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Molecules of Interest

Nitric oxide and nitric oxide synthase activity in plants

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Abstract

Research on NO in plants has gained considerable attention in recent years mainly due to its function in plant growth and development and as a key signalling molecule in different intracellular processes in plants. The NO emission from plants is known since the 1970s, and now there is abundant information on the multiple effects of exogenously applied NO on different physiological and biochemical processes of plants. The physiological function of NO in plants mainly involves the induction of different processes, including the expression of defence-related genes against pathogens and apoptosis/programmed cell death (PCD), maturation and senescence, stomatal closure, seed germination, root development and the induction of ethylene emission. NO can be produced in plants by non-enzymatic and enzymatic systems. The NO-producing enzymes identified in plants are nitrate reductase, and several nitric oxide synthase-like activities, including one localized in peroxisomes which has been biochemically characterized. Recently, two genes of plant proteins with NOS activity have been isolated and characterized for the first time, and both proteins do not have sequence similarities to any mammalian NOS isoform. However, different evidence available indicate that there are other potential enzymatic sources of NO in plants, including xanthine oxidoreductase, peroxidase, cytochrome P450, and some hemeproteins. In plants, the enzymatic production of the signal molecule NO, either constitutive or induced by different biotic/abiotic stresses, may be a much more common event than was initially thought.

Keywords: Nitrogen monoxide; Nitric oxide; NO; Nitric oxide synthase; NOS; NOS activity; Physiological function; Peroxisomal NOS; Biological function

1. Introduction

Nitrogen monoxide or nitric oxide ($NO^{\cdot}$) is a widespread intracellular and intercellular signalling molecule in mammals with a broad spectrum of regulatory functions in the central nervous, cardiovascular, and immune systems, as well as in platelet inhibition, programmed cell death, and host responses to infection, among others (Moncada et al., 1991; Jeffrey and Synder, 1995; Lloyd-Jones and Bloch, 1996; Wink and Mitchell, 1998; Ignarro, 2000). The widespread biological significance of nitric oxide was recognized by *Science* in 1992 which named the free radical $NO^{\cdot}$ 'Molecule of the year' (Koshland, 1992) and in 1998 the Nobel Prize in Physiology and Medicine was awarded for works that led to the discovery of NO as a biological mediator produced by mammalian cells.

Research on NO in plants has gained considerable attention in recent years and there is increasing evidence of a role of this molecule in plant growth and development (Gouvea et al., 1997; Leshem et al., 1998). Two milestone publications in 1998 demonstrated a regulatory role for NO as a plant defence signal against pathogen infection (Delledonne et al., 1998; Durner et al., 1998), and induced a burst in NO research. In plants, different reviews have been published in recent years on the biological function of nitric oxide (Bolwell, 1999; Wojtaszek, 2000; Beligni and Lamattina, 2001; Wendehenne et al., 2001; Neill et al., 2003; Lamattina et al., 2003) and particularly on nitric oxide signalling in the induction of cell death, defence genes, and interaction with ROS during plant defence against pathogen attack (Van Camp et al., 1998; Durner and Klessig, 1999; Klessig et al., 2000; Delledonne et al., 2001; Wendehenne et al., 2001; Neill et al., 2003; Romero-Puertas and Delledonne, 2003).

In this review, we describe the main physiological and biochemical functions reported for NO in plants. The

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different biochemical, immunological and molecular evidence of nitric oxide synthase-like activity in plants are presented, and the relevance of other alternative enzymatic sources of NO are analyzed with the conclusion that the enzymatic production of NO may be a much more common event in plants than was initially thought.

2. Detection of NO in plants

NO emission from plants was first observed by Klepper in 1975, much earlier than in animals, in soybean plants treated with herbicides (Klepper, 1979). Nitric oxide is a gaseous and highly unstable free radical and its detection and quantification involves methodological difficulties. In comparison with mammalian tissues, there are not many reports of direct measurement of NO in plants and the methods used came from studies in animal systems with some adaptations to different plant tissues. The main methods used to assay NO in plants include: gas chromatography and mass spectrometry (Neill et al., 2003); spectrophotometric measurement of the conversion of oxyhemoglobin to methemoglobin (Orozco-Cárdenas and Ryan, 2002); laser photo-acoustic spectroscopy (Leshem and Pinchasov, 2000); spin trapping electron paramagnetic resonance (EPR) spectroscopy (Caro and Puntarulo, 1999; Pagnussat et al., 2002; Corpas et al., 2002, 2004; Dordas et al., 2004; Huang et al., 2004); the nitric oxide electrode (Leshem and Haramaty, 1996; Yamasaki et al., 2001); and ozone chemiluminescence (Morot-Gaudry-Talarmain et al., 2002; del Río et al., 2003a).

Nevertheless, the use of 4,5-diaminofluorescein diacetate (DAF-2 DA) as fluorescent probe has become a common and very sensitive technique to detect NO in plant systems (Nakatsubo et al., 1998; Nagano and Yoshimura, 2002). This probe has been used in plant cells to obtain real-time bioimaging of NO with fine temporal and spatial resolution (Foissner et al., 2000; Pedroso et al., 2000; Neill et al., 2002b; Corpas et al., 2002; Lamattina et al., 2003; Gould et al., 2003). However, DAF-2 DA can also be used in a spectrofluorometric method which has a detection limit of less than 2–5 nM (Nakatsubo et al., 1998; Corpas et al., 2002, 2004). A new alternative to this fluorescent probe is 3-amino-4-(*N*-methylamino)-2′,7′-difluorofluorescein diacetate (DAF-FM DA) which fluoresces not only at neutral or basic pH but also at acidic pH (Zhang et al., 2003).

3. Physiological functions of NO

The application of exogenous NO to whole plants or cell cultures has allowed to obtain valuable information on how this molecule affects some physiological and biochemical processes. For example, the application of NO to plants has provided evidence of a mediating role of NO in the inhibition of catalase, ascorbate peroxidase and aconitase activities (Navarre et al, 2000; Clark et al., 2000), in cell wall lignification (Ferrer and Ros Barceló, 1999), the regulation of ion channels of guard cells (García-Mata et al., 2003), the mitochondrial and chloroplastic functionality (Yamasaki et al., 2001; Zottini et al., 2002; Takahashi and Yamasaki, 2002); cell death (Pedroso et al., 2000; Saviani et al., 2002; de Pinto et al., 2002), senescence (Leshem, 1996; Leshem and Haramaty, 1996; Hung and Kao, 2003), accumulation of ferritin (Murgía et al., 2002), wound signalling (Orozco-Cárdenas and Ryan, 2002), etc. Table 1 shows different processes in plants that can be regulated by NO.

Apparently, NO can mediate the biological effects of signalling molecules such as hormones, a similar role to that reported for H_2O_2 (Neill et al., 2002a). Cytokinin has been shown to induce NO synthesis in different plants and it is possible that NO can mediate the cytokinin-induced programmed cell death (PCD) process (Neill et al., 2003). It has been demonstrated that NO mediates ABA-induced stomatal closure, and ABA induces rapid NO synthesis in guard cells of pea (Neill et al., 2002b). Likewise, auxin was found to induce NO synthesis in cucumber roots (Pagnussat et al., 2002, 2003). On the other hand, the interaction between NO and ethylene in the maturation and senescence of plant tissues suggested an antagonistic action of both gases during these stages of plant development (Leshem et al., 1998; Lamattina et al., 2003).

Table 1
Summary of the functions postulated for NO in different plant physiological, biochemical and molecular processes

Processes
Growth and development
Germination
Root organogenesis
Stomatal movement
Senescence and programmed cell death (PCD)
Cell wall lignification
Nodule metabolism
Metabolism of subcellular compartments
Chloroplasts: chlorophyll biosynthesis, photophosphorylation
Mitochondria: cytochrome *c* oxidase, alternative oxidase
Peroxisomes: catalase regulation
Cytosol: aconitase modulation
Biochemical interactions
Protein nitration
Ferritin (iron homeostasis)
Haemoglobins (NO levels modulation)
ROS, GSH, ethylene, MAPKs, Ca^{2+}, ABA
Biotic stress: hypersensitive reaction (HR), systemic-acquired resistance (SAR)
Abiotic stress: wounding, salinity, high temperature, drought, hypoxia.

In the plant response to various abiotic stresses such as drought, low and high temperatures, UV, ozone exposure, and wounding, a mediating role for NO has been suggested (Neill et al., 2003). In biotic stress, a key signalling role for NO during the induction of the hypersensitive response (HR) following pathogen attack was demonstrated (Delledonne et al., 1998; Durner et al., 1998; Romero-Puertas and Delledonne, 2003). Different evidence indicated that NO synthesized by pathogens, via NOS, could interact with H_2O_2 to mediate the HR (Neill et al., 2003). In the development of systemic acquired resistance (SAR) a role for NO was suggested (Durner et al., 1998), and a signalling relationship probably takes places between H_2O_2, NO and salicylic acid during HR and SAR (Van Camp et al., 1998; Song and Goodman, 2001; Delledonne et al., 2001; Romero-Puertas and Delledonne, 2003). In pea plants, wilting intensified the NO emission (Leshem and Haramaty, 1996), and in tobacco cells under heat, osmotic and salinity stresses, by confocal laser scanning microscopy (CLSM) and fluorometric analysis a rapid increase in NO production was determined (Gould et al., 2003). In leaves of *Arabidopsis*, wounding induced a fast accumulation of NO, as checked by CLSM and spin trapping EPR (Huang et al., 2004). These data led to postulate that NO could be a useful marker of plant stress (Magalhaes et al., 1999), and that NO generation, like that of the ROS O_2^- and H_2O_2, can occur naturally as a generalized response to different types of stress (Magalhaes et al., 1999; Gould et al., 2003). According to this hypothesis, the generation and emission of NO could be an effective way to dissipate excess free radicals, supplementing other detoxification mechanisms (Gould et al., 2003).

A role for NO in the induction of apoptosis in two plant species (Magalhaes et al., 1999) and during pathogen-induced PCD in *Arabidopsis* has been proposed (Neill et al., 2003), and it appears that the induction of PCD is determined by the interaction between NO and the ROS O_2^- and H_2O_2 (Delledonne et al., 2001). In plant mitochondria, NO inhibits the cytochrome oxidase activity and the concomitant ATP synthesis, and altered mitochondrial activity stimulates PCD in plant cells (Yamasaki et al., 2001). It seems that the NO-induced PCD occurs by inhibition of respiration and the release of mitochondrial cytochrome c (Zottini et al., 2002; Neill et al., 2003). A substantial amount of research is being conducted on the role of NO in different signal transduction processes of plant cells. It appears that generation of cGMP, cADPR and elevation of cytosolic Ca^{2+} are involved in plant responses to NO, similarly to mammalian cells, although the detailed mechanism of these responses is not very well known yet (Wendehenne et al., 2001; Neill et al., 2003). Studies on the NO induction of gene expression in plants revealed a large number of genes that were induced by NO. Some of the characterized genes include *AOX1*a, *PAL*, *PR*-1, *CHS*, *AtNOS1* and those of several peroxidases, glutathione-*S*-transferases, ferritin, and key enzymes of jasmonic acid biosynthesis (Durner et al., 1998; Delledonne et al., 1998; Murgia et al., 2002; Huang et al., 2002a, 2004; Neill et al., 2003; Guo et al., 2003).

4. Nitric oxide synthase activity in plants

In animal systems most of the NO produced is due to the enzyme nitric oxide synthase (NOS; EC 1.14.13.39) (Moncada et al., 1991; Ignarro, 2000). This enzyme catalyzes the oxygen- and NADPH-dependent oxidation of L-arginine to NO and citrulline in a complex reaction requiring FAD, FMN, tetrahydrobiopterin (BH4), calcium and calmodulin (Knowles and Moncada, 1994; Alderton et al., 2001). Since 1996 there has been an increasing number of reports showing the presence of nitric oxide synthase activity in plants similar, to a certain extent, to mammalian NOS. A summary of the different plant species where NOS activity has been determined, is shown in Table 2. To demonstrate the existence of NOS in plants, essentially three different approaches were used, based on biochemical and physico-chemical, immunological and molecular methods.

4.1. Biochemical and EPR evidence

Cueto et al. (1996) and Ninnemann and Maier (1996) were the first to show the presence of NOS activity in higher plants by using the method of conversion of radiolabelled arginine, the substrate of NOS, into radiolabelled citrulline. Another method which has been widely used is the measurement in crude extracts incubated with L-arginine plus all the NOS cofactors, the NO production sensitive to NOS inhibitors by fluorometry or chemiluminiscence. In crude extracts from sorghum embryonic axis the NOS activity-derived production of NO in the reaction mixture has been recently determined by spin trapping electron paramagnetic resonance (EPR) spectroscopy, using as spin trap a complex formed by Fe(II) and dithiocarbamate [$Fe(MGD)_2$] (Simontacchi et al., 2004).

The occurrence of NOS activity was demonstrated in peroxisomes from pea plants (Barroso et al., 1999). In peroxisomes purified from pea leaves the NOS activity was determined using L-arginine as substrate plus all the NOS cofactors, and four different assays were employed: (a) Monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline; (b) Fluorometric detection with DAF-2 DA of NO produced in the enzymatic reaction; (c) Ozone chemiluminiscence detection of NO produced with a nitric oxide analyzer (NOA); and (d) Spin trapping EPR spectroscopy of NO generated during the enzymatic reaction, using the spin trap $Fe(MGD)_2$ (Barroso

Table 2
Nitric oxide synthase activity reported in some plant species

Species/Tissue or cell type	Assays	Reference
Pisum sativum/Leaves	Gaseous NO emission sensitive to NOS inhibitors	Leshem and Haramaty (1996)
Pisum sativum/Leaf peroxisomes	Arginine–citrulline assay	Barroso et al. (1999)
	Spin trapping EPR	Corpas et al. (2004)
Lupinus albus/Roots and nodules	Arginine–citrulline assay	Cueto et al. (1996)
Mucuna hassjoo	Arginine–citrulline assay	Ninnemann and Maier (1996)
Glycine max/*Ps. syringae*-infected cell suspensions	NO production sensitive to NOS inhibitors	Delledonne et al. (1998)
Nicotiana tabacum/TMV-infected leaves	Arginine–citrulline assay	Durner et al. (1998)
Glycine max/Embryonic axes	NADPH–diaphorase activity	Caro and Puntarulo (1999)
Zea mays/Root tips and young leaves	Arginine–citrulline assay	Ribeiro et al. (1999)
Taxus brevifolia/Callus	NO production sensitive to NOS inhibitors	Pedroso et al. (2000)
Nicotiana tabacum/Leaf epidermal cells	NO production sensitive to NOS inhibitors	Foissner et al. (2000)
Nicotiana tabacum/Cell cultures	NO production sensitive to NOS inhibitors	Tun et al. (2001)
Arabidopsis/Cell cultures		
Petrosilenum crispum/Cell cultures		
Glycine max/Cotyledons	Arginine–citrulline assay	Modolo et al. (2002)
Sorghum bicolour L./Seeds	NADPH-diaphorase activity	Simontacchi et al. (2004)
	Spin trapping EPR	

et al., 1999; Corpas et al., 2002, 2004). The specific activity of peroxisomal NOS was 5.6 nmol/min/mg and the enzyme was strictly dependent on L-arginine, NADPH, BH_4, and calmodulin, and required Ca^{2+} (Fig. 1). The effect of seven archetype inhibitors of different animal NOS isoforms was assayed and results showed a clear inhibition of the peroxisomal NOS activity of 59–100%, L-aminoguanidine being the most effective inhibitor (Barroso et al., 1999). Carboxymethoxylamine (CM; 200 μM) and aminoacetonitrile (AAN; 0.01%), two characteristic inhibitors of the P protein of the mitochondrial glycine decarboxylase complex (GDC) (Douce et al., 2002), and the nitrate reductase inhibitor azide (1 mM), did not have any effect on the peroxisomal NOS activity. Additionally, the incubation of peroxisomes with an antibody against murine iNOS, produced a 90% reduction of the NOS activity (Barroso et al., 1999). During natural senescence of pea leaves, the NOS activity of peroxisomes, measured as NO formation by chemiluminiscence, was strongly inhibited and this suggested that peroxisomal NO could be involved in the senescence process of leaves (Corpas et al., unpublished results).

4.2. Immunological evidence

Different antibodies raised against NOS from mammalian origin were used to study the existence of NOS in plants by Western blot analysis and immunogold EM. Antibodies to mouse brain NOS and rabbit nNOS were employed to show the presence of NOS in wheat germ and pea embryonic tissue, respectively (Kuo et al., 1995; Sen and Cheema, 1995). In maize roots and leaves, antibodies to mouse iNOS and rabbit nNOS showed the presence of immunoreactive bands (Ribeiro et al., 1999), and the same was found in pea leaves using an antibody against a synthetic peptide of the C-terminus of murine iNOS (Barroso et al., 1999). In the latter case the antibody was also found to largely inhibit the NOS activity of leaf samples measured by the arginine–citrulline assay.

However, Butt et al. (2003) in a proteomic study in extracts from maize embryonic axis with polyclonal rabbit antibodies against human nNOS and mouse iNOS found that many apparently NOS-unrelated proteins were recognized by the antibodies. On this basis, these authors cast doubts upon the results of NOS presence in plants obtained using immunological techniques with mammalian NOS antibodies. The lack of specificity of antibodies can be sometimes a problem in immunochemical assays and should always be carefully considered, but the results obtained by Butt et al. (2003) in maize extracts with two apparently non-specific commercial antibodies cannot disqualify all the results mentioned

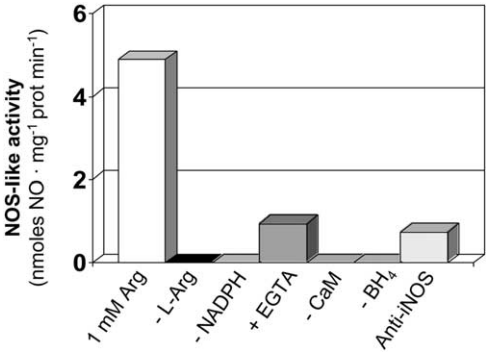

Fig. 1. Determination of NO production (NOS activity) in peroxisomes by ozone chemiluminiscence. Reaction mixtures containing peroxisomal fractions were incubated in the absence or presence of L-Arg (1 mM), NADPH (1 mM), EGTA (0.5 mM), calmodulin (10 μg/ml), BH_4 (10 μM), and antibody against murine iNOS (dilution 1/200). The NO produced was quantified by ozone chemiluminiscence using a nitric oxide analyzer (NOA). Results are means of samples from, at least, three different sucrose-density gradients.

above which were obtained with extracts from different plant species and distinct types of antibodies.

4.3. Molecular evidence

Following the publication of the *Arabidopsis* genome, not a single gene or protein with sequence similarity to the animal NOSs could be identified (The *Arabidopsis* genome initiative, 2000; Butt et al., 2003). This perhaps could explain why in plants until very recently neither the gene or cDNA, nor any protein with sequence similarity to animal NOSs could be found. Recently, in tobacco and *Arabidopsis* plants a variant of the P protein of the mitochondrial glycine decarboxylase complex (GDC) was found to be a pathogen-inducible plant NOS (Chandok et al., 2003). The P protein is part of the mitochondrial glycine decarboxylase multienzyme system which catalyzes the destruction of glycine molecules produced during the course of photorespiration (Douce et al., 2002). The variant of the P protein was designated as "plant iNOS" and was cloned and purified (Chandok et al., 2003). This protein which was induced by viral infection, used L-arginine as substrate like animal NOSs, and NADPH, BH_4, FAD, Ca^{2+} and calmodulin as cofactors, but its specific activity (20–47 μmol/min/mg) was about 30-times higher than that of animal iNOS (Chandok et al., 2003). The NOS activity of the variant P protein was suppressed by CM and AAN, two inhibitors of the P protein of the GDC. This "plant iNOS" shared very little sequence homology with animal NOSs and was postulated to be the major pathogen-induced NO synthesizing enzyme in plants.

Just five months after this surprising discovery, the identification of a nitric oxide synthase gene (*AtNOS*1) in *Arabidopsis* plants that regulates growth and hormonal signalling was described (Guo et al., 2003). The AtNOS1 protein had a much lower specific activity (5 nmol/min/mg) than the variant P protein mentioned above, and was of a similar order to that reported for the peroxisomal NOS (Barroso et al., 1999). The NOS activity of AtNOS1 did not depend on BH_4, FAD, FMN or heme as cofactors. Analysis of the amino acid sequence showed that AtNOS1 was very similar to a group of bacterial proteins with putative GTP-binding or GTPase domains but, like in the case of the variant P protein, did not have sequence similarities to any mammalian NOS (Guo et al., 2003).

5. Cellular localization of NOS activity

There is very little information about the subcellular localization of NOS activity in plants. In maize cells Ribeiro et al. (1999) by immunofluorescence with antibodies to mouse iNOS and rabbit nNOS found that the immunoreactive protein was localized in the cytoplasm of cells in the division zone and was translocated into the nucleus depending on the phase of cell growth.

The occurrence of NOS activity in leaf peroxisomes was demonstrated by biochemical methods and EPR spectroscopy, as indicated in Section 4.1, but NOS-like protein also was detected by Western blot analysis, and in intact leaves by electron microscopy immunogold labelling, using an antibody against the peptide PT387 from the C-terminus of the murine iNOS (Barroso et al., 1999). The EM immunolocalization of NOS showed the presence of the enzyme in the matrix of peroxisomes and also in chloroplasts, whereas no immunogold labelling was detected in mitochondria (Barroso et al., 1999). This contrasts with results obtained in mammalian tissues where NOS was found in mitochondria from rat and brain liver by cytochemical and immunocytochemical methods (Bates et al., 1995), and NOS activity was determined in mitochondria isolated from rat liver (Ghafourifar and Richter, 1997; Tatoyan and Giulivi, 1998). Using the same immunogold EM method, NOS was later also found in peroxisomes from olive leaves and sunflower hypocotyls (Corpas et al., 2004). The peroxisomal localization of NOS was ratified by confocal laser immunofluorescence microscopy using antibodies against murine iNOS and catalase, a characteristic marker enzyme of peroxisomes (Fig. 2). Both immunofluorescent markers co-localized indicating that NOS was present in peroxisomes (Corpas et al., 2002, 2004; del Río et al., 2003b). More recently, the localization of iNOS in animal peroxisomes has been reported, in cell organelles from rat hepatocytes (Stolz et al., 2002). This suggests that NOS could be a constituent enzyme of peroxisomes from different origins.

6. Other generating systems of NO

6.1. Non-enzymatic systems

The generation in vitro of NO by the reaction of H_2O_2 (10–50 mM) and L-arginine (10–20 mM) at pH 7.4 and 37 °C has been reported by Nagase et al. (1997). The non-enzymatic synthesis of NO has also been demonstrated, with short-time kinetics, by shock waves treatment of solutions containing 1 mM H_2O_2 and 10 mM L-arginine (Gotte et al., 2002).

In plants, nitric oxide can also be generated by non-enzymatic mechanisms. Nitrification/denitrification cycles provide NO as a by-product of N_2O oxidation into the atmosphere (Wojtaszek, 2000). It is known that the non-enzymatic reduction of nitrite can lead to the formation of NO, and this reaction is favoured at acidic pH when nitrite can dismutate to NO and nitrate (Stöhr and Ullrich, 2002). Nitrite can also be chemically reduced by ascorbic acid at pH 3–6 to yield NO and dehydroascorbic acid (Henry et al., 1997). This reaction

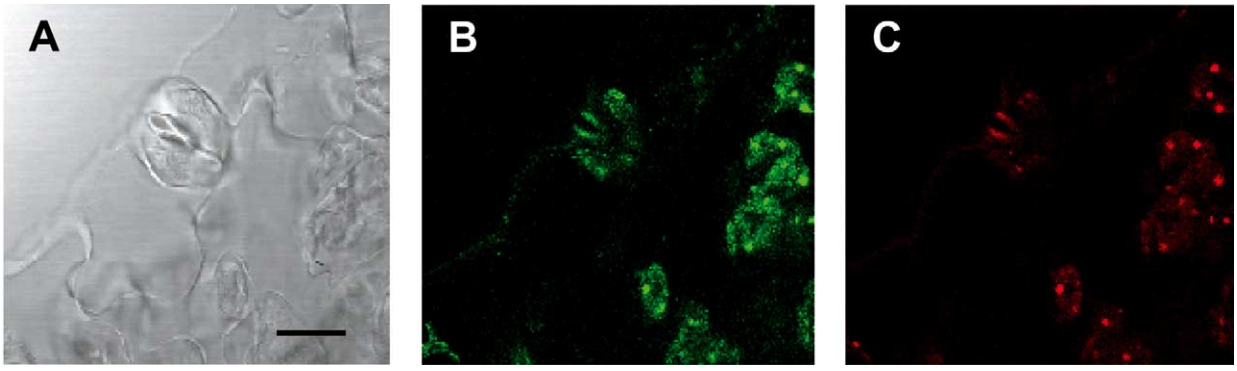

Fig. 2. Colocalization by CLSM of NOS and catalase in the guard cells of stomata of pea leaves. A, Pea leaf section showing a stoma guard cell. B, Immunolocalization of NOS showing the green Cy2-streptavidin immunofluorescence punctuates attributable to anti-iNOS. C, Immunolocalization of catalase showing the red Cy3 immunofluorescence punctuates attributable to anti-catalase. The pictures correspond to the same pea leaf section. Bar = 10 μm.

could occur at microlocalized pH conditions in the chloroplast and apoplastic space where ascorbic acid is known to be present (Horemans et al., 2000). In barley aleurone cells, NO can also be synthesized by reduction of nitrite by ascorbate at acidic pH (Beligni et al., 2002). Another non-enzymatic mechanism proposed of NO formation is the light-mediated reduction of NO_2 by carotenoids (Cooney et al., 1994).

6.2. Enzymatic systems

Nitric oxide can also be produced by other enzymes apart from nitric oxide synthase. A list of some established and potential enzymatic sources of NO in plant cells, with indication of the different substrates used, is presented in Table 3. Until very recently it was thought that the major origin of NO production in plants was through the action of NADH-dependent nitrate and nitrite reductases (Yamasaki et al., 1999; Lamattina et al., 2003), although the finding of new NOS activities in plants, mentioned above, perhaps might require to revise this idea. The production of NO by the molybdenum cofactor-containing enzyme nitrate reductase is known since the beginning of the 80s (Harper, 1981; Dean and Harper, 1988). This enzyme can generate NO from nitrite (NO_2^-) with NADH as electron donor and the catalysis site is probably the molybdenum cofactor (Moco) (Yamasaki et al., 1999; Rockel et al., 2002). Nitrate reductase also produces peroxinitrite simultaneously with NO (Yamasaki and Sakihama, 2000). However, the NO production capacity of nitrate reductase, at saturating NADH and nitrite concentrations, is about 1% of its nitrate reduction capacity, and in vivo the NO production depends on the total nitrate reductase activity, the enzyme activation state and the intracellular accumulation of NO_2^- and NO_3^- (Rockel et al., 2002). In plant cells, NO_2^- can be accumulated when the photosynthetic activity is inhibited or under anaerobic conditions (Rockel et al., 2002; Lamattina et al., 2003). On the other hand, in potato tubers infected by the fungus *Phytophthora infestans* the induction of nitrate reductase has been demonstrated (Yamamoto et al., 2003). This suggests the participation of nitrate reductase in pathogen-induced NO production. Thus, the nitrate reductase-

Table 3
Some established and potential enzymatic sources of NO in plant cells

Source	Substrates	Ref
Different crude extracts	L-Arg and NOS cofactors	reviewed by Neill et al. (2003)
Plant peroxisomes	L-Arg and NOS cofactors	Barroso et al. (1999) Corpas et al. (2002, 2004)
Variant P protein of the GDC	L-Arg and NOS cofactors	Chandok et al. (2003)
Arabidopsis protein AtNOS1	L-Arg and NOS cofactors except BH_4, FAD and FMN	Guo et al. (2003)
Nitrate reductase	NO_2^- and NADH	Dean and Harper (1988) Yamasaki et al. (1999)
Xanthine oxidoreductase	NO_2^- and NADH	Millar et al. (1998) Harrison (2002)
Horseradish peroxidase	Hydroxyurea + H_2O_2 NOHA + H_2O_2	Huang et al. (2002b) Boucher et al. (1992a)
Hemeproteins	NOHA + H_2O_2/ROOH	Boucher et al.(1992a)
Cytochrome P450	NOHA + NADPH + O_2	Boucher et al (1992b)
Plasma membrane-bound enzyme	NO_2^- + reduced Cyt *c*	Stöhr et al (2001)

NOHA, *N*-hydroxyarginine; ROOH, alkylhydroperoxides.

dependent generation of NO is expected to be enhanced under plant stress conditions (Rockel et al., 2002). Another enzyme that can generate NO from nitrite, is a plasma membrane-bound enzyme of tobacco roots (Stöhr et al., 2001). This enzyme has a higher molecular weight than nitrate reductase and still has to be characterized.

Xanthine oxidoreductase (XOR) is another Moco-containing enzyme which in animal systems has been recently demonstrated to produce NO (Harrison, 2002). Xanthine oxidoreductase occurs into two interconvertible forms: the superoxide-producing xanthine oxidase (form O; EC 1.1.3.22) and xanthine dehydrogenase (form D; EC 1.1.1.204) (Palma et al., 2002). XOR has been found present in pea leaf peroxisomes where the preponderant form of the enzyme is xanthine oxidase and only a 30% is present as xanthine dehydrogenase (Sandalio et al., 1988; Corpas et al., 1997). The enzyme XOR from animal origin can produce the free radicals $O_2^{\cdot-}$ and NO· during its catalytic reaction, depending on whether the oxygen tensions are high and low, respectively (Millar et al., 1998; Godber et al., 2000; Harrison, 2002). A model proposed for the catalytic reduction of nitrite to NO by animal XOR is shown in Fig. 3. The important property of producing $O_2^{\cdot-}$ and NO· radicals confers XOR a key role as a source of signal molecules in plant cells (Corpas et al., 2001).

However, apart from these cases, still there are other potential enzymatic sources of NO generation in plants that must be considered. The production of NO and citrulline by horseradish peroxidase from *N*-hydroxy-arginine (NOHA) and H_2O_2 was reported a decade ago (Boucher et al., 1992a). More recently, horseradish peroxidase was also demonstrated to generate NO from hydroxyurea and H_2O_2 (Huang et al., 2002b). This source of NO should be carefully considered taking into account that peroxidases are widespread enzymes involved in important physiologic processes of plant cells (Veitch, 2004). Another hemeproteins that have been proposed as good candidates for the enzymatic generation of NO are cytochromes P450. These proteins are present in plants, and in animal systems have been shown to catalyze the oxidation of NOHA by NADPH and O_2 with generation of NO (Boucher et al., 1992b; Mansuy and Boucher, 2002). Hemoglobin and catalase were also reported to produce NO and other nitrogen oxides by catalyzing the oxidation of NOHA by cumylhydroperoxide (Boucher et al., 1992a). These data emphasize the importance of hemeproteins as enzymatic generators of NO (Boucher et al., 1992b).

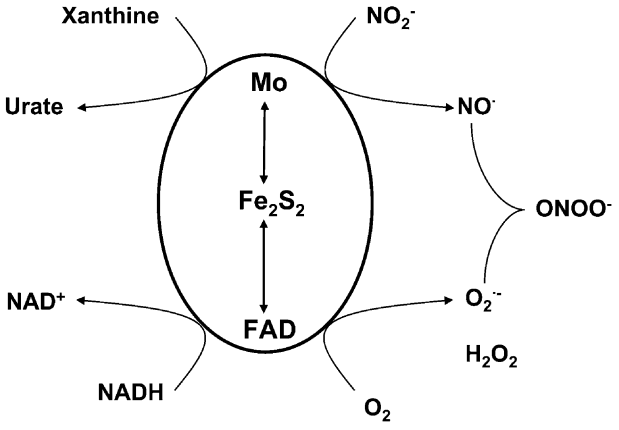

Fig. 3. Model proposed for the production of NO and peroxynitrite catalyzed by the enzyme XOR (Harrison, 2002). Under anaerobic conditions and in the presence of nitrite and a reducing substrate, such as xanthine or NADH, NO is generated at the Mo site. In the presence of O_2, this is reduced at the FAD site to give superoxide ($O_2^{\cdot-}$), which can react with NO to produce peroxynitrite. (*Free Radical Biology & Medicine* 33, 785; 2002. Copyright 2002 Elsevier Science Inc., USA.)

7. Concluding remarks

Since the discovery of NO emission by plants in the 1970s, this gaseous compound has emerged as a major signalling molecule involved in multiple physiological functions. The great interest arose by NO is even bigger than that produced by another gaseous molecule, the plant hormone ethylene, with which NO interplays. Much chemical, biochemical, cellular and molecular work is necessary to identify the different NO-producing enzymes in plants and understand the endogenous synthesis of NO, its detailed signalling mechanism, and the chemical changes induced by this molecule in vivo. Looking for the source of NO, in the past decade many plant biologists intensively searched for an enzyme similar to the nitric oxide synthase (NOS) identified in mammalian systems. However, the molecular evidence actually available indicate that the two "plant NOS" that have been cloned although use L-arginine as substrate to synthesize NO, are structurally different from the mammalian NOSs. In addition, plants also have other enzymatic sources for NO synthesis. Nitrate reductase can be an important supplier of NO, particularly under certain abiotic and biotic stress conditions, and NOS-like activity has been determined in crude extracts from different plants species and cell organelles. The peroxisomal NOS-like activity has been biochemically characterized and appears to be a constitutive enzyme different from both the pathogen-inducible iNOS activity detected in the variant P protein of the GDC and from the AtNOS1 protein. But horseradish peroxidase can also produce NO from NOHA/hydroxyurea and H_2O_2, and is a good example of how plant cells can have alternative sources of NO making use of the widespread and physiologically important enzymes peroxidases. Other enzymatic sources that must be considered are xanthine oxidoreductase, cytochrome P450, and other hemeproteins which are present in plants and have been shown to generate NO in animal systems. Additionally, an unknown plasma membrane-bound

enzyme different from nitrate reductase, was shown to catalyze the formation of NO from nitrite in plant roots. Taken together, this suggests that in plants the enzymatic NO production, either constitutive or induced by different biotic/abiotic stresses, may be a much more common event that was initially thought.

These examples show that the dated concept of one protein one function is too simplistic as far as NO generation is concerned. To improve our understanding of the physiological function of NO in the different plant cell compartments, we must realize that plant cells may not possess a unique enzymatic source of this versatile molecule but multiple generating systems. It appears that in plants there is a battery of multifunctional enzymes, able to catalytically produce NO, which structurally are unrelated to mammalian NOS. The burst of publications on "plant NOS activity" over recent years and the special interest in demonstrating that the origin of NO was due to a unique constitutive or inducible mammalian-type NOS, is a reminder to the discovery of the generation in biological systems of another important free radical, the superoxide anion ($O_2^{\cdot-}$) (McCord and Fridovich, 1968). It was then thought that $O_2^{\cdot-}$ radicals were only produced by the mammalian oxidative enzyme xanthine oxidase. Today it is known that there are many proteins and enzymes producing these radicals in many compartments of animal and plant cells, and it is well established that the generation of $O_2^{\cdot-}$ radicals may be unspecifically induced by different pathological or stress conditions (Bolwell, 1999; Halliwell and Gutteridge, 2000; Dat et al., 2000; Mittler, 2002; del Río et al., 2003b). In a similar way, concerning the enzymatic production of NO in plants, perhaps we are just starting to see the tip of the iceberg. In fact, it has already been proposed that NO emission in plants can be a generalized stress response similar to ROS (Gould et al., 2003).

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