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Induction of *Arabidopsis gdh2* Gene Expression during Changes in Redox State of the Mitochondrial Respiratory Chain

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Abstract—Expression of the *gdh2* gene encoding the α-subunit of mitochondrial glutamate dehydrogenase depends on redox state of the mitochondrial electron transport chain. Treatment of *Arabidopsis thaliana* cell suspension with antimycin A, a respiratory chain complex III inhibitor, resulted in an increase in *gdh2* transcripts within 2 h. Inhibition of complex I by rotenone did not influence the transcript level, but treatment with potassium cyanide, a complex IV inhibitor, also increased the transcript content. Thus, *gdh2* gene expression obviously responds to changes in the respiratory chain segment localized between complexes I and III. Lack of activation of gene expression after treatment of a cell suspension with hydrogen peroxide and the prooxidant paraquat and results of experiments with antioxidants suggest that *gdh2* gene expression is not associated with increased content of reactive oxygen species generated during inhibition of the electron transport chain. Protein phosphorylation by serine/threonine protein kinases is the essential step required for signal transduction into nucleus resulting in the induction of *gdh2* expression.

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Key words: glutamate dehydrogenase, alternative oxidase, respiratory chain, retrograde regulation, redox regulation, antimycin A

Glutamate dehydrogenase (EC 1.4.1.2) catalyzes the reaction of 2-oxoglutarate amination to L-glutamate; this enzyme links nitrogen and carbon metabolism in plant cells [1]. The reaction is easily reversible *in vitro* and it still remains unclear which direction dominates *in vivo* [2]. Numerous studies in this field have given contradictory results [3, 4]. It is possible that direction of this reaction depends on metabolic conditions. There is evidence that activity of this mitochondrial enzyme is controlled by many factors [2]. Recent studies have shown that osmotic and oxidative stresses activate glutamate dehydrogenase gene expression [5]; according to a scheme proposed by the authors, excess of glutamate generated by this enzyme under stress conditions is further used for proline biosynthesis. Nevertheless, signaling pathways responsible for activation of glutamate dehydrogenase expression remain unclear.

Results of recent studies indicate that expression of some plant nuclear genes encoding certain mitochondrial proteins depends on function state of mitochondria. Inhibition of the mitochondrial electron transport chain (ETC) or impaired functioning of respiratory chain complexes associated with mutations results in induction of genes encoding alternative oxidases (AO), alternative NAD(P)H-dehydrogenases, and components of mitochondrial systems responsible for protein import [6-8]. Detailed studies of mechanisms of such "retrograde" regulation were carried out only for AO gene expression. Numerous studies in this field have demonstrated complexity and specificity of activation of the expression process. For example, induction of each of three isoforms of maize alternative oxidase occurs in response to inhibition of a certain respiratory chain complex [9]. Induction of AO genes depends on the level of generation of reactive oxygen species (ROS) by the mitochondrial ETC, and it is suppressed by inhibitors of protein kinases and/or pro-

Abbreviations: AO, alternative oxidases; DMSO, dimethylsulfoxide; ETC, electron transport chain; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; ROS, reactive oxygen species.

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tein phosphatases (depending on the particular gene) [10, 11]. Evidently, information about signals involved in mitochondrial–nuclear interactions requires detailed study of regulatory pathways of expression of other "mitochondria-dependent" genes.

We have demonstrated that expression of the *gdh2* encoding the α-subunit of glutamate dehydrogenase is activated during inhibition of the mitochondrial ETC. Our results suggest that the primary signal resulting in induction of *gdh2* expression consists in altered redox state of a certain segment of the respiratory chain.

MATERIALS AND METHODS

Arabidopsis thaliana suspension culture cells were cultivated in darkness at 26°C in MS medium [12] supplemented with additions of thiamine (1 mg/liter), pyridoxine (0.5 mg/liter), nicotinic acid (0.5 mg/liter), inositol (100 mg/liter), sucrose (30 g/liter), and 2,4-dichlorophenoxyacetic acid (0.3 mg/liter). Each subcultivation was carried out for 14 days, and experiments were carried out on the tenth day of subcultivation. Acting compounds were dissolved in ethanol (antimycin A, KCN, rotenone, staurosporine, endothall) or in dimethylsulfoxide (DMSO) (paraquat, N-acetylcysteine). Final concentration of DMSO and ethanol in the suspension culture did not exceed 0.5 and 0.3%, respectively. Control preparations contained concentrations of ethanol or DMSO corresponding to their concentrations in the experimental preparations. In the case of combined addition of antimycin A with N-acetylcysteine, endothall, staurosporine, and FCCP (carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone), all these compounds were added to the cell culture 30 min before addition of antimycin A. Experiments were carried out in triplicate.

Cells were homogenized in liquid nitrogen and after that 450 μl of TE buffer, 50 μl of SDS (10%), and 500 μl of phenol (ICN, USA) were added. Subsequent isolation of total cell RNA was carried out by means of hot phenol extraction as described in [13]. Preparations of isolated RNA were analyzed in 1% agarose gel followed by staining with ethidium bromide.

Primers listed in the table were used for preparation of DNA probes. DNA amplification (35 cycles) employed Taq DNA polymerase (Fermentas, Lithuania) using the supplier's buffer and the following regime: denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and elongation at 72°C for 1 min 40 sec. The concentration of each of dNTP (Fermentas) was 100 μM. *Arabidopsis* cDNA obtained as described in [14] was used as a template for amplification. DNA fragments were labeled by means of PCR in the presence of [α-^{32}P]dATP (IRM, Russia) (100 μCi per sample). Reaction conditions were basically identical to the above-mentioned ones, except for the concentration of unlabeled dATP in the reaction mixture, which was reduced to 25 μM, and there was a final elongation step for 10 min. The labeled probe was purified from nucleotides using a Sephadex G-50 column (ICN).

Total cell RNA was separated in 1.2% agarose gel under denaturing conditions and transferred onto a Hybond N nylon membrane (Amersham, England) [15]. Pre-hybridization (42°C, 60 min) was carried out in buffer containing 5× SSC, 5× Denhardt's solution, 0.5% SDS, and 50% formamide. Hybridization was carried out at 42°C using the same buffer, containing a labeled DNA probe, which was pre-denatured on a boiling water bath for 5 min. Hybridization was carried out for 18 h. The membrane was washed twice for 5 min at 42°C using 5× SSC, twice for 10 min at 42°C using 1× SSC, and twice for 10 min at 65°C using 0.1× SSC (in all cases SDS concentration was 0.1%). The washed membrane was exposed to a Kodak film for 1-14 days. In the case of repeated hybridization with another DNA probe, the bound probe was washed by membrane incubation in 0.5% SDS at 95°C for 40-60 min.

Characteristics of DNA probes used in this study

Gene	Description of gene product	Locus number	Sequence of primers used for probe preparation
cox3	cytochrome oxidase *c* subunit 3	AtMg00730	L: ATTTCAAGGGGGTGCAACA R: CAAATGGGAATAACCGAACC
fro1	NADH dehydrogenase 18 kD subunit	At5g67590	L: ATGGCGCTTTGTGCTACTACT R: AATGGTGTGTTGGGCTTCTT
gdh2	glutamate dehydrogenase α-subunit	At5g07440	L: CATTACTGGTGCAATCAGGAA R: ACTTTGCCTTCACATCTCCA
aox1a	alternative oxidase 1a	At3g22370	L: AATCGCTTCGACGTTTTGAG R: GCAAAATGGTTTACATCACGG

Note: The letters L and R designate left and right primers, respectively.

RESULTS

The *A. thaliana* suspension culture has been chosen as a convenient system for studies of factors involved in mitochondrial–nuclear interactions. We have investigated the effect of treatment of a cell suspension with antimycin A, an inhibitor of mitochondrial respiratory chain complex III, on the level of transcripts of some genes (of both mitochondrial and nuclear localization) encoding mitochondrial proteins. The level of transcripts of most studied genes encoding subunits of the ETC, ATP synthase complex, and mitochondrial ribosomal proteins remained unaltered (data not shown). However, the study of the expression level of the *gdh2* gene encoding the α-subunit of glutamate dehydrogenase revealed a marked increase in the content of its transcripts. This increase detected after 2 h of treatment was maintained during at least 8 h (Fig. 1). Treatment for 24 h resulted in a decrease in the level of *gdh2* transcripts to nearly the control level. The level of transcripts of the mitochondrial gene *cox3* encoding a cytochrome oxidase complex subunit and the nuclear gene *fro1* encoding a NADH dehydrogenase complex subunit remained unchanged over the whole period of this cell treatment. The content of transcripts of these genes was used as control in all subsequent experiments. Studying the effects of other treatments, we used the time interval from 2 to 6 h because it provided a reliable time course for accumulation of transcripts. Thus, antimycin A-induced changes in the ETC induced *gdh2* gene expression. The question arises whether this effect is specific with respect to inhibition of the mitochondrial respiratory chain complex III, or inhibition of electron transfer at any segment of respiratory chain would cause the same effect.

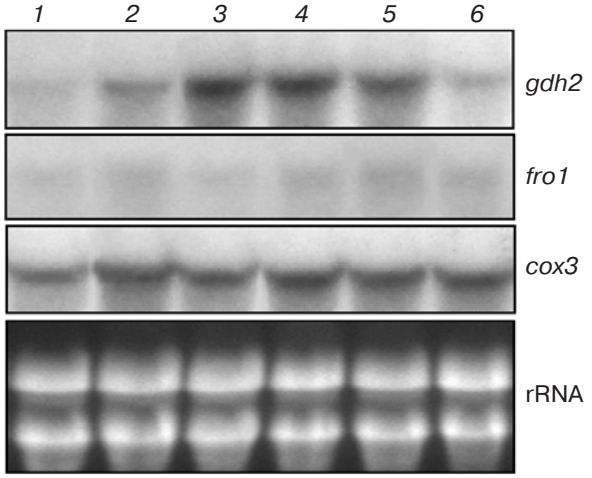

Fig. 1. Time course of accumulation of *gdh2* gene transcripts during the treatment of *Arabidopsis* suspension culture cells with 10 μM antimycin A. The levels of *fro1* and *cox3* gene transcripts were used as controls. *1*) Control; *2-6*) antimycin A (2, 4, 6, 8, and 24 h incubation, respectively).

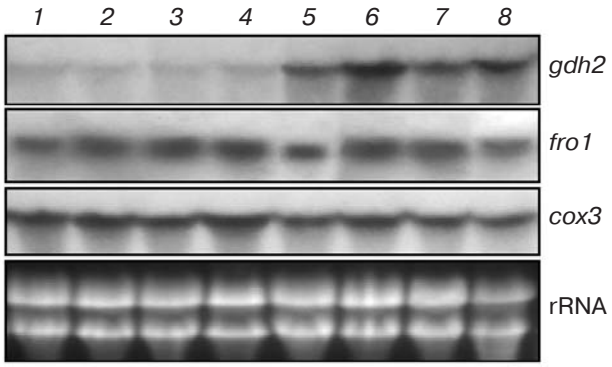

Fig. 2. Effects of inhibition of three complexes of the respiratory chain (40 μM rotenone, 10 μM antimycin A, 1 mM potassium cyanide) on the content of *gdh2* gene transcripts in suspension of *Arabidopsis* cells. The levels of *fro1* and *cox3* gene transcripts were used as controls. *1*) Control, 3 h; *2*) control, 6 h; *3*) rotenone, 3 h; *4*) rotenone, 6 h; *5*) antimycin A, 3 h; *6*) antimycin A, 6 h; *7*) KCN, 3 h; *8*) KCN, 6 h.

In the next series of experiments we compared the effects of inhibitors of ETC complexes I, III, and IV on expression of the *gdh2* gene. Inhibition of complex I by addition of 40 μM rotenone did not influence the level of the transcripts (Fig. 2). However, treatment with potassium cyanide, a complex IV inhibitor, increased *gdh2* transcripts. The degree of this induction of the gene expression was comparable with that observed during treatment with antimycin A (Fig. 2). Blockade of electron transfer in the ETC increases reduction of the respiratory chain segment located before the site of action of the corresponding inhibitor [16]. The data suggest that *gdh2* gene expression reacts to changes in the redox state of the respiratory chain segment located between complexes I and III. Since this ETC segment contains ubiquinone, we believe that the state of the ubiquinone pool is the most probable candidate for the role of primary signal for induction of *gdh2* expression.

On the other hand, the increase of ETC reduction, which appears after addition of corresponding inhibitors, results in the increased formation of reactive oxygen species (ROS) [16]. Since glutamate dehydrogenase is considered by some researchers as a stress-induced protein [5], it is reasonable to suggest that the increase in intracellular ROS occurring on ETC inhibition is a signal for activation of gene expression. To test this suggestion, we used N-acetylcysteine in our experiments. This antioxidant is widely used in experiments with plant cell cultures [11, 17]. A cell suspension was pretreated with N-acetylcysteine for 30 min before addition of antimycin A. If superoxide radical or hydrogen peroxide generated during ETC inhibition are really involved in signaling to the nucleus for the induction of expression, we would expect blockade of the activating effect of antimycin A. However, we did not observe this in our experiments (Fig.

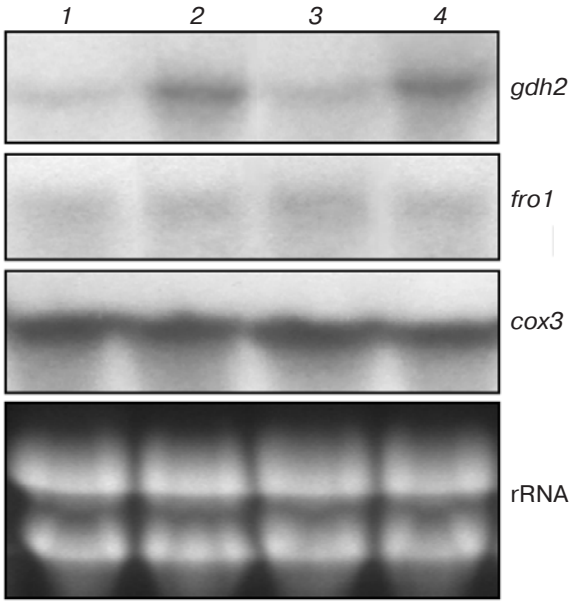

Fig. 3. Effect of N-acetylcysteine on the induction of *gdh2* expression by antimycin A. Cells were pretreated with 2 mM N-acetylcysteine for 30 min before addition of 10 μM antimycin A. After addition of antimycin A, cells were incubated for 2 h. The levels of *fro1* and *cox3* gene transcripts were used as controls. *1*) Control; *2*) antimycin A; *3*) N-acetylcysteine; *4*) N-acetylcysteine + antimycin A.

3). The pretreatment with antioxidant did not influence *gdh2* transcript content versus the corresponding control.

Subsequent experimental analysis of the hypothesis on the involvement of ROS in signaling for altered *gdh2* expression included treatment of the *Arabidopsis* suspension cell culture with hydrogen peroxide and paraquat. The herbicide paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride) is traditionally used as a prooxidant in studies of reactions of living system to oxidative stress. In darkness paraquat molecules are preferentially reduced in the plant cell by elements of the mitochondrial ETC, and then it undergoes spontaneous oxidation due to single electron transfer to an oxygen molecule; this results in superoxide radical formation [18]. Treatment of cells (over 6 h) with all concentrations of paraquat and hydrogen peroxide did not influence the level of *gdh2* transcripts (Fig. 4, lanes *2*-*5*). Thus, we suggest that the antimycin A-induced changes in ETC redox state rather than accompanying increase in ROS production is a primary signal for increased expression of the *gdh2* gene.

There is evidence that in plants, animals, and yeast cells changes in the electrochemical potential of mitochondrial membranes are signals for modulation of expression of nuclear genes, especially those encoding heat shock proteins [19, 20]. Since the antimycin A-induced inhibition of the ETC also results in a decrease in membrane potential, it is possible that *gdh2* induction also depends on changes in mitochondrial membrane potential. To test this hypothesis we treated the cells with the uncoupler of oxidative phosphorylation FCCP. This protonophore increases H^+ conductance of the inner mitochondrial membrane, resulting in a decrease in ATP production in the cell. Thus, treatments with antimycin A and FCCP cause similar effects on mitochondrial membrane potential and the level of cell ATP, but they cause opposite effect on redox state of the respiratory chain. Antimycin A blocks electron transfer and therefore increases reduction of the ETC, whereas FCCP causes oxidation of respiratory chain complexes. According to the results of our experiments (Fig. 4, lanes *6* and *7*), treatment with FCCP did not influence the level of *gdh2* transcripts. Thus, we conclude that induction of the *gdh2* gene caused by antimycin A is not related to the decrease in the electrochemical potential of the mitochondrial membranes and/or the decrease in ATP synthesis; it definitely depends on redox state of the respiratory chain.

Reactions of protein phosphorylation/dephosphorylation widely act as messengers in signal transduction. For evaluation of possible involvement of protein kinases and/or protein phosphatases in the signal transduction for altered expression of the nuclear *gdh2*, we pretreated the cell suspension with a protein kinase inhibitor, staurosporine, and a phosphatase inhibitor, endothall, for 30 min (before addition of antimycin A). Certain evidence exists that protein kinases and protein phosphatases are involved in retrograde regulation of AO genes in other plant species, and so we also investigated the level of transcripts of the alternative oxidase gene *aox1a*. In accordance with earlier data ([21] and our unpublished results), treatment with antimycin A was accompanied by induction of the *aox1a* gene. The presence of endothall, an inhibitor of serine/threonine phosphatase types 1 and 2A,

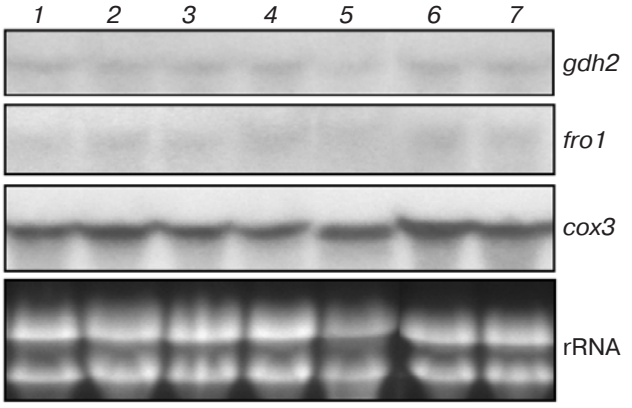

Fig. 4. Effects of 100 μM paraquat, 10 mM hydrogen peroxide, and 2 μM FCCP on the level of *gdh2* gene transcripts in *Arabidopsis* cell suspension. The levels of *fro1* and *cox3* gene transcripts were used as controls. *1*) Control; *2*) paraquat, 2 h; *3*) paraquat, 6 h; *4*) H_2O_2, 2 h; *5*) H_2O_2, 6 h; *6*) FCCP, 2 h; *7*) FCCP, 6 h.

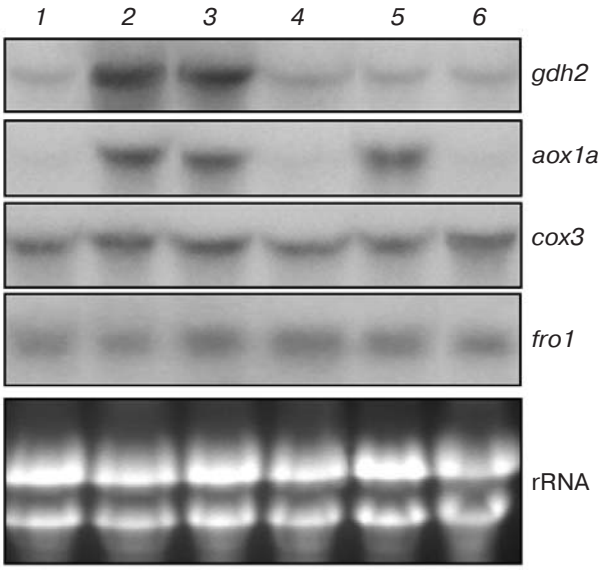

Fig. 5. Effect of protein kinase and protein phosphatase inhibitors on induction of the *gdh2* gene by antimycin A. Cells were treated with 50 μM endothall and 0.4 μM staurosporine for 30 min before addition of 10 μM antimycin A. After antimycin A addition, cells were incubated for 2 h. The levels of *fro1* and *cox3* gene transcripts were used as controls. *1*) Control; *2*) antimycin A; *3*) endothall + antimycin A; *4*) endothall; *5*) staurosporine + antimycin A; *6*) staurosporine.

did not influence antimycin A-induced accumulation of transcripts of *gdh2* and *aox1a* (Fig. 5). However, treatment with staurosporine (a serine/threonine protein kinase inhibitor) completely blocked the activating effect of antimycin A on *gdh2* gene expression, whereas the level of *aox1a* transcripts remained unchanged. Thus, we conclude that protein phosphorylation involving serine/threonine protein kinases is an ultimate step in transduction of the signal into the nucleus on change in *gdh2* expression.

DISCUSSION

We have demonstrated that treatment with inhibitors of complex III and complex IV of the respiratory chain was accompanied by significant increase in content of transcripts of the *gdh2* gene. (Inhibition of complex I did not influence the expression of this gene.) These data suggest that the redox state of the respiratory chain segment between complexes I and III plays a major role in generation of a signal causing the induction of this gene. Similar dependences of ETC inhibitors (activation by antimycin A and cyanide and lack of rotenone effect) were also shown earlier for expression of the *aox3* gene in maize seedlings [9]. Based on these data, it was hypothesized that the over-reduced state of the ubiquinone pool associated with inhibition of complexes III or IV is the major factor triggering a signaling cascade responsible for induction of *aox3* gene expression. We suggest that regulation of *gdh2* gene expression involves similar mechanisms. It should be noted that for chloroplasts modulation of expression of genes located in chloroplasts (*psaA*, *psbA*, etc.) and nucleus (*apx2*, *fed1*, etc.) depends on redox state of the plastoquinone pool [22, 23]. This supports the possibility of existence of similar regulatory pathways in mitochondria as well.

The observed gene activation could be alternatively explained by sensitivity of *gdh2* expression to intracellular level of ROS. According to most data, increased generation of ROS in the respiratory chain occurs during the increase in redox state of ETC; this would be a signal for activation of gene expression. Involvement of ROS in signal transduction for change in expression was demonstrated for AO genes [6]. Since induction of glutamate dehydrogenase expression has been found under stress (including oxidative stress) conditions [5] such a hypothesis is quite logical. However, results of our experiments argue against this suggestion. First, pretreatment with N-acetylcysteine did not influence antimycin A-induced *gdh2* expression. Interestingly, the presence of N-acetylcysteine caused almost total blockade of the activating effect of antimycin A on the level of AO transcripts in tobacco [17] and *Arabidopsis* cell suspensions (our unpublished results). Second, treatment of a cell suspension with hydrogen peroxide and paraquat did not influence gene expression within the time interval (6 h) characterized by active accumulation of *gdh2* transcripts induced by antimycin A and cyanide.

Our data on the lack of prooxidant effect on induction of *gdh2* expression are inconsistent with results obtained by Skopelitis et al.; they demonstrated that oxidative stress caused by menadione treatment increased the level of transcripts of the gene encoding the α-subunit of glutamate dehydrogenase [5]. However, Skopelitis et al. observed induction of this gene expression only 24-72 h after the beginning of such treatment. Existence of such a long time interval suggests that the observed effect is mediated by some secondary consequences of stress treatments.

Maxwell et al. [17] found seven nuclear genes of tobacco suspension culture cells that exhibited similar changes in expression during treatment with hydrogen peroxide and antimycin A. Results of that study suggest that in the case of both treatments the changes in ETC state were the primary signal to altered gene expression rather than the increase in ROS (as it was initially suggested). They showed that activity of the main pathway of electron transfer decreases under stress conditions induced by hydrogen peroxide or salicylic acid. Thus, ETC inhibition shares some similarity with processes induced by *in vivo* stress. Taking into consideration these data, we suggest that the increase in glutamate dehydrogenase transcripts found in [5] would be also attributed to stress-related changes in mitochondrial ETC functioning.

The role of the electrochemical potential of the inner mitochondrial membrane in signal transduction for induction of heat shock protein expression has been demonstrated [19, 20]. The other signaling system involved in regulation of AO gene expression includes ROS of mitochondrial origin [6, 10]. Results of the present study do not confirm involvement of these two signaling pathways in the regulation of *gdh2* gene expression. In generation of a signal for the change in *gdh2* expression, the redox state of ubiquinone obviously plays a central role. It should be noted that tobacco strains with impaired functioning of respiratory complex I are characterized by changes in expression of some antioxidant defense enzymes localized in various cell compartments and also by increased resistance to abiotic and biotic stresses [24]. Summarizing all the experimental data, we suggest that mitochondria play an active role in regulation of nuclear gene expression; mitochondria are the crossroads for various signaling pathways specific for certain genes encoding both mitochondrial and other proteins.

Protein kinases and protein phosphatases are components of signal transduction processes. Protein kinases that are activated in response to changes in redox state of the plastoquinone pool have been found in the thylakoids of *Arabidopsis* chloroplasts; these enzymes are involved in regulation of rearrangements of the photosynthetic machinery and possibly in signaling pathways regulating expression of chloroplast and nuclear genes [23, 25, 26]. In plant mitochondria, there are unidentified protein kinases and protein phosphatases involved in retrograde regulation of AO gene expression [10, 11]. For example, inhibition of serine/threonine phosphatase types 1 and 2A by treatment of cell suspension with endothall blocked the activating effect of cysteine on expression of the tobacco *aox1* gene [11]. In our experiments endothall did not influence the antimycin A-induced induction of both *gdh2* and *aox1a* gene expression. However, an inhibitor of serine/threonine protein kinases, staurosporine, completely abolished the activating effect of antimycin A on *gdh2* expression. A similar staurosporine effect was also found in soybean cells [10] in the case of citrate-induced activation of *aox1* gene expression. At the same time, the presence of staurosporine did not prevent induction of this gene caused by treatment with antimycin A [10]. Our experiments on the *Arabidopsis* cell suspension have also demonstrated lack of staurosporine effect on the antimycin A-dependent activation of *aox1a* gene expression. Thus, we conclude that various signaling cascades are involved in signal transduction pathways of alternative oxidase and glutamate dehydrogenase gene expression in response to treatment with antimycin A.

Our data suggest that serine/threonine protein kinases play a central role in the signal transduction process resulting in activation of *gdh2* expression. The localization of these protein kinases remains unknown. It should be noted that although study of plant mitochondrial protein kinases is just at the very beginning, there is evidence on the presence of some kinase activities phosphorylating several tens of proteins [27]. Employment of proteomic approaches resulted in identification of 10 protein kinases in *Arabidopsis* mitochondria [28]. Amino acid sequences of these proteins lack a mitochondrial signal peptide, but most of these proteins have transmembrane domains. It is tempting to suggest that protein kinases localized in the inner mitochondrial membrane may be involved in transduction of a signal on ETC redox state into the nucleus.

Thus, we have demonstrated that regulation of *gdh2* gene expression depends on mitochondrial redox state, and it can be considered as another example of retrograde regulation, which was earlier demonstrated for AO and some other proteins. Redox state of the ubiquinone pool is a primary factor of this regulation, which also involves serine/threonine protein kinases in signal transduction.

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