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Higher Plant Mitochondria as a Source for NO

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Abstract Higher plant mitochondria produce nitric oxide (NO) by two separate systems. One is a mitochondrial nitric oxide synthase (NOS), which catalyzes the synthesis of NO and L-citrulline from L-arginine using NAD(P)H. The other one is the respiratory electron transport chain, with the terminal oxidases, CytOx and AOX, which both reduce nitrite to NO. While oxygen is obligatory for the former reaction, the latter activity appears very low in air but high under oxygen deficiency. However, even under anoxia, the rate of nitrite:NO reduction rarely reaches ±1% of respiratory electron transport. For as yet unknown reasons, nitrite:NO reduction appears absent in mitochondria from green leaves. The contribution of NOS and of nitrite reduction to overall NO production, and possible functions of nirite:NO reduction under hypoxia/anoxia are discussed.

1 Introduction

Mitochondria are known as the organelles housing the citric acid cycle and membrane systems for respiratory electron transport and oxidative phosphorylation. The basic structural similarity of eukaryotic mitochondria reflects an overall similarity in these basic functions. However, in addition, mitochondria serve many more important purposes, such as synthesis of vitamins, cofactors, nucleotides, metabolism of organic acids, amino acids, lipids, and partial reactions of the photorespiratory cycle (Rebeille et al. 1997; Bartoli et al. 2000; Gueguen et al. 2000; Kowaltkowski 2000). Further, mitochondria appear to play an important role in programmed cell death (Balk et al. 1999; Balk and Leaver 2001; Lam et al. 2001; also compare the contributions in this book) and are a major source for reactive oxygen species (ROS) (for review see Møller 2001). These multiple functions are not expected to occur to the same extent in all plant organs, but are more or less expressed in specific organs, cell types, and developmental stages. Recently, evidence has been accumulating that mitochondria are also involved in two apparently opposed processes, namely the production, but also the consumption of nitric oxide (NO). As NO has gained increasing attention in plants as a signaling compound as well as a highly reactive modifying agent for biomolecules, including proteins, this new mitochondrial function appears potentially im-

portant. Thus, we will briefly summarize present knowledge on NO in context with mitochondria.

2 NO Production

Plants appear to produce NO by two basically different pathways (also compare Fig. 1):

1. The L-arginine-dependent pathway uses NAD(P)H and O_2 as cosubstrates and is catalyzed by nitric oxide synthase (NOS) according to reaction (1):

$$\text{L-Arg} + \text{NAD(P)H} + \text{H}^+ + \text{O}_2 \rightarrow \text{L-Citr} + \text{NAD(P)}^+ + \text{NO}\,. \quad (1)$$

2. The nitrite–dependent pathway uses NADH or "electrons" as reductands and is catalyzed by a number of different enzymes according to reaction (2):

$$\text{NO}_2^- + \text{e}^- + 2\text{H}^+ \rightarrow 2\text{NO} + \text{H}_2\text{O}\,. \quad (2)$$

In plants, reduction of nitrite to NO was originally thought to be only catalyzed by nitrate reductases (NR). Xanthine oxidase/dehydrogenase (XDH)

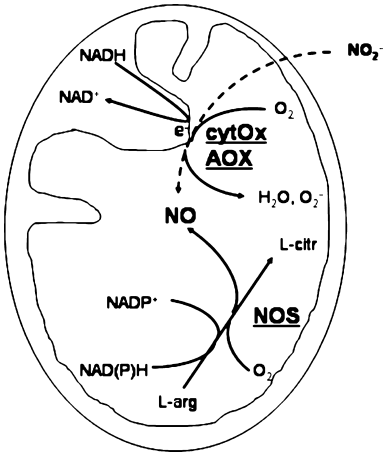

Fig. 1 Reactions producing NO in plant mitochondria. Nitric oxide synthase (NOS) has been shown in *Arabidopsis* to produce NO from L-arginine imported from the cytosol. NOS (AtNOS1) is probably integrated into the inner membrane. NO can be also produced by mitochondrial electron transport, reducing nitrite to NO (the formation of NO from NO_2^- does not require oxygen and is represented by a *dotted arrow*). The reaction is hardly detectable in air. One possible explanation is competition with oxygen. For further details see text. *Cyt ox* cytochrome oxidase, *AOX* alternative oxidase

has also been occasionally suggested as a source for NO using nitrite and xanthine as a substrate (Millar et al. 1998; Godber et al. 2000). However, our own experiments, using recombinant XDH, gave no evidence for NO production by the enzyme itself (Mendel and Kaiser, unpublished). Non-enzymatic NO production from nitrous acid according to Eq. 3 should occur at significant rates only at pH values below pH 5 (pK_a 3.2 of nitrous acid).

$$2HNO_2 \leftrightarrow NO + NO_2 + H_2O \leftrightarrow 2NO + 1/2O_2 + H_2O\,. \quad (3)$$

Conditions favoring non-enzymatic NO formation are probably rare, but occasionally they may be met in the apoplast of plant cells (Bethke et al. 2004) and perhaps also in the vacuoles.

Overall, the contribution of XDH and of non-enzymatic NO formation to overall plant NO production seems negligible compared to the contribution of NR. However, according to more recent research, mitochondria are another important source for NO in plants and indeed both reactions (1) and (2) appear to occur inside plant mitochondria, as will be shown.

2.1 Nitric Oxide Synthase is Located in the Mitochondria

The above-mentioned nitric oxide synthase (NOS) reaction was suggested as a source for NO in plants, mainly based on pharmacological evidence. Inhibition of NO formation or of NO-dependent reactions by chemical analogs of L-arginine is usually taken as an indication that the reaction was triggered by NOS-derived NO. Immunological evidence for NOS in plants was obtained with antibodies against animal NOS (Kuo et al. 1995; Sen and Chema 1995; Barroso et al. 1999; Ribiero et al. 1999), but those antibodies proved to be rather unspecific (Lo et al. 2000; Butt et al. 2003). As no *Arabidopsis* gene or protein homolog to the large and complex animal protein has yet been found, the existence of NOS in plants is still an enigma.

More recently, a breakthrough in NO research was achieved by the finding of the Crawford group (Guo et al. 2003; Crawford and Guo 2005) that *Arabidopsis* contains a gene with sequence similarity to a gene from *Helix pomatia* that is implicated in NO synthesis. The gene encodes a 60 kDa protein, which, when expressed in *E. coli*, increased NO synthesis in cell extracts. When the corresponding gene (*AtNOS1*) was knocked out in *Arabidopsis*, the resulting mutant had reduced NO production in roots (measured with DAF-2DA). Contrary to animal NOS (about 140 kDA), the much smaller AtNOS1 requires no flavin or tetrahydrobiopterin, but only Ca^{2+}, CaM and NADPH. AtNOS1 seems constitutively expressed. It has been suggested to be part of the signaling pathway involved in ABA-induced stomatal closure, germination, root and shoot growth, seed fertility (for review Crawford and Guo 2005), control of flower timing (He et al. 2004), senescence and protection against oxidative damage (Guo and Crawford 2005), and seems also involved

in NO production during plant–pathogen interactions, as derived from experiments with DAF-FM DA and EPR (Zeidler et al. 2004; Guo and Crawford 2005). Also, in the *atnos1* knock out mutant, induction of defence-related genes by *Pseudomonas syringae* was suppressed compared to the wild type (Zeidler et al. 2004). All these data suggest that AtNOS1, despite its different molecular properties, has functions analogous to animal NOS, but without the requirement for tetrahydrobiopterin as cofactor.

The first report on mitochondrial localization of NOS was by Giulivi et al. (1998), who detected NOS activity in purified animal mitochondria, mitochondrial homogenates, and submitochondrial particles, using EPR and oxyhemoglobin to detect NO. Indeed, NOS activity of animal mitochondria appears located in the inner mitochondrial membrane (Ghafourifar and Richter 1997).

Very recent work by Crawford's group indicates that plant AtNOS1, like the animal enzyme, is also located in the mitochondria (compare Fig. 1). This view was based on the following lines of evidence:

- Computational analysis of the NOS1 protein sequence reported a high probability of being targeted to the mitochondria
- Fluorescence from a p35S-NOS1cDNA-GFP construct strongly overlapped with MitoTracker fluorescence in mitochondria of roots and root hairs examined by confocal microscopy
- NO production in mitochondria isolated from *Arabidopsis* WT and AtNOS1 mutant plants was detected using DAF-fluorescence (Guo and Crawford 2005)

Whether AtNOS1, or (yet unknown) isoforms may be also located in other plant cell organelles, is not totally clear. Using an immunological approach, NOS-like activity in pea plants has been reported to be localized in both peroxisomes and chloroplasts (Barroso et al. 1999). The specificity of anti-NOS antibodies used for the experiments, however, has been questioned (Lo et al. 2000; Butt et al. 2003). Thus, at present it seems most probable that NOS-like activity in plants is exclusively located in the mitochondria. Sufficient supply of reductant in the mitochondria is assured by the citric acid cycle, and the second NOS substrate, L-arginine, may pass the mitochondrial membranes via a recently identified translocator for basic amino acids (Catoni et al. 2003; Hoyos et al. 2003). At this point it is also unknown whether AtNOS1 is actually exposed to the matrix side, or to the intermembrane space, as in animal mitochondria (Ghafourifar and Richter 1997). It is also not clear whether AtNOS1 can use NADH, as well as NAD(P)H, as substrate.

In the above-mentioned experiments with mitochondria purified from *Arabidopsis* leaves, Guo and Crawford (2005) surprisingly detected DAF-fluorescence indicative for NOS-dependent NO production without any reductand addition (NAD(P)H or others). However, plant mitochondria may contain 0.2–0.7 nmol NADP mg^{-1} protein, of which up to 40% may be in the reduced state, at least in vivo or in the presence of added substrate (Møller

2001 and literature cited). The NOS activity reported by Guo and Crawford (2005) for purified mitochondria was very low (0.1 pmol mg^{-1} protein min^{-1}), and thus the above NAD(P)H concentration might be sufficient to support NOS activity for some time.

A similar problem arises when considering the suggested protection against oxidative damage by NO. Reported rates of O_2^- formation in intact plant mitochondria vary considerably from about 100–1000 nmol mg^{-1} protein h^{-1} (Møller 2001 and literature cited). But in any case they appear much higher than even the maximum rates of NO production by mitochondria under anoxia, which are 5–10 nmol mg^{-1} protein h^{-1} (Gupta et al. 2005), and which may be much lower in aerobic conditions. If mitochondrial protein were 10% of the total leaf protein, the above mentioned NOS activity in leaf extracts from *Arabidopsis* WT plants (0.1 pmol mg^{-1} protein min^{-1}) would correspond to a NOS activity based on mitochondrial protein of 60 pmol mg^{-1} protein h^{-1}. This is far below estimated rates of ROS formation in the mitochondria. It is therefore not completely clear how intramitochondrial NO could contribute to ROS scavenging, as has been suggested (Millar et al. 2002; Guo and Crawford 2005; Crawford and Guo 2005).

2.2 Mitochondria also Produce NO by Reduction of Nitrite

Cytosolic nitrate reductase (NR) has been known for some time to reduce nitrite to NO with NADH as reductant, although with only a small fraction (about 1%) of its normal nitrate reducing capacity (Rockel et al. 2002; Planchet et al. 2005). In addition, in plant roots a PM-bound nitrite:NO reductase appears to catalyze a similar reaction, in close association with a PM-NR (Stöhr et al. 2001, also see the chapter by Stöhr in this volume). For a number of years, NR, together with the PM-bound enzyme, appeared to be the only source for nitrite-derived NO.

In 1999, Kozlov et al. demonstrated that animal mitochondria are able to produce NO from added nitrite in the absence of oxygen, and the reaction was abolished by the complex III inhibitor myxothiazol, indicating that the respiratory electron transport was donating electrons for nitrite reduction. In plants, a first hint on an involvement of the respiratory chain in nitrite-dependent NO production came from experiments with a *nia* mutant of the unicellular green alga, *Chlorella sorokiniana*. These mutant algal cells are not able to reduce nitrate to NO, and usually did not produce NO (measured as NO emission into the gas phase by chemiluminescence) when supplied with nitrate. When nitrite was added, however, they emitted NO under anoxia, but much less in air. Obviously, the algae could reduce nitrite to NO by means other than NR. The myxothiazol-sensitivity of the reaction (compare Fig. 2) was a hint that respiratory electron transport was the electron source (Tischner et al. 2004).

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