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METHODS

Determination of ROS Generation Rates in Plant Mitochondria in vitro Using Fluorescent Indicators: Non-Specific Effects of Inhibitors of Terminal Oxidases

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Abstract—A possibility of quantitative analysis of the ROS production by mitochondria isolated from etiolated winter wheat (*Triticum aestivum* L., cv. Mironovskaya 808) seedlings was studied by following hydrogen peroxide accumulation in medium of organelle incubation using a new highly sensitive test-system including the fluorogenic indicator Amplex Red and horseradish peroxidase. The rates of the process were about 100 pmol H_2O_2/(mg protein min) under state 3 conditions; they were almost doubled after mitochondria transition into state 4, and further increased by more than 50% after the addition of alamethicin, the pore-forming antibiotic inducing the organelle inner membrane permeabilization for low-molecular-weight compounds, H_2O_2 molecules among them. Experimental evidence is presented indicating that the classical inhibitors of the terminal oxidases of the plant mitochondrial respiratory chain, such as cyanide, salicylhydroxamic acid, and propyl gallate, can inactivate the ROS-detecting test-systems in vitro because of interactions with their functional components. These results are discussed in comparison with literature data obtained by similar test-systems and indicate that there are considerable limitations for obtaining reliable experimental evidence of the antioxidant role of alternative cyanide-resistant oxidase in plant mitochondria.

Keywords: Triticum aestivum, mitochondria, reactive oxygen species, fluorescent indicators, Amplex Red, alamethicin

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INTRODUCTION

Problems of ROS generation and the mechanisms of their metabolism regulation (generation and scavenging) are an object of intense interest during last decades due to the important role they play throughout the life of the cell, from division to death [1]. One of the most debated questions in biology and biomedicine are those associated with the dual function of ROS generated by cells under different types of stress. On the one hand, this is related to the elucidation of mechanisms of organism pathology developing because of oxidative stress arising from the disturbance in the cell redox balance, i.e., excessive ROS release and their damaging action on all key cell structures, including membranes, proteins, and DNA. On the other hand, this is determined by a possibility of ROS participation in the signal transduction, in particular by the functioning of signaling pathways triggered by ROS originated from mitochondria [1–3]. It is clear that such investigations require using of a relatively rapid and highly specific method for quantification of ROS generation. However, this task is greatly complicated by the low intracellular concentrations of ROS, transient character of their changes, and a relatively short of their life time, as well as the presence in the cells and some organelles sufficiently effective antioxidant defense systems [1, 4]. In this connection, the search and optimization of methods for adequate evaluation of the rates of ROS generation and rapid changes in their intracellular concentrations remain actual.

It is known that widely applied fluorescent dyes from the class of dichlorofluoresceins (DCF) suffer from serious failings, which are considered in detail in a number of recent reviews [5, 6]. Currently, one of the relatively new and most highly sensitive fluorogenic indicators for ROS determining in vitro is Amplex Red (AR), whose oxidation by H_2O_2 with the stoichiometry 1 : 1 is catalyzed by horseradish peroxidase (HP). Spectrally distinguish product of this reaction, resorufin, can be identified either by fluorescence or absorption [7]. It should be noted that, although this test-system is widely

Abbreviations: AOX—alternative oxidase; AR—Amplex Red; DCF—dichlorofluorescein; DMSO—dimethylsulfoxide; $\Delta\psi_m$—mitochondrial membrane potential; FCCP—carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; H_2DCFDA—2,7-dichlorodihydrofluorescein diacetate; HP—horseradish peroxidase; SHAM—salicylhydroxamic acid; V_{ROS}—the rate of mitochondria-generated ROS release.

used for determining the rates of ROS release in animal mitochondria [8–12], its application in similar studies carried out on plant organelles are rare [2].

The objective of this work was to determine the rates of hydrogen peroxide generation in dependence on metabolic state of mitochondria isolated from wheat seedlings using AR as an indicator. Another important task was to elucidate a possibility of application of ETC terminal oxidase inhibitors, in particular those of alternative cyanide-resistant oxidase (AOX), for studying their effects on ROS release by plant mitochondria, using this test-system.

MATERIALS AND METHODS

Plant material. Experiments were performed with etiolated seedlings of winter wheat (*Triticum aestivum* L., cv. Mironovskaya 808) grown hydroponically on tap water at 23–24°C for 3 days. Elite seeds were kindly presented by Acad. B.I. Sandukhadze (Moscow Agricultural Research Institute, Nemchinovka, Moscow oblast).

Isolation of mitochondria. Mitochondria isolation and the control of their functional activity were conducted as described by us in detail earlier [13]. Briefly, shoots (coleoptiles with embryonic leaves) were homogenized in isolation medium (1 : 6) containing 0.3 M sucrose, 18 mM potassium-phosphate buffer (pH 7.9), 1 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, and 0.1% FA-free BSA. Mitochondria isolated by differential centrifugation were resuspended in the small volume of medium containing 0.3 M sucrose, 18 mM potassium-phosphate buffer (pH 7.2), and 0.1% BSA and placed on ice during the entire period of experiment.

Oxygen uptake by mitochondria was measured in reaction medium: 0.3 M sucrose, 18 mM potassium-phosphate buffer (pH 7.2), 1 mM $MgCl_2$, 5 mM EDTA, and 0.1% BSA at 25°C using the Clark-type electrode (Hansatech Instruments, England). The rates of mitochondrial respiration in the presence of 10 mM malate and 10 mM glutamate were on average 120–130 and 35–40 nmol O_2/(mg protein min) in state 3 (after adding 200 μM ADP) and state 4 (after its exhausting in the process of phosphorylation), respectively. Coefficients of respiratory control and ADP/O ratio according to Chance [14] were on average 3.5 and 3.0, respectively. During the long period isolated mitochondria were capable of rapid and stable generation of the transmembrane potential on the inner membrane ($\Delta\psi_m$), followed using Safranin O [13].

ROS generation by mitochondria was tested immediately after organelle isolation using AR (N-acetyl-3,7-dihydroxyphenoxazin) in combination with horseradish peroxidase (HP). Measurements were carried out in reaction medium containing 0.3 M sucrose, 18 mM potassium-phosphate buffer (pH 7.2), 1 mM $MgCl_2$, 5 mM EDTA, 0.1% BSA, 5 mM malate, 5 mM glutamate, and 0.15–0.2 mg/mL of mitochondrial protein. The ratios of components in the test-system were chosen empirically; in most cases it was 10 mM AR and 1–10 units HP/mL. The formation of resorufin, the product of AR interaction with H_2O_2, was recorded spectrophotometrically following the change in the differential absorption ($\Delta A_{573-595}$) with the Hitachi-557 spectrophotometer [10]. The intensity of resorufin fluorescence was measured with the Hitachi-850 spectrofluorimeter (excitation wavelength – 564 nm, emission wavelength – 587 nm) [2]. In the case of another indicator application, e.g., H_2DCFDA (dichlorodihydrofluorescein diacetate), fluorescence of its deacetylated and oxidized product, DCF, was excited by the light with the wavelength of 480 nm and recorded at 552 nm [7, 15, 16]. At the end of each measurement, the sensitivity of these test-systems was checked by the addition of 50–100 nM freshly prepared H_2O_2.

The content of mitochondrial protein in samples was determined by the method of Lowry et al. [17] using BSA as a standard.

Reagents. The high quality KCN, KH_2PO_4, and other salts were produced in Russia; sucrose, substrates, nucleotides, peroxidase, EDTA, BSA, alamethicin, H_2DCFDA were purchased from Sigma (United States); Ampex Red was from Invitrogen (United States).

Statistics. Experiments were performed in 3–4 replicates with 2–3 recordings. Figures represent the results of typical experiments.

RESULTS

As it is known, potential possibilities of any test-system are determined by its sensitivity and specificity. Hence, first of all it was necessary to test the influence of H_2O_2 and other used reagents on the behavior of AR-based test-system per se, namely, fluorescence/absorption of resorufin in the reaction mixture without mitochondria to exclude side effects. Figure 1 shows that hydrogen peroxide addition to the reaction medium was accompanied by a rapid and almost proportional to its concentration increase in the absorption signal of resorufin, thus allowing the reliable evaluation of resorufin concentration below 45–50 nM, which is in agreement with characteristics mentioned in literature [5–7]. But the addition of KCN (the inhibitor of cytochrome oxidase) to the reaction mixture resulted in a visible decrease in a test-system sensitivity, as it was evident from the slowing-down of the kinetics of resorufin absorption signal induced by a subsequent hydrogen peroxide addition, and also from the expressed loss of its dependence on the H_2O_2 concentration (Fig. 1). Thus, this fact should be taken into account at the interpretation of obtained results, because an observed sharp drop in the level of ROS generation may be erroneously interpreted as a result of blocking the main cytochrome pathway of electron transport and activation of alternative oxidase (AOX)

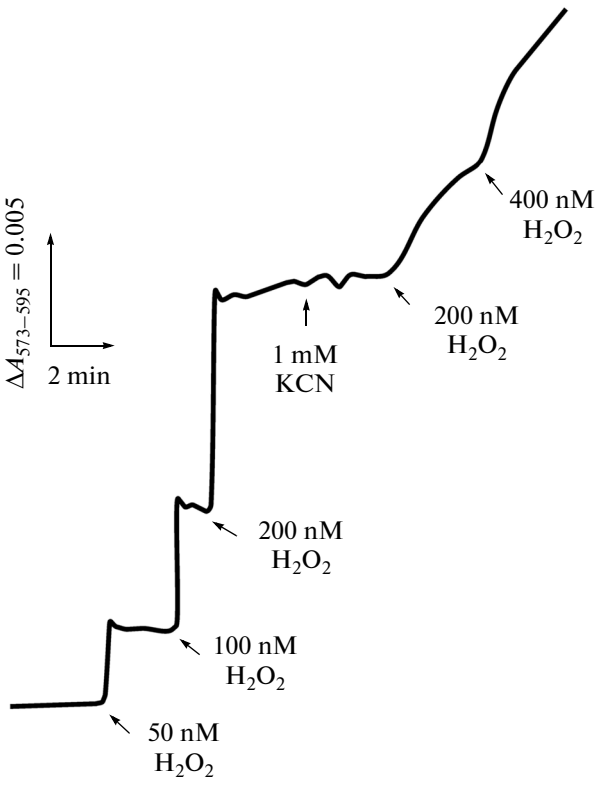

Fig. 1. Dependence of the resorufin absorption signal on the H_2O_2 concentration in reaction medium without mitochondria, and the effect of 1 mM KCN on this signal. Other conditions are described in the Materials and Methods section.

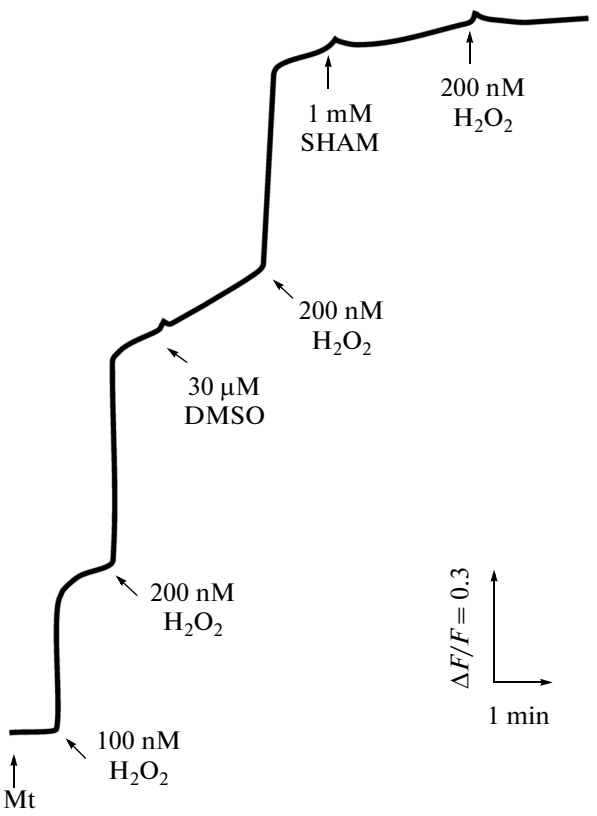

Fig. 2. Dependence of resorufin fluorescence on the H_2O_2 concentration in reaction medium in the presence of wheat mitochondria (Mt), and the effect of 1 mM SHAM on the signal. SHAM and its solvent DMSO were added in equivalent amount (2 μL/mL). Other conditions are described in the Materials and Methods section.

under these conditions. As compared with KCN, the addition to the reaction mixture of most often used AOX inhibitors, such as 1 mM salicylhydroxamic acid (SHAM) or 1 μM propyl gallate (data not shown), induced a more rapid inactivation of the ROS-detecting system; this effect is evident in the presence of mitochondria (Fig. 2) and without them (not shown). Figure 2 also shows that equivalent amounts of the solvent of these inhibitors, dimethylsulfoxide (DMSO), did not influence the sensitivity of the test-system checked by the subsequent H_2O_2 addition.

Significant side effects of SHAM were also observed at the attempt to elucidate the influence of AOX functioning on the rates of ROS release mitochondria using another indicator – dichlorodihydrofluorescein diacetate, whose deacetylated form (H_2DCF) is able in the presence of peroxidase and hydrogen peroxide to be oxidized to the fluorescent product DCF [5–7]. It was found that the response to the addition of SHAM of the test-system per se, i.e., in the absence of mitochondria in the reaction mixture, was dramatic fluorescence increase, which imitated quasi appearance of high H_2O_2 concentration in medium (Fig. 3). Non-specificity of this response evidently follows from the fact that it develops on background of complete loss of test-system sensitivity to H_2O_2 (Fig. 3). Also we suppose that the initial fluorescence of this test-system observed in our experiments is determined by the presence of some amount of deacetylated form of the dye because there was no response to the second addition of hydrogen peroxide (Fig. 3). Undoubtedly, the noted above, side, non-specific effects of the ETC blockers on the behavior of test-systems under study deserve special attention because they severely limit their ability to identify the relationship between the rate of ROS generation by mitochondria and changes of the ETC terminal oxidase activity under the influence of the respective inhibitors.

The experiments with AR probe showed that the rates of mitochondria-generated H_2O_2 release (V_{ROS}) under oxidation of malate in the presence of glutamate were approximately 110 pmol H_2O_2/(mg protein min) (Fig. 4). The transition of mitochondria from metabolic state 3 (after addition of ADP) into state 4 (after exhausting of added ADP in the process of phosphorylation) was accompanied by the increase in V_{ROS} more

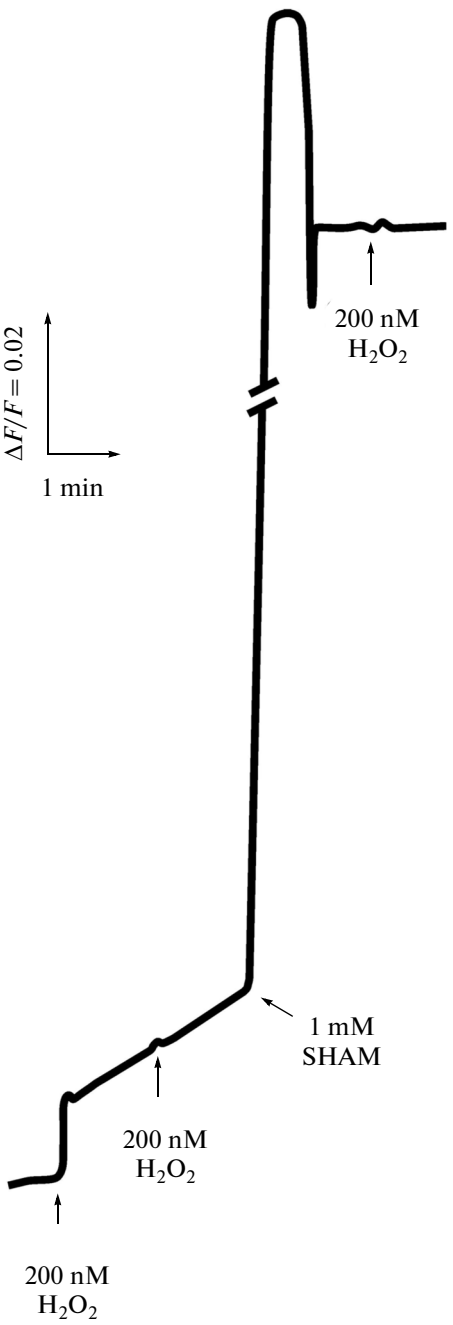

Fig. 3. Dependence of the DCF fluorescent signal on the presence of 200 nM H_2O_2 in medium, and the effect of 1 mM SHAM on the signal.

Other conditions are described in the Materials and Methods section.

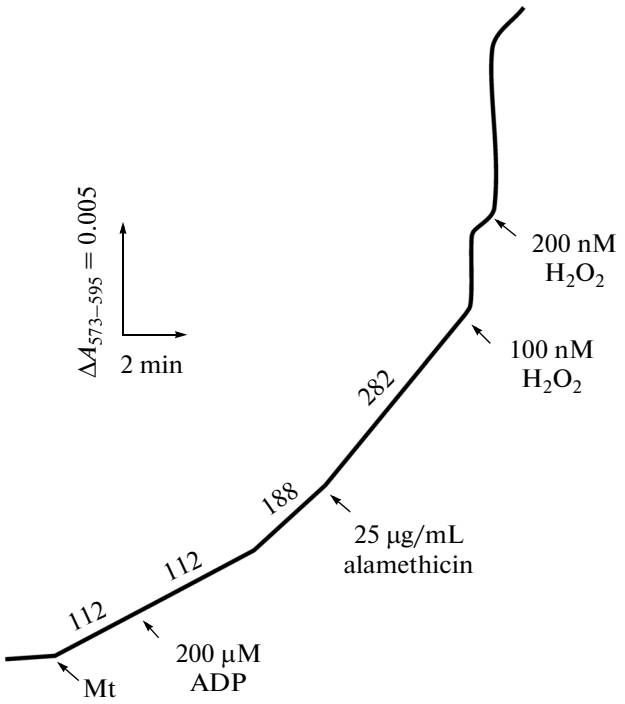

Fig. 4. Kinetics of hydrogen peroxide release from wheat mitochondria (Mt) during malate and glutamate oxidation in different metabolic states, and the influence of 25 μg/mL alamethicin on this process.

Figures above the curve designate the rates of H_2O_2 accumulation in the reaction medium (pmol/(mg protein min). Other conditions are described in the Materials and Methods section.

than 70% (Fig. 4). Furthermore, it was found that the real rates of ROS generation in wheat mitochondria were much higher than those of their accumulation outside organelles, which were revealed using the antibiotic alamethicin increasing the inner membrane permeability for small hydrophilic molecules, including ROS [18]. Thus, the addition of 25 μg/mL of alamethicin to mitochondria induced an increase in the V_{ROS} approximately by 50% (Fig. 4); in this case, test-system sensitivity to H_2O_2 was well preserved after organelle incubation in the presence of this antibiotic for more than 30 min (Fig. 4).

DISCUSSION

It is well known that ROS-detecting systems on the basis of fluorescent dyes widely applied in modern biological and biomedical studies are rather sensitive and permit determination of the levels of different ROS, hydrogen peroxide in particular, in both intact tissues and cells and in isolated organelles, including mitochondria. However, accumulating information on non-specific reactions, in which the system components may enter, requires a great caution in interpreting the results [10, 11, 19, 20]. There are data indicating clearly the artifact character of direct interaction of SHAM with H_2DCFDA, able to lead to a large increase in the fluorescence of the solution even under anaerobic conditions, which was interpreted as an esterase-like action of this AOX inhibitor [19].

In our experiments, it was also found that the addition of SHAM to H_2DCFDA + HP resulted in a sub-

stantial increase of reaction mixture fluorescence and further in a complete loss of indicator sensitivity to hydrogen peroxide (Fig. 3). In recent publication of El-Khoury et al. [11], the possibility of direct interaction of another AOX inhibitor propyl gallate with the AR-based ROS-sensing system was indicated. In this connection the work of Maxwell et al. [20] should be noted, where transgenic tobacco lines with genetically changed level of AOX expression and activity were used to elucidate the influence of AOX activity on ROS generation by mitochondria and preventing possible side effects of SHAM on H_2DCFDA.

The analysis of literature data and our own results indicate that the classical inhibitors of the terminal oxidases of plant mitochondria can affect substantially the ROS-detecting systems by both the inactivation of peroxidase or due to the direct interaction with the indicators. This is evidently indicated also by a considerable increase in the fluorescence of DCF after SHAM addition to isolated pea mitochondria, which was erroneously interpreted as substantial activation of ROS generation due to AOX inhibition because there was no control of H_2DCFDA + HP sensitivity to ROS in the presence of SHAM [15]. And vice versa, the absence of corresponding signal of this indicator in the presence of KCN was ascribed to the decrease in V_{ROS} as a result of cytochrome pathway inhibition and coupling AOX activation [21]. Similar results of experiments performed on mitochondria isolated from wheat seedlings [16] might be also erroneously interpreted as the activation of ROS generation because of AOX blockage, although in these experiments not SHAM but another derivative of hydroxamates , benzohydroxamic acid, was used. But similar affinity of HP for both hydroxamic acids was demonstrated earlier [22]. Thus, literature data and our results obtained with analogous test-systems (Figs.1–3) indicate substantial difficulties arising during the application of the inhibitory analysis to isolated plant mitochondria for obtaining reliable experimental evidence for AOX antioxidant role.

The application of the AR-based system for the determination of ROS generation rate (V_{ROS}) in mitochondria isolated from etiolated wheat seedlings (Fig. 4) showed that its values on the whole are comparable with those obtained for other plant and animal objects [2, 10, 12, 15]. Thus, in the work [15] cited above, the rates of ROS release by mitochondria isolated from etiolated pea seedlings during succinate oxidation were 130–150 pmol H_2O_2/(mg protein min). At the same time, the presented in the literature values of V_{ROS} varies widely, by tens times, even in organelles isolated from similar objects. For example, V_{ROS} values obtained by application of H_2DCFDA + HP for mitochondria from etiolated pea seedlings also oxidizing succinate were above 1.3 nmol H_2O_2/(mg protein min) [23]. In some recent publications, it was shown that animal mitochondria, in particular organelles of rat and porcine cardiac muscle cells, were characterized by V_{ROS} being not higher than 100–200 pmol H_2O_2/(min mg protein), which in turn corresponded to 0.1–0.2% from overall respiration rates at 37°C [10, 12]. In the detailed work of other authors [24], it was shown the high substrate and organ specificity of ROS generation in mitochondria, the rates of which varied from 0.1% (state 3) to 3% (state 4) from the rates or those of respiration.

Our calculations of V_{ROS} for mitochondria from tissues of winter wheat seedlings oxidizing malate showed that its values were approximately 0.1 and 0.5% for state 3 and 4 conditions, respectively. It is not excluded that this 5-fold difference can be determined by an increase in the mitochondrial membrane potential in state 4 approximately by 30% (Fig. 4, see also [13]). According to the available literature data, the stimulation of ROS generation determined by the increase in $\Delta\psi_m$ can be prevented by triggering the mechanism of "mild uncoupling" [25] based on the activation of the energy-dissipating systems favoring the maintenance of the lower $\Delta\psi_m$ [8, 25]. In this connection, one of the possible reasons of the relatively low rates of ROS generation by wheat mitochondria are a high constitutive activity of AOX, which depending on the used substrate of oxidation can achieve 30–50% of the rates of total respiration [13]. On the other hand, the relatively low measured values of V_{ROS} may be a consequence of the fact that they reflect the rates of ROS release from mitochondria but not the true rates of their generation inside organelles, as it is evidenced from the results of experiments with alamethicin. Using this antibiotic the substantial difference was revealed between the rates of ROS emission and generation in the matrix, indicating that V_{ROS} is markedly limited by the permeability of mitochondrial inner membrane for H_2O_2 (Fig. 4), which has been observed earlier also for animal mitochondria [18, 26].

Based on the obtained results, it has been concluded that new highly sensitive ROS-detecting systems on the basis of fluorogenic indicators, AR primarily, can be applied for the determination of the rates of hydrogen peroxide generation in plant mitochondria in vitro in dependence of their functional state. The application of the antibiotic alamethicin showed that the rates of H_2O_2 accumulation in the incubation medium measured by this method do not really reflect true and much higher rates of its generation in mitochondria. Found on revealed in this study pronounced side effects of the classic inhibitors of the terminal oxidases of ETC in plant mitochondria on the behavior of the studied test-systems associated with the loss of test-system sensitivity to ROS, allow a conclusion that the eliciting of AOX antioxidant role is considerably complicated and, apparently, requires the use of other approaches.

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