrate entry. The distribution of charges might play an essential role in binding of enzymes to the sites of their biological activity through electrostatic interactions. Differences in the glycosylation patterns and surface charges among the peroxidase protein family members could also potentially be involved in the determination of substrate specificity. In the case of the cationic peanut peroxidase, site-directed removal of each of the three N-linked complex glycans revealed that the N-60 and N-144 glycans influence the peroxidase catalytic activity, whereas the N-185 glycan is important for the thermostability of the enzyme (Lige *et al*., 2001). It has been postulated that glycans could affect substrate access because of their large size (Douroupi *et al*., 2005; Gabaldon *et al*., 2007), and could affect reaction dynamics due to a dampening of backbone motion (Nielsen *et al*., 2001).

The shared structural features of class III peroxidase proteins could indicate similar mechanisms but perhaps not similar specific functions. In addition, the data also seem to indicate that class III peroxidase genes with different specific functions could show a similar regulation pattern. Indeed, AtPrx21 that is regulated similarly to AtPrx62 and AtPrx71 has low structural homology with them. Notably, the conserved residue Asn70 is changed to Ser70, probably resulting in a significant change in the enzyme kinetics. Other authors reported that despite high protein sequence similarity, *AtPrx62* and *AtPrx69* displayed a different transcript profile in response to aluminium stress (Kumari *et al*., 2008). In contrast, *AtPrx62* and *AtPrx49* had comparable temporal expression profiles (Kumari *et al*., 2008), even though their protein sequences are evolutionarily distant (Welinder *et al*., 2002; Tognolli *et al*., 2002). These facts suggest that modulation of peroxidase activity and expression following internal and external stimuli must be rigorously controlled. Indeed, promoter sequences of *A. thaliana* are highly diversified (Fig. 3). Even genes resulting from recent tandem duplication such as *AtPrx33* and *AtPrx34* present a low level of promoter region similarity (35%)

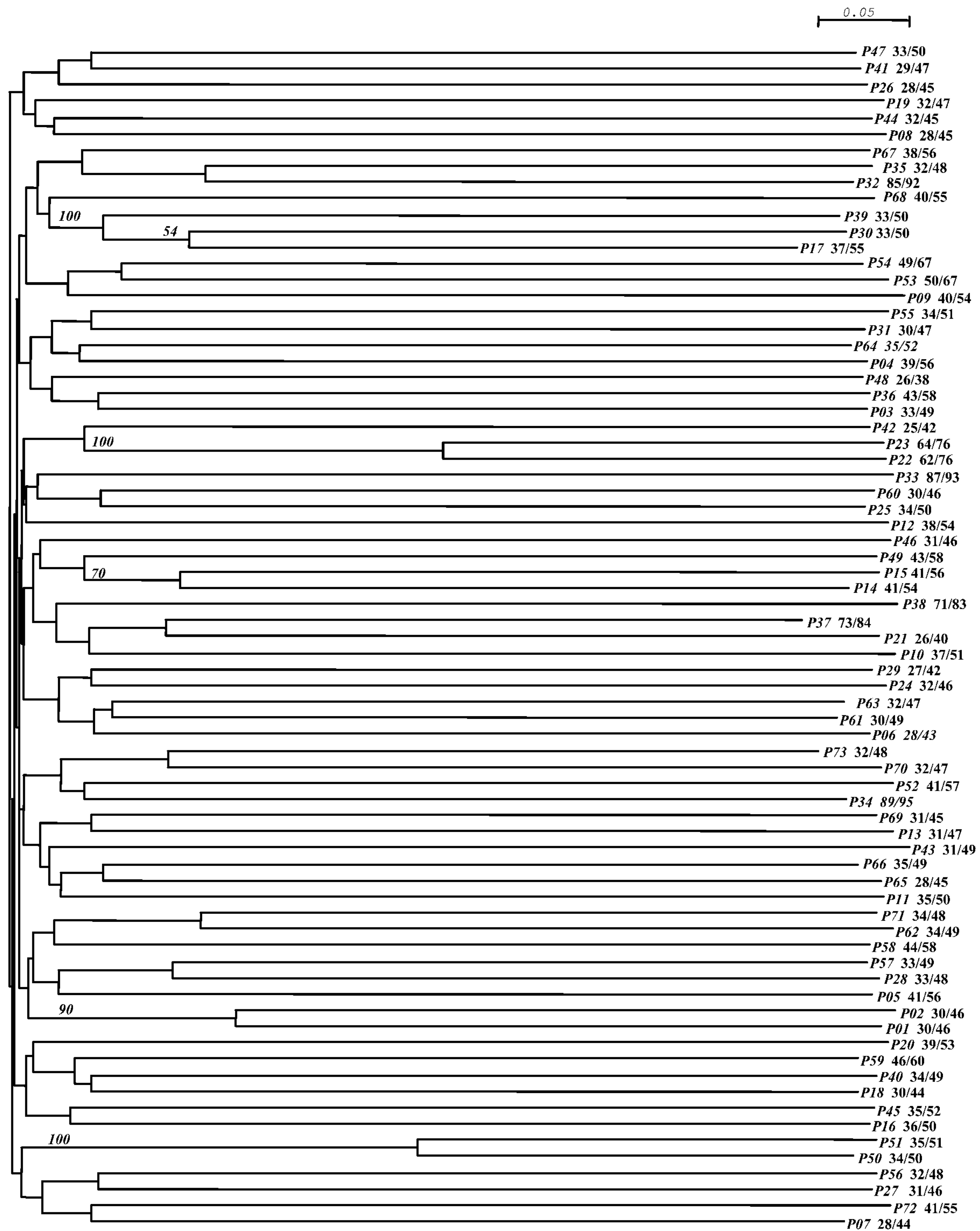

Fig. 3. Phylogenetic tree of the promoter sequences of *Arabidopsis* peroxidases. Protein sequence homology and identity (%) to the HRP-C amino acid sequence have been included next to the peroxidase name. The various groups (Duroux and Welinder, 2003; Passardi *et al.*, 2004*a*) classically found in phylogenetic analysis of class III peroxidases protein sequences do not appear in this tree, illustrating the diversification of the class III peroxidase regulatory sequences. The tree was constructed by the Neighbor–Joining method. Values at nodes indicate bootstrap supports >50%. All branches are drawn to scale and the scale bar represents 0.05 substitution per site. The 1000 bp regions upstream of the ATG were obtained from genomic sequences (www.arabidopsis.org).

and are differentially expressed (Passardi *et al*., 2006). In contrast, *AtPrx17* and *Atprx30* do not show close identity in their protein sequences (Welinder *et al*., 2002; Tognolli *et al*., 2002), but have both been identified together in different mechanisms (Ehlting *et al*., 2005; Cai and Lashbrook, 2008; Cosio *et al*., 2008*b*) and their two promoter sequences clustered together with a relatively high bootstrap support value when compared with the other levels (Fig. 3). These data support the idea that since class III peroxidase substrate specificity seems to be low (at least based on *in vitro* studies), targeting to a specific environment might also determine the role of individual peroxidase isoforms *in planta*. It seems unlikely therefore that classification based on sequence similarity alone will lead to the correct functional assignment for all individual peroxidase.

The isoelectric point (pI) is another feature that has been commonly used to characterize class III peroxidases and that—in contrast to the amino acid sequence—differs greatly (anionic and cationic forms) for the different isoforms. Nevertheless, to date, no simple correlation has been established between class III peroxidase pI and their putative enzymatic function (Welinder *et al*., 2002).

Class III peroxidases in other plants

Class III peroxidases are represented very widely in the scientific literature. As in *A. thaliana*, most functions (defence, lignifications, auxin catabolism, etc.) generally attributed to class III peroxidases as well as unexpected ones are represented, and few studies are completely conclusive.

Defence

A well documented analysis concerning defence has recently been performed in *Capsicum annum*, resulting in the identification of a peroxidase gene CaPrx02 (CaP02; Choi *et al*., 2007). Analysis of various transgenic plants suggested that CaPrx02 was involved in regulation of H_2O_2 levels during the defence response to pathogen invasion (Choi *et al*., 2007). The HR was also affected, indicating that H_2O_2 levels regulated by CaPrx02 had a role in generation of ROS involved in the HR. In addition, a correlation was found between the level of expression of CaPrx02 and *PR* genes. Although the link between the peroxidase and these genes is as yet unexplained, this could indicate that CaPrx02 is also involved in basal resistance in plants.

Infection of rice leaves by *Xanthomonas oryzae* pv *oryzae* (rice blight) induces the thickening of the secondary wall, reducing the access of the pathogen to the pit membrane, which is the pathogen's contact point in living cells (Hilaire *et al*., 2001). This thickening was correlated with strong induction in xylem vessels of a peroxidase, OsPrx114 (PO-C1), as shown by detection of specific antibodies. Nevertheless, further work will be necessary to demonstrate these relationships conclusively.

Another study describes that the presence of peroxidase activity in the growth medium of axenic *Spirodela punctata* (Lemnaceae) cultures is specifically enhanced in a concentration-dependent manner by phytotoxic halogenated phenols but not by other abiotic stress factors, elicitors, or plant metabolites (Jansen *et al*., 2004). An extracellular duckweed peroxidase (SpEx) was partially purified from *S. punctata* growth medium. *In vitro* studies showed that it is capable of catalysing the oxidative dechlorination of halogenated phenols. The authors proposed that the ability of *S. punctata* specifically to sense environmentally persistent phytotoxic chlorophenols, and to respond by increasing extracellular levels of a peroxidase capable of catalysing their oxidative dechlorination, is part of the protection strategy of this aquatic plant against xenobiotic stress.

In contrast, in tomato, a peroxidase, LePrx06, was identified that conferred susceptibility to *Pseudomonas syringae* pv tomato (Coego *et al*., 2005). This gene was induced by H_2O_2, and expression of the protein (GUS reporter gene) was limited to the area surrounding the site of infection. Antisense transgenic plants showed an increased resistance to pathogens. Nevertheless the overexpressing plants were not more susceptible than the wild type. Unfortunately, there was no characterization of lignin or ROS levels of these plants, limiting the precise function and mechanism of action of this peroxidase to pure speculation.

Another interesting study concerns a calcium-dependent peroxidase isolated from the latex of the Mediterranean shrub *Euphorbia characias* (ELP; Medda *et al*., 2003). Calcium plays a central role as a second messenger in the regulation of a number of physiological processes in plants, as in all eukaryotes. Among plant Ca^{2+}-sensing proteins, calmodulin (CaM) is increasingly appreciated as a critical player, but identifying its downstream target proteins and understanding their functions is still a work in progress. In this study, the authors isolated and sequenced the ELP cDNA and showed that the mature protein harbours two distinct CaM-binding sites (Mura *et al*., 2005). ELP–CaM interaction was also determined experimentally and by computer-assisted prediction methods. Subsequently, the authors demonstrated the presence of CaM in the latex. Finally, they showed that Ca^{2+}/CaM enhanced ELP activity. On the basis of these results, they proposed that Ca^{2+}/CaM participates in the regulation of *Euphorbia* latex peroxidase activity and, presumably, in the associated plant defence mechanisms. Additional research will be needed to determine the exact localization of ELP and CaM in *Euphorbia*.

Lignin

In Norway spruce, three genes of class III peroxidases were identified. One displays correct spatio-temporal localization for participation in the maturation of the

spruce tracheid secondary cell wall (Marjamaa *et al.*, 2006). In tobacco (*Nicotiana* spp.), a peroxidase, NtPrx60 (TP60), was identified whose antisense suppression led to a significantly lower lignin content and vascular tissue modification (Blee *et al.*, 2003). In aspen, antisense produced a decrease of G units of lignin (Li *et al.*, 2003). Nevertheless, as the antisense strategy is not totally specific and might also affect the expression of other genes, it would be desirable to complement these studies with overexpressing plants and *in vitro* studies in order to assess their function unambiguously.

Auxin

In *Vitis vinifera* a peroxidase (VvPrx07) with IAA oxidase activity was identified (Vatulescu *et al.*, 2004). This peroxidase seemed to control IAA levels during root initiation and development. In zucchini, the involvement of CpPrx01 in auxin catabolism was supported by the fact that CpPrx01 showed an elevated *in vitro* auxin oxidase activity (Cosio *et al.*, 2008*a*). CpPrx01 was found to be localized in the cell walls, an environment in which peroxidase activity and IAA are present (Kawano, 2003). Such localization was consistent with the measured changes in auxin levels as well as linked alterations in growth observed in CpPrx01 gain-of-function and loss-of-function plants (Cosio *et al.*, 2008*a*). It was therefore proposed that CpPrx01 participates in the local regulation of auxin level and consequently may control the hypocotyl elongation process.

Other functions

In Chinese red radish, a peroxidase RsPrx01 was purified from roots (Wang *et al.*, 2004). The optimum conditions for protein activity were investigated. It showed the highest activity at ~pH 4–5 and when anthocyanins were used as the substrate *in vitro*. The protein could therefore affect the tissue colour of the roots by oxidizing anthocyanins in cell vacuoles. Nevertheless, data *in planta* are not available and would be desirable to confirm at least the localization and, if possible, the function of this protein. Similarly, α-3′,4′-anhydrovinblastine (AVLB) is a dimeric monoterpenoid indole alkaloid, produced in the leaves of *Catharatus roseus* (L.) G. Don (previously *Vinca rosea*), believed to be the metabolic precursor of the anticancer drugs vinblastine and vincristine. These two compounds were the first anticancer agents and are still widely used as a complement in cancer chemotherapy (Leveque *et al.*, 1996). Due to its pharmaceutical relevance, the monoterpenoid indole alkaloid pathway has been intensively studied. It involves >20 enzymatic steps, nine of which have been characterized at the enzyme and gene level. The dimerizing step, thought to be particularly relevant from a regulatory point of view, consists of the coupling of the monomeric precursors catharantine and vindoline to yield AVLB. The search for the enzyme catalysing this reaction led to the finding of a basic peroxidase (CrPrx1; Costa *et al.*, 2008) showing an AVLB synthase activity. These two studies illustrate the great variety of possible substrates for class III peroxidases.

Specific localization

Other peroxidases show a very specific localization, suggesting a highly specific role in the corresponding organ. In the tropical legume *Sesbania rostrata*, the nodulation process involves a peroxidase gene, *SroPrx01* (*SrPrx1*), that is transiently and specifically expressed around bacterial infection pockets and infection threads (Den Herder *et al.*, 2007). The absence of the gene resulted in aberrant structure of the infection threads. Another example is the stigma-specific class III peroxidase gene, *SSP* (*stigma-specific peroxidase*) which is expressed exclusively in the stigmas of *Senecio squalidus* L. (Asteraceae; McInnis *et al.*, 2005, 2006). Expression of *SSP* is undetectable in small flower buds, but increases during flower development, to reach a maximum in newly opened flowers when stigmas are most receptive to pollen. *In vitro*, SSP did not show enzyme activity with the monophenolic substrates phenol and catechol, or any detectable IAA oxidase activity. This suggests that SSP is unlikely to be involved in cross-linking cell wall phenolic compounds or in auxin regulation within the stigma. These conclusions were supported indirectly by the absence of significant amounts of SSP in the cell walls of stigmatic papilla as revealed by immunolocalization studies. Interestingly, SSP showed its highest peroxidase activity *in vitro* at concentrations of H_2O_2 far in excess of that producing optimal enzyme activity for HRP. Based on the fact that *Senecio* stigmas show relatively high constitutive levels of H_2O_2, it is tempting to speculate upon a functional relationship between ROS/H_2O_2 production in stigmas and peroxidase activity. Perhaps SSP and other stigmatic peroxidases are important for regulating levels of H_2O_2 in stigmas or perhaps stigmatic peroxidases produce H_2O_2. An important next step will be to determine whether SSP can generate H_2O_2. Pollination is a critical stage in the life cycle of a flowering plant. It involves a complex series of cell–cell interactions that constitute the pollen–pistil interaction (McCubbin and Kao, 2000; Hiscock and Allen, 2008). Among the 73 peroxidase genes identified in *Arabidopsis*, three (*AtPrx28*, *AtPrx39*, and *AtPrx58*) have been identified as specifically expressed in stigmas and during pollen–pistil interaction (Swanson *et al.*, 2005; Tung *et al.*, 2005). Studying knock-out as well as overexpressing transgenic plants of these genes will certainly help to reveal the function of class III peroxidases in this process.

Discrepancies

As is the case in *A. thaliana*, some data are difficult to explain with the current level of knowledge. A report

showed *in vitro* that a tobacco anionic peroxidase that was putatively involved in lignification (Mader and Fussl, 1982; Lagrimini, 1991; Lagrimini *et al*., 1993, 1997*b*) also had an auxin oxidase activity (Gazaryan *et al*., 1996). Surprisingly, further studies *in planta* failed to show convincing direct evidence of such activities. Indeed, the antisense RNA mutant showed no significant effect on lignification (Lagrimini *et al*., 1997*a*) and auxin levels were not affected in the transgenic plants (Lagrimini *et al*., 1997*b*), although phenotypic changes observed in the mutants could be explained by changes in IAA metabolism. This tobacco peroxidase also had an unexplained role as the transgenic plants had a broad range mechanism of resistance to insects (Dowd and Lagrimini, 2006) and UV (Jansen *et al*., 2001). Similarly, in tomato, the *LePrx01* (*tpx1*) gene encodes a basic peroxidase in roots ionically bound to the cell wall. Previous studies on *LePrx01*, including the development of transgenic tomato plants overexpressing this gene, supported an involvement of this peroxidase in the synthesis of lignin and suberin after wounding (Botella *et al*., 1994; El Mansouri *et al*., 1999; Medina *et al*., 1999; Quiroga *et al*., 2000; Lucena *et al*., 2003; Talano *et al*., 2006). Another study with transgenic tomato hairy root cultures overexpressing *LePrx01* by transformation with *A. rhizogenes* of transgenic tomato plants that overexpressed *LePrx01* was reported (Oller *et al*., 2005). This study showed that the overexpression of LePrx01 basic peroxidase in a hairy root system increased phenol removal from aqueous solutions. These discrepancies are again most certainly explained by the low substrate specificity of class III peroxidase. Indeed class III peroxidases are capable of oxidizing a wide variety of hydrogen donors, but only minor differences in substrate specificity are observed *in vitro* among isoforms.

Future prospects for identification of the specific functions of plant peroxidases

A remarkable study reported the overexpression HRP-C and targeting to various compartments in tobacco plants. Subcellular targeting appeared to be a key determinant of the phenotype of the transgenic plant (Heggie *et al*., 2005). Information about the timing and tissue specificity of peroxidase gene expression therefore seems to be of the greatest importance, as it may indicate the putative function of a specific isoform encoded by that specific gene. Recently, a large number of studies have been conducted describing the temporal and spatial control of specific peroxidases genes (e.g. in *A. thaliana*, Valério *et al*., 2004; in wheat, Liu *et al*., 2005; in poplar, Sasaki *et al*., 2007). In the near future, it is anticipated that further microarray analysis and ultra high-throughput DNA sequencing and/or proteomic systems analysis will provide a more detailed understanding of the extent of peroxidase regulation and putative function of any plant life event, and also in different relevant mutants. These reports will allow a first screen of redundant peroxidase genes putatively involved in the same specific process to be performed. Further studies using transgenic plants with suppression or enhancement of each individual peroxidase gene will be needed to complete the data. Although the results obtained to date with transgenic plants indicate that the relationship(s) between peroxidase overexpression/silencing and function identification is not always straightforward, to date this type of study has been the most successful in attributing a function to a gene. As mentioned earlier, the difficulty in evaluating the function of each individual peroxidase is linked to the redundancy of genes in a single plant species. Studies by whole transcriptome analysis will indicate candidate genes for redundancy. In most cases it would be necessary to construct multiple mutant plants in order to produce conclusive data. Localization of isoforms *in planta* should also be confirmed by design of peroxidase-coding sequence fused to reporter genes such as GUS or green fluorescent protein (GFP). Finally, the regulatory sequences should be closely studied to identify potential regulators. The regulation pathway should also be investigated. Ideally, if the protein can be purified, an *in vitro* confirmation of the function should complete the study.

After >30 years of investigation and considerable interest in peroxidases, little is known concerning the function of individual genes *in planta*, and much remains to be investigated. This review emphasizes the importance of combining several approaches to assess unambiguously the precise role or process catalysed by the specific peroxidase in the identified functions. The complexity of the identification of individual peroxidase function is apparent. It seems that it is the lack of different evidence in the numerous reports concerning peroxidases in the literature that results in inconclusive data. Studies combining whole transcriptome studies, transgenic plants, physiology, and biochemistry seem the only way of gaining insight into the precise role in plants of the multigenic class III peroxidase family.

Concluding remarks

Increased understanding of the regulation of expression levels, biochemistry, and physiology of individual genes of the large peroxidase family is essential for a better understanding of the functions, regulation, and evolution of this key multifunctional enzyme family. Moreover, it is anticipated that the genetic engineering of peroxidase genes may assist future breeding efforts. The strong nature of the phenotype resulting from the vast majority of the genes identified to date generally impairs plant development, suggesting that the production of crop lines with less severe phenotypes will be necessary to apply this technology to agriculture (Ostergaard *et al*., 2006). Good candidates are

likely to be found in multigenic families such as peroxidase genes because they show a high redundancy and probably a functional specialization. Consequently, mutation of a single or a few isoforms should not affect general plant development and could be exploited to improve agriculturally and ecologically important traits.

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