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Editors
M. Nasir Khan
Mohammad Mobin
Faculty of Science,
Department of Biology
University of Tabuk
Tabuk
Saudi Arabia

Firoz Mohammad
Plant Physiology Section,
Department of Botany
Aligarh Muslim University
Aligarh
India

Francisco J. Corpas
Department of Biochemistry,
Cell and Molecular Biology of Plants
Estación Experimental del Zaidín Consejo
Superior de Investigaciones Científicas
Granada
Spain

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Professor M. M. R. K. Afridi (1931–2010)

Professor Muhammad Mahmudur Rahman Khan Afridi was born at Farrukhabad (U.P.), India on May 7, 1931. He did his graduation and post-graduation from Aligarh Muslim University (AMU), Aligarh, India. He completed his doctoral studies from University of Bristol, England. After completion of Ph.D., he joined AMU, as Lecturer of Botany in 1951. He became Reader in 1961 and Professor of Plant Physiology and Head of the Department of Botany in 1978. He assumed the charge of Dean, Faculty of Life Sciences, in 1989 and retired in 1991.

Professor Afridi started his research career in 1957 when he was awarded a Colombo Plan Fellowship for research in U.K. and joined the famous Long Ashton Research Station of the University of Bristol

with renowned Professor T. Wallace F.R.S. and Dr. E. J. Hewitt F.R.S. His pioneering work on the inducible formation of nitrate reductase, the key enzyme known today for nitric oxide synthesis in plants, was soon recognized internationally.

On his return to India, Professor Afridi devoted his energies to the applied aspects of the mineral nutrition of crop plants and established the first school on macro-nutrition. He successfully guided the research work of many students and published several research papers in the journals of national and international repute. Professor Afridi was one of the founding members and life member of Indian Society for Plant Physiology (I.S.P.P.). He was elected as the President of I.S.P.P. in 1979, and was awarded the Distinguished Scientists Medal in 1983 by the Academy for the Advancement of Agricultural Sciences, India and the J. J. Chinoy Medal in 1985 by I.S.P.P. Professor Afridi left all of us on 6th January, 2010 for heavenly abode at the age of about 79 years.

We dedicate this book to Professor M. M. R. K. Afridi for his marvelous contribution in the area of physiology and mineral nutrition of higher plants.

Preface

Nitric oxide (NO), a versatile gaseous free radical that diffuses readily through biological membranes, plays important role in diverse physiological processes in plants. A plethora of NO-generated events encompasses through germination to flowering and fruit ripening in a plant's life cycle. It alters flowering, stimulates germination, induces pollen tube re-orientation, breaks seed dormancy, triggers mitogen-activated protein (MAP) kinase signaling pathways, modulates the activity of certain enzymes, regulates stomatal closure, photosynthesis, cellular trafficking, cell death, expression of cell cycle genes, and other key metabolic processes. NO plays a key role as signaling molecule in biotic and abiotic stress signal transduction pathways in plants. NO acts as an antioxidant and confers resistance against detrimental consequences of stresses.

Acknowledging NO as a significant modulator of biological processes, renewed attention has been given to the mechanism of NO synthesis in plants. The reaction pathway of NO synthesis in animals has been employed to investigate the likely parallel in plants. In animal systems, NO is synthesized predominantly by the enzyme NO synthase (NOS) that converts L-Arginine into L-citrulline in a NADPH-dependent reaction, which releases one molecule of NO for each molecule of L-Arginine. Assays for Arginine to citrulline conversion and compounds that inhibit mammalian NOS have been used on several occasions to draw an analogy that NO synthesis by a NOS-type enzyme also occurs in plants. But still no direct homologs of any of the animal enzymes have been found in any of the fully sequenced plant genomes. This leaves us with many questions than answers related to NO biosynthesis, detection and mode of action in plants.

The research field of NO biology has transcended rapidly over the last few years, and a huge wealth of information has been accumulated in NO research arena. As a result, it became tangible that NO affects far more fundamental biological processes in plants, than originally anticipated.

Therefore, in our opinion, an overview of detection, biosynthesis and metabolism of NO and its role in stress physiology of plants is well timed.

This book "Nitric Oxide in Plants: Metabolism and Role in Stress Physiology" comprises of 17 chapters that covers the key features of NO molecule in a sequential manner starting from its metabolism, identification and detection in plants (Part I) to current understanding of NO molecule and its derivatives in terms

of chemical, physical, and biochemical properties, functional role, mode of action, signaling and interaction with phytohormones, mineral nutrients, biomolecules, ions and ion channels in plants under abiotic stresses (Part II).

Part I of the book comprises Chaps. 1–9. Chapter 1 presents an overview of NO metabolism with particular emphasis on the sources of NO in plants and their importance under abiotic stress conditions. Chapter 2 sheds light on the reductive and oxidative NO synthesis and their regulation. Chapter 3 discusses the peroxisomes as a source of NO and NO-derived species in response to abiotic stresses and detection of NO generation in peroxisomes. Chapter 4 is focussed on the role of mitochondrial NO homeostasis during hypoxic conditions. Chapter 5 deals with the detection methods and synthesis of NO in plants using marine unicellular red tide phytoplankton, *Chattonella marina,* as a model. Chapter 6 sheds light on the role of NO in nitrosylation of cystein thiol residues in proteins, and summarizes different methods developed to identify and quantify nitrosylated proteins. In this chapter authors also provided the first overview of plant nitrosylated proteome showing a wide range of functions and cellular compartments involved in NO signaling and/or targeting. Chapter 7 presents an overview of detection and measurement of NO and nitrosylated proteins, and various levels of regulation of NO on jasmonate signaling and biosynthesis pathway in response to abiotic stress. Chapter 8 sheds light on the function of *S*-nitrosoglutathione reductase (GSNO) as a natural reservoir of NO bioactivity and role of GSNO in plant development and stress response. Chapter 9 discusses nitro-fatty acids in the context of their biochemical activities and cell signaling actions.

Part II of the book includes Chaps. 10–17. Chapter 10 is focused on the properties of NO and its derivatives and their role as potent modulator of the redox regulation in various cell transduction pathways in response to abiotic stresses. Chapter 11 highlights the recent advances in NO signal transduction and its interactions with other signaling molecules in response to abiotic stress. Chapter 12 summarizes the role of exogenously applied NO on structural and functional parameters of plant cells under H_2O_2-induced oxidative stress. Chapter 13 focuses on the current knowledge of possible interactions between NO and phytohormones during plant abiotic stress responses. Whereas Chap. 14 presents an overview of the synergistic role of NO and calcium in the tolerance of plants to abiotic stress. Chapter 15 discusses functional links between the plant growth promoting action of humic substances and NO in response to abiotic stresses. Chapter 16 is focused on the role of chitosan-mediated induction of NO in plant defense responses against pathogen attack and crosstalk between abiotic and biotic stress responses is also discussed. Chapter 17 deals with the involvement of NO and other signaling molecules in signaling cascade and gene expression during biotic and abioitc stresses induced programmed cell death.

We collected contributions from various laboratories studying NO plant biology, and intended to present an overview of the contemporary challenges and possibilities in different areas of NO. We hope that this book will raise your interest in the field of NO research and will serve as a valuable reference.

We would like to express our gratitude to all the authors and reviewers who contributed to this book. Furthermore, we acknowledge Springer Science+Business Media, Heidelberg, with heartfelt gratitude to Dr. Christina Eckey, Editor, Plant Sciences and Dr. Andrea Schlitzberger, Project Coordinator for their professional support and cooperation during the preparation of the manuscript.

Tabuk, Saudi Arabia — M. Nasir Khan
Tabuk, Saudi Arabia — Mohammad Mobin
Aligarh, India — Firoz Mohammad
Granada, Spain — Francisco J. Corpas

Contents

Contributors

Dariusz Abramowski Department of Plant Physiology, Poznan University of Life Sciences, Poznan, Poland

Magdalena Arasimowicz-Jelonek Faculty of Biology, Department of Plant Ecophysiology, Adam Mickiewicz University, Poznan, Poland

E. Bacaicoa CIPAV-Roullier Group, Orcoyen, Spain

R. Baigorri CIPAV-Roullier Group, Orcoyen, Spain

Y. S. Bakakina Institute of Biophysics and Cell Engineering, National Academy of Sciences of Belarus, Minsk, Belarus

Juan B. Barroso Área de Bioquímica y Biología Molecular, Facultad de Ciencias Experimentales, Departamento de Biología Experimental, Universidad de Jaén, Jaén, Spain

Stefania Bonsegna CNR-ISPA, Institute of Sciences of Food Productions, Lecce, Italy

Mounira Chaki German Research Center for Environmental Health, Institute of Biochemical Plant Pathology, Helmholtz Zentrum München, Neuherberg, Germany

Francisco J. Corpas Department of Biochemistry, Cell and Molecular Biology of Plants, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Spain

Stefania De Domenico CNR-ISPA, Institute of Sciences of Food Productions, Lecce, Italy

L. V. Dubovskaya Institute of Biophysics and Cell Engineering, National Academy of Sciences of Belarus, Minsk, Belarus

Abasse Fares CEA Saclay DSV/iBiTec-S/SIMOPRO/LCV, Gif-sur-Yvette, France

Jolanta Floryszak-Wieczorek Department of Plant Physiology, Poznan University of Life Sciences, Poznan, Poland

Luciano Freschi Department of Botany, Institute of Biosciences, University of São Paulo, São Paulo, SP, Brazil

M. Fuentes CIPAV-Roullier Group, Orcoyen, Spain

Andrea Galatro School of Pharmacy and Biochemistry, Physical Chemistry-Institute of Biochemistry and Molecular Medicine (IBIMOL), University of Buenos Aires-CONICET, Buenos Aires, Argentina

J. M. Garcia-Mina CIPAV-Roullier Group, Orcoyen, Spain; Faculty of Sciences, Department of Chemistry and Soil Chemistry, University of Navarra, Pamplona, Spain

Ming-Hua Gu College of Agronomy, Guangxi University, Nanning, People's Republic of China

Hu-Yi He College of Agronomy, Guangxi University, Nanning, People's Republic of China

Long-Fei He College of Agronomy, Guangxi University, Nanning, People's Republic of China

Karolina Izbiańska Faculty of Biology, Department of Plant Ecophysiology, Adam Mickiewicz University, Poznan, Poland

M. Nasir Khan Faculty of Science, Department of Biology, University of Tabuk, Tabuk, Saudi Arabia

Daekyung Kim Jeju Center, Korea Basic Science Institute, Ara-dong, Jeju-Si, Jeju Special Self-Governing Province, Korea

E. V. Kolesneva Institute of Biophysics and Cell Engineering, National Academy of Sciences of Belarus, Minsk, Belarus

Yee-Min Kwan Faculty of Agriculture, Department of Plant Protection, Institute of Tropical Agriculture, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

Wei-Biao Liao College of Agronomy, Gansu Agricultural University, Lanzhou, China

Christian Lindermayr German Research Center for Environmental Health, Institute of Biochemical Plant Pathology, Helmholtz Zentrum München, Neuherberg, Germany

Eduardo López-Huertas Department of Biochemistry, Cell and Molecular Biology of plants, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Spain

Helenice Mercier Department of Botany, Institute of Biosciences, University of São Paulo, São Paulo, SP, Brazil

Paulo T. Mioto Department of Botany, Institute of Biosciences, University of São Paulo, São Paulo, SP, Brazil

M. Mobin Faculty of Science, Department of Biology, University of Tabuk, Tabuk, Saudi Arabia

Firoz Mohammad Plant Physiology Section, Department of Botany, Aligarh Muslim University, Aligarh, India

V. Mora CIPAV-Roullier Group, Orcoyen, Spain

Tatsuya Oda Faculty of Fisheries, Division of Biochemistry, Nagasaki University, Nagasaki, Japan

M. Olaetxea Faculty of Sciences, Department of Chemistry and Soil Chemistry, University of Navarra, Pamplona, Spain

Halley Caixeta Oliveira Departamento de Biologia Animal e Vegetal, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, PR, Brazil

José M. Palma Department of Biochemistry, Cell and Molecular Biology of plants, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Spain

Jean-Benoît Peltier Campus SupAgro-INRA, Montpellier, France

Palmiro Poltronieri CNR-ISPA, Institute of Sciences of Food Productions, Lecce, Italy

Susana Puntarulo Facultad de Farmacia y Bioquímica, Fisicoquímica-IBIMOL, Buenos Aires, Argentina

Luis A. del Río Department of Biochemistry, Cell and Molecular Biology of plants, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Spain

Michel Rossignol Campus SupAgro-INRA, Montpellier, France

Tamás Rőszer Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain; Zentrum für Biomedizinische Forschung, Institut für Allgemeine Zoologie und Endokrinologie, Universität Ulm, Ulm, Germany

Homero Rubbo Facultad de Medicina, Departamento de Bioquímica, Center for Free Radical and Biomedical Research, Universidad de la República, Montevideo, Uruguay

Mansour Salati Department of Plant Protection, Golestan Agricultural and Natural Resources Research Centre, Golestan, Iran

Ione Salgado Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, SP, Brazil

Angelo Santino CNR-ISPA, Institute of Sciences of Food Productions, Lecce, Italy

M. Ali Saqib Faculty of Science, Department of Biology, University of Tabuk, Tabuk, Saudi Arabia

Marco Taurino CNR-ISPA, Institute of Sciences of Food Productions, Lecce, Italy

Andrés Trostchansky Facultad de Medicina, Departamento de Bioquímica, Center for Free Radical and Biomedical Research, Universidad de la República, Montevideo, Uruguay

I. D. Volotovski Institute of Biophysics and Cell Engineering, National Academy of Sciences of Belarus, Minsk, Belarus

Mui-Yun Wong Faculty of Agriculture, Department of Plant Protection, Institute of Tropical Agriculture, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

Ji-Hua Yu College of Agronomy, Gansu Agricultural University, Lanzhou, China

A. M. Zamarreño CIPAV-Roullier Group, Orcoyen, Spain

Part I
Nitric Oxide: Metabolism, Identification and Detection

Chapter 1
An Update to the Understanding of Nitric Oxide Metabolism in Plants

Andrea Galatro and Susana Puntarulo

Abstract Nitric oxide (NO) is an inorganic free radical gaseous molecule which has been shown to play an unprecedented range of roles in biological systems. The potential reactions of NO are numerous and depend on many different factors. The site and source of production, as well as the concentration of NO collectively determine whether NO will elicit direct or indirect effects. In animals, NO is generated by the activity of nitric oxide synthase (NOS). In plants, neither the gene nor protein similar to known NOS has been found. However, different pathways producing NO in plants have been described, and can be classified as either oxidative or reductive steps. These sources of NO seem to cooperate to the growth and development, and to respond to several stress situations like abiotic stress. Chloroplasts are key organelles in plant metabolism and they seem to be involved in NO production, thus, proposed pathways for NO generation in chloroplasts are discussed.

Keywords Chloroplastic nitric oxide · Nitric oxide · Nitrogen active species · Nitric oxide sources

A. Galatro
School of Pharmacy and Biochemistry, Physical Chemistry-Institute of Biochemistry and Molecular Medicine (IBIMOL), University of Buenos Aires-CONICET, Buenos Aires, Argentina

S. Puntarulo (✉)
Facultad de Farmacia y Bioquímica, Fisicoquímica-IBIMOL, Junín 956, C1113AAD Buenos Aires, Argentina
e-mail: susanap@ffyb.uba.ar

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1.1 Introduction

1.1.1 Brief Review of the Chemistry of Nitrogen-Active Species

The broader chemistry of nitric oxide (NO) involves a redox array of species such as nitrosonium (NO^+), NO radical (NO) and nitroxyl anion (NO^-) (Fig. 1.1) which exhibit distinctive properties and reactivities (Gisone et al. 2004).

Neutral NO has a single electron in its 2p-π antibonding orbital and the removal of this electron forms NO^+ while the addition of one more electron to NO forms NO^- (Stamler et al. 1992). The chemistry of NO^+ is characterized by addition and substitution reactions with nucleophiles such as electron-rich bases and aromatic compounds. Nitrosation in aqueous phase can occur at –S, –N, –O, and –C centers in organic molecules and appears to involve NO^+ or related NO^+ equivalents. The biological relevance of NO^+ under weakly acidic or physiological conditions had been disputed, however a variety of nitroso-compounds that form effectively under neutral physiological conditions (Stamler et al. 1992) can be interpreted as reactions with NO^+ carriers. Important examples of such compounds are metal-nytrosyl complexes, thionitrites (RS–NO), nitrosamines (RNH–NO), alkyl and aryl nitrites (RO–NO) and dinitrogen tri- and tetra-oxides (N_2O_3 and N_2O_4). In biological systems, there are numerous nucleophilic centers whose potential susceptibility to nitrosative attack has been shown in in vitro studies (Stamler et al. 1992). The chemistry of NO^- has received significantly less attention, particularly in aqueous solution. NO^- converts rapidly to N_2O through dimerization and dehydration (Basylinski and Hollocher 1985) and it is known to react with Fe (III) heme (Goretski and Hollocher 1988). NO^- also undergoes reversible addition to both low molecular weight and protein-associated thiols, leading to sulfhydryl oxidation. Electron transfer and collisional detachment reactions are common and generally yield NO radical (NO) as the major product. *S*-nitrosothiols are believed to be a (minor) product of the reaction of NO^- with disulfides (Stamler et al. 1992).

From a biological point of view, the important reactions of NO are those with O_2 and its various redox forms and with transition metal ions. The reaction of NO with O_2 in aqueous solution is a second-order reaction in [NO] ($v = k\,[NO]^2\,[O_2]$) (Stamler et al. 1992), thus the biological half life of NO, generally assumed to be in the order of seconds, strongly depends on its initial concentration. NO also reacts rapidly with O_2^- in aqueous solution, yielding peroxynitrite ($ONOO^-$) (Saran et al. 1990). When discussing the chemistry and physiological effects of NO, it should be considered that NO is a highly diffusible second messenger that can elicit effects relatively far from its site of production. The concentration and therefore the source of NO are the major factors determining its biological effects (Wink and Mitchell 1998). At low concentrations (<1 μM), the direct effects of NO predominate. At higher concentrations (>1 μM), the indirect effects mediated by reactive nitrogen species (RNS) prevail. The direct effects of NO most often involve the interaction of NO with metal complexes. NO forms complexes with

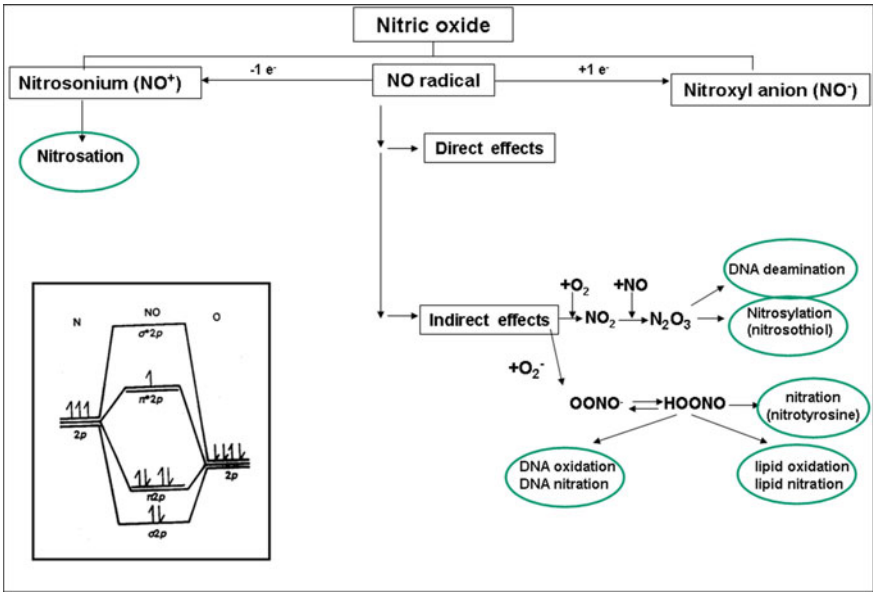

Fig. 1.1 Summary of chemistry of nitrogen-active species and some effects of the independent species. *Inset* Molecular orbital diagram for NO

the transition metal ions, including those regularly found in metalloproteins. The reactions with heme-containing proteins have been widely studied. NO also forms non-heme transition metal complexes and biochemical interest has been focused on its reactions toward Fe–sulfur centers in proteins, including several proteins involved in mitochondrial electron transport and enzymes (Henry et al. 1991). The reactions of NO with heme-containing proteins are the most physiologically relevant and include interactions with cytochrome P_{450} (Wink et al. 1993). Another established direct effect of NO on proteins is tyrosine nitration. Tyrosine nitration is selective and reversible and it has been shown that there are $ONOO^-$ dependent and independent pathways for the nitration in vivo (Davis et al. 2001). NO is also able to terminate lipid peroxidation (Rubbo et al. 1995). The indirect effects of NO, produced through the interaction of NO with either O_2 or O_2^-, include nitrosation (when NO^+ is added to an amine, thiol, or hydroxy aromatic group), oxidation (when one or two electrons are removed from the substrate), or nitration (when NO_2^+ is added to a molecule) (Wink et al. 1993). In aqueous solution NO can undergo autoxidation (i.e., reaction with O_2) to produce N_2O_3 and this compound can undergo hydrolysis to form nitrite (Ford et al. 1993). Since NO and O_2 are 6–20 times more soluble in lipid layers as compared to aqueous fractions, the rate of autoxidation is increased dramatically in the lipid phase (Ford et al. 1993) and the primary reactions of N_2O_3 are thought to occur primarily in the membrane fraction. In its reaction with O_2^-, NO generates $ONOO^-$ at a rate close to diffusion, and $ONOO^-$ acts as both nitrating agent and powerful oxidant to modify proteins

(formation of nitrotyrosine), lipids (lipid oxidation, lipid nitration), and nucleic acids (DNA oxidation and DNA nitration).

In summary, the potential reactions of NO are numerous and depend on many different factors. The site and source of production, as well as the concentration of NO collectively determine whether NO will elicit direct or indirect effects. In addition, a relative balance between oxidative and nitrosative stress exists, and it is a main aspect that should be carefully evaluated for understanding the complexity of biological effects of NO.

1.2 Sources of NO in Plants: An Overview

In animals, NO is generated by the activity of nitric oxide synthase (NOS). NOSs catalyze the conversion of L-Arginine to L-Citrulline and NO. The reaction requires O_2 and NADPH (Wendehenne et al. 2001) (Eq. 1.1).

$$\text{L} - \text{Arginine} + \text{NADPH}_2 + \text{O}_2 \rightarrow \text{L} - \text{Citrulline} + \text{NO} + \text{H}_2\text{O} \qquad (1.1)$$

While these mammalian NOSs are long known and well characterized, the plant community has not been successful in identifying corresponding genes or enzymes in higher plants so far (Fröhlich and Durner 2011). In plants, neither the gene or cDNA, nor any protein with high sequence similarity to known NOS, have been found (Lamattina et al. 2003). Despite this, several efforts have been made to improve this knowledge. Chandok et al. (2003) described the purification and characterization of a pathogen inducible NOS-like activity from tobacco plants and its identification as a variant form of P subunit of the glycine decarboxylase complex. However, this work was retracted by Klessig et al. (2004) due to difficulties in reproducing some data related to NO-synthesizing activity of the recombinant variant P.

A second approach was developed by Guo et al. (2003), with the identification of a plant NOS gene involved in hormonal signaling (*Atnos1*). *Arabidopsis* mutant (*Atnos1*) had impaired NO production, organ growth, and abscisic acid-induced stomatal movements. According to Guo et al. (2003), purified AtNOS1 protein employed arginine and NADPH as substrates, and was activated by Ca^{2+} and calmodulin, like mammalian endothelial and neuronal NOS. Thus, AtNOS1 was proposed as a distinct enzyme, with no sequence similarities to any mammalian isoform, and with a role in growth and hormonal signaling in plants (Guo et al. 2003). Later, due to the failure in the detection of NOS activity in purified AtNOS1 protein (Crawford et al. 2006; Zemojtel et al. 2006), it was suggested renaming AtNOS1 to AtNOA1 (nitric oxide associated 1), because it seems to be important for NO generation in the cell, but it is not a real NOS as defined for animal system. Although different research groups have independently confirmed the presence of decreased NOS activity and NO levels in the *Arabidopsis* mutant (*Atnos1*), other reports found that NO accumulation in response to different hormones or oxidative stress was similar in wild-type and *nos1* plants (Gas et al. 2009). Besides, not all the

phenotypes observed in the mutant can be rescued by NO supplementation (Gas et al. 2009). Thus, AtNOS1, renamed as AtNOA1, seems to have another function different from NO synthesis. Moreau et al. (2008) showed that AtNOA1 is a member of the circularly permuted GTPase family (cGTPase). AtNOA1 specifically binds GTP and hydrolyzes it. However, GTP hydrolysis is necessary but not sufficient for the physiological function of AtNOA1. Also, the C-terminal domain seems to play a crucial role *in planta*. cGTPases appear to be RNA-binding proteins, and the closest homolog of AtNOA1, the *Bacillus subtilis* YqeH, has been shown to participate in ribosome assembly and stability (Moreau et al. 2008).

Even though finally AtNOS1 is not a NOS, the discovery and development of the *Arabidopsis* mutant *Atnos1* was an important finding. The biological role of AtNOA1 or RIF1 (Flores-Pérez et al. 2008) is believed to be primarily associated with chloroplasts ribosome functions (Moreau et al. 2008; Gas et al. 2009; Liu et al. 2010). In *rif1* seedlings, not only chloroplast ultrastructure, but also the level of proteins encoded by the chloroplastic genome were affected (Flores-Pérez et al. 2008), suggesting that NOA1/RIF1 might bind plastidial ribosomes and is required for the normal function and proper protein synthesis in plastids (Gas et al. 2009). It has also been reported that NO accumulation in *Arabidopsis* is independent of NOA1 in the presence of sucrose (Van Ree et al. 2011). Thus, it is possible that the primary requirement for *noa1* activity is efficient chloroplast function to generate photosynthates. Provision of sucrose enables *noa1* to accumulate NO, raising the question why fixed carbon may be necessary for NO accumulation in *Arabidopsis* (Van Ree et al. 2011).

To add more complexity to this scenario, Foresi et al. (2010) have characterized the sequence, protein structure and biochemistry of NOS from the green alga *Ostreococcus tauri*. This NOS contains the main characteristics of animal NOS, and NO generation in this alga is dependent on light irradiance and growth phase. This single-cell alga is of particular interest because it shares a common ancestor with higher plants, providing compelling evidence that an active NOS functions in a photosynthetic organism belonging to the plant kingdom (Foresi et al. 2010).

NOS enzymes seem to be present in almost all organisms except plants. Despite the fact that NO plays a crucial role in plant physiology, higher plants seem to have lost the specific NOSs in the course of evolution (Fröhlich and Durner 2011). However, different pathways to produce NO in plants have been described, and they can be classified as either oxidative or reductive (Gupta et al. 2011a). Briefly, nitrate reductase (NR) as shown in Eq. 1.2, and mitochondrial or plasma membrane-associated NO production (NR:NiNOR system) are all reductive pathways and depend on nitrite as a primary substrate, whereas NO production from L-Arginine, polyamine or hydroxylamine are among the oxidative pathways (Gupta et al. 2011a).

$$2NO_2^- + NAD\ (P)H\ + 3H_3O^+ \overset{NR}{\rightarrow} 2NO\ + NAD(P)^+ + 5H_2O \qquad (1.2)$$

Although no NOS enzyme has been identified in plants, a NOS-like activity has been extensively reported. We have described L-Arginine-dependent NO generation in soybean leaves (Galatro et al. 2004) and soybean chloroplasts (Jasid et al. 2006), which were evaluated employing electron paramagnetic resonance (EPR). In both cases, NO generation was NADPH dependent and inhibited by known NOS mammalian inhibitors. Corpas et al. (2006) also described NO production from L-Arginine (NOS activity) in leaves, stems, and roots of pea seedlings during plant development, using a chemiluminescence-based assay and confocal laser scanning microscopy. Peroxisomes, have also been proposed as cellular source of RNS. NOS activity in peroxisomes was described employing several approaches (for a review, see del Río 2011). Also EPR measurements, employing isolated peroxisomes from pea leaves, clearly indicated the generation of NO as a result of the L-Arginine-dependent NOS activity (del Río 2011). Another candidate for NO production is the peroxisomal enzyme xanthine oxidoreductase (XOR). XOR from animal origin can produce superoxide (O_2^-) and NO free radicals during its catalytic reaction (del Río 2011).

Regarding polyamine (PA)-mediated NO generation, Tun et al. (2006) observed that addition of PAs to *Arabidopsis thaliana* seedlings caused rapid release of NO. A speculation could be the conversion of PA by as yet unknown enzymes or by PA oxidases to generate NO. PA oxidases are not known to generate NO in animal systems, and PA oxidase could be inhibited by L-NAME (L-nitroarginine methyl ester) (Tun et al. 2006).

L-Arginine and NR-dependent pathways have been the most reported (Rasul et al. 2012). Rasul et al. (2012) have investigated NO production in *Arabidopsis* elicited by oligogalacturonides (OGs) and have suggested that L-Arginine and NR pathways are co-involved in NO production and do not work independently. Recently, we also observed that cotyledons from soybean plants growing in the presence of ammonia as the unique source of nitrogen were physiologically nondistinguishable from control (nitrate-fed) cotyledons, and showed a similar NO accumulation, indicating that cotyledons are able to produce similar amounts of NO independently of the source of nitrogen supplied. These results led us assumed that different sources of NO could operate for NO accumulation in soybean cotyledons, e.g., nitrite- and L-Arginine-dependent sources. Thus, it is likely that under different conditions, for example the lack of a substrate, one pathway could result more operative to maintain NO generation and support the required NO levels in the cell to allow a normal function and development (Galatro et al. 2013). In this sense, NO production in *Arabidopsis* plants following pathogen attack may result from the interplay of L-Arginine- and nitrite-dependent pathways (Modolo et al. 2005). Rasul et al. (2012), suggested that L-NAME-sensitive NO production also affect NR-dependent NO production. NO can stimulate NR activity at the pos-translational level through a direct interaction or, alternatively, by affecting the activity of proteins involved in NR regulation. Part of the NO produced by L-Arginine-dependent pathway could be oxidized to nitrite, thus providing substrate for NR-triggered NO synthesis. Polyamines seem to be involved in NR

activity regulation. Rosales et al. (2012) studied the effect of PAs on NR activity in wheat leaves exposed to exogenously added PAs, and demonstrated that NO was involved in the inhibition or increase of NR activity. These findings point out the complexity of the study of NO generation in plants, as different pathways could be involved, and also work together for NO production in the plant cell under physiological or stress situations.

Evidence that plants oxidize hydroxylamines to NO has been described, open a new possibility for oxidative NO formation in plants. However, the existence and role of these reactions under physiological conditions are not clear (Rümer et al. 2009). Further experiments are required to find out whether any natural hydroxylamines can be formed under specific conditions by plants to serve as substrates for an endogenous oxidative NO generation (Rümer et al. 2009).

The mitochondrial electron transport chain is another proposed site for nitrite to NO reduction, operating significantly when the normal electron acceptor, O_2, is low or absent. Under these conditions, the mitochondrial NO production contributes to hypoxic survival by maintaining a minimal ATP formation (Gupta et al. 2011b).

1.2.1 Is Chloroplast a Source of NO?

The first reports describing chloroplasts as an NO source were based on studies developed with tobacco (Foissner et al. 2000; Gould et al. 2003). Foissner et al. (2000) described NO accumulation in epidermal tobacco leaf cells subjected to a proteinaceous elicitor from *Phytophthora cryptogea*. They evidenced an NO production in the cytosol, along plasma membrane, in chloroplasts, and organelles probably representing peroxisomes. NOS inhibitor N_G-mono-methyl-arginine monoacetate (L-NMMA) reduced NO levels but not as the NO scavenger cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide). These results suggested that other sources of NO could be operative.

In our laboratory, we have identified two independent pathways for NO generation in soybean chloroplasts, one pathway was dependent of the activity of a NOS-like enzyme employing L-Arginine and NADPH, and another pathway was dependent of nitrite (Jasid et al. 2006). NO generation in isolated chloroplasts was evaluated employing EPR in the presence of the spin trap (sodium-*N*-methyl-D-glucamine dithiocarbamate [MGD])$_2$-Fe(II), and the required cofactors described for assaying the activity of plant NOS (Galatro et al. 2004). The EPR signal corresponding to NO–MGD–Fe adduct was inhibited if the chloroplasts were incubated with NOS inhibitors, such as N_{ϖ}-nitro-L-Arg methyl ester hydrochloride (L-NAME) or N_{ϖ}-nitro-L-Arg (L-NNA). It is interesting to point out that Arginine was shown to be an abundant amino acid in chloroplast stroma, and that the reported synthesis of NO was not affected either by omission or addition of Ca^{2+} or by supplementation with calmodulin (Jasid et al. 2006). On the other hand, intact chloroplasts incubated under light conditions in the presence of sodium

nitrite also generated NO. However, this generation was detectable in the thylakoid fraction but not in the stroma, and was affected by the inhibition of photosynthetic electron flow by the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), that binds plastoquinone and blocks electron flow at the quinone acceptors of photosystem II. These results suggested that thylakoids were the main component of chloroplast involved in nitrite reduction (Jasid et al. 2006). Thus, chloroplasts seem to be able to produce NO in vitro, with the supplementation of adequate substrates. However, other alternative sources could be relevant under certain physiological or pathological conditions. Further experiments are required to assess the relative contribution of different sources, such as NO release from endogenous GSNO (*S*-nitrosoglutathione) (Barroso et al. 2006).

Arnaud et al. (2006), described NO generation in chloroplasts from *Arabidopsis* cells. They reported that NO accumulated in the chloroplasts after Fe treatment, and acts downstream of Fe to promote an increase of *AtFer1* (*Arabidopsis* Ferritin 1) mRNA level. This increase was inhibited by L-NMMA indicating that a NOS activity is involved in the pathway. However, since inhibition was not complete other pathways may lead to NO production in response to Fe (Arnaud et al. 2006). Tewari et al. (2013) also described endogenous NO, and $ONOO^-$ generation in protoplasts chloroplasts from *Brassica napus* L. cv. Bronowski plants. The inhibition of DAF fluorescence in the presence of NOS inhibitors suggests the involvement of NOS-like activity in NO generation in these chloroplasts. Moreover, protoplasts from *Atnoa1* mutants exhibited weak signal of NO generation (Tewari et al. 2013). Thus, AtNOA1 seems to be important for NO generation also in chloroplasts.

Recently, we explore the hypothesis that the content of NO in soybean cotyledons is related to chloroplast functionality *in planta*. Employing confocal fluorescence microscopy and EPR techniques, Galatro et al. (2013) showed that chloroplasts contribute to NO synthesis in vivo. Moreover, the level of NO in the whole tissue was related to chloroplasts functionality. The detection of NO in coincidence with cotyledon maximum fresh weight, chlorophyll content, and quantum yield of PSII, supported the hypothesis of a strong link between NO levels and chloroplast functionality. In addition, seedlings exposed in vivo to herbicides showed deleterious effects on chloroplast function (loss of photosynthetic capacity), and an impaired NO accumulation. The employment of the herbicide DCMU supports a role for the integrity of the photosynthetic electron chain in chloroplasts NO production in vivo, as was previously observed by Jasid et al. (2006) in the in vitro experiments with isolated chloroplasts. These results are consistent with the requirement of chloroplasts for NO generation in soybean cotyledons, both as a result of the active synthesis of NO in the organelle and/or because of an indirect requirement of some chloroplast products for NO synthesis in other areas of the plant, as it was described by Van Ree et al. (2011). Overall, these findings strongly suggest that chloroplasts are the organelles that contribute to NO synthesis in vivo, and that their proper functionality is essential for maintaining NO levels in soybean cotyledons (Galatro et al. 2013).

Chloroplasts are key organelles in plant metabolism, and seem to be strongly involved in NO synthesis. NO may function in chloroplasts as a regulator of

photosynthetic electron transport, and as an antioxidant preserving lipids, proteins (including D1) and nucleic acids from photooxidative damage (Jasid et al. 2006; Beligni et al. 2002), but also may be part of a complex network of regulation involved in processes that transcend chloroplasts, as its participation in Fe metabolism (Arnaud et al. 2006) through transcription of nuclear-encoded *AtFer1* gene.

1.2.2 NO Sources Under Abiotic Stress

Gould et al. (2003) have reported the impact of several abiotic stresses like, light, high temperatures, osmotic shock, salinity and mechanical injury on NO evolution from tobacco leaf cells. They tested the hypothesis that NO generation occurs as a general response to different environmental cues. However, they concluded that although different stressors can trigger NO synthesis (like high temperatures, osmotic stress, or salinity), it cannot be considered a universal plant stress response.

Several sources of NO would be involved in responses to abiotic stress. A NOS-like activity was detected in guard cells of *B. juncea*, which was enhanced by abiotic stress (Talwar et al. 2012). NOS-like activity has been involved in the induction of cadmium accumulation, cadmium-induced programmed cell-death, and protective responses against UV-B (Gupta et al. 2011a), salt stress, and phosphate deficiency (Fröhlich and Durner 2011). In addition, the NOS pathway is important for postharvest NO synthesis in tomato to avoid chilling injury (Zhao et al. 2011). NR as NO producer has been involved in cold, drought and osmotic stress (Gupta et al. 2011a; Fröhlich and Durner 2011). Ziogas et al. (2013) have studied nitrosative responses in citrus plants exposed to various abiotic stresses, including continuous light, continuous dark, heat, cold, drought, and salinity. They have shown that the expression of several genes potentially involved in NO production, was affected by the abiotic stress treatments, demonstrating that NO-derived nitrosative responses could be regulated by various pathways.

From these studies, it can be concluded that NO synthesis in response to abiotic stress could be achieved by different sources acting separately or jointly to deal with the stress for cell viability.

1.3 Concluding Remarks

It is clear that NO content in plants varies among tissues, and also depends on physiological status. The generated NO is widely accepted to cooperate for the growth and development of plants, and also to be a good candidate to participate in response to several stress conditions. Figure 1.2 briefly summarized proposed sources of NO in plants. Although the knowledge of NO functions in plants has been largely improved, the isolation and characterization of a single protein with

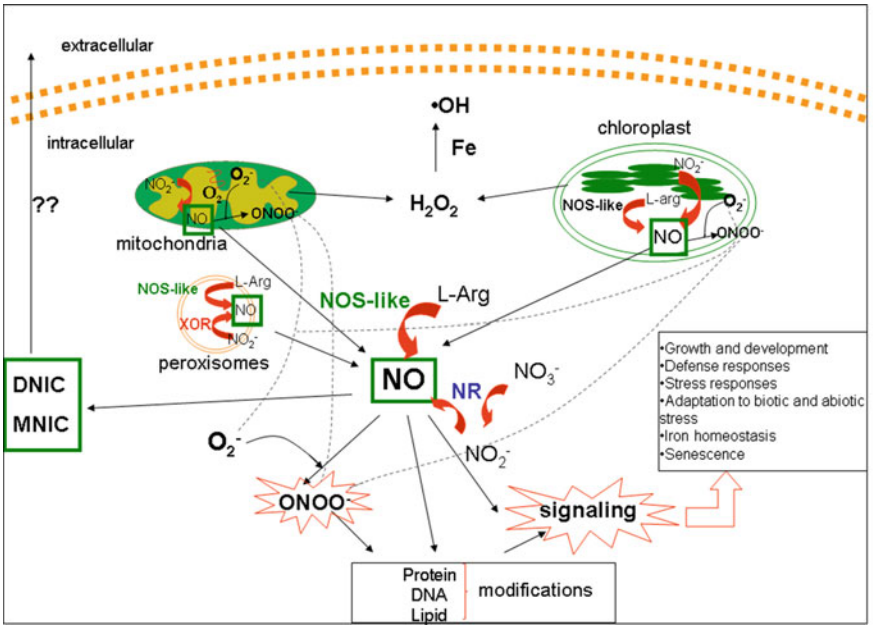

Fig. 1.2 Scheme of main proposed subcellular sources of NO in plants. Reaction with ROS and Fe, and biological effects are indicated. *NOS-like* nitric oxide synthase-like activity; *NR* nitrate reductase; *XOR* xanthine oxidoreductase; *MNIC* mononitrosyl Fe complexes, *DNIC* dinitrosyl Fe complexes; *L-Arg* L-Arginine; $ONOO^-$ peroxinitrite; H_2O_2 hydrogen peroxide. *Dotted lines* indicate the diffusion of the species. *Continuous lines* link species to their functions

NOS activity is still matter of active research and remains an issue to be fully elucidated. The complex scenario shown in the Fig. 1.2 reflects the participation of several organelles (chloroplasts, mitochondria, peroxisomes and cytosolic enzymatic activities) and reactive species that lead to the generation of not only NO but also $ONOO^-$. The dual effects of NO in the cellular biochemical steady state condition due to its capacity of both protect or damage bio-molecules require a careful analysis of each condition before designing any operative strategy. However, the possibility of affording laboratory protocols developed to change this versatile molecule functions in the inner of the cell could be considered as one of the intriguing issues and is nowadays the centre of an active debate and investigation.

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Chapter 2
Biosynthesis of Nitric Oxide in Plants

Tamás Rőszer

Abstract Nitric oxide (NO) regulates important events in plant physiology, disease resistance and stress tolerance. In plants, distinct enzymatic and chemical processes can generate NO from nitrite (NO_2^-), L-Arginine and possibly other *N*-compounds. Reduction of NO_2^- to NO is catalyzed by nitrate reductase and the mitochondrial electron transport chain. Deoxygenated heme-proteins also facilitate NO production from NO_2^-. NO may also be released in nonenzymatic processes from nitrous acid and *S*-nitrosoglutathione. Whether plants have a specific enzyme with primary oxidative NO synthesizing activity is an open debate. Although, NO synthase-homolog genes are present in green algae, and a protein (AtNOS1/AtNOA1) with regulatory effects on oxidative NO synthesis is known in vascular plants, integration of the multiple NO producing processes requires a complex regulatory network in the plant cell. However, our insight into the underlying molecular mechanisms is still limited. Plant hormones, stress and injury signals, modulation of intracellular Ca^{2+} levels are the potential drivers of plant NO synthesis under physiological and stress conditions.

Keywords Cell signaling · Nitrate reductase · Nitric oxide synthase · Plant hormones

2.1 Introduction

Nitric oxide (NO) is a bioactive molecule with multifaceted physiological roles in plants (Rőszer 2012b). Endogenous NO synthesis has been identified in cyanobacteria (Sturms et al. 2011), green algae (Foresi et al. 2010), lichens (Catala et al.

T. Rőszer (✉)
Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain
e-mail: troszer@cnic.es; tamas.roeszer@uni-ulm.de

T. Rőszer
Zentrum für Biomedizinische Forschung, Institut für Allgemeine Zoologie und Endokrinologie, Universität Ulm, Helmholtz Strasse 8/1 89081 Ulm, Germany

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