ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 6, pp. 609-621. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © I. V. Maksimov, E. A. Cherepanova, G. F. Burkhanova, A. V. Sorokan', O. I. Kuzmina, 2011, published in Biokhimiya, 2011, Vol. 76, No. 6, pp. 749-763.

REVIEW

Structural–Functional Features of Plant Isoperoxidases

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Received January 22, 2011
Revision received February 8, 2011

Abstract—Current data on structural–functional features of plant peroxidases and their involvement in functioning of the pro-/antioxidant system responding to stress factors, especially those of biotic origin, are analyzed. The collection of specific features of individual isoforms allows a plant to withstand an aggressive influence of the environment. Expression of some genes encoding different isoperoxidases is regulated by pathogens (and their metabolites), elicitors, and hormone-like compounds; specific features of this regulation are considered in detail. It is suggested that isoperoxidases interacting with polysaccharides are responsible for a directed deposition of lignin on the cell walls, and this lignin in turn is concurrently an efficient strengthening material and protects the plants against pathogens.

DOI: 10.1134/S0006297911060010

Key words: peroxidases, genes, expression, structural–functional features, pathogens, resistance

In 1898 Linossier isolated an enzyme from mammalian leukocytes that he called peroxidase (PO). The outstanding Russian scientist A. N. Bach considered this enzyme to be an essential component of the total respiratory oxidative system, determined optimal conditions for its activity, and described its substrates [1]. Among followers of A. N. Bach, B. A. Rubin must be mentioned, who considered PO as the most important enzyme involved in plant defense against pathogens.

In 1942 Theorell separated horseradish root PO into two components different in ability to oxidize phloroglucinol, and in 1966 Shanon et al. identified five isoforms with different contents of hydroxyproline and aromatic amino acids. Later it was found that horseradish, rice, and maize have up 42, 25, and 12 isoforms, respectively [2].

CLASSIFICATION OF PEROXIDASES

By substrate specificity, peroxidases are divided into 16 classes [3]. The most characteristic physiological role of the five classes of PO is considered: catalases (EC 1.11.1.6), true PO (EC 1.11.1.7), glutathione-PO (EC 1.11.1.9 and 1.11.1.12), ascorbate-PO (EC 1.11.1.11), and peroxiredoxins (EC 1.11.1.15).

Based on the structural and catalytic properties, POs are classified into three superfamilies [4, 5]. The first superfamily includes POs of animals, the second superfamily includes catalases of animals, plants, bacteria, and fungi, and the third superfamily includes POs of plants, fungi, and microorganisms. The third family is also subdivided into three large classes [3, 4, 6]. The first class includes plant ascorbate-POs (EC 1.11.1.11), catalase-POs of microorganisms (EC 1.11.1.6), and cytochrome *c*-POs (EC 1.11.1.5), and their main function is to utilize H_2O_2 [7]. Ascorbate-POs have a high affinity for ascorbate and are found in photosynthesizing organisms. In higher plants ascorbate-POs are located in chloroplasts (stroma and thylakoid-bound forms), peroxisomes, and cytoplasm [8]. Catalase-POs are located in mitochondria and use cytochrome as a donor of electrons. They can function as POs, but, similarly to catalase, can also utilize H_2O_2 and oxidize other substrates [9]. The second class combines manganese-PO (EC 1.11.1.13) and lignin-PO (EC 1.11.1.14) of fungi and also movable POs (EC 1.11.1.16) [10]. The third class is represented by a family of functionally similar proteins including classic plant POs, which are mainly located in the apoplast, cell wall, or vacuole [6, 11-13]. X-Ray crystallographic analysis revealed three-dimensional structures of some plant POs that suggested their conservative nature even in distant evolutionary taxons [14]. It is supposed that the class III POs have played a crucial role in the colonization of land by plants and in their adaptation to oxygen of the atmos-

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Abbreviations: PO, peroxidases; ROS, reactive oxygen species.
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phere [12]. The origin of these POs is thought to be associated with the necessity of protecting plants against the high level of peroxides, utilization of reactive oxygen species (ROS), and transformation of toxic phenolic compound into lignin and suberin. On one hand, this promoted the strengthening of the cell walls of plants and occupation of the Earth's surface, and, on the other hand, became an important contribution to biogenesis of soils [15-17].

There are no less than 73 genes encoding POs in the *Arabidopsis* genome and no less than 138 in the rice genome [4, 5, 12, 18-20]. Such a great number of PO-coding genes suggest that the homology between them in plant taxonomic series can be from 10 to 99% and be high in isoPOs from the evolutionarily remote plant genera, be virtually absent in related species, and display variability within the same species [21]. The homology of POs from taxonomically remote plant species can be assessed using a cross interspecific precipitation of antibodies against different PO isoforms [22] or by data of northern-hybridization using random primers to the PO genes [23].

From 50 genes of the rice POs (according to GenBank, EMBL, DDBJ) about 60% contain four exons interrupted with three introns. This structure is believed to be a classic one for genes of the class III plant POs, including those of *Arabidopsis* [5, 18] and wheat [20, 24]. The *TmPRX1* gene of spelt wheat PO has a 96% homology with the gene *TaPERO* of soft wheat. Comparison of expression profiles of the PO genes in a hybrid H-93-8 line of the wheat *Triticum aestivum* with those of *Aegilops ventricosa* resistant to the nematode *Heterodera avenae* and with those of a perceptive cultivar H-10-15 revealed the activation of about 20 genes encoding these enzymes [20]. Using a detailed analysis, among them the genes *TaPrx111*, *TaPrx112*, and *TaPrx113* were separated that encoded apoplastic POs of class III with a high expression level in the resistant plants.

Different isoforms can be products of the same gene modified on the post-transcriptional and post-translational levels [25-28]. Thus, the *PXD* gene located in the fifth chromosome of *A. thaliana* is actively expressed in the majority of plant organs and controls formation of three isoforms of anionic POs involved in lignification of the cell walls. This gene is represented by two copies, *AtPrx53* and *AtPrx54*, which seemed to have arisen as a result of a tandem duplication of the *PXD* gene [27]. The rice genes *poxA* and *poxN*, despite the complete identity of the translated region, play different roles in ontogeny of plants due to significant differences in the structure of the promoters. Of three complete nucleotide sequences of cDNA clones found in tobacco, one was identical to PO of pod (I02979), whereas the two others were identical to PO *TP60* (AF149251) [29]. Comparison of the transcriptional activity of the *TP60* genes and of the anionic PO *TP02* indicated that the activity of *TP60* gene dominates in lignified tissues of the stalk and roots.

Duroux and Welinder [6] studied the evolutionary relationship of the PO genes in higher plants and revealed noticeable differences in their structure in monocotyledonous and dicotyledonous plants. Using the Rise Genome Research program, rice was shown to have about 42 expressing PO genes [4] that were tissue specific and appeared at strictly determined periods of ontogenesis or under the influence of certain stress factors. By structural analysis of the rice gene cluster BAC q3037, a 22.5 kb PO gene group was identified that included *osp1*, *osp2*, *osp3*, *osp4*, and *osp5* [30]. The genes *osp4* and *osp5* each had three classic exons, whereas *osp1*, *osp2*, and *osp3* had two exons. The genes *osp1*, *osp4*, and *osp5* seemed to encode anionic POs. Comparative and evolutionary analysis of cluster genes indicated that they were represented by tandem gene duplications (*osp1* and *osp5*), whereas *osp5* was a potential ortholog of *ap1* and *prx7*. The *osp1-5* genes of rice represent an independent new evolutionary branch of the PO class III. Allelic variants of the anionic PO gene *ZmPox3* were studied in a series of 37 inbred lines and ecotypes of maize [31]. The gene was found to be of ~1.7 kb in length and to have in the structure two short introns with 127 and 111 bp. Thirty-one single-nucleotide substitutions (on average, 1 SNP per each 57 bp) and 17 insertions and deletions, which comprise 20% of the full length of the coding region, are detected. The most variable in the gene *ZmPox3* were noncoding regions, especially introns, which have on average one substitution per 14 bp. However, exons had one substitution per 82 bp.

Thus, plant POs are represented by genetically different proteins with the same enzymatic activity [32], and the highly variable genes encoding them are controlled by various *cis*-elements and *trans*-factors that are responsible for their differentiated expression activity depending on environmental conditions and stages of ontogenesis.

STRUCTURES OF PROTEINS WITH PEROXIDASE ACTIVITY

PO (donor: H_2O_2, oxidoreductase) belongs to the family of heme-containing glycoproteins which contains as a prosthetic group a protoheme which is a complex of trivalent iron with protoporphyrin IX (1,3,5,8-tetramethyl-2,4-divinylporphyrine-6,7-dipropionic acid). The protoheme is a component of the active center as a porphyrin ring of the heme and 10 α-helices, which represent its protein moiety [5, 33, 34]. All plant POs contain conservative amino acid residues as follows: Leu2, Tyr7, Pro12, Ile17, Phe41, Gly48, Glu64, Gly76, Phe77, Lys84, Glu88, Pro92, Val95, Ala98, Gly114, Pro115, Asp125, Phe152, Asp161, Leu166, Gly168, Gly173, Arg183, Gly242, Leu250, Phe273, Phe277, Met281, Gly295, Arg298, and Asn305 (they are numerated after PO of *Arabidopsis*) [32, 35]. A region between the proximal domain and the seventh cysteine residue is the most variable in the length and amino acid sequence. This region forms a substrate channel of PO and determines physiological functions of the isoforms. Models of *Arabidopsis* proteins AtPrx25, AtPrx62, AtPrx69, and AtPrx71, and also of PO of cotton and pepper based on their predicted primary structure revealed that a supposed matrix protein should possess important key functions of PO. Thus, for highly homologous proteins AtPrx62 and AtPrx71 no structural differences were detected except unevenness in the charge distribution on the surface of molecules [32]. But just this seems to be the key factor for the enzyme binding with other compounds, e.g. substrates or polysaccharides.

The structural conservativeness of the class III POs shows similarity of their enzymatic reactions but cannot explain specific functions of every isoform, which seems to be associated with the epigenetic regulation of their synthesis. In fact, the promoter sequences of *Arabidopsis* are very different [5]. Although the core part of the proteins AtPrx17 and AtPrx30 are not very similar in amino acid sequence, their combined functioning under different stress situations indicated a pronounced likeness of the promoter parts of their coding genes. Thus, the specific function of the enzyme depends on the environmental conditions, and, according to Cosio and Dunand [5], in determining the function of the enzyme not only the PO homology must be taken into account but also the efficiency of the promoter of the gene.

The free heme of PO forms complexes with different ligands and is the main participant in the electron transfer and catalysis during redox reactions, and possibly is responsible for the corresponding activities of the proteins. The porphyrin ring, which interacts hydrophobically with the apoprotein, is important for formation of the tertiary structure of the protein, stabilizes the protein globule of the enzyme, and sharply increases its resistance to the action of ultraviolet and heat. The binding of protoheme with the apoenzyme is provided for by a salt bridge between the propionic acid residue and one of the amino acid residues of the protein, and damaging this bond significantly decreases the catalytic activity of the enzyme [34]. Ferriporphyrin acting as an active center is involved in utilization of H_2O_2, which results in production of radicals of the corresponding substrates [33]. The key role of hemin in the enzymatic processes with the involvement of PO is supported by data on the addition of the PO activity to antibodies (abzymes) and RNA (ribozymes) upon its covalent binding [36, 37].

The iron ion in PO activates H_2O_2 and contributes to the substrate oxidation. Charged residues of arginine (Arg38) and histidine (His42) from the active center of the enzyme are involved in this process, whereas hydrophobic phenylalanine residues Phe41 and Phe143 are responsible for binding aromatic substrates. And the location of heme in the PO molecule allows its vinyl groups to be directed into the protein moiety, and propionic acid residues are directed outwards. The propionic acid residues bind the heme with the apoprotein by ionic bonds and also orient and fix the heme [34].

At low pH values most PO molecules disintegrates into the heme and apoprotein and loses their enzymatic properties. However, some recent works have shown that there are POs that retain their stability even at pH lower than 2.8 [38]. It seems that this is possible because the arginine and histidine residues (Arg38 and His42) are located near to the entrance into their active center, and a conformational transformation at low pH in the presence of Ca^{2+} results in closing of the substrate-binding channel [38]. Moreover, the presence of two calcium ions within the monomeric enzyme molecule ensures its thermostability [35]. Thus, in the presence of excess calcium in the catalytic medium the apoplastic moiety of PO retained its activity even on heating to 70°C.

Depending on the level of hormones and intensity of generation/utilization of reactive oxygen species (ROS) and also on the plant growth conditions, on the background of common constitutive isoPOs, some isoforms are expressed constitutive only to particular organs and phases of ontogenesis. Unfortunately, there are virtually no works detecting isoPOs with comparison of their activity profiles with the expression of the gene encoding them. It is rather difficult to find such connections because the knowledge of molecular geneticists based on comparative analysis of genes are insufficient for prediction of future dimensions and p*I* values of POs, and also there is no accounting for post-transcriptional modifications that determine the tertiary and quaternary structure of a specific isoform. Nevertheless, the generalizing reviews [5, 14, 16, 39] are extremely original; their authors analyze the role of various PO isogenes in plant physiology. Under these conditions products of particular genes could be most efficiently identified by creating transgenic plants capable of hypersynthesizing specific POs or, in contrast, containing knockout fragments [25, 40, 41]. However, in many cases even such genetic constructions cannot help to solve the problems [42], and thus the role of the majority of isoPOs in plants is still unknown.

FUNCTIONS OF PEROXIDASES IN PLANTS

Changes in the PO activity and in the spectra of its isoforms were followed on exposure to high and low temperatures, hormones, under conditions of insufficient of mineral nutrition, draught, salination, wounding, anaerobiosis, and in the presence of pathogens [5, 43-46]. The generality of PO activation under various stresses and its involvement in many physiological processes in plants are associated with a variety of genes encoding this enzyme [47, 48]. Although the heme-associated catalytic functions of PO are not numerous, its physiological functions are multiple. POs can realize their physiological functions due to the dynamic structure of the enzyme active center and its polyfunctionality.

The main function of PO is the protection of the plant against the deleterious action of ROS produced during photosynthesis and respiration [48-51]. The presence of the enzyme in chloroplasts and its ability to form an "active" complex with cytochrome *c* suggests its participation in redox reactions during photosynthesis and in mitochondria during respiration. The compensatory influence of PO on the level of ROS increased under the influence of various stress factors seems to be especially important [12, 48, 52-54]. Thus, it is known that an increased level of ROS promotes the growth of plant cells due to extension [12], but a sharp increase in ROS production resulting in an oxidative burst can induce in the plant cells hypersensitive reaction (HSR) [55]. However, suppression of PO synthesis promotes an increase in the plant sensitivity to oxidative stress [56].

The action mechanism of PO is based on its ability to interact with H_2O_2 with production of four intermediate complexes and, thus, due to oxygen of H_2O_2 to catalyze oxidation of compounds [12, 34]. Complex I is formed immediately upon the addition of H_2O_2 and usually is converted into complex II; complexes III and IV are compounds produced with excess of H_2O_2. PO activates H_2O_2, which interacts with cofactors and degrades producing hydroxy-radical forms of ROS [55]. Then these radicals interact with low molecular weight phenolic compounds, which bind chaotically and form poorly hydrolyzable lignin and suberin [54, 57, 58]. Sources of ROS during catalysis can be organic peroxides, including those of unsaturated acids. And PO has a very wide substrate specificity caused by the existence of several pathways of electron transport. The first group substrates (inorganic ions) interact directly with the heme, whereas the second group substrates (aromatic amines and phenols) interact with the protein. Substrates that are hydrogen donors are oxidized by PO significantly faster than electron donors. To explain the mechanism of the enzyme action, a model is used where the enzyme is represented as a conductor protein realizing a number of channels of electron transport from substrates contacting the protein onto the iron atom of the heme [34].

PO is characterized by the ability to catalyze oxidation by the oxidase pathway of many compounds including phloroglucinol, glutathione, hydro- and naphthoquinones, NADH and NADPH, dioxyfumaric acid, dicarbonic acids, indolylacetic acid (IAA), oxalate, phenylpyruvate, and ferrocytochrome [12]. This free radical oxidation occurs with involvement of Mn^{2+} and some phenols as cofactors. Extracellular POs are shown to produce ROS in the presence of oligosaccharide elicitors and monoamines, and POs generate highly toxic O_2^-. The reaction involves 2 moles of O_2^-, and 1 mole of H_2O_2 is produced [54]. POs play a significant role in realization of the oxygenase function of ribulose diphosphate carboxylase in the presence of copper ions. The enzyme is shown to be involved in the oxidative decarboxylation of amino acids and in conversion of tryptophan into IAA [59]. However, apoplastic POs can possess activities of NADPH oxidase and of IAA oxidase [13, 34, 47, 60-63].

Activity of POs, especially of those bound by ionic and covalent bonds with cell walls, usually increases with development of xylem tracheids. The activation of specific isoPOs in a culture of tobacco explants correlated with initiation of cell division, development of leaves and stamens, and with grain formation [64, 65]. The activation of PO positively correlated with aging and increased under the influence of unfavorable environmental factors. Correspondingly, the protective effect of PO against different stress agents is associated with its contribution to strengthening of the cell wall via the oxidative interaction of ferulic acid with polysaccharides, cross-linking of hydroxyproline-enriched glycoproteins, extensin, dityrosine, and polymerization of phenolic compounds with production of lignin [25, 41]. The enzyme is involved in the post-translational maturation of protein as chaperones and is able to dimerize tyrosine residues [66, 67].

Functional activity of PO proteins encoded by the genes *AtPrx33* and *AtPrx34* was studied using *Arabidopsis* mutants with decreased or suppressed expression of one of these genes [59]. These proteins were mainly accumulated in the cell wall of the root. Both *AtPrx33* and *AtPrx34* genes are involved in the reactions promoting elongation of root cells.

But mechanisms of the protective action of PO against unfavorable environmental factors are unclear. Thus, the activity of PO in the culture (apoplastic) fluid of the duckweed *Spirodela punctata* increased depending on the concentration of phytotoxic halogenated phenols, which characterized the universal ability of PO to protect plants also against xenobiotics [49]. However, tomato PO LePrx06 is considered as a factor promoting the plant's susceptibility to *Pseudomonas syringae* pv. *tomato* [68]. The gene of this enzyme was expressed under the influence of H_2O_2 in the infected zone. Antisense-mutations increased and hyperexpression did not induce resistance to the pathogen [68]. Accumulation of lignin and ROS and activity of catalases were not discussed in this work, so one can only imagine mechanisms of a decrease in the resistance under the influence of PO.

There are many data indicating appearance of new PO isoenzymes in plants under the influence of environmental factors; nevertheless, in the majority of cases it is not established whether their appearance is associated with *de novo* synthesis or with changes in the preexisting molecules of the enzyme. However, it is clear that their appearance can be important for adaptation. On speaking about possible mechanisms of changes in the PO activity under conditions of stress, one should note the molecular and functional heterogeneity of isoenzymes that permits their ratio to be changed for supporting the isoenzymes that are more "adapted" to the stress conditions. The physiological sense of changes in the activities of PO and its isoforms is especially interesting in connection with cell metabolism under stress conditions.

PEROXIDASES IN PLANT DEFENSE AGAINST PATHOGENS

When a plant is attacked by pathogens, its defense systems are activated. The synthesis *de novo* of defense proteins under the control of salicylic (SA), jasmonic (JA), abscisic (ABA) acids and ethylene in the plant starts [39, 69-72]. Induction of signaling systems by pathogens and elicitors leads to production of reactive species of oxygen and nitrogen, antimicrobial metabolites (phytoalexins), strengthening of the cell wall due to its lignification actively contributed by PO [73]. Thus, this enzyme occupies a crucial position in plant defense against pathogens [39, 74]. A direct biocidal action of products of the PO enzymatic catalysis was shown for virtually all living beings. Thus, in humans and other animals there are several POs, and among them myeloPO or lactoPO have strong antimicrobial efficiency [75].

PO is activated in plants in response to infection both due to constitutive synthesis and to expression of pathogen-inducible isoforms [46]. Activation of PO during pathogenesis correlates with formation in the infection zone of a spontaneous local hypersensitive reaction of plant cells surrounded by a zone impermeable for water and nutrients and resulting in suppression of growth and development of pathogens. This reaction can be induced not only by pathogens, but also by hydrolases produced by them. Consequently, this reaction can be initiated by polysaccharides possessing features of elicitors and also by some stress hormones [69-72, 76, 77].

In plants from different populations of the *Aegilops umbellulata* (42 specimens) the damage coefficient with septoriosis agent negatively correlated with the PO activity in the protein fraction bound with the cell walls [78], and this corresponded to hypotheses about the importance of POs located in the cell wall for the plant resistance to pathogens [79]. In the isoenzyme spectrum of uninfected leaves of resistant forms of egilops, a PO isoenzyme was detected with p*I* ~ 7.5 [78], i.e. with p*I* close to those of POs with antifungal properties against *Fusarium*-induced dry rot of roots [80].

Purified isoPOs from *Acorus calamus* AcPOX displayed a high antipathogenic activity to the fungi *Fusarium moniliforme*, *Macrophomina phaseolina*, *Trichosporium vesiculosum* [81], those from cotton were active against *Verticillium dahliae* [82], and isoPOs from beans were active against *Coprinus comatus*, *Mycosphaerella arachidicola*, *Fusarium oxysporum*, and *Botrytis cinerea* [83]. It should be noted that one of the isoPOs from sedge cane revealed homology with isoPOs from rice BAD61674, BAD61677, and BAD61671 expressed under the influence of bacteria [81]. The antifungal activity pronouncedly depended on the enzymatic activity. Similar antifungal activities were found in POs from cotton, which were bound with chitin of the pathogen cell wall [82]. Note that myeloPO of animals inhibited the growth of pathogenic fungi, including the plant pathogens *Fusarium solani*, *Colletotrichum gloeosporioides*, and *C. malvarum* in the presence of H_2O_2 [84]. Therefore, both plant and animal POs can be considered not only as mediators in the plant protective reactions associated with synthesis of ROS, lignin, and suberin, but also as enzymes with direct fungicidal activity.

The influence of POs on systems responsible for resistance against pathogens is specific. Thus, introduction of the ascorbate PO gene of the pepper *CAPOA1* into tobacco plants resulted in its hyperactivation and induced resistance to the oomycete *Ralstonia solanacearum* but not to the pathogenic bacterium *Pseudomonas siringae* pv. *tabaci* [85].

The production of H_2O_2 and formation of lignin are contributed both by alkaline and by acidic isoPOs located in the cell walls, and mainly the covalently bound fractions [53]. This is also confirmed by increased activities of other enzymes that are involved in modification of the plant cell walls – phytoalexins and polyphenol oxidases, which promote a rapid accumulation of primary compounds involved in polymerization of lignin with POs [43]. This provides conditions for effective and rational oxidation of phenolic compounds into lignin or suberin polymers and also for formation of phytoalexins, which are toxic for microorganisms [86].

Infection of seedlings of a resistant wheat cultivar with agents of septoriosis, root rots, and smut led to activation of PO isoforms with p*I* ~3.5, ~4.5, and ~9.8 that interacted with chitin and glucan from pathogenic fungi [87, 88]. Moreover, on infection with fungal pathogens in resistant plants and also under the influence of elicitors anionic POs were also activated in the enzyme fraction bound with the cell walls [89]. Therefore, some pathways can be suggested for regulation by elicitors of protective reactions in wheat plants infected with fungal pathogens. Thus, chitin induces a defensive response in plants, first by increasing the expression of anionic PO, and, second by inducing the translocation of isoPOs into the region of cell walls where they are involved in strengthening intermolecular bonds and lignification [89, 90] that later promotes a decrease in the damaging effect of pathogens. Thus, PO is likely to be a component of the active system of plant defense. The importance of POs in regulation of physiological characteristics of plants during their interaction with microorganisms is also observed in bean–rhizobial symbiosis [91, 92]. The plants are supposed to switch on regulatory mechanisms directed to limitation of further penetration of rhizobia and their multiplication or propagation through the tissues by changing the PO activities, which allows the optimal quantity of functional nodules to be formed [92, 93].

Thus, on studies of the plant resistance attention is accentuated on the importance of molecular multiplicity of enzymes for increasing the mobility of adaptation mechanisms and eliminating pathological consequences of stress factors. Certainly, the existence of isoenzymes enhances metabolic potentials of organisms exposed to stress factors, increases the plasticity of the cell metabolism and the capability of supporting the autoregulation system, and enlarges pathways for optimization of physiological processes disturbed in pathogenesis. Data on the direct antipathogenic influence of POs, including the constitutively synthesized ones, suggest that the enzyme could be an important constituent of the defense system that is efficiently functioning in the initial stages of the organism's defense against pathogens and during the immunity induced by them.

INVOLVEMENT OF PEROXIDASES IN SIGNALING REGULATION OF PLANT RESISTANCE

Determination of the role of low molecular weight compounds possessing hormonal and immunomodulating properties in the interrelations between the host and pathogens is now an actively developed trend in modern physiology and biochemistry of plants. The hormonal system is one of the most effective systems for regulation of various physiological and biochemical reactions in plants. Correction of hormonal balance in plants and, respectively, of their total biochemical status by using as modifiers biologically active natural compounds with hormonal functions and even of microorganisms as producers of such compounds is a current strategy in plant defense [71, 93].

In the plant, POs regulate levels and activities of endogenous and exogenous signaling molecules, including those that possess growth-regulating properties [41, 94, 95]. This is realized due to enzyme synthesis *de novo* and releasing of bound forms and also due to enzyme modification by proteases [60, 96, 97]. Thus, indolylacetic acid (IAA) is poorly oxidized in the transport system, but the cell wall and apoplast where POs are located are main sites of IAA activity [35]. The ability of POs to oxidize IAA [34, 98] allows them to regulate the amount of the auxin in plant tissues [54] and influence some processes within the cell by changing plant metabolism from cell growth to cell differentiation. IAA can be oxidized in plants through different pathways, and this is a free radical process. The PO functions as an IAA oxidase depends on the hormone location within the cell [60].

The important role of POs in the regulation of IAA level is also confirmed by the enzyme possessing sites which specific bind IAA [98]. Note that in the molecule of wheat anionic PO the domain adjacent to this site was capable of binding chitin. This was proved by inhibiting the IAA oxidase activity of the enzyme on addition of acetylated chitin into the preincubation or reaction medium with oligosaccharides [62]. These data allow us to consider otherwise the structural–functional organization of POs involved, on one hand, in the regulation of auxin levels and, on the other hand, capability of binding with poly- or oligosaccharides of the cell wall of a pathogen or even of a host. In addition to these findings, which suggest the involvement of PO in IAA catabolism, there are data on the contribution of PO to catalysis of oxidative decarboxylation of amino acids and to tryptophane conversion into IAA. The interesting observation of the involvement of PO in polymerization/depolymerization of phenolic compounds should also be mentioned.

Interesting data were obtained on mutant tomato plants with low level of abscisic acid (ABA), which were poorly colonized with arbuscular mycorrhiza, and the treatment with ABA promoted infecting the roots with fungi and bacteria [99, 100]. The authors consider this reaction of the plants to be associated with a negative influence of the hormone and pathogen on the PO activity. Note that the influence of ABA on the PO activity depended on both the dose and time of exposure. Although a short-term treatment with ABA increased the enzyme activity, long-term treatment with high concentrations decreased the enzyme activity [100, 101].

As far as PO is important for development of resistance under conditions of both abiotic and biotic stress, the multiplicity of PO forms should be taken into account because this multiplicity suggests a differentiated activation/suppression of individual isoPOs in which expression, in particular, of anionic isoPOs is directly controlled by ABA [27]. However, POs can efficiently function also in the absence of ABA. Thus, there is an ABA-independent pathway of activation of ascorbate PO in cotton plants. On the fluoridone-induced decrease in the activities of SOD, catalase, and glutathione reductase, the treatment of calluses with paraquat induced in the cells an ABA-independent activation of PO [101].

The nature of the enzyme catalyzing the last stage of ethylene biosynthesis is still under discussion. The involvement of PO in this process is supposed because production of ethylene correlates with PO activity in plant tissues. In a model system PO can *in vitro* convert 1-aminocyclopropane-1-carbonic acid (ACPC) into ethylene. However, because of low specificity, the model systems were supposed to generate ROS, which in turn nonenzymatically cleave ACPC with production of ethylene.

Pathogens and their metabolites, elicitors, and hormone-like compounds effectively regulate expression of genes encoding the class III isoPOs. Activities of POs and of their genes were shown to be induced in the presence of pathogenic fungi [102], bacteria [103], and viruses [4]. Numerous data indicate that there is differentiated transcription of genes controlling biosynthesis of certain PO isoenzymes in some tissues and organs or under the influence of various physical, chemical, and also biological stress factors that is associated with the presence of multiple *cis*-elements in the promoter part of the genes encoding this enzyme [5, 18, 24, 45, 50, 102].

However, the mechanism of interaction of the transcription regulating factors with DNA-dependent RNA polymerases and promoter regions of the PO-encoding genes is still a poorly studied problem in plant genome functioning. Information concerning the regulation of expression of PO genes is especially scarce. Only for 36-44 of 74 genes encoding POs of *A. thaliana* are products of transcriptional activity determined as individual isoPOs [5]. Eight genes with nonspecific and relatively high level of expression are contained in the promoter zone *cis*-elements sensitive to hormones and stress [27]. Thus, five of them contained *cis*-elements sensitive to auxin (ACTTTA, CATATG, TGTCTC) [27], gibberellin (TAACAAA and TTTTTTCC), ABA (AWTTCAAA). The level of PO mRNA in plants increased on treatment with abscisic, jasmonic, and salicylic acids [96, 99, 104]. A positive regulation of expression of anionic PO genes under the influence of ABA has been described. But it is also known that high concentrations of ABA exclude the hypersensitivity reaction of cells induced by elicitors, salicylic, and arachidonic acids [105] selectively suppressing activities of anionic POs in wheat [106] and expression of the pathogen-induced PO genes in tobacco and tomato [4]. Promoters of the genes of *Arabidopsis* peroxidase ABRE-elements were found [27].

A *trans*-factor ARF has been identified that is highly specific to an auxin-sensitive region of the promoter (TGTCTC) in the PO gene [93]. Many PO genes are found to have *cis*-elements (YTGTCWC) capable of reacting to water stress, hypothermia, or infection. It is supposed that expression of the zucchini gene encoding the pectin-bound PO is controlled by auxins [19]. Plants hyperproducing IAA are characterized by a high PO activity and, as a result, an increased resistance under stress [43]. However, mutants insensitive to endogenous auxin are susceptible to infection with pathogens [107]. Interestingly, reaction of plants to exogenous auxin is just the opposite. It is shown that IAA inhibits peroxidase activity by oxidation of a number of phenolic compounds but induces catalysis of its own oxidation [34]. It is worth noting that the PO molecule exposes on its surface a site of auxin reception. Homologous motifs of amino acid sequence on ABP1 protein binding to IAA but differing from amino acid sequence of the active site cleaving hydrogen peroxide are found in PO [98] and in proteins of the germin family [108]. On the surface of the PO molecule the site is located within the distal domain remote from the active site of the enzyme and contains the residue Trp117 [98]. Hence, at least two mechanisms regulating PO activity exist with involvement of auxins related to their effect on the enzyme activity and gene expression. Thus, although some regulatory mechanisms of PO gene expression are known, the interaction of auxins with PO genes and their products requires further studies.

Infection of the groundnut leaves with *P. syringae* induces in both susceptible and resistant plants a short-term expression of the *Ep5C* gene, the product of which resembles an extracellular cationic PO of horseradish and does not react to treatment with salicylic acid, ethylene, methyl jasmonate, and wounding [68]. Recently the *CaPrx02* gene was detected in *Capsicum annuum*, whose product regulates the H_2O_2 level during formation of the protective response to infecting with pathogens [109]. Infection of leaves of the rice *Xanthomonas oryzae* was associated with thickening of the cell wall, which prevented penetration of the pathogen at the point of infection [110]. The cell wall thickening correlated with the activity of *OsPrx114* PO.

From the latex of the Mediterranean shrub *Euphorbia characias* a Ca^{2+}-dependent PO was isolated that interacted with calmodulin at two binding sites [111], the PO *ELP* gene was sequenced, and positions of two calmodulin-binding sites were determined [112]. The interaction of calmodulin with ELP PO was shown both experimentally and using computerized programs. The authors have also shown that the Ca^{2+}/calmodulin ratio regulates (increases) the ELP PO activity and mechanisms of the protective response of the plant.

Seven PO genes in *A. thaliana* plants were expressed under the influence of *Pseudomonas* ssp., three genes in the leaves infected with fungi, and two genes on infection by insects [113-115]. Three genes (*AtPrx21*, *AtPrx62*, *AtPrx71*) were induced upon infection with fungi [113], on wounding [116], and also in response to other stresses, which suggests their involvement in the main mechanisms of plant defense. The expression of the *AtPrx62* gene caused an 80% increase in the resistance of transgenic plants, and the insertions of *AtPrx21* and *AtPrx71* caused a 30% increase [117]. The *AtPrx21* gene product is supposed to play a protective role on exposure to pathogens and cytodifferentiation of plants [5]. The *AtPrx71* gene is expressed during insufficiency of phosphorus [118] or oxygen [119], formation of secondary cell wall of vessels [120], under conditions of hyposmolality in cell culture, and increase in the level of H_2O_2 [121]. Wounding resulted in a pronounced activation of cationic and anionic isoPOs in seedlings of *Prosopsis* plants [44]. The activity of anionic POs in plants retained for a longer time than the activity of cationic POs in the presence of cordycepin, which is an inhibitor of mRNA polyadenylation. These findings suggest that either anionic POs are more stable than cationic ones or that plants have a certain reserve of mRNA.

The ability of the *shpx6b* gene from the lucerne *Stylosanthes humilis* to express on infection with the pathogen *Colletotrichum gloeosporioides* and under the influence of methyl jasmonate and wounding can be used as an effective test system for determination of systemic acquired resistance (SAR) of plants. Being inserted into the tobacco genome, this gene gave plants with tolerance to *Ph. parasitica* var. *nicotianae*, whereas tobacco plants hyperproducing anionic POs displayed an increased biosynthesis of lignin and were resistant to *Fusarium oxysporum* and to insects [122].

PEROXIDASES IN LIGNIFICATION AND SUBERINIZATION OF CELL WALLS OF PLANTS AND PATHOGENS

In addition to cellulose, vascular plants usually contain the unique biopolymer – lignin, which is specific only for them, and its fraction in some trees can reach 30% of their dry weight. The presence of lignin in moss and algae is still under discussion. Therefore, appearance of lignin is often considered to be associated with appearance of terrestrial plants because only they have a "supporting apparatus" and a vascular system necessary for transporting nutrients and water through the tissues [123]. Lignin is also a component of the secondary cell wall and of the intercellular plate in all cells of terrestrial plants that have virtually terminated their growth, and it is responsible for their rigidity required to support the plant's weight and counteract the negative hydrostatic pressure of lifting water across the xylem [124, 125]. The same biopolymer is responsible for the plant's resistance to pathogens and phytophages because it is not degraded by their hydrolases and, thus, creates a physical barrier in the path of their expansion and inhibits diffusion of toxins produced by them. Tissues with hypersynthesis of a pathogen- or phytophage-induced PO, as well as tissues of transgenic plants with high PO activity become not only unattractive for insects but also toxic for them [126].

Numerous literature data indicate that polymerization of phenolic monomers into lignin, suberin, and cutin is catalyzed by apoplastic anionic but not by cationic isoPOs [127]. By immunofluorescence their accumulation was shown upon wounding on the inner wall of the periderm, and this accumulation correlated with the accumulation of lignin in the same zone [128]. In the periderm of a potato tuber the suberin layer and anionic PO were located on an inner part of the suberinated cell wall, and some authors [129] think this to be a manifestation of its involvement in suberin production. Three genes encoding class III POs are known to be involved in the lignifications of cell walls in the spruce *Picea abies* [16]. Only two of five isolated POs of cottonwood are involved in lignification [11]. In transgenic tobacco inhibition of expression of the *NtPrx60* gene encoding the protein TP60 significantly decreased the content of lignin and production of vascular tissues [29].

Activities of PO isoenzymes and lignin contents change during cytodifferentiation in cell and tissue cultures. Thus, at the beginning of culture of lucerne cells, the PO activity increased due to activation of cationic isoforms, but in differentiation stage different isoPOs were present in neutral and acidic isoforms [35]. Similar changes in the activity of anionic PO were also observed in *Prosopsis* plants after wounding [44].

The PO genes of *AtPrx42*, *AtPrx64*, and *AtPrx71* were expressed during the formation of secondary cell wall of conducting elements [120]. The *AtPrx53* gene was expressed similarly in vascular bundles [25]. The *AtPrx66* gene was highly homologous to the PO *ZePrx01* gene of zinnia that was expressed only during the differentiation of tracheal elements, and the corresponding protein catalyzes the *in vitro* formation of lignin [130]. The *AtPrx66* gene is expressed in root vessels, and this confirms the involvement of this gene in the lignification of cell walls of the root. A detailed analysis of signaling regulation of the *AtPrx17* gene has shown that the transcription factors AGL15/18 are crucial regulators of its expression. Another three genes (*AtPrx13*, *AtPrx30*, and *AtPrx55*) were mainly expressed in flowers with participation of the *trans*-factors SHP1 and SHP2 regulating lignification of the pod dehiscence zone [131].

INTERACTION OF PEROXIDASES WITH CELL WALLS OF PLANTS AND PATHOGENS

The ability of plant oxidoreductases to interact with biopolymers of cell walls of plants and fungi has been under study during several decades [132, 133]. Cell wall polysaccharides, especially pectins, play a key role in formation of lignin [134]. Pectins are suggested to act as anchors for binding PO with the cell walls [135] and mediate the physiological functioning of the enzyme. In mesophilic cells of bean leaves infected with white rust, zones of penetration into the cell of the fungal haustorium are abundantly covered by polymeric diaminobenzidine (DAB), which indicated the development of lignification only in this area. Because DAB is intensively produced just on the surface of infectious products of a pathogen, this suggests concentration on them of plant proteins as ROS generators and their users, which are likely to also include POs.

Cellulose is the major polysaccharide of the plant cell wall, but there are also many other polysaccharides that were earlier called "hemicelluloses". Hemicelluloses include connecting glycans (xyloglycans, xylans, mannans, glucans (callose)) and pectins (polygalacturonic acid, rhamnogalacturonan, xylogalacturonan, arabinan, arabinogalactan) [134]. Note that these polysaccharides are subjected to cross-linking and are modified by methylation and acetylation in the cell wall [124]. But the functional role of acetylation of polysaccharides is still unclear [134]. Possibly, the acetylation of polysaccharides promotes their binding with some PO isoforms, similarly to the case of chitin from fungal pathogens [88]. However, zones with high electrostatic activity are found on the surface of some POs, and these zones can interact with the cell wall pectins in the presence of calcium [136]. Such isoPO genes were able to be activated in the zones of formation of meristemic tissues and to display a strict tissue transcriptional activity [136].

Using chitin as a biological matrix, we have developed an approach for isolating PO from protein extracts of plants [87]. Initially we found that a fraction from wheat extract absorbed on chitin contained a dark-brown pigment which could be eluted with 1 M NaCl and that manifested the enzymatic activity characteristic for PO in the presence of H_2O_2. For the first time proteins with pI ~ 3.5 and molecular weight of 37 kDa were found in plants, and these proteins could be sorbed by chitin due to ion exchange and manifest PO activity [88]. Analysis of matrices capable of PO sorption revealed that PO interaction with chitin decreased with deacetylation of the latter. It was suggested that anionic PO of wheat should more actively interact with acetylated derivatives of cellulose. In fact, acetyl cellulose sorbed anionic PO [88], whereas the proteins under study were not sorbed by cellulose, and this suggested an importance of polysaccharide acetylation for the binding with PO. The binding coefficient of these oxidoreductases with acetyl cellulose was much higher relative to its sorption onto chitin.

A similar sorption of PO but by plant pectins was also observed by other authors. Thus, researchers from Geneva University have shown sorption of PO isolated from zucchini and *Arabidopsis* by pectate in the presence of Ca^{2+} [19, 136]. The hypothesis about ionic interaction of these POs with pectates was proved using PO with a deletion of nucleotide sequence responsible for translation of polysaccharide-binding amino acid sequence, and the subsequent transgenosis of the mutant protein into tobacco plants [19].

Attachment of a pathogen to the surface of plant cells is the first step in formation of a multistep infection process. Unfortunately, it is difficult to observe these steps when the fungal growth and development are studied, and it is easier to study these steps using unicellular symbiotic bacteria. A 75-kDa protein found in carrot is immunochemically similar to human vitronectin and the elongation factor eEF-1a and is able to bind bacteria [137]. The authors indicated that the plant cells lost the ability for binding with the bacterial cells after treatment with ionic detergents, and this proved the ionic character of these bonds. Proteins with similar molecular weight and high-affinity binding with chitin were isolated in the microsomal fraction from rice cells [2, 138]. A vitronectin-like protein is involved in adhesion of plasma membrane to the cell wall and in extension of the fertilization tube. In this connection it is interesting that the peroxidase activity was manifested by the animal protein peroxinectin, which is similar to vitronectin. The 76-kDa protein peroxinectin can bind phage particles, and thus it is suggested to have lectin-like features. Both fibronectin and apolipoprotein E are found to possess a C-terminal heparin-binding domain [139]. Unfortunately, the gene encoding the vitronectin-like protein has not been detected in plants, and this leaves open the degree of its molecular likeness with human vitronectin. Antibodies to vitronectin-like protein and to the protein binding with bacterial ricadhesin did not give cross reaction; however, they could competitively suppress the binding of bacterial cells with plant cells, e.g. with pea cells. Consequently, the above-mentioned proteins are different in molecular structure, but their biochemical features are rather similar.

There is a hypothesis that an increase in PO activity upon interaction with an affinity sorbent can be associated either with releasing of the enzyme molecules from inhibitors or with conformational changes of the enzyme due to interaction of electrostatically active zones of PO and chitin [19]. We found that both anionic and cationic plant PO isoforms could bind with chitin; therefore, the sorption of isoPOs by this biopolymer should not be of classical ionic character. Anionic POs capable of binding with chitin in some cases have similar antigenic determinants, but nevertheless representatives of different families and of the same family can be different in structure. It seems that due to ability to interact with polysaccharides these isoPOs promote accumulation of lignin and are important enzymes involved in plant defense against pathogens.

The ability of PO to be sorbed to chitin without losing enzymatic activity suggests a possibility of oxidizing phenolic compounds on contact with a fungal mycelium with production of lignin. This reveals a new mechanism of PO involvement in protective reactions of plants. In this case only unshielded zones of the mycelium can be destroyed and be a source of chitin oligomers. This allows regulation of the entry of elicitors into the plant cell during pathogenesis.

The ability of PO to interact with acetyl residues of chitin allows us to compare them with monovalent lectins, i.e. extensins, the binding of which with hemicellulose is affected only in medium with high ionic strength [66]. As a rule, POs are bound with the plant cell wall and act as its modifiers. Note that proline-enriched regions similar to those in extensins are found in PO isoforms isolated from skullcap [140]. Some POs can form complexes with an extensin of cell walls [66]. Consequently, chitin-specific POs may have sites that interact with polysaccharides, for example, similar to membrane receptor binding sites [2] or analogous to domains of heparin-binding proteins [141].

Polysaccharide-specific POs, which are strongly activated in the cytoplasmic, cell wall, and extracellular zones by infection with pathogens, can be assigned to PR proteins. POs are detected in the protein fraction (ionically bound with the cell wall) of combined cultures and calluses treated with salicylic acid, and this suggests an active concentration of these enzymes in the zone of the plant cell wall, which is the cell component located most closely to the environment and pathogen.

Peroxidase performs in the plant cell vitally important functions mainly associated with oxidation of phenolic compounds and with formation and strengthening of the cell wall. Peroxidase is involved in the oxidative transformation of molecules with growth-regulating or signaling activities and, as a result, can also perform regulatory functions in the cell. These physiological functions of the enzyme are especially important in the case of cell damage under exposure to various stress factors, including infection with fungal pathogens. The molecular and functional heterogeneity makes it possible to change the ratio between the isoforms to the advantage of those that are more appropriate to specific stress conditions of the environment, and an increase in the PO activity can be considered as a protective function of the organism. Functions of PO can be associated not only with synthetic processes during cell differentiation and organogenesis, but also with regulation of plant cell metabolism and control of plant growth and development. However, it is still difficult to understand why the same PO isoforms can be responsible for plant growth and for the oxidative burst in leaves during fungal pathogenesis.

The available information points out the need for determination of role of oxidoreductase in plant resistance to unfavorable environmental factors and consideration of their role in the concentration, generation, and utilization of ROS in the infection zone. It is suggested that both quantitative and qualitative changes in the level and activity of oxidoreductases can lead to changes in reactions of free radical oxidation. Therefore, ROS and oxidoreductases involved in the system of their generation and degradation can be in total combined into the pro-/antioxidant system [142].

Genes encoding the class III POs are present in all higher plants, forming a multigenic protein family. But until now the relationship between isoPOs and genes encoding them has not been studied in detail, apparently because of methodical difficulties of such analysis. Nevertheless, such data recently began to appear, and this simplifies the explanation of structural–functional characteristics of PO.

Some POs are functionally associated with the plant cell wall and contribute to its modification due to a high affinity for hemicellulose (most likely, to pectin). Some isoPOs can electrostatically bind with components of the cell wall of pathogenic fungi and plants. It is likely that PO binding with polysaccharides performs a signaling and protective function in plants due to immediate involvement in the action of the pro-/antioxidant system and to triggering some signaling systems of the plants [142]. The possibility of regulating expression activity of PO genes by different regulators of plant resistance including oligosaccharides can reveal the role of the enzyme in plant immunity.

Obviously, defense reactions of plants in response to pathogen injection are complicated and multifactorial. An important physiological sense of these reactions is the strengthening of the cell wall and construction on the pathogens' paths of physical barriers consisting of lignin- and suberin-containing polymers, which also possess high toxicity. This defense reaction is developing more or less automatically, thus these barriers are formed just in the zone of pathogen penetration and concurrently with the beginning of the active pathogen expansion into the tissues of the host plant. However, mechanisms of development of these events are still unclear. Possibly, plants contain a whole subclass of isoPOs and their encoding genes. This subclass should be characterized by the ability for binding with polysaccharides, in particular with chitin, and indirectly display the defense functions directed to strengthen the cell wall of the host and to isolate with lignin the non-infected host tissues from the pathogen.

This work was supported by State contracts of the Ministry of Education and Science of the Russian Federation No. 113/09 (Development of Scientific Potential of the Highest School) and No. P339 (Research and Pedagogical Personnel of Innovative Russia).

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