Mitochondrial electron transport as a source for nitric oxide in the unicellular green alga *Chlorella sorokiniana*

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Abstract **Wild type (WT), and nitrate reductase (NR)- and nitrite-reductase (NiR)-deficient cells of *Chlorella sorokiniana* were used to characterize nitric oxide (NO) emission. The NO emission from nitrate-grown WT cells was very low in air, increased slightly after addition of nitrite (200 μM), but strongly under anoxia. Importantly, even completely NR-free mutants, as well as cells grown on tungstate, emitted NO when fed with nitrite under anoxia. Therefore, this NO production from nitrite was independent of NR and other molybdenum cofactor enzymes. Cyanide and inhibitors of mitochondrial complex III, myxothiazol or antimycin A, but not salicylhydroxamic acid (inhibitor of alternative oxidase) inhibited NO production by NR-free cells. In contrast, NiR-deficient cells growing on nitrate accumulated nitrite and emitted NO at very high equal rates in air and anoxia. This NO emission was 50% inhibited by salicylhydroxamic acid, indicating that in these cells the alternative oxidase pathway had been induced and reduced nitrite to NO.**

Keywords: Nitric oxide; Mitochondria; *Chlorella sorokiniana*

1. Introduction

Nitric oxide (NO) has emerged as a signalling molecule not only in animals but also in plants. This membrane permeable compound is involved in the regulation of plant metabolism, gene expression (review in [1]) and in pathogen responses causing hypersensitive cell death [2–7]. There are several potential sources of NO in plants, probably depending on plant organ and on the physiological situation of the organism. A well established NO producing system in plants is assimilatory nitrate reductase (NR), which is to some extent structurally similar to NOS from animal tissues in that it contains a heme and a NAD(P)H/FAD binding domain. Aside of reducing nitrate to nitrite, NR catalyzes (though with much lower capacity) the reduction of nitrite to NO at the expense of NADH. This reaction appears to occur not only in higher plants, but also in green algae and cyanobacteria [8–10]. A special form of plasma membrane-bound NR appears associated with a nitrite:NO oxidoreductase in plant roots. Xanthine oxidase/dehydrogenase has been suggested as another molybdenum cofactor (MoCo)-enzyme able to produce NO from nitrite under anoxic conditions [10–12]. Further, the long-discussed existence of an inducible plant nitric oxide synthase (NOS)-like enzyme appears now well established [13,14], although in algae evidence was negative [8]. Finally, a non-enzymatic chemical reduction of nitrite to NO has been proposed already decades ago, but should occur only under strongly reducing and/or acidic conditions, which may contribute, e.g., to NO production in the plant cell apoplast, as recently suggested [15].

Here, we present evidence for *Chlorella* mitochondria being an additional source of NO emission.

2. Materials and methods

2.1. Plant material

Chlorella sorokiniana cells (algae collection Göttingen, Germany) were cultivated as described earlier [16]. Briefly, the cells were resuspended in 10 mM KNO_3 or 5 mM $(NH4)_2SO_4$, 5 mM NaH_2PO_4, 0.5 mM Na_2HPO_4, 1 mM $MgSO_4$, 0.01 mM $CaCl_2$, 1 μM H_3BO_4, $MnSO_4$, $ZnSO_4$, 10 nM $CuSO_4$ and 1 μM $(NH_4)_6Mo_7O_{24}$ or 100 μM Na_2WO_4 and illuminated for 7 h followed by 5 h darkness. Under these conditions, the physiological state of the cells was maintained but still variations in physiological responses are obvious (see Fig. 2A and B vs. C and D). The *Chlorella* mutants (NR- or NiR-deficient) were obtained after X-ray irradiation followed by selection of pin-point colonies for further selection on specific media as described before [17,18]. The cells (mutants and wild type) were pre-cultivated on ammonium medium, washed twice with distilled water and transferred to nitrate medium for one life cycle. All cells were illuminated for 4 h before harvest for the experiments. In the case where molybdate (1 μM) was substituted by tungstate (100 μM) in the medium, the cells were cultivated for at least three life cycles in tungstate containing medium before the experiments.

2.2. Gas phase NO measurements

NO production by *Chlorella* was measured as NO emission from algal suspensions into the gas phase. An aliquot of the suspension (10 ml, containing algae equivalent to 60 μg chlorophyll) in a small glass beaker was placed in a transparent lid chamber (1 l total gas volume) on a magnetic stirrer.

A constant flow of measuring gas (purified air or nitrogen) of 1.5 l/min was pulled through the chamber and subsequently through the chemiluminescence detector (CLD 770 AL ppt, Eco-Physics, Dürnten, Switzerland, detection limit 20 ppt; 20 s time resolution) by a precision vacuum pump connected to an ozone destroyer. The ozone generator of the chemiluminescence detector was supplied with dry oxygen

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Abbreviations: AOX, alternative oxidase; COX, cytochrome *c* oxidase; L-NAME, L-N^6-Nitroargenine methyl ester; MoCo, molybdenum cofactor; NOS, nitric oxide synthase; SHAM, salicyl hydroxamic acid

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(99%). The measuring gas (air or nitrogen) was made NO free by conducting it through a custom-made charcoal column (1 m long, 3 cm internal diameter, particle size 2 mm). Calibration was routinely carried out with NO-free air and with various concentrations of NO (1–35 ppb) adjusted by mixing the calibration gas (100 ppb NO in nitrogen, Messer Griesheim, Darmstadt, Germany) with NO-free air. The reproducibility of the measurements was ±15–20% and the calibration varied by 2% in 24 h [19]. Flow controllers (FC-260, Tylan General, Eching, Germany) were used to adjust all gas flows. A microprocessor calculates the NO signal in ppb. Custom-made software based on Visual Designer (PCI-20901SS, Ver. 4.0, Tuscon, Arizona, USA) was used to process the data.

Light was provided by a 400 W Hqi-lamp (Schreder, Winterbach, Germany) above the cuvette. Quantum flux density was 250 μmol m^2 s^{-1} PAR. Air temperature in the cuvette was continuously monitored, and was usually about 20 °C in the dark and 23–25 °C in the light.

3. Results

3.1. WT Chlorella cells emit NO at high rates only when fed with nitrite under anoxia

In air and darkness, NO emission from a wild type (WT) *Chlorella* cell suspension was very low ($\leqslant 0.2$ nmol mg^{-1} chlorophyll h^{-1}, Fig. 1). Upon illumination, NO emission increased, reaching a steady rate of 0.5–1 nmol mg^{-1} chlorophyll h^{-1}. Upon flushing with nitrogen, NO emission in the dark remained low, contrary to what has been observed with leaves [20,21]. However, when cells were supplied with nitrite (200 μM) under anoxia, NO emission increased rapidly reaching rates up to 100 times higher than without nitrite addition (Figs. 1–3). A subsequent change from anoxia to aerobic conditions decreased NO emission almost immediately and strongly. Maximum rates of NO emission in different experiments varied depending upon the physiological state of the cells used (see: Section 2), although the responses were completely reproducible.

3.2. Mitochondrial electron transport can produce NO from nitrite

NR has been shown previously to be the major source for NO in higher plants [20,22,23] and in algae [8–10]. But as mentioned above, other NO sources have to be taken into account. To investigate the participation of NR in nitrite dependent, anoxic NO production, we used a *Chlorella* mutant lacking NR completely (Fig. 2A), and we also used these NR-deficient cells grown on tungstate instead of molybdenum, which results in cells without any other active MoCo-enzyme, e.g., xanthine dehydrogenase (XDH) (Fig. 2B). With both, the NR-deficient mutant and the tungstate grown mutant cells supplied with nitrate only, practically no emission of NO was detected in air or in nitrogen. Unexpectedly, however, after addition of nitrite, both emitted NO under anoxia in the dark at similar or even higher rates than WT cells (Fig. 2A and B).

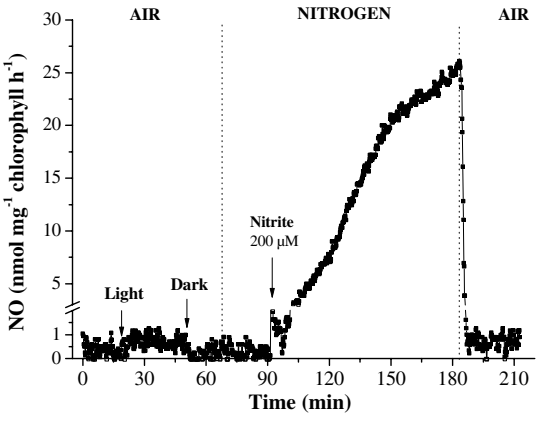

Fig. 1. NO emission from *Chlorella* wild type cells. The dotted lines indicate the changes from normoxia to anoxia and vice versa. A representative curve out of four repetitions is presented.

Generally, nitrite-dependent NO emission by the algae was totally insensitive to the NOS-inhibitor L-NAME (10 mM, not shown).

When the algae were illuminated while producing NO from exogenous nitrite under anoxia in darkness, NO emission was largely prevented (Fig. 2A and B), whereas a small increase of NO emission after illumination was found without nitrite addition (Figs. 1, 2B and C). NO emission increased again in darkness following a short light phase (Fig. 2A and B).

The unknown NO-source was completely and rapidly inhibited by cyanide, a rather unspecific inhibitor of heme containing enzymes including NR and cytochrome *c* oxidase (COX) in both genotypes [24]. While this cyanide effect is not helpful for identifying the unknown source of NO, it enabled exclusion of chemical conversion of nitrite as a source for NO, under these conditions.

Due to the efficient inhibition of nitrite dependent, but NR- (and XDH)- independent NO production by cyanide, and also because of the high oxygen sensitivity of this process, we assumed mitochondria to participate in NO production. Therefore, we used more specific inhibitors of the mitochondrial electron transport chain to verify this possibility. We found no inhibition of NO production by rotenone (1 μM), which blocks complex 1 of the respiration chain, but not external/internal NADH oxidases (data not presented). Two other inhibitors of the respiratory electron transport chain at complex III, myxothiazol (3 μM, Fig. 2C) or antimycin A (100 μM, Fig. 2D), efficiently inhibited NO production.

3.3. In nitrite reductase-deficient cell lines with continuous high NO production, the AOX pathway is induced which also reduces nitrite to NO

It has been previously suggested that NO could induce the alternative oxidase (AOX) pathway by inhibiting COX [25]. In order to test for a participation of the alternative electron transport pathway, we used salicylhydroxamic acid (SHAM, 1 mM) or propylgallate (20 μM), both inhibitors of the AOX and found no inhibition of NO emission by WT cell lines (not shown). However, we also examined the nitrite reductase (NiR)-deficient-mutant, which, when grown on nitrate, always accumulates and releases nitrite into the medium where concentrations may reach up to 3–4 mM [18]. Accordingly, these cells continuously emitted NO with very high rates, even in air (Fig. 3). Here, SHAM caused a partial inhibition (50%) of NO emission. The remaining SHAM-insensitive NO emission was only slightly sensitive to myxothiazol, but was completely abolished by cyanide. As in the nitrite-fed WT cells (Fig. 2B), the high NO emission of NiR-deficient mutants in the dark was partly decreased by light (not shown).

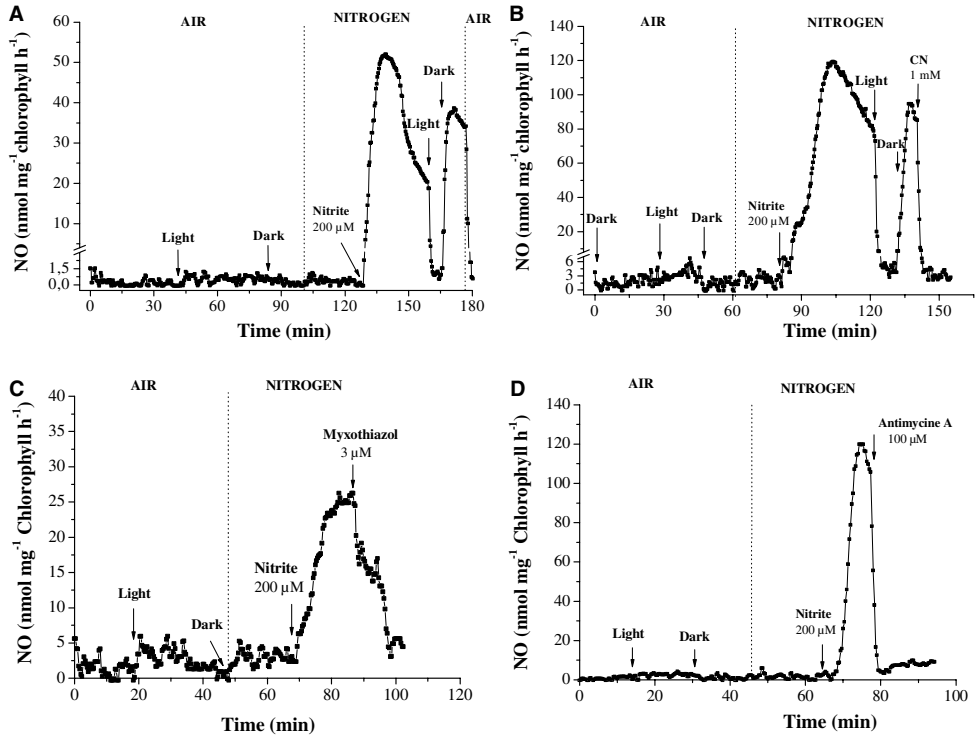

Fig. 2. (A) NO emission from *Chlorella* mutant lacking NR. The dotted lines indicate the changes from normoxia to anoxia and vice versa. (B) NO emission from *Chlorella* mutant lacking NR grown in the presence of tungstate, which replaced molybdate in the medium, for three life cycles. The addition of cyanide completely inhibited NO emission. (C) NO emission from *Chlorella* mutant lacking NR. The addition of myxothiazol inhibited NO production completely. (D) NO emission from *Chlorella* mutant lacking NR. The addition of antimycin A inhibited NO production completely. The dotted line indicates the change from normoxia to anoxia. All data are representatives out of 3–5 repetitions.

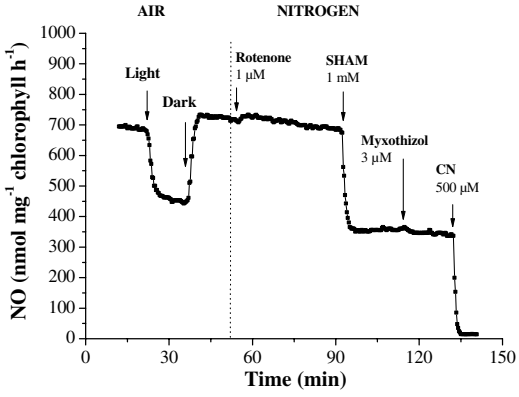

Fig. 3. NO emission from *Chlorella* mutant lacking NiR. These cells accumulate high nitrite concentrations in the medium (up to 5 mM). This is due to an intact nitrate uptake system and NR. The addition of rotenone and myxothiazol had only small effects. That of SHAM and cyanide inhibited strongly and additively. In these cells the contribution of both, mitochondria and NR, to NO emission is obvious. The dotted line indicates the change from normoxia to anoxia. Presented is a representative measurement out of three repetitions.

4. Discussion

Previous reports have indicated that in unicellular algae NO production was exclusively due to NR activity [8–10] under normoxia. A similar conclusion has been reached for leaves of higher plants [20,22,23], although in *Arabidopsis* a NOS has been presented recently [14]. Another NOS-like activity has been identified as being part of the mitochondrial glycine-decarboxylase complex [13]. In addition, in tobacco roots yet another enzyme linked to the plasma membrane (not NR) exists that has been suggested to produce NO from nitrite and NADH [26,27].

Chlorella cells produced a considerable amount of NO only under anaerobic conditions. This NO production occurred in darkness and was strictly dependent on nitrite (200 μM) supply (Fig. 1). As the NOS-inhibitor L-NAME (10 mM) had no effect on (nitrite-dependent) NO emission (not shown), we conclude that *Chlorella* cells could not produce NO from any other source except nitrite, and that a NOS-like activity was not involved. This is consistent with previous observations on NO production by algae [8–10].

A change from anoxia to aerobic conditions reduced NO emission from WT and from NR-deficient-mutant cells immediately and drastically (Fig. 1). Reduction of NO emission in air or by light might be either due to an inhibition of NO production or to an oxidative quenching of NO. In that context, it is important that with the NiR deficient-mutant nitrite-saturated NO emission was as high in air as in nitrogen (Fig. 3). This suggests that oxidative NO quenching was too low to be responsible for the very low rates of aerobic NO emission observed with WT or NR deficient-cells.

Another possibility might be a substrate competition of oxygen with nitrite. Such a competition could occur on the

MoCo-domain of NR, which reduces not only nitrate to nitrite, but also – with somewhat lower rates – nitrite to NO [23] and oxygen to superoxide [28], which might cause oxidative quenching. This situation might be especially valid in the NiR deficient-mutant. A competition could also occur at the mitochondrial COX or AOX, which preferentially reduce oxygen (see below). The fact that NiR-deficient cells with very high endogenous nitrite concentrations (up to 5 mM, not shown) gave no apparent inhibition of NO emission in air suggests that the effect of oxygen (air) was a competitive one and not based on NO quenching. As another explanation, we speculate that reduction of nitrite to NO may be co-limited by NADH and nitrite under normoxia. Under anoxia, where NO emission is at maximum, NADH and nitrite concentrations should be much higher than in air as indicated by the production of fermentation products.

Results obtained with the NR deficient-mutant [17] indicate that at least under anoxia NR is not the only source of NO. Inhibition of NO emission by antimycin A and myxothiazol suggests that mitochondrial electron transport also reduces nitrite to NO, and that the flow of electrons from complex III to complex IV via cytochrome *bc* is involved in nitrite reduction, at least in WT and NR deficient-cells. Anoxia inhibits COX reaction and an electron leakage occurs upstream of complex III [29].

Further, plant mitochondria possess two terminal oxidases, COX and an AOX. These compete for electrons, with inhibition of one pathway redirecting e^--flux to the other. NO emission was by far highest from a mutant lacking NiR. The major reason was the high concentration of nitrite accumulating in these cells, which still have an intact NR (Fig. 3). This very high NO emission was inhibited to about 50% by SHAM, and myxothiazol had no effect, suggesting that here, nitrite was reduced to NO mainly via the AOX pathway. Under anoxia, cells produce NADH via glycolysis and should accumulate pyruvate. These conditions induce AOX activity [30–33] and remarkably, NO has been shown to induce AOX expression [34]. As NO is an potent inhibitor of COX but not of AOX [25], NO may contribute to AOX induction by inhibiting COX. An inactivation of NR, here in the case of a lack of NR, also stimulates AOX expression in Chlamydomonas [35]. In addition, inhibition of aconitase by NO will lead to accumulation of citrate which in turn stimulates AOX synthesis [32].

From these data, we conclude that plant mitochondria can produce NO under anoxia and sufficient nitrite supply. These prerequisites may be faced, e.g., by plant roots under water logging conditions, or by aqueous plants and algae in eutrophic waters. Under hypoxic or anoxic stress, increasing NO levels have been reported for alfalfa roots [36] and for darkened leaves [21]. Limited oxygen supply activates NR and partially inhibits nitrite reduction [37], which appears a prerequisite for high anoxic NO production. As we demonstrate, mitochondria can reduce nitrite to NO which may be a signal triggering metabolic adaptation to low oxygen tension, including induction of AOX. Recent reviews on NO signalling in plants have been presented in [1,38]. Thus, plant mitochondria may be more important for cellular NO production and NO signalling than previously thought.

The reaction type of nitrite reduction to NO is similar to that found in denitrifying bacteria, which have cytochromes (*cd1*, *bc*) in their internal membrane. Such cytochromes can be found in eukaryotic mitochondria and thus this type of mitochondrial nitrite reduction may be considered as a heritage from bacterial ancestors.

NO has been shown to function as an endogenous regulator of mitochondrial electron transport and oxidative phosphorylation in mammalian cells [39]. NO production by mammalian mitochondria under normoxia has also been described [40] and the sensitivity to NOS inhibitors and to L-arginine pointed to a mitochondrial NOS as an NO source. However, Nohl et al. and Kozlov et al. [41,42] demonstrated the production of NO by isolated rat liver mitochondria under strictly controlled anoxia after nitrite supply. Myxothiazol but not L-NAME inhibited NO production. Also, in these experiments an electron leak from succinate fed mitochondria (state 3) to external nitrite was observed. The nitrite necessary for these reactions came from inflammatory or ischemic processes.

Further research will focus on intact higher plant mitochondria as such a preparation is not successfully possible from *Chlorella* due to the thick cell wall.

References

[1] Lamattina, L., García-Mata, C., Graziano, M. and Pagnussat, G. (2003) Annu. Rev. Plant Biol. 54, 109–136.
[2] Yamamoto, A., Katou, S., Yoshioka, H., Doke, N. and Kawakita, K. (2003) J. Gen. Plant Pathol. 69, 218–229.
[3] Polverari, A., Molesini, B., Pezzotti, M., Buonaurio, R., Marte, M. and Delledonne, M. (2003) Mol. Plant Microbe Interact. 16, 1094–1105.
[4] Romero-Puertas, M.C. and Delledonne, M. (2003) IUBMB-Life 55, 579–583.
[5] Delledonne, M., Xia, Y., Dixon, R.A. and Lamb, C. (1998) Nature 394, 585–588.
[6] Delledonne, M., Polverari, A. and Murgia, I. (2003) Antioxid Redox Signal 5, 33–41.
[7] Zhang, C., Czymmek, K.J. and Shapiro, A.D. (2003) Mol. Plant Microbe Interact. 16, 962–972.
[8] Mallick, N., Mohn, F.H., Rai, L.C. and Soeder, C.J. (2000) J. Plant Physiol. 156, 423–426.
[9] Mallick, N., Mohn, F.H., Rai, L. and Soeder, C.J. (2000) J. Microbiol. Biotechnol. 10, 300–306.
[10] Sakihama, Y., Nakamura, S. and Yamasaki, H. (2002) Plant Cell Physiol. 43, 290–297.
[11] Godber, B.L.J., Doel, J.J., Saptoka, G.P., Blake, D.R., Stevens, C.R., Eisenthal, R. and Harisson, R. (2000) J. Biol. Chem. 275, 7757–7763.
[12] Li, H., Samouilov, A., Liu, X. and Zweier, J.L. (2001) J. Biol. Chem. 276, 24482–24489.
[13] Chandok, M.R., Ytterberg, A.J., van Wijk, K.J. and Klessig, D.F. (2003) Cell 113, 469–482.
[14] Guo, F.Q., Okamoto, M. and Crawford, N.M. (2003) Science 302, 100–103.
[15] Bethke, P.C., Badge, M.R. and Jones, R.L. (2004) Plant Cell 16, 332–341.
[16] Koltermann, M., Moroni, A., Gazzarini, S., Nowara, D. and Tischner, R. (2003) Plant Mol. Biol. 52, 855–864.
[17] Knobloch, O. and Tischner, R. (1989) Plant Physiol. 89, 786–791.
[18] Burhenne, N. and Tischner, R. (2000) Planta 211, 440–445.
[19] Planchet, E., Stoimenova, M., Sonoda, M., and Kaiser, W.M. (in press) in: Magalhaes, J.P., Singh, R.P., Passos, L.P., (Eds.), Nitric oxide signalling in higher plants. LCC, Houston, Texas, USA: Studium Press.
[20] Rockel, P. and Kaiser, W.M. (2002) In: Prog. Botany, 63, pp. 246–257, Springer-Verlag, Berlin.
[21] Rockel, P., Strube, F., Rockel, A., Wildt, J. and Kaiser, W.M. (2002) J. Exp. Bot. 366, 103–110.

[22] Desikan, R., Griffiths, R., Hancock, J. and Neill, S. (2002) PNAS 99, 16314–16318.
[23] Yamasaki, H. and Sakihama, Y. (2000) FEBS Lett. 468, 89–92.
[24] Dean, J.V. and Harper, J.E. (1988) Plant Physiol. 88, 389–395.
[25] Millar, A.H. and Day, D.A. (1996) FEBS Lett. 398, 155–158.
[26] Stöhr, C., Strube, F., Marx, G., Ullrich, W.R. and Rockel, P. (2001) Planta 212, 835–841.
[27] Meyer, C. and Stöhr, C. (2002) in: Advances in Photosynthesis and Respiration (Foyer, C.H. and Noctor, G., Eds.) Photosynthetic Nitrogen Assimilation and Associated Carbon and Respiratory Metabolism, Vol. 12, pp. 49–62, Kluwer Academic Publishers, New York.
[28] Barber, M.J. and Kay, C.J. (1996) Arch. Biochem. Biophys. 15, 227–232.
[29] Hatefi, Y. and Yagi, T. (1982) Biochemistry 21, 6614–6618.
[30] Hoefnagel, M.H.N., Rich, P.R., Zhang, Q. and Wiskich, J.T. (1997) Plant Physiol. 115, 1145–1153.
[31] Szal, B., Jolivet, Y., Hasenfratz-Sauder, M.P., Dizengremel, P. and Rychter, A.M. (2003) Physiol. Plant. 119, 494–502.
[32] Vanlerberghe, G.C. and McIntosh, L. (1996) Plant Physiol. 111, 589–595.
[33] Valerberghe, G.C. and Ordog, S.H. (2003) in: Advances in Photosynthesis and Respiration (Foyer, C.H. and Noctor, G., Eds.) Photosynthetic Nitrogen Assimilation and Associated Carbon and Respiratory Metabolism, Vol. 12, pp. 173–191, Kluwer Academic Publishers, New York.
[34] Huang, X., von Rad, U. and Durner, J. (2002) Planta 215, 914–923.
[35] Baurain, D., Dinant, M., Coosemans, N. and Matagne, R.F. (2003) Plant Physiol. 131, 1418–1430.
[36] Dordas, C., Hasinoff, A.U., Manach, N., Rivoal, J. and Hill, R.D. (2003) Plant J. 35, 763–770.
[37] Botrel, A. and Kaiser, W.M. (1997) Planta 201, 496–501.
[38] Neill, S.J., Desikan, R. and Hancock, J.T. (2003) New Phytol. 159, 11–35.
[39] Bates, T.E., Loesch, A., Burnstock, G. and Clark, J.B. (1996) Biochem. Biophys. Res. Commun. 218, 40–44.
[40] Giulivi, C., Poderoso, J.J. and Boveris, A. (1998) J. Biol. Chem. 273, 11038–11043.
[41] Nohl, H., Staniek, K., Sobhian, B., Bahrami, S., Redl, H. and Kozlov, A.V. (2000) Acta Bioch. Pol. 47, 913–921.
[42] Kozlov, A.V., Staniek, K. and Nohl, H. (1999) FEBS Lett. 454, 127–130.