Biochemistry (Moscow), Vol. 68, No. 7, 2003, pp. 747-751. Translated from Biokhimiya, Vol. 68, No. 7, 2003, pp. 910-916.

Effect of Electron-Transport Inhibitors on the Generation of Reactive Oxygen Species by Pea Mitochondria during Succinate Oxidation

V. N. Popov[1]*, E. K. Ruuge[2], and A. A. Starkov[3]

[1]Department of Plant Physiology and Biochemistry, Voronezh State University, Universitetskaya pl. 1, Voronezh 394693, Russia; fax: (0732) 78-9755; E-mail: pvn@main.vsu.ru
[2]Russian Cardiology Research and Production Association, Ministry of Public Health of the Russian Federation, 3-ya Cherepkovskaya ul. 15a, Moscow 121552, Russia; E-mail: ruuge@bior.phys.msu.ru
[3]Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow 119992, Russia; fax: (095) 939-0338; E-mail: stark@izba.ru

Received May 21, 2002
Revision received July 1, 2002

Abstract—The effect of inhibitors of the cytochrome pathway and alternative oxidase on the rate of respiration and generation of reactive oxygen species by pea mitochondria was studied. Respiration of mitochondria from pea cotyledons was inhibited by 70-80% by salicylhydroxamate (SHAM). The rate of hydrogen peroxide production by pea cotyledon mitochondria during succinate oxidation was 0.15 nmol/min per mg protein. SHAM considerably accelerated the hydrogen peroxide production. The SHAM-dependent H_2O_2 production was stimulated by 2 μM antimycin A and inhibited by 5 mM KCN and 1 μM myxothiazol. The study of the rate of $O_2^{\cdot-}$ generation by pea mitochondria using EPR spin traps and epinephrine oxidation showed that H_2O_2 accumulation can be accounted for by a significant increase in the rate of $O_2^{\cdot-}$ production.

Key words: reactive oxygen species, mitochondria, electron transport, alternative oxidase, myxothiazol, antimycin A, cyanide

Oxidation of respiration substrates via the cytochrome pathway requires the presence of ADP, which is involved in oxidative phosphorylation. When the energy demands of the cell are low, the ADP concentration decreases, and the cytochrome pathway is inhibited. This may result in an increase in the concentration of ubisemiquinone, which is capable of the parasitic one-electron process of superoxide radical ($O_2^{\cdot-}$) generation. Superoxide radical is rapidly converted by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). The latter may be either detoxified by catalase or peroxidase in mitochondria or converted to hydroxyl radical ($OH^{\cdot}$), a very aggressive oxidant that can oxidize almost all cell components including DNA. The protection against this process may include a decrease in the intramitochondrial oxygen concentration or a decrease in the ubisemiquinone lifespan [1, 2].

It was shown earlier that the production of $O_2^{\cdot-}$ is negligible at low transmembrane potential ($\Delta\psi$) and exponentially increases with an increase in $\Delta\psi$. It was also found that the rate of $O_2^{\cdot-}$ generation is correlated with the degree of cytochrome b_{566} (b_l) reduction [3]. In view of this, it is interesting to note that plants have a complex system of uncoupled electron-transport pathways that bypass the generators of H^+ electrochemical-potential gradient ($\Delta\mu_{H^+}$) in the electron-transport chain (ETC). For example, in mitochondria of higher plants, NADH is oxidized not only by the ETC complex I, but also by a system of uncoupled NADH dehydrogenases located in the inner and outer mitochondrial membranes. The cyanide-resistant oxidase (alternative oxidase) is one of the best-studied systems of uncoupled respiration in plants [2, 4]. This enzyme, located in the inner mitochondrial membrane, catalyzes four-electron oxygen reduction at the expense of ubiquinol oxidation. During this process, energy is not saved in the form of $\Delta\mu_{H^+}$. Interestingly, the uncoupled alternative oxidase functions at higher $CoQH_2$-to-CoQ ratios than cytochrome oxidase [5]. This ratio is usually increased upon the transition from state 3 to state 4, when the risk of parasitic reactions of one-electron oxygen reduction increases [3]. The

Abbreviations: ETC) electron transport chain; SHAM) salicylhydroxamate; SOD) superoxide dismutase; ROS) reactive oxygen species.

* To whom correspondence should be addressed.

affinity of the alternative oxidase for oxygen is much lower than that of cytochrome oxidase [6, 7]. Apparently, alternative electron transport in plant mitochondria is activated when the oxygen concentration becomes greater than that optimum for the energy-conserving process. V. P. Skulachev assumed that one of the functions of uncoupled oxidation is to maintain the oxygen concentration at relatively low level [2]. This is one of the protective mechanisms preventing excessive generation of reduced oxygen species. For this reason, the data on the involvement of reactive oxygen species (ROS) in the induction of alternative oxidase are very important [8].

Thus, earlier it was postulated that the alternative oxidase plays a protective role under the conditions of oxidative stress [2, 8-10] and shown that salicylhydroxamate (SHAM) activates the H_2O_2 production by plant mitochondria [11]. However, data on a combined effect of the cytochrome-pathway inhibitors and alternative oxidase on the rate of generation of reduced oxygen species in plant mitochondria were missing. For this reason, the purpose of this work was to study the effect of the inhibitors of certain ETC components on the rate of generation of H_2O_2 and $O_2^{\cdot-}$ in plant mitochondria.

MATERIALS AND METHODS

Mitochondria were isolated from 8-9-day-old etiolated pea (*Pisum sativum* L.) seedlings. The plants were grown hydroponically in distilled water at 25°C. Mitochondria were isolated by differential centrifugation and centrifugation in a Percoll gradient. The plant material (50 g) was homogenized in 100 ml of isolation medium containing 20 mM HEPES-Tris buffer (pH 7.5), 1 mM EGTA, 0.4 M sucrose, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 0.2% fatty-acid-free BSA [8].

The rate of mitochondrial respiration was determined using a closed-type Clark electrode (Rank Brothers, UK). The incubation medium contained 20 mM HEPES-Tris (pH 7.5), 0.4 M sucrose, 5 mM KH_2PO_4, 150 μM ATP (to activate succinate dehydrogenase), 1 mM EGTA, and 1 mM $MgCl_2$. After addition of mitochondria (1-2 mg protein) to the 1-ml polarographic cell, their respiration was initiated by addition of the substrate, 5 mM succinate. Complex I of the ETC was inhibited by 2 μM rotenone; complex III, 1-2 μM antimycin A and 1 μM myxothiazol; cytochrome oxidase, 5 mM potassium cyanide; alternative oxidase, 0.1-1 mM SHAM; and ATPase, 2 μM oligomycin.

The rate of H_2O_2 production by the mitochondrial fraction was estimated fluorometrically using 2 μM 2,7-dichlorodihydroxyfluorescein diacetate (H_2DCF-DA) on an MPF-4 fluorimeter (Hitachi, Japan) [11]. The incubation medium contained 20 mM HEPES-Tris (pH 7.5), 0.4 M sucrose, 5 mM KH_2PO_4, 1 mM EGTA, 1 mM $MgCl_2$, 2 μM rotenone, and 1 μM oligomycin. Peroxidase (3 U per 1 ml incubation medium) conjugated with the dye was used as an auxiliary enzyme for the determination of hydrogen peroxide, and hydrogen peroxide was used as a substrate. The calibration curve was plotted based on the logarithmic dependence of the fluorescence intensity of the H_2O_2 concentration.

The rate of $O_2^{\cdot-}$ production was determined judging by the oxidation of epinephrine (assessed by the difference in absorbance at 480 and 579 nm) [12]. For this purpose, 1 mM epinephrine was added to the incubation medium (whose composition was similar to that of the medium for H_2O_2 determination). The tables show the SOD-sensitive component of the epinephrine oxidation.

All EPR spectra were recorded using an E-109E spectrometer (Varian, USA) under conditions of varied gas medium at room temperature (approximately 25°C). The mitochondria were incubated directly in the resonator of the spectrometer under continuous aeration of the specimen. The oxygen content was determined by the width of the components of the EPR spectrum of the nitroxyl radical TEMPONE-D-^{15}N (4-oxo-2,2,6,6-tetramethylpiperidine-D16-1-oxyl-^{15}N). The spectra were recorded under the following conditions: SHF power, 5 mW (for TRION) and 0.5 mW (for TEMPONE-D-^{15}N); SH frequency, 9.15 GHz; and amplitude of HF modulation, 0.05 mT. The absolute values of the rate of $O_2^{\cdot-}$ generation by mitochondria were determined using a model system of xanthine oxidase–xanthine by the intensity of the EPR signal from the spin trap TIRON. The xanthine oxidase–xanthine system was calibrated by measuring the rate of the cytochrome *c* reduction by $O_2^{\cdot-}$ on a Beckman spectrophotometer (USA). The mitochondrial protein concentration was determined using the biuret reaction.

The results were statistically processed using Student's *t* test. The differences between the experimental data were regarded significant at $p < 0.05$. The results are expressed as the mean ± standard error of the mean ($M \pm$ SEM).

RESULTS AND DISCUSSION

When studying the effect of the electron transport inhibitors on the rate of respiration and generation of ROS in pea cotyledon mitochondria, we found that respiration of mitochondria proceeds predominantly via the alternative oxidase. When 5 mM succinate was used as a substrate, 1 mM KCN decreased the respiration rate in pea mitochondria by only 20%. An inhibitor of the alternative oxidase, SHAM, completely suppressed residual oxygen consumption. The study of the extent of pea mitochondria coupling showed that their respiration

was activated by ADP or uncouplers. However, the uncoupling agent SF_{6847} at the concentration of 69 nM accelerated the rate of oxygen consumption only 2.5-fold. This finding may be accounted for by a high content of endogenous fatty acids, which are effective uncouplers, and a high proportion of uncoupled electron transport.

The study of the effect of the ETC inhibitors on the rate of hydrogen peroxide production by pea mitochondria showed that, in the case of utilization of endogenous respiration substrates, the rate of H_2O_2 production during respiration of pea mitochondria is 0.02 nmol/min per mg protein (Table 1). Addition of an exogenous substrate (5 mM succinate) to the incubation medium increased the initial rate of H_2O_2 production by mitochondria. Addition of 2 μM antimycin A increased the rate of H_2O_2 production 1.5-fold, and 2 μM rotenone only slightly changed the rate of this process.

Inhibition of the alternative oxidase with SHAM increased H_2O_2 production. In the presence of 5 mM succinate and 0.5 mM SHAM, it reached 2.8 nmol/min per mg protein, i.e., increased more than 15-fold compared to H_2O_2 production during succinate oxidation. Under these conditions, 2 μM antimycin A accelerated H_2O_2 production, whereas 1 mM cyanide blocked it (Table 1).

The production of H_2O_2 by pea mitochondria after partial heat-induced denaturation (70°C, 10 min) served as a control. This denaturation regimen was used to prevent alteration of the lipid composition of the membranes. The heat-treated mitochondria generated H_2O_2 at a low rate (0.05 nmol/min per mg protein). In this case, addition of rotenone, antimycin A, or SHAM only slightly changed the rate of H_2O_2 generation. Therefore, H_2O_2 was produced by mitochondria by the functioning of the enzyme systems that underwent denaturation as a result of heat treatment.

The study of the rate of $O_2^{\cdot-}$ generation by pea mitochondria using adrenochrome showed that addition of SHAM and antimycin A induced the production of $O_2^{\cdot-}$, whereas myxothiazol inhibited it. We also found that the transition of mitochondria to state 3 after addition of 150 μM ADP also significantly decreased the rate of $O_2^{\cdot-}$ generation (Table 2).

The analysis of $O_2^{\cdot-}$ generation by pea mitochondria using epinephrine was hampered by the high rate of background SOD-insensitive epinephrine oxidation. For this reason, $O_2^{\cdot-}$ was also detected using the spin-trap method. The rate of basal generation of $O_2^{\cdot-}$ during succinate oxidation was 0.24 nmol/min per mg protein. As shown in the figure, after addition of antimycin A the rate of $O_2^{\cdot-}$ generation increased to 0.8 nmol/min per mg protein, and the subsequent addition of the alternative oxidase inhibitor, SHAM, resulted in an almost fourfold increase in the rate of $O_2^{\cdot-}$ production (3.3 nmol/min per mg protein).

Table 1. Effect of electron transfer inhibitors on the rate of hydrogen peroxide production (nmol/min per mg protein) by pea mitochondria with 5 mM succinate as exogenous substrate

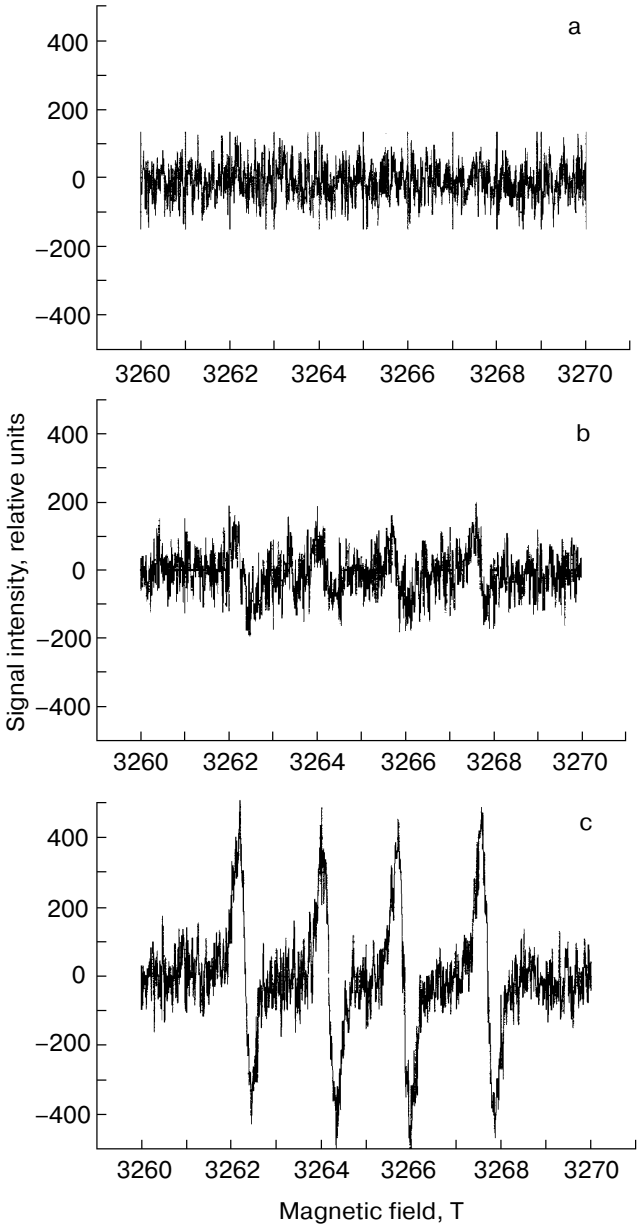
Inhibitor	Control	+ 2 μM antimycin A	+ 5 mM KCN
Without substrate and inhibitors	0.04 ± 0	0.03 ± 0	0 ± 0
Without inhibitors	0.18 ± 0.03	0.25 ± 0.04	0 ± 0
5 μM rotenone	0.15 ± 0.04	0.23 ± 0.06	0 ± 0
5 μM rotenone, 0.1 mM SHAM	0.55 ± 0.07	0.65 ± 0.08	0.10 ± 0
5 μM rotenone, 0.5 mM SHAM	2.80 ± 0.40	4.30 ± 0.54	0.50 ± 0.09

Table 2. Effect of inhibitors of electron transfer and transition from state 3 to state 4 on SOD-sensitive epinephrine oxidation by pea mitochondria with 5 mM succinate as exogenous substrate

Inhibitor	Respiration rate, nmol O_2/min per mg protein	Superoxide radical production, nmol/min per mg protein
Without inhibitors	41 ± 4	1.10 ± 0.18
5 μM rotenone	43 ± 4	1.02 ± 0.13
5 μM rotenone, 150 μM ADP	107 ± 14	0.36 ± 0.04
5 μM rotenone, 2 μM antimycin A	28 ± 2	2.40 ± 0.31
5 μM rotenone, 0.5 mM SHAM	13 ± 13	3.35 ± 0.23
5 μM rotenone, 1 μM myxothiazol	32 ± 5	0.46 ± 0.07

DISCUSSION

It was shown earlier that the production of reduced oxygen species by plant mitochondria is dramatically intensified when the alternative oxidase is inhibited [11]. However, the authors of [11] performed only qualitative determination of H_2O_2 and did not demonstrate that the H_2O_2 accumulation observed was directly associated with the $O_2^{\cdot-}$ generation. The experimental data presented in



EPR spectrometry analysis of the rate of the superoxide radical production by pea mitochondria oxidizing 5 mM succinate, after addition of (a) 5 μM rotenone, (b) 5 μM rotenone and 2 μM antimycin A, and (c) 5 μM rotenone and 0.5 mM SHAM

the work cited only quantitatively characterize the $O_2^{\cdot-}$ generation and its dismutation to H_2O_2.

The use of various methods of detection of H_2O_2 and $O_2^{\cdot-}$ showed that these measurements are hampered by many factors. First, we observed marked SOD-insensitive epinephrine oxidation by plant mitochondria, which led to a decrease in the signal-to-noise ratio at low rates of $O_2^{\cdot-}$ production. Under these conditions, it is more preferable to detect the EPR signal intensity using the TIRON spin trap. Upon an increase in the $O_2^{\cdot-}$ concentration, the data obtained using different methods were consistent. Second, the linear mode of dependence of the signal intensity during calibration of the systems H_2O_2–peroxidase–fluorescein diacetate and xanthine–xanthine oxidase–epinephrine was disrupted when measuring $O_2^{\cdot-}$ and H_2O_2 in isolated mitochondria. For example, after addition of SHAM to the incubation medium, the rate of H_2O_2 production increased 23-fold, whereas the rate of $O_2^{\cdot-}$ production increased only 12.8-fold (judging by the EPR data) and 3.6-fold (judging by the rate of epinephrine oxidation). We think that this fact may be due to the competition for $O_2^{\cdot-}$ between the probes used and SOD. An increase in the concentration of $O_2^{\cdot-}$ may result in the saturation of SOD, thereby increasing the probability of interaction between the radicals and the probe. In addition, a high activity of the systems of H_2O_2 detoxication, such as ascorbate peroxidase, catalase, and peroxidases are characteristic of plant mitochondria [13]. A considerable increase in the rate of $O_2^{\cdot-}$ production may results in exhaustion of endogenous antioxidant systems, thus leading to a nonlinear acceleration of H_2O_2 accumulation.

The presence of a complex system of detoxication of ROS may account for the observed deviation of the data from the ideal superoxide radical-to-hydrogen peroxide ratio of 2 : 1. Note that at low rates of $O_2^{\cdot-}$ generation, this value is greater than the predicted one. This means that either not all $O_2^{\cdot-}$ is transformed to H_2O_2 (e.g., due to its interaction with ascorbate) or H_2O_2 is rapidly detoxified by the functioning enzyme systems or interaction with antioxidants, which decreases the probability of interaction between $O_2^{\cdot-}$ and the detection system. After reaching the maximum rates of $O_2^{\cdot-}$ production, this ratio is close to 1.2-1.3, which is suggestive of accumulation of a considerable amount of H_2O_2 and increase in its accessibility for the peroxidase–fluorescein diacetate system.

When studying the effect of the ETC inhibitors on respiration of pea cotyledon mitochondria, we detected high content of alternative oxidase and confirmed the data that inhibition of this enzyme leads to a considerable increase in the rate of both H_2O_2 and $O_2^{\cdot-}$ production. Taking into account the aforementioned methodological problems, the analysis of these data led us to conclude that that main pathway of H_2O_2 production in plant mitochondria is the superoxide radical dismutation. Pea cotyledons exhibit a high rate of respiration processes, related to the involvement of mitochondria in the processes of gluconeogenesis from reserve fats. Gluconeogenesis requires high ATP/ADP and $NADH/NAD^+$ ratios, i.e., *in vivo* such mitochondria are apparently in state 4. This process can be accompanied by intensive generation of ROS for the following reasons. First, the rate of oxygen consumption by mitochondria under the conditions of inhibited cytochrome pathway decreases; as a result, the intramitochondrial concentration of $O_2^{\cdot-}$ increases. Second, flavins, ubiquinone, cytochromes *b*, and non-heme ferroproteins, which are

the main donors for one-electron reduction of oxygen, are in the reduced state [3]. Thus, the level of oxygen and reduced electron carriers increases, leading to accelerated production of the product of one-electron oxygen reduction, superoxide radical [14], which, as was shown earlier, can result in induction of the alternative oxidase. Wagner showed that H_2O_2 can induce the expression of the genes encoding alternative oxidase in petunia cells [8]. Later, a similar regulatory path was shown in tobacco cells [15]. The induction of the expression of the genes encoding alternative oxidase was also revealed when growing plant cells and yeast in the presence of antimycin A [16] (i.e., under the conditions when $O_2^{\cdot-}$ is actively produced).

A significant difference in the ROS generation in mitochondria from pea cotyledons and mitochondria isolated from animal tissues was observed when studying the inhibition of the ETC complex I with rotenone. In porcine heart mitochondria oxidizing succinate, rotenone inhibited the rate of ROS production [17]; however, in our experiments we observed no marked effect of this inhibitor. This may be accounted for by the fact that a considerable portion of NADH is oxidized in pea cotyledon mitochondria by the rotenone-insensitive NADH dehydrogenases [18], and the complex I content in them is much lower than in mitochondria from animal tissues.

The effect of the cytochrome pathway inhibitors on the ROS production by mitochondria from pea cotyledons and mitochondria isolated from animal tissues was similar. Antimycin A blocked the Q cycle functioning at the stage of electron transfer from cytochrome b_l to cytochrome b_h and, correspondingly, induced $CoQ^{\cdot-}$ accumulation. For this reason, in both animal and plant tissues antimycin A accelerated the ROS production. Interestingly, in pea mitochondria this effect was most pronounced in the case of the alternative oxidase inhibition, when both potential pathways of the electron transfer to oxygen by the ETC components are blocked. Turrens and Boveris [14] showed that the H_2O_2 production by porcine heart mitochondria is negligible in state 3 or in the presence of uncouplers but increases in state 4. In the case of pea cotyledon mitochondria, the transition from state 4 to state 3 significantly suppressed the H_2O_2 accumulation. Thus, it was shown that the rate of the reduced oxygen species generation depends not only on the overall level of reduction of the ETC carriers, but also on the $CoQ^{\cdot-}$ lifespan. The inhibitory effect of cyanide is apparently due to its ability to completely inhibit cytochrome oxidase, thus leading to overreduction of the FeS_{III} cluster of complex III. In this case, ubisemiquinone is not produced, and ubiquinol is a less effective one-electron oxygen reducer.

Based on these data, we conclude that, under the conditions of simultaneous functioning of the cytochrome pathway and alternative oxidase in plant mitochondria in state 4, ubiquinol ($CoQH_2$) is preferentially oxidized by the alternative oxidase. This process prevents the ubisemiquinone accumulation and, therefore, prevents intensive ROS generation in plant mitochondria.

Thus, we showed that the ETC inhibitors markedly affect the rate of ROS production in plant mitochondria. The inhibition of alternative oxidase and block of the Q cycle function between cytochromes b_l and b_h results in significant activation of ROS production. Conversely, myxothiazol and cyanide decrease the intensity of this process.

This study was supported by the CRDF, the Russian Foundation for Basic Research (project No. 01-04-48431), and the Program "Universities of Russia" (No. UR.07.01.043).

REFERENCES

1. Skulachev, V. P. (1994) *Biochemistry* (Moscow), **59**, 1433-1435.
2. Skulachev, V. P. (1996) *Quart. Rev. Biophys.*, **29**, 169-202.
3. Liu, S. S., and Huang, J. P. (1996) *Proc. Int. Symp. on Natural Antioxidants: Molecular Mechanisms and Health Effects* (Moores, D., ed.) AOCS Press, Champaign, IL, pp. 112-118.
4. Wagner, A. M., and Moore, A. L. (1997) *Biosci. Rep.*, **17**, 319-333.
5. Van den Bergen, C. W., Wagner, A. M., Krab, K., and Moore, A. L. (1994) *Eur. J. Biochem.*, **226**, 1071-1078.
6. Solomos, T. (1997) *Ann. Rev. Plant Physiol.*, **28**, 279-297.
7. Millar, A. N., Bergersen, F. J., and Day, D. A. (1994) *Plant Physiol. Biochem.*, **32**, 847-852.
8. Wagner, A. M. (1995) *FEBS Lett.*, **368**, 339-342.
9. Purvis, A. C., and Shewfelt, R. L. (1993) *Physiol. Plant.*, **88**, 712-718.
10. Purvis, A. C. (1997) *Physiol. Plant.*, **100**, 165-170.
11. Popov, V. N., Simonyan, R. A., Skulachev, V. P., and Starkov, A. A. (1997) *FEBS Lett.*, **415**, 87-90.
12. Purvis, A. C., Shewfelt, R. L., and Gegogeine, J. W. (1995) *Physiol. Plant.*, **94**, 743-749.
13. Mackenzie, S., and McIntosh, L. (1999) *Plant Cell*, **11**, 571-579.
14. Turrens, J. F., and Boveris, A. (1980) *Biochem. J.*, **191**, 421-427.
15. Vanlerberghe, G. C., and McIntosh, L. (1994) *Plant Physiol.*, **105**, 867-874.
16. Minagawa, N., Koga, S., Nakano, M., Sakajo, S., and Yoshimoto, A. (1992) *FEBS Lett.*, **302**, 217-219.
17. Boveris, A., and Chance, B. (1973) *Biochem. J.*, **134**, 707-716.
18. Moller, I. M. (2002) *Trends Plant Sci.*, **7**, 235-237.