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Nitric Oxide – A Product of Plant Nitrogen Metabolism

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Abstract Nitric oxide is an intermediate product of inorganic nitrogen assimilation. In plants, it can be formed either by reducing inorganic nitrogen by the nitrite-dependent pathway or by oxidation of organic nitrogen by the arginine-dependent pathway. Both pathways require adequate nitrogen supply to the plant and may not operate under nitrogen deficiency. However, the pathways are differently regulated in relation to oxygen availability and, therefore, have a different importance for underground organs like roots, than for above-ground organs like the shoot.

1 Introduction

A confusing bulk of information is available about possible functions and synthesis of nitric oxide (NO) in plants. New roles played by NO in plant systems are identified constantly. It has been suggested that NO plays important roles in such diverse physiological processes as growth and development, plant disease resistance, abiotic stress, and signal transduction in above and underground plant organs (see selected reviews: Lamattina et al. 2003; Neill et al. 2003; Wendehenne et al. 2004; Shapiro 2005; Crawford and Guo 2005; Lamotte et al. 2005; Crawford 2006). On the cellular level NO was proven to have definite roles in various compartments, such as cytoplasm, mitochondria, peroxisomes, and chloroplasts and as an "extracellular compartment" the apoplast. Specificity demands subcellular targeting or generation of NO.

Formation of NO by plants is necessarily closely linked to nitrogen assimilation and metabolism, since it is produced from inorganic or organic nitrogen sources. Either reduction of the oxidized form forms nitrate via nitrite, or a five-electron oxidation of reduced nitrogen in the form of the guanidine nitrogen of the amino acid L-arginine can lead to release of nitric oxide. Nitrogen is the mineral nutrient required in the highest amounts by plants and is most frequently limiting to growth and yield.

For most plants, nitrate is the inorganic nitrogen source available to roots, especially in temperate agricultural soils (Cookson et al. 2005). Once taken up by the root system, nitrate can be reduced to nitrite in the cytosol, stored in the vacuole, or transported to the shoot. Nitrate incorporation into bi-

ological molecules such as amino acids and amino acid-derived molecules (Fig. 1) involves reduction of nitrate by nitrate reductase (cNR) in the cytosol. Under normal growing conditions the resulting nitrite is further reduced to

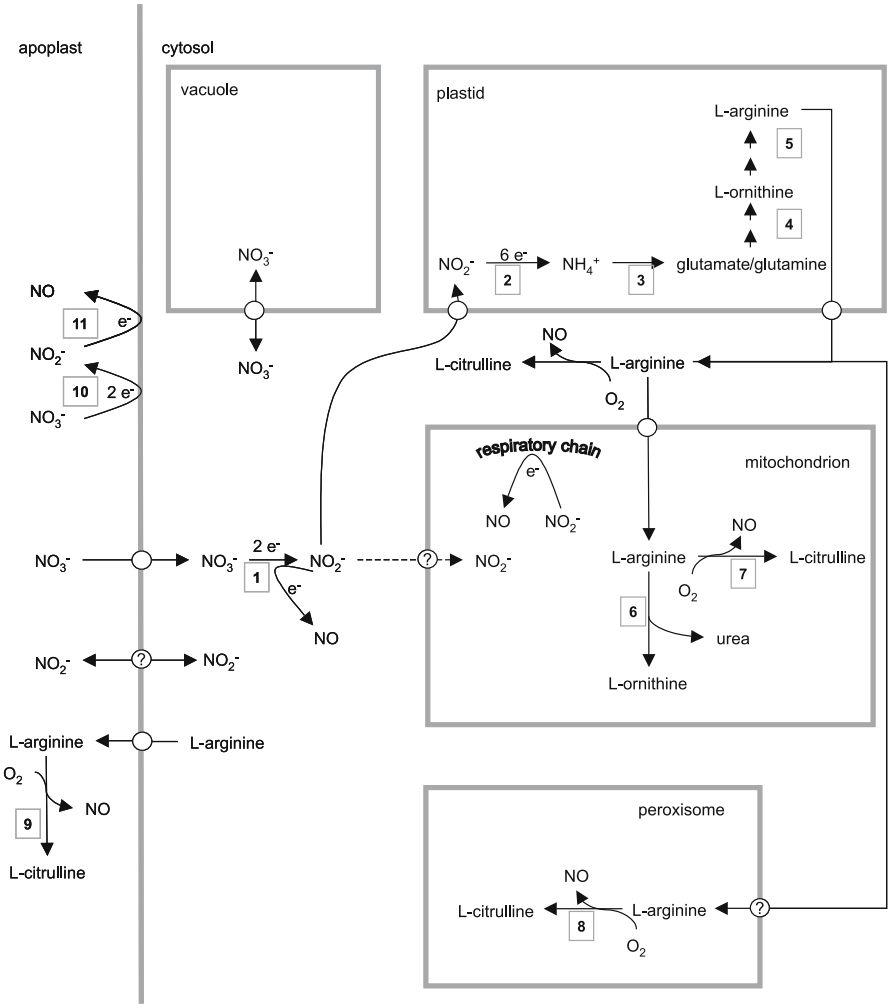

Fig. 1 NO-forming pathway in a root cell in relation to nitrogen assimilation. Enzymes that catalyze the indicated reactions are: *1* cytosolic nitrate reductase; *2* nitrite reductase; *3* glutamine synthetase and glutamine-2-oxoglutarate aminotransferase; *4* the "ornithine pathway": *N*-acetylglutamate synthase, *N*-acetylglutamate kinase, *N*-acetylglutamate-5-P ductase, *N*2-acetylornithine aminotransferase, *N*2-acetylornithine:glutamate acetyltransferase; *5* the "arginine-pathway": ornithine transcarbamoylase, argininosuccinate synthase, argininosuccinate lyase; *6* arginase; *7* mitochondrial nitric oxide synthase1; *8* peroxisomal nitric oxide synthase; *9* apoplastic nitric oxide synthase; *10* plasma membrane-bound nitrate reductase; *11* nitrite:NO reductase

ammonia in the plastid by nitrite reductase (NiR). In both the chloroplasts and the non-photosynthetic plastids, reduced ferredoxins supply the necessary six electrons (Matsumura et al. 1997; Emes and Neuhaus 1997). Only if the toxic nitrite accumulates may it serve as a second substrate for cNR, to become reduced to NO. Cytosolic NR holds a key position in the nitrate assimilation pathway and is under complex regulation at both transcriptional and posttranscriptional level. Yet, nitrate is not only a nutrient. It serves also as signal for rapid changes in metabolism, which include the induction of the synthesis of nitrate assimilatory enzymes and the shift from starch biosynthesis to the production of organic acids to assimilate ammonium (for review see Crawford 1995; Stitt 1999; Foyer et al. 2003). More than 1000 genes are found to respond to low levels of nitrate after only 20 min (Wang et al. 2003). As a direct descendant of nitrate, NO may even trigger some of its effects.

This report does not review the characteristics of the different NO-producing enzymes found in plants nor summarize all of the putative functions of NO (the reader is referred to the excellent reviews available [see above selection] and to the other chapters in this book). This review will focus exclusively on NO production and specificity in regard to nitrogen assimilation and metabolism as well as to oxygen availability.

2
Nitrite as Substrate for Plant NO Formation

Nitrite and NO are intermediates of nitrate assimilation in plants and are also products in bacterial nitrification and denitrification processes (Stewart 1988). Nitrite accumulation in the soil may occur while microbial nitrite oxidation is inhibited by nitrifying and denitrifying bacteria (Burns et al. 1996). In fact, much of the natural NO emission from soil was originally deduced to be of microbial origin and the possible contributions of plants were largely neglected (reviewed by Stöhr and Ullrich 2002).

Nitrite serves as substrate for plant cell NO formation in apoplast, cytosol, and mitochondria (Fig. 1). To reduce nitrite to NO an accumulation of nitrite is necessary, relative to the various enzyme affinities. Generally, cell nitrite concentration is kept very low because of its toxic properties (Sinclair 1987). It is a strong oxidant in neutral and acidic solutions (Hinze and Holzer 1985) and is withdrawn by immediate reduction by nitrite reductase to ammonia in the plastid. Two possibilities allow transport of cytoplasmically formed nitrite to the plastid. The nitrite anion exists in equilibrium with the protonated form, nitrous acid ($p\mathrm{K_a}$ 3.1 – 3.5), setting up a small concentration of HNO_2 under physiological conditions (Yamasaki 2000). This would allow a free diffusion across membranes, as proposed by Shingles and coauthors (1996). Alternatively, nitrite might be transported as an anion by a saturable nitrite transporter (Brunswick and Cresswell 1988a,b). Only in *Chlamydomonas* has

the plastid transport protein Nar1 been identified (Rexach et al. 2000), but homologs have been found in *Arabidopsis*. Yet, the function of Nar1 orthologs as nitrite transporters in higher plants has to be established. Due to nitrite reductase (e.g., K_m of 0.3 mM for spinach NiR, Bellissimoa and Privalle 1995) the plastidic nitrite concentration is estimated to be in the submillimolar range (Yamasaki 2000).

For the most part, nitrite accumulation in plant cells has been only noted in plants supplied abundantly with nitrate, when nitrite reduction to ammonia is limited. Nitrite accumulates in the cytosol if reduction equivalents are not available due to inhibited photosynthesis or to reduced respiration combined with a delayed negative regulation of nitrate reductase, usually via phosphorylation (Kaiser and Huber 2001). In leaves, nitrate reductase is rapidly inactivated in the dark or when CO_2 is removed (for a recent review the reader is referred to Meyer et al. 2005) leading to a transient nitrite accumulation. Under natural conditions a sudden CO_2 limitation in photosynthesis caused by stomatal closure or reduced light conditions due to clouding may be events causing short-term nitrite build-up in leaves.

In roots, nitrite accumulates upon anaerobiosis (Botrel et al. 1996), whereas hypoxia of leaves has been only reported in aquatic plants (Schlüter and Crawford 2001). Roots of higher plants might be frequently exposed to fluctuations in oxygen availability in their local environment. These variations can range from 21 kPa, which is the value of the pO_2 in air, and represents a maximum probably never met even in well aerated soils, to values close to zero in flooded soils (Saglio et al. 1984). In contrast to marsh plants, internal O_2 transport in non-adapted mesophytes plays only a limited role (Vartapetian et al. 1978; Saglio et al. 1983) and cannot meet the respiratory requirements of buried organs, which draw most of their O_2 from the rhizosphere (Saglio et al. 1984). Under such conditions nitrite accumulates in root cells and is secreted into the rhizosphere (Botrel and Kaiser 1997), probably by-passing nitrate transporters in the plasma membrane. In *C. reinhardtii*, four high affinity nitrate/nitrite transporters have been described (Rexach et al. 1999 and references therein).

In higher plants, nitrite has been found to inhibit nitrate influx in a competitive manner, which suggests that both ions share at least some transport systems (Siddiqi et al. 1992). Nitrite influx and efflux across the plasma membrane may involve a combination of nitrous acid and nitrite ions (Meyer et al. 2005). In addition, nitrite can be formed enzymatically in root apoplast. A plasma membrane-bound nitrate reductase (PM-NR) reduces apoplastic nitrate with succinate as electron donor (Stöhr and Ullrich 1997) in the root apoplast. This enzyme activity is diurnally regulated (Stöhr and Mäck 2001) and highly influenced by external nitrate availability (Stöhr 1999). So far, transport or formation of nitrite in mitochondria remains unclear. Nitrite as a source for nitric oxide might be important mainly for roots in transient hypoxic environments under sufficient nitrate nutrition.

3
L-Arginine as Substrate for NO Formation

L-Arginine-dependent NO formation is catalyzed by NO synthases in various locations in the cell, using NADPH and molecular oxygen as cosubstrates and employing calmodulin as cofactor (see review by Crawford 2006). Besides mitochondrial *At*NOS1 (Guo and Crawford 2005) the protein nature of the enzymes is unknown and they are mainly identified by their activity (see review by Crawford 2006). The biosynthesis of L-arginine (Fig. 1) is primarily dependent on ammonia and, ultimately, on the inorganic nitrogen supply of the plant. Ammonia is assimilated by glutamine synthetase and glutamine-2-oxoglutarate aminotransferase (GS/GOGAT cycle) into the organic forms, glutamine and glutamate serving as nitrogen donors in the biosynthesis of essentially all amino acids (Coruzzi 2003). Glutamine and glutamate can then be used to form aspartate and asparagine, and these four amino acids are used to translocate organic nitrogen from sources to sinks (Lea and Miflin 1980; Peoples and Gifford 1993).

For L-arginine synthesis, two distinct processes are necessary (reviewed by Slocum 2005) leading first to the synthesis of L-ornithine from glutamate, and second to the synthesis of L-arginine from L-ornithine via the urea cycle in plants. Prediction and subcellular fractionation data indicate plastid localization for all arginine pathway enzymes (Slocum 2005). Plastid-localized basic amino acid transporters permit exchange of arginine from the plastid to the cytosol. Catabolism of arginine seems to occur in the mitochondria (Goldraij and Polacco 2000), where two amino acid transporters have been proven to be involved in the exchange of L-arginine (and also L-lysine, L-ornithine, and L-histidine in order of decreasing affinity) between cytoplasm and mitochondria (Catoni et al. 2003; Hoyos et al. 2003). These data point out that arginine might be present in mitochondria where NO formation from L-arginine has been observed. Yet, it remains unclear whether and how L-arginine is delivered to peroxisomes.

As an essential amino acid for protein synthesis and substrate for NOS, arginine has even more roles in plant metabolism. It is the precursor of the diamine putrescine from which polyamines and many important plant alkaloids are derived (reviewed by Slcocum 2005). In turn, polyamines induce NO-biosynthesis in *Arabidopsis* seedlings (Tun et al. 2006). The NO-generating activity of added compounds increased from arginine to putrescine, spermidine, and spermine with an as-yet unknown mechanism (Tun et al. 2006). Most interestingly, the presence of free arginine might also inform about the global nitrogen status of plant tissues. A universal mechanism has been detected by which the activity of *N*-acetylglutamate kinase, the second enzyme of the ornithine pathway and therefore also in arginine synthesis (Fig. 1), may be modulated in response to arginine availability in concert with PII proteins (Chen et al. 2006). These are highly conserved signal transduction proteins in-

volved in sensing the carbon and nitrogen status of cells. PII targets include transcriptional regulators and enzymes of nitrogen metabolism in bacteria and plants (Burillo et al. 2004; Maheswaran et al. 2004).

The concentration of L-arginine in plant cells obviously depends on the developmental state. Its function as a storage amino acid is well known. Since it contains four N atoms per six C atoms, arginine may represent as much as 30% of the total nitrogen in seed storage proteins (Van Etten et al. 1963). It is the most abundant free amino acid in the cotyledons of pea seeds, where it is catabolized as a nitrogen source during the early stages of germination (de Ruiter and Kollöffel 1985). Under environmental conditions, significant changes in the content of various free amino acids in all examined plant parts during the course of a year can be followed. Their content rises in autumn, remains stable during winter and declines quickly at the beginning of spring. The most abundant amino acids in the end of winter storage period – asparagine, arginine and glutamine – made up about 90% of nitrogen in the fraction of free amino acids (Gloser 2002). This also includes a high availability of arginine for NO production under conditions of germination and primary developmental stages.

Arginine as a signaling molecule for high nitrogen availability might be only available for NO production in plants well supplied with nitrogen or under certain developmental conditions when storage proteins are degraded. Together with the view on nitrite, this points to NO as a signal molecule only for plants with good nitrogen nutrition, which is not always the case in a natural environment.

4 Subcellular Location of NO

Cellular formation of NO in plant cells has been followed using fluorescent dyes such as the compounds of the diaminofluorescein (DAF) group. Formation of the fluorescent dyes is not reversible and only reflects a build-up of NO over time. It cannot reveal fluctuations in NO concentrations. Although control experiments were mostly run, these observations have to be judged carefully since DAF compounds are rather unspecific (Stöhr and Stremlau 2006). Moreover they react strongly with ascorbate present in plant tissue. In photosynthetic tissue, intracellular concentrations of ascorbate were estimated at 1–10 mmol g^{-1} FW (Noctor 2006) facilitating chemical reduction of nitrite by ascorbate to yield NO. Beside fluorescent dyes, subcellular synthesis was indicated by the presence of NO-forming enzymes in various organelles. Quantitative measurements of NO concentrations in plant cells or even organelles are still lacking.

Nitric oxide as a non-polar molecule is supposed to cross membranes without restriction. Yet, by unlimited diffusion of NO passing all membranes,

the questions arise: how can the specificity of the NO signal be achieved and, moreover, how are the dangerous and toxic reactions of NO restricted? Toxicity of NO is a consequence of its reactivity with transition metal proteins and oxygen and of its ability to form adducts with amines and thiols of varying stability (Van der Vliet et al. 1998). Considering the behavior of NO in membranous environment it has to be taken into account that membranes are not homogenous lipid layers. Moreover, they consist of different areas caused mainly by varying lipid compositions (Meder and Simons 2005; Bérczi and Horvath 2003). Because of their physical properties, NO cannot easily traverse membranes as often assumed. When membranes are in the gel phase, no significant membrane penetration was observed for NO and nor for O_2. In the fluid phase, the transmembrane profiles of NO and O_2 are similar, but that of NO is less steep and shifted towards the center of the membrane, relative to O_2 (Nedeianu et al. 2004). NO seems to be trapped in the hydrophobic core of the membrane. This might explain the observation that fluid-phase membranes were also strong barriers to NO transport, whereas sterols significantly increased NO diffusion (Subczynski et al. 1996). This points to the diffusion coefficient of NO being highly dependent on membrane composition, which is flexible and dynamic (Meder and Simons 2005).

Also, NO reacts differently within membranes in comparison to the aqueous environment. Autoxidation proceeds about 240-fold faster in membranes than in aqueous phases (Lancaster 2000; Shapiro 2005). Whereas NO terminates lipid peroxidation in aqueous medium, it induces lipid peroxidation in a non-aqueous environment (Hiramoto et al. 2003). This might explain conflicting observations on the effects of NO on lipid peroxidation. Protective effects of NO on lipid peroxidation were explained by terminating the radical chain reaction by the reaction of NO with the lipid peroxyl radical (O'Donnell et al. 1997, 1999). However, NO itself can drive lipid peroxidation (Hiramoto et al. 2003).

Possible trapping of NO in the hydrophobic core of membranes, as well as probable chemical reactions with lipids or proteins, raises the question of whether NO is as mobile as usually assumed. Regarding the well-ordered subcellular organization of NO formation (Fig. 1) it seems that a certain local available amount of NO is responsible for its correct function.

4.1 Cytosol

Ascertaining the cytosolic location of proteins is difficult, since the cytoplasm cannot be isolated as easily as chloroplast or mitochondria. Visualized by fluorescence microscopy, NO production in the cytosol has been assumed to be mediated by the cytosolic nitrate reductase. In vitro NO production by purified cNR with NADH as electron source was measured by Yamasaki et al. (1999) and confirmed for different species (reviewed by Meyer et al. 2005). *In*

planta NO production by cNR is dependent on enzyme activity and the availability of nitrite and reduction equivalents. The expression of cNR is induced by small amounts of nitrate, and the activity of the protein is altered in response to changes in environmental conditions, such as light, dark, anoxia, pH, and carbon dioxide concentration (for review see Kaiser et al. 1999, 2001; Kaiser and Huber 2001; Meyer and Stitt 2001; Stitt et al. 2002).

As mentioned before, nitrite accumulation in leaves can be achieved during hypoxia or abrupt darkness, resulting in a more immediate cessation of photosynthetic electron transport than in down-regulation of nitrate reductase activity (Kaiser et al. 2002). The K_m of cytosolic nitrate reductase for nitrite ranges from 100 to 300 μM. Besides, nitrate competitively inhibits nitrite reduction by cNR with a K_i of 50 μM for nitrate (Rockel et al. 2002). This implies that only under conditions when nitrite accumulates to concentrations far above those of nitrate, will enzymatic reduction of nitrite to NO occur. However, cytosolic nitrate concentration is maintained at a constant level under many environmental conditions (Miller and Smith 1996; Cookson et al. 2005). A large range of values have been reported for cytosolic nitrate concentrations, but microelectrode measurements suggest that in mature root cells this parameter is regulated at a value independent of changes in the external concentration (Miller and Smith 1996). The vacuolar nitrate pools change with the external nitrate supply and this store is remobilized to maintain the cytosolic concentration of nitrate (van der Leij et al. 1998). In *Arabidopsis* mesophyll cells, cytosolic nitrate was maintained at approximately 1.5 and 2.0 mM during the light and dark treatment, respectively (Cookson et al. 2005). Different from leaves, the roots of many plant species commonly meet hypoxic environmental conditions. Then, cNR activity increases (Stoimenova et al. 2003) and as a consequence, the usually low cytosolic nitrite content builds up and nitrite is excreted into the apoplast (Botrel et al. 1996). Under these conditions NO is formed by cNR.

4.2 Apoplast

The apoplast is an important space for storage, mineral nutrition, certain enzyme activities, stress reactions, and defence responses (Sattelmacher 2001). It consists of an aqueous phase, which (apart from the xylem) is usually a rather thin film adjacent to or within the cell walls. Whereas most transport in and out of cells has to cross this aqueous film and is influenced by its ionic milieu, in the non-aqueous part gas exchange takes place (Felle 2005). Besides secretion from the cytosol, nitrite in the root apoplast can also be formed by local nitrate reduction by plasma membrane-bound nitrate reductase (Stöhr and Ullrich 1997). A root-specific form of PM-NR uses succinate as electron donor and is highly regulated by external nitrate availability (Stöhr 1999) and light (Stöhr and Mäck 2001) in a manner different from cytosolic NR. It is still

under discussion whether this enzyme may act as nitrate sensor in combination with exudated succinate, reflecting the carbon status of a cell (Meyer and Stöhr 2002).

Nitrite as substrate is used by root-specific plasma membrane-bound nitrite:NO reductase (NI-NOR), whose nitrite-reducing activity markedly differs from that of cytosolic NR (Stöhr et al. 2001). It does not use reduced nicotine adenine nucleotides; instead reduced cytochrome c can serve as electron donor in vitro. However, a participation of cytochrome c at the plasma membrane in vivo seems unlikely and the physiological electron donor has not been identified. One of the most prominent differences from cNR is its high affinity for nitrite, ranging between 2 and 30 μM (unpublished data). Results of solubilization studies suggest a tight association between NI-NOR and PM-NR (Meyer and Stöhr 2002) indicating a step-by-step reduction of nitrate via nitrite to NO.

Under certain conditions, as an acidic pH and the presence of reduced antioxidants (ascorbate) as are met in barley aleurone layers, NO might be formed via non-enzymatic reduction of apoplastic nitrite (Bethke et al. 2004).

NOS activity in the apoplast has been reported by Zhang and coauthors (2003) and can be blocked by an arginine substrate analog. The authors report a putative activating lipid signal for apoplastic NO production, which might derive from enzymatic breakdown of plasma membrane lipids during the HR response, and probably also during wounding (Shapiro 2005). The question arises: to what extent are the substrates arginine and NADPH available in the apoplast? It is known that amino acids are secreted in the rhizosphere and are important for root colonization by mircoorganisms or fungi (Simons et al. 1997). Arginine was frequently detected in root exudates of various species and may therefore also serve as substrate for NOS in the apoplast (Simons et al. 1997; Phillips et al. 2006). Cell-wall-bound malate dehydrogenase has been early identified as providing NADH at the expense of secreted malate (McNeil et al. 1984) and recently confirmed by cell wall proteome analysis (Zhu et al. 2006). Also, NADH has been detected in solutions obtained from the cell wall free space by a low-speed centrifugation technique (Shinkle et al. 1992) and may serve as electron donor. However, the apoplastic presence and use of NADPH for NO production has to be demonstrated.

4.3 Mitochondria

Nitric oxide affects mitochondrial functionality in plant cells and reduces total cell respiration due to strong inhibition of the cytochrome pathway. The residual respiration depends on the alternative pathway and de novo synthesis of alternative oxidase. These adjustments are associated with depolarization of the mitochondrial membrane potential and release of cytochrome c from mitochondria and will finally lead to induction of about 20% of cell

death (Zottini et al. 2002). Thus, it is surprising that mitochondria of all organelles seem to be the major NO producing site in plant cells.

NO formation by mitochondria is both oxygen dependent and independent, with arginine or nitrite, respectively, as substrates. Enzymatic reduction of nitrite was first observed by mitochondria of mammals (Kozlov et al. 1999). It is now proved that the unicellular green alga *Chlorella sorokiniana* (Tischner et al. 2004) and tobacco suspension cells reduce nitrite at the expense of NADH under anoxic conditions, but not in air (Planchet et al. 2005). Obviously, in all higher plants, only root mitochondria, but not leaf mitochondria are able to reduce nitrite to NO, both in vitro (isolated mitochondria) and in situ (Gupta et al. 2005). From inhibitor experiments, it was concluded that electrons from the mitochondrial electron transport chain are transferred to nitrite by cytochrome c oxidase as well as by alternative oxidase. However, the authors cannot exclude the participation of further enzymes.

Production of NO from L-arginine by mitochondrial NOS has been reported in roots and leaves. So far *At*NOS1 is the only protein that has been discovered as NOS in plants (Guo and Crawford 2005). It is targeted to mitochondria where it is required for arginine-dependent NO synthesis. Analysis of a knockout mutant demonstrated that it is responsible for 75% of the basal NOS activity in *Arabidopsis* leaf extracts and most of the ABA-induced NOS activity in roots.

4.4 Peroxisomes

As with the plastid, the role of peroxisomes is different in leaves and roots. Functions of peroxisomes in plant cells are photorespiration, β-oxidation of fatty acids, glyoxylate cycle, metabolism or ureides, and metabolism of ROS (del Río et al. 2002). Peroxisomes contain NOS activity with L-arginine, oxygen, and NADPH as substrates (Barroso et al. 1999; Corpas et al. 2001, 2004; Prado et al. 2004). It was originally discovered in pea seedlings and requires all the cofactors and cosubstrates, as they are necessary for the mammalian enzyme (Barroso et al. 1999). A regulatory role in the glyoxylate cycle, photorespiration, or oxidative catabolism is assumed, but it has not yet been confirmed (Corpas et al. 2001). Beside mitochondria, peroxisomes could be a major site of L-arginine-dependent NO synthesis in plants (Corpas et al. 2001); however, so far no data are available concerning the presence and transport of L-arginine in peroxisomes.

4.5 Plastid

Despite the fact that all various substrates (nitrite, arginine, reduction equivalents, oxygen) for NO formation are present in the plastid, it seems not be

a major source of NO in plant cells, maybe because it is particularly sensitive towards NO. With less NO concentrations than required for inhibition of mitochondrial respiration, decline of net photosynthesis is achieved (Saxe 1986; Wodala et al. 2005). Yet, NO formation in the chloroplast has been observed with DAF (Foissner et al. 2000; Gould et al. 2003) and the effect of NOS-inhibitors points to arginine-derived activity in chloroplasts. As mentioned above, the use of DAF has to be judged carefully. Different studies following lipopolysaccharide stimulation of NO formation state clearly that NO was absent from the chloroplast (Zeidler et al. 2004). The involvement of plastidic nitrite reductase (NiR) in NO_x formation was studied in transgenic tobacco plants that express an antisense NiR construct (Vaucheret et al. 1992) and have very low NiR activities, and hence accumulate nitrite (Goshima et al. 1999). In these plants emission of N_2O occurred, but not in the wild type or in transgenic plants grown on ammonium. When NR activity was blocked, no evolution of N_2O was found (Goshima et al. 1999). In vivo NO production by NiR seems to be negligible.

Plastids function not only as the site of photosynthesis and carbon fixation but also as the location of many metabolic biosynthetic pathways, including assimilation of NO_2^- into organic forms, and synthesis of nucleotides and amino acids. As such, the chloroplast is the primary site for the interface of carbon and nitrogen metabolism and is also the compartment where plant PII resides (see above). With that, it might not be a major source of NO formation, but it contains the major prerequisites.

5 Oxygen – The Critical Factor in NO Formation and Action

Regarding the different enzymes for NO formation in plants and their substrates, nitrite together with various reduction equivalents or L-arginine together with oxygen and NADPH, it becomes obvious that NO formation seems to be regulated differently according to the oxygen availability. This may not necessarily concern the final subcellular NO concentration rather than the kinetics of formation, eventually leading to specific "NO signatures" (following the term used for Ca^{2+} signaling). Although discussed controversially (McAinsh and Hetherington 1998; Plieth 2005), specific Ca^{2+} signatures exist for various environmental signals, regarding length, amplitude, and frequency of Ca^{2+} oscillation in the cytosol. To achieve these fluctuations, Ca^{2+} buffers are required that quickly bind free Ca^{2+}. Similarly NO buffer substances seem to exist in the form of plant hemoglobins, as they have been found in the nucleus and cytoplasm (Seregélyes et al. 2000; reviewed by Perazzolli et al. 2006). Together with further reaction partners, cell NO concentration is probably highly controlled. These chemical reactions differ in their dependence on oxygen partial pressure.

Nitric oxide itself is not a particularly reactive molecule nor it is highly toxic (Brunelli et al. 1995; Beckman 1996). The reactivity of NO is comparable with that of molecular oxygen, and like molecular oxygen it becomes toxic by conversion to more strongly oxidizing species. The most pronounced chemical property of NO is an unpaired electron, leading to a high reactivity with O_2 and O_2^- and with several nitrogen compounds. The rate of reaction of NO with O_2 is second-order in NO and first-order in O_2, independent of pH (between pH 4.9 and 7.4) and leads to nitrite; no nitrate was detected (Lewis and Deen 1994). Shapiro (2005) estimated a high diffusion rate for 1 μM NO in air-saturated water. It was 3400 $\mu m^{2\,-1}$ for plant leaves demonstrating that autoxidation would hardly limit NO action within one single cell. However, the combined unpaired radical electrons on NO and the reactive oxygen species superoxide form a stable bond to produce the peroxynitrite anion.

Peroxynitrite is not a free radical and is stable in alkaline solution. It has a pK_a of 6.8 and can decay to produce hydroxylradical, nitrogen dioxide, and bicarbonate radical. The hydroxylradical is a very strong oxidizing species and can rapidly attack biological membranes and all types of biomolecules, such as DNA and proteins, leading to irreparable damage, metabolic dysfunction, and cell death (del Rio et al. 2003). By itself, peroxynitrite is responsible for tyrosine nitration and oxidation of thiol residues to sulphenic and sulphonic acids (Lamattina et al. 2003). Radical oxygen species are produced in mitochondria and peroxisomes, and there, reaction with NO is likely to occur (Vanin et al. 2004; del Río et al. 2002). Yamasaki (2005) included sulphur radicals in this view of oxygen and nitrogen radicals in the combined "ONS" hypothesis to demonstrate that only the balance of all different radicals facilitate the correct operation of plant systems.

The NO chemistry changes in absence of oxygen. NO reacts directly with metal complexes or with thiols in an alkaline environment to yield disulfide and N_2O (Williams 2004). The reaction of NO with secondary amines leads to the formation of nitrosamines, and that with aromatic amines leads to deamination. An example is the irreversible deamination of deoxynucleotides in DNA, thereby causing point mutations. Yet, it also leads to S-nitrosylation of proteins (also in the presence of oxygen), which are discussed as a posttranslational modification to activate or inhibit protein activity (Lindermayr et al. 2005).

Since the NOS-type enzymes (L-arginine-dependent) need oxygen for NO production, maybe more NO-producing enzymes (nitrite-reducing-type) that remain active under oxygen deprivation had to be developed in plant roots. Because of the higher oxygen concentration in green tissue originated by photosynthesis and by direct contact to ambient air, the NOS-type enzymes may function preferentially in the shoot (Fig. 2). The nitrite-reducing-type in mitochondria (Gupta et al. 2005) and that present in apoplast (Stöhr and Stremlau 2006) are both root-specific. They are not only independent of oxygen but rather they are reversibly inhibited by oxygen in a dose-

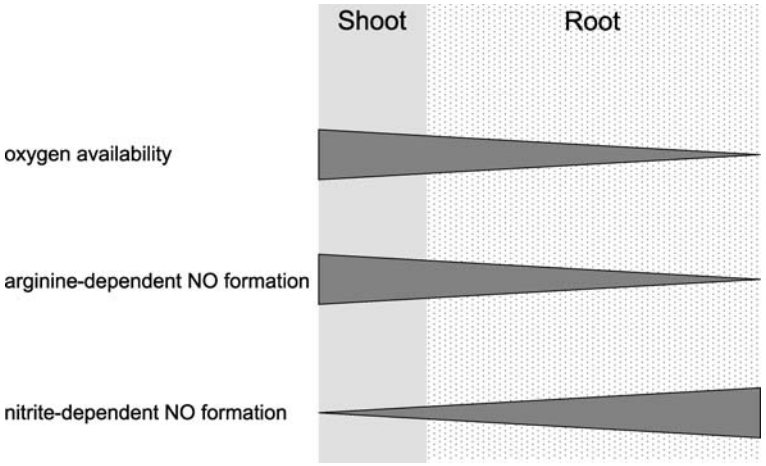

Fig. 2 Dependence of nitrite- and L-arginine-dependent NO production on oxygen availability. High oxygen availability, as mainly found in green tissue, leads to NO formation by NOS-type enzymes that use L-arginine, oxygen, NADPH, and cofactors. Oxygen deprivation, which mainly occurs in root tissue, leads to activation of the nitrite-dependent NO-forming enzymes in the apoplast, cytosol, and mitochondria.

dependent manner, indicating a strong regulation of NO formation by oxygen availability. NO formed by cNR, which is present in roots and leaves, may play a role when the cells are in transition to the unfavorable anaerobic conditions.

Under low oxygen concentrations, plant cells cannot sustain respiration due to lack of an electron acceptor for the terminal oxidases (Geigenberger 2003) and possible functions of NO formed during plant hypoxia have been proposed:

1. Nitrate and nitrite may act as alternative electron acceptors for regeneration of NAD^+ to maintain glycolysis under hypoxic conditions, as an alternative to the use of alcohol dehydrogenase (Crawford 1978). The hereby formed NO might be detoxified by stress-induced hemoglobins (Dordas et al. 2003), which have also been implicated in regeneration of NAD^+ during hypoxia (Hill 1998). In the same context, the induction of stress-induced hemoglobin in *Arabidopsis* by elevated nitrate (Wang et al. 2000) has been also related to modulation of NO levels (Igamberdiev et al. 2004).
2. NO may either accelerate or inhibit programmed cell death depending on its concentration and the combination of additional factors (Kim et al. 2001; Pedroso et al. 2000; Zhang et al. 2003). As observed during pathogen defence, the effects may be either local by inducing cell necrosis or systemic by regulatory pathways. It may also be selective in relation to the responding cells. A similar type of reaction could be responsible for selected

cell death during aerenchyma formation in roots exposed to waterlogging (Drew 1997; Drew et al. 2000; Igamberdiev et al. 2005).

3. Igamberdiev and Hill (2004) discussed the role of nitrate, NO, and hemoglobin in maintaining plant cell viability under anoxic stress. Nitrate as an intermediate electron acceptor during oxygen deficiency leads finally to production of NO by the root plasma membrane-bound enzymes or by cytosolic nitrate reductase. A cycle is proposed whereby NO is oxygenated by hypoxically induced class 1 hemoglobin. The turnover of this reaction is maintained by a methemoglobin reductase. This cyclic reaction may help to maintain the redox status of the cell at very low oxygen tension as an alternative to fermentative pathways. NADH that accumulates under hypoxia due to the lack of electron acceptors is oxidized in an alternative type of respiration to mitochondrial electron transport under limited oxygen (Igamberdiev et al. 2005).
4. NO production as signal for oxygen deficiency was postulated for apoplastic (Stremlau and Stöhr 2006) and intracellular NO by binding to hemoglobin, and to regulate the citric acid cycle and respiration (Millar et al. 2002).

6 Conclusions

Several NO-producing systems operate in plants and are differentially influenced by oxygen partial pressure. Subcellular location of NO-forming enzymes, the chemistry of NO, and the presence of NO scavengers (e.g. hemoglobins) indicate a narrow field of NO activity within a cell. Probably, the NO formation pathways operate in an organ-specific manner and are dependent on plant nitrogen supply. Further data have to be collected to prove whether various kinetic features could lead to specific NO signatures as postulated for the Ca^{2+} signaling pathways.

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