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RESEARCH PAPERS

Activities of Catalase and Peroxidase in Wheat and Rice Plants under Conditions of Anoxia and Post-Anoxic Aeration

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Abstract—Effects of anoxia and subsequent reaeration on formation of hydrogen peroxide, and activities of catalase (EC 1.11.1.6) and guaiacol peroxidase (EC 1.11.1.7) were studied in wheat and rice plants that are contrasting in their tolerance to hypoxia. It was found that anoxia and reaeration, especially long-term, strongly increased the H_2O_2 production in hypoxia-intolerant wheat plants. Meanwhile, such treatment did not significantly entail peroxide production in rice, which is tolerant to oxygen lack. In these tolerant plants, the activities of catalase, as well as intra- and extracellular guaiacol peroxidases, rapidly increased under anoxia and the following oxidative stress. By contrast, wheat did not display stimulation of catalase activity; activation of its peroxidase occurred only in the shoots after a short anoxia and long reaeration. The increased or stable activities of catalase and peroxidase in the rice seedlings, under anoxia and reaeration, were inhibited by cycloheximide and, to smaller extent, actinomycin D. In rice, the stimulation of catalase activity was accompanied by the increased expression of the corresponding genes, in particular, *OsCatB* and *OsCatC*. The activities of apoplastic peroxidases were suppressed by brefeldin A. Electrophoresis did not reveal the emergence of new isoforms of guaiacol peroxidase under anoxia and post-anoxic aeration. Meanwhile, the activities of the majority of preexisting isoforms in rice and individual isoforms in wheat were considerably stimulated under these conditions. It is suggested that, in the tolerant plants subjected to anoxia and post-anoxic aeration, the activation of apoplastic peroxidases fulfills an adaptive function decomposing hydrogen peroxide in the cell wall and preventing its penetration into the cytosol. Under long-term reaeration, detoxification of ROS in the rice cytosol may be favored by activation of catalase and intracellular peroxidase. Unlike rice, the absence of activation of the antioxidant enzymes in the wheat cell wall allows ROS to penetrate inside the cell where they are deactivated by cytoplasmic forms of guaiacol peroxidase. The efficient work of antioxidant enzymes under anoxia, followed by post-anoxic aeration, prevents ROS accumulation in the tolerant plant and, thus, represents an important adaptation to both lack of oxygen and subsequent oxidative stress.

Keywords: *Triticum aestivum*, *Oryza sativa*, anoxia, reaeration, oxidative stress, hydrogen peroxide, catalase, peroxidase

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INTRODUCTION

Lack of oxygen is a common unfavorable factor impacting both agricultural and wild plants upon their flooding, especially with stagnant water, under asphalt cover in towns, in soil covered with ice crust, in germinating seed, and even in improperly stored fruits and vegetables. Seed potential to germinate in environment deprived of oxygen is fulfilled by an array of morpho-anatomical and physiological-biochemical adaptations of plants that are detailed in several reviews [1–9]. In brief, there are two adaptive strategies in the hypoxia-tolerant plants: active avoidance of oxygen lack (Low Oxygen Escape Syndrome, LOES) and passive quiescent strategy (Low Oxygen Quiescence Syndrome, LOQS) [7–11]. The first strategy includes (1) rapid growth of the shoots, hyponastic bend of the leaves, formation of the aerenchyma, emergence of the adventitious roots, rearrangement of leaf anatomy, and appearance of gaseous film on their hydrophobic surface to improve gas diffusion, etc. Owing to these adaptations, mainly morphoanatomical, the above-ground part of the plant transports oxygen to the submerged roots maintaining their metabolism. The second strategy (LOQS) is associated with growth retardation and primarily acts as a metabolic adaptation. Its constituents are (2) the efficient providing of the plant organism with energy due to storage of carbohy-

drates, intensification of glycolysis and fermentation, and employing alternative respiratory substrates; (3) detoxification of poisonous metabolites of the anaerobic metabolism through their removal or reoxidation of reduced coenzymes with the help of alternative metabolic pathways; and (4) sustaining of stability of the tissues (mainly meristem), cells, and subcellular membrane structures (mitochondria and plastids) [1–5, 9]. These adaptations allow tolerant plants to obtain energy in amounts sufficient for vital activity, to support the water–mineral balance, and to grow even in the absolute absence of free molecular oxygen.

As a matter of fact, a transfer of a plant from an oxygen-deficient medium is fraught with more danger. Hypoxic (low oxygen) or anoxic (zero oxygen) conditions are tightly coupled with oxidative stress. It emerges already in anaerobic environment and maximally increases upon returning of the plants to normal aeration. The lack of oxygen entails accumulation of reduced substrates that are thereafter oxidized by air oxygen to yield reactive oxygen species (ROS). Post-anoxic damages also develop and can lead to plant death [2, 10, 11]. In nature, oxidative stress is often a direct consequence of hypoxia and anoxia. The post-anoxic damage was found in German bearded iris (*Iris germanica*) [2, 12], rice (*Oryza sativa*) [13], wheat (*Triticum aestivum*), oat (*Avena sativa*) [12, 13], soybean (*Glycine max*) [14], yellow lupine (*Lupinus luteus*) [15], and other plants. Nevertheless, plant specimens differently tolerant to oxygen deficiency are very seldom compared as to degree of their damage with this factor. We earlier revealed that lipids and proteins of wheat are more sensitive to oxidative damage than those of rice [13, 16]. Post-anoxic reaeration elevates the levels of ROS [14, 15, 17–20] and free radicals [21] in the plants.

To control an adverse action of the oxidative stress, plants possess the multilevel system protecting from the ROS-originated damage. The essential part of the antioxidant system is composed of the enzymes preventing the formation of ROS or decomposing them. Among these enzymes, various peroxidases play the chief role.

Catalase (EC 1.11.1.6) accomplishes a disproportionation of two molecules of hydrogen peroxide yielding water and free molecular oxygen. This enzyme does not require auxiliary substrates and carries out an oxidation of one H_2O_2 molecule by oxygen of another H_2O_2 molecule. A major part of the enzyme is situated in the peroxisomes, where it is responsible for antioxidant protection, including that related to photorespiration. Its role is notably important under the action of adverse environmental factors. It is reported that reaeration stimulates catalase activity in the plants of yellow lupine [15, 21], Indian lotus (*Nelumbo nucifera*) [22], *Arabidopsis thaliana* [19] and rice [23]. The action of post-anoxic oxidative stress was compared by Monk et al. [24] in the plants contrasting in their tolerance to hypoxia. It is revealed that reaeration significantly increases catalase activity in the rhizomes of the less tolerant plants. Meanwhile, there was no published comparison between wheat and rice concerning the changes in the enzymatic activity in the roots and green shoots of these plants.

Guaiacol peroxidase (class III secreted peroxidase EC 1.11.1.7) catalyzes oxidation of various chemical compounds by oxygen of hydrogen peroxide [25]. It is involved in phenolic metabolism and cell wall lignification. The adaptive role of peroxidases is essential under harsh conditions, in particular, pathogenesis. Besides, many of their isoforms participate in oxidation of auxin; hence, this enzymatic activity is related to regulation of growth processes. Antioxidant function of guaiacol peroxidase consists in detoxification of hydrogen peroxide as one of ROS. The enzyme is localized in the vacuoles, the system of vesicular transport, and the cell wall [26].

Hypoxia and anoxia are known to stimulate peroxidase activity and bring about appearance of its new isoforms [20, 27–29]. The activation of the enzyme follows from with the necessity of utilization of the excessive products of anaerobic metabolism, including phenolic compounds. The involvement of guaiacol peroxidase in the process of H_2O_2 decomposition is reported for the soybean [14] and *A. thaliana* [19] cell cultures under post-anoxic aeration. Meanwhile, peroxidase does not significantly contribute to the ROS detoxification under reoxygenation of lupine roots [21]. Similarly, this enzyme is not stimulated by hypoxia in cowpea (*Vigna unguiculata*) [30]. Furthermore, the activity of guaiacol peroxidase even decreases in the hypoxic rice seedling shoots, which may be related to the growth acceleration of these organs [31].

Therefore, the literary data on the influence of oxygen lack on activities of plant catalase and guaiacol peroxidase are scarce and ambiguous. Our experiments with the plant species contrasting in their tolerance to oxygen deficiency revealed the peroxidase activation caused by anoxia [1, 29] that occurred to a larger extent in the apoplast of the root cells of the tolerant plants [32]. However, these studies did not probe into the role of guaiacol peroxidase as an antioxidant enzyme during the post-anoxic period and did not compare its activity and accumulation of hydrogen peroxide.

The goal of the present work was to compare the effects of anoxia and post-anoxic oxidative stress on the production of hydrogen peroxide and the activities of catalase and guaiacol peroxidase in the plants of wheat and rice contrasting with each other in their tolerance to oxygen shortage.

MATERIALS AND METHODS

Plant material and growth conditions. 7-day-old wheat (*Triticum aestivum* L., cv. Leningradka) seedlings were used as hypoxia-sensitive plants. Hypoxia-

tolerant plants were represented by 10-day-old rice (*Oryza sativa* L., cv. Liman) seedlings. The seeds were surface-sterilized with 5% sodium hypochlorite and were grown in hydroponic culture described earlier [33]. Anaerobic conditions were created by passing gaseous nitrogen (with less than 0.01% oxygen impurity) through growth chambers with the plants. Thereupon, the chambers were hermetically sealed and were transferred to the dark to prevent photosynthetically originated oxygen formation. The anaerobic conditions were verified with an Anaerotest indicator (Merck, Germany). The treated plants were exposed to nitrogen atmosphere for 12, 24, or 72 h. The control plants were normally aerated in the dark for the same time.

For reaeration of the anaerobically-treated plants, they were taken from the chambers and were left in air in the darkness for 1, 12, or 24 h after each of three exposures in gaseous nitrogen. Hydrogen peroxide was assayed after shorter reaeration—5, 15, 30, or 60 min. Relative levels of transcripts of catalase genes were assessed after the reaeration for 1 or 24 h.

Hydrogen peroxide was analyzed spectrophotometrically with xylenol orange [34]. The rate of H_2O_2 production was derived from the changes in A_{560} that were measured with an SP-26 spectrophotometer (LOMO, Russia) calibrated with standard solutions of hydrogen peroxide. The specificity of the analysis was validated by inhibition of the peroxide production by the addition of catalase at a final concentration 500 units/mL.

Preparation of apoplastic fluid (to measure the activity of cell wall guaiacol peroxidase). Plant material (1 g) was rinsed with distilled water, dried on a filter paper, and cut into 30-mm in length pieces which were vacuum-infiltrated for 20 min at –70 kPa in 0.05 M MES-buffer (pH 6.0) containing 1 M NaCl [35]. The ratio of tissue : buffer was 1 : 10 (w/v). After the infiltration, the plant material was dried and centrifuged at 1000*g* for 15 min over a net. This preparation brought about 180–250 μL of apolastic fluid, in which the activity and isoenzyme composition of guaiacol peroxidase were determined. A possible contamination of the resultant apoplastic fraction with cytoplasmic proteins was tested by analyzing glucose-6-phosphate dehydrogenase as a marker cytosolic enzyme. In all cases, its activity was not revealed in the examined fractions.

Extraction of symplastic guaiacol peroxidase and catalase. All preparative procedures were carried out at the temperature from zero to +4°C. The plant tissue remaining after the centrifugation of the infiltrate (for guaiacol peroxidase) or the 1 g sample of the plant material (for catalase) was homogenized and then was extracted with cold 0.02 M sodium-phosphate buffer, pH 6.5, for 30 min at a 1 : 10 ratio, w/v. After centrifugation at 5500*g* for 15 min, the supernatant was collected. The sediment was resuspended in a half of an initial buffer volume followed by new 20-min extraction and centrifugation. The sediment was washed again. In the combined fraction, the activities of cytoplasmic enzymes were analyzed together with an isoenzyme spectrum of guaiacol peroxidase.

Content of protein in the extracts was assayed according to Bradford [36].

Activity of catalase was determined in a medium of 0.02 M K, Na-phosphate buffer (pH 6.5), and 40 μL of the enzymatic extract containing 7 to 25 μg protein. The reaction was initiated by the addition of 10 mM H_2O_2 (or water in the control) to the final volume of 3 mL. The optical density at 240 nm was measured every 20 s for 2 min using a SP-46 spectrophotometer (LOMO). The enzymatic activity was expressed as a number of micromoles of peroxide decomposed over 1 min per 1 g of tissue fresh weight. The extinction coefficient 39.4 mM^{-1} cm^{-1} was used for hydrogen peroxide [37].

Activity of guaiacol peroxidase was measured using guaiacol, which is converted into tetraguaiacol in peroxidase reaction. The reaction mixture consisted of 0.5 mL 0.05 M guaiacol (Sigma, United States), 0.5 mL 0.03% H_2O_2, and 25 μL of the enzymatic extract containing 7–25 μg protein in 0.02 M phosphate-citrate (pH 5.0 for the apoplastic fraction) or 0.02 M K, Na-phosphate (pH 6.5 for intracellular fraction) buffer. The final volume of the mixture was 3 mL. The reaction was started by the addition of hydrogen peroxide. The optical density at 470 nm was read over 2 min with 20-s intervals using a SP-26 spectrophotometer. The enzymatic activity was expressed as a rate of guaiacol oxidation, namely, the number of its micromoles per 1 g FW per 1 min. The extinction coefficient 26.6 mM^{-1} cm^{-1} was used for tetraguaiacol.

Isoenzyme spectrum of peroxidases was analyzed by native electrophoresis in plates with polyacrylamide gel in an acidic buffer system [38]. The separation was performed in Mini-PROTEAN chambers with a PowerPac Universal current source (Bio-Rad Laboratories, United States) at the voltage 300 mV for 40 min. After the separation, the gels were washed in 0.2 M acetate buffer (pH 5.0) three times for 10 min each. Peroxidases were developed in a medium of 35 mM guaiacol and 3.3 mM H_2O_2 in 0.1 M acetate buffer, pH 5.0. The developed gels were scanned on a HP ScanJet 2000C scanner (Hewlett-Packard, United States). The obtained images were analyzed using the ImageJ 1.53k software. The densitograms of the electrophoretic lanes were digitized, and the intensity of spot coloration was evaluated. The plots of the Supplementary Information (Figs. 1, 2) represent means of three replications.

Inhibitory analysis. To elucidate possible mechanisms controlling activities of the examined enzymes, the plants were treated with the inhibitors of transcription (6×10^{-5} M actinomycin D, Reanal, Hungary), translation (3×10^{-5} M cycloheximide, Sigma), or vesicular transport (7×10^{-5} M brefeldin A, Sigma).

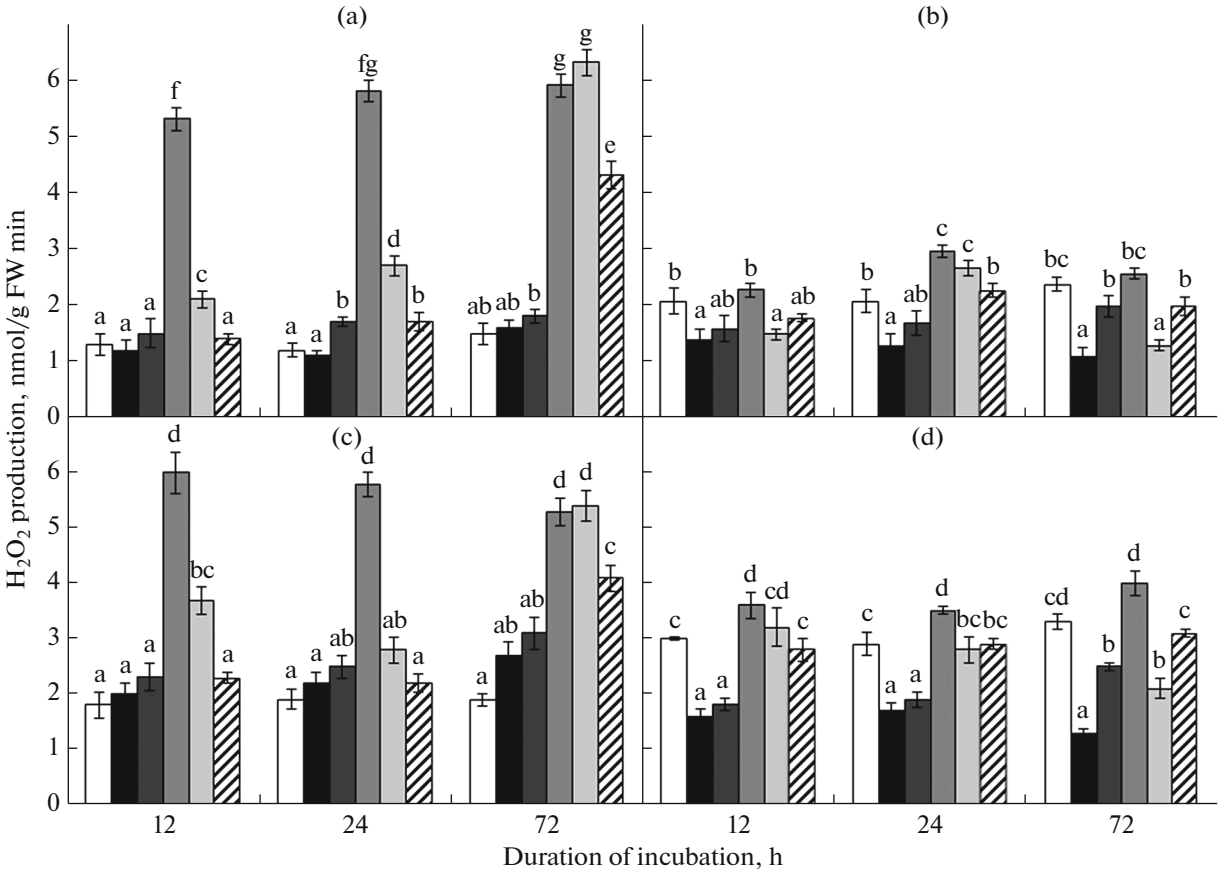

Fig. 1. Production of hydrogen peroxide in the (a, b) shoots and (c, d) roots of the (a, c) wheat and (b, d) rice seedlings incubated under anoxia followed by reaeration. Conditions of incubation: □—aeration; ■—anoxia; ■—5-min reaeration; ■—15-min reaeration; ■—30-min reaeration; ▨—60-min reaeration. The means significantly different at $P < 0.05$ according to LSD test are indicated with different letters.

Before the experiments, the plants were incubated for 3 h in beakers containing the inhibitors dissolved in 20 mL of 20% Knop's solution.

Design of primers for catalase genes. Expression of catalase genes was studied in rice seedlings, because only this plant species exhibited the rise in this enzymatic activity. The class III peroxidases (guaiacol peroxidase) of rice are encoded with 125–160 ORF, and the study of their expression requires special transcriptomic exploration. To design the appropriate primers, the electronic databases of the annotated rice genome (Rice Genome Annotation Project, http://rice.plantbiology.msu.edu/, and The Rice Annotation Project Database, http://rapdb.dna.affrc.go.jp/tools/search/) were analyzed. In addition, the rice reference genome IRGSP-1.0 of *Oryza sativa* var. *japonica*, cv. Nipponbare, available at http://www.ncbi.nlm.nih.gov/, was considered. The nucleotide sequences of the three genes encoding catalase were found. The coding sequences of DNA (CDS), determining the variants of alternative splicing (isoforms) of one type of the enzymes (Cat A and B, correspondingly), were studied. By means of the program MegAlign 5.05 from the DNAStar software package, all CDSs of the corresponding isoforms were aligned through the ClustalW algorithm. In the program Beacon Designer 8, the primers were designed for the consensus regions (Table 1). Therefore, the primers we worked out allow for assessing expression of any variants of alternative splicing on the examined genes. The *OsTUB4* (Os01g0805900) gene, which encodes beta-4-tubulin, was used as a reference. In preliminary experiments, it demonstrated the most stable expression. The specificity of the primers was tested by means of searching homologies with the rice genome and transcriptome using the BLASTn algorithm in the site of the NCBI database (http://blast.ncbi.nlm.nih.gov/). The primers were purchased from the Beagle company (Russia, http:// www.biobeagle.com/).

For isolation of RNA and synthesis of cDNA, rice seedlings were used. The samples of shoots (150 mg) or roots (200 mg) were frozen in liquid nitrogen. Thereafter, they were disrupted three times for 1 min at 50 strikes per second in a Tissue Lyser LT bead mill

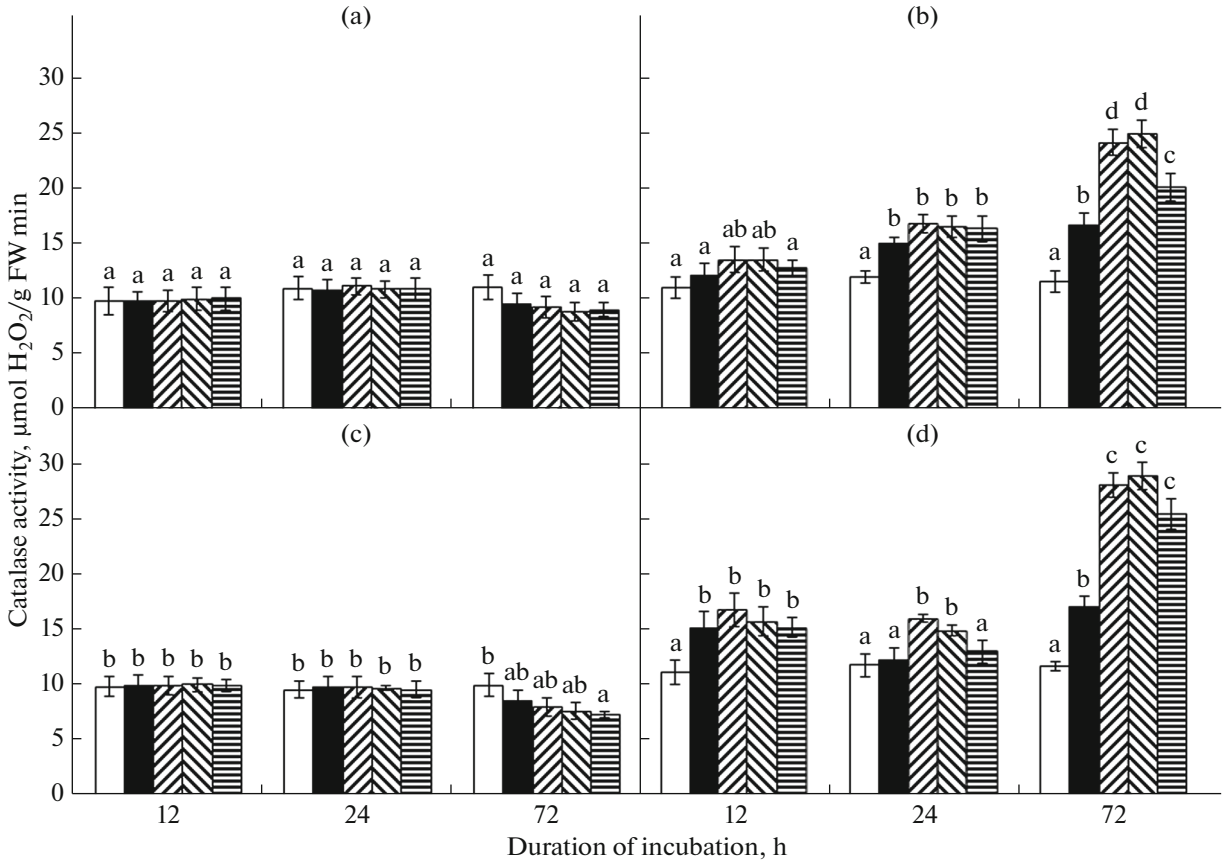

Fig. 2. Activity of catalase in the (a, b) shoots and (c, d) roots of the (a, c) wheat and (b, d) rice seedlings incubated under anoxia followed by reaeration. Conditions of incubation: □—aeration; ■—anoxia; ▨—1-h reaeration; ▧—12-h reaeration; ▤—24-h reaeration. The means significantly different at $P < 0.05$ according to LSD test are indicated with different letters.

(Qiagen, Germany). One mL of PureZOL RNA-isolation reagent (Bio-Rad Laboratories) was added to the homogenate followed by further isolation according to the manual of the manufacturer. The isolated RNA was separated from DNA impurity using DNase I (Thermo Fisher Scientific, United States). The quality of the treatment with DNase was tested with PCR using RNA as a matrix followed by electrophoretic separation of the products in 1% agarose gel in TAE buffer at 120 V for 40 min with a subsequent staining with 0.5 μg/mL ethidium bromide. None of polymerization products with RNA was found with any used pairs of primers. The RNA concentration was measured using a NanoDrop 1000 spectrophotometer

Table 1. Primers for the rice genes encoding catalases and beta-4-tubulin

Gene	RAP-DB	NCBI	Primers (5'-3')	Product length, bp	Protein location
OsCatA	Os02g0115700	LOC4328073	GGAGGCAGAAGGCGACGATACA AACGACTCATCACACTGGGAGAGG	187	peroxisomes glyoxysomes mitochondria
OsCatB	Os06g0727200	LOC4342124	CAAGGAGAACAATTTCCAACAGGC CACTGCGACCAGTAGGAGATCCAG	153	peroxisomes
OsCatC	Os03g0131200	LOC4331509	GTGATTGCCAAGGAGAACAACTTCA GCCAGTTTCTGACCCAGAGACCTG	188	peroxisomes
OsTUB4	Os01g0805900	LOC4327550	GAACCATTTGATTTCTGCCACCA CGGTACTGCTGGGAGCCACG	171	tubulin-beta 4 reference gene

(Thermo Fisher Scientific). After the measurement, the samples were aligned to the RNA concentration 2.5 μg/mL that was necessary for quantitative analysis of the expression.

Complementary DNA (cDNA) was synthesized by means of a RevertAid Reverse Transcriptase kit (Thermo Fisher Scientific) using a RiboLock inhibitor of RNases (Thermo Fisher Scientific). All manipulations were strictly corresponded to the protocol of the manufacturer. Synthesis of cDNA was carried out with Oligo(dT) primers (Beagle, Russia). The cDNA samples were precipitated with 0.1 M sodium acetate in ethanol, dissolved in sterile deionized water, and divided into aliquots for storage at −80°C before analysis.

Quantitative real-time PCR was performed in 12.5 μL of a reaction mixture containing 50 ng cDNA, forward and reverse primers (F and R, 10 pmol each, Table 1), 0.75 μL $MgCl_2$, and 5 μL of 2.5× SYBRGreen reaction mixture (Sintol, Russia, http://www.syntol.ru). The total mixture was adjusted to a desired volume with sterile bidistilled water taken from the kit. Each reaction was carried out in three analytic replications. A sample without cDNA was a control for each primer. Real-time PCR was conducted in a C1000 thermal cycler equipped with a CFX96 optical module (Bio-Rad Laboratories). The hardware of the Research Park of the Center for Molecular and Cell Technologies (St. Petersburg State University) was used. The amplification program included 45 cycles: melting at 95°C for 15 s, annealing at 60°C for 15 s, and elongation at 72°C for 30 s. The start of amplification was preceded by 7-min incubation at 95°C. Before the finish of amplification, the melting curves for the products were obtained during progressive heating from 65 to 95°C. Relative amounts of transcripts were derived from a threshold cycle of amplification (Ct) by the $2^{-\Delta Ct}$ method. The degree of changes in relative amounts of transcripts of each gene, the $2^{-\Delta\Delta Ct}$ method was used [39]. The changes in the expression level were normalized to the control (normoxia) values.

Statistics. All experiments contained four to eight biological replications. The data were statistically processed using the GraphPad Prism 5 software for Windows. The graphs (Figs. 1–5) represent means and their SEs. Different letters indicate means that significantly differ at $P < 0.05$ according to Least Significant Difference Test. In Tables 2–4, the data of inhibitory analysis are given as a percentage of a corresponding inhibitor-free control; means and their SEs are reported. Asterisks show statistically significant differences from the control at $P < 0.05$ according to Wilcoxon's test.

RESULTS

Rice plants, especially their roots, more intensively produced hydrogen peroxide than wheat plants (Fig. 1). This parameter was not significantly affected by anoxia in the wheat seedlings. By contrast, anoxia decreased the rate of the peroxide formation in rice. Reoxygenation intensified the H_2O_2 production in both plant species. In the wheat seedlings deprived of oxygen for 12 or 24 h, either shoots or roots manifested almost the fivefold enhancement in the H_2O_2 production after 15-min contact with air; afterwards, this index decreased down to the control level (Figs. 1a, 1b). After 72 h of anoxic exposure followed by not only 15 but also 30 min of reaeration, the rate of peroxide formation remained high and did not decrease down to the initial level even after 1 h contact with air. In the reaerated shoots and roots of rice, the hydrogen peroxide production did not exceed the control values so much as in wheat. This process increased stronger in the rice roots in comparison with shoots (Fig. 1d). In both plant species, the peroxide production was maximal after 15 min of reaeration preceded by anoxia of any duration.

Catalase is one of the essential enzymes detoxifying hydrogen peroxide. We found that its initial level was nearly the same in wheat and rice plants (Fig. 2). Short-term anoxia and the subsequent reaeration did not change its activity in the wheat seedlings, but long (72 h) anoxia followed by 24 h of reoxygenation diminished this parameter (Figs. 2a, 2b). In rice, catalase, on the contrary, was activated after 12 h of anoxia in the roots and 24 h of anoxia in the shoots. Deprivation of oxygen for 72 h stimulated the enzymatic activity in both organs (Figs. 2c, 2d). During reaeration of the rice seedlings subjected to any anoxic exposure, their catalase activity exceeded the control level. Maximal (by two to three times) activation of catalase occurred in the reaerated rice seedlings after their 72 h of anoxia. For this reason, effects of inhibitors on the activity of rice catalase were tested under these particular conditions. To compare, the inhibitory analysis included the measurements on wheat whose activity, on the contrary, decreased. The inhibitors of transcription (actinomycin D) or translation (cycloheximide) were applied to the plants. Pretreatment of wheat seedlings with actinomycin did not entail any pronounced changes in the activity, while cycloheximide inhibited it after 12–24 h of reaeration (Table 2). The catalase activity of rice was more strongly suppressed by the inhibitors, and cycloheximide was also more efficient. The strongest suppression was observed after 12 h of reaeration (Table 2). In this regard, we studied the expression of the genes encoding catalases. It was found that, before the experiment, in the shoots of 10-day-old seedlings, the *OsCatA* gene was mainly expressed (80% of total expression of catalase genes was estimated against the reference *OsTUB4* gene). In the roots, the *OsCatB* gene was expressed with the same predominance. The expression of the *OsCatC* did not exceed 5% in the shoot and 1% in the root. In the rice seedlings, short-term (12 h) anoxia hardly influenced the expression of the catalase genes,

Table 2. Effects of inhibitors of transcription and translation on the catalase activity in the wheat and rice seedlings incubated under anoxia followed by reaeration

Conditions of incubation	Wheat			
	shoot		root	
	actinomycin	cycloheximide	actinomycin	cycloheximide
Anoxia	99.5 ± 7.3	92.1 ± 10.1	98.4 ± 11.3	91.9 ± 11.2
1 h reaeration	98.9 ± 7.0	92.2 ± 9.1	96.8 ± 10.5	83.4 ± 9.1
12 h reaeration	96.8 ± 6.0	81.6 ± 7.2*	98.0 ± 9.9	83.1 ± 8.4
24 h reaeration	97.7 ± 7.1	68.4 ± 7.6*	98.0 ± 3.9	71.3 ± 13.1*
	Rice			
	shoot		root	
	actinomycin	cycloheximide	actinomycin	cycloheximide
Anoxia	97.0 ± 7.4	73.6 ± 6.7*	95.9 ± 6.4	72.6 ± 7.7*
1 h reaeration	87.7 ± 4.0*	62.1 ± 4.8*	75.1 ± 2.5*	58.9 ± 4.1*
12 h reaeration	83.6 ± 2.4*	59.6 ± 3.9*	72.4 ± 2.7*	55.2 ± 4.0*
24 h reaeration	91.5 ± 5.6	72.7 ± 3.4*	79.0 ± 6.4*	62.4 ± 6.0*

The activity values are represented as a percentage of the counterpart without inhibitors. Asterisks indicate statistically significant difference from the control estimated with Wilcoxon's test at $P \leq 0.05$.

while reaeration stimulated accumulation of the transcripts of all three genes; this happened to *OsCatB* and *OsCatC* after 1 h but to *OsCatA* only after 24 h (Figs. 3a–3c). After the longer anoxia (24 and 72 h), the *OsCatA* expression was diminished under oxygen-free conditions and were restored only after 24 h of reaeration in the rice shoots. By contrast, the *OsCatB* and *OsCatC* expressions were stimulated under anoxia and remained active under reaeration. In the rice roots, the short-term (12 h) anoxia stimulated the transcript accumulation of all catalase genes. The process remained highly active also during reaeration and, similarly to the shoots, the maximal accumulation of mRNA of the *OsCatB* and *OsCatC* occurred after 1 h of post-anoxia, and that of the *OsCatA* after 24 h (Figs. 3d, 3e). In the rice roots, as in the shoots, long-term anoxic exposure inactivated the expression of the *OsCatA* but stimulated those of the *OsCatB* and *OsCatC* so that the changes in the catalase gene expression in the root were almost entirely associated with the *OsCatB* all the time of the stressor's action. Under reaeration, the *OsCatA* transcript level approached to the control values, while those of the *OsCatB* and *OsCatC* were still above the control. Therefore, all the catalase genes were found to be sensitive to anoxia and post-anoxic oxidative stress.

Guaiacol peroxidase is one of the most common enzymes in the symplast and apoplast of plant cells. It catalyzes oxidation of phenolic compounds by hydrogen peroxide. Its catalytic activity is brought into action upon simultaneous binding of two phenolic molecules and one H_2O_2 molecule in the active center of the enzyme. The apoplastic form of peroxidase first encounters with extracellular ROS and, therefore, special attention is paid to it in our study. We found that the activity of cell-wall peroxidase was almost the same in the initial control plants of both species (Fig. 4). In wheat, the activity of the enzyme decreased over the first 12 h of anaerobiosis in either shoots or roots. Under 24 h of anoxia, the activity was still low in the shoots and almost invariable in the roots as in both organs under 72 h of anoxia (Figs. 4a, 4c). In rice, the activity of guaiacol peroxidase was suppressed in the shoots only during short-term (12 h) anoxic exposure. In the roots, the enzymatic activity even rose, especially, under 24 and 72 h of exposure (Figs. 4b, 4d). In wheat, certain activation of apoplastic guaiacol peroxidase occurred in both shoots and roots during post-anoxic reoxygenation, most of all, after 12 h of contact with air (Figs. 4a, 4c). Nevertheless, the activation was far less pronounced than that in rice (Figs. 4b, 4d), in which the apoplastic guaiacol peroxidase was activated as early as the first hours of reoxygenation and was maximal in the shoots after long-term anoxia (Fig. 4d).

The symplastic form of guaiacol peroxidase was much more active than apoplastic one in either plant species, in particular, in their roots (Fig. 5). As a result of anaerobiosis, the enzymatic activity decreased in the seedlings of both plants (to larger extent in the shoots). Restoration of oxygen supply stimulated this enzyme in wheat stronger than in rice (Fig. 5a). In wheat, the maximal activation of intracellular peroxidase was found in the shoots after 12 to 24 h of reaeration preceded by the shortest (12 h) incubation in an oxygen-free environment. In rice, the activity of the symplastic form of the enzyme attained maximum after 24 h of normal aeration in all cases (Figs. 5b, 5d).

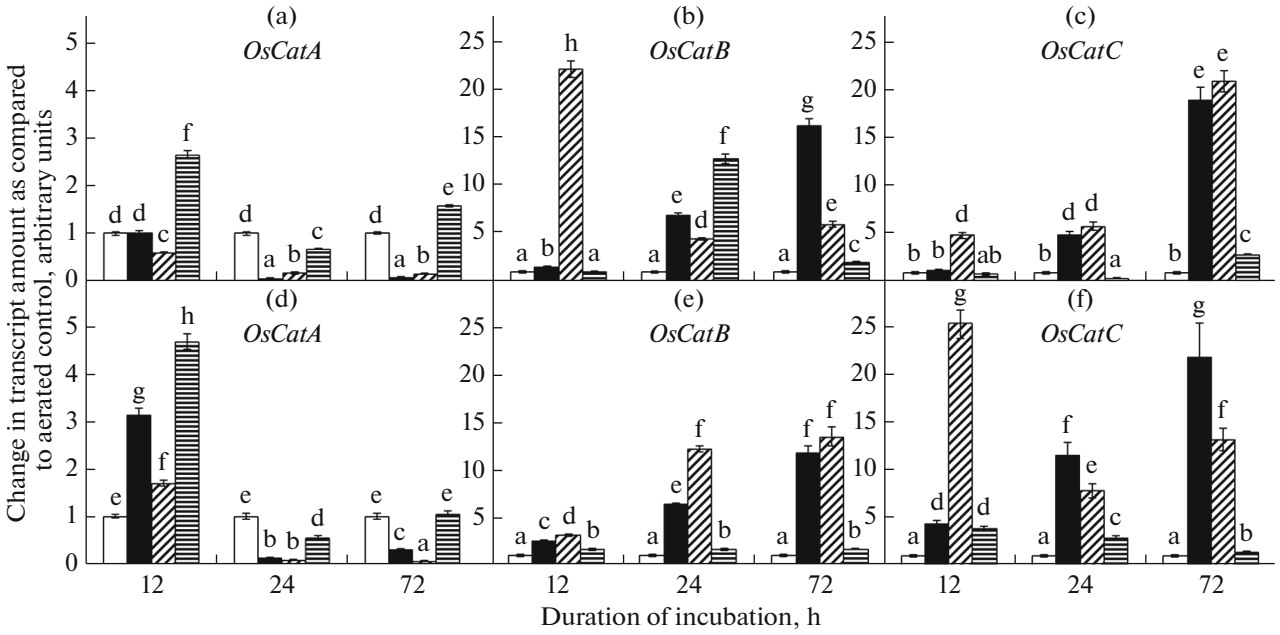

Fig. 3. Relative level of transcription of the (a, d) *OsCatA*, (b, e) *OsCatB*, and (c, f) *OsCatC* catalase genes in the (a–c) shoots and (d–f) roots of the rice seedlings incubated under anoxia followed by reaeration. The expression level in the control (aeration) is defined as one unit. Conditions of incubation: □—aeration; ■—anoxia; ▨—1-h reaeration; ▤—24-h reaeration. The means significantly different at $P < 0.05$ according to LSD test are indicated with different letters.

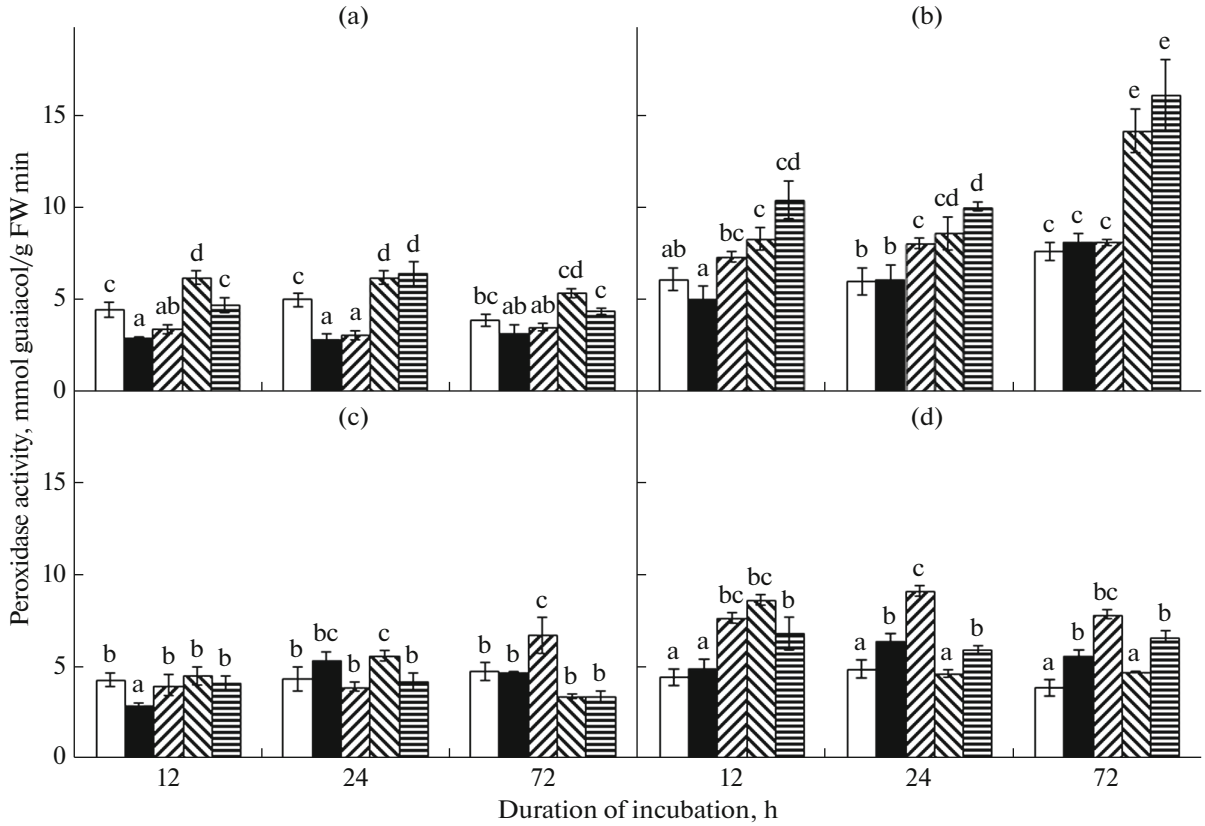

Fig. 4. Activity of apoplastic peroxidase in the (a, b) shoots and (c, d) roots of the (a, c) wheat and (b, d) rice seedlings incubated under anoxia followed by reaeration. Conditions of incubation: □—aeration; ■—anoxia; ▨—1-h reaeration; ▧—12-h reaeration; ▤—24-h reaeration. The means significantly different at $P < 0.05$ according to LSD test are indicated with different letters.

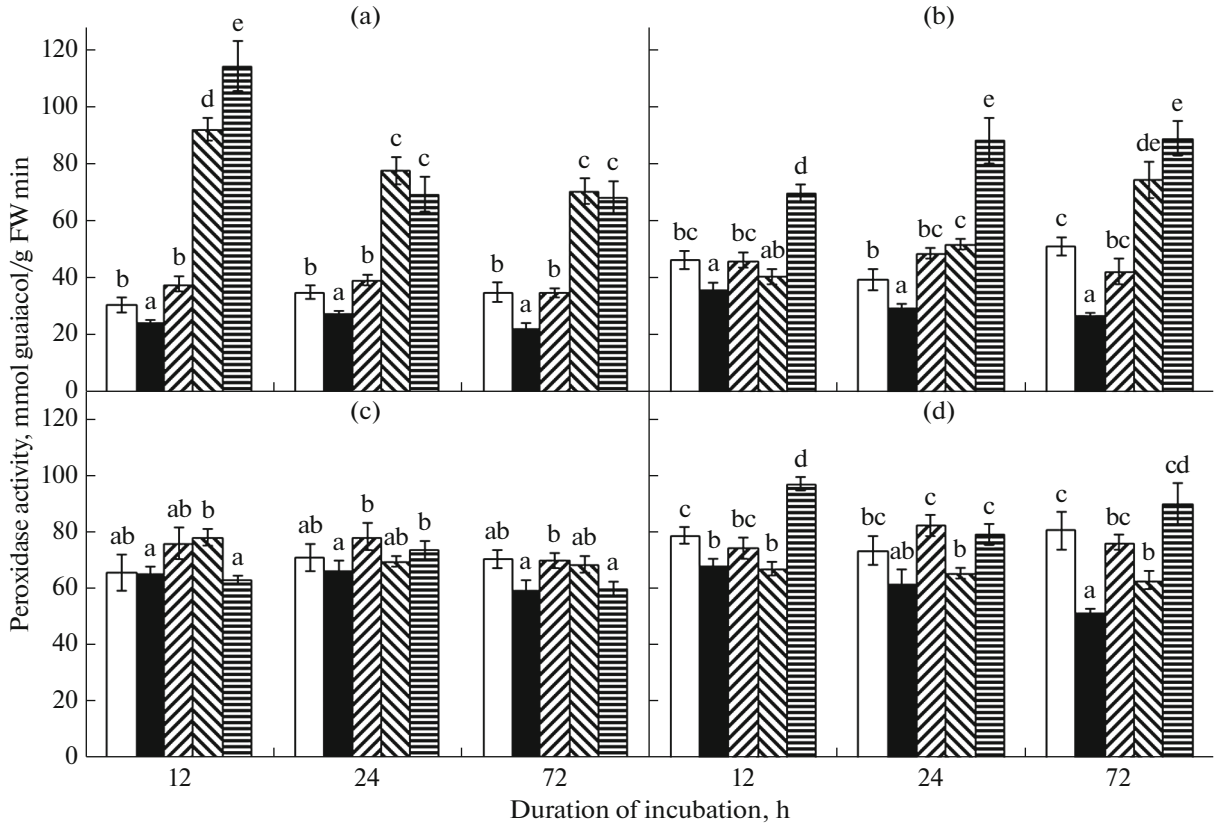

Fig. 5. Activity of intracellular peroxidase in the (a, b) shoots and (c, d) roots of the (a, c) wheat and (b, d) rice seedlings incubated under anoxia followed by reaeration. Conditions of incubation: □—aeration; ■—anoxia; ▨—1-h reaeration; ▧—12-h reaeration; ▤—24-h reaeration. The means significantly different at $P < 0.05$ according to LSD test are indicated with different letters.

Since the experiments have shown the activation of the enzyme in the apoplast of rice and symplast of both plant species under post-anoxic aeration, it was interesting to elucidate whether this activation is related to the processes of biosynthesis of mRNA and protein of the enzyme. In addition, we examined the effect of an inhibitor of vesicular transport (brefeldin A) on the rate of reaction of guaiacol peroxidase in the apoplast of the plant cells.

It was rather difficult to select the optimal duration of anoxia to assess the effects of inhibitors on the activity of guaiacol peroxidase. In fact, there were no considerable differences in the enzyme activation between different times of anoxia. However, the activity of the enzyme increased only in the apoplast of rice roots after 24 and 72 h of anoxia and hardly changed after 12 h of anoxia. Nevertheless, only 12 h of anoxia led to activation of both apoplastic and intracellular guaiacol peroxidases during subsequent reaeration. In the wheat seedlings, the activation of intracellular enzyme occurred under reaeration only after 12 h of anoxia and decreased after 24 and 72 h of exposure. Therefore, the regimen of 12-h anoxia followed by reaeration was selected to test effects of inhibitors on the activities of guaiacol peroxidases in both wheat and rice. Actinomycin D was found to be almost indifferent towards either apoplastic or symplastic enzymes during the post-anoxic period in both plant species. As an exception, the rice seedlings manifested significantly decreased activity of the cell-wall enzyme in the roots after 12-h reaeration (Table 3) that also happened to the symplastic form in both shoots and roots after 24 h of reaeration (Table 4). Application of cycloheximide inhibited both forms of peroxidase (apoplastic form to larger extent) in the tolerant plant (Tables 3, 4). Brefeldin A also reduced activation of the apoplastic enzymes in both plant species (Table 3).

Since class III peroxidases of rice (guaiacol peroxidases) are encoded by approximately 150 ORF, investigation of their expression requires a separate transcriptomic study. Here, native electrophoresis of peroxidases was carried out. In rice, six apoplastic isoforms were revealed in the shoot and five in the root; five and three intracellular isoenzymes were found, respectively, in the shoots and roots. In wheat, five isoforms of cell wall peroxidase were found in the shoot and three in the root; respectively, five and two intracellular isoforms were found in the shoot and the root (Fig. 6). In general, rice possessed more isoform types and they, especially the cell wall enzymes, pro-

Table 3. Effects of inhibitors of transcription, translation, and vesicular secretion on the activity of apoplastic peroxidase in the wheat and rice seedlings incubated under anoxia followed by reaeration

Conditions of incubation	Wheat					
	shoot			root		
	actinomycin	cycloheximide	brefeldin	actinomycin	cycloheximide	brefeldin
Anoxia	95.0 ± 5.4	79.3 ± 9.6	100.0 ± 8.1	96.8 ± 4.0	133.8 ± 18.5	96.0 ± 6.2
1 h reaeration	107.6 ± 9.0	119.4 ± 12.9	71.6 ± 6.2*	99.5 ± 9.8	104.2 ± 3.2	90.2 ± 2.2*
12 h reaeration	98.6 ± 2.3	96.1 ± 0.2*	47.1 ± 4.2*	94.9 ± 7.1	106.0 ± 2.4	78.5 ± 4.2*
24 h reaeration	96.0 ± 7.0	102.8 ± 6.8	51.4 ± 4.2*	103.3 ± 9.0	91.0 ± 7.5	97.2 ± 6.2
	Rice					
	shoot			root		
	actinomycin	cycloheximide	brefeldin	actinomycin	cycloheximide	brefeldin
Anoxia	99.2 ± 8.3	96.8 ± 9.5	94.7 ± 6.2	94.1 ± 4.3	76.9 ± 10.2*	76.5 ± 7.4*
1 h reaeration	98.7 ± 3.9	91.6 ± 2.4*	72.0 ± 3.9*	94.4 ± 7.0	66.3 ± 6.4*	64.9 ± 7.8*
12 h reaeration	95.6 ± 5.0	77.2 ± 5.3*	62.9 ± 4.3*	69.7 ± 0.7*	48.1 ± 4.6*	47.6 ± 2.7*
24 h reaeration	93.0 ± 5.4	73.0 ± 7.7*	57.9 ± 4.5*	98.4 ± 1.6	68.9 ± 5.6*	67.7 ± 5.6*

The activity values are represented as a percentage of the counterpart without inhibitors. Asterisks indicate statistically significant difference from the control estimated with Wilcoxon's test at $P \leq 0.05$.

Table 4. Effects of inhibitors of transcription and translation on the activity of symplastic peroxidase in the wheat and rice seedlings incubated under anoxia followed by reaeration

Conditions of incubation	Wheat			
	shoot		root	
	actinomycin	cycloheximide	actinomycin	cycloheximide
Anoxia	112.8 ± 5.0	136.5 ± 19.1	96.8 ± 2.1	104.9 ± 14.7
1 h reaeration	92.2 ± 6.1	116.2 ± 11.5	98.8 ± 4.0	117.4 ± 3.4
12 h reaeration	98.2 ± 2.3	92.5 ± 2.4 *	97.4 ± 3.3	102.2 ± 3.8
24 h reaeration	95.4 ± 9.3	88.4 ± 5.7	96.6 ± 9.7	108.6 ± 7.1
	Rice			
	shoot		root	
	actinomycin	cycloheximide	actinomycin	cycloheximide
Anoxia	99.0 ± 13.8	69.4 ± 8.0 *	92.6 ± 5.2	106.3 ± 6.3
1 h reaeration	92.7 ± 11.9	89.3 ± 7.3	104.6 ± 7.5	96.0 ± 4.5
12 h reaeration	95.0 ± 7.8	91.5 ± 8.2	99.9 ± 3.2	90.9 ± 3.5*
24 h reaeration	67.4 ± 8.9*	51.9 ± 5.2 *	57.0 ± 3.5 *	90.4 ± 7.3

The activity values are represented as a percentage of the counterpart without inhibitors. Asterisks indicate statistically significant difference from the control estimated with Wilcoxon's test at $P \leq 0.05$.

duced more intense staining. The apoplast of both plant species comprised more peroxidase isoforms than the symplast with the exception of wheat shoots that contained equal isoform numbers in both parts of the cell.

The intolerant plants, subjected to anoxia and reaeration, did not change the number of isoforms. In the majority of apoplastic and intracellular isoperoxidases, the staining intensity faded under the lack of oxygen and restored or exceeded the control upon post-anoxia (Fig. 6; Supplementary Information, Figs. 1, 2). Meanwhile, the root apoplastic isoform 2 (Rf 0.09, Fig. 6c; Supplementary Information, Fig. 1d) was the most inducible by anoxia, and the shoot sym-

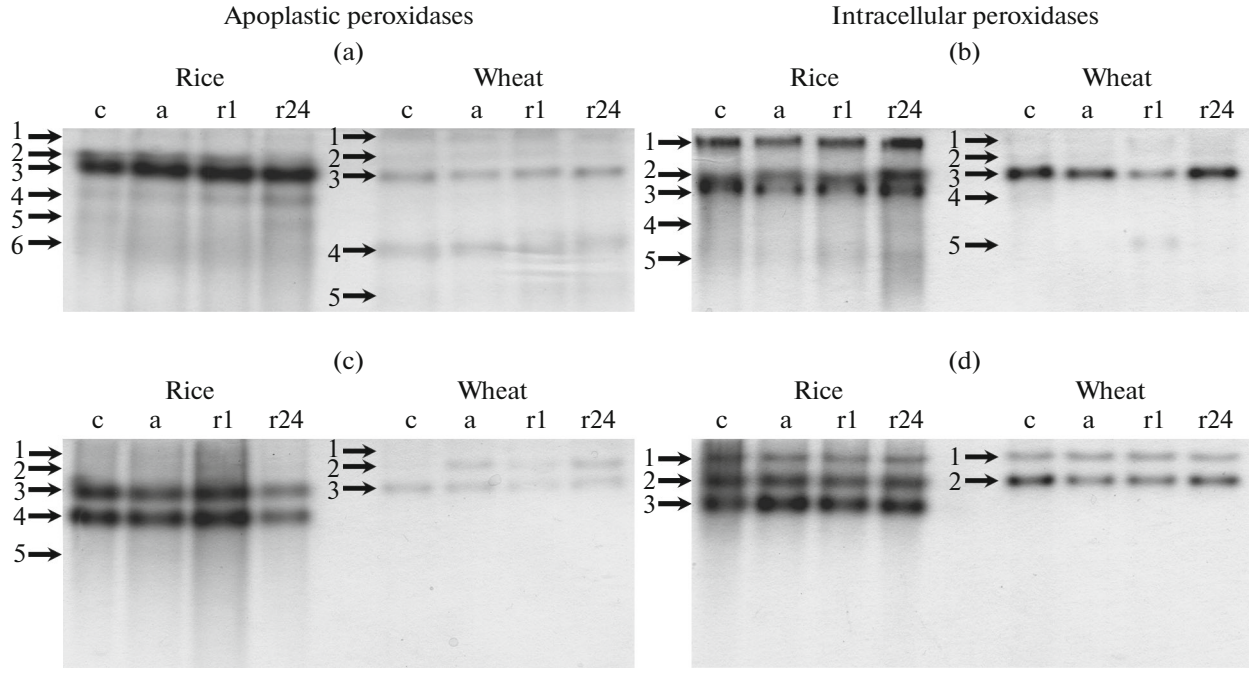

Fig. 6. Isozyme spectrum of (a, c) apoplastic and (b, d) symplastic peroxidases in the (a, b) shoots and (c, d) roots of the wheat and rice seedlings incubated under anoxia followed by reaeration. Conditions of incubation: (c) control (aeration); (a) 12-h anoxia; r1—1-h reaeration; r24—24-h reaeration.

plastic isoform 5 (Rf 0.42, Fig. 6b; Supplementary Information, Fig. 2b) similarly responded to reaeration. In the rice seedlings, the staining intensity of the cell wall isoforms increased in both shoots and roots (Figs. 6a, 6c; Supplementary Information, Figs. 1a, 1c). The changes in staining of the shoot symplastic isoforms in rice were close to that of wheat (Fig. 6b; Supplementary Information, Fig. 2a) and there were no significant changes in the roots (Fig. 6d; Supplementary Information, Fig. 2c). The emergence of new isoforms and disappearance of the existing ones were not observed.

DISCUSSION

In the present study, it was found that the hypoxia-tolerant plant (rice) produced more amounts of hydrogen peroxide than the intolerant one (wheat) under regular aeration (Fig. 1). However, rice did not accumulate this ROS under anoxic conditions. During post-anoxic aeration, this process took place but was weaker and attained a maximum later than in the wheat plants (Fig. 1). Meanwhile, both shoots and roots of wheat seedlings intensively accumulated H_2O_2, especially after 15–30 min of reaeration.

The examples of peroxide overproduction by plants during anoxia and post-anoxic reaeration are known. Thus, H_2O_2 accumulation was reported in both roots and leaves of different plant species under hypoxic and anoxic conditions [14, 17]. Transmission electron microscopy revealed accumulation of hydrogen peroxide in the apoplast and the region of plasma membrane under anoxia and, especially, during post-anoxic aeration in the plants that are tolerant or sensitive to oxygen deficit. Blokhina et al. [18] analyzed the H_2O_2 formation in the roots and rhizomes of the hypoxia-tolerant (rice and yellow flag iris *I. pseudacorus*) and hypoxia-sensitive (wheat and German bearded iris *I. germanica*) plants. It was demonstrated that the tolerant plants accumulate peroxide mainly in the apoplast and the sensitive ones in the cytosol during anoxia and particularly post-anoxic reoxygenation.

Interactions of ROS with components of membrane lipids, proteins, and DNA yield various hydroperoxides indicating lipid peroxidation (LP) and result in disturbance in integrity of cellular membranes. Literature reports the facts that the plants adapted to hypoxia in comparison with not adapted ones undergo weaker LP under anoxia and, especially, during post-anoxic aeration. This is evidenced by corresponding differences in accumulation of the intermediates (diene and triene conjugates) and the final product (malonic dialdehyde) of LP [12, 13, 16]. In the plants tolerant to the lack of oxygen, the reduced LP reflects the higher stability of their membranes. This well conforms to our results demonstrating lower the accumulation of hydrogen peroxide by rice upon anoxia and reoxygenation. This may point to more efficient regulation of delivery and utilization of ROS in the tolerant plants.

The relatively lower H_2O_2 accumulation in rice, shown in our study, may, in turn, be a consequence of

more effective work of antioxidant enzymes in the hypoxia-tolerant plants. This suggestion is also supported by the fact that the initial ROS level was higher in the rice than in wheat tissues. Presumably, the antioxidant system of rice is better prepared for elimination of ROS. In rice, the reduction in the H_2O_2 level may be a consequence of its more intense metabolization in the reactions performed by catalases and peroxidases that are responsible for its decomposition or using peroxide as a substrate in biosynthesis of phenolic polymers.

Catalase participates in detoxification of the cytosol-originated peroxide or that translocated into the cell from the apoplast. In the seedlings of wheat, which is intolerant to anoxia, we did not find significant changes in the catalase activity. Furthermore, we revealed inactivation of the enzyme in the wheat roots after long (72 h) anoxia and 24 h of reaeration (Fig. 2c). In search of the causes of the enhanced catalase activity in rice subjected to reaeration after 72-h anoxia, we managed to show that it occurs at the transcription and translation levels (Figs. 2, 3; Table 2). There were no effects of the inhibitors on the synthesis of wheat catalase mRNA (Table 2). Garnczarska et al. described activation of catalase in the reaerated roots of yellow lupine but only after a short-term (less than 1 day) hypoxia [15, 21]. In the cell culture of *Arabidopsis*, application of anoxic conditions suppresses the enzymatic activity and return to normal aeration elevates it 1.4 times above the control [19]. In the rice seedlings grown under water, the catalase activity is tenfold lower than in the aerobically grown specimens and increases manifold during post-hypoxic reaeration to reach the level exceeding 1.5 times the control [23]. A similar effect is exhibited by seedlings of Indian lotus (*Nelumbo nucifera*) [22]. Oxygen deficiency significantly activates catalase in the leaves of *Potamogeton anguillanus*; in this regard, anoxia is more effective than hypoxia [20]. The elevation of catalase activity in the submerged rhizomes of southern cattail (*Typha domingensis*) appears to be related to a yield of molecular oxygen upon decomposition of hydrogen peroxide [40]. In our experiments, the catalase activity in the rice seedlings, which have been aerobically grown and exposed to anoxia, either coincided with the control or briefly increased. During post-anoxia, a further activation proceeded, in particular, after long-term (72 h) anaerobic conditions so that the activity exceeded the control by two to three times (Figs. 2b, 2d). In this case, the catalase gene expression (particularly, *OsCatB* and *OsCatC*) also increased (Fig. 3). Considerable augmentation of catalase activity is shown in the rhizomes of some hypoxia-sensitive plants (*Glyceria maxima*, *Juncus effusus*, and *I. germanica*) under post-anoxia [24]. This activation was stimulated to a large extent by ethanol, which is accumulated under oxygen deficit. It is interesting here that catalase is involved not only in antioxidant protection but also in conversion of ethanol into acetaldehyde. This product is toxic and contributes to the post-anoxic injury leading to rapid death of the rhizomes of the intolerant species [11, 24]. In more tolerant plants (*Schoenoplectus lacustris, Acorus calamus*, and *I. pseudacorus*), the catalase activity did not change or only slightly rose in the reaerated rhizomes. Therefore, catalase appears to be an essential component of antioxidant protection decomposing hydrogen peroxide under post-anoxic conditions. However, the degree of its activation in the plants adapted to oxygen shortage depends on particular plant species, their organs, and the stage of their development.

We demonstrated the participation of apoplastic guaiacol peroxidase in protection of plants from post-anoxic oxidative impact. Until recently, this enzyme has not been considered as a member of the antioxidant protective system. Its major functions are thought to be confined to lignin synthesis, phenolic metabolism, and regulation of growth processes. Nevertheless, our experiments disclosed its activation under conditions of reaeration. In the anoxia-tolerant plant, this occurred as early as after 1 h of reaeration and was more intense in the shoot, which first encounters oxidative processes upon return to normoxic conditions. As a result, hydrogen peroxide is detoxified in the cell wall, where its formation, upon anoxia and reaeration, was showed by Blokhina et al. [18]. This barrier should hinder H_2O_2 from movement into the cytoplasm and prevent the accompanying impairment of cellular metabolism. Such stimulation was not observed in the sensitive plant.

Our data on the symplastic form of guaiacol peroxidase showed that its activity decreased to some extent under anoxia in both tolerant (rice) and intolerant (wheat) plants (Figs. 3, 4). This fact disagrees with some earlier publications [27, 29]. Those authors, in contrast to us, evaluated the total activity of guaiacol peroxidase without differentiation into the intracellular and apoplastic forms; they also used hypoxic rather than rigorous anoxic (O_2 content below 0.01%) regimen. Under the conditions close to ours, the activities of intracellular peroxidases do not change in the rice coleoptiles and roots [31]. This may be related to elimination of molecular oxygen from the ambient and, consequently, decrease in the level of ROS, including H_2O_2 as a substrate (Fig. 1). Restoration of a regular aeration led to accumulation of these radicals and compounds that stimulated the activation of intracellular peroxidases (Figs. 3, 4). Rise in activity of symplastic peroxidases may be interpreted as the second (after the apoplastic) line of antioxidant defense. Its function is related to detoxification of hydrogen peroxide originating in cytoplasm or coming from the apoplast despite the activation of the cell wall peroxidases. In the sensitive plant, peroxidase became activated only by 12 h of reaeration and only inside the cells, presumably, because of the absence of an extracellular apoplastic barrier detoxifying peroxide yet in

the cell wall. Moreover, after prolonged anaerobiosis, the activation capacity of symplastic guaiacol peroxidase progressively declined in wheat (Fig. 3) but remained rather stable after any terms of anoxia in rice (Fig. 4).

Our study did not reveal an influence of the transcription inhibitor (actinomycin D) on activities of guaiacol peroxidases of the apoplast and symplast but showed the possibility of biosynthesis of the enzymes based on the preexisting mRNAs in the cell (Tables 3, 4). The enzyme, which was synthesized this way, was transported to the apoplast within the vesicles, because brefeldin A effectively suppressed its activity in both plant species (Table 3).

Our electrophoretic analysis of peroxidases did not reveal emergence of their novel isoforms under anoxia and post-anoxic aeration (Fig. 6; Supplementary Information, Figs. 1, 2). However, marked stimulation of most preexisting isoforms (under anoxia and post-anoxia) was found in rice as well as stimulation of some isoforms in wheat. Similar data were already reported, for instance, increasing in the preexisting isoforms of soybean apoplastic peroxidase without emerge of new forms [14].

Therefore, the activation of cell wall peroxidase, which we found in rice, may evidence that this enzyme is a component of the antioxidant system of hypoxia-tolerant plants. It prevents penetration of ROS from the cell wall into the cytoplasm. The obtained data allow us to infer that the tolerant plants do not yield considerable amounts of hydrogen peroxide, probably because of rapid and intense activation of apoplastic guaiacol peroxidases detoxifying peroxide already in the cell wall and, consequently, preventing its spread into the cytoplasm. Peroxide of cytoplasmic origin, together with that coming from the apoplast, are degraded by intracellular enzymes. They preserve high activity in all experimental variants in rice, unlike wheat. This scheme is valid for both short and long terms of anaerobic and post-anoxic influences.

In contrast to rice, wheat does not seem to possess so much distinct functional coordination of antioxidant enzymes under the lack of oxygen and subsequent aeration. In response to enhanced peroxide production, we did not observe rapid activation of catalase or apoplastic guaiacol peroxidase. The second enzyme was activated only after long reaeration. In this case, apoplastic H_2O_2 could penetrate into the cytoplasm of the cell to be detoxified by intracellular guaiacol peroxidases. However, such protection seems to be inadequate because these enzymes were rapidly inactivated that allowed ROS to destabilize membranes and destroy the cells of wheat.

Therefore, the tolerant plant possesses efficiently controlled antioxidant system, including catalase and guaiacol peroxidase. Under anoxia and post-anoxic aeration, this system prevents ROS accumulation and, thus, fulfills an important adaptation to both lack of oxygen and subsequent oxidative stress. In the sensitive plant, these enzymes are not so efficient. Thus, they less intensively detoxify reactive oxygen species and permit them to accumulate and cause damage and rapid death of the plants due to not only oxygen deficiency but also oxidative stress.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any work conducted on animal or human participants.

AUTHOR CONTRIBUTIONS

The authors V.V. Yemelyanov and T.V. Chirkova devised and worked out the experimental design. V.V. Lastochkin and E.G. Prikaziuk cultivated plant material. The experiments on hydrogen peroxide production and enzymatic activities were carried out by V.V. Lastochkin, those on isozymic spectrum of peroxidases by V.V. Lastochkin and V.V. Yemelyanov, and those on catalase gene expression by E.G. Prikaziuk and V.V. Yemelyanov. The data processing was performed by V.V. Lastochkin, E.G. Prikaziuk, and V.V. Yemelyanov. The text of the paper was written by V.V. Yemelyanov and T.V. Chirkova. All the authors were involved in discussions of the results.

SUPPLEMENTARY INFORMATION

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