Вавиловский журнал генетики и селекции. 2019;23(5):615-623
DOI 10.18699/VJ19.533

Prediction of some peroxidase functions in *Arabidopsis thaliana* L. by bioinformatic search

A.S. Tugbaeva ✉, A.A. Ermoshin, I.S. Kiseleva

Ural Federal University named after the first President of Russia B.N. Yeltsin, Ekaterinburg, Russia
✉ e-mail: anastasia.tugbaeva@gmail.com

Peroxidases of class III are common in various organisms. They are involved in lignin biosynthesis and plant protection against stressors. Peroxidases are presented in many isoforms, whose role is not always clear. The aim of this study is to analyze the amino acid sequences of reference peroxidases with known functions and peroxidases from *Arabidopsis thaliana* L. whose functions are unknown and to consider their putative roles in lignin biosynthesis. The structural and functional organization of peroxidases was analyzed by bioinformatical methods applied to open Internet sources. Seven reference peroxidases were chosen from four plant species: *Zinnia* sp., *Armoracia rusticana* P.G. Gaertn., *Lycopersicon esculentum* L. и *Populus alba* L. Twenty-four amino acid sequences of homologous peroxidases from *A. thaliana* were selected for the analyses with the BLAST service. Their molecular weights and isoelectric points were calculated. Multiple alignments of amino acid sequences and phylogenetic analysis were done. Sites of binding to monolignol substrates were identified in seven peroxidases from *A. thaliana*, and the enzymes were assigned to the groups of S- or G-peroxidases. Amino acid replacements in the primary structures of peroxidases were analyzed. Peroxidases from *A. thaliana* were clustered with reference peroxidases. They formed six clusters on the phylogenetic tree, three of which contained only *A. thaliana* peroxidases. Peroxidases within each cluster had similar molecular weights and isoelectric points, common localization of expression, and similar functions. Thus, the use of bioinformatics, databases, and published data bring us to assumptions as to the functions of several *A. thaliana* class III peroxidases. AtPrx39 peroxidase was shown to be affine to sinapyl alcohol; AtPrx54, to *p*-coumaryl and coniferyl alcohols. They are likely to participate in lignin biosynthesis.
Key words: peroxidase; lignification; *Arabidopsis thaliana* L.; bioinformatics; multiple alignments.

For citation: Tugbaeva A.S., Ermoshin A.A., Kiseleva I.S. Prediction of some peroxidase functions in *Arabidopsis thaliana* L. by bioinformatic search. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(5):615-623. DOI 10.18699/VJ19.533

Предсказание функций некоторых пероксидаз *Arabidopsis thaliana* L. на основе биоинформатического поиска

А.С. Тугбаева ✉, А.А. Ермошин, И.С. Киселева

Уральский федеральный университет им. первого Президента России Б.Н. Ельцина, Екатеринбург, Россия
✉ e-mail: anastasia.tugbaeva@gmail.com

Пероксидазы III класса являются распространенными в разных группах организмов ферментами, участвуют в биосинтезе лигнина, защите растений от патогенов и абиотических стрессоров. Пероксидазы имеют множество изоформ, роль которых в клеточных процессах не всегда ясна. В работе проанализированы аминокислотные последовательности референсных пероксидаз с известными функциями и пероксидаз *Arabidopsis thaliana* L., функции которых неясны, выдвинуто предположение о роли последних в биосинтезе лигнина. Проведен биоинформатический анализ информации о структурно-функциональной организации пероксидаз из открытых интернет-источников. Было выбрано семь референсных пероксидаз из травянистых и древесных растений четырех видов (*Zinnia* sp., *Armoracia rusticana* P.G. Gaertn., *Lycopersicon esculentum* L. и *Populus alba* L.), для которых показано их участие в лигнификации клеточной стенки. С помощью сервиса BLAST были отобраны 24 аминокислотные последовательности гомологичных пероксидаз из *A. thaliana*. Для каждого фермента рассчитаны молекулярная масса и изоэлектрическая точка. Построены множественные выравнивания аминокислотных последовательностей и проведен филогенетический анализ. Для семи пероксидаз *A. thaliana* выявлены сайты связывания с субстратом (синаповый, *пара*-кумаровый и конифериловый спирты), их принадлежность группам S- или G-пероксидаз. Проанализированы аминокислотные замены в первичной структуре белка. Пероксидазы *A. thaliana* кластеризуются с референсными пероксидазами и образуют шесть групп на филогенетическом дереве, три из которых образованы исключительно пероксидазами *A. thaliana*. Пероксидазы, объединенные в общий кластер, характеризуются близким значением молекулярной массы и величиной

изоэлектрической точки, имеют общую локализацию экспрессии и выполняют схожие функции. Таким образом, использование биоинформатических методов, анализ литературы и материалов в базах данных позволили предположить не известные ранее функции для некоторых пероксидаз *A. thaliana*, относящихся к III классу. Показано, что пероксидаза AtPrx39 обладает сродством к окислению синапового спирта; AtPrx54 – к окислению *napa*-кумарового и конифирилового спиртов, и, предположительно, они могут участвовать в биосинтезе лигнина.
Ключевые слова: пероксидаза; лигнификация; *Arabidopsis thaliana* L.; биоинформатика; множественные выравнивания.

Introduction

Peroxidases are the group of enzymes that catalyze the oxidation of a substrate with the presence of hydrogen peroxide. The superfamily of "plant" peroxidases (those of plants, fungi, and bacteria) is divided into three classes based on their structural and catalytic properties. All peroxidases contain 10 homologous α-helixes. Class I and class II have one specific α-helix, and class III peroxidases have three specific α-helixes (Hiraga et al., 2001).

Living organisms contain many peroxidase isoforms, and their amino acid sequences are similar by less than 20 %. A high level of conservation characterizes five amino acid positions essential for the folding of α-helixes, assembly of subunits, and catalytic properties of the enzymes (Hiraga et al., 2001).

Higher plants contain class I and III peroxidases, which differ in structure, function, and location in the plant cell. Ascorbate peroxidase (EC 1.11.1.11) and glutathione peroxidase (EC 1.11.1.9) belong to class I. They are located in chloroplasts, peroxisomes, and cytoplasm. Class I peroxidases are distinguished by high specificity to an oxidizable substrate. Class III peroxidases (EC 1.11.1.7) include enzymes that are located in vacuoles and secreted into the apoplast. They oxidize various substrates. Class III plant peroxidases are encoded by a large family of genes: 73 have been identified in *Arabidopsis thaliana* L. and 138 in *Oryza sativa* L. (Welinder et al., 2002; Passardi et al., 2004a). Class III peroxidases act as components of the antioxidant system of plants and, at the same time, can form reactive oxygen species (Passardi et al., 2004a). The dual functions of peroxidases allow them to take part in many physiological processes: protection against pathogens (Passardi et al., 2004b), wound healing, auxin and anthocyanin catabolism, and porphyrin metabolism (Cosio, Dunand, 2009; Jovanovic et al., 2018).

Apoplastic peroxidases are involved in the biosynthesis of cell wall components, such as lignin and suberin. Lignin is an aromatic phenolic heteropolymer with a disordered structure, covalently associated with polysaccharides of the secondary cell wall and responsible for its strength and hydrophobicity. The composition and amount of lignin in the cell wall change in the course of plant ontogenesis (Boerjan et al., 2003) and in response to different stress factors (Liu et al., 2018).

Peroxidase and laccase are involved in the formation of lignin precursors: *p*-coumaryl, coniferyl, and sinapyl radicals. Laccases (EC 1.10.3.2) catalyze the formation of guaiacyl (G) units, whereas peroxidases are involved in the generation of syringyl (S), *p*-hydroxyphenyl (H), and guaiacyl (G) units of lignin (Berthet et al., 2012).

Despite the large amount of research focusing on class III peroxidases, only few isoforms have been shown to participate in lignin biosynthesis in herbaceous (*Zinnia* sp., *Armoracia rusticana* P.G. Gaertn., *Lycopersicon esculentum* L.) and woody *(Populus alba* L.) plants (Quiroga et al., 2000; Aoyama et al., 2002; Sasaki et al., 2004; Sato et al., 2006; Marjamaa et al., 2009). Class III peroxidases can oxidize three monolignols; however, most isoforms oxidize coniferyl and *p*-coumaryl alcohols and only few of them use sinapyl alcohol as a substrate *in vitro* (Barcelo et al., 2007).

The structures of peroxidases ZePrx34, ZPO-C, CWPO-C, HRP, HRP-A2A, HRP-C1C, and TPX1, which can be considered reference ones, have been studied in detail (Quiroga et al., 2000; Aoyama et al., 2002; Sasaki et al., 2004; Gabaldon et al., 2005; Sato et al., 2006). The attention to peroxidases is due to their function in the formation of plant resistance to oxidative stress caused by both abiotic and biotic factors, as well as to their participation in lignin biosynthesis and plant growth. Bioinformatic analysis of peroxidases with unknown functions is of fundamental (determination of the enzyme functions) and practical (design of genetic constructs to create resistant plants or plants with a modified cell wall) significance. The purpose of this work is to analyze the functions of *A. thaliana* peroxidases based on the similarity to the amino acid sequences of reference plant peroxidases for which the involvement in cell wall lignification is known.

Materials and methods

Amino acids sequences of plant peroxidases from *Zinnia elegans* Jacq. (ZePrx34.70, NCBI identifier – Q4W1I8.1), *Zinnia violacea* Cav. (ZPO-C, BAD93164.1), *P. alba* (CWPO-C, BAE16616.1), *A. rusticana* (HRP, CCJ34837.1; HRP-A2A, CCJ34825.1; HRP-C1C, P15233.1) and *L. esculentum* (TPX1, NP_001289850.1) were sought in the Protein NCBI database (https://www.ncbi.nlm.nih.gov/protein/). They were aligned with the amino acid sequences of *A. thaliana* with the Protein BLAST tool (https://blast.ncbi.nlm.nih.gov). Search route: database – model organism (landmark), organism – *A. thaliana* (taxid: 3702), algorithm PSI-BLAST (Position-Specific Iterated BLAST). A library (Suppl. 1)[1] was formed from 24 amino acid sequences of *A. thaliana* peroxidases with high levels of similarity to reference peroxidases (E-value less than $1e^{-80}$). The online program EMBOSS Pepstats (https://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats/) was applied to calculate the molecular masses and isoelectric points of the proteins.

The phylogenetic tree based on *A. thaliana* peroxidase proteins was built by the Neighbor-Joining method (Sanou, Nei, 1981) in the MEGA 7 program based on sequence alignments of the encoded protein. The evolutionary distances were

[1] Supplementary Materials 1–3 are available in the online version of the paper: http://www.bionet.nsc.ru/vogis/download/pict-2019-23/appx11.pdf

computed by the *p*-distance method (Nei, Kumar, 2000). The bootstrap test included 1000 replicates, and the results are shown nearby the branches (Kumar et al., 2016).

Information about the expression of *A. thaliana* peroxidase genes at different stages of development was obtained from the bio-array resource for plant functional genomics (http://bar.utoronto.ca). Analysis of peroxidase functions was carried out with regard to information stored in the Arabidopsis Information Resource (www.arabidopsis.org) by gene identifiers in TAIR. We considered information from the Annotations, GO Biological Process section on the involvement of peroxidases in stress reactions, growth, and lignification of the cell wall. Amino acid sequence alignments were built with the CLUSTAL algorithm for multiple sequence alignment in MUSCLE 3.8 (https://www.ebi.ac.uk/Tools/msa/). Highly conservative and semiconservative domains, structural motifs were identified.

Results

The bioinformatic search with Protein BLAST showed that plant peroxidases from *Z. elegans* (ZePrx34.70, ZPO-C), *P. alba* (CWPO-C), *A. rusticana* (HRP, HRP-A2A, HRP-C1C) and *L. esculentum* L. (TPX1) have high levels of similarity to 24 *A. thaliana* peroxidases. Molecular weights and isoelectric points were calculated for these enzymes (Table 1).

The isoelectric points (pIs) and molecular weights of *A. thaliana* peroxidases differ from those of reference enzymes. In particular, the pI value of peroxidase AtPrx36 is in the more acidic pH range compared to ZePrx34.70, and the protein has a higher molecular weight (38.24 vs. 34.24 kDa, respectively). AtPrx13 peroxidase is characterized by an acidic pI value (4.74), whereas pI for HRP is 8.35. AtPrx32, 37 and 23 peroxidases have pIs within 6.62–7.97, and their molecular weights vary from 38.10 to 38.85 kDa, whereas the pI and molecular weight of HRP_A2A protein are 4.62 and 35.03 kDa, respectively.

It is seen that *A. thaliana* peroxidases differ in pI values and molecular weights from reference enzymes and they are expected to differ in their affinity to the substrate and in functions. It is known that basic peroxidases (isoelectric point > 7.0) can oxidizing *p*-coumaryl, coniferyl and sinapyl alcohols (Kukavica et al., 2012), while acidic peroxidases (isoelectric point < 7.0) are poorly capable of oxidizing sinapyl alcohol (Barcelo et al., 2004). Therefore, the roles of basic and acidic peroxidases in cell wall lignification may be different. Plant peroxidases with high ability to oxidize coniferyl alcohol (CWPO-A, HRP-C1C and AtPrx53) or sinapyl alcohol (CWPO-C from *P. alba*, ZePrx from *Z. elegans*, AtPrx4) are described.

The phylogenetic tree of these peroxidases shown in Figure is constructed by amino acid alignment. The reference peroxidases and *A. thaliana* enzymes form six clusters. The first one includes HPR-C1C, AtPrx33, 34, and 32 peroxidases with a high bootstrap support value of 72–100 %. The second cluster groups peroxidases, homologous to HRP_A2A: AtPrx2 and 54 (bootstrap support 100 %). AtPrx52 and 4 peroxidases, homologous to ZePrx34.70, form the third cluster with bootstrap support of 98–99 %. AtPrx47, 64, and 66 peroxidases group in the fourth cluster together with ZPO-C peroxidase (92–100 % bootstrap support). The fifth cluster on the phylogenetic tree combines peroxidases TPX1 and AtPrx3 and 39 with a bootstrap support of 100 %. The sixth cluster consists of HRP, CWPO-C, AtPrx71, 62, and 69 peroxidases with bootstrap support value of 72–100 %.

Separate clusters on the phylogenetic tree include *A. thaliana* peroxidases: AtPrx38 and AtPrx37 (cluster A), AtPrx22 and AtPrx23 (cluster B), AtPrx36 and AtPrx72 (cluster C) with 100 % bootstrap support.

With materials from the BAR and TAIR databases, the peroxidase functions and expression sites were identified for the enzymes of clusters 1–6 (Table 2). The Table 2 does not include clusters A, B, or C, formed by homologous proteins of *A. thaliana.*

Functions of some *A. thaliana* peroxidases have been studied in mutants with knocked-out genes and transgenic plants. According to experimental studies, peroxidase HRP-C1C from *A. rusticana* most effectively oxidizes coniferyl alcohol *in vitro* (Sasaki et al., 2004). The most homologous HRP-C1C peroxidases AtPrx33 and AtPrx34 are involved in root growth and cell elongation (Irshad et al., 2008) and in an oxidative burst, when pathogens penetrate into the cell (Bindschedler et al., 2006). AtPrx32 peroxidase is involved in cell elongation (Irshad et al., 2008). Thus, there is no data on the participation of cluster 1 enzymes in cell wall lignification.

Purified peroxidase HRP_A2A from *A. rusticana* efficiently oxidizes guaiacol *in vitro* (Krainer et al., 2014). According to the BAR database, the *AtPRX2* and *AtPRX54* genes are expressed in its seedling roots and hypocotyl and in the roots of juvenile plants (see Table 2). Mutants of *A. thaliana atprx2* are characterized by a reduced total lignin content, changes in lignin composition, and plant biomass decrease (Shigeto et al., 2013).

The isoform ZePrx34.70 from *Z. elegans*, catalyzing the oxidation of sinapyl alcohol, is expressed in roots and hypocotyl and involved in lignification (Gabaldon et al., 2005). Among the analyzed peroxidases, AtPrx4 and AtPrx52 from *A. thaliana* are homologous to ZePrx34.70, as confirmed in (Herrero et al., 2013a). According to (Fernandez-Pereza et al., 2015), the *AtPrx4* gene is expressed in roots, stems, and leaves, and it affects the plant growth on long days. The product of its expression is involved in syringol polymerization.

Purified ZPO-C peroxidase from *Z. violacea* uses both synapyl and coniferyl alcohol as a substrate *in vitro* (Sato et al., 2006). Homologous AtPrx66 takes part in cell wall lignification of forming vessels (Sato et al., 2006). It was shown that the homolog AtPrx64 also plays a role in xylem lignification (Yokoyama, Nishitani, 2006).

The gene for the basic peroxidase TPX1 of *L. esculentum* is specifically expressed in root xylem and involved in lignification and suberization (Quiroga et al., 2000). An increase in lignin content has been shown in transgenic *L. esculentum* plants with overexpression of TPX1 (Mansouri et al., 1999). Homologous peroxidase AtPrx3 is involved in lignification (see Table 2). AtPrx3 cationic peroxidase transcripts were found in the seedlings and roots, and their participation in the response of plants to salt stress and drought was shown (Llorente et al., 2002). The role of AtPrx39 peroxidase in cell wall lignification has not been studied. However, the *AtPRX39* gene is expressed in the root transport zone. It affects the development of the root system (Tsukagoshi et al., 2010).

Table 1. Annotation of class III peroxidases from *A. thaliana* with high levels of similarity (Score and E-value) to reference peroxidases. The TAIR acc. no., NCBI acc. no., molecular weight, and isoelectric point are indicated for each protein

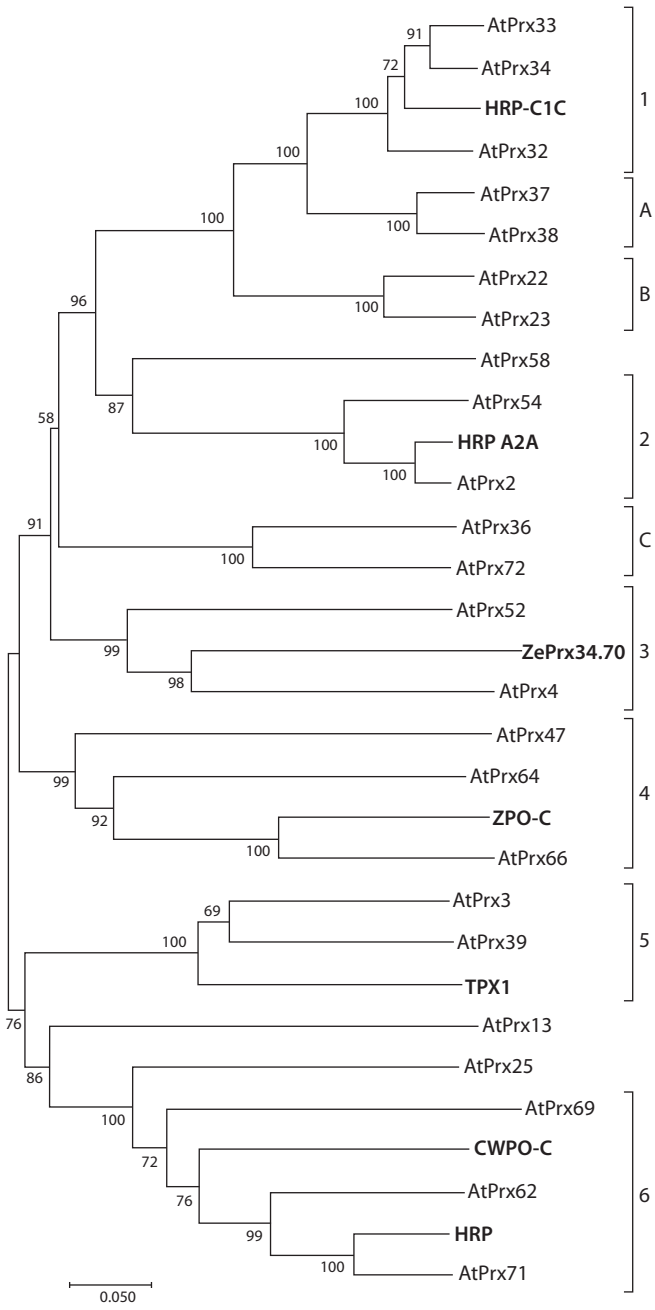
Reference peroxidase, protein ID, pI, molecular weight, kDa	*A. thaliana* peroxidase, protein ID, pI, molecular weight, kDa	TAIR ID	Score	E-value
ZePrx34.70, *Zinnia elegans* Q4W1I8.1 8.22 34.24	AtPrx4 NP_172906.1 7.74 34.41	AT1G14540	342	$1e^{-117}$
	AtPrx52 NP_196153.1 8.30 34.21	AT5G05340	327	$3e^{-111}$
	AtPrx36 NP_190668.2 4.66 38.24	AT3G50990	262	$1e^{-85}$
ZPO-C, *Zinnia violacea* BAD93164.1 8.64 34.79	AtPrx66 NP_200002.3 9.76 35.56	AT5G51890	452	$5e^{-161}$
	AtPrx64 NP_199033.1 9.04 34.71	AT5G42180	328	$5e^{-112}$
	AtPrx47 NP_001320124.1 8.29 35.97	AT4G33420	283	$2e^{-94}$
CWPO-C, *Populus alba* BAE16616.1 8.30 34.63	AtPrx71 NP_201217.1 8.18 34.89	AT5G64120	425	$8e^{-150}$
	AtPrx25 NP_181679.4 7.53 35.89	AT2G41480	417	$6e^{-147}$
TPX1, *Lycopersicon esculentum* NP_001289850.1 7.51 35.99	AtPrx3 NP_172018.1 8.41 34.91	At1G05260	449	$2e^{-159}$
	AtPrx39 NP_192868.1 6.92 35.60	AT4G11290	430	$9e^{-152}$
	AtPrx72 NP_201440.1 8.47 37.43	AT5G66390	261	$3e^{-85}$
HRP, *Armoracia rusticana* CCJ34837.1 8.35 34.79	AtPrx62 NP_198774.1 8.42 34.13	AT5G39580	459	$2e^{-163}$
	AtPrx69 NP_201215.1 9.71 35.68	AT5G64100	326	$8e^{-111}$
	AtPrx13 NP_177835.3 4.74 34.76	AT1G77100	278	$3e^{-92}$

End of Table1

Reference peroxidase, protein ID, pI, molecular weight, kDa	*A. thaliana* peroxidase, protein ID, pI, molecular weight, kDa	TAIR ID	Score	E-value
HRP_A2A, *Armoracia rusticana* CCJ34825.1 4.62 35.03	AtPrx2 NP_196290.1 4.52 34.99	AT5G06720	635	0.0
	AtPrx54 NP_196291.1 4.27 37.29	AT5G06730	578	0.0
	AtPrx22 NP_181372.1 5.76 38.11	AT2G38380	380	$1e^{-131}$
	AtPrx58 NP_197488.1 4.92 35.43	AT5G19880	363	$2e^{-125}$
	AtPrx37 OAP01113.1 7.85 38.20	AT4G09970	357	$8e^{-123}$
	AtPrx23 NP_181373.1 7.97 38.10	AT2G38390	356	$3e^{-122}$
	AtPrx32 NP_850652.1 6.62 38.85	AT3G32980	354	$2e^{-121}$
HPR-C1C, *Armoracia rusticana* P15233.1 6.62 36.54	AtPrx33 NP_190480.1 6.80 38.94	AT3G49110	629	0.0
	AtPrx34 NP_190481.1 7.56 38.83	AT3G49120	624	0.0
	AtPrx38 NP_192618.1 7.57 38.09	AT4G08780	526	0.0

Peroxidase CWPO-C from *P. alba* is a cationic isoform of the enzyme efficiently polymerizing sinapyl alcohol *in vitro* (Aoyama et al., 2002). HRP is a cationic isoform with high ability to oxidize coniferyl alcohol. Peroxidase isoenzymes, such as HRP and CWPO-C, have been shown to catalyze single-electron oxidation of sinapyl alcohol using coniferyl alcohol as a radical mediator (Aoyama et al., 2002). AtPrx71 peroxidase is the closest homolog of HRP. It participates in the formation of secondary xylem (Yokoyama, Nishitani, 2006) and in the response to biotic factors (Chassot et al., 2007). AtPrx62 peroxidase expression increases in response to heavy metal ions and plant pathogens (Cosio, Dunand, 2009).

The genes encoding peroxidase AtPrx32 and 37 are expressed in the root and hypocotyl and involved in lignification (see Table 2). Overexpression of *AtPRX37* in transgenic *A. thaliana* causes a decrease in plant growth rate, affects the development of xylem, and ultimately leads to the formation of a dwarf phenotype. Presumably, AtPrx37 peroxidase is involved in the regulation of plant growth through the cell wall lignification process (Pedreira et al., 2011). AtPrx72 and 36 peroxidases differ in localization in plant tissues and their functions. The *AtPRX72* gene is expressed in roots and stems (Valerio et al., 2004). The *AtPRX36* gene is expressed in the hypocotyl, where it takes part in cell elongation (Irshad et al., 2008); in the endosperm; and the seed coat (Kunieda et



The peroxidase protein phylogenetic tree from *A. thaliana* based on sequence alignments of the encoded protein (Sanou, Nei, 1981).

The evolutionary history was reconstructed by the Neighbor-Joining method. The optimal tree with the sum of branch length 5.06962574 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown nearby the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to construct the phylogenetic tree. The evolutionary distances were computed by the *p*-distance method (Nei, Kumar, 2000). They are presented as numbers of amino acid differences per site. The analysis involves 31 amino acid sequences. All positions containing gaps and missing data are eliminated. A total of 268 positions are present in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). For protein database NCBI accession numbers of the sequences used for the building of phylogenetic tree see Table 1, Suppl. 1.

al., 2013). Analysis of *A. thaliana* mutants for the *AtPRX72* gene showed a decrease in lignin content and the number of syringyl units. In addition, the mutants were characterized by slow growth, decrease in stem diameter, and smaller numbers of shoots and leaves (Herrero et al., 2013b). Thus, several peroxidases, clustered in groups 2–6, can oxidize monolignols and participate in the lignification of cell walls.

We anticipate that peroxidases combined into a common cluster on a phylogenetic tree are predominantly expressed in the same plant organs and perform similar functions (see Table 2). Of all peroxidases, the participation of AtPrx54 and 39 in physiological processes is least understood. We infer from the results of data analysis that AtPrx54 peroxidase performs functions similar to AtPrx2: it is involved in lignification, growth, and response to abiotic stress. AtPrx39 peroxidase is involved in lignification and responses to biotic and abiotic stress. Assuming the concept of the evolutionary origin of proteins (Gabaldon, Koonin, 2013), we conjecture the participation of AtPrx54 and 39 peroxidases in cell wall lignification processes, since they are orthologs of HRPA2A and TPX1, respectively. AtPrx69 and 62 peroxidases, combined into a common cluster with AtPrx71 peroxidase, are also highly likely to be involved in cell wall lignification.

The functions of homologous proteins can be determined based on their domain structure and substrate binding sites. Multiple alignment of amino acid sequences was previously performed (data not shown). It included reference and *A. thaliana* peroxidases combined into a common cluster (clusters 1–6 on the phylogenetic tree, see Figure). AtPrx54 peroxidase was shown to have structures like AtPrx4 and HRP_A2A; and AtPrx39 peroxidase was highly homologous to TPX1, CWPO-C, and HRP proteins. Highly conservative and semiconservative sections, structural motifs characteristic of the analyzed class III peroxidases were identified (see Suppl. 2, 3).

Thus, peroxidases are distinguished by structural motifs, and, accordingly, affinity to oxidizable substrates. The structural motifs that are necessary and sufficient to polymerize G-monolignols are V78, 95-VSCSD, S98, 105-SEA, F185, and N281. S-Peroxidases have motifs absent from G-peroxidases: I80, 95-VSCAD, A98, 105-ARD, Y178, and K268. The 95-VSCAD motif determines the ability of peroxidases to polymerize both syringaldazine and sinapyl alcohol (Barcelo et al., 2007). In addition, the affinity of peroxidase to the substrate is affected by hydrophobic interactions between the substrate and the enzyme, which involve the amino acids at positions P69, I138, P139, S140, R175, and V178 (Barcelo et al., 2007). Studies of the ATP A2 peroxidase structure from *A. thaliana* have shown that hydrophobic interactions between the sinapyl alcohol and the amino acid residues in position I138 and P139 do not allow the enzyme to use sinapyl alcohol as a substrate (Ostergaard et al., 2000).

The G-peroxidases whose amino acid sequences were analyzed include AtPrx2 and 54, HRP_A2A. They are characterized by structural motifs V78, 95-VSCSD, S98, 105-SEA, F186, and N281. The hydropathicity of the site of substrate binding is determined by amino acid substitutions increasing hydrophobicity: proline to alanine at position 96, isoleucine to leucine at position 138, and isoleucine to

Table 2. The function of class III peroxidases from *A. thaliana*

Cluster	Peroxidase	Function	Localization of expression
1 Homology HRP-C1C	AtPrx33	Cell elongation, biotic stress	Root, hypocotyl, 2nd internode, leaves, stage 15 flowers, sepals
	AtPrx34	Cell elongation, biotic and abiotic stress	Root, hypocotyl, 2nd internode, leaves, stage 15 flowers, sepals
	AtPrx32	Cell elongation	Root 1-day-old-seedling
2 Homology HRP A2A	AtPrx54	No data	Root, hypocotyl
	AtPrx2	Lignification, cell elongation, abiotic stress	Root, hypocotyl, 1st node, carpels, sepals, stamen
3 Homology ZePrx34.70	AtPrx52	Lignification, abiotic and biotic stress	Flowers, sepals
	AtPrx4	Lignification, cell elongation	Root, cotyledon, hypocotyl, 2nd internode, leaves, stage 10–15 flowers, sepals, stamen
4 Homology ZPO-C	AtPrx47	Lignification	Root, petals, stage 15 flowers
	AtPrx64	Lignification, abiotic stress	Seeds, 1st node, 2nd internode
	AtPrx66	Lignification	Leaves, stage 3–4 seeds, sepals, petals, stamen
5 Homology TPX1	AtPrx3	Lignification, abiotic and biotic stress	Root, hypocotyl, stage 4-7 seeds
	AtPrx39	No data	Root, hypocotyl, carpels
6 Homology CWPO-C и HPR	AtPrx69	Cell elongation, abiotic and biotic stress	Root, hypocotyl
	AtPrx62	Abiotic and biotic stress	Root, hypocotyl, 24 h imbibed seed
	AtPrx71	Lignification, abiotic and biotic stress	Leaves, hypocotyl, sepals, petals, carpels, stage 3 seeds

Note: The data about peroxidases functions were obtained from the Arabidopsis Information Resource (www.arabidopsis.org). Localization of expression on different stages of development was analyzed using the bio-array resource for plant functional genomics (http://bar.utoronto.ca).

phenylalanine at position 142. Replacements at sites with hydrophilic amino acids (glycine to proline at position 68, isoleucine to leucine at position 138, arginine to glutamine at position 175, glycine to valine at position 177, or valine to threonine at position 178) do not change the properties of the substrate-binding sites. The 138-IPS hydrophobic motif determines the conformation of the protein and the hydrophobicity of the substrate-binding site. Thus, AtPrx54 peroxidase has sites that enable it to polymerize *p*-coumaryl and coniferyl alcohols.

S-Peroxidases include reference enzymes CWPO-C, TPX1, HPR, and AtPrx3, 39, 62, 69, and 71 from *A. thaliana*. Their catalytic properties are determined by the motifs I78, 92-VSCAD, A96, 103-ARD, Y182, and K282. Amino acid substitutions of leucine for isoleucine at position 135 and tyrosine for phenylalanine at position 231 ensure the hydrophobicity of the substrate-binding site. Presumably, peroxidases AtPrx39, 69, and 62 are involved in the polymerization of sinapyl alcohol.

Conclusion

Plant peroxidases of class III from different plant families are similar to each other in amino acid sequences, tissue localization, and functions. Structure-functional regions were identified in the peroxidases on the base of amino acid sequence homology. These regions allow inferences as to the substrate specificity of the peroxidases. The results show that AtPrx39 oxidizes sinapyl alcohol and belongs to S-peroxidases; AtPrx54 oxidizes *p*-coumaryl and coniferyl alcohols and belongs to G-peroxidases. Therefore, AtPrx39 and 54 peroxidases can participate in the polymerization of monolignols in lignin biosynthesis. Thus, the use of bioinformatic methods

and the analysis of literature and materials in databases suggest previously unknown functions of *A. thaliana* peroxidases belonging to class III.

References

Aoyama W., Sasaki S., Matsumura S., Mitsunaga T., Hirai H., Tsutsumi Y., Nishida T. Sinapyl alcohol-specific peroxidase isoenzyme catalyzes the formation of the dehydrogenative polymer from sinapyl alcohol. J. Wood Sci. 2002;6(48):497-504. DOI 10.1007/BF00766646/.

Barcelo A.R., Gomez Ros L.V., Carrasco A.E. Looking for syringyl peroxidases. Trends Plant Sci. 2007;12(1):486-491. DOI 10.1016/j.tplants.2007.09.002.

Barcelo A.R., Gomez Ros L.V., Gabaldon C., Lopez-Serrano M., Pomar F., Carrion J.S., Pedreño M.A. Basic peroxidases: the gateway for lignin evolution? Phytochem. Rev. 2004;3:61-78. DOI 10.1023/B:PHYT.0000047803.49815.1a/.

Berthet S., Thevenin J., Baratiny D., Demont-Caulet N., Debeaujon I., Bidzinski P., Leple J.C., Huis R., Hawkins S., Gomez L.D., Lapierre C., Jouanin L. Role of plant laccases in lignin polymerization. Adv. Botan. Res. 2012;61:145-172. DOI 10.1016/B978-0-12-416023-1.00005-7.

Bindschedler L.V., Dewdney J., Blee K.A., Stone J.M., Asai T., Plotnikov J., Denoux C., Hayes T., Gerrish C., Davies D.R., Ausubel F.M., Bolwell G.P. Peroxidase-dependent apoplastic oxidative burst in *Arabidopsis* required for pathogen resistance. Plant J. 2006;47:851-863. DOI 10.1111/j.1365-313X.2006.02837.x.

Boerjan W., Ralph J., Baucher M. Lignin biosynthesis. Annu. Rev. Plant Biol. 2003;54:519-546. DOI 10.1146/annurev.arplant.54.031902.134938.

Chassot C., Nawrath C., Metraux J.P. Cuticular defects lead to full immunity to a major plant pathogen. Plant J. 2007;49:972-980. DOI 10.1111/j.1365-313X.2006.03017.x.

Cosio C., Dunand C. Specific functions of individual class III peroxidase genes. J. Exp. Bot. 2009;2(60):391-408. DOI 10.1093/jxb/ern318.

Fernandez-Pereza F., Vivara T., Pomarb F., Pedrenoa M.A., Novo-Uzal E. Peroxidase 4 is involved in syringyl lignin formation in *Arabidopsis thaliana.* J. Plant Physiol. 2015;175:86-94. DOI 10.1016/j.jplph.2014.11.006.

Gabaldon C., Lopez-Serrano M., Pedreño M.A., Barcelo A.R. Cloning and molecular characterization of the basic peroxidase isoenzyme from *Zinnia elegans*, an enzyme involved in lignin biosynthesis. Plant Physiol. 2005;3(139):1138-1154. DOI 10.1104/pp.105.069674.

Gabaldon T., Koonin E.V. Functional and evolutionary implications of gene orthology. Nat. Rev. Genet. 2013;14(5):360-366. DOI 10.1038/nrg3456.

Herrero J., Esteban-Carrasco A., Zapata J.M. Looking for *Arabidopsis thaliana* peroxidases involved in lignin biosynthesis. Plant Physiol. Biochem. 2013a;67:77-86. DOI 10.1016/j.plaphy.2013.02.019.

Herrero J., Fernandez-Perez F., Yebra T., Novo-Uzal E., Pomar F., Pedreño M.A., Cuello J., Guera A., Esteban-Carrasco A., Zapata J.M. Bioinformatic and functional characterization of the basic peroxidase 72 from *Arabidopsis thaliana* involved in lignin biosynthesis. Planta. 2013b;6(237):1599-1612. DOI 10.1007/s00425-013-1865-5.

Hiraga S., Sasaki K., Ito H., Ohashi Y., Matsui H. A large family of class III plant peroxidases. Plant Cell Physiol. 2001;5(42):462-468. DOI 10.1093/pcp/pce061.

Irshad M., Canut H., Borderies G., Pont-Lezica R., Jamet E. A new picture of cell wall protein dynamics in elongating cells of *Arabidopsis thaliana*: confirmed actors and newcomers. BMC Plant Biol. 2008;8:94. DOI 10.1186/1471-2229-8-94.

Jovanovic S.V., Kukavica B., Vidovic M., Morina F., Menckhoff L. Class III peroxidases: functions, localization and redox regulation of isoenzymes. Antioxidants and Antioxidant Enzymes in Higher Plants. 2018;269-300. DOI 10.1007/978-3-319-75088-0_13.

Krainer F.W., Pletzenauer R., Rossetti L., Herwig C., Glieder A., Spadiut O. Purification and basic biochemical characterization of 19 recombinant plant peroxidase isoenzymes produced in *Pichia pastoris*. Protein Expr. Purif. 2014;100(95):104-112. DOI 10.1016/j.pep.2013.12.003.

Kukavica B., Veljovic-Jovanovic S., Menckhoff L., Lüthje S. Cell wall-bound cationic and anionic class III isoperoxidases of pea root: biochemical characterization and function in root growth. J. Exp. Bot. 2012;63:4631-4645. DOI 10.1093/jxb/ers139.

Kumar S. Stecher G., Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datesets. Mol. Biol. Evol. 2016;33(7):1870-1874. DOI 10.1093/molbev/msw054.

Kunieda T., Shimada T., Kondo M., Nishimura M., Nishitani K., Hara-Nishimura I. Spatiotemporal secretion of PEROXIDASE36 is required for seed coat mucilage extrusion in Arabidopsis. Plant Cell. 2013;4(25):1355-1367. DOI 10.1105/tpc.113.110072.

Liu Q., Luo L., Zheng L. Lignins: biosynthesis and biological functions in plants. Int. J. Mol. Sci. 2018;19(2):335. DOI 10.3390/ijms19020335.

Llorente F., Lopez-Cobollo R.M., Catala R., Martinez-Zapater J.M., Salinas J. A novel cold-inducible gene from Arabidopsis, RCI3, encodes a peroxidase that constitutes a component for stress tolerance. Plant J. 2002;32:13-24. DOI 10.1046/j.1365-313X.2002.01398.x.

Mansouri I.E., Mercado J.A., Santiago-Domenech N., Pliego-Alfaro F., Valpuesta V., Quesada M.A. Biochemical and phenotypical characterization of transgenic tomato plants overexpressing a basic peroxidase. Physiol. Plant. 1999;106:355-362. DOI 10.1034/j.1399-3054.1999.106401.x.

Marjamaa K., Kukkola E.M., Fagerstedt K.V. The role of xylem class III peroxidases in lignification. J. Exp. Bot. 2009;60(2):367-376. DOI 10.1093/jxb/ern278.

Nei M., Kumar S. Molecular Evolution and Phylogenetics. New York: Oxford University Press, 2000.

Ostergaard L., Teilum K., Mirza O., Mattsson O., Petersen M., Welinder K.G., Mundy J., Gajhede M., Henriksen A. *Arabidopsis* ATP A2 peroxidase. Expression and high-resolution structure of a plant peroxidase with implications for lignification. Plant Mol. Biol. 2000;44:231-243. DOI 10.1023/A:1006442618860.

Passardi F., Longet D., Penel C., Dunand C. The class III peroxidase multigenic family in rice and its evolution in land plants. Phytochemistry. 2004a;65:1879-1893. DOI 10.1016/j.phytochem.2004.06.023.

Passardi F., Penel C., Dunand C. Performing the paradoxical: how plant peroxidases modify the cell wall. Trends Plant Sci. 2004b;9:534-540. DOI 10.1016/j.tplants.2004.09.002.

Pedreira J., Herrera M.T., Zarra I., Revilla G. The overexpression of *AtPrx37*, an apoplastic peroxidase, reduces growth in *Arabidopsis*. Physiol. Plant. 2011;141:177-187. DOI 10.1111/j.1399-3054.2010.01427.x.

Quiroga M., Guerrero C., Botella M.A., Barcelo A., Amaya I., Medina M.I., Alonso F.J., Milrad de Forchetti S., Tigier H., Valpuesta V. A tomato peroxidase involved in the synthesis of lignin and suberin. Plant Physiol. 2000;122:1119-1127. DOI 10.1104/pp.122.4.1119.

Sanou N., Nei M. The Neighbor-Joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 1981;4:406-425. DOI 10.1093/oxfordjournals.molbev.a040454.

Sasaki S., Nishida T., Tsutsumi Y., Kondo R. Lignin dehydrogenative polymerization mechanism: a poplar cell wall peroxidase directly oxidizes polymer lignin and produces *in vitro* dehydrogenative polymer rich in beta-O-4 linkage. FEBS Lett. 2004;562:197-201. DOI 10.1016/S0014-5793(04)00224-8.

Sato Y., Demura T., Yamawaki K., Inoue Y., Sato S., Sugiyama M., Fukuda H. Isolation and characterization of a novel peroxidase gene *ZPO-C* whose expression and function are closely associated with

lignification during tracheary element differentiation. Plant Cell Physiol. 2006;4(47):493-503. DOI 10.1093/pcp/pcj016.

Shigeto J., Kiyonaga Y., Fujita K., Kondo R., Tsutsumi Y. Putative cationic cell-wall-bound peroxidase homologues in *Arabidopsis*, AtPrx2, AtPrx25, and AtPrx71, are involved in lignification. J. Agric. Food Chem. 2013;16(61):3781-3788. DOI 10.1021/jf400426g.

Tsukagoshi H., Busch W., Benfey P.N. Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root. Cell. 2010;4(143):606-616. DOI 10.1016/j.cell.2010.10.020.

Valerio L., De Meyer M., Penel C., Dunand C. Expression analysis of the *Arabidopsis* peroxidase multigenic family. Phytochemistry. 2004;65:1331-1342. DOI 10.1016/j.phytochem.2004.04.017.

Welinder K.G., Justesen A.F., Kjaersgard I.V.H., Jensen R.B., Rasmussen S.K., Jespersen H.M., Duroux L. Structural diversity and transcription of class III peroxidases from *Arabidopsis thaliana.* Eur. J. Biochem. 2002;269:6063-6081. DOI 10.1046/j.1432-1033. 2002. 03311.x.

Yokoyama R., Nishitani K. Identification and characterization of *Arabidopsis thaliana* genes involved in xylem secondary cell walls. J. Plant Res. 2006;119:189-194. DOI 10.1007/s10265-006-0261-7.

ORCID ID

A.S. Tugbaeva orcid.org/0000-0001-9231-3650
A.A. Ermoshin orcid.org/0000-0003-2784-4898
I.S. Kiseleva orcid.org/0000-0002-8709-5203

Acknowledgements. The work was supported by the Ministry of Science and Higher Education of the Russian Federation (No. 075-02-2019-980).

Conflict of interest. The authors declare no conflict of interest.

Received March 14, 2019. Revised May 13, 2019. Accepted May 15, 2019.