ISSN 1021-4437, Russian Journal of Plant Physiology, 2017, Vol. 64, No. 1, pp. 1–14. © Pleiades Publishing, Ltd., 2017.
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REVIEWS

Redox Reactions in Apoplast of Growing Cells

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Received May 20, 2016

Abstract—Redox reactions affecting the cell wall extensibility proceed in the apoplast of growing cells. The reactions involve dozens of oxidoreductases localized in cell walls (Class I and III heme peroxidases, FAD- and Cu-dependent amine oxidases, oxalate oxidase, ascorbate oxidase, superoxide dismutase, etc.) together with NADPH oxidase and quinone reductase of the plasma membrane. The cell wall extensibility decreases due to peroxidase-catalyzed phenolic cross-links of polymers. Cell growth is proven to be directly dependent on production of reactive oxygen species (ROS) in the apoplast. A special value is attached to hydroxyl radical $OH^{\bullet}$, which is able to locally cleave polysaccharides and, thus, increase wall extensibility. Generation of $OH^{\bullet}$ results from one-electron reduction of H_2O_2 and, consequently, is related to the complex of enzymatic and spontaneous reactions of H_2O_2 turnover in the apoplast. The extensibility also depends on an ascorbate concentration in the apoplast and on a ratio of its oxidized to reduced forms. This dependence is expressed not only in the well-known down-regulation of phenols oxidation but also through pro-oxidant and signal activities. There is only indirect evidence of a role of apoplast-originated redox signaling in the cell growth regulation. In addition to ascorbate, the signaling may supposedly involve ROS, glutathione recycling reactions, numerous redox-sensitive peptides, and proteins localized in the cell wall and the plasma membrane.

Keywords: apoplast, ascorbate, cell wall, glutathione, growth regulation, oxidoreductases, phenols, polyamines, redox reactions, ROS

DOI: 10.1134/S1021443717010149

INTRODUCTION

Plant cell growth depends on a potential of primary cell walls to irreversibly extend under the force of turgor pressure. In turn, the extensibility of cell walls is determined by structures of polymers, of which the walls consist. Besides, it is regulated by interpolymer cross-links—the stronger the links, the lower the extensibility. Hydrogen bonds integrate main structural components of cell walls—cellulose microfibrils and surrounding them hemicellulose. Ionic bonds are the most significant for pectin network building, while covalent bonds are involved in maintenance of hemicellulose and protein networks.

Redox reactions occurring in the apoplast create or disrupt covalent bonds between wall polymers, as well as destroy the polymers themselves. These reactions and their roles in growth regulation of plant cells have been studied as long as a half a century. In the last decade, postgenomic and cell technologies have helped expressing a concept of a multicomponent redox system of cell walls. This is the system of interacting oxidants and reductants. They participate in reactions catalyzed by dozens of various enzymes of the oxidoreductase class. They also undergo diverse nonenzymatic conversions, in which ROS are frequent participants. The cell wall is an oxidative compartment of the plant cell, where oxidation of organic compounds predominate. Its redox balance is sustained owing to a constant supply of reductants from intracellular sources. This review discusses the apoplast redox system in an aspect of its involvement in regulation of plant cell growth.

Abbreviations: AA—ascorbic acid; AO—ascorbate oxidase; APX—ascorbate peroxidase; CRK—cysteine-rich receptor-like protein kinases; DHA—didehydroascorbic acid; DAO—diamine oxidase; GABA—γ-aminobutyric acid; GGT—γ-glutamyl transferase/transpeptidase; GSH—reduced glutathione; GSSG—oxidized glutathione; MDA—monodehydroascorbic acid; OO—oxalate oxidase; PAO—polyamine oxidase; PRX—guaiacol peroxidase; SOD—superoxide dismutase.

GROWTH RETARDATION CAUSED BY OXIDATION OF PHENOLIC COMPONENTS

Primary cell walls are poor in phenolics. In dicotyledonous plants, dry mass of the walls contains up to 10% of a structural protein extensin, which, in turn, contains 8–10 mol % of phenolic amino acid tyrosine. Within the first hours after secretion, extensin is easily extractable with salt solutions from cell walls. However, with time, it insolubilizes due to oxidative cross-links between tyrosine residues; among them, isodityrosine cross-links are the most common. The forma-

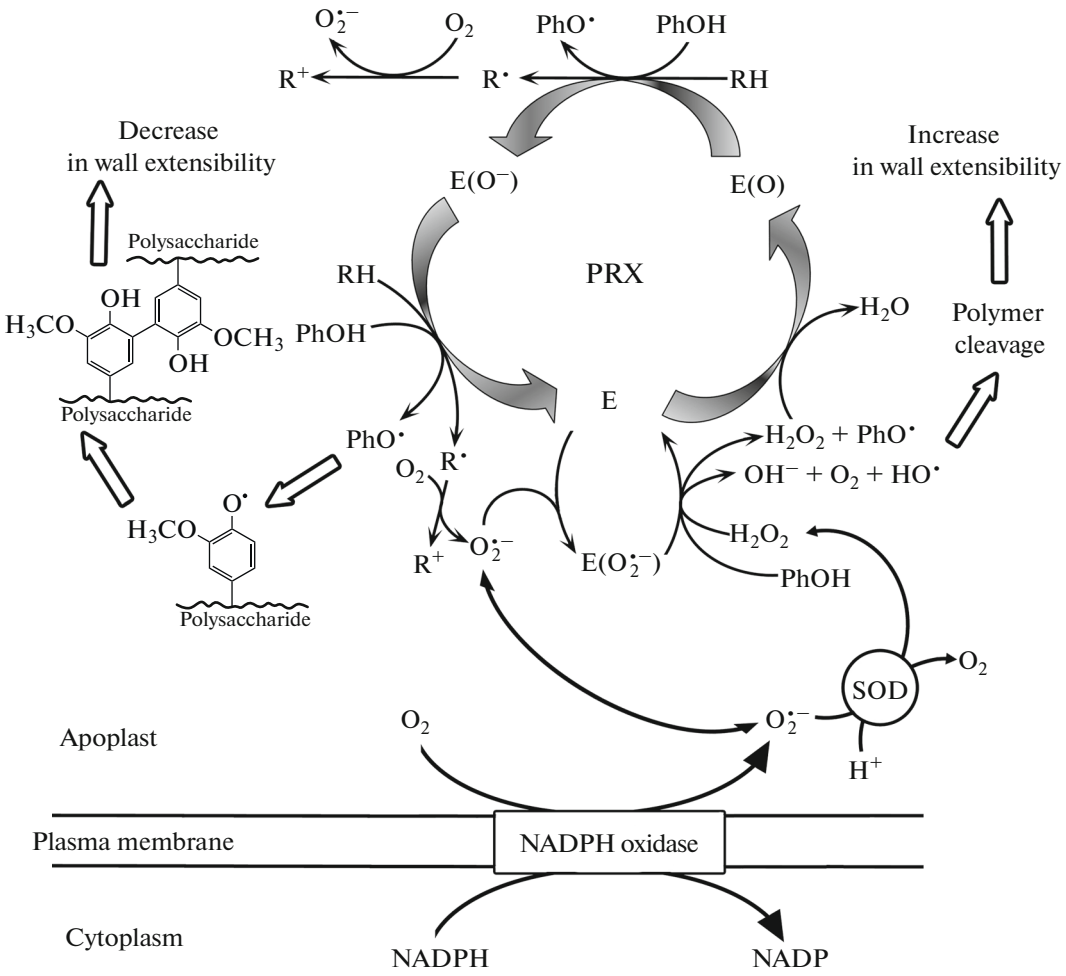

Fig. 1. Participations of PRX, NADPH oxidase, and SOD in ROS formation, oxidation of phenolic compounds, and regulation of cell wall extensibility. E—PRX in a basic state; E(O)—Compound I; E(O$^-$)—Compound II; E($O_2^{\bullet-}$)—Compound III; PhOH—phenolic reductant; PhO$^\bullet$—phenoxyl radical; RH, R$^\bullet$, R$^+$—different reductants (NADH, IAA, etc.) and their oxidized forms.

tion of these bonds involves specific "extensine peroxidase" [1]. For many years, since its discovery in the early 1960s, extensin was considered as a possible growth regulator that gave its name to it. However, another idea has received the reliable experimental proof in the sequel. It is the role of the extensin insolubilization in a cell wall reinforcement, which takes place under stress, for example, upon attacks of pathogens [2].

In cereals, phenolic substances of primary cell walls are mainly hydroxycinnamic acids (ferulic, coumaric, and sinapic) present in arabinoxylans. In oxidation by peroxidases, phenolic acids form cross-links between hemicelluloses (Fig. 1). Diferulate bridges (5-5, 8-8, 5-8, 4-O-8, etc.) are the most usual. Their role in seedling growth retardation of various cereals is well substantiated. The rise in levels of ferulic acid and diferulates was reported for the cases of coleoptile senescence [3], light-dependent and basipetal growth retardation of mesocotyls [4], under hypergravitation [5], and salinization [6].

GROWTH ACCELERATION CAUSED BY ROS

Many plant treatments with oxidants and reductants were performed in order to conceive roles of redox processes in growth regulation. The compounds tested included hydrogen peroxide, NAD(P)H, ascorbate, glutathione, dithiothreitol, inhibitors of oxidases and those of peroxidases. The usual outcome of such treatments was growth retardation as an obvious stress response to dramatic changes in a redox balance.

Some attempts achieved the growth stimulation by evoking free radical reactions in plants. In the 1940s–1960s, as related to the progress in nuclear energetics, effects of ionizing radiation on plants were intensively studied. Paradoxically strong positive effects were recorded; this promoted development of "radioactive fertilizers" for growth stimulation. These investigations were summarized by Sax [7]. In his opinion, only repeating exposures of growing plants to small doses of ionizing radiation might authentically accelerate the growth. Hydroxyl radical OH$^\bullet$ is a potent initiator of free radical processes, and growth stimulation by it was

found (Fig. 1). The accelerated elongation of coleoptiles of maize, hypocotyls of cucumber, soybean, sunflower, and pine [8] and maize roots [9] occurred in the medium where the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$) proceeded yielding hydroxyl radical. The effects were caused by the sharp increase of cell wall extensibility. In vitro, incubation of xyloglucans in this medium decreased viscosity of their solution because of depolymerization of these hemicelluloses [10].

The ^3H-fingerprinting is used to detect oxidative damages of polysaccharides in cell walls [11, 12, 13]. The method is based on reduction by NaB^3H_4 of carbonyl groups that appeared in polysaccharide molecules due to their oxidation; this way, tritium labels oxidized fractions of polysaccharides. Such damages were revealed in cell walls in ripening pear fruits [11], in ruptured endosperm cup during radicle emergence in cress, in rapidly growing maize coleoptiles [12], and also in abscission layer in frond of water fern *Azolla* [13].

Many assays, applicable to cell walls, were developed to detect particular or total ROS. The oxidation of 2',7'-dichlorofluorescin that yields the fluorescing product 2',7'-dichlorofluorescein is often used to determine the total ROS level [14, 15]. The apparently most usable assay for superoxide radical $O_2^{\cdot-}$ is based on its ability to reduce tetrazolium salts: nitroblue tetrazolium to produce insoluble formazan or XTT (Na_2, 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid) to yield soluble formazan [9, 12, 14–16]. To detect hydrogen peroxide, a FOX method based on Fe^{2+} oxidation to Fe^{3+} [17] and peroxidase methods based on enzymatic oxidations of phenolic substrates by H_2O_2 to colored products [9] are developed. Other methods employ the capability of H_2O_2 to oxidize luminol causing its chemiluminescence and to oxidize $CeCl_3$ to yield an electron-dense sediment [15, 18]. Hydroxyl radical is assayed by ESR method, which senses long-living free radicals of $OH^{\cdot}$-traps that appear in their reactions with $OH^{\cdot}$ [9, 10, 14].

With these methods, the ROS are assayed in incubation media of plant objects or in apoplastic fluid that is extracted from tissues by low-speed centrifugation. Histochemical tests are accomplished with probes, which are adsorbed on organ surfaces or in cell walls. It should be noted that ROS release into incubation media strongly depends on permeability of plant surface that is usually higher in young rapidly growing tissues. The luminescence intensity of histochemical probes depends on particular sites of their concentration.

The above-mentioned methods ascertained the $O_2^{\cdot-}$ discharge from elongating maize coleoptiles into incubation medium, and the process was more intense if the growth was stimulated by auxin [14]. Generation of $O_2^{\cdot-}$ and H_2O_2 in the cell wall and $O_2^{\cdot-}$ release into incubation medium are maximal in the elongation zone of maize leaves [15]. If the leaf growth is retarded by salinization, ROS level in cell walls decreases [16]. In maize mesocotyls, the apoplastic H_2O_2 concentration was the highest in a zone of rapid cell elongation [17].

In maize roots, the maximal $O_2^{\cdot-}$ and $OH^{\cdot}$ generation was observed on the surface of the elongation zone, and the IAA-dependent growth inhibition was accompanied by a decrease in the ROS level [9]. An increased extensibility of cell walls favoring maintenance of maize root growth under salinization is associated with an increased H_2O_2 production in the apoplast of the elongation zone [18]. However, Joo et al. [19], in experiments on gravitropic bend of the maize root, revealed IAA-dependent ROS accumulation in cells of the lower, slowly growing part of a horizontally placed root. Besides, ROS were found to accumulate during the IAA-dependent growth inhibition of a vertically placed root. Unlike Liszkay et al. [9], who reported the opposite effect on the same object, Joo et al. used 2',7'-dichlorofluorescin diacetate, which enabled registration of ROS level rather inside cells than on their surfaces.

A high rate of $O_2^{\cdot-}$ generation was found in an elongation zone of radices of germinating pea seeds [20]. Rapidly growing radices also generate $OH^{\cdot}$ but not H_2O_2, on their surfaces. The fluorescent probe for $O_2^{\cdot-}$ brings about a positive result in root tips of germinating seeds of cress [12] and willow [21].

In *Arabidopsis*, ROS are concentrated in a tip of a root hair at early stages of its development; these products are involved in the formation of axial gradients of calcium ions [22]. However, tests with the OxyBURST probe, to which cells are impermeable, revealed that during elongation of the root hair the cell wall of its growing tip produces much lesser ROS amounts than non-growing side walls [23]. Besides, during growth oscillations, the acceleration of hair growth is preceded by decrease in ROS level in cell walls while retardation is preceded by its increase. An intense $O_2^{\cdot-}$ formation occurs on a surface of the growing cotton seed hair [24].

Therefore, H_2O_2, $O_2^{\cdot-}$, and $OH^{\cdot}$ emerge in the apoplast of growing cells. Of them, hydrogen peroxide is the least reactive and, hence, it can accumulate, diffuse at distances comparable with cell sizes, and readily penetrate cell membranes. In the apoplast, H_2O_2 may originate not only from in muro reactions but also at the expense of intracellular sources though the contribution of the latter case is poorly estimated. Without stresses, H_2O_2 level in the apoplast is approximately 0.01–0.1 mM. The detectable in the apoplast $OH^{\cdot}$ and $O_2^{\cdot-}$ are produced exactly in the cell walls, since they are so reactive that unable to both diffuse at long distances and accumulate in cells. Thus, not concen-

trations of these ROS but rates of their generation are usually evaluated and discussed. Superoxide radical as its protonated form $HO_2^{\bullet}$ can penetrate membranes. The protonation occurs in an acid medium (pK 4.8). Since cell walls are acidic (pH 5–6), $O_2^{\bullet-}$ can move from the apoplast into cells. For $OH^{\bullet}$, the diffusion distance does not exceed 1 nm, so this species oxidizes organic molecules exactly in the site of its origin.

REDOX PROTEOMICS OF CELL WALLS

The cell wall proteome numbers several hundred of diverse proteins and peptides. More than half of them possess signal N-peptide and, thus, reach the cell wall via classical vesicular secretion, by passing through the Golgi apparatus [25]. Some proteins have signals of so-called non-classical secretion, which is not mediated by the Golgi apparatus and insensitive to brefeldin [26]. Many cell wall proteins are alkaline glycoproteins extractable by salt solutions and, therefore, linked to a wall matrix by ion bonds. Some proteins are very tightly incorporated in the wall and may be isolated only after polysaccharide hydrolysis. But other proteins are present in apoplastic fluid and easily diffuse into incubation medium of plant tissues.

The proteomes of *Arabidopsis* and rice cell walls are the best studied and contain nearly 500 and 270 proteins, respectively [27]. Approximately 10–15% of them are oxidoreductases, in which Class III heme peroxidases or so-called guaiacol peroxidases (PRXs) are the most common. In *Arabidopsis*, they are encoded by 73 genes and, in most cases (71 of 73), have a signal N-peptide [28]. Seven PRXs, in addition to the N-peptide, possess a C-terminal signal of vacuolar localization. The proteomic analysis of the cell wall fraction of *Arabidopsis* revealed 32 PRXs. Additionally, 17 PRXs of a plasma membrane fraction might also belong to the cell wall, since they do not have hydrophobic transmembrane domains and are supposedly adsorbed on the membrane by means of certain membrane proteins. Ascorbate peroxidases (APXs)—Class I heme peroxidases without a signal N-peptide—were also identified in cell walls [29, 30].

Among flavin-containing oxidases, the best-characterized enzymes are polyamine oxidases [31]; other proteins similar to reticuline oxidase participating in biosynthesis of alkaloids of the benzylisoquinolinium group are also found [27, 32].

The group of metal-dependent oxidoreductases is rather large. It includes copper-containing ascorbate oxidases (AOs), phenol oxidases, diamine oxidases, Cu-Zn-superoxide dismutases (SODs), Mn-dependent germins possessing oxalate oxidase activity, and germin-like proteins [29, 30].

NAD-dependent dehydrogenases (malate dehydrogenase, alcohol dehydrogenase, lactate dehydrogenase, etc.) are present in cell walls [29, 30, 33]. Many workers explain their occurrence as a result of cytoplasmic contamination, because these enzymes do not have N-peptide, and NAD or other pyridine dinucleotides are not found in cell walls so far. All the same, substrates of these dehydrogenases are present in the apoplast. Malic acid is among the main apoplastic organic acids, and its concentration reaches 3–4 mM [17, 34–36]. Apoplast of many plants contains lactic acid at up to 0.8–1.3 mM [34]. In *Arabidopsis* suspension cells, the lactate level in the apoplast diminishes in response to a biotic attack [36]. We recently showed that the lactate content of the apoplast of maize mesocotyls is especially high in rapidly elongating cells adjacent to the coleoptile node and sharply falls towards the basal direction (unpublished data).

Over the last decade, enzymes requiring glutathione were found in cell walls. For instance, glutathione-S-transferase showed up in the *Arabidopsis* leaf apoplast [32]. There were records of glyoxalases in apoplasts of maize roots [29] and of poplar leaves [30]; using reduced glutathione, these enzymes transform methylglyoxal—a highly toxic by-product of carbohydrate and amino acid metabolism—to lactic acid. The role of apoplastic γ-glutamyl transferases in a recycling of oxidized glutathione and, in general, in the redox regulation was thoroughly investigated [37]. One of eight glutathione peroxidases of *Arabidopsis* (AtGPX6) bears attributes of a protein of non-classical secretion and was found in cell walls [38]. This enzyme reduces not only hydrogen peroxide but also organic hydroperoxides ROOH, including lipid ones, and utilizes glutathione or thioredoxin as reductants.

Cell walls contain certain redox-sensitive proteins whose activities are associated with SH-groups reversible oxidation. One of glutaredoxins is present in the apoplast of maize roots [29]. These proteins are more diverse in plants than in animals and fulfill a glutathione-dependent reduction of oxidized SH-groups of different proteins [39]. In their catalytic cycle, glutaredoxins get glutathionilated themselves or glutathionilate their protein substrates. The profile of glutaredoxin content in maize root tips shows that it peaks in the walls of cells accomplishing elongation [29].

Peroxiredoxin (Prx) [30] and thioredoxin [30, 40] were also found in cell walls. Peroxiredoxins reduce H_2O_2 at the expense of oxidation of their own SH-groups: H_2O_2 + Prx-SH → H_2O + Prx-SOH [41]. These groups are rereduced by thioredoxins, which, in turn, are reduced involving NADPH-dependent thioredoxin reductases. Therefore, these thioredoxin-dependent peroxidases participate in the chain of sequential oxidations that accomplish H_2O_2 reduction at the expense of NADPH: H_2O_2 → peroxiredoxin → thioredoxin → thioredoxin reductase → NADPH. Thioredoxins reduce SH-groups of not only peroxiredoxins but also those of many different proteins, for example, plant glutathione peroxidases [38]. Experiments with transgenic rice plants, whose level of the

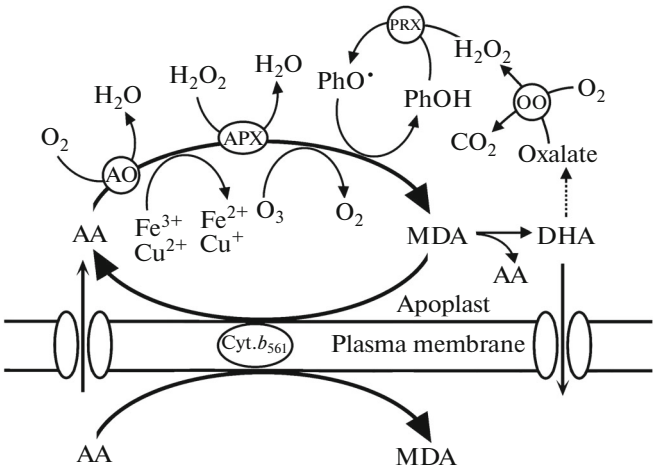

Fig. 2. Metabolism of ascorbate and oxalate in apoplast. PhOH—phenolic reductant; PhO·—phenoxyl radical.

gene *OsTRXh1* expression was modified, showed that thioredoxin OsTRXh1 diminishes ROS production in the leaf apoplast under a salt stress [40].

Diverse cysteine-rich secretory peptides [25] and cysteine-rich receptor-like protein kinases (CRK) of the plasma membrane [42] may also be participants of the cell wall redox metabolism.

The enzymes lipoxigenases, glutathione peroxidases, monodehydroascorbate reductases, and quinone reductases that are localized in the plasma membrane substantially contribute to the redox metabolism of cell walls [43]. The most studied of them are NADPH oxidases; they generate superoxide radical in cell walls due to an oxidation of cytoplasmic NADPH [44].

Dynamics of the cell wall proteome was mostly studied under stress conditions. The proteomic analysis of cell walls of elongating cells was carried out on maize seedling roots [29]. It revealed that the significant increase in root cell wall extensibility, allowing root elongation in conditions of high salinity despite the decrease in osmotic pressure, was associated with a manifold rise in content of oxalate oxidase, Cu-Zn-SOD, APX, and several PRXs in cell walls.

CLASS III HEME PEROXIDASES

Approximately half of cell wall oxidoreductases are guaiacol peroxidases. The enzyme molecule consists of one polypeptide chain of approximately 300 amino acid residues and includes heme composed of Fe^{3+} and protoporphyrin IX [45]. Most PRXs are glycoproteins differing in number and composition of oligosaccharide chains. In the course of peroxidase catalytic cycle (Fig. 1), enzyme (E) is at first oxidized by hydrogen peroxide: Fe^{3+} gives oxoferryl $Fe^{4+} = O$, and porphyrin ring gives its cation radical. This way, the enzyme turns into Compound I—E(O). After two sequential one-electron oxidations of the substrate, the enzyme converts into Compound II—E(O^-) and then returns to its ground state.

PRXs mainly oxidize phenolic compounds and exhibit low substrate specificity. As a result, every PRX interacts with a wide spectrum of phenols and other reductants. However, according to the substrate specificity, PRXs may be divided into two groups [46]. G-peroxidases display the most activity towards guaiacol-like low-molecular-weight phenols and are less active against larger molecules, for example, S-lignans or syringaldazine simulating S-lignans. On the contrary, S-peroxidases prefer syringaldazine to guaiacol. Presumably, only S-peroxidases are involved in lignification, for example, cationic PRX of white poplar CWPO-C, AtPrx2, AtPrx25, and AtPrx71 of *Arabidopsis* [47]. S-peroxidases are able to oxidize large molecules, because the radical migrates in the Compound I from iron porphyrin to surface-located tyrosine residues, which turn into phenoxyl radicals taking electrons from the substrate [48].

Related to the peroxidase cycle, chemical transformations are extremely diverse, since the main peroxidase products are phenoxyl radicals (PhO·) entering into spontaneous reactions (Fig. 1). Among them, the best-studied one is a quenching of phenoxyl radicals through their dimerization and oligomerization resulting in oxidative phenolic cross-linkages or lignin formation. Phenoxyl radicals are reduced due to an oxidation of ascorbic acid (AA) or NAD(P)H. Consequently, if the reaction mixture contains AA (or NAD(P)H), only this substrate is oxidized, while phenols play a role of cofactors of ascorbate peroxidase reaction (Fig. 2). Phenoxyl radicals can undergo disproportionation leading to reduction of one radical at the expense of deeper oxidation of another one. Spontaneous reactions are especially complex in mixtures of phenolic compounds. Here, some compounds in forms of phenoxyl radicals mediate oxidation of other

compounds, which are incapable of direct oxidation by peroxidase.

PRXs display not only peroxidase but also oxidase activity coupled with generation of ROS $O_2^{\bullet -}$, H_2O_2, and $OH^{\bullet}$ (Fig. 1). Two chief factors underlie this activity. The first, some substrates of PRX, after being oxidized to free radicals, are spontaneously oxidized by molecular oxygen to yield superoxide later on. The second, in the presence of strong reductants or $O_2^{\bullet -}$, the enzyme turns into the Compound III—$E(O_2^{\bullet -})$, the return from which to the ground state is coupled with production of H_2O_2 and $HO^{\bullet}$.

In vitro, PRXs display the maximum oxidase activity towards NAD(P)H, which, in the presence of Mn^{2+} and phenolic cofactors, is oxidized following the total equation $NAD(P)H + O_2 \rightarrow NAD(P) + H_2O_2$. Peroxidases can also generate ROS via oxidation of IAA, aromatic monoamines, glyoxalic, dihydroxyfumaric and salicylic acids, chitooligosaccharides, cysteine, and fatty acids [36, 49] but with a negligible yield in comparison with that of NAD(P)H oxidation. The ROS-producing oxidase reactions can also involve some phenolic compounds, acetosyringone for example, as substrates [50, 51].

The utterly important feature of PRXs is their production of hydroxyl radical (Fig. 1). In a form of the Compound III, they interact with hydrogen peroxide and actually carry out the Haber–Weiss reaction $E(O_2^{\bullet -}) + H_2O_2 \rightarrow E + O_2 + OH^{\bullet} + OH^-$ [52].

Numerous demonstrations of PRX involvements in the oxidative bursts that occur in response to attacks of phytopathogens were reported [36, 53], although the nature of reductants affording ROS generation is still uncertain under these conditions.

To figure out PRX functions, it essential to know microdomains of their localization in cell walls. For instance, upon formation of Casparian strips in the root endodermal cells, lignification occurs only in radial and transverse but not tangential cell walls. In *Arabidopsis*, the principal participants of the process—NADPH oxidase of the plasma membrane, SOD, and PRX (Atprx64) of the cell wall—are assembled with the help of special transmembrane proteins in zones of Casparian strips' formation [54]. Therefore, both formation of H_2O_2 and its utilization for lignin synthesis are focused in a special site. Ion bonds with pectins may be maintained due to clusters of arginine residues that are present in some PRXs, for example, in prx32/34/37/38 of *Arabidopsis* [28]. Localization of PRXs participating in $OH^{\bullet}$ generation is of special interest, because this radical is barely diffusible.

Relations between cell growth rate and PRX activities in cell walls have been studied over many decades. For instance, a progressive retardation of cell elongation in axial growth zones in shoots and roots is usually accompanied by a considerable rise in peroxidase activity. Similar PRX activation is often observed during growth retardation under stress conditions. The function of PRXs in growth suppression is explained by the enzymatic catalysis of oxidative cross-links and lignin synthesis [55].

All the same, PRXs that are localized in rapidly expanding cell walls exist as well. Such PRXs were discovered by proteome analysis in cell walls of maize root tips [29]. In an emerging hair of a cotton seed, the positive correlation between growth rate, ROS formation, and PRX GhPOX1 activity takes place [24]. PRX inhibitor (salicylhydroxamic acid) suppresses both hair elongation and ROS generation. In *Arabidopsis*, enzymes prx33 and 34, capable of ROS production, are apparently involved in root growth maintenance [56]. Mutations in these PRXs lead to shorter roots, while roots of overexpessors are longer than in a wild type.

METABOLISM OF ASCORBIC ACID

Ascorbic acid is an only representative of a "Big Three" of biological reductants (NAD(P)H, glutathione, and ascorbate), which is present at considerable amounts in cell walls. The apoplast contains 2–8% of the total cellular ascorbate, where its concentration is 0.1–1 mM [32, 57]. Therefore, the ascorbate level in the apoplast is approximately 100 times lower than in cytoplasm and plastids (10–20 mM) and lower than in a total plant tissue homogenate (1–3 mM). Inside cells, nearly 90% of the ascorbate exists in a reduced form (AA), whereas the ratio of AA to dehydroascorbate (DHA) in cell walls strongly varies from 0 to 60–70%. Media containing both AA and DHA always contain monodehydroascorbic acid (MDA) as a product of a spontaneous disproportionation AA + DHA $\leftrightarrow$ 2 MDA. The equilibrium of this reaction is strongly shifted leftward ($K_{eq} = 10^{-8}$); hence, MDA concentration does not exceed 0.1 μM.

The reduced AA is translocated into cell walls by means of specific carriers, of which the DHA/AA exchange carrier is the most investigated [58]. In cell walls, AA is oxidized through PRX, APX, AO activities, as well as nonenzymatically by Fe^{3+}, Cu^{2+}, or ROS (Fig. 2).

The contribution of APXs is the least. These Class I heme peroxidases do not possess signs of cell wall proteins, since they are not glycosylated and do not have signal N-peptide. However, as was already mentioned, they are often found in proteome of cell walls. There, they were revealed also by enzymatic methods [59], using specific features enabling to distinguish APX from PRX. Firstly, APXs are irreversibly inactivated by even microamounts of hydrogen peroxide if AA is absent in the medium. Thus, for revealing of APXs, extracting buffers should contain ascorbate. Secondly, they are inhibited by thiol reagents, for example, *p*-chloromercuribenzoate [60].

APX activity in cell walls is negligible as compared with activities of PRXs, which are capable of AA intense oxidation mediated by phenoxyl radicals [61]. Therefore, the triad AA–phenols–PRX displays an APX activity and scavenges the apoplast from H_2O_2 (Fig. 2). Besides, AA prevents phenol oxidation and, consequently, lignification and formation of phenolic oxidative bridges between wall polymers [62–64].

AO contributes significantly to ascorbate metabolism in cell walls (Fig. 2). This is homodimer; its subunits are glycosylated, they have a signal N-peptide and a claster of four copper ions, which are held in histidine-rich sites. AO is found in many plant species, it is the most active in Cucurbitaceae and Solanaceae.

The substantial evidence of the apoplastic ascorbate participation in the regulation of cell elongation rests on studies of AO activity. It is maximum during cell elongation in tobacco suspension culture [65] and rises in *Arabidopsis* roots upon their gravistimulation [66]. In addition, AO activity and expressions of the encoding genes are enhanced many times upon exposure to IAA [67].

The following possible mechanisms of AO relationship with elongation growth are considered:

(1) The enzyme decreases O_2 level in the apoplast and, thus, inhibits oxidative reactions, including those yielding ROS. This function is supported by facts of an increased AO activity in nitrogen-fixing nodules [68].

(2) AO produces MDA, which may be reduced in expense of cytoplasmic AA (Fig. 2) with an involvement of plasma membrane cytochrome b_{561} [58]. Therefore, MDA functions as an electron acceptor. MDA is known to cause sustained hyperpolarization of the plasma membrane associated with proton pump activation and K^+ influx to cells [69, 70].

(3) AO activity results in DHA accumulation and decrease in AA/DHA ratio [58]. Presumably, DHA may interact with lysine and arginine residues of proteins and oxidize SH-groups. DHA is spontaneously hydrolyzed and oxidized yielding threonic and oxalic acids. Oxalate accumulation in cell walls leads to calcium chelation and, hence, to its elimination from the wall structure. In addition, several chemically active intermediates appear in the course of DHA oxidation [71]. AA/DHA ratio, apparently, influences the degree of reduction of SH-groups in redox-sensitive proteins (for example, CRK of the plasma membrane) and, thus, can play a signaling role in growth regulation [58].

Comparison of dynamics of oxidized and reduced ascorbate in cell walls with cell growth dynamics shows that, as a rule, the total apoplastic content of ascorbate increases and AA/DHA ratio decreases during elongation. This means that apoplast contains relatively high amounts of DHA and MDA [58, 65]. Treatment of growing tissues with AA does not affect elongation or suppresses it [72]. Stimulation takes place only if AA is rapidly oxidized, for example, in the presence of Cu^{2+} or H_2O_2 [70, 73].

In summary, among many hypotheses explaining growth-regulating role of apoplastic ascorbate, the best-substantiated ones are:

(1) AA hinders oxidations of phenols by guaiacol peroxidases;

(2) MDH causes the plasma membrane energization;

(3) AA reduces Fe^{3+} and Cu^{2+} and, thus, promotes the Fenton reaction and $OH^{\cdot}$ generation; and

(4) AA/DHA ratio is perceived by redox-sensitive receptors of the plasma membrane as a signal controlling growth rate.

OXALATE OXIDASES

Among oxalate oxidases (OOs), the enzymes of cereal cell walls are the most investigated. In these plants, they are especially active at early phases of germination that gave them the name germins [74]. All plants, including cereals, house germin-like proteins with poorly studied functions. Germins are homohexamers; their subunits are glycosylated, contain Mn^{2+}, and have signal N-peptide. Although expressions of germin genes is a striking marker of an onset of cereal germination, germin activities are focused not in growing zones but in dermal and vascular tissues and in differentiated root regions. Activity of OOs enhances under stress caused, for example, by salinization or attacks of pathogenic fungi. These enzymes participate in the reactions of oxidative burst and hypersensitivity. They yield hydrogen peroxide, which can be utilized by peroxidases (Fig. 2), for instance, to produce diferulates retarding growth of wheat shoots [75]. In addition, OOs help maintenance of maize root growth under salinization [18].

NAD(P)H OXIDASES OF PLASMA MEMBRANE

Plant cells have long been known to reduce electron acceptors, to which the cells are impermeable. The best-studied example is ferricyanide which represents chelated Fe^{3+}. This phenomenon is related to a capability of the plasma membrane to oxidize NADH and NADPH. However, proteinaceous components of NAD(P)H–ferricyanide reductases are so far not identified in spite of the intense investigations of this activity in the 1980s–1990s.

Plants can oxidize NAD(P)H on both internal and external sides of the plasma membrane. Ecto-NAD(P)H oxidase activity is reported, which differs from a similar activity of PRX. It is not sensitive to cyanide, which inhibits PRX, and is not related to ROS generation [76]. Ecto-NAD(P)H oxidase activity is highest in elongating cells and is stimulated by

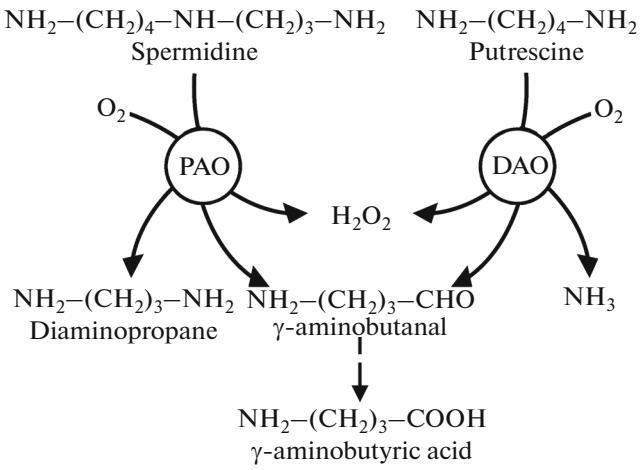

Fig. 3. Metabolism of polyamines in apoplast. DAO—diamine oxidase; PAO—polyamine oxidase.

auxins; its molecular bearers are not identified. Quinone reductase activity, which is coupled with $O_2^{\bullet-}$ production, was recently found in the plasma membrane and is not still explored in detail. In addition, there is no evidence of quinone presence in the plasma membrane [49].

Superoxide-generating NAD(P)H oxidase is the most fully characterized at molecular level (Fig. 1). It is homologous to the catalytic subunit $gp91^{phox}$ of the NADPH oxidase protein complex of the plasma membrane of mammalian neutrophils, which produces the oxidative burst in response to alien cells. The neutrophilic enzyme is strictly NADPH-specific. Enzymatic characteristics of its plant analog are poorly studied; some facts suggest that the enzyme prefers NADH [52]. Plant NAD(P)H oxidase is an integral membrane flavohemoprotein, and its polypeptide chain crosses the membrane six times. NAD(P)H oxidase, with mediation of FAD and two cytochromes b_{558} present in its content, accomplishes transmembrane electron transfer. The latter starts from NAD(P)H, which is oxidized on a cytoplasmic side of the membrane, and finishes at molecular oxygen, which is reduced to $O_2^{\bullet-}$ at a side facing the cell wall [44].

In plants, functions of the plasma membrane NAD(P)H oxidase are not restricted to the oxidative burst. In *Arabidopsis*, the enzyme is encoded by ten genes *RBOH* (respiratory burst oxidase homologs). They are involved in defense responses, lignification, suberinization, stomatal closure, and growth regulation. Thus, the AtRBOH-C activity is necessary to establish Ca^{2+} gradient in tips of root hair at early stages of its formation [23]. AtRBOH-H and AtRBOH-J commit the same function in tips of growing pollen tubes [77]. Activities of AtRBOH-D and AtRBOH-F are required for ROS generation under stresses, as well as for ABA-dependent stomatal closure [44]. The xylem vessel lignification involves AtRBOH-E [78], and AtRBOH-F participates in formation of Casparian strips in the root endoderm [54]. Gene expressions of *AtRBOH-A/G/I* are enhanced in the root elongation zone [44].

Roles of NAD(P)H oxidases in various processes are often interpreted according to results of inhibitor-based trials with the use of diphenylene iodonium—the inhibitor of flavin-containing enzymes. NAD(P)H oxidases are much more sensitive to it than FAD-dependent amine oxidases of cell walls [16]. The inhibitor analysis shows that NAD(P)H oxidases are involved in lignification, suberinization, and oxidative burst in many plants [78]. They are also needed for growth of cotton hair [24] and growth of soybean hypocotyls [52].

POLYAMINES AND AMINE OXIDASES

Polyamines are polycations possessing reducing activity. Their contents in primary cell walls are 0.1–0.5 mM [16, 79]. The most common polyamines are spermine, spermidine, and putrescine. Their links with the cell wall is sustained by ion interactions with acid pectin, from which they displace Ca^{2+} and, thus, destroy the calcium-dependent rigid conformation [79, 80]. In addition, they form amide bonds with phenolic acids (ferulic and coumaric). Young tissues are known to contain more polyamines than mature ones. But this pattern, presumably, is not valid for cell walls, since their polyamines content is either retained [79, 80] or increased [81] with age.

In cell walls, polyamines are oxidized by amine oxidases [31]. Primary amino groups are oxidized by Cu^{2+}-dependent amine oxidases, which are the most active against diamine putrescine and are often called diamine oxidases (DAOs) for this reason (Fig. 3). In cell walls, their activity is much higher in dicotyledonous plants than in monocotyledonous. Legumes are especially rich in DAOs.

FAD-dependent amine oxidases or polyamine oxidases (PAOs) oxidize secondary amino groups and, therefore, their substrates are spermine and spermidine but not putrescine. PAOs are the most active in cereals.

Products of reactions of these enzymes are hydrogen peroxide, γ-aminobutanal, and ammonium (with DAO) or diaminopropane (with PAO) (Fig. 3). Aminobutanal spontaneously cyclizes into 1-pyrroline, the oxidation of which yields γ-aminobutyric acid (GABA). Usually, only trace amounts of GABA are present in the apoplast. But its concentration achieves 3 mM under stresses [82]. Interestingly, GABA at concentrations below 0.5 mM can stimulate growth [83] but inhibit it at higher (1–10 mM) concentrations [84].

Most works concerning functions of amine oxidases of cell walls address their ability to produce hydrogen peroxide. Amine oxidase involvements in processes of lignification, suberinization, oxidative burst [31], ethylene-dependent stomatal closure [85],

and insolubilization of the structural protein extensin that is associated with formation of dityrosine bridges [86] are documented.

There is an evidence of a negative correlation between amine oxidase activity and growth rate. Thus, retardation of growth of maize mesocotyls by red light is accompanied by an increased PAO activity, while auxin decreases it [87].

Positive correlations between growth and activities of these enzymes were also known. For example, a maintenance of maize leaves elongation under salinization depends on $O_2^{\cdot -}$ and $OH^{\cdot}$ whose formation involves PAOs [16]. Zones of intense growth possess highly active fraction of PAO, which is readily extractable from cell walls. It was demonstrated on roots [88] and mesocotyls [17] of maize. A very tight interconnection between cell extension growth and DAO activity was found in soybean seedlings [89]. It is observed in growth zones of roots and hypocotyls. It was also found during the reaction of hypocotyl straightening, which depends on high DAO activity in the lower rapidly growing half. Therefore, amine oxidases, as well as PRXs, are involved in reactions that are capable of strong and multidirectional effects on growth.

GLUTATHIONE

In animal cells, removal of H_2O_2 is fulfilled by the glutathione peroxidase cycle to a large extent. Consequently, the reduced glutathione (GSH) is regarded as the most important water-soluble antioxidant. In plants, activities of glutathione peroxidases are low, and glutathione mainly plays its antioxidant role as a substrate of dehydroascorbate reductase in the ascorbate-glutathione cycle [90]. Glutathione is indispensable for detoxification of xenobiotics and heavy metals. It is a stored and transportable form of reduced sulfur. Its content in plant cells many times exceeds levels of all other thiols (SH-containing compounds) and is found to be 3–10 mM in cytoplasm, cell organelles, and phloem sap [91]. Glutathione regulates activities of redox-sensitive proteins, whose SH-groups are prone to reversible oxidation. These proteins include enzymes (for instance, protein phosphatases and protein kinases), CRK, transcription factors, ion channels, and proton pumps [92].

Interest in the apoplastic SH-metabolism emerged long ago. Soon after the discovery of first proteins—extensins—bound to cell walls, Lamport [93] wrote that the problem of growth regulation by auxin boils down to a question of how auxin reduces labile disulfide bridges of extensin. Later on, it was ascertained that extensins do not contain cysteine, but there are another cysteine-rich proteins in cell walls.

The first convincing evidence of glutathione presence in the apoplast was obtained in investigation of γ-glutamyl cycle involved in a glutathione recycling. In cells of yeasts and mammals, the enzyme of this cycle

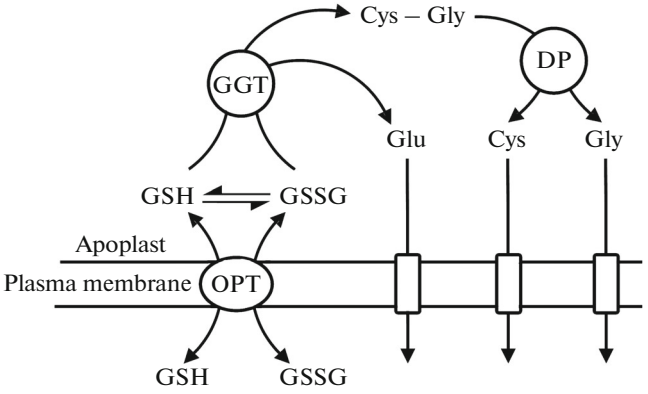

Fig. 4. Apoplastic loop of glutathione recycling. OPT—oligopeptide transporter; DP—dipeptidase.

γ-glutamyl transferase/transpeptidase (GGT) resides on the outer side of the plasma membrane. Therefore, to close the γ-glutamyl cycle, glutathione should move into the extracellular matrix [94]. In plants, the cycle closes through the apoplast, where GGTs are concentrated (Fig. 4). GGT activity was found in cell walls of a whole range of plants (radish, onion, tomato, barley, maize, and *Arabidopsis*). The activities in barley roots [95, 96], in all organs and at all developmental stages of *Arabidopsis* [37, 94, 97] are described in detail.

Arabidopsis contains three homologous GGTs: GGT1 and GGT2 in the cell wall and GGT3 in the vacuole. GGT1 is responsible for almost 80–90% of total γ-glutamyl transferase activity of cells [97]. This glycoprotein is extractable from cell walls with 1 M NaCl. Affected by GGT, glutathione decomposes into Glu and dipeptide Cys-Gly, which is then hydrolyzed by a little-studied dipeptidase. The resulting amino acids are easily reabsorbed by cells (Fig. 4). GGT can also display transpeptidase activity. It transfers Glu residue from glutathione not only to water but also to α-NH_2 of other amino acids and to that of di- or tripeptides [94]. GGT is active towards either oxidized or reduced glutathione as well as to its conjugates [95].

The usual glutathione concentration is 0.3–0.4 mM in leaf homogenates. The substance is irregularly distributed among cell compartments. Its highest concentration is in mitochondria, while the lowest in vacuoles and the apoplast. Thus, subcellular glutathione levels in *Arabidopsis* leaves compose the row: mitochondria 9–10 mM, nucleus 6–10 mM, cytosol and peroxisomes 3–5 mM, chloroplasts 1–1.5 mM, vacuoles 0.01–0.14 mM [98]. In cell walls of *Arabidopsis* leaves, glutathione concentration does not exceed 0.03 mM [32, 97]. The apoplast is especially poor in reduced glutathione, whose concentration is two to three times higher than that of the oxidized one; meanwhile, inside cells, glutathione is reduced for more than 95%. Glutathione moves into cell walls by means of oligopeptide transporter that carries both reduced and oxidized glutathione and its conjugates [90].

Plant deprivation of GSH by means of a complete supression of synthetases responsible for its synthesis is lethal at the embryonic stage. For example, manifold decrease in a GSH biosynthesis rate in the *Arabidopsis* mutant *rml1* (*rootmeristemless 1*) negatively affects functions of the root apical meristem, formation of lateral roots, and polar transport of auxin. Weaker suppression of the GSH biosynthesis is not usually expressed phenotypically [91]. Increase in GSH level by overexpression of the gene encoding γ-glutamylcystein synthetase, which limits GSH biosynthesis in cells, usually weakly affects plants under normal conditions but strengthens their stress resistance. It is unknown how GSH biosynthesis, mainly occurring in plastids, influences the glutathione status in the apopalst. Therefore, experiments on mutants or transgenic plants with modified activities of γ-glutamylcysteine synthetase and glutathione synthetase do not allow judging about the physiological importance of the apoplastic glutathione.

Plant treatment with exogenous glutathione may elevate the level of this compound in the apoplast. Exogenous GSH usually affords negative effects on plants. It increases ROS generation and inhibits growth. This, for instance, manifests in barley roots submerged in 6–10 mM solution of glutathione [96]. Treatment of *Arabidopsis* seedlings with this substance also causes oxidative stress and growth suppression [99]. Simultaneously, expression of genes involved in ROS detoxification and those necessary to sustain growth (for instance, cell wall biosynthesis) were suppressed.

The glutathione level in the apoplast depends on GGT. In *Arabidopsis*, GGT1 predominates and is the most expressed in rapidly growing seedlings [94]. In developed organs, it is concentrated in conducting tissues, especially, the phloem. In leaves of the *ggt1* mutant, GGT activity is suppressed as strong as 90% [97]. It does not change the glutathione balance of entire cells but several times increases its apoplastic level [32, 97, 100]. The mutant plants have smaller leaves, whose yellowing and senescence occur faster because of the oxidative stress that is associated, in particular, with a higher rate of lipid peroxidation [97]. Presumably, in response to the oxidative stress, the *ggt1* mutant mobilizes protective antioxidant enzymes [37]. Proteomic analysis shows that this effect also spreads over the apoplast where content of SOD and PRX (prx34), which is increased involved in H_2O_2 production, is increased [32]. Necessity of GGT for a normal development is evidenced by an inability of *Arabidopsis* seeds to germinate in the presence of GGT inhibitor—acivicin [94].

Therefore, apoplastic glutathione may affect growth and development of plants. This influence takes place at a level of gene expression. It is suggested that glutathione commits the action by regulation of activities of certain redox-sensitive proteins localized in the apoplast or in the plasma membrane. However, dynamics of oxidized and reduced glutathione in the apoplast of growing cells, as well as targets of its activity, are not yet explored.

CONCLUSIONS

For many decades, the rate of plant cells extension growth has been considered as a function of cell wall extensibility. Obviously, the extensibility is controlled by a secretion together with various biochemical and physicochemical events occurring in walls themselves. The cell wall metabolism is generally catabolic with a predominance of hydrolysis and oxidation of organic molecules. Hydrolytic and physicochemical processes had long remained in the focus of attention. Oxidation has been regarded only as a factor of formation of oxidative cross-links between polymers. This process occurs constantly, and lowering its rate may augment the extensibility that was evidenced for many cases (Fig. 1). These reactions depend on the content of PRXs, H_2O_2, and phenolic substrates: the lower the levels of these compounds, the larger distensibility.

In the late 20th century, the knowledge of the plant cell wall metabolism grew up, and the situation became not so unequivocal. The active growth was discovered to be associated with an intensive ROS formation in cell walls. The direct relation between growth rate and rates of $O_2^{\bullet -}$ and $OH^{\bullet}$ generation seems especially distinct. These ROS are intermediates of the pathway of molecular oxygen reduction. But what reductants are available for that? There is no direct reliable evidence of NAD(P)H existence in cell walls. However, owing to the NAD(P)H oxidase activity, the reduction potential of cytoplasm is recruited for $O_2^{\bullet -}$ production in cell walls (Fig. 1).

It was proven that PRXs produce ROS as well, but the question of reductants used is also open so far [36]. Main PRX substrates are phenolic compounds that quench rather than initiate free radical reactions. Formation of strong oxidants in interactions of certain phenoxyl radicals was discovered just recently [50, 51]. In addition, PRX interactions with NADPH oxidase and SOD in the context of control of redox processes have been discussed in recent years (Fig. 1). Interestingly, this joint activity may result in either excitation or quenching of free radical processes.

Hydrogen peroxide production in cell walls also employs reduction potentials of oxalate and polyamines as substrates of oxalate oxidases and amine oxidases, respectively (Figs. 2 and 3). It is worth noting that all these ROS-producing enzymes are able to both accelerate and retard the growth. On the one hand, if H_2O_2 is consumed in the PRX peroxidase cycle creating oxidative cross-links between polymers, then activities of H_2O_2-yielding enzymes will retard the growth (Fig. 1). However, on the other hand, if H_2O_2 participates in the Haber–Weiss reaction ($O_2^{\bullet -}$ +

$H_2O_2 \rightarrow O_2 + {}^{\cdot}OH + OH^-$) catalyzed by PRX or transition metals (Fe or Cu), then the cell wall extensibility will increase. In this reaction, another reductant available in the cell wall may play a very important role. It is AA that is able to directly reduce Fe^{3+} and Cu^{2+} (Fig. 2) and, consequently, to support the Fenton reaction that promotes the Haber–Weiss reaction. However, the prooxidant role of AA in cell walls is not proven unlike its antioxidant role. It is established that AA inhibits peroxidase-dependent phenols oxidation (Fig. 2) and, thus, prevents lignin synthesis and cross-link formation between polymers.

In recent years, new participants of the redox metabolism of cell walls, glutathione and redox-sensitive proteins, were reported. Their involvement in cell growth-controlling redox signaling is supposed. It is interesting in this regard that cell walls contain not only glutathione but also glutaredoxin and thioredoxin regulating reduction of protein SH-groups. The AA to DHA ratio in the apoplast appears to play a significant role in the redox signaling. Therefore, a great deal of evidence substantiates the potent influence of the apoplastic redox reactions on cell growth rate, but particular mechanisms of this influence are not sufficiently studied. It is unclear, for example, how the pro-/antioxidant activities of the chief members of the redox metabolism is regulated and how redox signals are originated and perceived in cell walls.

ACKNOWLEDGMENTS

This work was supported by the Russian Science Foundation, project no. 16-16-00026.

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Translated by A. Aver'yanov