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Foreword

Plants are essential to life on Earth, and they have been harnessed by humans for food, fuel, and many other purposes. The need to increase crop production is becoming more urgent due to increasing population and diversion of crops to biofuels production. Furthermore, this increase in production needs to be done sustainably, with reduced inputs, and in the face of global environmental change. It is also notable that at least one-third of the world's food production is grown under irrigation—much of this irrigation is unsustainable, using water supplies that are both overexploited and under threat from changing weather patterns resulting from global climate change. To meet the consensus targets for increased food production by 2050, significant increases in historical annual increases in production are required, which requires substantial innovations in agricultural production. Many of these innovations must ultimately come from, among other sources, plant science research. One important and relevant area of this plant science research is covered in-depth in this book.

The gap between potential yield and actual yield is primarily due to the effects of abiotic stresses on crop production. It is therefore an imperative to improve our ability to maintain crop production in environments with suboptimal conditions such as low water or nutrient supplies, or high salinity. One of the key components of plant responses to these abiotic stresses is nitric oxide (NO). Understanding the role of NO in the control of plant reactions provides fundamental information to underpin the much-needed innovations to come in global food production.

As such, the book edited by Drs. Khan, Mobin, Mohammad, and Corpas provides a useful and timely compilation of up-to-date overviews of advances in the important area of plant sciences, "Nitric Oxide Action in Abiotic Stress Responses in Plants." In this volume, a range of papers have been brought together which address the multifarious roles of NO in plant function and the responses of plants to many abiotic stresses. The breadth of areas covered in this volume highlights well the importance of this simple molecule and thus the utility of this compilation. The highly international set of contributors, including many from developing countries, is also both valuable and encouraging.

The editors and contributors are to be congratulated on their efforts, and readers are commended to use this volume for a long time to come, for both teaching and research.

February 2015

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Preface

Nitric oxide (NO), a small gaseous signaling molecule, has attracted huge interest during recent times. The journey of nitric oxide, which started as a harmful air pollutant to the molecule of the year in 1992, has been both interesting and arduous. The discoveries of the enzymes that produce NO with NADPH and L-arginine have altogether shifted the NO paradigm from being a toxic air pollutant to a prolific signaling molecule. Now it is acclaimed to be involved in the modulation of various physiological processes during the entire life of the plant. Several studies have revealed the involvement of NO as a control signal during various abiotic stress responses, including salinity and osmotic stress, temperature, UV light stress, and anoxia. Enhanced NO production by plant tissue has been noted in response to various abiotic stresses. Recent developments in the field of NO biology in plants have indicated that NO closely interacts with several signaling molecules linked with plant adaptive stress responses, such as ABA, cytosolic Ca^{2+}, and H_2O_2.

Abiotic stresses, such as drought, flood, problem soils (salinity and alkalinity), extreme temperatures, and heavy metal toxicity, impose a serious threat to sustainable agricultural production. The production of reactive oxygen species is a unifying commonality in a large number of abiotic stresses. NO interacts with ROS in various ways and might serve as an antioxidant during various stresses. Modulation by NO of superoxide formation and inhibition of lipid peroxidation also illustrates its potential antioxidant role.

This book "Nitric Oxide Action in Abiotic Stress Responses in Plants" is a collection of 15 chapters and is divided into two parts. Part I of the book comprises Chaps. 1–4, which vividly describes the properties of NO and gives an au courant information available on the mitigating effect of NO in stress-induced alterations in plants. Part II, which includes Chaps. 5–15, mainly focuses on the role of NO in environmental- and soil-related abiotic stresses including postharvest stress and wounding.

Chapter 1 describes the current recognition of the roles of reactive nitrogen species such as peroxynitrite or *S*-nitrosothiols in the plant metabolism under both physiological and stress conditions as these molecules are known to react with a

wide spectrum of biomolecules and may act as a transporter and reservoirs for NO in a broad range of plant cell signaling affairs.

Chapter 2 elucidates the significance of nitric oxide as phytohormone and its involvement in alleviation of various abiotic stresses such as drought, salinity, heavy metal stress, high or low temperature stress, and ultraviolet radiation.

Chapter 3 illustrates the antioxidant role of NO and its ability to maintain the cellular redox homeostasis and regulate the toxicities of oxidative stress-induced ROS. Chapter 4 manifests the involvement of nitric oxide in stress-induced morphogenic response (SIMRs). However, the precise nature of involvement of nitric oxide in SIMRs must await clarifications from the likely complex interactions among NO, auxin, and possibly with other plant hormones and reactive oxygen species.

Chapter 5 delves into the current understanding of the involvement of NO in high temperature stress and in the mechanism of thermotolerance. This chapter also highlights the significance of nitric oxide, abscisic acid, H_2O_2, Ca^{2+}, and calmodulin (CaM), as signaling cues in heat stress response in plants.

Chapter 6 highlights the physiological and molecular basis of plant adaptive response to drought regulated by the NO. The chapter discusses how NO aids the regulation of the antioxidative systems, stomatal closure, and adventitious root formation under drought conditions. Additionally, the involvement of NO in plant acclimation to water deficit by activating stress defense genes via post-translational modifications is also elucidated.

Chapter 7 emphasizes the function of plant hemoglobin with particular emphasis on nonsymbiotic hemoglobin and their role in hypoxic tolerance in plants and the involvement of NO. It has also been postulated that class 1 nonsymbiotic hemoglobin (nsHb-1s) possibly acts as NO dioxygenase in the nsHb/NO cycle which consumes NADH and maintains ATP levels via an as yet unknown mechanism.

Chapter 8 provides a synthetic view of our current knowledge on the biology of NO in the context of plant response to low temperature. This includes the specific features of NO production in cold-stressed plants and the functions it may undertake during cold acclimation. A particular attention is paid to the involvement of cold-induced NO in the regulation of cold-responsive gene expression.

Chapter 9 addresses the cytoskeleton-related nitric oxide signaling events under ultraviolet-B (UV-B) exposure. The putative biochemical mechanisms of the protective effects of exogenous NO donors on plant cells and the input of NO-synthase-like activities and nitrate reductase under UV-B exposure are also highlighted.

Chapter 10 describes the effect of transition metal stress on endogenous NO level and how it alters the cellular and metabolic responses. The biological actions of NO and its derivatives exerted through the binding to transition metals of metalloproteins and covalent modifications of cysteine and tyrosine residues. Furthermore, it also examines the effect of exogenous application of NO on transition metal stress responses.

Chapter 11 presents an overview of the current knowledge on the involvement of NO in plant response to nutritional stress, with special emphasis on salinity, calcium and iron homeostasis, and heavy metal stress.

Chapter 12 reviews the effects of heavy metals (HMs) on endogenous NO content. It also provides insight into the enzymatic and nonenzymatic pathways of nitric oxide generation. In addition, the role of exogenous-applied NO in alleviating HMs toxicity is summarized and discussed.

Chapter 13 underlines the current state of the art of the physiological and molecular aspects of NaCl-induced NO signaling in plants and discusses the roles of endogenous and exogenous NO in NaCl toxicity and salt tolerance mechanisms. Particular attention is paid to the role of NO and NO-induced protein modifications in the activation of specific steps of PCD during salt stress.

Chapter 14 illustrates the prospects of managing postharvest handling of horticultural produce by exogenous application of NO. The chapter portrays the beneficial effects of exogenous NO application in lowering the ethylene level, ion leakage, and lipid peroxidation and enhancing natural antioxidant defense systems. It suggests that postharvest application of NO may be a potential new technology to reduce losses in horticultural produce during handling and marketing.

Chapter 15 contrives the current knowledge on the possible role of nitric oxide as a modulator in response to wounding. Furthermore, it is elucidated the involvement of extracellular ATP (eATP) and NO in a complex sequence of events occurring downstream wounding in plants. A brief discussion of the interplay between ROS/RNS and Ca^{2+} as counterpart signal molecules is also presented.

We wish to express profound appreciation to our experienced and well-versed contributors, who cordially accepted our invitation to write their chapters. Nonetheless, we would like to thank Springer Science+Business Media, Heidelberg, especially Dr. Christina Eckey, Editor, Plant Sciences and Dr. Andrea Schlitzberger, Project Coordinator for their professional support and cooperation during the preparation of the manuscript.

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Contents

Part I
Nitric Oxide: Properties and Functional Role

Chapter 1
Reactive Nitrogen Species and Nitric Oxide

Dagmar Procházková, Naďa Wilhelmová and Milan Pavlík

Abstract Free radical nitric oxide (NO) is a biological messenger with diverse functions in plant physiology, including in stress physiology. Together with NO, related molecules called reactive nitrogen species (RNS), e.g. peroxynitrite or *S*-nitrosothiols, are associated with plant metabolism under both physiological and stress conditions. These molecules are able to react with wide spectrum of biomolecules, and they may act as a transporters and reservoirs for NO in a broad range of plant cell signalling affairs. It is possible that some of these reactions, nitration and *S*-nitrosylation, have the same importance as phosphorylation. In this chapter, the current recognition of both the properties, chemical reactions and physiological roles of NO and reactive nitrogen species in plants is reviewed.

Keywords Nitration · Nitrosothiols · Peroxynitrite · *S*-nitrosylation

1.1 Introduction

Originally, nitric oxide (NO) was recognized as a component of environmentally polluting NO_x complex (NO_2 and NO) from combustion processes such as fossil fuel burning or automobile combustion engines. Surprisingly, in 1998, the Nobel Assembly awarded the Nobel Prize in Medicine and Physiology to Robert Furchgott, Louis Ignarro and Ferid Murad for their discoveries concerning NO as a signalling molecule in the cardiovascular system. Since then, NO has become one of the most vigorously researched molecules of biological chemistry. Now, it is well known that this bioactive molecule is involved in many animal physiological processes, such as vasorelaxation, platelet inhibition, neurotransmission,

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cytotoxicity, smooth muscle contraction and relaxation, egg fertilization, immunoregulation and apoptosis (Schmidt and Walter 1994; Stamler 1994; Jeffrey and Snyder 1995; Lloyd-Jones and Bloch 1996; Wink and Mitchell 1998; Gonzales-Zulueta et al. 2000; Hess et al. 2005; Siddiqui et al. 2011).

With the finding of these roles of NO in animal cells, various studies have reported its presence in the plant kingdom and its diverse function in plant cells. As the list of physiological functions of NO has been growing, it has become evident that our knowledge of the pathways and molecular mechanisms responsible for NO effects is poorly understood (Astier et al. 2012). In addition, the term reactive nitrogen species (RNS) was introduced to designate other NO-related molecules, such as *S*-nitrosothiols, *S*-nitrosoglutathione, peroxynitrite, among others, which have relevant roles in multiple physiological processes in animal and plant cells (Halliwell and Gutteridge 2007). With the aim of promoting interest of plant biologists in NO, the following chapter will endeavour to summarize current recognition of both properties and physiological roles of NO and RNS in plants.

1.2 Nitric Oxide

1.2.1 Properties of Nitric Oxide

NO or nitrogen monoxide (systematic name) is a gaseous free radical, existing—due to its ability to adopt an energetically more favourable electron structure either by gaining or by losing an electron—in three redox-related species: nitric oxide radical ($NO^{\cdot}$), nitrosonium cation (NO^{+}) and nitroxyl anion (NO^{-}) (Stamler et al. 1992; Wojtaszek 2000). It is one of the smallest diatomic molecules which contains an unpaired electron in its π_2 orbital but remains uncharged. It is soluble in water (0.047 cm^3/cm^3 H_2O at 20 °C, 1 atm), with increasing solubility in the presence of ferrous salts (Anderson and Mansfield 1979; Neill et al. 2003). NO may not only easily migrate in the hydrophilic regions of the cell, such as the cytoplasm, but also due to its lipophilic character also freely diffuse through the lipid phase of membranes without the aid of specific membrane transporters (Arasimowicz and Floryszak-Wieczorek 2007) at a rate of 50 μm per second (Corpas et al. 2010).

Being the reactive free radical, it has a relatively short half-life which is estimated to be <6 s (Bethke et al. 2004). However, its half-life is dependent on many factors. For example, at low concentrations (<1 μmol l^{-1}), NO can have half-life of minutes to hours and could thus diffuse over several cell layers or over longer distances in intercellular spaces. At higher concentrations, NO has shorter half-life, in order of seconds (Henry et al. 1997). In addition, NO half-life depends on the local concentrations of its targets, i.e. oxygen, hydrogen peroxide, proteins,

haemoproteins, bound iron and copper, cysteine, and ascorbic acid (Stöhr and Ullrich 2002).

Due to its instable nature, NO has a very rich chemistry (Mengel et al. 2013). It reacts with molecules, which are likely to be produced temporally and spatially alongside NO (Neill et al. 2003): for example, very well documented is a simultaneous generation of NO and superoxide radical (O_2^{-}) in plant peroxisomes, mitochondria and chloroplasts (Corpas et al. 2001; del Río et al. 2006; Jasid et al. 2006; Blokhina and Fagerstedt 2010).

1.2.2 Various Roles of NO in Plant Physiology

NO first came to prominence within the context of plant defence regulation during plant–pathogen interactions (Delledonne et al. 1998; Durner et al. 1998). Now, it is well known that NO is involved in the stimulation of seed (Beligni and Lamattina 2000) and pollen (Šírová et al. 2011) germination, modulation of plant growth and development (Durner and Klessig 1999), regulation of cell elongation during primary root growth (Fernández-Marcos et al. 2012), plant maturation and senescence (Leshem et al. 1998; Guo and Crawford 2005; Wilhelmová et al. 2006), floral regulation (He et al. 2004), mediation of stomatal movement (García-Mata and Lamattina 2001; Neill et al. 2002; Guo et al. 2003; Desikan et al. 2004; Bright et al. 2006), gravitropism (Hu et al. 2005), mitochondria functionality (Zottini et al. 2002), photosynthesis regulation (Takahashi and Yamasaki 2002) or involvement of light-mediated greening (Zhang et al. 2006a). In addition, NO is involved in responses to various stresses, such as drought, salt, and heat stresses, risk element stress, disease resistance and apoptosis (Durner and Klessig 1999; García-Mata and Lamattina 2002; Zhao et al. 2004, 2007; Zhang et al. 2006b; Procházková et al. 2012). In addition, NO plays an important role in symbiotic organisms, particularly between legumes and *Sinorhizobium* (Baudouin et al. 2006). NO also acts as a regulator of gene expression at the transcriptional-level regulation of disease resistance processes (Polverari et al. 2003) and the expression of stress-related transcription factors and signalling-related kinases (Parani et al. 2004), and by the interaction with other signalling molecules such as salicylic acid and jasmonic acid (Grün et al. 2006; Lozano-Juste et al. 2011).

Recently, the differential intracellular role of NO has been described as well. For example, NO has recently been shown to modulate mitochondrial alternative oxidase activity to influence the generation of reactive oxygen species (ROS), net NO production and shift primary metabolism towards amino acid biosynthesis via inhibition of aconitase (Cvetkovska and Vanlerberghe 2012; Gupta et al. 2012). In peroxisomes, NO nitrosylates proteins such as catalase, glyoxylate oxidase and malate dehydrogenase are involved in photorespiration, β-oxidation and the detoxification of ROS (Ortega-Galisteo et al. 2012).

1.3 Peroxynitrite

1.3.1 Properties of Peroxynitrite

Reaction between NO and O_2^- results in spontaneous formation of peroxynitrite ($OONO^-$) by a diffusion-limited reaction (Huie and Padmaja 1993), as shown in Eq. 1.1.

$$ON^{\cdot} + O_2^{\cdot -} \rightarrow ONOO^- \tag{1.1}$$

The rate constant of the reaction has been determined by several methods to be within the range of 4–16 $\times$ 10^9 M^{-1} s^{-1} (Arasimowicz-Jelonek and Floryszak-Wieczorek 2011). In essence, NO and superoxide form $OONO^-$ every time they collide. No enzyme is required to produce $OONO^-$ because no enzyme can possibly catalyse any reaction as fast. NO is the only known biological molecule that reacts faster with superoxide and is produced in high enough concentrations to outcompete endogenous levels of superoxide dismutase. Consequently, the kinetics and thermodynamics of the reaction of superoxide with NO make the formation of $OONO^-$ inevitable in vivo (Pacher et al. 2007). In addition, $OONO^-$ can be also produced by an enzyme nitrate reductase in the presence of oxygen and NAD(P)H (Bethke et al. 2004). In plants, low levels of $OONO^-$ are likely to be formed continuously in photosynthesizing chloroplasts, whereas higher levels are likely to be synthesized in response to stress, which induces the production of both NO and ROS (Vandelle and Delledonne 2011).

Direct scavenging of O_2^- towards $OONO^-$ may suggest the antioxidant role of NO in relation to this ROS, since in this situation, NO may disrupt the chain of reactions leading to more toxic ROS. On the other hand, $OONO^-$, as will be mentioned later, may cause serious damage to cell structures.

$OONO^-$ is a relatively short-lived reactive nitrogen species at the physiological pH range and temperature, which may readily migrate through biological membranes and interact with target molecules also in surrounding cells within the radius of one or two cells (~5–20 μm) (Szabó et al. 2007). This molecule is surprisingly stable in solution, considering its strong oxidizing potential, and that it is 36 kcal/mol higher in energy than its isomer nitrate. The unusual stability of $ONOO^-$ results in part because it folds into a stable *cis*-conformation where the negative charge is localized over the entire molecule (Tsai et al. 1994). The molecules are further stabilized by forming strong hydrogen bonds with two or three waters (Tsai et al. 1995).

The first papers suggesting that $ONOO^-$ could be a biological oxidant were shown on animal cells in 1990 (Beckman 1990; Beckman et al. 1990). In plants, at first, the generation of $ONOO^-$ was demonstrated during plant response to biotic stress. Saito et al. (2006) observed intracellular time-dependent $ONOO^-$ production in tobacco BY-2 cells treated with INF1 elicitin secreted by *Phytophthora infestans*. Gaupels et al. (2011) found a significant accumulation of $ONOO^-$ at 3–4 h in *Arabidopsis* challenged with an avirulent *Pseudomonas syringae* pv.

tomato, which significantly increased at 7–8 h after pathogen treatment. Corpas et al. (2009b) described the generation of $ONOO^-$ also during abiotic stress: they found $ONOO^-$ production in *Arabidopsis* roots exposed to salinity stress.

At physiological pH, $ONOO^-$ equilibrates rapidly with peroxynitrous acid (HOONO) that rapidly decomposes to the highly reactive hydroxyl radical ($HO^{\cdot}$) as shown below in Eq. 1.2.

$$OONO^- + H^+ \leftrightarrow HOONO \rightarrow HO^{\cdot} + NO_2^{\cdot} \quad (1.2)$$

It has been reported that this reaction is a far more effective in producing hydroxyl radical than the Fenton reaction or the iron-catalysed Haber–Weiss reaction (Beckman et al. 1990). In biological systems, the reaction may be relevant mainly in hydrophobic phases to initiate lipid peroxidation and nitration processes (Radi et al. 1991; Szabó et al. 2007).

$ONOO^-$ interacts with proteins, lipids and DNA via direct one- or two-electron oxidation reactions or indirectly through the formation of highly reactive radicals. Surprisingly, in plants, $ONOO^-$ does not appear to be as toxic as in animal tissues (Delledonne et al. 2001). Why $ONOO^-$ is not very toxic to plant cells is still unclear. One hypothesis could be the existence of specific detoxifying mechanisms absent in animals. Among them, flavonoids that are known to display a strong antioxidant capacity attracted an attention. However, *transparent testa* mutants impaired in flavonoid biosynthesis are not susceptible to $ONOO^-$ treatment (Vandelle and Delledonne 2011).

$ONOO^-$ reacts with target molecules through two possible pathways. First, peroxynitrite anion or peroxynitrous acid can react directly with a certain target molecule in an overall second-order process (e.g. thiol oxidation). Second, peroxynitrous acid can first homolyse to form nitrogen dioxide and hydroxyl radicals, which in turn react with the target molecule. The latter processes are first order in peroxynitrite but zero order in target, because the formation of the radicals is rate-limiting. To this last type of reaction belong tyrosine nitration and lipid peroxidation (Alvarez and Radi 2003).

The main product from peroxynitrite decay in the absence of targets is nitrate (Anbar and Taube 1954), while secondary reactions of the radicals can also lead to nitrite and dioxygen, particularly at alkaline pH (Pfeiffer et al. 1997; Coddington et al. 1999; Alvarez and Radi 2003).

1.3.2 Reactions of $ONOO^-$ with Proteins

The reaction of $ONOO^-$ with proteins occurs through three possible pathways:

1. $ONOO^-$ reactions with prosthetic group, particularly transition metal centres:

   The reactions of $ONOO^-$ with transition metal centres, particularly those containing haeme and non-haeme iron, copper and manganese ions, are some of the fastest known for $ONOO^-$ (Alvarez and Radi 2003).

2. In the same way as with other Lewis acids (LA), such as the proton or carbon dioxide, the reaction proceeds to form a Lewis adduct which in turn homolyses to yield NO_2 and the corresponding oxyradical ($^{\cdot}O–LA^{-}$) (Radi et al. 2000), as shown in Eq. 1.3.

$$\begin{aligned} ONOO^{-} + LA \rightarrow ONOO - LA^{-} \rightarrow {}^{\cdot}NO_2 + {}^{\cdot}O - LA^{-} \\ \rightarrow {}^{\cdot}NO_2 + O = {}^{\cdot}LA^{-} \end{aligned} \tag{1.3}$$

3. Peroxynitrite can also oxidize reduced metal centres by two electrons yielding the oxyradical or oxo-compound accompanied by the formation of nitrite instead of nitrogen dioxide. This is particularly relevant in the case of reduced cytochrome c oxidase. In the case of the one electron oxidation of cytochrome c, which has all six coordination positions occupied, peroxynitrite reacted with the reduced but not the oxidized form, oxidizing the Fe^{2+}–Fe^{3+} possibly through an outer sphere electron transfer process (Thomson et al. 1995).

Jasid et al. (2006) showed that $ONOO^{-}$ oxidizes chloroplastic proteins and diminishes both the oxygen evolution and the fluorescence yield of photosystem (PS) II in a dose-dependent manner.

1.3.3 Reactions of $ONOO^{-}$ with Amino Acids

(a) Cysteine, methionine, tryptophan and histidine oxidation

Peroxynitrite may alter protein structure and function by reacting with various amino acids in the peptide chain. The predominant and the fastest (10^{3}–10^{7} M^{-1} s^{-1}) reaction is with cysteine (Alvarez and Radi 2003). It has been described in animal cells that $ONOO^{-}$ directly oxidizes methionine, forming methionine sulphoxide, and to a lesser extent ethylene and dimethyldisulphide (Szabó 2003). $ONOO^{-}$ can also oxidize tryptophan yielding *N*-formylkynurenine, oxindole, hydropyrroloindole and nitrotryptophan (Alvarez and Radi 2003). The physiological role of this modification, if any, is unclear (Vandelle and Delledonne 2011). The proteomic analysis of inflamed neurons has shown that several nitrotryptophan-containing proteins contain functional tryptophan residues that interact with other molecules. These proteins appear to be involved in energy metabolism, protein synthesis and stress responses, and it has been suggested that tryptophan nitration may modulate specific interactions between these proteins and their targets (Ikeda et al. 2007; Vandelle and Delledonne 2011). In plants, Galetskiy et al. identified 138 tyrosine and tryptophan nitration sites, mainly in PSI, PSII, cytochrome b6/f and ATP-synthase complex indicating that protein nitration belongs to one of the prominent posttranslational protein modifications in photosynthetic apparatus (Galetskiy et al. 2011a).

In animal cells, $ONOO^{-}$ modifies histidine through a radical mechanism, forming a histidinyl radical, a mechanism involved in the inactivation of Cu, Zn-SOD

by $ONOO^-$ (Alvarez et al. 2004). In plant cells, Gonzalez-Perez et al. (2008) revealed that the specific inhibition site of $ONOO^-$ in PSII is in the plastoquinone Q_AFe^{2+} niche of the PSII acceptor side. $ONOO^-$ interacts with the non-haeme Fe^{2+}; however, the type of the redox reaction between peroxynitrite and the non-haeme Fe^{2+} is not known. They suggested that the products of the one- or two-electron oxidation are able to oxidize or to nitrate the Fe^{2+}-coordinated histidine residues or to induce Fe^{2+} release and as a consequence to destroy the magnetic coupling between Q_A and the non-haeme Fe^{2+} (Gonzalez-Perez et al. 2008).

(b) Tyrosine nitration

Protein tyrosine nitration is a covalent protein modification resulting from the addition of a nitro ($-NO_2$) group adjacent to the hydroxyl group on the aromatic ring of tyrosine residues (Gow et al. 2004). A stable product 3-nitrotyrosine is formed by the addition of −NO2 to the ortho position of tyrosine (Dixit et al. 2009). This biochemical event induces change of the tyrosine molecule into a negatively charged hydrophilic nitrotyrosine moiety and causes a marked shift of the local pKa of the hydroxyl group from 10.07 in tyrosine to 7.50 in nitrotyrosine impinging on the protein function (Turko and Murad 2002). Tyrosine nitration is considered to be a selective process, and proteins have usually approximately 3–4 mol% of tyrosine, but only one or two of these tyrosines may become preferentially nitrated, this depending on several factors, such as protein structure, nitration mechanism and environment, where the protein is located (Bartesaghi et al. 2007; Corpas et al. 2009a). Bayden et al. (2011) suggested that despite the moderately hydrophilic nature of tyrosine, its relatively high degree of surface exposure (only 15 % of tyrosine residues are buried) and the fact that most proteins contain tyrosine (natural abundance 3.2 %), only a limited number of proteins are nitration targets and this does not depend on their abundance.

Tyrosine nitration has been shown to be capable of changing the function of a protein in several ways: (1) gain of function as well as no effect on function; and (2) inhibition of function, which is much more common result of protein tyrosine nitration (Radi 2004; Corpas et al. 2009a). For example, Alvarez et al. (2011) reported the inhibition of *Arabidopsis* O-acetylserine(thiol)lyase A1 by tyrosine nitration. It has been also demonstrated that nitration of tyrosine residue may either prevent further phosphorylation or stimulate phosphorylation (Shi et al. 2007; Rayala et al. 2007). In animal cells, nitrotyrosine has been used as a biomarker of nitrosative stress. In plants, nitrotyrosine is often used as a marker of nitrosative stress during abiotic stress in the same way as lipid peroxidation or protein carbonylation, and like this, it was used, e.g., for salinity stress (Valderrama et al. 2007) and during sunflower–mildew interaction (Chaki et al. 2009). Also under high light, which is a major stress factor often leading to over-excitation of the photosynthetic apparatus and production of ROS, tyrosine nitration was demonstrated (Galetskiy et al. 2011a).

However, evidence accumulates that this modification also has a signalling function in plant cells. For example, Cecconi et al. (2009) reported that tyrosine nitration, as a key process of redox signalling, could be involved in Rubisco

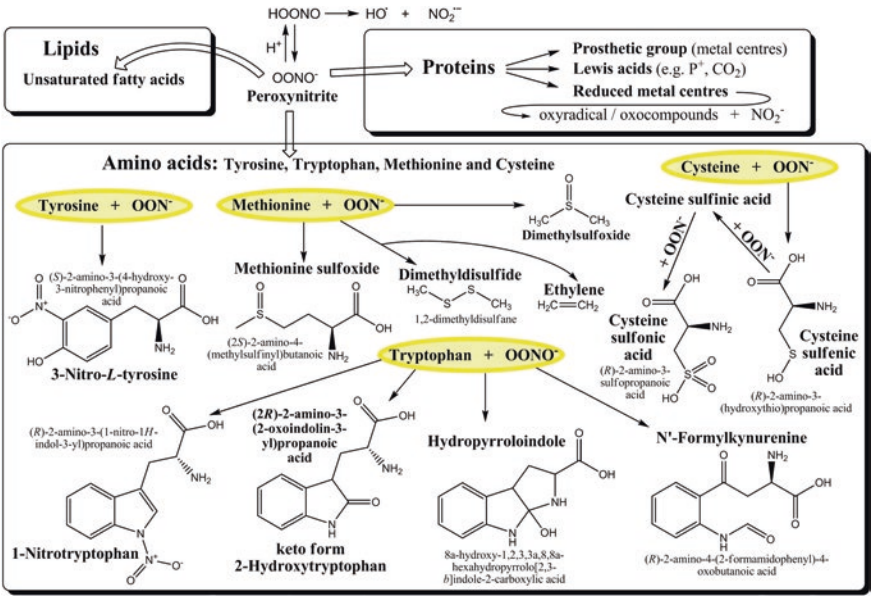

Fig. 1.1 Summary of some reactions of peroxynitrite

large subunit and Rubisco activase modulation during hypersensitive response. Similarly, Gaupels et al. (2011) proposed that $ONOO^-$ transduces the NO signal by modifying protein function via tyrosine nitration during the hypersensitive defence response. In addition, the conformation change in the stromal loop between membrane α-helices IV and V due to nitration might provide a signal for the degradation of D1 protein (Galetskiy et al. 2011b). The reactions of peroxynitrite with amino acids are described in Fig. 1.1.

1.3.4 Reactions of $ONOO^-$ with Lipids

A major aspect of peroxynitrite-dependent cytotoxicity relies on its ability to trigger lipid peroxidation in membranes (Radi et al. 1991). Exposure of low-density lipoprotein to $ONOO^-$ results in the oxidation of unsaturated fatty acids. The mechanism of initiation is unclear but may involve either abstraction of a bis-allylic hydrogen by the 'the hydroxyl radical-like' activity $ONOO^-$ or induced homolysis of $ONOO^-$ by the unsaturated fatty acid (Hogg et al. 1992).

$ONOO^-$ can also rapidly oxidize tocopherols: α-tocopherol is oxidized by two electrons to α-tocopheryl quinone, a form that is not easily repaired by cellular reductants (Hogg et al. 1993). Desel et al. (2007) found that *Brassica napus*, *Nicotiana tabacum* and *Arabidopsis thaliana*, with high levels of endogenous γ-tocopherol, produced 5-nitro-γ-tocopherol during seed germination. It has

been speculated that nitration of γ-tocopherol in plant tissues may be an important mechanism for in vivo modulation of NOx levels (Arasimowicz-Jelonek and Floryszak-Wieczorek 2011). However, research in nitrolipids is in an early stage of investigation, and there is virtually no information available regarding these two nitration processes in plant systems, this being a new area of reactive nitrogen species metabolism that needs to be explored (Corpas et al. 2009a).

1.3.5 Reactions of $ONOO^-$ with DNA

$ONOO^-$ can also react with DNA (Radi 2004). DNA can be damaged by $ONOO^-$ by both introducing oxidative modifications in nucleobases and in sugar–phosphate backbone (Pacher et al. 2007). $ONOO^-$ is able to attack the sugar–phosphate backbone by abstracting a hydrogen atom from the deoxyribose moiety, resulting in the opening of the sugar ring and the generation of DNA strand breaks (Burney et al. 1999; Niles et al. 2006; Pacher et al. 2007). Rubio et al. (1996) have also reported that treatment of deoxynucleosides with $ONOO^-$ leads to the formation of 2-thiobarbituric acid-reactive substances.

Among the nucleobases, guanine is the most reactive with $ONOO^-$ because of its low reduction potential (Yu et al. 2005). The major product of guanine oxidation is 8-oxoguanine, which further reacts with peroxynitrite, yielding cyanuric acid and oxazolone (Niles et al. 2006). Following the formation of abasic sites that can be cleaved by endonucleases in vivo, it gives DNA single-strand breaks (Burney et al. 1999). Unfortunately, similarly as in the case of nitrolipids, research in 8-nitroguanine is in an early stage of investigation in plant systems (Corpas et al. 2009a).

However, increasing studies conducted mainly under in vitro conditions have reported that $ONOO^-$ cannot only be considered as a cytotoxic agent (Altug et al. 1999) but might also act as a potent modulator of the redox regulation in various cell signal transduction pathways (Liaudet et al. 2009; Arasimowicz-Jelonek and Floryszak-Wieczorek 2011).

1.4 Nitrosothiols

The formation of nitrosothiols is still debated. The direct reaction of thiol groups with NO is too slow to occur in vivo; instead, it is assumed that N_2O_3 is the main nitrosylating species in aerobic conditions although the formation of dinitrogen trioxideis controversially discussed (Folkes and Wardman 2004; Ridnour et al. 2004). Other reactive nitrogen species described to mediate *S*-nitrosothiol formation are nitrosonium and nitroxyl ions (Ridnour et al. 2004). Nitroso groups can also be transferred between thiols in a process termed as transnitrosylation. Transnitrosylation occurs between proteins and between proteins and low molecular weight nitrosothiols (e.g.

S-nitrosylated glutathione GSNO) in animals; however, in plants, evidence for this mechanism is lacking (Hogg 2002; Nakamura and Lipton 2013).

S-nitrosothiols are generally more stable in solution compared to NO; therefore, they can participate in the transport, storage and delivery of NO and consequently contribute to posttranslational modifications involved in cell signalling and in stress processes (Foster et al. 2003; Benhar et al. 2006; Leterrier et al. 2011).

One of the families of the most abundant low molecular mass *S*-nitrosothiols is GSNO. GSNO results from the reaction between NO and reduced glutathione (GSH) in a process called *S*-nitrosylation or by a process of transnitrosation from other *S*-nitrosothiols with GSH. *S*-nitrosylation appears to take place through either the formation of N_2O_3 or the addition of NO to a glutathionyl radical formed during this reaction (Broniowska et al. 2013). *S*-nitrosylation of proteins is rapidly reversible, making it an attractive candidate for involvement in signal transduction (Grennan 2007). Increasing evidence suggests that *S*-nitrosylation plays a regulatory role in plant physiology. For example, it can play a negative regulatory role in ethylene biosynthesis via *S*-nitrosylation of S-adenosylmethionine, which is a precursor for ethylene biosynthesis (Lindermayr et al. 2006). In *Arabidopsis* leaves exposed to NO gas, 52 *S*-nitrosylated proteins, including stress and redox-related, metabolic, signalling and cytoskeletal proteins, were identified (Lindermayr et al. 2005). In addition, it was reported that the activity of peroxiredoxin IIE, which detoxifies peroxynitrite and hydrogen peroxide, was inhibited by *S*-nitrosylation (Romero-Puertas et al. 2007). Similarly, isoforms of glyceraldehyde dehydrogenase were inhibited after treatment with GSNO in *Arabidopsis* (Holtgrefe et al. 2008). In the same way, the Rubisco activity was inhibited in *Kalanchoe pinnata* (Abat et al. 2008).

GSNO may function both as an intracellular NO reservoir and as a transporter for NO throughout the cell (Singh et al. 1996) and therefore can affect the process of transnitrosation equilibrium between GSNO and *S*-nitrosylated proteins. In this sense, it has been proposed a mechanism of GSNO formation mediated by cytochrome *c* (Basu et al. 2010; Leterrier et al. 2011).

GSNO seems to be an important molecule during plant responses to various abiotic and biotic stresses. For example, under treatment of heavy metals, GSNO content decreased in pea and *Arabidopsis* (Barroso et al. 2006; Leterrier et al. 2012). On the other hand, its content increased under high temperature in sunflower (Chaki et al. 2011a) and after mechanical wounding in sunflower and *Arabidopsis* (Chaki et al. 2011b; Espunya et al. 2012).

The key enzyme regulating GSNO pools is nitrosoglutathione reductase (GSNOR). GSNOR reduces GSNO to ultimately produce glutathione disulphide and ammonia (Mur et al. 2012). Because of the ubiquitous nature of GSNOR, it has been suggested that this enzyme serves more to protect against nitrosative stress than as cell signalling factor (Lindermayr and Durner 2009).

The family of low molecular mass *S*-nitrosothiols includes other molecules such as *S*-nitrosocysteine and *S*-nitrosocysteinylglycine.

Most proteins possess cysteine residues, but the affinity of this amino acid residue to NO can be very different (Stamler et al. 1997). The vicinity of acid

and base catalysts may help in *S*-nitrosylation formation, although this does not account for all the *S*-nitrosylation sites already founded. Inspection of known *S*-nitrosylated proteins revealed that the presence of a hydrophobic environment, which enables the formation of *S*-nitrosylating species via the reaction between oxygen and NO, also promotes *S*-nitrosylation (Stamler et al. 2001).

Linking nitrosothiol on cysteine residues mediates NO signalling functions of a broad spectrum of mammalian proteins, including caspases, the main effectors of apoptosis. Plant metacaspases can be kept inactive through *S*-nitrosylation of a critical cysteine residue but are insensitive to *S*-nitrosylation when matured (Belenghi et al. 2007). This discrepancy could be related to differences in NO householding between plants and mammals. Whereas mammals control internal NO levels very strictly by fine regulation of activities of the various NO synthase isoforms, plants must deal with atmospheric NO as well as with internal leakage of NO that accumulates under physiological growth conditions due to its production from nitrite (Belenghi et al. 2007).

In addition, there is another group of SNOs called high molecular mass SNOs which are produced by NO binding to sulfhydryl (–SH) groups present in specific cysteine residues of proteins (Corpas et al. 2013).

1.5 Conclusion

Research in the field of NO in plant systems is a challenge; however, it is evident that these researches lag behind the research in the animal system. For example, there is a considerable lack of knowledge regarding, e.g., transnitrosylation, signalling functions of various RNS and explanation of a lesser $ONOO^-$ toxicity comparing to animal cells. Hence, additional research is necessary to explain all these doubts.

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Chapter 2
Functional Role of Nitric Oxide Under Abiotic Stress Conditions

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Abstract Nitric oxide (NO), a free radical in living organisms, is considered a phytohormone and a key signalling molecule functioning in various physiological processes of plants. These physiological processes include germination, growth, senescence, and photosynthesis as well as response mechanisms to specific environmental stresses. Plants under abiotic stress conditions experience oxidative and nitrosative stress; the latter mainly elicited by regulation of NO production. Nitrosative stress describes the molecular or cellular damage promoted by imbalance in NO homeostasis and other reactive nitrogen species. Additionally, depending on its concentration and location in plant cells or tissues, NO might function as an antioxidant and scavenge some other reactive intermediates. Direct or indirect involvement of NO in response mechanisms under water stress, drought, salinity, heavy metal stress, high or low temperature extremities, and ultraviolet radiation has been reported. In this work, the recent findings and current knowledge on the function of NO in plants under abiotic stress conditions are reviewed briefly.

Keywords Nitrosative stress · Oxidative stress · Phytohormone · Signalling

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2.1 Introduction

Nitric oxide (NO) is a gaseous signalling molecule which has attracted much attention because of its diverse functional roles in physiological processes and response mechanisms to various environmental stresses. NO functions in cellular protection against toxicity of reactive oxygen species (ROS), defense response, and tolerance to abiotic stress (Lamattina et al. 2003; Corpas et al. 2007; Besson-Bard et al. 2008a; Neill et al. 2008). In plant cells, endogenous NO can be produced by either L-arginine-dependent nitric oxide synthase (NOS)-like activity or nitrate reductase (NR) activity (Moreau et al. 2008). There are also few other enzymatic and non-enzymatic processes which have been proposed to contribute to cellular NO content. NO and a family of related molecules are designated as reactive nitrogen species (RNS) which include *S*-nitrosothiols (SNOs), *S*-nitrosoglutathione (GSNO), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3) and nitrogen dioxide (NO_2) (Corpas et al. 2007).

To clarify the effects of NO in plants under environmental stresses, extensive studies have been conducted using indirect approaches such as exogenous application of NO donors [e.g. sodium nitroprusside (SNP)], NO scavengers [e.g. 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO)] and enzyme inhibitors. Under adverse environmental circumstances, imbalance of RNS leads to nitrosative stress (Corpas et al. 2011). Certain downstream signalling pathways, employing calcium ion (Ca^{2+}), cyclic guanosine monophosphate (cGMP), and cyclic adenosine diphosphoribose (cADPR), were proposed to be involved in NO-mediated molecular events in plant cells (Neill et al. 2003; Besson-Bard et al. 2008b). NO can exert its effects through regulating the generation of ROS or modulating components of antioxidative system (Laspina et al. 2005). NO can also modulate biological responses by direct modification of proteins, reacting with cysteine residues (*S*-nitrosylation), tyrosine residues (nitration), or iron and zinc in metalloproteins (metal nitrosylation) (Besson-Bard et al. 2008a). Schematic representation of functional roles of NO under abiotic stresses is displayed in Fig. 2.1.

Available data indicates that plant response to stressors such as drought, high or low temperature, salinity, heavy metals and oxidative stress, is regulated by NO (Uchida et al. 2002). The recent findings and current knowledge on the function of NO in plants under different abiotic stress conditions are reviewed in this chapter.

2.2 Nitric Oxide and Abiotic Stress

2.2.1 Heavy Metal Toxicity

Heavy metals include a group of metals and metalloids that are toxic to plants at extremely low concentrations. Although some members of this group, specifically the micronutrients, are required for normal growth and development, others do

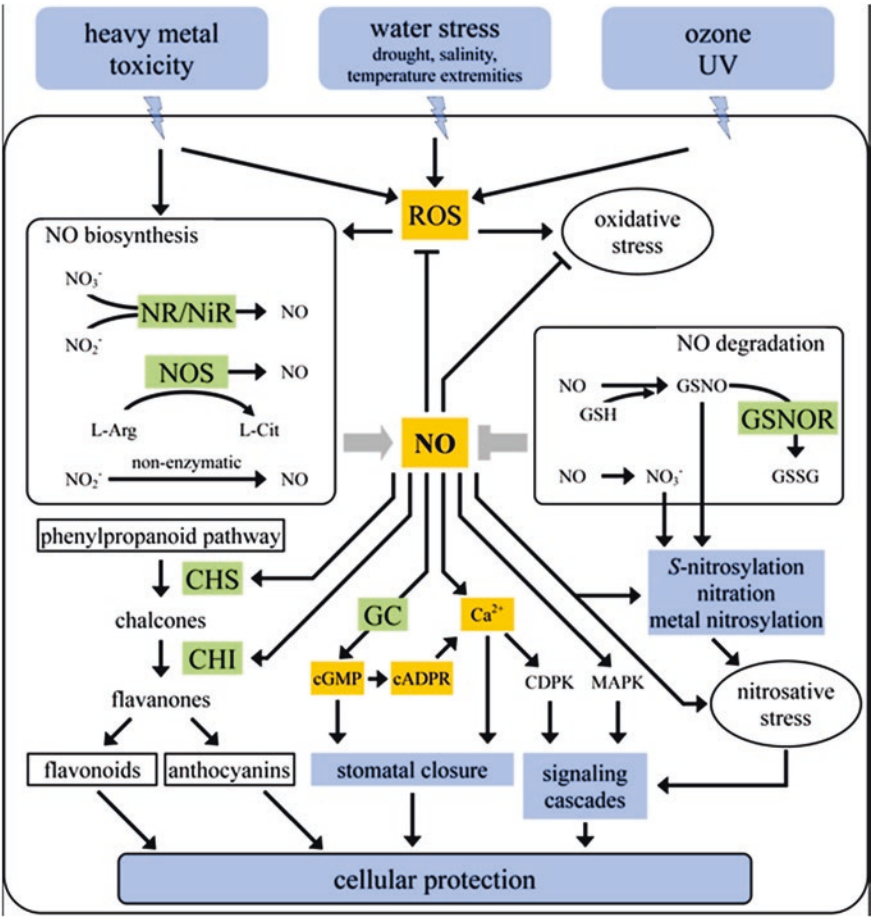

Fig. 2.1 Schematic representation showing functional role of nitric oxide under abiotic stresses. The signalling molecules include nitric oxide (NO), reactive oxygen species (ROS), calcium ion (Ca^{2+}), cyclic guanosine monophosphate (cGMP), and cyclic adenosine diphosphoribose (cADPR). The enzymes include nitric oxide synthase (NOS), nitrate reductase (NR), nitrite reductase (NiR), *S*-nitrosoglutathione reductase (GSNOR), guanylyl cyclase (GC), chalcone synthase (CHS), and chalcone isomerase (CHI). *Arrows* and *T-bars* indicate activation and inhibition, respectively. [Ultra violet (UV), nitrate (NO_3^-), nitrite (NO_2^-), L-arginine (L-Arg), L-citrulline (L-Cit), reduced glutathione (GSH), oxidized glutathione (GSSG), *S*-nitrosoglutathione (GSNO), calcium-dependent protein kinase (CDPK), mitogen-activated protein kinase (MAPK)]

not perform any known physiological function in plant cells. Nevertheless, at high concentrations all heavy metals can accumulate over time, inhibit growth, and cause toxicity in plant tissues (Benzarti et al. 2008; Rascio and Navari-Izzo 2011).

In order to cope with various heavy metal stresses, plants possess mainly two defense strategies: (1) They adjust cellular free metal content by metal exclusion,

cell wall binding, chelation in the cytosol, and compartmentation in the vacuole; and (2) regulate cellular and molecular responses by induction of antioxidative defense, up-regulation of defensive gene expression, and recovery of stress-damaged proteins (Hall 2002). Accumulating data clearly indicated involvement of NO in cellular response against heavy metal toxicity. Although the information on molecular mechanisms is limited, it has been well established that NO functions in tolerance as a key signalling molecule besides ROS, Ca^{2+}, GSH, and others (Fig. 2.1) (Thapa et al. 2012).

Cadmium (Cd) is one of the most common heavy metals found in soil and considered one of the most phytotoxic anthropogenic pollutant (Benavides et al. 2005; Arasimowicz-Jelonek et al. 2011). Cd toxicity limits growth, photosynthetic attributes, and ultimately yield components. However, exogenous application of NO improves all these parameters in Cd-treated plants (Jhanji et al. 2012). The molecular mechanisms of Cd cytotoxicity in plants are not fully understood, however, certain studies reported participation of NO under Cd stress and alleviation of Cd-induced oxidative damage by application of exogenous NO. Cd-induced increase in endogenous NO level has been observed in cell suspension cultures of soybean (Kopyra et al. 2006) and *Arabidopsis thaliana* (De Michele et al. 2009) and roots of yellow lupine (*Lupinus luteus* L.) seedlings (Arasimowicz-Jelonek et al. 2012).

However, several studies reported reduction of NO or GSNO, a natural reservoir for NO, under Cd toxicity (Barroso et al. 2006; Rodríguez-Serrano et al. 2009). Furthermore, Barroso et al. (2006) observed a decrease in the glutathione (GSH) content with concomitant reduction in the activity and expression level of the catabolic enzyme GSNO reductase (GSNOR) in pea leaves. In a similar study, Rodríguez-Serrano et al. (2009) observed a severe decline in the NOS-dependent NO production under Cd stress.

According to another approach interestingly, exogenous NO counteracts Cd toxicity by regulating cellular distribution of excess Cd and accumulation in cell wall. Xiong et al. (2009) attributed the NO-induced Cd tolerance to the distribution of Cd in the cell walls of rice (*Oryza sativa*) roots. NO also activates the Osmotic Stress-Activated Protein Kinase (OSAK) (Kulik et al. 2012) and Mitogen-Activated Protein Kinase (MAPK) during programmed cell death (Ye et al. 2013), through regulation of gene expression and signal transduction (Zago et al. 2006). Moreover, NO protects plants from Cd-induced oxidative stress (Hsu and Kao 2004; Laspina et al. 2005) either by directly scavenging ROS or by activating antioxidative enzymes (Arasimowicz-Jelonek et al. 2011). Wang et al. (2013) observed that exogenous application of NO donor SNP significantly decreased the level of ROS and lipid peroxidation and increased the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and ascorbate peroxidase (APX) in ryegrass (*Loliumperenne* L.) seedlings exposed to Cd.

Although a certain number of studies indicated a role for exogenous NO in protection against Cd toxicity, it was also claimed that endogenous NO production is necessary for implementation of toxicity (Groppa et al. 2008; Besson-Bard et al. 2009; De Michele et al. 2009). It has been noticed that a short-term

Cd treatment in cell suspension might promote NO burst whereas a long treatment might decrease NO generation. Additionally the concentration, subcellular or tissue localization, and site of action of NO could also account for the observed variations. Alleviation of Cd toxicity by NO also depends on Cd concentrations, duration of treatment, and developmental stage of the plant (Groppa et al. 2008). Arsenic (As) is a potentially pollutant metalloid component of a wide range of minerals. Under As toxicity, plants suffer alterations at various levels including uptake and transport of As, intracellular metabolism, and gene expression (Verbruggen et al. 2009). Strategies employed by plants against As toxicity include reduction of As to arsenite (AsIII), chelation of AsIII with glutathione and phytochelatins, and sub-sequestration of complexes in vacuoles (Gupta et al. 2011). Accumulating data suggest that metabolism of ROS and oxidative stress might be involved in As toxicity (Sharma 2012). Additionally, protein *S*-nitrosylation induced by NO was demonstrated to be key in regulating the activity of proteins involved in the metabolism of ROS, such as glycolate oxidase, CAT (Ortega-Galisteo et al. 2012), and NADPH oxidase (Yung et al. 2011). Recently it was suggested that As might cause oxidative stress by inducing glycolate oxidase. However, NADPH oxidase did not appear to participate in ROS overproduction but could be critical in regulating antioxidant defenses as well as the transport and translocation of As and other macro- and micro-nutrients (Gupta et al. 2013).

Studies analyzing function of NO under As toxicity in higher plants are limited. In roots of rice (*O. sativa*), exogenous application of SNP provided tolerance against As toxicity and ameliorated the As-induced decrease in length. Furthermore, SNP reduced As-induced accumulation of malondialdehyde (MDA), superoxide ion ($O_2^{\cdot-}$), and H_2O_2 (Singh et al. 2009; Jin et al. 2010), and an increase in the activities of SOD, CAT and APX was recorded in the presence of SNP under As stress (Jin et al. 2010). In mung bean (*Vigna radiata*) exogenous application of NO as SNP (75 μM) significantly improved seed germination, growth and CAT activity and decreased As accumulation and activity of SOD (Ismail 2012).

In *Arabidopsis*, exposure to As resulted in a significant growth reduction and caused lipid peroxidation. The changes in growth parameters were accompanied by an alteration of antioxidative enzymes including CAT and glutathione reductase. Additionally, changes in NO metabolism such as a significant increase in NO content, GSNOR activity and protein tyrosine nitration as well as a reduction in GSH and GSNO content were observed. Thus, it was concluded that As stress in *Arabidopsis* provoked both oxidative and nitrosative stress (Leterrier et al. 2012).

It was proposed that As stress is transduced through MAPK signalling cascade in rice (Rao et al. 2011). Toxic As concentration produced ROS and NO in rice roots and severely retarded growth of seedlings. Activity assays were used for determination of As-mediated activation of a 42 kDa MAPK in leaves and 42 and 44 kDa MAPK in roots. Transcript analysis of MAPK family and immunokinase assay in As-treated rice seedlings revealed significant level of induction in OsMPK3 transcripts in leaves and OsMPK3 and OsMPK4 transcripts in roots. Among MAPK kinase (MKKs) gene family, OsMKK4 transcripts were induced

in As-treated rice leaves and roots. The results together with in silico analysis indicated that OsMPK3 and OsMPK4 were the kinases responsible for the signal transduction along with OsMKK4 as an upstream element of the cascade (Rao et al. 2011). However, genetic and mutant analyses need to be carried out to confirm these results.

Although aluminum (Al) is not a heavy metal, it limits crop growth and yield in acid soils by inhibiting cell division and uptake of water and nutrients (Ma et al. 2002). In *Hibiscus moscheutos*, exposure to Al rapidly inhibited the root elongation, but the inhibitory effect of Al on root elongation was alleviated by application of SNP. Further, it was proposed that Al toxicity might inhibit NOS-like activity and might disrupt NO homeostasis, leading to low levels of endogenous NO and inhibition of root elongation in plants (Tian et al. 2007; He et al. 2012a). Accumulating evidences point towards the alleviation of Al toxicity by exogenous NO treatments through activation of antioxidative capacity that may eliminate ROS (He et al. 2012a). Exogenous SNP prevented Al-induced accumulation of MDA in roots and leaves, and alleviated inhibition of growth and impairment of photosynthetic electron transport chain in sour pummelo (*Citrus grandis*) seedlings (Yang et al. 2012) and improved photosynthetic efficiency of Al-stressed *Artemisia annua* (Aftab et al. 2012). Wang et al. (2010) observed a significant role of NR-dependent NO production in alleviating Al-induced oxidative stress in the roots of red kidney bean (*Phaseolus vulgaris*). However, He et al. (2012b) proposed that NO might reduce Al accumulation and enhance tolerance to Al toxicity by regulating hormonal equilibrium in root apices of rye (*Secale cereale* L.) and wheat (*Triticum aestivum*).

The effects of different heavy metals on endogenous NO content in different plant species and tissues as well as the effects of exogenous NO supply on tolerance to different heavy metals are a matter of continuous research. An increase in NO accumulation has been observed in *Arabidopsis* cell suspension cultures under stress imposed by 300 μM iron (Fe) (Arnaud et al. 2006). Zinc (Zn)-induced NO production promoted ROS accumulation in roots of *Solanum nigrum* primarily by modulating the expression and activity of antioxidative enzymes (Xu et al. 2010). In *Pogonatherum crinitum* root cells, lead (Pb) treatment enhanced nitrate reductase activity and induced rapid NO generation (Yu et al. 2012). On the other hand, NO donor, SNP, effectively reduced copper (Cu)-induced toxicity in the mountain ginseng (*Panax ginseng*) adventitious roots (Tewari et al. 2008) and manganese (Mn) toxicity in excised rice leaves (Srivastava and Dubey 2012).

In most of the studies exogenous NO was proposed to alleviate heavy metal toxicity and attenuate oxidative stresses by decreasing the H_2O_2 content and enhancing the activity of antioxidant enzymes like SOD, CAT, POX and APX. On the other hand, heavy metal-induced accumulation of NO was reported to be responsible for heavy metal toxicity. Further detailed studies may facilitate understanding of the networks involved in plant defenses against heavy metal stress and the roles of NO in regulating both ion homeostasis and cellular responses to heavy metals (Xiong et al. 2010).

2.2.2 Drought Stress

Drought is an important stress affecting crop productivity and yield. It was exhibited that exogenous NO improved drought tolerance and enhanced net photosynthetic rate in wheat (Garcia-Mata and Lamattina 2001; Boyarshinov and Asafova 2011) and rice (Farooq et al. 2009) by improving stability of membrane, enhancing activities of antioxidant enzymes and reducing H_2O_2 and MDA contents. It has been suggested that protective effect of NO on cell membranes might depend on activation of antioxidant enzymes (Boyarshinov and Asafova 2011) as NO has a high affinity for iron-containing enzymes. There are several studies that support NO-induced stimulation of major antioxidant enzymes such as APX, CAT and SOD under stress conditions (Sang et al. 2008; Uchida et al. 2002; Tian and Lei 2007). Hao et al. (2008) reported that treatment with NO prevented water loss and oxidative damage by enhancing SOD activity in maize leaves under water deficit conditions. Application of exogenous SNP prevented drought-induced decrease in growth performance, relative water content and membrane stability by enhancing proline accumulation and activation of antioxidant enzymes accompanied by a decrease in lipid peroxidation and H_2O_2 content under drought stress (Lei et al. 2007; Nasibi and Kalantari 2009). Transgenic *Arabidopsis* lines expressing rat neuronal NOS showed higher drought tolerance with reduced rate of water loss, reduced stomatal aperture, and altered proline and MDA contents (Shi et al. 2012).

The presence of NO donors and ROS increased the abscisic acid (ABA) synthesis in wheat roots under water deficit (Zhao et al. 2001). Additionally, NO accumulation was shown to be essential in ABA-induced closure of stomata in *Vicia faba* plants (Garcia-Mata and Lamattina 2002). It was also indicated that stress-induced ABA and NO accumulation and stomatal closure were additionally dependent on H_2O_2 synthesis in *Arabidopsis* plants (Bright et al. 2006; Neill 2007; Neill et al. 2008). Neill et al. (2008) suggested that cADP ribose (cADPR) might be involved in NO synthesis and signal transduction pathways. Since the changes in stomatal conductance seemed to be closely related to both ABA and NO increase after drought stress in grapevine plants, it was assumed that NO has a potential role in signalling pathway in grapevines under drought (Patakas et al. 2010). Treatment with NO scavenger, cPTIO, increased the NOS activity while the application of NO donor, SNP, reduced NOS activity in maize seedlings which further confirms involvement of NO in drought signalling pathway and function of NO as a cellular messenger to mediate the adaptive responses to drought stress (Hao et al. 2008).

2.2.3 Salinity

Salt stress takes place in the presence of excessive accumulation of soluble salts in the soil. It suppresses plant growth and productivity due to its negative effects

on ion homeostasis and osmotic balance (Munns and Tester 2008). In addition to ionic and osmotic components, salt stress, like other abiotic stresses, leads to oxidative stress through an increase in ROS. Plant adaptations to salinity are mainly of three distinct types; (1) osmotic stress tolerance, (2) ion exclusion, and (3) tissue tolerance to accumulated ions (Munns and Tester 2008). The involvement of NO in salt tolerance has recently been investigated extensively, and function of NO in tolerance has been demonstrated in many plant species (Uchida et al. 2002; Zhang et al. 2006; Tanou et al. 2012; Khan et al. 2012).

Exogenous application of NO increases tolerance to salinity by inducing the activities of antioxidative enzymes in *Citrus aurantium* L. (Tanou et al. 2012) and tomato (Hayat et al. 2012). Besides, enhancing the activities of antioxidative enzymes, NO also protects plants from salinity by boosting the contents of antioxidant metabolites, e.g. ascorbate and reduced glutathione, and the osmolytes, e.g. proline and soluble sugar (Wu et al. 2011). It has been shown that combined application of NO donor, SNP, and calcium chloride ($CaCl_2$) plays a role in enhancing the tolerance of plants to salt stress by improving antioxidative defense system, osmolyte accumulation and ionic homeostasis (Khan et al. 2012).

Maintenance of K^+ to Na^+ ratio is another strategy to counter the damaging effects of salt stress. In the calluses of reed (*Phragmites communis*) under 200 mM NaCl treatment, addition of NO donor, SNP, stimulated the expression of plasma membrane H^+-ATPase and induced salt resistance by increasing the K^+ to Na^+ ratio (Zhao et al. 2004). In sunflower seedlings, it was shown that NO provoked biochemical adaptation during seedling growth under salinity conditions. The Na^+/K^+ ratio increased 4-fold in roots, and Na^+ was rapidly transported to the cotyledons (David et al. 2010). *Arabidopsis* mutant *Atnoa1* with reduced endogenous NO level was more sensitive and exhibited lower survival rates compared to wild type under NaCl stress (Guo et al. 2003; Zhao et al. 2007). *Atnoa1* mutants displayed a greater Na^+/K^+ ratio in shoots than the wild type after exposure to NaCl, but SNP treatment attenuated this elevation of the Na^+/K^+ ratio (Zhao et al. 2007).

The available data on the involvement of NO in response mechanism to salinity are contradictory, solely due to the plant species and the severity of the salinity treatment used. A proteomic analysis was used to determine 85 leaf proteins that underwent quantitative variations in citrus plants directly exposed to salt stress. The results exhibited a crosstalk between signalling pathways of H_2O_2 and NO in acclimation to salinity (Tanou et al. 2009). Existence of crosstalk between ROS and NO has been documented by others as well (Zago et al. 2006; Neill 2007; Rodríguez-Serrano et al. 2009). Recently it was reported that oxidative and nitrosative signalling and associated post-translational modifications including protein carbonylation, nitration and *S*-nitrosylation orchestrate the acclimation of citrus plants to salinity stress (Tanou et al. 2012). Family of protein kinases was proposed as a key component of signalling process mediated by NO during salinity response. In cell suspensions of tobacco exposed to salt stress, *Nicotiana tabacum* Osmotic Stress-Activated Protein Kinase (NtOSAK) was activated by NO. Analysis of NtOSAK revealed that the activation was not due to a process of

S-nitrosylation, but instead due to a process of phosphorylation of two residues located in the kinase activation loop. Additionally, it was demonstrated that NtOSAK interacted with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which underwent a process of *S*-nitrosylation which affected neither GAPDH activity nor interaction with NtOSAK. Overall, the results indicated that both proteins were regulated directly or indirectly by NO (Wawer et al. 2010).

The interaction between NO and polyamines, and their role in defense responses against abiotic and biotic stress conditions have been investigated (Wimalasekera et al. 2011). It was reported that NO enhanced the tolerance of cucumber seedlings to NaCl stress by regulating the content and proportions of different types of free polyamines (Fan et al. 2013).

2.2.4 Heat Stress

High temperature is one of the most important abiotic stresses that negatively affect plant growth and development. Heat stress results in overproduction of ROS (Kotak et al. 2007) and NO. The rate of NO emission was increased under heat stress in adaxial epidermal cells and cell suspensions of tobacco (Gould et al. 2003). NO donor SNP activated antioxidant enzymes (SOD, CAT, and soluble POX) and elevated heat tolerance in wheat coleoptiles. SNP-induced heat tolerance was dependent on Ca^{2+} and ROS, whose production was probably boosted by activation of NADPH oxidase (Karpets et al. 2011). It was suggested that the activation of already available NADPH oxidases was initiated by NO (Karpets et al. 2012).

However, in sunflower seedlings, heat treatment (38 °C for 4 h) reduced NO production, inhibited GSNOR activity, increased protein nitration, and caused accumulation of SNOs and peroxynitrite (Chaki et al. 2011). Studies with *Arabidopsis* mutant *hot5* deficient in GSNOR activity showed the correlation of this enzyme with heat stress response. It was indicated that GSNOR modulates the intracellular level of SNOs, enabling thermo tolerance (Lee et al. 2008). When pea plants were exposed to heat stress (38 °C for 4 h), NO content was reduced in leaves but NOS-like activity was not affected significantly. It was also shown that the SNO content increased in stressed pea plants (Corpas et al. 2008). After heat treatment of tobacco suspension cells at 35 °C or 55 °C, it was determined that NO production in cells shocked at 35 °C was lower compared to ones shocked at 55 °C (Locato et al. 2008). Song et al. (2006) reported that calluses of two ecotypes of reed (*P. communis*) showed much higher activities of SOD, CAT and APXin the presence of SNP under heat stress than those under heat stress alone. On the other hand, the activity of lipoxygenase was down-regulated accordingly in calluses of reed (Song et al. 2006). Although exogenous application of H_2O_2 or SNP prior to treatments with salinity or heat alleviated the deleterious effects of salt or heat stress in young rice plants, high concentrations of H_2O_2 or SNP treatment decreased growth rate (Uchida et al. 2002).

The effects of NO on non-photochemical fluorescence quenching (NPQ) of chlorophyll *a* fluorescence were investigated under heat stress (Hossain et al. 2011). When intact leaves of wild type and ΔGLB3 (lacks truncated hemoglobin gene) *Arabidopsis thaliana* were compared under heat stress, it was found that NPQ of ΔGLB3 was severely declined. The effect was also found to be mimicked by chemical NO donors, and it was completely prevented by the NO scavenger, cPTIO. It was suggested that NO can be involved in the decline of NPQ under heat stress (Hossain et al. 2011). Overall, it can be concluded that the modulation of intracellular NO depends on the degree of heat treatment and plant tissues or species used.

2.2.5 Cold Stress

Changes in temperature have important effects on plant growth and development. In nature, some plants acquire enhanced freezing tolerance by cold acclimation, which increases the chance of survival at subsequent freezing temperatures. Although low temperature induces changes in expression of hundreds of genes (Seki et al. 2002; Shinozaki et al. 2003; Sharma et al. 2005; Zhu et al. 2007; Winfield et al. 2010), the information related with the effect of NO and nitrogenous molecules on tolerance to cold stress is very limited.

When NO was applied exogenously, tolerance to cold conditions was improved in various plant species such as wheat, maize and tomato (Neill et al. 2003). Both NR and NOS-like enzyme have been suggested as sources of NO in response to cold (Corpas et al. 2008; Zhao et al. 2009, 2011; Cantrel et al. 2011). The synthesis of NO under low temperature stress has been reported in *Lotus japonicus*, *Pisum sativum* and *Arabidopsis thaliana* (Shimoda et al. 2005; Corpas et al. 2008; Cantrel et al. 2011). However, Airaki et al. (2012) reported a significant decrease in NO content in pepper leaves under low temperature (8 °C for 24 h) stress. It was proposed that NO induction under cold stress played a role in freezing tolerance through proline synthesis (Zhao et al. 2009). In *Brassica juncea* low temperature-induced *S*-nitrosylation caused inactivation of carboxylase activity of Rubisco, and this may indirectly indicate a role for NO after chilling exposure (Abatand Deswal 2009). It was suggested that NOS-like activity and subsequent NO accumulation participates in decreased cold-responsive pollen germination, inhibited tube growth, and reduced proline accumulation, partly via cGMP signalling pathway in *Camellia sinensis* (Wang et al. 2012).

NO was proposed as a critical regulator of synthesis of phosphatidic acid, a phospholipid derived signal, in plants (Laxalt et al. 2007). While exogenous application of NO donors led to phosphatidic acid synthesis in tomato (*Solanum lycopersicum*), *Vicia faba* and cucumber (*Cucumis sativus*), phosphatidic acid synthesis in response to xylanase and auxins was impaired by treatments with the NO scavenger, cPTIO (Laxalt et al. 2007; Distefano et al. 2008; Lanteri et al. 2008). Cantrel et al. (2011) demonstrated that the rise in phosphatidic acid levels was not

mediated by cold-triggered NO production. It was shown that sphingolipids were transiently phosphorylated in response to cold exposure and NO served as a negative regulator of this phosphorylation. It was also indicated that phytosphingosine phosphate and ceramide phosphate were transiently synthesized after chilling, and synthesis was negatively regulated by NO in *A. thaliana*. It was proposed that NO might be considered as an intermediate in gene regulation and lipid-based signalling during cold transduction (Cantrel et al. 2011). Since NO plays a central role in modulating the synthesis of sphingolipid signals, it might be a key player in the plant response to cold stress.

2.2.6 Ozone

Ozone (O_3) is an extremely active form of oxygen. Air pollutants can liberate ozone during photochemical reactions. Ozone has negative effects on plants as well as protective effects on cellular damage by ultraviolet (UV) radiation. Air pollutant ozone enters to the leaf through open stomata. The effects of ozone on plants depend on concentration and exposure time. While a low level of ozone exposure reduces photosynthesis and growth, a high concentration of ozone provokes cell death with visible injuries in the leaves. After ozone treatment, NOS activity was induced, salicylic acid was accumulated, and cell death was observed (Rao and Davis 2001; Ahlfors et al. 2009). High concentrations of ozone treatment in *Arabidopsis* caused a burst of ROS and NO (Ahlfors et al. 2009) but decreased contents of ascorbate and glutathione (Mahalingam et al. 2006).

When tobacco plants were exposed to ozone, accumulation of H_2O_2 and NO was observed. Up-regulation of alternative oxidase gene (*AOX*) expression, decrease in cytochrome-c content and accumulation of ethylene were also detected under ozone exposure. Depending on the results, it was suggested that NO is coordinated together with ethylene for the regulation of *AOX* expression (Ederli et al. 2006). NO was indicated as indispensable for activation of *AOX1a* encoding a protein involved in scavenging of mitochondrial ROS (Ederli et al. 2006, 2008). Ozone and NO caused transcriptional activation of *AOX1a*, glutathione peroxidase (*GPX*) and induction of ethylene production through aminocyclopropancarboxylic acid synthase (*ACS2*) which were cGMP independent. However, NO-induced accumulation of transcripts of phenylalanine ammonia lyase (*PALa*) and pathogenesis-related protein (*PR1a*) was cGMP dependent (Pasqualini et al. 2009). Additionally, cGMP has been reported to increase in response to NO (Durner et al. 1998). Treatment with ozone induced increases in NO levels in plants (Ahlfors et al. 2009). Ozone also stimulates a salicylic acid induced protein kinase (SIPK) in a NO-dependent manner and Ca^{2+} is essential, but not sufficient, to promote NO accumulation in ozone-treated tobacco (Pasqualini et al. 2012).

Ozone exposure induced flavonol accumulation and NO generation in *Ginkgo biloba* cells, however, pretreatment with NO specific scavenger cPTIO inhibited ozone-induced flavonol production. It was also indicated that NR activity

was enhanced with ozone exposure. NR-mediated NO signalling is found to be involved in ozone-induced flavonol accumulation in *G. biloba* cells (Xua et al. 2012). It can be concluded that ozone prompts a general response where NO, ROS and several hormones interact. NO can modify signalling, hormone biosynthesis and gene expression in plants exposed to ozone.

2.2.7 UV-B Radiation

Ultraviolet B (UV-B) radiation (280–320 nm) is absorbed by stratospheric ozone, but a small proportion is transmitted to the earth surface. Since atmospheric pollutants cause the destruction of ozone layer, UV-B radiation on the earth surface rises. High doses of UV-B radiation induce production of ROS, causing damage to proteins, lipids and DNA, and hence affect the cell integrity, morphology, and physiology of plants. Under UV-B stress, the NO produced in plants is able to protect the cells from the deleterious effects of ROS and oxidative stress (Frohnmeyer and Staiger 2003).

Besides oxidative pathways, UV-B radiation strongly induced NOS-like activity and caused a decrease of both leaf biomass and exo- or endo-β-glucanase activities (An et al. 2005). In contrast, an NOS inhibitor and a NO donor largely decreased the activity of NOS in non-irradiated seedlings. Tossi et al. (2009a) observed that apocynin (an inhibitor of NADPH oxidase) reduced UV-B-induced oxidative damage by reducing chlorophyll breakdown caused by H_2O_2, and this was correlated with NO production mediated by NOS activity. Pretreatment with diphenyleneiodonium (an inhibitor of NADPH oxidase) and NG-nitro-L-arginine methyl ester (L-NAME, an inhibitor of NOS) partially blocked the NO accumulation (Tossi et al. 2009b). In bean seedlings subjected to UV-B radiation, exogenous NO partially alleviated the UV-B effect characterized by a decrease in chlorophyll content and oxidative damage to the thylakoid membrane (Shi et al. 2005).

Various NOS-like enzymes were characterized in plants, but their molecular basis is still doubtful in higher plants (Moreau et al. 2008). Zhang et al. (2011) have proved that NO derived from NR plays a role in conferring UV-B stress tolerance. Direct association between NR activity, NO emission and nitrite (NO_2^-) content was determined in silver birch (*Betula pendula*) under UV-B radiation. It was confirmed that NR activity and NO emission are required for flavonoid accumulation in silver birch, and NO scavenger cPTIO abolished NO. Although a role for an NOS-like source for NO during UV-B stress was suggested in maize and *G. biloba* (Hao et al. 2009; Tossi et al. 2009a), the effect of mammalian NOS inhibitors on UV-B-induced NO generation cannot be shown by Zhang et al. (2011).

The involvement of NO in response to UV-B radiation (An et al. 2005; Qu et al. 2006) appears to act through ABA-mediated pathways (Tossi et al. 2009b). Tossi et al. (2012a) proposed that UV-B perception in plant cells leads to an increase in ABA levels which triggers elevation of cytosolic Ca^{2+} concentration leading to NO production through induction of NOS and/or NOS-like activities.

This enhancement of NO production contributes to tolerance to high doses of UV-B by protecting cell redox homeostasis from uncontrolled generation of ROS and associated deleterious effects provoked by UV-B radiation. In *A. thaliana* an NOS inhibitor and a NO scavenger partially blocked UV-B-mediated induction of chalcone synthase (*CHS*) gene involved in producing chalcones that participate in defense mechanisms and production of protective pigments such as flavonoids (Fig. 2.1) (Mackerness et al. 2001). It was found that irradiation of silver birch leaves with UV-B induces both flavonoid accumulation and NO generation (Zhang et al. 2011). Systemic accumulation of NO through UV-B radiation in maize seedlings was also reported by Tossi et al. (2012b). Up regulation of maize P (*ZmP*) gene expression and its target genes *CHS* and chalcone isomerase (*CHI*) by NO, leads to an increased flavonoid biosynthesis in non-irradiated leaf regions. This systemic response to UV-B perception involves enhanced levels of NO and flavonoids. It was also indicated that NO is involved in secondary metabolite production. Recently, it was suggested that UV-B-enhanced NO levels in plant cells can also protect microtubule organization as well as microtubule-related processes of root growth and development against disrupting effects of UV-B (Krasylenko et al. 2012).

2.2.8 Flooding

Flooding is a major obstacle affecting plant survival in various regions of the world. Non-enzymatic formation of NO depends on acidic pH and extracellular NO_2^- which are conditions that occur during anaerobiosis (Dat et al. 2004). When cells are under anaerobic conditions like flooding, NO_2^- can accumulate intracellularly. Subsequently, NO_2^- is used as a substrate by cytosolic NR for generation of NO (Botrel et al. 1996; Rockel et al. 2002).

Various reactions involved in production of NO in roots are stimulated under anaerobic conditions. It was shown that side-reaction of NR is the reduction of NO_2^- to NO (Yamasaki et al. 1999). Since NO formation by NR requires high NO_2^- concentration, it is suggested that NO can be formed by NR and accumulates only when the cells are in transition to the unfavorable anaerobic conditions (Sairam et al. 2008). Dordas et al. (2003) proposed that in hypoxic maize cell cultures and alfalfa root cultures, one of the functions of hypoxic stress-induced haemoglobin (Hb) is to modulate NO levels in the cell. High nitrate (NO_3^-) concentrations together with the induction of stress-induced Hb in *Arabidopsis* may also relate to a requirement to modulate NO levels (Wang et al. 2000). When plant roots were exposed to hypoxia, NR activity was increased, with NO_2^- reduction being suppressed at the nitrite reductase step (Botrelet al. 1996). For the immediate survival of plant root cells, Hb may be pivotal to regulate the levels of NO. Hypoxia results with a decline in mitochondrial respiration, an increase in NADH and a drop in ATP levels. Then, Hb gene expression and activation of NR occurs, together with production of NO (Igamberdiev et al. 2005). This would prevent

the cell death and maintain ATP levels. Sanchez et al. (2010) suggested that NO formed by copper-containing nitrite reductase in soybean nodules after flooding has a negative effect on expression of nitrogenase. They proposed that plant oxygen carrier leghemoglobin (Lb) has a major role in detoxifying NO and NO_2^- in response to flooding conditions.

2.2.9 Wounding

Different types of abiotic and biotic stresses, including herbivores, wind or rain, can produce mechanical injury in plants. In the damaged zone, plants respond with a cascade of events and signals that induce various genes and molecular responses (Schilmiller and Howe 2005). There are studies providing evidence for the involvement of NO and other nitrogenous metabolites in response to wounding. In *A. thaliana*, mechanical wounding induced a rapid accumulation of NO which was proposed to be involved in jasmonic acid-associated defense response (Huang et al. 2004). In pea (*P. sativum*) leaves, mechanical wounding induced accumulation of NO after 4 h. This was accompanied by induction of NOS and GSNOR activities and an increase in the content of SNOs. However, pattern of tyrosine nitration of proteins was not affected by mechanical injury (Corpas et al. 2008). It was reported that wounding triggered accumulation of certain SNOs, specifically GSNO, but did not affect NO content in sunflower hypocotyls. GSNO accumulation was indicated as an outcome of down-regulation of GSNOR activity in sunflower (Chaki et al. 2011). Overall, involvement of NO or other reactive nitrogen species (RNS) might be critical for induction of responses to mechanical injury in plants.

2.3 Conclusion

It is well known that abiotic stresses (salinity, water deficit, extreme temperatures, toxic metals, air pollutants etc.) limit plant growth and productivity. Abiotic stress is estimated to be the primary cause of worldwide crop loss. Several studies have been performed to understand tolerance mechanisms of plants in order to overcome the negative effects of these stresses on yield. There are also studies in literature supporting the relevance of NO in plants under abiotic stress conditions. Application of exogenous NO provides certain level of resistance against several types of stresses by activating different biochemical pathways. NO may help plants to survive stressful conditions through its function as a signalling molecule in the activation of antioxidative enzymes or its direct reaction with active oxygen, nitrogen and lipid radicals. Further genetic and proteomic analyses and additional physiological approaches will be required to understand the details of NO metabolism and function in plants. The acquired data will shed light on the sources of NO

and factors affecting its synthesis under abiotic stress, and also will provide in depth information on different strategies which this multifaceted molecule adopts in facing the detrimental effects of abiotic stress.

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Chapter 3
Nitric Oxide and Abiotic Stress-Induced Oxidative Stress

Sunita Sheokand and Anita Kumari

Abstract Abiotic stresses such as temperature, salinity, drought, heavy metals and UV induce ROS production and oxidative stress. NO is a free radical generated in plant cells. NO has been reported to be induced rapidly by various environmental stresses and regulates the plant responses to abiotic stresses. The antioxidant role of NO is mainly based on its ability to maintain the cellular redox homeostasis and regulate the toxicity of ROS. Moreover, NO has also been proposed to play an important role as a signalling molecule. In this chapter, we will discuss the different environmental stresses-induced oxidative stress and the effect of NO on the oxidative stress in plants.

Keywords Drought · Reactive oxygen species · Salinity · Temperature stress · UV stress

3.1 Introduction

3.1.1 Oxidative Stress and Reactive Oxygen Species

All life exists in an oxidizing environment where oxygen supports aerobic life with great energy output. The very molecule which sustains aerobic life can act as lethal contaminant in mildly reduced cellular environment through endless formation of ROS (reactive oxygen species). "ROS" comprises of ions or small molecules consisting of oxygen ions or free radicals of inorganic or organic form. Oxygen itself is a strong oxidant as it possesses two unpaired electrons in its outermost π orbital.

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The reduction of oxygen by non radical species needs transfer of two electrons having parallel spins to oxygen in order to fit with parallel spins of two unpaired electrons. Oxygen, therefore, gets converted into ROS by univalent reduction (transfer of electron) or by energy transfer. The common ROS produced in plants include superoxide anion ($O_2^{\cdot-}$), perhydroxy radical ($HO_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), alkoxy radical ($RO^{\cdot}$), peroxy radical ($ROO^{\cdot}$), singlet oxygen (lO_2), organic hydroperoxide (ROOH), and so forth (Sharma et al. 2012). They are generated endogenously during certain developmental transitions such as seed maturation and also as a result of normal, unstressed, photosynthetic, and respiratory metabolism. Any circumstance in which cellular redox homeostasis is disrupted can lead to oxidative stress via the generation of ROS. Environmental stresses like temperature, salinity, drought, heavy metals, herbicides and pathogen attack greatly affect plant metabolism and productivity (Mittler and Blumwald 2010). One of the common responses to different environmental stresses either biotic or abiotic is the accelerated generation of ROS, thus causing oxidative stress and exacerbating cellular damages (Gill and Tuteja 2010).

ROS are also generally produced as a by-product of normal aerobic metabolism, involving largely the membrane-linked electron transport processes, redox cascades and metabolisms, whose production is aggravated under the influence of unfavourable environmental cues. In all aerobic organisms, the concentration of ROS is tightly controlled by ROS scavenging pathways that metabolize ROS. However, an imbalance in generation and metabolism of ROS leads to a variety of physiological challenges by disrupting redox homeostasis of cell, which is collectively known as "oxidative stress".

Superoxide radical is generated in plant cell at the onset of oxidative burst of cell. It is a charged molecule and cannot pass through the biological membranes. Due to subcellular compartmentation of anitioxidative defence mechanisms, the efficient removal of superoxide anions at their sites of generation is crucial. Protonated form of $O_2^{\cdot-}$, $HO_2^{\cdot}$ is more reactive than superoxide anion itself, but in plant cells at physiological pH, a very small proportion of $O_2^{\cdot-}$ would be in this form (Eltsner 1987; Bhattacharjee 2010) as superoxide anions are readily dismutated to H_2O_2. O_{2-} can either transfer its excitation energy to other biological molecules or continue with them, thus forming endoperoxides or hydroperoxides (Halliwell and Gutteridge1989). Superoxide radical is a moderately reactive, short-lived ROS with a half-life of approximately 1–4 μs (Dat et al. 2000) in water and 100 μs in polar solvent (Foyer and Harbinson 1994). Hydrogen peroxide, on the other hand, is capable of diffusing across membranes and is thought to fulfil a signalling function in defence responses (Mullineaux et al. 2000). Much more reactive hydroxyl radical ($OH^{\cdot}$) can be formed from $O_2^{\cdot-}$ and H_2O_2 through Fe-catalysed Haber–Weiss reaction (Eltsner 1987; Bhattacharjee 2010). Singlet oxygen, an electronically excited species of O_2, is also very toxic, and its significance has been realized due to the development of methods for its generation, free from other contaminants as well as its detection (Halliwell and Gutteridge 1989). In addition, peroxy and alkoxy radicals formed as intermediates in membrane lipid peroxidation are also very toxic at high concentration and poses threat to several biomolecules.

3.1.2 Site of ROS Production

It has been estimated that 1–2% of total oxygen consumed by plants is sidetracked to produce ROS in various subcellular loci. The main sites of production are mitochondria, chloroplast, peroxisomes, plasma membrane, and apoplast, either by excitation or reduction of oxygen (Fig. 3.1). Under stress conditions, limited CO_2 fixation leads to a decrease in carbon reduction by the Calvin cycle and to a decrease in oxidized $NADP^+$ to serve as electron acceptor in photosynthesis. While ferredoxin is over-reduced during photosynthetic electron transfer, electrons may be transferred from PS-1 to oxygen to form superoxide radicals ($-O_2^{-\cdot}$) by the process called Mehler reaction (Hsu and Kao 2003). This triggers a chain reaction that generates more aggressive oxygen radicals. Photorespiration has evolved to protect over-reduction of electron transfer chain by the regeneration of $NADP^+$. H_2O_2 is produced in peroxisome during photorespiration, and it is also produced from β oxidation of fatty acids as a by-product. Xanthine oxidase is the other source of ROS in peroxisomes, which generates $O_2^{-\cdot}$ during the catabolism of purines. In the mitochondria, the intracellular generation of ROS occurs due to leakage of the electrons at the ubiquinone: cytochrome b region and at the matrix side of complex I (NADH dehyrogenase) (Moller 2001). H_2O_2 generation and regulation by uncoupling of ETC and oxidative phosphorylation have also been demonstrated. At endoplasmic reticulum, $O_2^{-\cdot}$ is formed as a result of detoxification reactions catalysed by cytochrome, particularly cytochrome P450. ROS is also generated by NADPH-dependent oxidases at the plasma membrane level or extracellularly in the apoplast.

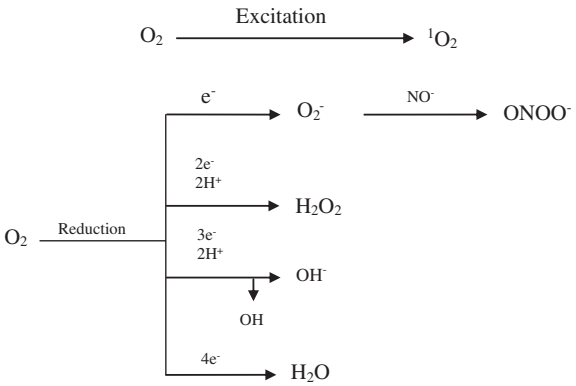

Fig. 3.1 Reactive oxygen species (ROS) formed in plant cell replaced with indifferent states. Excitation of ground-state oxygen gives $^1O_2$, singlet oxygen. Partial reduction of oxygen resulted in the formation of superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical. Superoxide anion can further react with NO^- to produce peroxynitrite ($ONOO^-$), and hydroxyl radical (OH) can further react with chloride anions to produce hypochlorite anion

3.2 Nitric Oxide and Oxidative Stress

Nitric oxide (NO) is a small, water- and lipid-soluble gas that has emerged as a major signalling molecule of ancient origin and ubiquitous importance (Durner et al. 1999). It contains an unpaired electron in its π_2 orbital, but remains uncharged. However, because of its free radical nature, it can adopt an energetically more favourable electron structure by gaining or losing an electron, so that NO can exist as three interchangeable forms: the nitro oxide radical ($NO^{\cdot}$), the nitrosonium cation (NO^{+}), and the nitroxyl radical (NO^{-}) (Stamler et al. 1992; Wojtaszek 2000). NO is able to move by diffusion in aqueous parts of the cell, such as the cytoplasm, as well as it can also move freely through the lipid phase of membranes, i.e., once produced, it can move from one cell to another or within a cell. Typically, NO rapidly reacts with O_2 to form nitrogen dioxide (NO_2) and rapidly degrades to nitrite and nitrate in aqueous solution (Fig. 3.2).

In recent years, the term reactive nitrogen species (RNS) was introduced in the biological literature to designate NO and other NO-related molecules, such as *S*-nitrosothiols (RSNOs), *S*-nitrosoglutathione (GSNO) and peroxynitrite ($ONOO^{-}$), among others, which have relevant roles in multiple physiological processes of animal and plant cells (Corpas et al. 2007b; Halliwell and Gutteridge 2007). Like oxidative stress, a term nitrosative stress was introduced to describe a similar process caused by RNS in plants under stress conditions. Consequently, it is considered that under a specific situation, the plant undergoes nitrosative stress when there is a de-regulated synthesis or overproduction of NO and NO-derived products that can have toxic physiological consequences. Figure 3.3 explains that NO and abiotic stresses are involved in triggering the nitrosative stresses. In this context, it is also important to define a reliable marker or footprints of this type of stress (Corpas et al. 2007a). Due to high $ONOO^{-}$ concentration protein tyrosine nitration increased which is a good marker to evaluate a process of nitrosative stress (Valderrama et al. 2007; Corpas et al. 2008b; Chaki et al. 2011). *S*-nitrosoglutathione is formed by the reaction of NO with reduced glutathione (GSH) in the presence of oxygen and it can function as a mobile reservoir of NO bioactivity in animal and plant cells (Durner and Klessig 1999; Ng

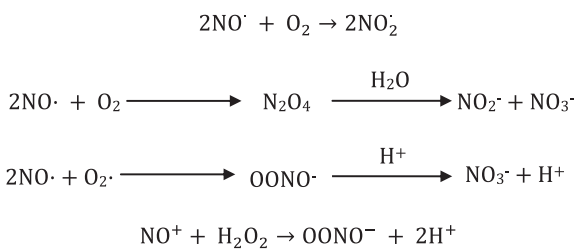

Fig. 3.2 Reactions of nitric oxide (NO); NO can react with oxygen to give nitrite and in aqueous solution lead to the generation of nitrite and nitrate. NO, either as the radical or NO^{+} ion, can react with superoxide and hydrogen peroxide, respectively, to produce peroxynitrite

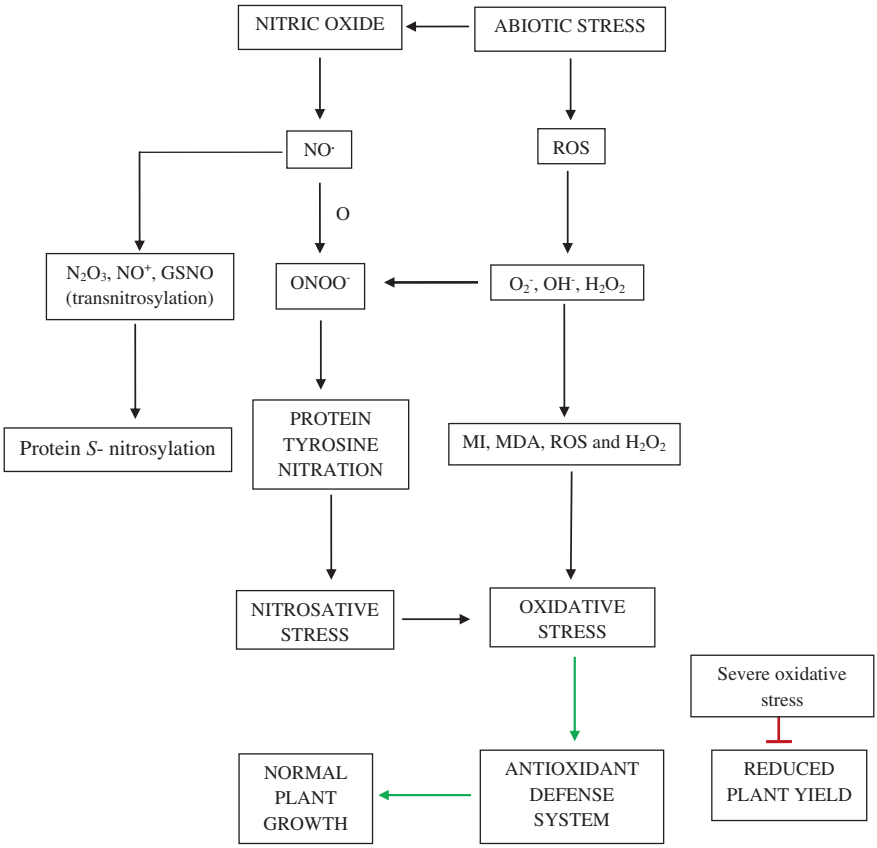

Fig. 3.3 Abiotic stresses and nitric oxide are involved in oxidative stress. Nitric oxide resulted in the production of nitro oxide anion ($NO^{\cdot}$), which leads to the formation of peroxynitrite ($ONOO^{-}$), causing nitrosative stress via protein tyrosine nitration (*black arrows).* Abiotic stresses also cause oxidative stress by ROS and $NO^{\cdot}$ production. Antioxidative defence system activated under oxidative stress leads to normal plant growth (*green arrow*), but under severe stress, antioxidative system is not effective and plant yield decreased (*red arrow*)

and Kubes 2003; Diaz et al. 2003; Wang et al. 2006a). Liu et al. (2001) reported that the enzyme designated as class III alcohol dehydrogenase (ADH3) is available to catalyze the NADH-dependent reduction of GSNO to oxidized glutathione (GSSG) and NH_3, being also designed as glutathione-dependent enzyme formaldehyde dehydrogenase (FALDH; EC 1.2.1.1) or GSNO reductase (GSNOR) activity. So far, the presence of GSNOR activity in plants has been reported in different plant species (Leterrier et al. 2011) including *Arabidopsis* (Sakamoto et al. 2002) pea (Barroso et al. 2006; Corpas et al. 2008a) and sunflower (Chaki et al. 2009) among others.

NO has been reported to be induced rapidly by several different types of chemical, mechanical and environmental stressors in a variety of plant species, and

regulates the plant responses to the abiotic stresses. NO acts as signalling molecule in defending plants from damage under stress conditions. For a signalling molecule to be effective, it needs to be produced quickly and efficiently on demand, to induce defined effects within the cell and to be removed rapidly and effectively when no longer required. There are several potential cellular sources of NO in plants, while its chemical structure enables it to move and relay a signal and be removed efficiently, terminating the message it originally sent to convey. NO generated under signalling conditions is in the picomolar and nanomolar range, and effects of NO are dependent on its source and concentration (Davis et al. 2001). In the aqueous solution, the half-life of NO is 0.09 to >2 s, when its decomposition depends on reactions with oxygen. In the aqueous phase, NO is auto-oxidized by oxygen to N_2O. NO can also react with other potential signalling molecules that are likely to be produced temporally and spatially alongside NO. One such chemical is the free radical superoxide anion ($O_2^{\cdot-}$) (Pryor and Squadrito 1995). Superoxide will readily dismutate to hydrogen peroxide (H_2O_2), especially at low pH, or in a reaction catalysed by superoxide dismutase (SOD). In fact, if superoxide is produced, the presence of hydrogen peroxide becomes virtually inevitable. Both superoxide and hydrogen peroxide have been suggested as signalling molecules in plants (Jabs 1999; Neill et al. 2002). Consequently, if NO reacts with superoxide or H_2O_2, this could potentially abrogate superoxide/hydrogen peroxide signalling. The product of the reaction between superoxide and the NO radical, or H_2O_2 and NO^+, is the ion peroxynitrite, itself a reactive and destructive compound. Such interaction between NO and reactive oxygen species (ROS) has been reported particularly during plant–pathogen interactions (Delledonne et al. 2001). NO can also react with proteins, particularly with thiol side groups, or low molecular weight thiols. High level of NO enhances superoxide production in mitochondria by inhibiting electron flow from cytochrome C oxidase (Millar and Day 1996).

The antioxidant role of NO is mainly based on its ability to maintain the cellular redox homeostasis and regulate the toxicity of ROS. Moreover, NO was also proposed to eliminate the excess nitrite from plant cells, as high concentration of nitrite is toxic to plant cells (Wellburn 1990; Shingles et al. 1996). Another important role of NO in abiotic stress responses relies on its properties as a signalling molecule. It has been reported to be involved in the signalling pathway of jasmonic acid synthesis and H_2O_2 synthesis, and also has been reported to regulate the expression of some stress tolerance genes (Orozco-Cardenas and Ryan 2002; Wendehenne et al. 2004). Furthermore, NO influences Ca^{2+} level in response to either salinity or osmotic stress caused by sorbitol (Gould et al. 2003; Zhao et al. 2004).

3.3 Salinity and Nitric Oxide

The involvement of NO in response to salinity can sometimes be contradictory, depending on the plant species and the severity of the salinity treatment. NO can provoke both beneficial and harmful effects, which depend on the concentration

and location of NO in the plant cells. Abiotic stresses alter (promote or suppress) NO production, while externally applied NO donors enhance or inhibit plant tolerance to specific stresses (Delledonne et al. 1998; Garcia-Mata and Lamattina 2002; Uchida et al. 2002; Zhao et al. 2007a). Uchida et al. (2002) found that low levels of NO conferred increased salt stress tolerance in rice, while high concentrations inhibited growth, which may be due to NO-induced oxidative or osmotic stress. Uchida et al. (2002) demonstrated that sodium nitroprusside (SNP, a NO donor) induces not only expression of transcripts for stress-related genes (e.g., sucrose-phosphate synthase, Δ'-pyrroline-5-carboxylate synthase, small heat-shock protein 26) but also enhances ROS scavenging enzymes activities in salt-stressed rice seedlings. Liu et al. (2007) reported that the increase of nitrate reductase-dependent NO production in red kidney bean roots under salt stress enhanced the activities of antioxidant enzymes by controlling the NADPH levels via glucose-6-phosphate dehydrogenase activation. The antioxidant-based protective effect of NO in salt stress was also confirmed in citrus plants. Tanou et al. (2009a) showed that exogenously introduced NO effectively induced antioxidant enzymes activity, promoted the maintenance of the cellular redox homeostasis, and mitigated the oxidative damage produced by Fenton-like reaction-mediated $OH^{\cdot}$ generation under salinity. A body of evidence suggests that the majority of NO-affected proteins seem to be regulated by S-nitrosylation, which occurs by oxygen-dependent chemical reactions or by the transfer of NO from a nitrosothiol to a sulfhydryl group of a cysteine residue (Moreau et al. 2009). Cysteine residues may be also oxidized upon oxidative stress (Spadaro et al. 2009), and this oxidative carbonylation of proteins could contribute to cellular signalling (Oracz et al. 2007) In olive plants grown under in vitro conditions, salt stress (200 mM NaCl) augmented the l-arginine-dependent production of NO, total SNOs, and the number of proteins that underwent tyrosine nitration in the molecular mass range between 44 and 60 kDa. Moreover, confocal laser scanning microscopy analysis using either specific fluorescent probes for NO and SNOs or antibodies to GSNO and 3-nitrotyrosine also showed a general increase in these RNS mainly in vascular tissue (Valderrama et al. 2007). Thus, these findings appear to indicate that in olive leaves, salinity induces nitrosative stress, while vascular tissues could play an important role in the redistribution of NO-derived molecules throughout the different organs of the plants. In *Arabidopsis* wild type and mutants expressing green fluorescent protein through the addition of peroxisomal targeting signal 1 (PTS1), which enables peroxisomes to be visualized in vivo, it has been shown that under salinity stress (100 mM NaCl), peroxisomes are required for NO accumulation in the cytosol of root cells, thereby participating in the generation of peroxynitrite ($ONOO^{-}$) and in increasing protein tyrosine nitration. In this case, the generation of NO in peroxisomes seems to be mediated by a putative calcium-dependent NOS activity (Corpas et al. 2009).

NO alleviates the hazards of the ROS, reacts with other target molecules, and regulates the expression of stress-responsive genes under salt stress conditions. Exogenous NO treatment significantly stimulated seed germination and

noticeably decreased the MDA content under salt stress in bell pepper (Nalousi et al. 2012). A similar antioxidative stress function of exogenous NO was also observed in salt-stressed barley plants (Li et al. 2008). Therefore, exogenous NO treatment could be effective in protecting plants/germinating seeds against oxidative damage caused by salt stress. It is well known that the antioxidant enzymes such as SOD and POD play a significant role in scavenging ROS in salt-stressed plants (Tseng et al. 2007; Tuna et al. 2008; Ashraf 2009); although, SOD activity decreased with higher SNP levels in the salt stress. Pretreatment of plant species with NO donor resulted in better growth and viability in rice (Uchida et al. 2002) and promoted seed germination (Kopyra and Gwóźdź 2003). *Arabidopsis* mutant *Atnoa1* with an impaired in vivo nitric oxide synthase (NOS) activity and a reduced endogenous NO level was more sensitive to NaCl stress than wild type (Zhao et al. 2007b). When grown under NaCl stress, the wild-type *Arabidopsis* plants exhibited higher survival rate than *Atnoa1* plants. Mutant plants were more sensitive to salt and oxidative stress than wild-type plants (Zhao et al. 2007a). Treatment with exogenous NO (SNP) to *Arabidopsis* mutants *Atnoa1* alleviated the oxidative damage caused by NaCl stress. *Atnoa1* mutants displayed a greater Na^+/K^+ ratio in shoots than wild type when exposed to NaCl, but SNP treatment attenuated this elevation of Na^+/K^+ ratio (Zhao et al. 2007b). Similarly, NO induced salt resistance of calluses from *Populus euphratica* under salt stress by increasing the K^+/Na^+ ratio, and this process was mediated by H_2O_2 and dependent on the increased plasma membrane H^+-ATPase activity (Zhang et al. 2007). In addition, NO was observed to stimulate the expression of plasma membrane H^+-ATPase in both salt-tolerant and salt-sensitive reed calluses (Zhao et al. 2004). Expression of a rice gene *OsNOA1* homologous to *Arabidopsis AtNOA1* can re-establish diminished NO synthesis in *Atnoa1* and induced the expression of plasma membrane Na^+/H^+ antiporter gene *AtSOS1* and H^+-ATPase gene *AtAHA2*, resulting in restoration of *Atnoa1* in terms of Na^+/K^+ ratio and salt tolerance phenotypes (Qiao et al. 2009), and this phenomenon can be mimicked by exogenous application of NO donor. Tanou et al. (2009b) have reported that NO and H_2O_2 elicited long-lasting systemic primer-like antioxidant activity in the leaves of citrus plants under NaCl stress and stress-free conditions. An ameliorative effect of exogenous NO treatments on oxidative metabolism in NaCl-treated chickpea plants was reported by Sheokand et al. (2008, 2010). The protective role of NO is mainly based on its ability to maintain the cellular redox homeostasis and regulate the level and toxicity of ROS induced by NaCl stress. NO decreases membrane permeability, rate of ROS production, malondialdehyde (MDA), H_2O_2, and intercellular CO_2 concentration (Ci) under salt stress by inducing ROS scavenging enzyme activities of CAT, peroxidase (POD), SOD, ascorbate peroxidase (APX), and proline (Pro) accumulation (Kopyra and Gwóźdź 2003; Fan et al. 2007; Shi et al. 2007). A protective role of NO under salinity stress has been reported by a number of workers (Kopyra and Gwóźdź 2003; Fan et al. 2007; Shi et al. 2007; Sheokand et al. 2008, 2010; López-Carrión et al. 2008; Zheng et al. 2009).

3.4 Drought and Nitric Oxide

Recently, much evidence has been accumulated indicating that NO appears to regulate the cellular redox homeostasis, acting either as a powerful oxidant or as a potent antioxidant. It is evidenced that various abiotic stresses such as drought, low and high temperatures, and UV and ozone exposure induce the NO generation in a variety of plant species (Neill et al. 2002). Moreover, a number of reports available support the potential role of NO as a regulator of plant drought tolerance. NO donor SNP treatment of 0.2 mM enhanced wheat seedling growth and kept high relative water content and alleviated the oxidative damage under drought conditions (Tian and Lei 2006). NO induced by polyamines (PAs) and CK has been shown to improve plant drought resistance in cucumber (Arasimowicz-Jelonek et al. 2009), *Zea mays* (Shao et al. 2010). Arasimowicz-Jelonek et al. (2009) reported that cucumber roots subjected to mild (5–10 h) water deficit showed slightly enhanced NO synthesis in cells of root tips and in the surrounding elongation zone compared with severe water stress (17 h), which resulted in an intensive NO production. Also drought-promoted NO production in pea, wheat, and tobacco was reported by Leshem and Haramaty (1996), Gould et al. (2003), and Kolbert et al. (2005). Liao et al. (2012) reported that NO and H_2O_2 alleviate drought stress in marigold explants and promote its adventitious root development. They suggested that the protection of mesophyll cell ultrastructure by NO or H_2O_2 under drought conditions improves the photosynthetic performance of leaves and alleviates the negative effects of drought on carbohydrate and nitrogen accumulation in explants, thereby adventitious rooting being promoted. Using pharmacological, physiological, and genetic approaches, Desikan et al. (2004) demonstrated that nitrate reductase (NR)-mediated NO synthesis in *Arabidopsis* guard cells was responsive to treatment with ABA and was required for ABA-induced stomatal closure (Desikan et al. 2004). Zhao et al. (2001) reported that NO induced ABA biosynthesis in wheat root tip under osmotic stress, and this NO induction was inhibited by carboxy-2-(4-carboxy-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) (c-PTIO) or NOS inhibitor L-NNA but enhanced by ROS. Xing et al. (2004) have also observed that NO and ROS stimulate ABA biosynthesis for maintaining leaf water under drought stress. Although NO is characterized by its inherent toxic nature and is known to potentially be damaging to cells, depending on its concentration and on the situation (Beligni and Lamattina 1999a), NO can also have a direct, protective effect against abiotic stress factors, as it alleviates the deleterious effects of ROS in establishing stress resistance responses (Qiao and Fan 2008) partly by increasing the activity of antioxidant enzymes. Application of NO donor SNP enhanced plant tolerance to drought stress by reducing water stress, ion leakage, and transpiration rate and inducing stomatal closure (García-Mata and Lamattina 2001). Exogenous SNP alleviated oxidative damage, accelerated protein synthesis, enhanced photosynthesis rate, increased the activities of SOD and CAT, maintained higher relative water content, and lowered water loss in leaves of wheat seedlings exposed to polyethylene glycol (PEG). Interestingly,

such effects of SNP were reversed by the addition of c-PTIO, a specific NO scavenger (Tan et al. 2008). These results suggested that application of SNP might confer on plants an enhanced resistance to drought stress. Sang et al. (2008) demonstrated that water stress induced the synthesis of NO in the maize mesophyll cells and activity of NOS in cytosolic and microsomal fractions of maize leaves, and this NO production was blocked by the pretreatment with inhibitors of NOS and nitrate reductase (NR), suggesting that NO is produced from NOS and NR in leaves of maize plants exposed to water stress. The pretreatments of NOS and NR inhibitors inhibited activities of chloroplast and cytosolic antioxidant enzymes, i.e., SOD, APX, and GR, and the decreased activities of these enzymes were enhanced by the exogenous application of NOS, thereby decreasing the accumulation of H_2O_2 induced by water stress. Hao et al. (2008) reported that NO dependence on NOS-like activity participated in the signalling of drought-induced protective responses in maize seedlings. Both NOS activity and the rate of NO release increased substantially under dehydration stress. The high NOS activity induced by c-PTIO as NO scavenger and NO accumulation blocked by NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME) in dehydration-treated maize seedlings indicated that most NO production under water deficit stress may be generated from NOS-like activity. After dehydration stress, detached maize leaves pretreated with SNP maintained more water content by decreasing transportation rate. Exogenous application of SNP alleviated the membrane permeability, a cell injury index when c-PTIO as a specific NO scavenger was applied; the effects of applied SNP were counteracted. Treatment L-NAME on leaves also led to higher membrane permeability, higher transpiration rate, and lower SOD activity than those of control leaves, indicating that NOS-like activity was involved in the antioxidative defence under water stress. These results suggested that NO dependence on NO-like activity serves as a signalling component in the induction of protective response and is associated with drought tolerance in maize seedlings. The protective effect of NO in osmotic stress was recently confirmed in two ecotypes of reed suspension cultures. The findings of Zhao et al. (2008) suggested that PEG-60000 induced NO release and activities of antioxidant enzymes in stress tolerant, but not sensitive, ecotype reed can effectively protect against oxidative damage and confer an increased tolerance to osmotic stress. NO could counteract oxidative damage under drought stress in *Phragmites communis* by regulating activities of antioxidant enzymes and lipoxygenases (Zhao et al. 2008). NO alleviates the ROS-mediated cytotoxic process in potato leaves (Beligni and Lamattina 1999b). The ROS-mediated damages caused by drought, including cell death, ion leakage, and DNA fragmentation, were inhibited by exogenous NO, and all those protective effects were abolished by the treatment with PTIO (Beligni and Lamattina 1999b). The protective effect of NO in osmotic stress was confirmed in two ecotypes of reed suspension cultures recently; Zhao et al. (2008) suggested that polyethylene glycol (PEG-60000)-induced NO release in stress tolerant but not sensitive ecotype reed can effectively protect against oxidative damage and conferred an increased tolerance to osmotic stress (Zhao et al. 2008).

3.5 Low Temperature and Nitric Oxide

In many studies, researchers have reported the role of NO in alleviating low-temperature stress (Siddiqui et al. 2010; Corpas et al. 2011). Zhao et al. (2009) reported that cold acclimation induced an increase in endogenous NO production in wild-type *Arabidopsis thaliana* and mutant Atnoa1/rif1 (for NO associated 1/resistant to inhibition by fosmidomycin 1) leaves. Cold acclimation stimulated NR activity and induced up-regulation of NR-dependent NO synthesis. Zhao et al. (2009) have also demonstrated that NO production in plants is involved in acquiring cold acclimation or cold tolerance. Guillas et al. (2011) have evidenced that NO is produced immediately as a plant response to cold stress, and it participates in the regulation of cold-responsive gene expression with novel downstream elements identified as phosphosphingolipid metabolic species. Liu et al. (2011) reported that exogenous SNP treatment exerted its protective effect under low-temperature stress through the activation of antioxidant enzymes. The application of exogenous NO induced cold tolerance in tomato, wheat, and maize (Neill et al. 2003). An analysis of NO and other RNS in pea plants exposed to low temperature (8 °C for 48 h) showed activation of l-arginine NOS and GSNOR activities as well an increase in the content of SNOs. Therefore, these results indicate that metabolism of RNS is triggered in response to low temperature implying a connection between NO and this stress (Corpas et al. 2008b). In leaves of pepper plants, low temperature (8 °C for 24 h) caused cold stress characterized by a general imbalance of the ROS and RNS metabolism, triggering a rise in the lipid oxidation and the protein tyrosine nitration. This points towards an induction of oxidative and nitrosative stress promoted by low temperature (Airaki et al. 2011). Similar behaviour has been observed in *Arabidopsis thaliana* exposed to 4 °C for 1–4 h (Cantrel et al. 2011) or during cold acclimation (Zhao et al. 2009), where the NO content increased. In *Brassica juncea* seedlings, low-temperature stress (4 °C for 1–48 h) inactivated Rubisco carboxylase by a process of S-nitrosylation which is well correlated with the photosynthetic inhibition detected under this type of stress (Abat and Deswal 2009).

3.6 High Temperature and Nitric Oxide

NO production under HT has been reported by a number of workers (Corpas et al. 2011). For example, in alfalfa sprouts, heat stress (37 °C for 2 h) resulted in a doubling of the rate of NO emission (Leshem et al. 1998). In tobacco, HT generates a rapid and significant rise in NO in adaxial epidermal cells after 7 min of heat treatment at 40 °C and in suspension cells after 5 min of heat treatment at 45 °C, evaluated by the fluorescence probe DAF-2 DA (Gould et al. 2003). Conversely, pea plants exposed at 38 °C for 4 h reduced the NO content of leaves, but it did not significantly affect the NOS-like activity. However, it was found that the SNO content increased 3-fold and that some nitrated proteins intensified (Corpas et al.

2008b). Sunflower seedlings under the same experimental conditions (38 °C for 4 h) have been reported to undergo oxidative stress, which was accompanied by a reduction in NO production, inhibition of GSNOR activity, accumulation of *S*-nitrothiols, formation of peroxynitrite, and rise of protein nitration. A proteomic analysis of some specific targets identified nitration as causing an inhibition of the activities both of carbonic anhydrase and of ferredoxin-NADP reductase, proteins involved in photosynthesis (Chaki et al. 2011). In tobacco Bright Yellow-2 (TBY-2) suspension cells exposed for 10 min to 35 °C or 55 °C and then 27 °C, the analysis of the released NO showed that at 35 °C, heat-shocked cells had a low production of NO compared to cells exposed to 55 °C, the latter showing fast-increasing NO production. This production was well correlated with the cell viability and DNA integrity as well with the presence of oxidative stress markers (Suzuki and Mitler 2006). In *Arabidopsis*, several mutants have been identified to have impairment in the *GSNOR1* gene, showing the involvement of this gene in the mechanism of response against HT. Thus, the mutant HOT5 (sensitive to hot temperatures) showed that GSNOR modulates the intracellular level of SNOs, enabling thermo-tolerance as well as the regulation of plant growth and development (Lee et al. 2008). In calluses of reed (*P. communis*), the exogenous application of SNP or ABA elevated thermo-tolerance by alleviating ion leakage, lipid peroxidation, and growth suppression induced by heat stress (45 °C for 2 h). However, the pretreatment with L-NNA or cPTIO aggravated the damages caused by heat stress and blocked the protective effect of exogenous ABA. On the other hand, exogenous ABA notably activated NOS activity and increased NO release, maintaining the heat tolerance (Song et al. 2008). Exogenous supply of NO protects wheat seedlings from high temperature-induced oxidative stress by up-regulating antioxidant defence and methyl glyoxal (MG) detoxification system (Hasanuzzaman et al. 2012). Song et al. (2006) found that application of SNP and SNAP, both NO donors, dramatically alleviated heat stress-induced ion leakage increase, growth suppression, and cell viability decrease in callus of reed under heat stress and also elevated the activities of SOD, APX, CAT, and POD. These results suggest that NO can effectively protect callus from oxidative stress induced by heat stress and that NO might act as a signal in activating active oxygen scavenging enzymes under heat stress. Decreased lipid peroxidation and increased antioxidative enzyme activities have also been reported in Chrysanthemum (Yang et al. 2012) and in mung bean leaf discs (Yang et al. 2006). Taken together, the data indicate that the heat stress affects NO metabolism, but the increase or decrease of this molecule depends on the degree of the treatment and plant tissues or species.

3.7 UV-B Radiation and Nitric Oxide

UV-B radiation promotes ROS formation significantly and exerts oxidative stress in the plant. Recently, various studies have pointed to the involvement of NO in response to UV radiation (An et al. 2005; Shi et al 2005; Qu et al.2006; Gupta

et al 2011; Corpas et al. 2011). UV radiation has been reported to increase NO production (Mackerness et al. 2001). NO may act as a prooxidant or antioxidant depending on the concentration. Mechanisms of NO action in UV protection are now emerging, with a very recent study showing an effect by inducing chalcone synthase (CHS) and chalcone isomerase (CHI) genes linked to polyphenylpropanoid production (Tossi et al. 2012). The results of Wang et al. (2006b) supported that NO generated from NOS-like activity appeared to act in the same direction or synergistically with ROS to induce ethyl synthesis in defence response under UV-B radiation in maize leaves. Zhang et al. (2003) and An et al. (2005) reported that UV-B induced increase of NOS activity in maize hypocotyls, indicating that NO may act as a second messenger and perform antioxidant response to UV-B radiation, and SNP-exposed maize plants exhibited increased activity of glucosidase and protein synthesis. The accumulation of endogenous NO in maize leaves in response to UV-B radiation is ABA dependent and is paralleled by greater tolerance to high doses of UV-B radiation (Tossi et al. 2009b). In another study, Tossi et al. (2009a) reported that when maize seedlings were UV-B-irradiated, cellular damage occurred and ROS were found widely distributed in chloroplasts and mesophyll cells. Pretreatment with apocynin and coinciding NO accumulation prevented this damage. In excised leaves of kidney bean (*Phaseolus vulgaris*) under UV-B stress, NO production is mediated by H_2O_2 through greater NOS activity (Zhang and Zhao 2008). In stems of pea (*Pisum sativum* L.) seedlings exposed to UV-B radiation, the release of NO through the induction of a NOS activity has been shown (Qu et al. 2006).

In bean seedlings subjected to UV-B radiation, exogenous NO partially alleviated the UV-B effect characterized by a decrease in chlorophyll contents and oxidative damage to the thylakoid membrane (Shi et al. 2005). In soybean plants exposed to low UV-B doses, it has been shown that ROS mediates haeme-oxygenase (HO) up-regulation, which exerts a protective action against oxidative damage. Moreover, it has been observed that the induction mechanism is mediated by NO, this being produced by a NOS-like activity (Santa-Cruz et al. 2010). A notable finding was that the exogenous application of NO to the rhizosphere of pea seedlings mimicked the responses of stems to UV-B radiation (Qu et al. 2006). It has also been reported that UV-B treatment can trigger up-regulation of antioxidant enzymes (Shi et al. 2005, Beligni and Lamattina 1999a; Chen et al. 2003). On the other side, it has also been shown that NO can down-regulate expression/activity of some antioxidant enzymes under various conditions (De Pinto et al. 2002; Clark et al. 2000).

3.8 Heavy Metal Stress and Nitric Oxide

There are variable reports on the effect of heavy metals on endogenous NO content in different plant species and tissues, and possible reasons for this discrepancy have been proposed (Xiong et al. 2010). In the cell suspensions, this discrepancy

is attributed to differences in the cell responses to short and long periods of metal treatment (Rodríguez-Serrano et al. 2006), a short heavy metal treatment period promoting a NO burst and a long treatment directly or indirectly decreasing NO generation. Groppa et al. (2008) propose that these opposite findings could be explained by the use of different heavy metal concentrations, the age of the plants, the duration of treatment used, and the variety of plant tissues used. An increase in NO content with heavy metal treatment has been reported by many workers (Bartha et al. 2005; Arnaud et al. 2006; Mahmood et al. 2009; De Michele et al. 2009; Xu et al. 2010). De Michele et al. (2009) reported that NO is actually required for cadmium-induced cell death. NO also modulated the extent of phytochelatin content, and possibly their function, by S-nitrosylation. These results shed light on the signalling events controlling cadmium cytotoxicity in plants. However, a decrease in NO content has also been reported widely (Illéš et al. 2006; Tian et al. 2007).

The protective role of NO under heavy metal stress is based on its ability to regulate the level and toxicity of ROS. The protective effect has been widely reported (Kopyra and Gwóźdź 2003; Hsu and Kao 2004; Wang and Yang 2005; Yang et al. 2006; Sun et al. 2007; Singh et al. 2008; Xu et al. 2009; Kumari et al. 2010; Saxena and Shekhawat 2013). Pretreatment with NO could significantly improve wheat seed germination and alleviate oxidative stress against Cu toxicity (Hu et al. 2007). In soybean plants exposed to $CdCl_2$, the exogenous application of NO protected against oxidative stress. The main aspects of antioxidant capabilities of the molecule seem to be a direct scavenging of ROS, mobilization of SOD, and reducing the level of oxidized proteins (Kopyra et al. 2006). It was also observed that pretreatment of seedlings with SNP protected sunflower leaves against Cd-induced oxidative stress (Laspina et al. 2005). Using fluorescent and laser scanning confocal microscopy, Kopyra and Gwóźdź (2003) found that NO pretreatment significantly reduced O_2^--induced specific fluorescence in *Lupinus luteus* roots under heavy metal treatment. These results suggest that NO antioxidant function may be carried out by scavenging of O_2^-. The detoxifying effect and antioxidant role of NO were also found in Cu-treated *chlorella* (Singh et al. 2004). Application of the NO donor SNP efficiently alleviated the Cu toxicity effects in roots of *Vicia faba* (Zou et al. 2012). NO prevented Cd-induced increase in the contents of H_2O_2 and MDA, decreased the contents of GSH and ascorbate, and increased the specific activities of antioxidant enzymes in rice leaves (Hsu and Kao 2004). NO up-regulates the component of antioxidant defence machinery and thiol status to cope better with Cu toxicity in the adventitious root of *Panax ginseng* (Tewari et al. 2008). NO reverses Cd-induced increase in the activities of antioxidant enzyme in wheat roots (Singh et al. 2008). NO also improves Cd tolerance in *Medicago truncatula* roots by the increase in the production of Pro and total GSH content (Xu et al. 2010). Application of SNP-promoted ROS scavenging enzymes reduced the accumulation of H_2O_2 and induced the activity of H^+ATPase and H^+PPase in plasma membrane or tonoplast and also significantly alleviated the growth inhibition induced by $CuCl_2$ in tomato plants. These results suggested that exogenous NO could effectively induce tomato seedlings to adjust

physiological and biochemical mechanisms against Cu toxicity and maintain metabolic capacity and normal growth under heavy metal stress (Cui et al. 2009). Xiong et al. (2010) also indicate that exogenous application of NO decreases both ROS accumulation in rice roots and H_2O_2 accumulation in leaves under Cd stress. SNP (250 μM) was effective in decreasing MI (%), MDA, H_2O_2, and ROS content, increasing the activity of antioxidant enzymes such as SOD, CAT, POX, APX, DHAR, MDHAR, and the GSH/GSSG and ASC/DHA ratio under Cd stress in chickpea (Kumari et al. 2010). Thus, the anti-toxicity mechanisms of NO could be largely explained by its augmentation of antioxidant content and antioxidative enzyme activity, which affects cell wall components and regulates the expression of stress-related genes.

3.9 Conclusions and Future Projections

The generation of ROS in abiotic stress has been well established in the last years. Increasing amount of evidence suggests that the generation of specific types of ROS in defined subcellular compartments is an important component of the stress response. Rather than being just cytotoxic by-products of biochemical processes, ROS are likely to play central roles as regulators of stress adaptation. While ROS are biochemically simple molecules, the intricacies of ROS signalling are still insufficiently understood. Multiple lines of evidence discussed above suggest that there is a biologically active interplay between ROS and NO signals in plants that modulates responses to abiotic stresses (for an overview see Fig. 3.3). However, most of the work has still to be done in respect of (a) NO functions as a signalling molecule in interaction with ROS, (b) NO-mediated defence gene regulation in plants, and (c) NO production in plant and its relation to environmental stimulus and cellular redox homeostasis regulation.

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Chapter 4
Regulatory Role of Nitric Oxide in Alterations of Morphological Features of Plants Under Abiotic Stress

David W.M. Leung

Abstract Previous studies have identified that plants under different abiotic stresses appear to exhibit some common morphogenic responses termed as stress-induced morphogenic response (SIMRs). One of the best examples is the inhibition of primary root elongation with accompanying initiation of new lateral roots in response to sub-optimal growth conditions. A central research question revolves around the signal transduction pathway in SIMRs. Nitric oxide (NO) is now recognized as an important signal molecule in plant growth and development. Here, an examination of its connection to the various morphological features of plants known to be affected under abiotic stress supports that NO is likely to be involved in the regulation of SIMRs, but the precise nature of its involvement must await clarifications from the likely complex interactions among NO, auxin, possibly other plant hormones and reactive oxygen species (ROS).

Keywords Nitric oxide donors · Plant hormones · Plant morphogenesis · Root elongation · Signal transduction

4.1 Introduction

Plants are sessile organisms that do not have the option of taking flight from their immediate environment even in the event of imposing adverse conditions or abiotic stress. Total collapse and death of the whole plants is by no means an inevitable consequence for plants under abiotic stress (Potters et al. 2007). For example, a substantial reduction in stem size and severe symptoms of iron deficiency (leaf chlorosis) were observed following exposure of 9-day-old seedlings of *Brassica juncea* and *Brassica napus* to $CuSO_4$ for 7 days in a concentration-dependent manner (Feigl

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et al. 2013). Although these effects of Cu^{2+} ions in excess were very pronounced, there were no accompanying visible signs of tissue necrosis. Moreover, there seems to be natural variation in plants' ability to survive and recover from non-lethal or sub-optimal environmental conditions (Leung 2013a). A fundamental research problem in stress biology of plants is to gain a better understanding of the signal transduction pathway involved in the response of plants to abiotic stress (Potters et al. 2009).

A literature survey found many studies on plants under abiotic stress at the biochemical and molecular levels. Adverse effects of abiotic stress on the morphology of plants were often limited to simple quantifications in terms of reduction of the fresh or dry weights of roots and shoots, or shoot and root lengths. Relatively little detailed descriptions on the morphological responses in terms of alterations in morphological features under abiotic stress were provided. Nonetheless, from the limited literature, many morphological features of plants are known to be affected following exposure to different forms of abiotic stress (Potters et al. 2009). Based on a literature analysis, it has become clear that there appear to be some generic morphogenic responses, termed as SIMRs, common to different forms of abiotic stress (Potters et al. 2007, 2009). This suggests that there might be a broad-spectrum strategy for plants to cope with non-lethal levels of different abiotic stresses. From this, it was argued that this strategy could serve the purpose of redistribution of resources for new growth under abiotic stress. In addition, the signal transduction pathway for SIMRs was being deduced based on the extensive literature on plant hormone research and the recent perspectives of some reactive oxygen species (ROS) in relation to plant growth and development. Auxin, ethylene, and ROS have already been identified likely to be the key drivers in SIMRs (Potters et al. 2009).

Nitric oxide (NO) has emerged as an important messenger molecule in the regulatory pathways of plant growth and development (Hebelstrupa et al. 2013). There are numerous recent research papers, mainly from investigations at the biochemical and molecular levels, showing a relationship between NO and tolerance of plants to abiotic stress (Siddiqui et al. 2011). These studies have contributed toward a general understanding of the mechanistic aspects of abiotic stress tolerance. However, our understanding of the signal transduction pathway involved is still incomplete.

In this chapter, based on the limited data available, the possibility is raised that NO might be part of the regulatory pathways underpinning SIMRs in different plant species under abiotic stress.

4.2 Root and Stem Growth

One of the best examples of SIMRs or morphological changes in response to different abiotic stresses is the inhibition of primary root elongation, while there is a redirection of resources for increased lateral root initiation (Potters et al. 2009). Also, some common changes in stem growth have been observed under different stresses. Based on the underlying cellular changes of these generic phenotypes in different plant species, it seems reasonable that the dynamic interactions among ROS, auxin,

and ethylene are strongly implicated in the signal transduction pathway upstream of many known molecular mechanisms associated with stress tolerance.

A recent survey of the literature broadly supported that NO may also be considered an important candidate messenger for SIMRs in different plant species (Table 4.1). The relevant studies mainly relied on the extensive use of NO donors such as sodium nitroprusside (SNP) and/or detection of changes in endogenous NO production. There are many studies showing alleviation of heavy metal effects in different plant species by exogenous application of NO in the form of a NO donor (Gill et al. 2013; Leung 2013b), but there are relatively fewer similar studies in relation to other forms of abiotic stress. For example, root elongation was retarded in the seedlings of *B. juncea* (Verma et al. 2013) and *Arabidopsis thaliana* (Phang et al. 2011) exposed to Cd^{2+} and Pb^{2+} in excess, respectively (Table 4.1). The effect of Cd^{2+} in excess on root elongation was correlated with an increased endogenous NO level in the metal-treated roots, while that of Pb^{2+} in excess was reversed partially by exogenous SNP (Table 4.1). Therefore, the participation of NO in regulation of root elongation is suggested, but the precise nature of its involvement remains unclear. In these studies, lateral root formation was not reported, presumably because the duration of the experiments was not sufficiently long enough for this observation to be made. Nevertheless, this should be necessary for studies to implicate a role for NO in this SIMR phenotype in different plant species. Interestingly, there was no difference between wild-type (WT) and a NO-overproducing mutant *A. thaliana* seedlings in response to exposure to Cu^{2+} in excess (Peto et al. 2011), suggesting a very minor role, if any, for NO being an endogenous signal in the morphological response to heavy metal stress. However, this interpretation might be complicated by the auxin homeostasis in the same tissue under the same stress (Peto et al. 2011; Kolbert et al. 2012).

Formation of lateral roots in wheat seedlings (Wang et al. 2013) and adventitious roots in marigold de-rooted seedlings (Liao et al. 2012) under osmotic and drought stress, respectively, was inhibited. However, exogenous application of SNP was found to counteract these adverse effects (Table 4.1). While these studies did not show evidence for a redistribution of resources as part of the survival strategy under water deficit conditions, the involvement of NO as a messenger in response to the abiotic stresses was implicated.

Inhibition of stem elongation was observed in pea seedlings under UV-B stress (Qu et al. 2006) and cucumber plants under NaCl stress (Fan et al. 2013). In the latter study, there was evidence for the development of thicker stems under the stress, suggesting that there was a redirection of resources under abiotic stress (Table 4.1). Furthermore, exogenous application of NO alleviated partially the observed effects of NaCl on cucumber. In contrast, exogenous application of SNP inhibited pea stem elongation, mimicking the effect of UV-B stress (Table 4.1). Other experiments in the study have implicated NO as an endogenous signal in response to UV-B stress in pea (Table 4.1). Unfortunately, there was no information in this study on the thickness of the stem or the development of any new axillary branching, and thus, it was not certain if NO was a signal involved in a typical SIMR phenotype of pea.

Table 4.1 Examples of studies implicating the involvement of nitric oxide (NO) in abiotic stress-induced morphological responses (SIMRs)

Stress	Plant	Morphology affected by stress	NO investigations	References
Heavy metal stress: Cadmium (50 μM Cd^{2+})	*Brassica juncea* L. (15-d-old seedlings)	Root elongation was severely inhibited under Cd stress	NO produced under Cd stress	Verma et al. (2013)
Heavy metal stress (lead, Pb^{2+}, 100 μM)	*Arabidopsis thaliana* (7-d-old seedlings)	Shorter and thickened primary roots resulted under Pb stress. These roots turned from creamy color to brown and had reduced root hair formation	The morphological effects of Pb stress on the primary roots and cotyledons were less evident if the seed were pretreated with 0.5 mM SNP for 3 h before germination	Phang et al. (2011)
		In addition, the edge of the cotyledons was wilted and brown		
Heavy metal stress: Copper (5, 25, and 50 μM Cu^{2+})	*Arabidopsis thaliana* (7-d-old seedlings)	Primary root elongation: In wild type, primary root length was shorter in the treatment with 25 or 50 μM Cu^{2+}	Response of NO-overproducing or NO-deficient mutant plants to Cu treatment was very similar to that of WT plants	Peto et al. (2011)
NaCl (50 mM)	Cucumber seedlings (*Cucumis sativus* L.) grown hydroponically (age of seedlings not specified)	Plant height, stem thickness, and fresh and dry weights of plants: All reduced by NaCl stress	Exogenous NO (100 μM SNP), 8 days of treatment: Exhibited partial reversal of NaCl inhibition	Fan et al. (2013)
NaCl (200 mM)	*Limonium bicolor*, seedlings were used for experiments after the development of the third leaf	(a) Leaf area: The expansion of leaf 3 and 4 (not 5 or older leaf) was negatively affected under NaCl stress	NaCl + 50 μM SNP: Exogenous NO appeared to counteract the NaCl stress-induced inhibition of leaf expansion	Ding (2013)
		(b) Density of salt glands (number of salt glands per leaf area) which was the same in leaf 3–5 in the absence of NaCl stress: NaCl stress-induced two- to threefold higher salt gland density	NaCl+SNP: No change in salt gland density compared to the NaCl alone treatment	

(continued)

Table 4.1 (continued)

Stress	Plant	Morphology affected by stress	NO investigations	References
Increased ultraviolet-B radiation (UV-B, 4.8 kj m^{-2} per day)	Pea (*Pisum sativum*), seedlings were used for experiments when the second trifoliate leaf had fully expanded	Inhibition of stem elongation in the seedlings exposed to enhanced UV-B throughout 5 days of growth compared to the control	Exogenous NO (300 µM SNP applied to the root zone in the absence of increased UV-B treatment) resulted in shorter stems	Qu et al. (2006)
			A NO-specific scavenger + UV-B radiation: Inhibition of stem elongation was prevented	
			More endogenous NO released in UV-B-treated seedlings than in the non-irradiated control	
Osmotic stress (100 mM mannitol)	Tomato seed (*Solanum lycopersicum*): Wild type (WT)	Seed germination (% of seeds with visible radicle protrusion through the seed coat): WT under white or blue light, osmotic stress completely inhibited seed germination	Mannitol + a NO donor (SNG, S-nitrosoglutathione, 200 µM), overcame the osmotic stress-induced inhibition of WT seed germination slightly (close to 20 % of the seeds germinated compared to 0 % in the treatment with mannitol alone)	Piterkova et al. (2012)
Osmotic stress (15 % PEG*−6000, about −0.3 MPa for 5 days) (PEG = polyethytlene glycol)	*Triticum aestivum* (wheat), 1-week-old seedlings	Lateral root formation and growth were reduced under osmotic stress	The osmotic stress effect was less severe in the presence of a nitric oxide donor in a dose-dependent manner (20–50 µM SNP)	Wang et al. (2013)

(continued)

Table 4.1 (continued)

Stress	Plant	Morphology affected by stress	NO investigations	References
Drought stress (PEG 6000) for 5 days: (a) mild level (0.1 % PEG), (b) moderate level (0.5 % PEG), (c) severe level (1 % PEG)	5-d-old marigold seedlings with their original primary root removed as explants (the starting experimental materials)	Number of adventitious roots per explants dropped from 6.6 to 0.82 in the treatments with 0.1–1 % PEG	5 μM SNP + 0.1 % PEG… completely reversed the effect of drought stress in terms of root number and root fresh weight	Liao et al. (2012)
			Reduction of the endogenous NO level by a NO-specific scavenger or synthesis inhibitor under drought stress: more severe inhibition of root formation and reduction in root fresh weights	
Cold stress (4 °C)	*Camellia sinensis* (tea) pollen	Pollen germination and pollen tube growth (pollen tube length): Inhibited by cold stress	The effect of cold stress was partly reversible by a NO-specific scavenger or a NO synthesis inhibitor; an increase in NO generation under cold stress; a NO donor applied exogenously inhibited pollen germination and tube growth at 25 °C (mimicking the effect of cold stress)	Wang et al. (2012)
Heat stress at 48 °C for 18 h	*Zea mays* (maize seedlings, 2.5 days old)	Survival of seedlings: 40 % of the seedlings that were under heat stress regrew and turned green during recovery for a week at 26 °C in a growth room with 12-h photoperiod	Pretreatment with 150 mM SNP for 6 h increased the survival of seedlings (close to 80 %)	Li et al. (2013)

4.3 Germination and Survival

Tomato seed germination was prevented under osmotic stress which could be overcome to a significant extent by exogenous application of a NO donor (Piterkova et al. 2012). In another study, tea pollen germination and tube growth were inhibited by another abiotic stress, namely cold, which was counteracted partially by exogenous application of a NO-specific scavenger or NO synthesis inhibitor (Wang et al. 2012), suggesting that cold stress could induce endogenous NO production. This was substantiated by other experiments in the same study. It is likely that NO is an endogenous signal in the response of tomato seed and tea pollen to abiotic stress, although the nature of its involvement might be complex as it has been discussed in relation to plants under cadmium stress (Arasimowicz-Jelonek et al. 2011).

The link between survival of maize seedlings and NO was revealed when the vitality of the seedlings was compromized by a period of heat stress, and their ability to recover after the heat treatment was promoted significantly by pre-treatment with SNP (Li et al. 2013). Taken the findings of all the three studies here into consideration, it would seem that there was no overt sign of redistribution of resources for the development of other organs, while some growth processes were simultaneously inhibited under different abiotic stresses. However, survival and temporary suspension of growth processes under sub-optimal environmental conditions might be meritorious, and conservation of resources took priority over redirection of resources for new development under abiotic stress. It is worthwhile to pursue further the mechanisms for participation of NO in this survival strategy in different plant species under abiotic stress.

4.4 Specialized Morphological Features

Some plants or halophytes can tolerate and grow in high-salinity soils with the help of some specialized morphological features (Ding 2013). For example, *Limonium bicolor* has salt glands scattered on leaf surfaces to aid salt secretion out of the leaves to avoid adverse effects of accumulation of salts in the leaves. Under NaCl stress, expansion of the developing leaves of *L. bicolor* was inhibited, but exogenous application of SNP alleviated this effect (Ding 2013). Interestingly, the density of salt glands on the leaf surfaces was increased in response to high salinity although exogenous SNP did not affect this response. This is consistent with the notion of a redistribution of resources associated with typical SIMRs although the involvement of NO is not clear.

4.5 Morphological Response of Cotyledons Under Abiotic Stress

The morphological response of cotyledons under abiotic stress has not been studied extensively. Under Pb stress, the cotyledons of 1-week-old wild-type *A. thaliana* seedlings showed visible symptoms of injury and wilting of the edges

which were preventable with exogenous application of SNP (Phang et al. 2011). In response to different concentrations of $CuSO_4$ (0, 5, 25, 50 μM), the cotyledon area was promoted at 25 μM but was decreased at 50 μM (Peto et al. 2011). Furthermore, only in response to 5 μM $CuSO_4$, there was a threefold increase in NO-specific fluorescence over that of the control (no copper added to the growth medium), but there was no change in the cotyledon size compared to that of the control. There were no changes in endogenous NO levels, while there were morphological alterations in the cotyledons of the wild-type seedlings treated with higher concentrations of $CuSO_4$. Superficially, it is not clear if the apparent difference in the possible involvement of NO in the morphological response of the cotyledons was a response to the effects of different metals in excess. It would also seem that NO might play a minor role in the morphological response of cotyledons under abiotic stress. However, other parallel experiments studying auxin metabolism and transport in the wild-type, NO-overproducing, and NO-deficient *A. thaliana* seedlings in response to Cu^{2+} treatments revealed a more complex network of antagonistic relationships between NO levels and auxin homeostasis (Peto et al. 2011; Kolbert et al. 2012). This has added implications to the conceptual framework that there are cross talks among ROS, auxin, and ethylene, which have already been considered as the important signals in the SIMRs (Potters et al. 2009). This is perhaps not surprising given that during normal plant growth and development, for example, NO is known to interact with auxin in the regulation of lateral root and root hair formation (Correa-Aragunde et al. 2004) and with ABA in regulation of stomatal functions (Bright et al. 2006).

4.6 Conclusion

There is some evidence suggesting the involvement of NO in SIMRs. It seems necessary to consider the implications of cross talk among NO, auxin, possibly with other plant hormones and ROS in plants under abiotic stress. A better understanding of the nature of these complex interactions would shed some light on the precise nature of the participation of NO in the regulatory pathways of SIMRs.

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Part II
Nitric Oxide and Plant Adaptation to Abiotic Stresses

Chapter 5
Nitric Oxide and High Temperature Stress: A Physiological Perspective

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Abstract High temperature stress is one the devastating abiotic stresses, causing severe damage to each stage of plant growth and development. However, to cope with detrimental effects of heat stress plants are equipped with a system of defense orchestrated by a combination of several sensor proteins, heat shock factors (HSFs), heat-shock proteins (HSPs), antioxidant enzymes and signaling molecules. Precise assessment of rising temperature at cellular level is of vital importance to induce defense system of plant before the onset of heat damage. Plants sense heat stress primarily by plasma membrane-enclosed sensors which generate a heat stress response that induces a downstream signal leading to the expression of stress-responsive genes. Nitric oxide (NO), along with abscisic acid, H_2O_2, Ca^{2+}, and calmodulin (CaM), acts as an important signaling molecule in heat stress response in plants. Heat stress elevates synthesis of NO which in association with these molecules stimulate the DNA-binding activity of HSFs as well as the accumulation of HSPs leading to resistance to heat stress. Moreover, exogenous application of NO has also been shown to have an ameliorative effect on heat stress, which has been confirmed by several studies using NO scavengers and inhibitors. In the present chapter, the current understanding of the involvement of NO in heat stress and in the mechanism of thermotolerance is reviewed.

Keywords Heat-shock proteins · Heat stress · Nitric oxide · Thermotolerance

5.1 Introduction

Extreme exploitation of natural resources and uncontrolled anthropogenic activities are the major causes behind the global climate change, one of the discussed topics among the scientific community, which is predicted to warm the earth's

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climate with an average of 2–4 °C by the end of the twenty first century (IPCC 2007). It has been estimated that the annual mean maximum and minimum temperatures have increased by 0.35 and 1.13 °C, respectively, for the period 1979–2003 (Peng et al. 2004). Plants are integral part of our ecosystem and being sessile in nature, they have no choice to escape of high temperature stress; thus in order to survive, plants must have to combat these abiotic stresses by altering their physiological and biochemical metabolism.

High temperature or heat stress (HS) is a condition when the temperature is high enough to damage plant tissues and substantially influence growth and metabolism of plants (Balla et al. 2009). Plant species possess an inherent threshold of thermosensitivity to perform optimally only under certain temperature range; therefore, an increase in temperature beyond the particular threshold can adversely affect the plants at morphological, physiological, biochemical as well as at the molecular level. HS damages plant tissues (Pareek et al. 1997) and causes burning of leaves, branches and stems, leaf senescence and abscission, shoot and root growth inhibition, abortion of buds, fruit discoloration and damage, and reduced yield (Guilioni et al. 1997; Ismail and Hall 1999; Vollenweider and Gunthardt-Goerg 2005). Excessive generation of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) (Mittler 2002; Yin et al. 2008) is one of the detrimental effects of HS. Overproduction of ROS induces oxidative stress and causes peroxidation of membrane lipids, denaturation of proteins, damage to nucleic acids, chlorophyll, leakage of electrolytes and ultimately cell death (Scandalios 1993; Mittler 2002) which reflects in the form of losses in biomass production and crop yield. HS causes alterations in the structural organization of thylakoids (Karim et al. 1997), swelling of stromal lamellae, clumping of vacuolar contents, disruption of cristae and draining of mitochondrial contents which collectively culminate in the depletion of photosystem II in the antenna and ultimately reduced photosynthesis and respiration (Zhang et al. 2005).

Simple structure, small dimensions, and high diffusivity and reactivity make NO a key signaling molecule (Arasimowicz and Floryszak-Wieczorek 2007), which directly or indirectly interacts with other signaling molecules such as H_2O_2, salicylic acid, and cytosolic Ca^{2+} (Neill et al. 2003; Lamotte et al. 2004; Wendehenne et al. 2006). NO plays important roles in mediating responses to abiotic stresses such as salinity, drought, heavy metal, UV radiations, water logging, and low and high temperatures. Exogenous NO enhances tolerance against various abiotic stresses by inducing antioxidant enzymes, osmoprotectant accumulation, and water content and by lowering the levels of lipid peroxidation and ROS (Corpas et al. 2011; Siddiqui et al. 2011; Khan et al. 2012).

This chapter is an attempt to unravel the role of NO in plants growing under high temperature stress. The chapter also presents an overview of physiological and biochemical aspects of NO-mediated responses to heat stress.

5.2 Effect of High Temperature Stress on Plants

An abrupt increase in temperature may cause denaturation of membrane proteins and fatty acids, leading to increased electrolyte leakage and ultimately loss of cellular functions. High temperature stress is one of the abiotic stresses that affect most of the crops during some stages of their life cycle from seed germination to harvest.

5.2.1 Germination

The successful completion of a plant's life cycle begins with the germination and establishment of the seed, thus, any alteration in the process of germination will directly affect plant growth and development. Temperature has significant influence on germination, and high temperature significantly affects germination and grain formation. Effect of temperature on seed germination was observed as early as in 1933 by Livingston and Haasis, they observed that 90 % germination at 25 °C requires incubation for 6 days, while at 31–36 °C requires 2-day incubation. High temperature adversely affects development of embryo, seed germination, and vigor leading to reduced emergence and seedling establishment (Barnabás et al. 2008; Ren et al. 2009). Extended exposure of high temperature during seed germination may slow down or totally inhibit germination (Wahid et al. 2007). High temperature lowers water content and induces excessive accumulation of malondialdehyde and conjugated dienes in coleoptile which results in reduced seedling growth and development (Mahan and Mauget 2005; Savicka and Škute 2012). Temperate cereal crops are particularly affected by HS during flowering and grain filling and exhibit a decline in grain weight, grain number, starch, protein, and total oil yield in various crops (Banowetz et al. 1999; Mahmood et al. 2010). Moreover, seed reserve provides essential nutrition during germination, but proper nutrition needs mobilization and utilization of seed reserve which has been shown to decrease under HS leading to poor seed germination (Essamine et al. 2010; Blum and Sinmena 1994).

5.2.2 Morphology

The symptoms of HS are primarily reflected by the alterations in morphological features of plant such as scorching of leaves, twigs, branches and stems, leaf senescence and abscission, inhibition of shoot and root growth, damage to fruit, and discoloration (Rodríguezet al. 2005). As leaves are the most delicate organs of the plants, thus, HS causes significant damage to leaves which appear in the form of rolling and drying of leaves, damaged leaf-tip and margins, and necrosis (Omaeet al. 2012). Phenology determines the performance of crop plants under

particular climatic conditions. High temperatures may alter the total phenological duration and results in shorter grain filling periods and negatively affect yield components of cereals (Zhang et al. 2006; Nahar et al. 2010). High temperature reduces total growth duration by reducing the germination period, days to anthesis booting and maturity (Yamamoto et al. 2008). Loss of water is one of the adverse effects of high temperature stress which reduces water-use efficiency and ultimately growth of crop plants; therefore, adaptation of plants to high temperature is a function of the interaction of phenology with the pattern of water use. Hence, under reduced availability of water, maximization of water-use efficiency will be of key importance for the endurance of crops under elevated temperatures (Vadez et al. 2012). Different techniques of increasing water-use efficiency have been proposed (see Siddique et al. 2001; Davies and Hartung 2004).

5.2.3 Flowering

Flowering is considered as the most susceptible stage to high temperature stress, which causes abnormalities in male and female reproductive organs and ultimately reduces crop yield. Exposure of plants to high temperature at pre-anthesis stage suppresses growth of ovaries, water-soluble carbohydrate leading to decreases in grain number and weight (Wardlaw and Wrigley 1994; Calderini et al. 1999; Talukder et al. 2013, 2014). A variation in the response of reproductive organs to high temperature has been observed, male gametophyte is reported to be more susceptible to heat stress than pistil and female gametophyte which are considered to be more tolerant (Hedhly 2011), this is the reason that a sudden decline in yield with temperature is mainly associated with pollen infertility (Young et al. 2004; Zinn et al. 2010). High temperature stress induces male sterility seems to be associated with various changes in anther tissues such as tapetum, epidermis, endothecium and stomium (Peet et al. 1998; Sato et al. 2000, 2002), arrested cell proliferation, inflated vacuoles, altered development of chloroplast and mitochondrial abnormalities (Sakata et al. 2010). It is well established that sugars play significant role in pollen viability; however, heat stress has been shown to reduce the concentration of soluble sugars in the anther walls, developing pollen grains, and in the locular fluid which result in decreased sugar concentration in the mature pollen grains and decreased pollen viability (Ismail and Hall 1999).

5.2.4 Photosynthesis

Photosynthesis is not only considered as one of the vital processes in plant system but also the main source of ROS generation in plants. It is considered as the most sensitive physiological process to HS and damage to photosynthesis and respiration are prominent effects of heat stress. Among the components of photosynthetic apparatus, photosystem II (PSII) has been described as the most

thermolabile (Berry and Björkman 1980; Schrader et al. 2004). High temperature impairs photosynthesis by adversely affecting the light harvesting capacity, PSII- and PSI-mediated electron transfer, and Calvin cycle activity (Stasik and Jones 2007). Moreover, moderate heat stress stimulates reduction of plastoquinone, cyclic electron flow and Rubisco activity (Sharkey 2005).

Heat stress causes reduction in photosynthetic pigments, particularly chlorophyll, which may lead to the impairment in electron transport and hence reduced photosynthetic capacity (Ashraf and Harris 2013). Heat stress-induced reduction in chlorophyll content may occur due to impairment in pigment biosynthetic pathways and/or pigment degradation. However, impaired chlorophyll biosynthesis has been suggested as the first of the processes in plastids affected by high temperature stress. High temperature reduces chlorophyll biosynthesis by damaging various enzymes involved in the biosynthetic pathway of chlorophyll (Dutta et al. 2009; Reda and Mandoura 2011). Heat stress inhibits 5-aminolevulinate dehydratase, the first enzyme of pyrrole biosynthetic pathway, 5-aminolevulinic acid (ALA), protochlorophyllide, orphobilinogen deaminase, and protochlorophyllide oxidoreductase (Tewari and Tripathy 1998, 1999; Mohanty et al. 2006).

5.3 Source of NO in Plants

In mammals, NO is formed exclusively by the enzyme nitric oxide synthase (NOS). NOS 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 (Cueto et al. 1996; Alderton et al. 2001; Butt et al. 2003; Mohanty et al. 2006). Growing body of evidences suggest the existence of NOS-like enzyme in plants that can be inhibited by NOS inhibitors. The occurrence of NOS activity was reported in pea peroxisomes (Barroso et al. 1999; del Río et al. 2002), roots, stems and leaves of pea seedlings (Corpas et al. 2006). An Arabidopsis protein AtNOS1 with NOS activity was identified which produced NO in response to hormonal signals; however, AtNOS1 has NOS activity dependent on NADPH, Ca^{2+}, and CaM, but not on FAD, FMN or BH4 (Guo et al. 2003). Conversely, these studies were challenged when Zemojtel et al. (2006) and Crawford et al. (2006) were unable to reproduce earlier results. Moreover, no protein or gene has been identified with any sequence similarity to the complete animal NOS.

Besides NOS, there are several other enzymes in plants which are involved in NO synthesis. These enzymes include nitrate reductase (NR), nitrite reductase (NiR), xanthine oxidoreductase, mitochondrial cytochrome c oxidase and horseradish peroxidase. Of these, NR is considered as the core enzymes for NO synthesis in plants with rigorously confirmed NO-producing activity both in vivo and in vitro (Kaiser et al. 2002). NR is a molybdenum cofactor-containing (Moco) enzyme (Yamasaki et al. 1999; Lamattina et al. 2003) with the capacity of NADPH-dependent reduction of nitrite (NO_2^-) to NO (Kaiser et al. 2002). NR-dependent NO production has been recorded in cucumber (de la Haba et al. 2001), maize (Rockel et al. 2002), Arabidopsis (Desikan et al. 2002), and tobacco (Planchet et al. 2005).

Synthesis of NO by NiR is another way by which NiR reduces NO_2^- to NO using reduced cytochrome c as an electron donor. NiR-dependent NO production has been observed in *Helianthus annuus* (Rockel et al. 2002), *Glycine max* (Delledonne et al. 1998), and *Chlamydomonas reinhardtii* (Sakihama et al. 2002). Another possible NO_2^-NO oxidoreductase (Ni-NOR) has been identified in the root plasma membrane of the tobacco. Ni-NOR reduces NO_2^- to NO using reduced cytochrome c as an electron donor (Stöhr and Stremlau 2006).

Xanthine oxidoreductase (XOR), a Moco-containing enzyme, has been also suggested to participate in NADH-dependent reduction of NO_2^- to NO (Harrison 2002). XOR has been reported in pea leaf peroxisomes and exists in two interconvertible forms: the superoxide-producing xanthine oxidase and xanthine dehydrogenase (Palmaet al. 2002).

Horseradish peroxidase is another enzyme involved in the generation of NO from N-hydroxyarginine (NOHA) and H_2O_2 (Huang et al. 2002; Veitch 2004). Other enzymatic sources of NO include cytochrome P450 (Boucher et al. 1992b; Mansuy and Boucher 2002), hemoglobin, and catalase which have been shown to generate NO by catalyzing the oxidation of NOHA (Boucher et al. 1992a).

Apart from enzymatic sources, plants also synthesize NO using non-enzymatic sources. Nagase et al. (1997), reported in vitro generation 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. Acidic conditions have been shown to favor non-enzymatic synthesis of NO in plants. Yamasaki (2000) proposed such a mechanism of NO synthesis from NO_2^- under acidic conditions (Eq. 5.1).

$$2NO_2^- + 2H^+ \leftrightarrow 2HNO_2 \leftrightarrow NO + NO_2 + H_2O \leftrightarrow 2NO + \frac{1}{2}2O_2 + H_2O \quad (5.1)$$

Other reactions of NO synthesis under acidic conditions include dismutation of NO_2^- to NO and nitrate (Stöhr and Ullrich 2002), and reduction of NO_2^- to NO and dehydroascorbic acid by ascorbic acid (Henry et al. 1997). Carotenoids and polyamines also contribute to non-enzymatic synthesis of NO (Cooney et al. 1994; Tun et al. 2006).

Although existence of NOS in plants is mysterious, plants are able to synthesize NO by several other enzymatic and non-enzymatic ways. But how these mechanisms of NO synthesis orchestrate the complicated NO signaling network needs to be unraveled, and how this network is operated in response to environmental stimuli will be of considerable interest in plants.

5.4 Heat Stress and NO Synthesis in Plants

Properties of NO such as simple structure, small dimensions, and high diffusivity have been well established NO as an important endogenous signaling molecule in plant system that mediates resistance responses to several stimuli of biotic and abiotic stresses. Heat stress induces increase in endogenous NO level, which

plays a protective role against high temperature stress (Xuan et al. 2010). Song et al. (2006) observed that in calli of stress-sensitive and stress-tolerant ecotypes of reed, high temperature induced endogenous generation of NO in the tolerant ecotype, while in the sensitive ecotype, it was not observed. NO enhanced more stress tolerance in sensitive than tolerant ecotype, and depletion of endogenous NO by its scavenger 2–4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) reduced survival in the tolerant ecotype. An increase in NO production was observed in heat-stressed alfalfa (Leshem 2001) and Arabidopsis (Xuan et al. 2010). Similar results were reported by Karpets et al. (2015), they observed that application of NO to the plants under heat stress induces endogenous levels of NO and increases resistance to heat stress, whereas application of NO scavengers prevents development of heat resistance. On the contrary, the work of Beard et al. (2012) was not in agreement with these findings, as they reported inhibition of NO accumulation in cultured guard cell protoplasts subjected to heat stress.

The relation between NO formation and stress resistance is not well explored and a significant variation in studies is observed in NO-mediated thermotolerance. NO-mediated thermotolerance involves abscisic acid (ABA) and ABA has been reported to induce H_2O_2-dependent generation of NO (Song et al. 2008; Neill et al. 2008), whereas ABA and H_2O_2 mediated increase in MAPK and antioxidant gene expression are dependent on endogenous NO generation. A transient increase in NO, ABA, and H_2O_2 contents were recorded in the plants subjected to heat stress (Song et al. 2008; Karpets et al. 2015) and exogenous application of H_2O_2 has also been shown to increase NO levels and vice versa (Neill et al. 2008; Karpets et al. 2015).

5.5 NO and Thermotolerance

As discussed in the preceding pages, high temperature stress adversely affects plants at almost all levels of their life cycle starting from seed germination to growth, flowering, fruiting, and harvesting. High temperature also causes excessive loss of water and set the plants under water deficit conditions. Therefore, to combat high temperature stress, plants must possess the heat stress tolerance mechanism which could maintain the high relative water content and manage the normal cellular functioning.

Thermotolerance is the ability of plants to resist severe temperatures. Plants have evolved various strategies of thermotolerance at morphological, physiological as well as at molecular level. Such strategies include ion transporters, osmoprotectants, antioxidants, late embryogenesis, abundant proteins, and factors involved in signaling cascades and transcriptional control which are of crucial importance to counteract the detrimental effect of heat stress. However, assessment of stress stimulus by plants is of prime importance to activate various defense systems against heat stress. The alterations caused by the heat stress serve as a signal for the induction of various defense mechanisms within the plants. Of these, changing

in membrane fluidity plays a central role in sensing and influencing gene expression which facilitate to reestablish homeostasis and to protect and repair damaged proteins and membranes (Vinocur and Altman 2005).

Ameliorating effect of NO on several abiotic stresses has been well established, and heat stress is not untouched with the same. NO possesses the ability to protect plants against heat stress either by acting directly as an antioxidant and scavenging ROS or acts as a signal molecule in inducing thermotolerance by up-regulating the expression of heat-responsive genes. Exogenous application of NO induces heat tolerance in several plants through inducing antioxidant enzymes and by reducing peroxidation of membrane lipids, ion leakage, and levels of ROS. Ameliorating effect of NO on high temperatures induced damage was recorded in *Oryza sativa* (Uchida et al. 2002), tobacco (Gould et al. 2003), Arabidopsis (Xuan et al. 2010; Wang et al. 2014), *Festuca arundinacea* (Chen et al. 2013), and wheat (Karpets et al. 2015). Application of NO to heat-treated wheat seedlings reduces lipid peroxidation, H_2O_2 content and increases the content of chlorophyll and activities of enzymatic as well as non-enzymatic antioxidant. Both exogenous as well as endogenous NO alleviates heat stress, but in both the cases, they adopt almost same strategy of defense, i.e., modulation of ROS accumulation, expression of genes encoding for antioxidant enzymes, and accumulation of osmolytes leading to membrane thermostability and cell viability.

Heat-shock proteins (HSPs) are of vital importance in the protection of plants against high temperature stress, and NO has been shown to induce the expression of genes encoding HSPs. NO protects chloroplast against oxidative damage under heat stress by inducing expression of genes encoding small HSP26 (Uchida et al. 2002). Xuan et al. (2010) observed the evidence for the involvement of NO in thermotolerance, and they observed that NO, through stimulating DNA-binding activity of heat shock transcription factors (HSFs) and the accumulation of HSP18.2, contributed positively to thermotolerance in Arabidopsis. It has been shown that NO together with ROS takes part in the regulation of production and accumulation of HSP70 under heat stress (Piterkova et al. 2013). NO stimulates DNA-binding activity of HSFs and the accumulation of HSPs through H_2O_2, and H_2O_2 acts upstream of NO in thermotolerance, which requires increased DNA-binding activity of HSF and HSP accumulation (Wang et al. 2014). Therefore, a cross talk between H_2O_2 and NO exists which stimulate the DNA-binding activity of HSFs as well as the accumulation of HSPs to confer thermotolerance in plants (Fig. 5.1).

5.6 NO Signaling: Heat Perception and Mechanism of Thermotolerance

Plants adopt various strategies of defense for survival under the conditions of high temperature stress, but precise assessment of heat stress by plants is imperative for the timely execution of defense system of plants prior to the commencement of heat damage. Significant number of evidences suggest that a stimulus of rising

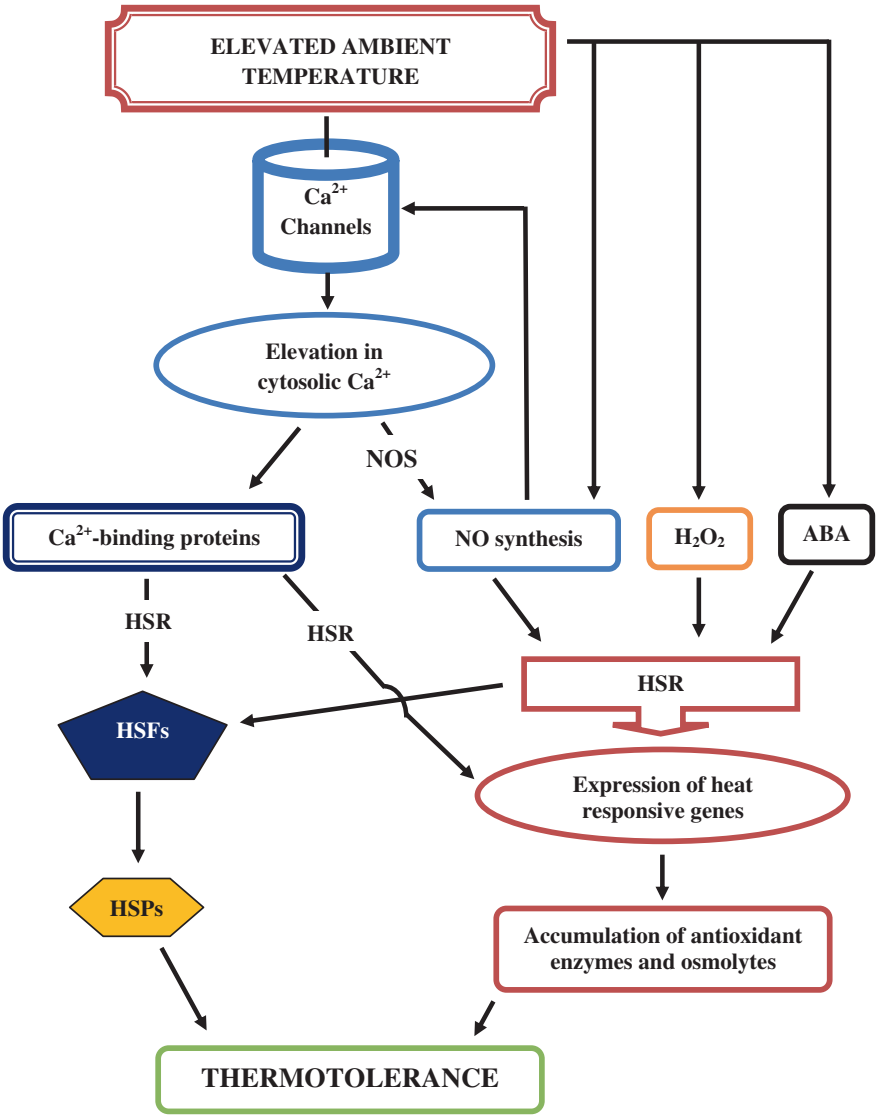

Fig. 5.1 Elevated ambient temperature alters the fluidity of membrane which causes influx of Ca^{2+} through Ca^{2+} channels, as a result cytosolic Ca^{2+} concentration increases. Elevated cytosolic Ca^{2+} serves as HSR which is sensed by CaBPs that stimulate DNA-binding activity of HSFs and expression of antioxidant and osmolyte encoding genes leading to accumulation of HSPs, antioxidant enzymes, and osmolytes, respectively. All these together contribute to enhanced thermotolerance. Elevated temperature induces NO generation directly or through cytosolic Ca^{2+}-mediated activation of NOS-like activity, and elevated level of NO in turn mobilizes Ca^{2+} release via targeting Ca^{2+} channels. Increase in ambient temperature also induces H_2O_2 and ABA levels which along with NO trigger HSFs and other heat-responsive genes leading to thermotolerance

ambient temperature is sensed by membrane-enclosed sensory devices of plants and are transduced into appropriate gene expression (Vígh et al. 1998; Los and Murata 2004) leading to the activation of a series of protective reactions. In order to control the defense network, plants should possess a system which could regulate the signaling network as per the requirement.

Being sessile organism, plants are forced to face the damaging effects of heat stress, which adversely affect almost all aspects of plant life. In order to survive under heat stress, plants should execute some strategies other than deployed under normal environmental conditions. Any change in ambient temperature is sensed by plants with a complicated set of sensors positioned in various cellular compartments. High temperature increases fluidity of the membrane which leads to activation of lipid-based signaling cascades and to an increased Ca^{2+} influx through membrane-associated cyclic nucleotide-gated Ca^{2+} channels (Saidi et al. 2009) which results in the increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in response to heat stress. It has been shown that NO serves as Ca^{2+} mobilizing agent and causes elevation in $[Ca^{2+}]_{cyt}$ in plant cells exposed to biotic and abiotic stresses. Moreover, several studies suggested the requirement of Ca^{2+} during NO synthesis in plants (Corpas et al. 2004, 2006; del Río et al. 2004). Elevated levels of $[Ca^{2+}]_{cyt}$ trigger an optimal heat shock response (HSR) ,which initiate downstream events leading to changes in gene expression and plant tolerance to heat stress. Signaling between these routes leads to the production of osmolytes and antioxidants in response to heat stress (Fig. 5.1).

Plants possess highly conserved HSR to a gradual or a sharp rise of temperature, above the physiological growth range (Wahid et al. 2007; Mittler et al. 2012). The HSR involves multiple pathways, cellular compartments, and up-regulation of hundreds of specific genes and HSPs which induce thermotolerance in plants. Sensing of heat stress by membrane sensors causes activation of HSFs that is capable of binding to promoter regions of HSP genes known as heat shock elements (HSEs) and exhibits transcriptional activity (Charng et al. 2007). Plants use different routes for sensing heat stress, which include various putative sensors such as plasma membrane channels, nuclear histone sensors, and unfolded protein sensors in the endoplasmic reticulum and cytosol (Mittler et al. 2012). Of these, plasma membrane has been considered as the most sensitive and earliest responder to mild increase in temperature (Horváth et al. 1998). Recently, in the moss *Physcomitrella patens*, it has been shown that specific calcium channels in plasma membrane regulate the onset of the acquired thermotolerance in the plant by controlling the intensity of the HSR via a preceding Ca^{2+} influx (Saidi et al. 2009). Later on, it was demonstrated that that membrane lipid composition regulates the calcium-dependent heat-signaling pathway and temperature differences, but not absolute temperature, determines the extent of the plant HSR.

The NO plays a significant role of a signaling molecule in the stress responses in plants. Several reports suggest the central role of NO in mediation of stress responses under high temperature stress, since endogenous NO levels have been shown to increase within half an hour of heat shock (Gould et al. 2003; Xuan et al. 2010). Arabidopsis mutant *noa1* impaired in NO synthesis exhibited reduced NO

levels and impaired thermotolerance as compared to the wild type (Xuan et al. 2010). Moreover, expression of AtHsp18.2 following temperature elevation is less pronounced in mutants impaired in NO synthesis; however, pretreatment of *noa1* mutant with NO restored temperature-induced AtHsp18.2 expression and induced thermotolerance. NO-induced thermotolerance has been shown to be mediated by ABA, H_2O_2 and HSFs, and HSPs.

It has been well established that Ca^{2+} acts as second messenger and was found to be involved in regulation of several responses of plants to stresses. Clmodulin (CaM), a calcium-binding protein, is believed to play a significant role as a sensor protein in plants and has been shown to be up-regulated by heat stress (Gong et al. 1997a, 1997b) and involved in the accumulation of HSPs (Liu et al. 2003; Li et al. 2004). Currently, AtCaM3 has emerged as a key factor in heat shock signal transduction, which signifies a relationship between NO and Ca^{2+}-CaM under heat stress (Sang et al. 2008; Xuan et al. 2010), because NO has been shown to function as signal molecule acting upstream of AtCaM3, stimulating DNA-binding activity of HSFs as well as the accumulation of HSPs (Zhao et al. 2010).Thus, it can be concluded that NO together with CaM plays significant role in the protection of plants against heat stress (Fig. 5.1).

Exposure of plants to heat stress causes excessive generation of ROS, of these, H_2O_2 along with NO function in heat shock signaling pathway in plants. Heat shock treatment has been shown to enhance the endogenous levels of NO and H_2O_2, however, NO specific scavenger cPTIO suppressed the HS-induced H_2O_2 and NO generation (Xu et al. 2008). These findings were further confirmed when exogenous application of NO to heat-shocked plants enhanced the concentration of H_2O_2 and vice versa. Electrophoretic mobility shift assays, Western blotting, and real-time reverse transcription polymerase chain reaction proved that NO stimulates the DNA-binding activity of HSFs and the accumulation of HSPs through H_2O_2 and Ca^{2+} channels leading to thermotolerance (Wang et al. 2014; Jia et al. (2014). Moreover, H_2O_2 application has been shown to trigger an increase in intracellular free Ca^{2+} (Rentel and Knight 2004). Karpets et al. (2015) reported an increase in NO and H_2O_2 content within two hours and half hour, respectively, after heating of wheat seedlings, while this effect was neutralized when seedlings were treated with the NO scavenger cPTIO and the inhibitor of NO synthase L-NAME (N^G-nitro-L-arginine methyl ester).

5.7 Conclusion

Endurance of plants under heat stress depends on the activation of plants' defense system, orchestrated by a network of sensors, genes, proteins, and enzymes. Accurate assessment of the rising ambient temperature by the plant cell is of vital importance for the timely activation of defense system and the establishment of an optimal thermotolerance. The rise in ambient temperature is sensed by a set of plasma membrane sensors which trigger a heat shock response that causes a

signaling cascade leading to the expression of several heat-responsive genes leading to the activation of antioxidant defense system, and accumulation of osmolytes and HSPs which collectively results in the enhanced tolerance of plants to heat stress. NO functions as a signaling molecule in response to heat stress, and a cross talk exists among NO, H_2O_2, and Ca^{2+} which stimulates the DNA-binding activity of HSFs and the accumulation of HSPs and activation of antioxidant enzymes leading to thermotolerance. Although the existence of protective mechanisms against heat stress are repeatedly suggested but the pathway of defense mechanism against high temperature stress needs further investigation so that heat stress-induced temporal and spatial alterations at cellar, subcellular and molecular level could be explored more precisely.

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Chapter 6
Nitric Oxide in Drought Stress Signalling and Tolerance in Plants

Shivam Sidana, Jayakumar Bose, Lana Shabala and Sergey Shabala

Abstract The limited availability of water for agricultural usage and recurrent droughts resulting from the changes in the climatic patterns has significantly affected agriculture worldwide. In the light of the current global scenario, understanding physiological and molecular mechanisms adapted by plants to combat water deficit becomes essential to minimize multibillion dollar losses to the industry. The knowledge acquired would be further employed to enhance the tolerance of the agricultural crops to drought by adopting gene-manipulating techniques. This present chapter highlights the physiological and molecular basis of plant adaptive response to drought regulated by the nitric oxide (NO). Being a bioactive molecule, NO is involved in many physiological processes in animals and has also been known as a mediator of biotic and abiotic stress responses in plants. In recent years, various enzymatic and non-enzymatic pathways for its synthesis have been elucidated. Several important advancements have been made to reveal the roles NO plays in plant growth and development, and the role of NO as a signal molecule in activating ROS scavenging enzymes under abiotic stresses including drought has been established. In this review, we discuss how NO aids to the regulation of the antioxidative systems, stomatal closure and adventitious root formation under drought conditions. Apart from direct regulatory role at the physiological level, NO also plays an important role in plant acclimation to water deficit by activating stress defence genes via post-translational modifications.

Keywords Abscisic acid · Late embryogenesis abundant proteins · Nitrosative stress · Oxidative stress · Stomatal regulation · Water deficit

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6.1 Introduction

Drought is defined as a "condition of moisture deficit sufficient to have an adverse effect on vegetation, animals and man over a sizeable area" (White and Brinkmann 1975). Agricultural drought occurs when the available water is unable to support average crop production which could be due to low precipitation, dry and hot condition or when soil and agricultural practices require more water than is available from usual precipitation. The water requirement of plants is related to weather conditions, species of the plant, the growth stage, and physical properties of the soil. The meteorological drought of precipitation shortage leads to agricultural drought conditions due to water deficit in soil and decrease in groundwater or reservoir levels.

Recent trends in climate change have increased the frequency and severity of drought stress to become the largest environmental global concern. African continental countries have faced drought almost every year for the past 12 years (Zwaagstra et al. 2010). Multiple drought events in Australia between 2002 and 2010 also had a negative impact on the economy by reducing the household income of the farmers by 10 % (Alston and Kent 2004). The devastating drought of 2012 in south-western China and USA affected wheat, corn, soya bean and livestock producing areas leading to a surge in commodity prices (Henderson 2012).

Drought poses a detrimental effect on physiological and biochemical processes in the plants due to the reduction in availability of soil moisture (Jaleel et al. 2007). Decrease in the water potential, stomatal closure due to turgor loss in leaves (Yordanov et al. 2000) and decrease in cell growth (Wu et al. 2008) are a few negative effects of drought stress. Severe water stress causes inhibition of photosynthesis (Flexas and Medrano 2002), via multiple mechanisms. One of them is a decrease in the leaf area (Zhang et al. 2004). Adverse effects of drought on respiration, ion uptake, nutrient translocation and metabolism have also been reported (Farooq et al. 2008; Heuer and Nadler 1995; Sankar et al. 2007; Wu et al. 2008; Specht et al. 2001).

Plants grown under water-deficit conditions have evolved various mechanisms to reduce the stress by drought which will be discussed in detail in the next section. Some of the vital biomolecular adaptations are regulation of stomatal closure, counteracting the oxidative stress and nitrosative stress arising in water-deficit conditions all of which involve nitric oxide (NO). NO is a free radical that has been researched extensively in the recent years. Acting as a signalling molecule, NO was shown to be involved in various cell processes such as growth, respiration, maturation and senescence (Graziano and Lamattina 2005) as well as in signalling of abiotic and biotic stresses (Arasimowicz and Floryszak-Wieczorek 2007).

The knowledge of pathways mediated by NO under water stress can be used to improve crop production under drought conditions.

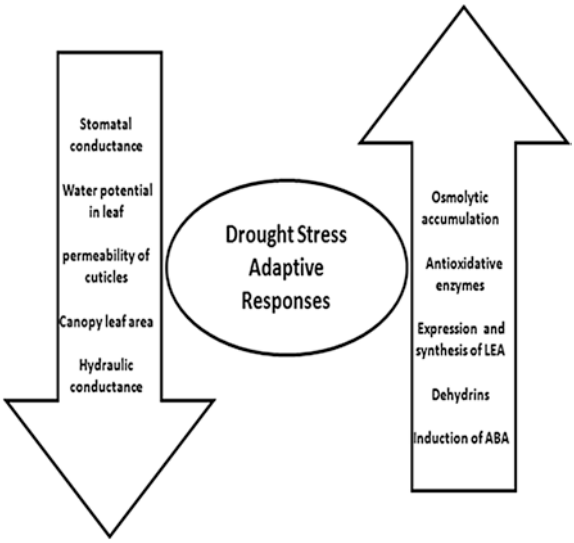

Fig. 6.1 Drought tolerant mechanisms adopted by plants at physiological and molecular level

6.2 Mechanisms of Adaptation to Drought

Plants employ adaptive features to endure water deficit caused by drought at whole plant and cellular level (Fig. 6.1). They are generally categorized into (1) drought escape mechanism and (2) drought tolerance mechanism (Bartels 2005). Under the first strategy, native plants of arid regions show high developmental plasticity with reproduction being set before severe stress resulting in completion of their life cycle before physiological water deficit occurs (Mooney et al. 1987). Other plants such as cereals use the second strategy and store reserves during stress, which are mobilized during fruit production (Yang et al. 2000). The drought tolerance leads to a number of anatomical modifications in plants such as development of extensive root systems to increase water uptake, reduction of foliar area, changes in the cuticle permeability, developing of the sunken stomata, rolling of leaves to limit evaporation, and developing spongy tissues acting as water reservoirs. The presence of leaf pubescence and epicuticular wax increases the reflectance of radiation from the leaf surface decreasing the leaf temperature and transpiration loss. The loss of water is also controlled by restricting the water loss through shrunken stomata due to transpiration (Ehleringer and Cooper 1992). The stomatal closure as well as lowering of the mesophyllic conductance of CO_2 hampers the process of photosynthesis by deactivating the Rubisco (Bohnert and Sheveleva 1998). NO has been also shown to be involved in regulation of stomata operation by increase of the cyclic guanosine monophosphate (cGMP) level which acts as the second messenger activating the plant defence genes. The defence genes are also activated via the cyclic adenine dinucleotide phosphate ribose (cADPR), another second messenger of NO.

Generation of reactive oxygen species (ROS) is one of the consequences of drought stress. The decrease in photosynthetic rate under drought stress has been

observed to divert the electron flow towards the generation of ROS leading to oxidative burst (Loggini et al. 1999). ROS impose their detrimental effect through lipid peroxidation, damaging the photosynthetic products. Plants have evolved efficient enzymatic and non-enzymatic antioxidant systems to handle the resultant oxidative burst. The non-enzymatic antioxidants include vitamin C, E, glutathione, flavonoids, polyamines, and carotenoids, and enzymatic scavenging pathways against ROS are executed by catalases,superoxide dismutase (SOD), peroxidase and metallothionein which have been discussed in later sections (Seki et al. 2001).

One of the strategies employed by plants to counteract water deficit is through osmotic adjustments. For osmotic adjustment, plants need to synthesize and/or accumulate compatible solutes that are low molecular weight organic osmolytes which are neutral at physiological pH. These osmolytes have been categorized under four major classes: (1) sugars (trehalose), (2) polyols (glycerol, inositols, mannitol, sorbitol), (3) amino acids (proline, glycine betaine), (4) quaternary ammonium compounds (β-alanine betaine, choline O-sulphate,proline betaine and hydroxyproline betaine) (Rontein et al. 2002). The overexpression of activator of ABA-dependent regulatory systems—Myb4—was found to accumulate compatible solutes such as glucose, sucrose, proline, and glycine betaine in high levels under drought stress (Mattana et al. 2005).

The above-mentioned osmolytes do not interfere with normal biochemical reactions and execute their highly specific protective mechanism by maintaining the membrane structures and integrity of the enzymes under water deficit. For example, mannitol enhances tolerance to water-deficit stress primarily through osmotic adjustment and scavenging of OH^-, and thus protecting ferredoxin and glutathione from the effects of hydroxyl radicals. A considerable increase in water stress tolerance was observed on introduction of a mannitol dehydrogenase (mtlD) gene in wheat (Abebe et al. 2003). Another osmolyte, trehalose, is present as a reserve carbohydrate in plants that stabilizes membranes from denaturation. The transgenic plants expressing trehalose genes conferred drought stress tolerance (Avonce et al. 2004). Being an osmolyte itself, proline increases the concentration of other osmolytes and detoxifies ROS. The response of proline to water deficits has been demonstrated in transgenic tobacco overexpressing proline genes (Kavi et al. 1995). Transgenic plants with pyrroline-5-carboxylate reductase (P5CS) gene, which interconverts glutamate, ornithine and proline, in the antisense direction leading to suppression of proline synthesis, demonstrated sensitivity to water deficit (de Ronde et al. 2000).

Plants confer biochemical drought adaptation through the accumulation of heat-shock proteins (HSP) also known as chaperones which are involved in the folding of proteins (Campalans et al. 2001). They prevent the denaturation of proteins and facilitate the recovery of the functions after abiotic stress. The oxidative stress and nitrosative stress mediated by NO by oxidized glutathione stimulate the induction of HSP 70 which protects cells from apoptosis (Mosser et al. 1997). One of the chaperon-cyclophilin overexpressed during the drought stress confers resistance to multiple abiotic stresses responding in a pattern similar to the application of ABA (Sekhar et al. 2010).

6.3 Regulation of Genes Under Drought

The adaptability of plants to water deficit at the cellular and physiological level is implemented by the induction or repression of various genes. The genetic profiling of Arabidopsis revealed the involvement of dehydration responsive element/C-repeat (DRE/CRT) in the ABA-independent regulatory systems operational during abiotic stress. The overexpression of protein DREB1/CBF which binds to DRE/CRT changes the expression of stress-inducible genes leading to enhancement in drought tolerance (Seki et al. 2001). The modulation of expression of the stress-inducible genes by NO has been also observed (Courtois et al. 2008). NO was shown to act upstream of ABA-dependent SnRK2-induced pathways combating osmotic stress in Arabidopsis by up-regulating stress-inducible genes with the help of transcription factors—DREB1A/CBF3 involved in abscisic acid-independent and abscisic acid-dependent stress-response pathways.

Microarray and RNA blot analysis have identified the genes in the transgenic plants overexpressing CBF3 and ABF3 (Oh et al. 2005). Transgenic maize plants expressing transcription factor (NF-YB class CCAAT-binding transcription factor) from Arabidopsis had enhanced tolerance to drought in the field. The transcriptional activators of ABA signalling such as auxin-binding factors (ABF3/ABF4) altered expression of ABA or stress-responsive genes such as rd29B and rab18 and led to drought tolerance (Kang et al. 2002). The expression of ABA synthesis genes (zeaxanthin epoxidase (ZEP), aldehyde oxidase (AAO3) and sulfurase with molybdenum cofactor (MCSU)) was shown to be up-regulated by drought (Zhu et al. 2002). The generation of ABA under drought stress causes stomatal closure and expression of stress-inducible genes in plants. The regulation of stomatal movements was observed to be carried out by two transcription factors expressed in guard cell—AtMYB60 and AtMYB61 whose expression is negatively modulated during drought (Zhu et al. 2002). The engineering of stomatal activity through these factors would help plants to survive water-deficit conditions.

The first genes identified in seed development expressed at the stage of maturation and desiccation phases are called LEA (late embryogenesis abundant proteins) (Close 1996; Xu et al. 1996) which find expression in vegetative tissues under water deficit. The expression of LEA was observed in parallel to increase in water retention. These proteins are hydrophilic in nature and have been proposed to confer water stress tolerance. During maturation of embryo in dehydrated seedlings of angiosperms, the rise in the expression of LEA proteins is reported (Swire-Clark and Marcotte 1999). Expression of barley HVA1 gene encoding three LEA proteins in wheat resulted in an improved biomass productivity and efficiency in water use under drought conditions (Sivamani et al. 2000). Xu et al. (1996) reported that increased level of barley HVA1 protein (LEA 3) and PMA80 (wheat LEA gene) in transgenic rice led to enhanced plant tolerance to water deficit by providing protection to cell membranes from injury.

The sdd1 (stomatal density and distribution) gene involved in stomata development was also observed to be down-regulated under drought. Increased

number of stomata in *sdd* mutants in Arabidopsis pointed towards the function of this gene as a negative regulator of the guard cell formation (Von Groll et al. 2002). Expression of another gene, GRP (glycine-rich protein), has been also observed in plants under dehydration stress (Wang et al. 2009). The localization of these proteins has been observed in the cell wall of higher plants and was shown to be important for maintenance, reinforcement, and repair during the dehydration–rehydration process (Mousavi and Hotta 2005). Drought adaptation regulates several genes such as HSP1 (heat-shock protein) and a DH3 (dehydrin) and CLP1 (Calpain) which codes for an ATP-dependent calpain–cysteine protease. HSP stabilizes the three-dimensional structure of the proteins by binding to the unfolded proteins and prevents intermolecular interactions. Protein analysis has confirmed the involvement of HSP1, DH3 and CLP1 in protein–protein interactions, assembly and folding, intracellular localization as well as secretion, prevention of protein aggregation and degradation of damaged proteins in drought endurance in plants (Demirevska et al. 2008). The proteolytic activity of cysteine proteases increases drastically in response to water deficit in wheat (Zagdariska and Wisniewski 1996), and some investigations suggest that drought-tolerant species have suppressed proteolytic activity compared to the sensitive ones (Hieng et al. 2004).

The inactivation of gene-PDH1 (prephenate dehydrogenase protein) catalysing the aromatization of prephenate to phenylalanine under drought leads to suppression of phytoalexins, lignans and pro-anthocyanidins involved in signalling and defence as phenylalanine serves as initial molecule of the phenylpropanoid metabolic pathway.

6.4 Nitric Oxide Generation in Plants

Of many NO generation pathways observed, only few have been thoroughly investigated in plants. It is believed that NO is synthesized by either enzymatically different pathways using nitrate/nitrite or arginine as a substrate (Besson-Bard et al. 2008) or non-enzymatically through nitrification/denitrification processes (Bethke et al. 2004; Neill et al. 2003; Rockel et al. 2002). The main suggested enzymatic sources for NO (1) nitrate reductase (NR) is present in cytosol; (2) nitric oxide synthase (NOS) is present in peroxisomes; (3) Nitrite-NO reductase (Ni-NOR) is plasma membrane bound; and (4) xanthine oxidoreductase (XOR) is present in peroxisomes (Fig. 6.2) (Corpas et al. 2004).

Experimental studies have highlighted the role of NR in the primary level of NO synthesis in plant leaves and roots (Vanin et al. 2004).NR was linked to NO generation as early as 1988 when it was observed that, unlike wild-type plants, mutants of soya bean deficient in NR did not synthesize NO, indicating that NR was responsible for NO generation (Dean and Harper 1988). NR is now recognized as a key enzyme of nitrate uptake in higher plants, often catalysing the rate-limiting step. NR reduces nitrate to nitrite using NADPH as an electron source, further catalysing electron transfer from NAD(P)H to nitrite resulting in NO formation (Neill et al. 2003). The ability of NR to generate NO has been established both in vitro (Yamasaki and Sakihama 2000) and in vivo (Rockel et al. 2002).

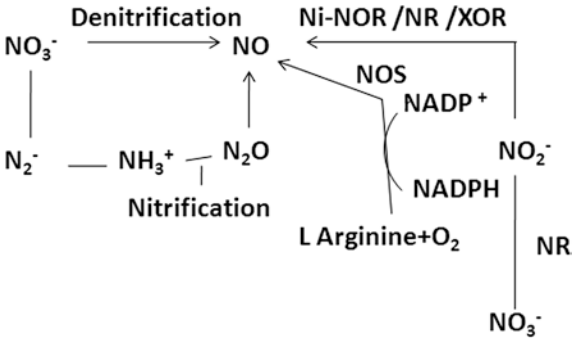

Fig. 6.2 Generation of nitric oxide from nitrate by enzymatic pathways comprising nitrate reductase (NR) Nitrite-NO reductase (Ni-NOR), xanthine oxidase (XOR) and nitric oxide synthase (NOS). The non-enzymatic generation includes nitrification/denitrification process

NR-derived NO was shown to be a signalling molecule when ABA failed to stimulate NO production and stomatal closure in NR-deficient *Arabidopsis thaliana* mutants of *nia1* (nitrate reductase Arabidopsis thaliana) and *nia2* (Bright et al. 2006; Desikan et al. 2004).

Another enzyme Nitrite-NO reductase (Ni-NOR) is present in roots and is involved in the NO formation from nitrite accumulated at significant levels during anaerobic conditions, or conditions inhibiting photosynthetic activity. Ni-NOR has been observed to utilize reduced nicotine adenine nucleotides as electron donors during NO generation (Stöhr et al. 2001). An association between the plasma membrane-bound Ni-NOR and NR generates NO by reducing nitrate to nitrite using succinate as electron donor in the root apoplast (Meyer and Stöhr 2002). NOS are a family of enzymes, which have been studied extensively and well characterized for their role in generation of NO in animal systems (Fröhlich and Durner 2011). In animals, NO is produced when NOS catalyses L-arginine to L-citrulline through electron oxidation using NADPH (Neill et al. 2003). NOS-like enzyme was genetically identified for the first time in *A. thaliana* and named AtNOS1 (Guo et al. 2003).

XOR (xanthine oxidase or xanthine dehydrogenase), peroxidases and cytochrome P450 are all involved in NO synthesis. Horseradish peroxidase is shown to produce NO from NOHA (N-hydroxy-L-arginine) by using hydroxyurea and hydrogen peroxide (Huang et al. 2002). Cytochrome P450, haemoglobin (Hb) and catalase, present in both plants and animals, catalyse oxidation of NOHA using NADPH generating NO in the process (Boucher et al. 1992).

The non-enzymatic pathways involve nitrification/denitrification cycles occurring in the roots, releasing NO into the atmosphere as a by-product of N_2O oxidation (Wojtaszek 2000). In acidic condition occurring in chloroplast and apoplastic spaces, non-enzymatic reduction of nitrite can also lead to the formation of NO (Stöhr and Ullrich 2002; Beligni et al. 2002). This reduction can also be brought about by ascorbic acid at a pH of 3–6 (Henry et al. 1996).

A light-mediated reduction of NO_2 by carotenoid pigments present in chloroplast of the cells was shown to significantly reduce formation of nitrosating intermediates

and release NO into the gas phase into the cytosol (Cooney et al. 1994). Nitrate has been seen as an intermediate electron acceptor under oxygen deficiency leading to the production of NO by the root plasma membrane-bound Ni-NOR or by cytosolic NR. Further oxygenation of NO is carried out by Hb, and the turnover of reaction is maintained by the methaemoglobin reductase enzyme. This reaction maintains redox state of the cell at low oxygen level (Igamberdiev and Hill 2004).

NO generation also takes place through polyamines such as spermidine and spermine. Application of polyamines to Arabidopsis seedlings increases NO synthesis in root tip, elongation zone and young primary leaves (Tun et al. 2001). The effect of polyamines and their crosstalk with NO is discussed in more detail in one of the following sections.

6.5 Nitric Oxide Signalling in Plants

Cyclic guanosine monophosphate is the second messenger molecule executing intracellular signalling. The concentration of cGMP is dependent on the external stimulus and is regulated by the action of biosynthetic enzyme guanylyl cyclase (GC) and degrading phosphodiesterases (PDE). cGMP mediating the NO signal is produced when NO reacts with the haeme moiety of membrane-bound soluble form of GC causing a conformational change and activates the enzyme (Söderberg 2005). The identification and quantification of cGMP in plants was for the first time carried out in corn (Janistyn 1983) and bean (Newton et al. 1984) by mass spectroscopy and radioimmunoassay techniques. NO has been shown to increase cGMP levels in plants which stimulate stress-related gene expression and biosynthesis of secondary metabolites implicated in defence responses (Fig. 6.3) (Perazzolli et al. 2006).

Another second messenger molecule in the NO signalling pathway, cADPR, works by activating the release of calcium from membrane vesicles such as endoplasmic reticulum and vacuoles (Leckie et al. 1998). The production of cADPR is activated by cGMP which in turn has been produced from NO activation of GC (Wendehenne et al. 2001). cADPR performs vital functions in signalling pathways such as NO-induced stomatal closure (García-Mata and Lamattina 2001).

The effect of cADPR to mimic the manifestation of defence-related genes encoding pathogenesis-related 1 protein (PR-1) and phenylalanine ammonia-lyase (PAL) by NO in tobacco is found to be inhibited by the ruthenium red, an inhibitor of intracellular Ca^{2+} release (Durner et al. 1998). Treatment with cADPR in *Commelina communis* has been shown to induce stomatal closure, while treatment with nicotinamide, which inhibits the synthesis of cADPR, lowers ABA-induced stomatal closure and vacuolar ion efflux (Leckie et al. 1998). Nicotinamide has been shown to inhibit stomatal closure induced by ABA or NO in pea plant (Neill et al. 2002).

Both synthesis and signalling of NO involve regulation via protein phosphatases as well. Cantharidin which acts as a protein phosphatase 2A-inhibitor demonstrated an increase in NO synthesis when applied to soya bean suspension cultures (Delledonne et al. 1998). However, NO synthesis in spinach by NR was observed to be impeded by cantharidin (Rockel et al. 2002).

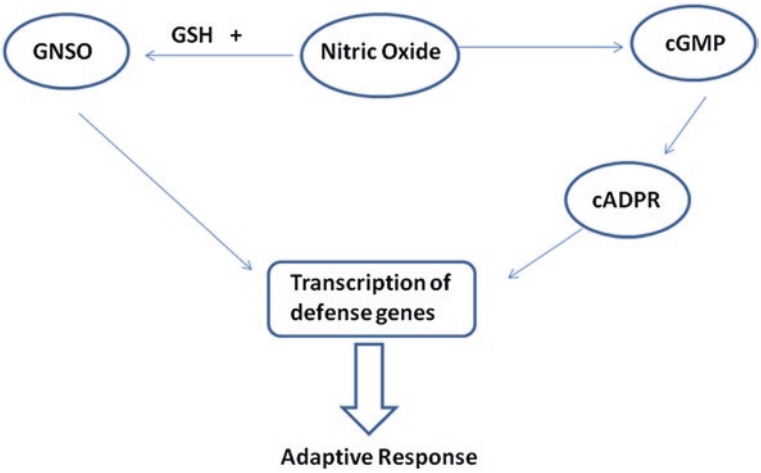

Fig. 6.3 Nitric oxide induces its stress-responsive effect through second messengers—cGMP and cADPR levels which further activate the expression of genes involved in defence. The stress-responsive genes also get activated by NO in signalling through *S*-nitroso-*l*-gluthione (GNSO) (Durner et al. 1998)

NO also reacts with the transition metal in plant proteins to form complex called metal nitrosyl complex which forms an important part of plant signalling mechanisms. Most of the work on NO–metal complexes in plant signalling has been carried out with plant Hb which produces NO_3 from NO in aerobic environment, oxidizing the haem centre of Hb to produce methaemoglobin which further releases NO_3 following reduction by NADPH or a flavin-containing reductase (Perazzolli et al. 2004). Besides Hb, NO also reacts and forms metal nitrosyl complex with cytosolic and mitochondrial aconitases, ascorbate peroxidase, lipoxygenase, catalase and cytochrome c oxidase (COX) (Klessig et al. 2000).

6.6 Effect of Nitric Oxide in Plant Hormone-Mediated Signalling

Phytohormones such as abscisic acid (ABA),ethylene, auxin and cytokinins (CK) are intricately involved in plants' drought responses (Khan et al. 2012). Drought stress induces production of ABAwhich regulates many key processes in plants including seed germination and stomatal conductance (Hubbard et al. 2010) through the involvement of signalling molecules such as NO and ROS. The role of NO in hormonal signalling pathways in plants is well established (Neill et al. 2003). One of the most known cases is the role of NO as a key signalling molecule mediating ABA-induced stomatal closure under hyperosmotic stress conditions (Zhang et al. 2007).

ABA is produced under osmotic stress conditions and plays an important role in drought stress response and tolerance of plants (Nakashima and Yamaguchi-Shinozaki 2013). It modulates turgor pressure in the guard cells surrounding stomata and regulates stomatal opening. ABA also inhibits cell elongation and causes seed dormancy (Nambara and Marion-Poll 2003; Kushiro et al. 2004). NO acts as a breaker of seed dormancy (Bethke et al. 2004). Thus, the role of ABA and NO in seed germination appears to be antagonistic. Another plant hormone ethylene also causes stomatal closure in the presence of NO and ABA during wilting. It is assumed that during rapid wilting, ABA signalling bypasses the need for NO synthesis (Neill et al. 2008).

Drought stress also causes accelerated leaf senescence and leaf abscission as a means to decrease the canopy size. It also induces premature senescence and suppresses nitrogen fixation in leguminous nodules. Both the above processes are also known to be controlled by the level of endogenous CK (Sakakibara 2006). Meanwhile, exogenous application of CKs results in a rapid release of NO, suggesting the role of NO signalling in CK-mediated morphogenetic responses (Tun et al. 2001). NO donors have been also shown to induce similar effects as that of CKs through betacyanin accumulation in Amaranthus seedlings. Also, NOS inhibitor and NO scavenger have been shown to block the action of CKs on betacyanin accumulation (Scherer and Holk 2000).

Adventitious roots have been observed to aid seedling establishment by providing effective drought avoidance through greater water uptake capacity as compared with primary roots (Briske and Wilson 1980). Auxin has been shown to promote adventitious root formation (Davis and Haissig 1994), and physiological stages of the rooting process are linked to endogenous auxin concentrations (Heloir et al. 1996). The induction of adventitious roots development by auxin is also mediated by NO, as the transient increase in the level of NO in the basal region of hypocotyls is observed after IAA treatment causes adventitious roots formation (Pagnussat et al. 2002).

6.7 Crosstalk Between Polyamines and NO

A number of reports showed that plant pretreatment with polyamines improved drought tolerance and reduced water loss (Arasimowicz-Jelonek et al. 2009; Yamaguchi et al. 2007). At the same time, in wheat (Tian and Lei 2006) and maize leaves (Hao et al. 2008), SNP treatment reduced transpiration rate and conserved water under drought stress, suggesting that under water stress conditions NO may act as a downstream signal of polyamines. Polyamines have been observed to have positive influence on the membrane structures of cell, as spermidine-treated tissues maintained structure and prevented ion leakage resulting from oxidative burst during drought (Amri and Shahsavar 2010). A similar effect on stability of membrane was observed after exogenous application of NO suggesting that optimal concentration of NO plays a key role in plant fitness and adaptive responses to water deficit during the initial phases of stress. NO infers the protective effects on membranes by quenching the radicals, and this effect is dose-dependent (Stöhr 2007).

6.8 Oxidative Stress Alleviation by Nitric Oxide

During drought stress, the limitation on CO_2 fixation decreases $NADP^+$ regeneration from Calvin cycle, thereby causing downsizing of photosynthetic electron transport chain due to leakage of electrons during photosynthesis (Smirnoff 1993), and it was estimated that the above leakage may amount up to 50 % under drought stress conditions (Biehler and Fock 1996; Sgherri et al. 1996). Lipid peroxidation and membrane damage by the ROS have been reported to escalate under drought stress in plants (Halliwell and Gutteridge 1999). The superoxide radicals or hydroxyl radicals generated in the oxidative burst during water deficit are responsible for the oxidation of carbonyl groups of amino acid residues (Moran et al. 1994). Antioxidative defence system comprising of several antioxidant enzymes such as SOD, guaiacol peroxidase (GPX), catalase (CAT), dehydroascorbate reductase (DHAR), ascorbate peroxidase (APX) and glutathione reductase (GR) makes significant contribution to the drought tolerance trait in crops (Fig. 6.4) (Jung et al. 2000). The antioxidant system is induced by NO present in mitochondria where it is involved in two respiratory electron transport pathways (Zottini et al. 2002). NO has been observed to alleviate effects of oxidative stress such as chlorosis, DNA fragmentation and apoptotic cell death (Beligni and Lamattina 1999). The damage due to oxidative stress posed by drought is tackled by NO by playing a role of a chain breaker limiting the lipid peroxidation and activates genes for antioxidant

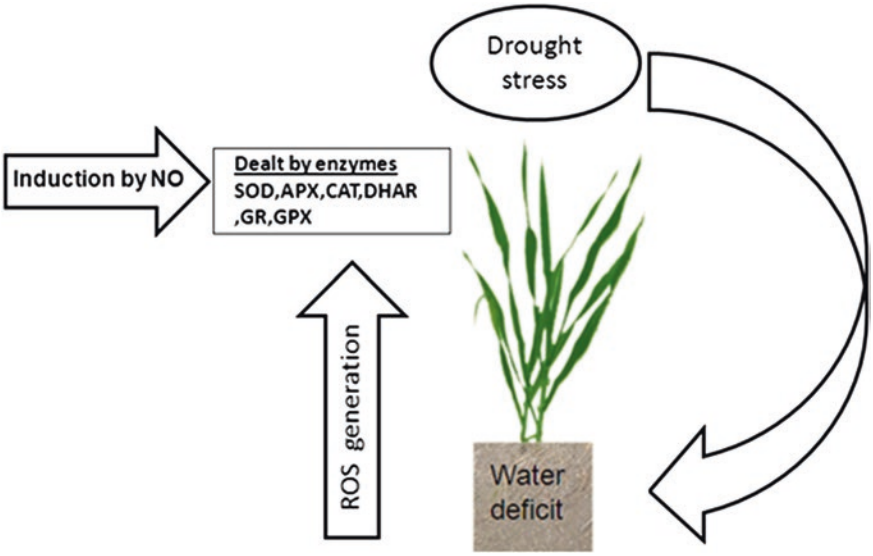

Fig. 6.4 Activation of antioxidant enzymes comprising superoxide dismutase (*SOD*), guaiacol peroxidase (*GPX*),catalase (*CAT*), dehydroascorbate reductase (*DHAR*), ascorbate peroxidase (*APX*) and glutathione reductase (*GR*) by NO to counteract reactive oxygen species (*ROS*) generation under water-deficit condition arising in roots under drought

enzymes, thus protecting plant cells from injury (Lipton et al. 1993; Nunoshiba et al. 1993). The exogenous application of NO through its donors such as sodium nitroprusside (SNP) has been observed to activate antioxidant enzyme specially (SOD) which ultimately eliminates superoxide anion and lipid radical (Shi et al. 2007). NO has been seen to induce synthesis of catalase, glutathione, SOD, s-transferase and alternative oxidase which also result in programmed cell death in barley aleurone cells in aid of gibberellins (Beligni et al. 2002; Polverari et al. 2003).

The effects of ROS and NO are concentration dependent, with their high levels resulting in extensive oxidative damage, while low levels acting as signalling molecules in many metabolic and physiological pathways. Higher levels of NO were shown to enhance Fenton reaction causing damage to membranes by generation of OH^- (Fukuto et al. 2000). Leaf peroxisomes are considered to be a source of NO due to the presence of NOS, and the possibility of interaction of NOS with ROS metabolites has been hypothesized. It was suggested that the requirement of NADPH for executing the NOS reaction for NO generation is fulfilled by NADP-dependent dehydrogenases present in the peroxisomal matrix (Corpas et al. 1999). The formation of peroxynitrite takes place in the peroxisome by xanthine oxidase when NO reacts with superoxide radicals generated in the peroxisomal matrix (Del Río et al. 1992). The formation of peroxynitrite in cytosol also takes place on the reaction of superoxide radicals with NO diffused through peroxisomal membrane (Del Río et al. 1998).

It has been reported that NO stimulated SOD production which lowered lipid peroxidation in rice leaves, preventing their early senescence (Cheng et al. 2002). In another study, SNP application has been reported to significantly induce activities of APX, DHAR and GR, enabling chloroplasts to avoid oxidative damage (Gao et al. 2012). The application of NO donor SNP resulted in the induction of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) in both rice (Uchida et al. 2002) and wheat seedlings (Hung et al. 2002) under oxidative stress.

The activities of antioxidant enzymes were shown to be enhanced after ABA application as reported for a broad range of plant species such as maize (Jiang and Zhang 2001), rice (Lin et al. 2001), legumes (Zhou et al. 2005) and turf grass (Lu et al. 2003). This increase is believed to rely on ABA-induced generation of NO in mesophyll cells which, in turn, activates MAPK and up-regulates the expression of antioxidant enzymes requires H_2O_2 (Zhang et al. 2007).

6.9 NO Mediation of ABA-Induced Stomatal Closure

NO generation in guard cells is stimulated by ABA produced endogenously during drought stress with its effect on stomatal closure being mediated by cGMP and cyclic ADP ribose (cADPR) (Wendehenne et al. 2004). This NO-induced stomatal closure requires calcium which is also an important component of stomatal ABA signalling pathways (Webb et al. 2001). The model for ABA signalling pathways in modulation of guard cells has been proposed during water deficit

which encompasses the functions of ion channels and messengers contributing to increase in cytosolic Ca^{2+}, guard cell's turgor loss and ultimately stomatal closure (McAinsh 1990). ABA action requires mobilization of calcium from both inside and outside of the cell (MacRobbie 2000).

In the signal transduction pathway, Ca^{2+} is located downstream of cADPR as activation of a few defence genes by it was inhibited by a Ca^{2+} channel blocker (Durner et al. 1998). Lower concentration of NO decreased the degree of stomata opening. The chelation of Ca^{2+} led to the reversal of the NO effect suggesting that NO could be acting upstream of Ca^{2+}. The studies indicate towards the potential of NO to regulate stomatal opening by influencing Ca^{2+} signal transduction pathway (García-Mata and Lamattina 2001).

The osmotic stress has been seen to induce a fast elevation in the content of cyclic nucleotides, and cGMP was suggested to be a target of the stress-responsive signalling of NO (Donaldson et al. 2004). The inhibition of cGMP synthesis after treating pea guard cells with inhibitor attenuated ABA and led to stomatal closure induced by NO (Neill et al. 2002). The prerequisite of NO generation by ABA induction in guard cells of *A. thaliana* is the presence of NR as illustrated by the failure to induce stomatal closure in NR mutants (García-Mata and Lamattina 2002). Guard cells of wild-type Arabidopsis generated NO in response to treatment with ABA and nitrite, a substrate for NR. In the NR double mutant, *nia1–nia2* that has diminished NR activity, guard cells could not synthesize NO nor cause stomatal closure in response to ABA or nitrite (Desikan et al. 2002). It has also been demonstrated, in ABA-insensitive (ABI) *abi1-1* and *abi2-1* mutants, that the ABI1 and ABI2 protein phosphatases are downstream of NO in the ABA signal transduction cascade (Desikan et al. 2002).

6.10 Promotion of Adventitious Root Growth

The water-deficit conditions induced NO production in root tips of cucumber (Arasimowicz-Jelonek et al. 2009), pea (Leshem and Haramaty 1996), wheat (Gould et al. 2003) and tobacco (Kolbert et al. 2005), and the involvement of NO in promotion of the root growth has been concluded from many studies (Gouvêa et al. 1997; Forde 2002) by inducing cell elongation similar to auxins. The transient rise in NO concentration was implicated in the adventitious roots development induced by indole acetic acid (Pagnussat et al. 2002), suggesting the crosstalk between NO and auxin in root formation.

In soya bean roots, gravitropic bending due to non-uniform NO accumulation has been demonstrated (Hu et al. 2005). NO-releasing substances have been shown to promote root elongation (Gouvêa et al. 1997). The treatments with SNP, NO scavenger cPTIO and an NO synthase (NOS) inhibitor revealed in mountain ginseng that NO is involved in adventitious root growth. The NO released from SNP activates NADPH oxidase activity, resulting in increased generation of superoxide radicals which subsequently induces growth of adventitious roots (Tewari et al. 2008). The

endogenous NO and H_2O_2 play crucial roles in rooting under drought conditions, and H_2O_2 may be involved in rooting promoted by NO under drought stress. NO or H_2O_2 treatment of marigold attenuated the destruction of mesophyll cells ultrastructure by drought stress thus improving the photosynthetic performance of leaves and alleviates the negative effects of drought on carbohydrate and nitrogen accumulation in explants, and promoting adventitious rooting (Liao et al. 2012).

6.11 Conclusion and Future Prospects

Nitric oxide confers tolerance against drought by affecting a range of physiological and biochemical processes in plants. It aids in the elimination of superoxide radicals by inducing enzymatic antioxidant system in plants under drought stress, thus alleviating detrimental effects of ROS on cellular structures. It influences many adaptive physiological processes acting in synergy with plant hormones in key processes such as stomatal closure and adventitious root formation and also regulates stress-inducible genes by second messenger molecules such as cGMP and cADPR. The involvement of NO is suggested to be at the beginning of stress-response signal transduction, but the mechanism of transfer of stress stimulus to NO still has to be elucidated. NO generation in plants and its signalling under drought stress have been supported by the pharmacological studies through the use of NO donors/scavengers. Of the several generation pathways suggested, biochemical details of the cooperation between different pathways remain ambiguous. Polyamines and NO confer drought tolerance through prevention of ion leakage during oxidative burst, but their mutual effects are yet to be determined. More experimental studies are needed to support the effect of NO on stress genes counteracting intense water loss during long-term drought.

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Chapter 7
Nitric Oxide and Plant Hemoglobins Improve the Tolerance of Plants to Hypoxia

Christos Dordas

Abstract Hemoglobins (Hbs) are heme proteins that are found in most organisms and they have the property to react with O_2, CO, and NO. Their structure, size, and function are quite diverse among the different organisms. In plants, three different types of hemoglobins were found: symbiotic (sHb), nonsymbiotic (nsHb), and truncated hemoglobins (trHb). Nonsymbiotic hemoglobins are divided into two classes: class 1 hemoglobins (nsHb-1s), which have a very high affinity for oxygen, and class 2 hemoglobins (nsHb-2s), which have lower affinity for oxygen and are similar to the sHbs. nsHb-1s are expressed under hypoxia and were found to improve the tolerance of plants to hypoxia and maintain the energy status of the plant cells. Class 1 nsHbs improve the tolerance of plants to hypoxia and provide an alternative type of respiration to mitochondrial electron transport. Therefore, nsHb-1 possibly acts as NO dioxygenase in the nsHb/NO cycle which consumes NADH and maintains ATP levels via an as yet unknown mechanism. However, other possible functions of nsHbs cannot be precluded as Hbs have many different functions in other organisms.

Keywords Hypoxic stress · Nitrate reductase · Nitric oxide · Nonsymbiotic hemoglobins

7.1 Introduction

Hemoglobins are present in all five kingdoms of living organisms (Wittenberg and Wittenberg 1990) and are heme proteins that reversibly bind with oxygen (Hardison 1998; Weber and Vinogradov 2001). In the animal kingdom, apart from vertebrates, hemoglobins are found in about 33 % of the presently known lower animal classes (Terwilliger 1998). Moreover, hemoglobins are present in plants, algae, and

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also in bacteria. Hbs are also found in chimeric form as the NH_2-terminal globin domain is linked covalently to flavoproteins in bacteria and yeast (Gardner 2005). Hemoglobins can have different sizes, structures, and functions, can range from monomers to giant multi-subunit structures, and can occur in widely different anatomical sites (Weber and Vinogradov 2001).

Hemoglobins have a heme domain and the ability to react with gases such as CO, O_2, and NO. The most well-studied reaction of hemoglobins is with O_2, but they also react with other gasses such as CO and NO and also with several organic molecules such as phospholipids and other membrane lipids (Bonamore et al. 2003; D'Angelo et al. 2004; Ollesch 1999; Rinaldi et al. 2006). Most organisms contain several different hemoglobins like plants which have symbiotic, nonsymbiotic, and truncated hemoglobins. The fact that hemoglobins are present in most living organisms suggests that they are some of the most multi-functional proteins found in living organisms and that are vital for the survival of the species and the adaptation under different environmental conditions (Weber and Vinogradov 2001). Hbs have several different functions as they can transfer electrons from NADPH to heme iron, protect from oxidative and nitrosative stress, and be part of terminal electron acceptors during respiration. In addition, Hbs can also control NO levels, use NO to control the levels of O_2, bind and transport sulfides, transport O_2, control dehaloperoxidase activity, detoxify chlorinated materials, and act as O_2 sensors and O_2 storage molecules (Weber and Vinogradov 2001).

This review describes the function of plant hemoglobins with particular emphasis on nonsymbiotic hemoglobins and their role in hypoxic tolerance in plants and the involvement of nitric oxide (NO). In addition, the readers can check a number of excellent reviews over the last several years which cover specific aspects of plant hemoglobin research. Hill (2012) emphasizes the possible function of nonsymbiotic hemoglobins on hormone signaling, while Gupta et al. (2011) discuss the structure function and the evolutionary basis of the three classes of plant hemoglobins and its function with O_2 and NO. Vazquez-Limon et al. (2012) discuss the evolution of plant hemoglobin, while Hebelstrup et al. (2013) emphasize the role of plant hemoglobin in plant development. Moreover, Dordas (2009) emphasizes the function of hemoglobin in stress tolerance, while Garrocho-Villegas et al. (2007) provide an overview of the last six decades of research on plant hemoglobins. A comprehensive review about the structural properties of the plant hemoglobins, their oxygen-binding properties, and the likely physiological function is given by Smagghe et al. (2009), while two other reviews portray the structure and ligand-binding characteristics of plant hemoglobins (Hoy and Hargrove 2008; Kakar et al. 2010).

7.2 Plant Hemoglobins: Categories and Function

In the plant kingdom, highly reputable hemoglobins are the symbiotic hemoglobins, found only in nodules of plants capable of symbiotic nitrogen fixation. Symbiotic hemoglobins have the function to regulate oxygen supply in

root nodules at optimal levels for bacterial function (Appleby 1992). The most interested properties of sHbs are that they have higher oxygen association and lower oxygen dissociation rate constants than myoglobins, but those values are still within the bounds of partial saturation required for binding and release of oxygen allowing the oxygen gradient within the nodule (Kundu et al. 2003).

Nonsymbiotic hemoglobins are not involved in symbiotic nitrogen fixation, and they exist throughout the plant kingdom, including plants capable of symbiosis. In addition, nsHbs are expressed in seed, root, and stem tissue of both dicot and monocot plants (Hill 1998). They are present in much lower concentrations in plant tissues than the sHbs, and this is the reason why they were not found earlier. In addition, nsHbs are divided into two different classes (Classes 1 and 2) based on the O_2-binding characteristics and the different patterns of expression (Duff et al. 1997). nsHbs differ from sHbs, mammalian Hbs, and myoglobins as they are partially "hexacoordinate" in both ferric and ferrous states due to a histidine in the distal pocket that reversibly binds the sixth coordination site of the heme iron (Arredondo-Peter et al. 1997, 1998; Duff et al. 1997). Another class of recently discovered hemoglobins in plants is the truncated hemoglobins (trHbs), appear to be ubiquitous and share some characteristics with nonsymbiotic hemoglobins (Watts et al. 2001). trHbs are so named because the typical "3-on-3" alpha helical "sandwich fold" of most globins is truncated to a "2-on-2" fold (2/2). However, the full primary sequence of trHbs is not necessarily shorter than other Hbs as happens with higher plant trHbs (Watts et al. 2001). TrHbs in *Arabidopsis* are expressed in root and shoot tissues, and their expression is reduced by hypoxia (Watts et al. 2001). The protein is pentacoordinate, but forms a transient hexacoordinate state upon reduction with sodium dithionite. Also, plant trHb has a moderate oxygen affinity that does not preclude oxygen transport (Hoy and Hargrove 2008).

7.3 Properties of Nonsymbiotic Hemoglobins

Class 1 nonsymbiotic hemoglobins are characterized by a very high affinity for O_2, whereas Class 2 nonsymbiotic hemoglobins have a much lower affinity (Arredondo-Peter et al. 1998). This property is very crucial for its physiological role. Rice nsHb-1 has a 78 times higher O_2 affinity than the soybean leghemoglobin. The high affinity is mainly due to the very low O_2 dissociation constant ($K_{off} = 0.038\ s^{-1}$). Therefore, this high affinity of nsHb-1 for O_2 makes it incapable of being O_2 sensor, O_2 carrier, and O_2 storage molecules, which are three of the initially proposed functions of nsHb-1 in plants (Arredondo-Peter et al. 1998). Since nsHb-1 has very high affinity for oxygen, it will remain oxygenated under very low oxygen tension which is not found in living organisms (Hill 1998). In addition, nsHb-1s have high redox potential together with the high O_2 affinity indicates that they are also unlikely to function in electron transport (Hill 1998; Dordas et al. 2003b). Similar properties were found for nsHb-1s from other plant species such as barley, maize, and *Arabidopsis*.

For AtnsHb-1, it was proposed that a system of cavities allows for internal mobility of ligands and might be involved in the NO dioxygenase activity (Perazzolli et al. 2004; Abbruzzetti et al. 2007). The reaction requires the sequential binding of two substrates, NO and O_2, to heme. Also, secondary binding sites could contribute to the regulation of multi-substrate reaction and to the confinement of unstable intermediates. In the case of neuroglobin, truncated hemoglobins , and myoglobin, the metabolism of NO to nitrate seems to be accomplished through docking of NO to an internal cavity, until oxygen is bound to the heme Fe (Abbruzzetti et al. 2007). In the case of AtnsHb-1, the presence of the kinetic traps gives ground to the hypothesis that the NO dioxygenase activity takes advantage of a similar system of cavities. The characterization of the accessibility of these docking sites and the reactivity of each intermediate is a fundamental step in gaining into the possible involvement of these reactant reservoirs in the metabolism of NO (Abbruzzetti et al. 2007).

Class 2 nsHbs are much less studied than class 1 nsHbs. nsHb-2 is hexacoordinate, and there is a reduced interaction between the distal histidine and the exogenous ligand in *Arabidopsis thaliana* nsHb-2 (AtnsHb-2) (Bruno et al. 2007). There is a hydrophobic channel and internal ligand docking site that allow rapid ligand migration in AtnsHb-1, while AtnsHb-2 relies on protein fluctuation to regulate the exchange of ligands from solvent to distal pocket.

7.3.1 Expression of nsHb-1

When the nsHb-1 was first described in barley, it was found in aleurone tissues and later it was found in almost all plant organs (Taylor et al. 1994). The barley nsHb-1 is expressed in the roots of seedlings, and nsHb-1 is induced in oxygen-stressed aleurone tissues, in the roots under flooding stress, and in NO_3-treated plants (Trevaskis et al. 1997; Parent et al. 2008; Hill 1998; Nie and Hill 1997; Taylor et al. 1994). Soybean nsHb-1 is expressed in stems, seeds, roots, leaves, cotyledons, and in nodules (Andersson et al. 1996). nsHb-1 is repressed by fungal infection (Qu et al. 2005, 2006) and is induced by osmotic stress (Trevaskis et al. 1997), cold stress (Sasakura et al. 2006; Shimoda et al. 2005), P, K, and Fe nutrient deficiencies (Wang et al. 2003), darkness (Lira-Ruan et al. 2001), exposure to nitrate (Nie and Hill 1997; Wang et al. 2003), respiratory inhibitors (Nie and Hill 1997), rhizobial infection (Sasakura et al. 2006; Shimoda et al. 2005), H_2O_2 (Qu et al. 2005, 2006), nitric oxide (Qu et al. 2005, 2006; Sasakura et al. 2006; Shimoda et al. 2005), or plant hormones such as ABA and cytokinin, ethylene, and methyl jasmonic acid (Qu et al. 2005, 2006; Sasakura et al. 2006). Such findings are not universal. Some researchers could not find increased nsHb-1 expression in oxidative, nitrosative, and hormonal stresses in rice (Lira-Ruan et al. 2001). It is possible that rice did not respond so strongly as *Arabidopsis* or that plants responded differently during development. Differences were also observed between two species differing in flood tolerance, as there was a much earlier

expression of the nsHb in the flood-tolerant oak species than in the flood sensitive oak species (Parent et al. 2008). Their up-regulation in stress response may involve energy maintenance and NADH reduction as hypothesized for barley nsHb-1 in hypoxic stress (Hebelstrup et al. 2007). These observations suggest that nsHbs take part in plant responses to some stress conditions.

Lotus japonicus nsHb-1 is expressed in roots and was also found in nodules, and its expression is enhanced by rhizobial infection (Shimoda et al. 2005). nsHb-1s are induced in symbiotic rhizobial at infection, which indicates that nsHb-1 can play a significant role in N_2 fixation. The internal environment of root nodules is hypoxic, and this could have induced nsHb-1. It is also possible that when the nodules are very active, there is a shortage of ATP, which can induce the expression of nsHb-1. In addition, it is possible that the NO produced in these tissues at high levels together with nsHb-1 detoxifies and lowers the NO levels.

Nonsymbiotic Hb-1 is expressed in cotton roots under fungal infection, while overexpression of nsHb-1 in *Arabidopsis* leads to enhanced disease resistance and nitric oxide tolerance (Qu et al. 2005, 2006). Overproduction of nsHb-1 in *Arabidopsis* leads to constitutive expression of the defense genes PR-1 and PDF 1.2 and confers enhanced disease resistance to *Pseudomonas syrignae* and tolerance to *Verticillium dahliae* (Qu et al. 2006). Transgenic *Arabidopsis* plants overexpressing nsHb-1 are more tolerant to exogenous NO, contain lower amounts of NO, and develop spontaneous hypersensitive lesions on the leaves in the absence of pathogen inoculation. These data show that nsHb-1 proteins can play a role in the defense responses against pathogen invasions by modulating the NO level and the ratio of H_2O_2/NO in the defense process (Qu et al. 2005). However, there are also studies where the expression of nsHb-1 does not have a significant effect on disease resistance (Perazzolli et al. 2004).

7.4 Effect of Hypoxic Stress on Metabolism

Hypoxia is major stress that can occur under different conditions and the most common is soil water logging and submergence (termed flooding). Flooding results in reduced growth and leads to plant death for many plant species. A major effect of flooding is the reduced supply of oxygen (hypoxia) to submerged tissues as the diffusion rate of oxygen through water is 10^4-fold slower than in air (Armstrong and Drew 2002). In addition, to hypoxia, flooding leads to changes in the soil environment that influence plant growth such as increase in the levels of ethylene and production of metabolites from anaerobic metabolism of soil microorganisms (such as Mn^{2+}, Fe^{2+}, S^{2-}, H_2S, and carboxylic acids) (Jackson 1985). Plants have developed a number of adaptations and acclimations such as physical escape from flooding (Voesenek et al. 2003), avoidance of oxygen deficiency through effective internal aeration (Jackson and Armstrong 1999), tolerance to anoxia (Gibbs and Greenway 2003), and a capacity to prevent, or repair, oxidative damage during re-aeration (Blokhina et al. 2003).

Hypoxia causes energy deficit and inhibition of respiration as O_2 is the terminal acceptor of electrons in oxidative phosphorylation and indirectly provides the plant with ATP. This energy deficit is one of the major problems encountered by plants under hypoxia. Furthermore, the increased NAD(P)H/NAD(P) ratio inhibits glycolysis, the only pathway to produce energy under anaerobiosis. In order to overcome the stress, they induce the alcohol dehydrogenase and lactic dehydrogenase for recycling NAD(P)H to NAD(P). Moreover, another significant pathway that recycles NAD(P)H to NAD(P) is nitrate reductase which forms NO and together with hemoglobins helps maintaining the energy status of the plant (Dordaset al. 2004).

Together with the above mechanism, there are other genes that are induced under hypoxia and are involved with the detoxification of reactive oxygen species (peroxidase, ascorbate peroxidase, monohydroascorbate, glutathione reductase, and superoxide dismutase) (Klok et al. 2002). It was found that that the products of these genes are involved in the protection against postanoxia injury (Monk et al. 1989).

A second strategy in plants that developed to cope with the low O_2 supply is the formation of aerenchyma formation. The aerenchyma is formed from programmed cell death of the cortical area and creates longitudinally interconnected pathways of gas spaces which allow diffusion of O_2 from the air to the root tips. NO is possible involved in the formation of aerenchyma and together with the nsHb-1 can play another role in the adaptation of plants to hypoxia (Dordas et al. 2004).

7.5 Nitric Oxide

Nitric oxide (NO) is a water and lipid soluble gas and is found in all biological systems (microorganisms, animals, and plants) (Gupta et al. 2011). In microorganism, such as bacteria and yeast, a flavohemoglobin was found to act also as dioxygenase, which breaks down toxic NO to protect the microorganism from the toxic concentrations of NO (Gardner et al. 1998). NO was found to be involved in many physiological processes in animals, including the central nervous, cardiovascular, and immune systems; in platelet inhibition and programmed cell death (apoptosis), in host responses to infection and many others (Moncada et al. 1991; Jeffrey and Synder 1995; Lloyd-Jones and Bloch 1996; Wink and Mitchell 1998; Ignarro 2002). Its biological significance was recognized by Science Magazine in 1992 in designating it the “Molecule of the Year.” Moreover, the Nobel Prize for Medicine in 1998 was awarded to three scientists who discovered that NO is a biological mediator produced by mammalian cells.

In plants, NO is involved in seed germination, root organogenesis, stomatal movement, senescence and programmed cell death (apoptosis), cell wall lignification, nodule metabolism, chlorophyll biosynthesis, hormone signal transduction, hypersensitive response, systemic acquired resistance, wounding, salinity, high temperature, drought, hypoxia, induction of plant growth and development, plant maturation and senescence, suppression of floral transition, mediation of

stomatal movement, xylogenesis, gravitropism, and involvement of light-mediated greening (He et al. 2004; Bright et al. 2006; Desikan et al. 2002; Garcia-Mata and Lamattina 2002; Neill et al. 2002; Crawford and Guo 2005; Leshem et al. 1998; Guo et al. 2003; Durner and Klessig 1999; del Rio et al. 2004; Neill et al. 2003; Lamotte et al. 2005; Delledonne 2005; Lamattina et al. 2003; Zhang et al. 2006).

7.5.1 Production of NO Under Hypoxic Stress

The origin of NO under hypoxic conditions is still unclear, and there are probably different NO sources depending on the pathway. In plants, NO synthesis is still a matter of debate (Besson-Bard et al. 2008; Moreau et al. 2010; Corpas et al. 2009). Beside a nonenzymatic conversion of NO_2 to NO in the apoplast (Bethke et al. 2004), various NO-generating systems have been proposed, such as nitric oxide synthase (NOS)-like proteins (Corpas et al. 2009), nitrate reductase (NR) (Dean and Harper 1988; Rockel et al. 2002), mitochondrial electron transport chain (ETC), (Gupta et al. 2005; Igamberdiev and Hill 2009), polyamine oxidase (Yamasaki and Cohen 2006), and nitrite–NO reductase (Stöhr and Stremlau 2006). Under N_2-fixing symbiosis, several plant NO sources have been found such as a NOS-like activity has been measured for the first time in lupine (*Lupinus albus*) nodule extract (Cueto et al. 1996) and a NOS-type activity was found in *M. truncatula–S. meliloti* (Baudouin et al. 2006; Leach et al. 2010). In addition, a potential NOS in *Arabidopsis*, AtNOS1, was reported by Crawford and co-workers which was based on a homology to a hypothetical snail NOS (Guo et al. 2003). However, the function of the AtNOS1 was called into question (Zemojtel et al. 2006) and this resulted in renaming AtNOS1 as NO-associated protein, AtNOA1 (Crawford et al. 2006).

Nitric oxide was found to be produced in relatively large amounts in maize suspension cell cultures and also in alfalfa roots cultures grown under hypoxic conditions (Dordas et al. 2003b, 2004; Igamberdiev et al. 2004). The level of NO, based on results for Hb-underexpressing maize cells, is about 0.4 nmol/g fresh weight/min (Dordas et al. 2004). This is lower than the 1.0–1.5 nmol/g fresh weight/min found upon bacterial treatment of *Arabidopsis* cell suspension cultures (Clarke et al. 2000), but within the range where the NO effect on plant tissue might be similar. Since plant cells have the ability to scavenge amounts of NO as high as 4–5 μmol NO g^{-1} fresh weight min^{-1} (Igamberdiev et al. 2004), the measurements of NO formation in situ are probably an underestimation of the actual amount of NO that is produced by plant cells. An effective system to break down NO in plant tissue possible through its reaction with Hb has been postulated (Dordas et al. 2003a).

Nitrate reductase is another potential contributor to nitric oxide production (Yamasaki and Sakihama 2000) and is also activated during hypoxia (Glaab and Kaiser 1993). Moreover, nitrate was found to provide protection to a number of higher plants under hypoxia (Fan et al. 1988). Another advantage that nitrate reductase has compared with that of NOS is that it does not require oxygen for the reaction which consumes two moles of NAD(P)H per mole of NO formed.

Experiment carried out production in hypoxic maize cell suspension cultures supplemented with NH_4^+ in place of NO_3^- as the nitrogen source where detection of ^{15}NO in the suspension cells when $^{15}NO_3^-$ was supplied has shown that NO comes from NO_3^- and possibly through the nitrate reductase (Dordas et al. 2004).

NO can also be formed in acidic and reducing environment by nonenzymatic reduction of nitrite to nitrous acid, which reacts with ascorbate producing dehydroascorbate and NO (Weitzberg and Lundberg 1998). In aleurone layers, NO is formed nonenzymatically (Bethke et al. 2004). Furthermore, xanthine oxidoreductase (an enzyme located in peroxisomes) has been reported to produce NO (Hardison 1998).

7.6 Interaction of Nitric Oxide with nsHb-1s

Nonsymbiotic Hb-1 has found to maintain the energy levels of the cell under hypoxic conditions with the involvement of NO (Dordas et al. 2003b; Igamberdiev and Hill 2004; Igamberdiev et al. 2004, 2006a, b; Sowa et al. 1998; Perazzolli et al. 2004). The mechanism of the interaction of nsHb-1 with NO results in higher ATP levels in nsHb-1 overexpressing lines of a number of plant species (maize, alfalfa, and Arabidopsis), and the plant cells and the root cultures performed better when they were under hypoxic stress (Dordas et al. 2003b; Igamberdiev and Hill 2004; Igamberdiev et al. 2004, 2006a, b; Sowa et al. 1998; Perazzolli et al. 2004). In addition, NO was found to be produced under hypoxic stress and the level of NO was inversely correlated with the nsHb-1 expression with the underexpressing lines to have higher levels of NO (Dordas et al. 2003a, b, 2004; Perazolli et al. 2004). It was suggested that nsHb-1 regulates the NO level and with this way affects the NO-dependent physiological processes in plants (Dordas et al. 2003a, b, 2004).

The mechanism of NO with nsHb-1 was named as nsHb/NO cycle (Igamberdiev et al. 2005) and involves the reduction of nitrate to NO. Following, NO reacts with HbO_2 and produces NO_3 (Fig. 7.1). This reaction is coupled with the oxidation of NAD(P)H; the redox status is maintained under hypoxic stress; and this is a possible reason why the nsHb1 overexpressing lines had higher ATP levels and higher NAD(P) levels. The recycling of NAD(P)H maintains glycolysis and keeps the metabolism running under hypoxic stress. From this reaction, nsHb-1 was proposed to act as a NO dioxygenase (Gardner et al. 1998) which involves utilization of oxyhemoglobin as a substrate from oxygenating NO. A problem to this reaction is that nsHb(Fe^{3+}) that is generated must be converted to nsHbFe^{2+} to maintain the nsHb/NO cycle. A possible mechanism is the involvement of the cytosolic monodehydroascorbate reductase (MDHAR) which can reduce nsHb(Fe^{3+}) (Igamberdiev et al. 2006b). However, other mechanisms can act like the one found in nodules where leghemoglobin can be reduced to ferrous leghemoglobin either enzymatically by ferric leghemoglobin reductase or nonenzymatically (Becana and Klucas 1992).

It was found that in maize, lines overexpressing nsHb-1 show lower activity of alcohol dehydrogenase which indicates that nsHb/NO cycle can substitute the mechanism NADH with the ADH (Hebelstrup et al. 2007; Sowa et al. 1998). The

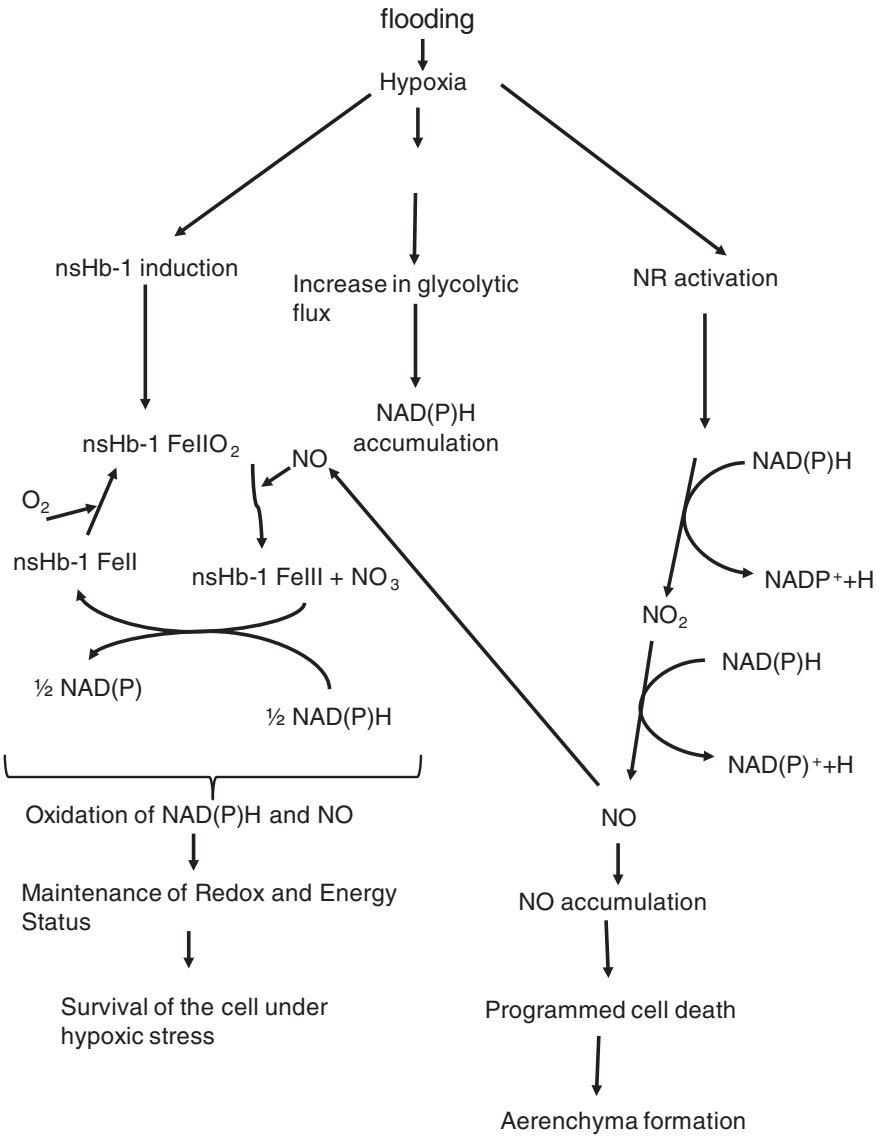

Fig. 7.1 Suggested pathways of the function of nsHb/NO cycle which results in increasing the tolerance of plants to hypoxia

overexpressing lines show lower NADH/NAD and NADPH/NADP ratios possibly because the nsHb/NO cycle uses two NAD(P)H per NO turned over compared with the one NADH per ethanol/CO_2 produced from glycolysis. Therefore, nsHb-1 maintains the growth of the plant and increases in the survival of the plants under hypoxic conditions (Dordas et al. 2003a; Sowa et al. 1998; Perazzolli et al. 2004).

7.7 Concluding Remarks and Future Directions

Despite the fact that considerable research was conducted during the last ten years, there are still several aspects that need to be determined. The regulation of gene expression needs to be determined together with the O_2 affinity in vivo for nsHb-1, a very important property for Hbs. In addition, the involvement of nsHb-1 with other stress response such as osmotic, nutrient, and cold stresses needs to be determined. The efficiency of the nsHb/NO cycle was not determined in vivo which can be done by measuring the glycolytic flux of carbon in the different lines of expression on nsHb-1. The expression of nonsymbiotic hemoglobins can be affected by many factors, including hormones. This aspect should be investigated more thoroughly as there is evidence for the involvement of hormones in the function of nsHb-1. In addition, there is a need to design careful studies that can elucidate the multiple pathways involving nsHbs occurring in specific plant tissues or growth stages or under various physiological and stress conditions. There are evidence suggesting that nonsymbiotic hemoglobins may have an important function in the signal transduction pathways plant hormone such as auxin, ethylene, jasmonic acid, salicylic acid, cytokinin, and abscisic acid. Therefore, there is a strong need for research on hemoglobin gene expression at the cellular level in relation to hormone signal transduction. In the longer term, it is anticipated that the advances in nsHb-1 research may provide novel opportunities for breeding and rational crop design to improve plant stress tolerance.

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Chapter 8
Nitric Oxide as a Mediator of Cold Stress Response: A Transcriptional Point of View

Emmanuel Baudouin and Sylvain Jeandroz

Abstract Low temperature constitutes a major constraint for plant development and spreading and imprints plant biodiversity. Plant tolerance towards cold is a complex matter that relies on deep metabolic reprogramming principally governed by transcriptomic changes themselves controlled through multiple signalling pathways. A set of recent reports points out nitric oxide (NO) as a key element of signalling networks underlying plant response to low temperature. Based on the identification of several cold-regulated NO-dependent genes, that play key functions during plant response to temperature lowering, NO might sustain major functions during cold stress. This chapter summarizes our current knowledge on NO availability and bioactivity during low-temperature response, with a special emphasis on its implication in the regulation of gene expression.

Keywords Antioxidants · *CBF* genes · Cold stress · Nitrate reductase · Osmoprotectant metabolism

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8.1 Introduction

Low temperature is a major determinant of phytogeography and restricts plant species spreading in wild environments on the basis of their relative cold sensitivity/tolerance. A classic distinction is made between exposure to sub-zero temperature (also called freezing) and exposure to sub-optimal but positive temperature (also called chilling). Under temperate climates, most of the species are chilling tolerant but might exhibit a large spectrum of sensitivity towards freezing. This trait is modulated by the ability of certain plants to cold acclimate, i.e. improve their survival capacity to freezing temperature as long as they had been exposed to a chilling pre-treatment. This phenomenon—referred as acclimation—is naturally occurring with the seasonal temperature drop.

Having the ability to maintain cell integrity and metabolism under low temperature and acquiring the capacity to overcome freezing periods are therefore major adaptive traits for wild plants. They also represent main concerns for crop culture and selection (Chew and Halliday 2011). Indeed, even though the average temperature rose over the last century, the frequency of prolonged and acute cold periods also increased. In this context, unravelling the molecular mechanisms governing plant tolerance to cold and their regulation is particularly pertinent to eventually make available tolerant crop hybrids to farmers. Recent reports using high-throughput technologies have provided general maps of the metabolic modifications triggered by low temperature. Combined with genetic approaches using natural or engineered resources from a range of plant models and crops, these analyses pointed out conserved mechanism and molecular players required for efficient chilling and/or freezing tolerance. We advise the reader to refer to a series of recent reviews depicting the diverse aspects of plant response to cold, from the perception of temperature lowering to the set-up of long-term adaptive processes (Ruelland et al. 2009; Ruelland and Zachowski 2010; Knight and Knight 2012; Krasensky and Jonak 2012; Theocharis et al. 2012). As underlined in these publications, low-temperature exposure leads to a profound reprogramming of gene expression in all the plant species and constitutes a key determinant for plant tolerance to chilling and/or freezing, but also a meeting point for a range of signalling pathways.

Recent data have suggested that nitric oxide (NO) is an important element of signalling cascades transducing low-temperature stress. As illustrated in the different chapters of this book, NO is a pleiotropic actor of (a)biotic stress signalling networks and impacts plant cell physiology via the modification of protein activities, gene expression and/or by participating in the overall modulation of cell redox status (for review, Besson-Bard et al. 2008; Wilson et al. 2008; Baudouin 2011).

This present chapter aims at providing a synthetic view of our current knowledge on the biology of NO in the context of plant response to low temperature. This includes the specific features of NO production in cold-stressed plants and the functions it may undertake during cold acclimation. A particular attention will be paid to the involvement of cold-evoked NO in the regulation of cold-responsive gene expression.

8.2 NO Bioavailability During Plant Response to Low Temperature: More than a Way to Skin a Cat?

The formation of NO following exposure to low temperature has been observed in a range of plant species belonging to different families from both monocots and dicots (Table 8.1) (Shimoda et al. 2005; Corpas et al. 2008; Zhao et al. 2009; Liu et al. 2010; Cantrel et al. 2011; Airaki et al. 2012; Bai et al. 2012; Majláth et al. 2012; Xu et al. 2012). NO generation is therefore a frequent response of plants submitted to cold stress. It should be noted that some plant species accumulate similar (*Helianthus annuus*) or even lower NO content (*Capsicum annuum*) after cold exposure (Chaki et al. 2011; Airaki et al. 2012). NO production has been observed in different plant material, e.g. leaf, root, fruit or cultured cells produce NO when submitted to low temperature. Although the temperature range over which NO is formed has not been precisely defined yet and may vary among species, it appears rather large. For instance, NO generation was observed after cold treatment at 8 °C in pea leaves, at 4 °C in Arabidopsis leaves or at 0 °C in *Chorispora bungeana* cultured cells (Corpas et al. 2008; Zhao et al. 2009; Liu et al. 2010). A striking feature of the NO formation reported in chilled plants is the apparent duration of NO generation. Indeed, Zhao et al. (2009) measured a sustained formation of NO in chilled Arabidopsis leaves over the two-week-long

Table 8.1 NO detection in plants exposed to low temperature

Plant species	Plant organ	Chilling temperature (°C)	Duration	Method of detection	References
Arabidopsis thaliana	Leaf	4	1–14 days	Fluorescent dye (DAF-FM DA)	Zhao et al. (2009)
Arabidopsis thaliana	Leaf	4	1–4 h	Fluorescent dye (DAF2-DA) chemiluminescence	Cantrel et al. (2011)
Capsicum annuum	Leaf	8	24 h	Fluorescent dye (DAF-FM DA)	Airaki et al. (2012)
Eriobotrya japonica	Fruit	1	4–24 days	Haemoglobin assay	Xu et al. (2012)
Chorispora bungeana	Cultured cells	0 and 4	3 days	Griess reagent	Liu et al. (2010)
Triticum aestivum	Root	5	3 h	Fluorescent dye (DAF2-DA)	Majlath et al. (2012)
Pisum sativum	Leaf	8	2 days	Fluorescent dye (DAF-FM DA)	Corpas et al. (2008)
Lotus japonicus	Root	4	24 h	Fluorescent dye (DAF-FM DA)	Shimoda et al. (2005)
Baccaurea ramiflora	Seed	2	5–120 h	Fluorescent dye (DAF-FM DA) haemoglobin assay	Bai et al. (2012)

period of the experiment. In complement, NO formation was also reported within the first hours following temperature shift (Cantrel et al. 2011; Majláth et al. 2012). So far, only indirect information suggests that NO generation could happen at earlier time points (Cantrel et al. 2011).

Whereas NO formation is a common response of plants exposed to low temperature, at least two sources might be involved in its synthesis (for further information on NO synthesis in plants, we advise readers to refer to the corresponding chapter of this issue). On the one hand, the use of NO synthase (NOS) inhibitors indicates that NO synthase-like activities are responsible for cold-responsive NO generation in pea leaves and *Chorispora bungeana* cultured cells (Corpas et al. 2008; Liu et al. 2010). This is in accordance with a higher NO synthase-like activity in extracts from chilled plants (Corpas et al. 2008). On the other hand, nitrate reductase (NR) activities have been implicated in NO formation in chilled Arabidopsis, loquat fruit and *Baccaurea ramiflora* embryos (Zhao et al. 2009; Cantrel et al. 2011; Bai et al. 2012; Xu et al. 2012). The determination of the overall NO content of chilled plants may underestimate the quantity of NO produced as it integrates turnover processes. For instance, the activity of *S*-nitrosoglutathione (GSNO) reductase, the enzyme that reduces the reaction product between glutathione (GSH) and NO into ammonia and oxidized glutathione, is stimulated concomitantly with NOS activity in chilled pea leaves (Corpas et al. 2008). Finally, the reaction of NO with O_2^- and GSH to form $ONOO^-$ and GSNO, respectively, generates alternative NO derivatives during cold response, which are not measured by conventional NO detection methods, but likely account in the NO signalling network (Corpas et al. 2008; Airaki et al. 2012).

8.3 NO and Plant Tolerance to Low Temperature

Several studies have shed light on several functions for NO during plant response to low temperature. Early evidences were provided in a seminal study by Zhao et al. (2009). Using the Arabidopsis NR double mutant line *nia1nia2*, which is not producing NO upon cold exposure, the authors demonstrated that NO is required for freezing tolerance in non-acclimated plants but also participates in the acquisition of freezing tolerance in acclimated plants. Similarly, wild-type plants treated with the NO scavenger cPTIO[2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide] or inhibitors of NO synthase and NR were more sensitive to freezing than untreated plants. Finally, the freezing-sensitive phenotype of the *nia1nia2* mutant was rescued by a pre-treatment with the NO donor sodium nitroprusside (SNP). Correlatively, the synthesis of proline (Pro), which is accumulated during acclimation and participates in the protection of cell structures, was impaired in *nia1nia2* mutant as well as by treatment with cPTIO and NR inhibitors, indicating that it accounts at least partially for the NO-dependent acclimation and subsequent freezing tolerance. Recent data also indicate that NO participates

in chilling response. Indeed, an Arabidopsis transgenic line overexpressing the non-symbiotic haemoglobin AHb1 presented less root growth inhibition when cultured at 12 °C than wild type (Guillas et al. 2012). Moreover, this mutant did not accumulate anthocyanin when cultivated at 4 °C (Guillas et al. 2012). Together with a strong impairment of cold-responsive gene expression in AHb1 overexpressor (see next paragraphs), these data suggest that NO is required for an array of cold-induced responses.

Using fumigation with NO gas or treatments with NO-releasing chemicals, several studies have correlated the capacity of NO to prevent chilling injury with the up-regulation of the antioxidant defence (Singh et al. 2009; Liu et al. 2010; Zaharah and Singh 2011; Bai et al. 2012; Xu et al. 2012; Esim et al. 2012). NO treatment has been shown to enhance GSH and ascorbate contents and to increase superoxide dismutase, catalase and ascorbate peroxidase activities, while decreasing O_2^- and H_2O_2 levels. The regulation of antioxidant capacity by NO might be particularly important for the post-harvest physiology of fruits and seeds conserved at low temperature (Singh et al. 2009; Zaharah and Singh 2011; Bai et al. 2012; Xu et al. 2012). It is to note that this effect might proceed via the alleviation of ethylene formation (Singh et al. 2009; Zaharah and Singh 2011). These observations reinforce the hypothesis of an ethylene/NO balance prompting fruit conservation or senescence (Leshem et al. 1998) and extend it to cold-induced fruit injury. Although preliminary reports suggest that it might also participate in the response of vegetative organs to chilling (Esim et al. 2012), further investigations are required to unravel how antioxidant defence integrates into the NO-dependent regulatory network in cold-stressed plants.

8.3.1 Identification of Cold-Responsive NO-Dependent Genes: From Specific to Holistic

Recent data have shed light on the implication of NO in regulating the expression of cold-responsive genes (Table 8.2). The information currently available relies on the analysis of well-known cold-responsive gene markers. For instance, cold-evoked NO participates in the regulation of the expression of *C*-repeat *B*inding *F*actor (*CBF*) genes (Cantrel et al. 2011; Zhao et al. 2011). Indeed, Cantrel et al. (2011) reported that the cold-responsive expression of *CBF1* and *CBF3* is dramatically reduced in Arabidopsis *nia1nia2* mutant and in an *AHb1* overexpressing line. In good agreement, the NO scavenger cPTIO also impaired cold-triggered *CBF1* and *CBF3* expression (Cantrel et al. 2011). Moreover, treatments with SNP stimulated *LeCBF1* expression in tomato fruits, when the NO synthase inhibitor L-NNA blocked cold-triggered *LeCBF1* expression (Zhao et al. 2011). CBFs are the best-characterized transcription factors controlling cold-responsive gene expression and cold acclimation (Chinnusamy et al. 2007; Thomashow 2010; Medina et al. 2011). Indeed, 12 % of the cold-regulated genes would be under the control of CBFs and constitute the so-called CBF regulon (Gilmour et al. 2004; Vogel et al. 2005).

Table 8.2 NO-regulated genes characterized in plants exposed to low temperature

Plant species	Methodologies used to modulate NO level	NO-dependent genes	Other NO-dependent parameters	References
Arabidopsis thaliana	Mutants (*nia1nia2; atnoa1*)	*P5CS1*	Freezing tolerance/ acclimation	Zhao et al. (2009)
	NR and NOS inhibitors; cPTIO	*ProDH*	Proline synthesis	
	SNP			
Arabidopsis thaliana	Mutants (*nia1nia2*; *AtHB1* overexpressor)	*CBF1/CBF3/ COR15a/LTI30/ LTI78*	Sphingolipid metabolism	Cantrel et al. (2011)
	NR and NOS inhibitors; cPTIO	*AtHB1*		
Solanum lycopersicum	NOS inhibitor	*LeCBF1*	Oxidative damages	Zhao et al. (2011)
	SNP		Antioxidant enzymatic activities	
Medicago falcata	PTIO	*AtMIPS1*		Tan et al. (2013)
	SNP			

As expected, NO depletion also impaired CBF-dependent cold-responsive genes such as *COR15*, *LTI30* and *LTI78* (Cantrel et al. 2011). This study highlighted some interesting and unexpected features of NO-dependent regulation of cold-responsive gene expression. Firstly, whereas the activation of *CBF1* and *CBF3* expression by cold required cold-evoked NO, that of *CBF2* did not. As proposed previously, this observation supports the hypothesis that the regulation and function of different CBFs is only partially redundant (Novillo et al. 2007). Secondly, ZAT12, another cold-induced transcription factor which regulates distinct cold-responsive genes from CBF regulon (Vogel et al. 2005), was not affected by NO depletion, which suggests that only a subgroup of cold-regulated genes is NO dependent (Cantrel et al. 2011).

A second line of evidence links cold-evoked NO to the regulation of genes involved in the metabolism of compatible compounds, i.e. Pro and *myo*-inositol (Table 8.2) (Zhao et al. 2009; Tan et al. 2013). As mentioned earlier, Pro accumulates in acclimated plants via a NO-dependent mechanism. Pro accumulation might result from the regulation of Pro synthesis, Pro degradation or both. The modulation of Pro synthesis and Pro degradation is mainly achieved by the transcriptional regulation of $\Delta(1)$-pyrroline-5-carboxylate synthase (P5CS) and Pro dehydrogenase (ProDH), respectively (Szabados and Savouré 2010). Zhao et al. (2009) evidenced that cold-triggered Pro accumulation is achieved by the up-regulation of *P5CS1* and the down-regulation of *ProDH*. Interestingly, cold-responsive *P5CS1* expression was strongly reduced in *nia1nia2* mutant as well as in leaves treated with cPTIO or with the NR inhibitor okadaic acid. Conversely, *ProDH* expression was maintained to a high level in cold-treated *nia1nia2* plants.

Treatments with SNP do not modify ProDH expression, and it is therefore unclear how cold-evoked NO might down-regulate *ProDH* expression. On the other hand, *P5CS1* is up-regulated by SNP, which makes *P5CS1,* a likely NO-dependent cold-responsive gene. Previous evidence pointed out the induction of *P5CS1* by NO in copper-stressed *Chlamydomonas* (Zhang et al. 2008), which suggests that the regulation of *P5CS1* might constitute a pleiotropic mechanism controlled by NO signalling under stress conditions. A recent report on *myo*-inositol synthesis provides another example of a link between the synthesis of compatible solutes triggered by cold and the regulation of cold-responsive gene expression by NO (Tan et al. 2013). Finally, it should be noted that genes related to NO metabolism are themselves up-regulated in response to low temperature. These include the two NR genes, *NIA1* and *NIA2*, the NO-associated 1 gene, *NOA1* (Zhao et al. 2009), and the non-symbiotic haemoglobin, *AHb1* (Cantrel et al. 2011). It is noteworthy that although *NIA2* and *NOA1* genes are induced upon cold exposure, they are probably not involved in cold-regulated NO metabolism (Zhao et al. 2009). In contrast, the transcriptional regulation of *NIA1* and *AHb1* might afford for NO bioavailability during cold response. Strikingly, *AHb1* expression is induced by cold via a NO-dependent mechanism (Cantrel et al. 2011). Whether a similar feedback loop also regulates *NIA1* expression is currently unknown.

As pointed out above, cold-evoked NO has the capacity for being a major regulator of cold-responsive gene expression. Nevertheless, the identification of NO-dependent genes regulated in the context of low-temperature response remains fragmented and probably incomplete. Indeed, this has been addressed so far only by analysing well-known cold-responsive genes, i.e. relying on the a priori selection of genes to be studied. Getting a holistic view of the "NO regulon" at work during low-temperature response therefore requires new experimental procedures to identify cold-regulated NO-responsive genes with no functional prerequisite. On the one hand, almost no overlap was observed in the NO-dependent genes identified using treatments with various NO-releasing molecules or when different plant organs were used, which made impossible to define a list of robust NO marker genes. On the other hand, exogenous NO treatments poorly mimic endogenously evoked NO signals, therefore making correlations risky. Current strategies therefore favour the comparison between plant materials in which NO is conventionally produced with similar material in which endogenous NO accumulation is blocked by chemical treatments or by genetic means. This approach led to the successful identification of Cd-responsive NO-dependent genes (Besson-Bard et al. 2009). We recently initiated a similar strategy to identify cold-responsive NO-dependent genes. In these experiments, cold-evoked NO was alleviated by the use of the NO scavenger cPTIO (Baudouin and Jeandroz, unpublished data). As observed by others (Hannah et al. 2005; Lee et al. 2005; Vogel et al. 2005; Usadel et al. 2008), short exposures to low temperature (4 °C, 1–6 h) impacted the expression of hundreds of genes (>3000) in our conditions. Out of these, almost 35 % (>1000) were not regulated by cold in cPTIO-treated leaves. This high proportion supports the hypothesis that NO has a major impact on the transcriptional reprogramming triggered by low temperature. Furthermore, the cold-responsive

transcriptional regulation of >150 transcription factors appeared NO dependent, suggesting that additional genes might be indirectly regulated by NO at later time points. Clustering and functional analysis of the cold-responsive NO-dependent genes is currently under way and will provide insights into the cellular mechanisms controlled through NO signalling during cold response.

8.4 How NO Regulates Cold-Responsive Gene Expression? The Missing Links

Whereas compelling data plead for an important function for NO in regulating cold-responsive gene expression, how such regulation is achieved remains unknown. The simplest way would be the direct post-translational modification of cold-responsive TFs by NO, thereafter prompting gene expression. No such cold-responsive TF has been identified yet, although an array of TFs play essential functions during low-temperature response. As previously reported for genes activated by NO donors (Palmieri et al. 2008), we expect that the analysis of the *cis*-elements present in the promoter of NO-dependent cold-responsive genes and overrepresented compared to the whole genome will provide clues on possible candidate TFs. Parallel to the analysis of the promoter regions of cold-regulated NO-dependent genes, the identification of TFs undergoing NO-based post-translational modifications could bring valuable information. So far, only rare examples of transcriptional regulators affected by NO-based post-translational modifications have been reported in plants (Serpa et al. 2007; Tada et al. 2008; Lindermayr et al. 2010). It is now assumed that the current strategies to isolate such modified proteins from a total protein extract poorly identify low-abundant proteins such as TFs. Ongoing approaches integrating sub-cellular fractionations should permit to overcome these limitations.

Besides the hypothetical NO-based modification of specific TFs, it is likely that more complex signalling cascades link NO production and gene expression. This last scheme has been illustrated in several physiological models where NO is evoked and introduces additional elements, e.g. Ca^{2+}, H_2O_2, cGMP, PtdOH, protein kinases and cytoskeleton, that might operate in the transduction of NO signal (Laxalt et al. 2007; Besson-Bard et al. 2008; Gaupels et al. 2011; Yemets et al. 2011). Information about the downstream elements of NO in cold signalling pathways is currently scarce. Cantrel et al. (2011) reported that PtdOH is not a relay for cold-evoked NO. In contrast, they pointed out the interplay between NO and sphingolipid signalling and in particular phospho-phytosphingosine (PHS-P) that has recently been involved in cold signal transduction (Dutilleul et al. 2012). Nevertheless, PHS-P has a limited impact on plant response to cold and in particular on cold-responsive gene expression (Dutilleul et al. 2012). It is therefore unlikely that the regulation of PHS-P synthesis represents per se a major mechanism for NO-dependent gene regulation during plant response to low temperature. Further investigations to interconnect cold-evoked NO with the overall cold

signalling events are now required to further unravel the mechanisms linking NO production and gene expression in this physiological context.

8.5 Concluding Remarks

Seminal reports published during the last years have shed light on the role of NO during plant response to low temperature. Strikingly, NO appears as a conserved cold-evoked signal in a range of plant species and plant organs. Future works should provide further information on the degree of conservation shared by NO metabolism and NO-dependent responses in these different models. In particular, investigations are required to decipher the possible correlation between NO signalling and plant tolerance to cold. In that sense, the comparison of the genes regulated by cold via NO-dependent processes in a range of plant materials exposed to various low temperature regimes will bring valuable clues. A major challenge will be the integration of such data to provide a picture of cold-activated NO-based signalling network. By now, large-scale analyses are required to identify a full set of NO-regulated genes and proteins during cold response. Together with the study of the crosstalks between NO signalling and the overall cold signalling network, this information should help understand how NO participates to plant acclimation to temperature lowering.

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Chapter 9
Nitric Oxide and UV-B Radiation

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Abstract Over the past few years, nitric oxide (NO) has emerged as a key player in plant adaptive/hypersensitive responses to abiotic (stress) factors, since the content of endogenously generated NO increases in adverse environmental conditions. This chapter addresses to the signalling events with the involvement of the reactive nitrogen species under ultraviolet-B (UV-B) exposure. Solar UV-B has long been recognized as merely deleterious environmental factor; however, rapidly increasing evidence indicates that it plays regulatory role as photomorphogenic trigger for plants. In turn, NO is supposed to be the crucial signalling molecule under plant response to UV-B. The putative biochemical mechanisms of the protective effects of exogenous NO donors on plant cells and the input of NO-synthase-like activities and nitrate reductase under UV-B exposure are discussed. The involvement of cytoskeleton-related NO signalling pathways (especially based on involvement) of microtubules in realization of intracellular UV-B effects is highlighted in the review.

Keywords Cytoskeleton · Intracellular signalling · Nitrosative stress · Reactive nitrogen species · Tyrosine nitration

9.1 Introduction

For long years, nitrogen monoxide (NO), nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3) have been supposed to be merely toxic compounds and a nitrous components of harmful air pollutants, though at present NO has been emerging to be an ubiquitous "jack-of-all-trades" signalling molecule and a key second messenger in (a) biotic stress redox signalling pathways in plant cell (Delledonne

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et al. 1998; Durner et al. 1998; Pedroso and Durzan 2000). NO mediates wide spectrum of physiological processes in plants covering not only developmental processes from root formation to flowering, but also different responses to environmental factors leading even to programmed cell death (Durzan and Pedroso 2002; Besson-Bard et al. 2008; Astier et al. 2010; Mur et al. 2013). Despite the list of cell processes with the participation of NO has been unswervingly growing, the understanding of NO synthetic and signalling pathways in plants is still in its infancy. Currently, at least seven enzymatic and non-enzymatic NO sources in plant cell have been identified: cytosolic nitrate reductase as the main source of NO under aerobic conditions, peroxisomally located xantine oxidoreductase, which reduces nitrite to NO at the expense of NADH under anaerobic conditions, plasma membrane-bound nitrite: NO reductase, nitric oxide synthase (NOS)-like arginin-dependant enzymatic activities as well as oxidative pathways utilizing polyamine and hydroxylamine (Mur et al. 2013).

Notwithstanding that different developmental and/or (a)biotic factors could potentially activate distinct, but overlapping NO sources, the precise conditions required for the switch between NO sources need to be designated. NO plays a role of secondary messenger in common cascades together with such signalling molecules as cytosolic Ca^{2+} (Lamotte et al. 2006), cyclic adenosine diphosphate ribose (cADPR) (Astier et al. 2010), salicylic and jasmonic acids, hydrogen peroxide (H_2O_2), and cyclic guanosine 5′-monophosphate (cGMP) as well as with MAPK kinases, namely salicylic acid-induced protein kinase (SIPK) (Wilson et al. 2008; Astier et al. 2010; Hasanuzzaman et al. 2013). Furthermore, numerous genes involved in jasmonic acid and ethylene metabolism, photosynthesis, organelles motility, hypersensitive response, programmed cell death, etc., were up-and/or down-regulated by NO that was revealed by microarray analysis (Grün et al. 2006; Wilson et al. 2008).

Synergistic interplay between NO and reactive oxygen species (ROS) is also well established both under influence of environmental stimuli and during the regulation of cell metabolism under physiological conditions (Wrzaczek et al. 2010) including peroxyntrite ($ONOO^-$)-mediated signalling (Vandelle and Delledonne 2011). Direct signalling pathways of NO and NO-related molecules are realized via *S*-nitrosylation (or transnitrosylation) of thiol group (for review see Lindermayr et al. 2005; Romero-Puertas et al. 2013), nitration of tyrosine (for review see Blume et al. 2009; Lozano-Juste et al. 2011) as well as Fe-S binding (Astier et al. 2010); however, physiological significance of these NO-mediated posttranslational modifications remains to be elucidated (Baudouin 2011).

It is well-known fact that in eukaryotic cell, NO acts as a Janus depending on its intracellular concentration defined by spatio-temporal kinetics of NO production, distribution and scavenging (Noriega et al. 2007). NO reveals antioxidant cytoprotecting properties at concentrations <100 μM (Beligni and Lamattina 1999; Lamattina et al. 2001; Shi et al. 2005; Krasylenko et al. 2011) and prooxidative cytotoxic—at concentrations >500 μM (Beligni and Lamattina 1999). Thus, the excess of NO is able to inhibit plant growth and division (Corpas et al. 2011) and injure photosynthetic electron transport and DNA (Pedroso et al. 2000). NO

exhibits antioxidant properties and is able to protect plants from a diverse abiotic challenges such as high temperatures, drought, salt stress or phytotoxic metals pollution (Garcia-Mata and Lamattina 2002; Noriega et al. 2007) accompanied by the oxidative stress (Beligni and Lamattina 2001). Protective effects of NO donors could be realized through the direct detoxification of ROS by NO in the reaction between NO and $O_2^{\cdot -}$ with $ONOO^-$ formation and/or due to the activation of antioxidative machinery. Indeed, it was recently demonstrated that NO could stimulate the expression of a heme oxygenase, which catalyses the conversion of heme to biliverdin IX with the concomitant release of CO and iron, and acts against oxidative stress in plants (Shi et al. 2005; Noriega et al. 2007). One of the possible pathways for the realization cytoprotective effects of NO could be also directed via in vivo constitutive *S*-nitrosylation of *Arabidopsis thaliana* metacaspase 9 (AtMC9) that inhibits its auto processing and proteolytic activities (Belenghi et al. 2007).

Many abiotic environmental challenges as well as pathogen invasions induce fast accumulation of endogenously generated nitrosonium cation (NO^+), nitroxyl anion (NO^-), free radical ($NO^{\cdot}$), $ONOO^-$ and/or *S*-nitrosothiols (GSNOs) generally referred to as reactive nitrogen species (RNS), leading to the progression of such metabolic cell status named nitrosative stress (Valderrama et al. 2007; Corpas et al. 2008, 2011) that is frequently accompanied by oxidative stress in terms of an increased level of ROS (Mittler 2002; Mazid et al. 2011). One of the nitrosative stress proteomic markers is the increase of tyrosine nitration level (Valderrama et al. 2007; Corpas et al. 2008), though this NO-mediated posttranslational modification gains recognition as regulatory under physiological conditions leading to loss/gain or no effect on protein function (Abello et al. 2009).

It was found that NO is involved in plant response to a number of abiotic factors such as extreme temperatures, drought, flooding, phytotoxic metals accumulation, excessive illumination, mechanical injury, ozonation as well as ultraviolet-B exposure (Arasimowicz and Floryszak-Wieczorek 2007; Qiao and Fan 2008; Corpas et al. 2008, 2011). Ultraviolet-B (UV-B, 280–315 nm)—not photosynthetically active non-ionising radiation—is one of the abiotic factors that lead to the increase of endogenous NO and ROS content in plant cells (Mackerness et al. 2001; Zhang et al. 2003). From the one hand, its proportion in total solar flux increases due to climate and/or anthropogenic (via halogen compounds and nitrogen oxides) depletion of the protective stratospheric ozone (Ballaré et al. 2011; McKenzie et al. 2011) causing various deleterious effects such as growth/division inhibition, tissue necrosis and severe oxidative stress progression and, finally, decrease of plant productivity and crop yields (Frohnmeyer and Staiger 2003). One of common UV-B effects on plant cell is DNA damage that has also signalling meaning because of the activation of reparation enzymes genes expression (Hollósy 2002; Jiang et al. 2012). Secondary metabolites synthesis and accumulation such as coumestrol, anthocyanin and flavonoids are triggered by UV as well (Jansen 2002; Hao et al. 2009). However, from the other hand, ambient and ecologically relevant low doses of UV-B trigger photomorphogenic signalling pathways aimed to realize adaptive morphogenic responses and are the components of light perception (for review see Jansen 2002, 2012; Frohnmeyer and Staiger 2003;

Jenkins 2009; Heijde and Ulm 2012). It has become evident that UV-B-mediated stress in plants is a relatively rare event (Ballaré et al. 2011; Jansen and Bornman 2012; Jansen et al. 2012) and the UV-B response is defined by the dose and duration of the exposure, plant developmental stage, immune status, ROS/RNS homoeostasis and overlap with other environmental factors.

The identification of the secondary messengers and key target molecules involved into UV-B signalling is important for the development of the new strategies to modulate plant responses to this environmental abiotic factor, explore suitable crop improvement or ways to alleviate stress. For instance, such highly reactive molecules as ROS and RNS are involved into the signalling cascades under the influence of adverse environmental factors and UV-B is not exclusion.

9.2 NO Reveals Protective Effects Under UV-B Influence in Dose-Dependent Manner

First evidence of the interrelations between NO and UV irradiation in plant cells was obtained by Wright and Murphy (1975). It was found that UV (254 nm) exposure of tobacco cells suspension inhibited the production of nitrate reductase, though the subsequent photoreactivation with white light partially restored the synthesis of this enzyme. Later, Hari et al. (2003) revealed that NO can be synthesised and emitted by plants as a consequence of UV exposure. Together with O_2^- and H_2O_2, NO is involved in early UV-B signalling responses as it causes up-regulation of the chalcone synthase (*chs*) gene responsible for flavonoids production during UV-B exposure of *A. thaliana*. In turn, the induction of the *chs* was ROS independent, because O_2^- up-regulated defencin gene *pdf1.2*, while H_2O_2 up-regulated pathogene-related protein 1 gene *pr-1* and down-regulated light harvesting complex binding protein gene *lhcb* (Mackerness and Jordan 1999; Mackerness et al. 2001).

NO might function as both a second messenger (endogenous) in corn (*Zea mays* L.) mesocotyl growth inhibition and simultaneously as antioxidant (exogenous) during UV-B irradiation of growing and developing mesocotyls (Zhang et al. 2003). The NOS activity in maize hypocotyls was significantly increased by UV-B irradiation, supporting that NO may act as a secondary messenger and perform antioxidant responses to UV-B. The treatment with sodium nitroprusside (SNP) at concentration 100 μM reduced the exo- and endoglucanase activities, increased protein content and mimicked the responses of the mesocotyl to UV-B radiation (Zhang et al. 2003). Soon after, Qu et al. (2006) found the participation of NO in UV-B-induced inhibition of *Pisum sativum* L. stem elongation. Exogenously applied SNP (100 μM) diminished the toxic effects of UV by 80 % increase of flavonoids content, shielding waxes and anthocyanins in *Solanum tuberosum* sp. cv. Pampeana, thereby enhancing plant tolerance to the excessive UV exposure (Beligni and Lamattina 2001). Protective effects of exogenous NO donors (SNP, *S*-nitroso-*N*-acetylamine (SNAP), spermine-NO, diethilamine-NO, DETA-NO (NOC 18), PAPA-NO, SIN-1 chloride,

etc.) used at low concentrations (<100 μM) might be realized via scavenging of superoxide anion O^{2-} radicals with the formation of peroxyntrite and/or activation of antioxidant enzymes (Lamattina et al. 2001).

Extra evidences about the existence of *L*-Arg-dependent NO synthetic pathway in plant cell and its involvement in UV-B response were obtained by several authors. The first published results for that NOS are most likely to be the source of NO in response to UV-B exposure, since *L*-NAME, the inhibitor of one of the mammalian NOS, effectively prevented the induction of *chs* expression, was provided by Mackerness et al. (2001). Moreover, UV-B radiation strongly stimulated NOS activity, but decreased leaf biomass in *Z. mays* L. seedlings. Both endogenously produced (by means of NOS as the mammalian NOS inhibitor LNMA was effective) and exogenously applied NO is important for leaf UV-B-induced photomorphogenic responses because it regulates exo- and endo-β-glucanase activities that could make the cell wall loose, increases its extensibility and changes its composition (An et al. 2005). According to these results, it is more likely that NO functions as a signalling molecule rather than reveals antioxidative properties in leaves during their growth and development (An et al. 2005).

The pretreatment of kidney bean (*Phaseolus vulgaris* L.) seedlings with SNP at concentrations 50 and 100 μM partially alleviated the UV-B deleterious effects revealed by ion leakage as a reflection of membrane injury as a result of the oxidative damage, chlorophyll loss; the decrease of maximum efficiency of PSII photochemistry (Fv/Fm) and the quantum yield of PSII electron transport φPSII, thylakoid membrane protein damage in terms of thiol group content; and increase of superoxide dismutase, catalase and ascorbate peroxidase content. These protective effects of SNP were annihilated by the NO scavenger, 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) (Shi et al. 2005). Therefore, NO might prevent oxidative stress progression by the decrease of H_2O_2 content with the simultaneous increase of the thiol groups contents, and its protective effects may be mediated by up-regulation of active oxygen scavenging genes expression under UV-B irradiation (Shi et al. 2005).

UV-B-induced stomatal closure was mediated by H_2O_2 and NO generated by a NOS-like activity in *Vicia faba* L. (He et al. 2005). NO of NOS origin acts synergistically with ROS in the induction of ethylene production under UV-B exposure in *Z. mays* L. (Wang et al. 2006). UV-B exposure (0.4 and 0.8 W m^{-2}) for 2 h reduced pollen germination and tube growth of *Paulownia tomentosa* Steud. in vitro, enhanced NOS activity and NO generation in pollen grains and tubes. It indicates that exogenous NO donors SNP and *S*-nitrosoglutathione inhibited both pollen germination and tube growth in a dose-dependent manner, while NOS inhibitor *L*-NAME and NO scavenger *c*-PTIO not only largely prevented the NO generation, but also partially reversed the UV-B-inhibited pollen germination and tube growth. These results indicate that UV-B radiation inhibits pollen germination and tube growth partly through the promotion of NO production in pollen grain and tube by a NOS-like enzyme. As both pollen germination and tube growth inhibited by UV-B and NO donors were prevented by a cGMP inhibitor LY-83583, NO signalling under UV-B exposure is likely to be mediated by cGMP. Since the effects of *c*-PTIO,

L-NAME and LY-83583 on the UV-B-inhibited pollen germination and tube growth were only partial, the existence of NO-independent UV-B signalling pathways has to be taken into the account (He et al. 2007). Recently, it was shown that, apart from H_2O_2 and NO, GPA1, the Ga-subunit of heterotrimeric G proteins is involved into the process of UV-B-induced stomatal closure in *A. thaliana* (He et al. 2013).

In excised leaves of kidney bean, H_2O_2 produced under UV-B stress, played a role of second messenger required for the induction of NOS as its inhibitor *L*-NNA blocked the NO synthesis (Zhang and Zhao 2008). Apocynin, a natural vanillin-like compound and an inhibitor of NADPH oxidase, weakened UV-B-induced oxidative damage by the prevention of chlorophyll degradation under H_2O_2 influence and enhanced NO synthesis by *L*-Arg-dependent pathway (Tossi et al. 2009). For the NO-mediated attenuation of deleterious UV-B effects, early increase of the abscisic acid (ABA) content is required as it was found using *vp14* maize mutant defective in ABA synthesis (Tossi et al. 2011, 2012). The elegant experiment described by Tossi et al. (2011) with leaves of the UV-B-susceptible plants of sunflower (*Helianthus annuus* L.) protected by sprayed SNP solution (100 μM) preventing chlorophyll decay supports the statement that exogenous NO inputs in general mechanisms of plant protection against UV-B damage.

Kim et al. (2010) demonstrated that SNP pretreatment enhanced catalase and ascorbate peroxidase activities and blocked H_2O_2 and malondialdehyde content increase in *Z. mays* L., thus alleviating UV-B-induced growth inhibition, preventing chlorophyll content loss and increased quantum yield for photosystem II. In turn, NOS inhibitor (*L*-NNA) significantly increased H_2O_2 and malondialdehyde accumulation and decreased antioxidant enzyme activities in maize leaves under UV-B stress. These results suggest that NO may up-regulate ROS-scavenging system that alleviates UV-B-induced oxidative stress (Kim et al. 2010). It has been reported that SNP (800 μM, 12 h) increased catalase and ascorbate peroxidase activities, lowered H_2O_2 and O^{2-} content and prevented chlorophyll loss and ion leakage in UV-B-exposed soybean (*Glycine max* L.) (30 kJ/m^2, 100 min) (Santa-Cruz et al. 2010). NO produced by NOS-like activity under UV-B exposure regulates heme oxygenase gene (HO-1) expression that protects cells from oxidative damage (Santa-Cruz et al. 2010). The decrease of leaf length and seedlings biomass in *Triticum aestivum* L. cv. Linyou 7287 irradiated with UV-B (10.08 kJ\m^2 d) was alleviated by 100 μM SNP treatment as well as photochemical efficiency, ATPase activity, chlorophyll and carotenoid content (Fv/Fm) (Yang et al. 2013).

It has to be mentioned that not only NO generated by NOS-like sources, but also nitrate reductase-derived NO participates in UV-B response (Gupta et al. 2011). Even more, the inconsistency that different species use different NO sources under the same type of the environmental stress is proposed to be explained by some inaccuracies of NO content measurements by fluorescent assays and non-specificity of the mammalian NOS inhibitors (Gupta et al. 2011). Thus, UV-B-induced flavonoid production was mediated by NOS-like NO production in silver birch (*Betula pendula* L.) that was defined by relevant oxyhaemoglobin assay, and the inhibitors of the mammalian NOS were not effective in this case testifying that NOS-like activity does not input in total NO content (Zhang et al. 2011).

Indeed, NO is an important signalling molecule in UV-B stress not only in higher plants, but also in green algae and cyanobacteria. Thus, SNP (100–300 μM) renovated the activity of numerous UV-B-inhibited enzymes such as nitrate/nitrite reductase, glutamine synthetase, plasma membrane H^+-ATPase, 5′-nucleotidase, peroxidase, ascorbate peroxidase and glutathione reductase in cells of UV-B-irradiated *Chlorella pyrenoidosa* (Chen et al. 2010). Moreover, SNP (500 μM) could markedly alleviate the damage caused by enhanced UV-B-exposure of *Spirulina platensis* by the elevation of chlorophyll *a* and malondialdehyde content, superoxide dismutase (0.95-fold) and catalase (6.73-fold) activities as well as by the increase of biomass. Besides that, SNP pretreatment eliminated the excess of O_2^- and stimulated accumulation of the reduced glutathione (GSH) (Xue et al. 2007). UV-B exposure of $N_{(2)}$-fixing cyanobacterium *Spirulina platensis* caused intensity-dependent inhibition of nitrogenase activity; however, pretreatment with 500 μM SNP increased it almost twofold (Xue et al. 2006, 2011).

Thus, the accumulating evidences of the protective effects of NO donors under UV-B exposure indicate that this secondary messenger could be involved into the signalling events, though the detailed mechanisms of NO-mediated signal transduction remain mainly unclear.

9.3 The Role of NO-dependent Regulatory Cascades in UV-B Perception by Plant Cell

The starting point of UV-B photomorphogenic sensing is its perception by newly identified photoreceptor UV RESISTANCE LOCUS8 (UVR8) present in homodimeric state (Jenkins 2009; Rizzini et al. 2011). For signal transduction, it has to gain monomeric form to interact with COP1 protein (CONSTITUTIVELY PHOTOMORPHOGENIC 1) (Jenkins 2009; Rizzini et al. 2011; Heijde and Ulm 2012; Wu et al. 2012) and induction of the expression of the ELONGATED HYPOCOTYL5 (HY5) transcription factor regulating genes involved in photomorphogenic UV-B responses such as chalcone synthase, phenylammonia lyase and cyclobutane pyrimidine dimer photolyase (Ioki et al. 2008; Rizzini et al. 2011; Jiang et al. 2012). Several negative regulators such as BBX24/STO, RCD1 and RUP1/2 finely tune UV-B morphological responses; however, UV-B pathways in light signalling are far from the completion (Jiang et al. 2012). Among the UV-B-responsive genes those encoding DNA repair, antioxidative defence and flavonoid biosynthesis enzymatic machinery were identified (Zhou et al. 2007; Jenkins 2009). As plants are mainly photosynthetic organisms and light is vital energy source, they developed constitutively expressed and well-conserved sophisticated mechanisms of differential light perception during evolution, e.g. specific families of photoreceptors (Jenkins and Brown 2007; Wu et al. 2012). Thus, in *Arabidopsis*, red/far red is perceived by phytochromes (PHYA, PHYB, PHYC, PHYD and PHYE), UV-A and blue light—by phototropins (PHOT1, PHOT2), cryptochromes (CRY1, CRY2 and CRY3) and members of the Zeitlupe family (ZTL, FKF1, and LKP2) (Jenkins 2009; Rizzini et al. 2011; Wu et al. 2012).

Exposure to UV-B leads to the generation of ROS such as O_2^-, H_2O_2 and $OH^·$ that could cause oxidative damage of DNA, membrane lipids and proteins; however, it was supposed recently that these molecules can play pivotal role in signalling (for review see Brosche and Strid 2003; Jenkins 2009). Both ROS of different biochemical origin and NO act parallel to regulate the expression of stress-related genes such as chalcone synthase to UV-B radiation (Mackerness et al. 2001). As the inhibitor of the mammalian NO-synthase N^{ω}-nitro-*L*-arginine (*L*-NAME) was effective at preventing the induction of chalcone synthase, it can be assumed that the source of NO in response to UV-B exposure is most likely to be NOS (Mackerness et al. 2001). Though the NO-synthase from the marine picoplankton unicellular microalgae *Ostreococcus tauri* has been revealed recently and the activity of NO-synthase-like enzyme was detected in the cytosol, nucleus, peroxisomes and chloroplasts of many plant species, no gene/s or protein/s complex responsible for NO generation from *L*-arginine has not been identified and isolated yet in higher plants (Correa-Aragunde et al. 2013) possibly because the plant NO-synthases possess the inherent structure non-homologous to animal NO-synthases. Moreover, endogenous NO may be the second messenger required for UV-B-induced growth inhibition (Zhang et al. 2003) and during developmental growth of the leaves (Kim et al. 2010).

Numerous proteins are *S*-nitrosylated and tyrosine nitrated under nitrosative stress conditions (Corpas et al. 2008; Lozano-Juste et al. 2011). The cataloguing of UV-B-responsive nitro- and nitrosoproteome would enhance understanding of the NO-dependent mechanisms of UV-B signal transduction. One of the intracellular targets of UV-B is main cytoskeletal proteins from the integrated networks of microtubules and microfilaments, microtubule- and actin-related proteins and others. It is assumed that tubulin and actin as UV-B targets in vitro are UV-B *downstream* effectors able to percept amplify and/or transduce UV-B signal per NO-mediated pathways (Krasylenko et al. 2011). Microtubules and actin filaments reorganization is supposed to be one of the mechanisms of UV-B-induced plant morphological responses (Staxèn et al. 1993; Guo et al. 2010; Chen et al. 2011), however, in dose-dependent manner (Staxèn and Bornman 1994; Jacques et al. 2011; Krasylenko et al. 2012).

In pioneer work of Staxèn et al. (1993), it was reported that the enhanced UV-B (4–24 mmol photons/m^2) reversibly fragmented cortical microtubules in *Petunia hybrida* Vilm. mesophyll protoplasts and inhibited cell cycle progression in G_1/S/G_2 phases. However, these alterations in microtubule organization and the arrest of cell division observed in vitro were not revealed in the epidermal cells of *P. hybrida* leaves grown under UV-B radiation (9 kJ\m^2 day) (Staxèn and Bornman 1994). Cytoskeleton rearrangements are supposed to underlie UV-B-induced changes of anisotropic mechanical properties of barley (*Hordeum vulgare* L.) cell walls and root hair initiation, since 100 μM colchicine treatment resulted in root swelling (Ktitorova et al. 2006). Later, it was shown that the intense UV-B exposure (10.08 kJ/m^2 day) caused stick-and-spot depolymerization of microtubules in *T. aestivum* L. mesophyll protoplasts (Guo et al. 2010). Moreover, *F*-actin arrays in wheat root tip cells under the enhanced UV-B (10.08 kJ\m^2 d) became

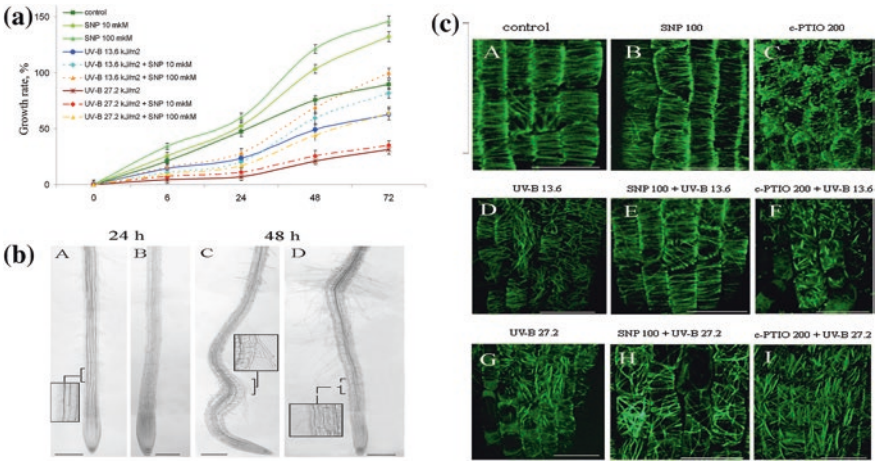

Fig. 9.1 Combined influence of UV-B exposure and SNP pretreatment on growth rate, morphology and microtubules organisation in primary root cells of *Arabidopsis thaliana* (expressing GFP-MAP4) (Krasylenko et al. 2011). **a** Growth rate of primary roots after SNP pretreatment (1 h) with further UV-B radiation (13.6 and 27.2 kJ/m^2); **b** morphological responses of primary roots after 100 μM SNP treatment for 1 h (*A*, *B*) and 100 μM SNP pretreatment with further 27.2 kJ/m^2 UV-B irradiation (*C*, *D*), Bar: 200 μm; **c** microtubular organization in epidermal cells of primary root tips (48 h): control (*A*); 100 μM SNP (*B*); 200 μM *c*-PTIO (*C*); 13.6 kJ/m^2 UV-B, (*D*); 100 μM SNP + 13.6 kJ/m^2 UV-B (*E*); 200 μM *c*-PTIO + 13.6 kJ/m^2 UV-B (*F*); 27.2 kJ/m^2 UV-B (*G*); 100 μM SNP + 27.2 kJ/m^2 UV-B (*H*); 200 μM *c*-PTIO + 27.2 kJ/m^2 UV-B (*I*), Bar: 20 μm

disordered in interphase cells and disintegrated into short fragments of random orientation during prophase/metaphase cells (Chen et al. 2011).

Recently, it was revealed that the interphase and mitotic microtubules in epidermal/cortex cells of all primary root zones of *A. thaliana* (L.) Heynh. seedlings were randomized, depolymerized and/or stabilized after the UV-B exposure (13.6–68 kJ/m^2) that was accompanied by cell swelling and excessive root hair formation. Moreover, NO donor SNP at the level of 10 and 100 μM recovered UV-B (13.6 and 27.2 kJ/m^2)-inhibited root growth (Fig. 9.1a) and altered morphology (Fig. 9.1b) as well as alleviated UV-B damaging effects on microtubular organization (Fig. 9.1c), while the treatment with NO scavenger *c*-PTIO revealed no protective effects (Krasylenko et al. 2011).

It is generally accepted that stimulatory/destructive effects of UV-B as well as their ecological relevance are determined by its acute or ambient doses. Exactly, the recent findings of Jacques et al. (2011) suggest that despite the reduction of *Arabidopsis* leaf epidermal cells elongation induced by chronic UV-B doses (0.1646 W m^{-2}), microtubule arrangements remained unaffected because of unaltered deposition of cellulose microfibrils.

The increased content of exogenous NO in plant cells can protect microtubules organization as well as microtubule-related processes of root growth and development from the disrupting UV-B effects. Thus, we have found recently

UV-B-induced *A. thaliana* primary root growth inhibition and such morphology alterations as root swelling and excessive root hair formation, and also the disturbed organization of microtubules in root cell could be restored by SNP pretreatment at concentration 100 μM (Fig. 9.1) (Krasylenko et al. 2011).

Furthermore, the finding that indirect UV-B-induced dose-dependent randomization and depolymerization of microtubules in cells of transition zone of shielded *A. thaliana* primary roots gives extra evidence for their importance as one from intracellular targets in the perception of UV-B irradiation and further transduction of generated signals to form adequate answers (Krasylenko et al. 2013). Indeed, microtubules are not just a scaffold and anchorage for intracellular organelles, but also common participants in responses of plant cell to such environmental stimuli (Gardiner et al. 2012) as light, gravity, cold, heat, touch and wind (Nick 2008; Yemets et al. 2011). As microtubules in plant cell reorient/reorganize (become randomized, fragmented or depolymerised) in a response to direct UV-B exposure, these cytoskeletal components could be involved into UV-B signalling pathways as a highly responsive players, possibly via posttranslational α-tubulin tyrosine nitration—recently discovered regulatory modification of this cytoskeletal component in plant cell and one of the direct mechanisms of NO signal transduction (Yemets et al. 2009; Krasylenko et al. 2012; Blume et al. 2013). Further investigations will shed light on the NO-mediated signalling through cytoskeleton components as NO downstream effectors and UV-B targets.

9.4 Conclusions and Future Perspectives

Taking into account all existing data, mechanisms of NO input in UV-B signalling are now just emerging. Although several actual NO synthetic pathways in plants have been found, the detailed mechanisms of their activation by environmental challenges including UV-B as well as tissue specificity remain obscure. Proteomic tools are accelerating the discovery of definite sites of NO-modified proteins to establish biotechnological strategies aimed to increase plant stress tolerance and crop productivity. The comparative analysis of the UV-modulated proteins with the nitroproteome (*S*-nitrosylated, tyrosine nitrated and *S*-glutathionylated proteins) as well as the expression profiles of UV-B- (Casati and Walbot 2004) and NO-responsive genes (Grün et al. 2006) is required to reveal the overlapping sets of genes up-or-down-regulated both by UV-B exposure and NO.

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Chapter 10
Nitric Oxide Impact on Plant Adaptation to Transition Metal Stress

Mohammad Mobin, M. Nasir Khan and Zahid Khorshid Abbas

Abstract Nitric oxide (NO), a gaseous bioactive messenger molecule, regulates diverse array of physiological processes ranging from seed germination, growth, flowering to the biotic and abiotic stress responses. Transition metals such as manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni) copper (Cu), and zinc (Zn) are essential micronutrient for normal growth and development of living organisms. Although essential in trace amounts, at higher levels these metals can be toxic to cells because they directly or indirectly influence DNA, protein, and membrane integrity and function. The biological actions of NO and its derivatives exerted through the binding to transition metals of metalloproteins, and covalent modifications of cysteine and tyrosine residues. The presence of transition metal influences the endogenous NO levels and thereby modulates several metabolic processes. Depending on the type of plant tissue and concentrations, the NO can elicit both beneficial and deleterious effects. Recently, several articles have proclaimed the alleviation of transition metal toxicity by exogenous application of NO. The present article discusses the recent understanding of the endogenous and exogenous NO to essential transition metal tolerance.

Keywords Transition metal · Nitric oxide · Iron · Copper · Exogenous application

10.1 Introduction

Earlier studies by Mill and Bennett (1970) reported the toxic action of nitric oxides (NO_2, N_2O_3, NO_2^-, NO_3^-) on the photosynthetic apparatus and chlorophyll levels in selected forest tree species near park and industrial area. But it

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was not until 1998, when the studies of Delledonne et al. (1998) and Durner et al. (1998) proved NO as a signaling molecule in an array of plant physiological functions. Later on, the works of Leshem et al. (1998), Durner and Klessig (1999), Garcia-Mata and Lamattina (2001), Hung and Kao (2003), and Prado et al. (2004) confirmed the participation of NO in various cellular metabolic activities such as a growth and development, respiratory metabolism, senescence, maturation, and abiotic and biotic stress responses.

NO as a very reactive gaseous molecule can combine with the atmospheric oxygen. The reaction between oxygen and NO is of second order of magnitude, whereas molecules of NO react with oxygen to generate various forms of nitrogen oxides such as NO^*, N_2O_3, and N_2O_4. Further, due to hydrolysis or reaction with thiols and cellular amines, these oxide species may get converted into NO_2^- and NO_3^-. The free radical form of NO^* may be transformed by one electron oxidation or one electron reduction into nitrosonium cation (NO^+) and nitroxyl radical (NO^-), respectively (Wojtaszek 2000; Garcia-Mata and Lamattina 2003). It has been noted that NO^* readily reacts with transition metals, especially haem iron and iron–sulfur centers of proteins (Stamler et al. 1992; Wojtaszek 2000). Nonetheless, nitrosonium cation, as an electrophile, may attack sulfur, iron, nitrogen, and carbon centers of various biomolecules. The reversible nitrosylation of sulfhydryl groups of proteins is a very important event as such modifications as *S*-nitrosylation/denitrosylation of proteins affect biological activity of these biomolecules, thus constituting an important element of signal transduction. The mechanisms by which NO help plants to resist the challenges posed by stressful conditions include one of the two by either: (a) upregulating the antioxidant enzyme activity (Hsu and Kao 2004); or (b) chelating the metals in the root cell wall and decreasing the accumulation of the metal to the toxic level (Xiong et al. 2009).

Transition metals such as manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), and zinc (Zn) are essential micronutrient for normal growth and development of living organisms. Degtyarenko (2000) suggested that over half of all proteins in every organism are metalloproteins. Although essential in trace amounts, at higher levels, these metals can be toxic to cells because they directly or indirectly influence DNA, protein, and membrane integrity and function. The biological actions of NO and its derivatives exerted through the binding to transition metals of metalloproteins, covalent modifications of cysteine and tyrosine residues. Considering the importance of transition metals in various physiological actions of plants, this chapter is focused upon recent progress in understanding the function of NO in transition metal toxicity and tolerance in plants.

10.2 Transition Metals in Plants: An Exquisite Balance

The transition metals are a geochemically and isotopically complex group of elements clustered in the center of the periodic table. The term transition metal, according to the International Union of Pure and Applied Chemistry (IUPAC),

refers to an element whose atom has an incomplete d sub-shell or which can give rise to cations with an incomplete d sub-shell. A characteristic of transition metals is that they generally show two or more oxidation states, which leads to differences in the stable isotope composition of the chemical compounds and aqueous species they constitute. As an essential element for nearly all living organism, transition metals play an indispensable role in normal growth and development of the plant. Basic cellular functions are crucially dependent on the redox reactions of these metals. Most of them are cofactors of enzymes and are involved in important processes such as photosynthesis (Mn, Cu), DNA transcription (Zn), hydrolysis of urea into carbon dioxide and ammonia (Ni), and legume nodulation and nitrogen fixation (Co, Zn). Some are involved in flowering and seed production and in plant growth (Cu, Zn), especially when their availability is very low. Fe is an integral part of haem proteins (cytochromes, catalase and ferredoxin). Cu is an indispensable component of electron transfer proteins in photosynthesis (plastocyanin) and respiration (cytochrome c oxidase) and is involved in lignification. Mn a less redox active element is involved in oxygen evolution in photosynthesis. Zn a non-redox-active element has a key structural and/or catalytic role in many proteins and enzymes. Other transition metals such as Ni and Mo are also essential micronutrients for plant function.

Cu, Fe, and Mn are the elements of redox active group, can catalyze the formation of activated oxygen species in a Haber–Weiss or Fenton-type reaction. The Irving–Williams series illustrates that the preferential binding affinities of the divalent forms of transition metals for ligands is Mn < Fe < Zn < Cu. Thus relative to Mn and Fe, the potential for Zn and especially Cu to displace other metals in binding sites of essential proteins is relatively high. Deficiency of any of these metals can seriously hamper the growth of the plant (Marschner 1995). However, although essential, these metals can harmful when present in excess as it will initiate a series of reactions generating reactive oxygen species (ROS) and oxidative damage (Schützendübel and Polle 2002). Therefore, the concentrations of transition metals within the cells must be assiduously regulated for the sustainable growth and development.

10.3 Mechanism of Transition Metal Toxicity

Several reports suggest that the transition metals can interact with nuclear proteins and DNA and trigger the oxidative deterioration of biological macromolecules (Leonard et al. 2004). According to Nieboer and Richrdson (1980), transition metals can readily bind to oxygen, nitrogen, and sulfur atoms of the cysteine residues of the enzymes and finally inactivate them. In a more remarkable way, the transition metal can take part in Fenton-type reaction and redox active metals such as Fe^{2+} or Cu^{+} auto-oxidize to form superoxide anion (O_2^{-}) with subsequent production of hydrogen peroxide (H_2O_2) and OH^{-}. Gallego et al. (1999) and Sharma and Dietz (2009) reported that metals of transition series modulate both

the activities of antioxidative enzymes and the content of soluble antioxidants in plants, followed by an increase of oxidative stresses that includes hydroxylation, DNA fragmentation, lipid peroxidation, and haem protein oxidation.

10.4 Nitric Oxide in Transition Metal Stress

Current understanding of the effects of NO on different types of cell has revealed that NO can act as prooxidant or antioxidant, based on the endogenous level. NO at low concentrations acts as a signal molecule for the activation of defense response, but at higher concentration, it causes severe injury due to uncontrolled ROS generation (Wink and Mitchell 1998). NO molecule has an unpaired electron which enable them to interact with ROS especially O_2^-. Ferrer and Barcelo (1999) and Clarke et al. (2000) proposed that the dual role of NO might be incumbent upon the differences in related cellular processes, developmental stages, or species. In fact, the restricting ROS level directly correlated with survival response under abiotic stress. NO interacts with ROS in various ways and serves as an antioxidant during several stresses, directly scavenging ROS, such as O_2^-, to form peroxynitrite ($ONOO^-$) (Misra et al. 2011), comparatively less toxic than peroxides and thus restrict the cellular damage.

10.5 Transition Metal Stress Alters the Endogenous Level of Nitric Oxide

A considerable amount of nitric oxide is generated or released by plants in certain physiological processes or under stressful conditions (Neill et al. 2003). Endogenous production of NO in plants is commuted, both enzymatically using either nitrite or arginine as substrates (Crawford 2006), and non-enzymatically (Bethke et al. 2004). The results obtained by Zhao et al. (2007) demonstrated that an *Arabidopsis* mutant (Atnoa1) with defective in vivo NOS activity has lower endogenous NO levels than wild-type plants and was more sensitive to salt stress than wild-type plants. However, the reported results signify the importance of metal-induced alterations of endogenous NO levels in different plant species, but the conclusive evidence yet to be achieved as several unexplained queries warrant answers.

The generation of NO is an initial response to iron deficiency in many plant species. Graziano and Lamattina (2007) observed a rapid increase in the endogenous level of nitric oxide in the root epidermis of tomato plants (*Solanum lycopersicum*), under iron deficient conditions. Jin et al. (2011) also noted an increase in the endogenous NO level in Fe-deficient tomato plants (*Solanum lycocarpum*) cv. Micro-Tom. In the cluster roots of white lupin (*Lupinus albus*), deficiency of Fe resulted in enhanced accumulation of NO in the pericycle and rootlet primordia

(Meng et al. 2012). NO production in roots has been linked to the actions of a NOS-type enzyme (Corpas et al. 2006; Guo et al. 2003; Hu et al. 2005) and/or the NR (Hu et al. 2005). The excess of iron resulted in a nitric oxide burst in *Arabidopsis* which proceeded with the ubiquitin-dependent protein degradation for *AtFer 1* ferritin gene expression (Arnaud et al. 2006). In another study, Martin et al. (2009) found an exceptionally high iron content and ROS in mitochondria that corresponded with the elevated endogenous NO level in both the frataxin-deficient *Arabidopsis* plants and yeast.

Copper, a redox-active metal, is an essential element involved as an integral component for the function of key metabolic enzymes of electron transfer chains of mitochondria and chloroplasts (Van Assche and Clijsters 1990; Maksymiek 1997). Cu concentrations are toxic to plant cells, and thus, its uptake and turnover have to be tightly regulated (Yruela 2005). Cu toxicity resulted in elevated NO concentration in the adventitious roots of *Panax ginseng* when exposed to 50 μM Cu for 24 h (Tewari et al. 2008). Furthermore, Bartha et al. (2005) also noted remarkably higher generation of nitric oxide in Cu-treated *Brassica juncea* and *Pisum sativum* plants than with Zn and Cd treatments.

Zn, an essential micronutrient for the normal growth and development of plants, may turn toxic to plants when accumulated to disproportionate level that may hamper the plant growth and development (Todeschini et al. 2011). Accumulation of NO has been implicated in the mechanism of Zn tolerance too. Feigl et al. (2014) noted an enhanced level of NO in the Zn tolerant *Brassica juncea* which and help them to tolerate the excess Zn. Autogenous level of NO was increased in the root tissues of Zn hyperaccumulator *Solanum nigrum* when supplemented with the excess Zn which was correlated well with the enhanced ROS generation (Xu et al. 2010). Furthermore, it was suggested that Zn-induced NO production could be related to Fe deficiency as the excess Zn decreases the Fe uptake (Wintz et al. 2003; Wang et al. 2009) and upregulates IRT1 expression (van de Mortel et al. 2006). In wheat (*Triticum aestivum*) root tissue, the 3 mM Zn treatment evoked an enhanced generation of NO (Duan et al. 2015). The NOS activity was significantly elevated in Zn-treated wheat roots. Additionally, the NOS enzyme activity was depleted and enhanced when NO scavenger PTIO and NADPH oxidase inhibitor DPI was added together with Zn, respectively.

10.6 Exogenous Application of Nitric Oxide Alters the Transition Metal Tolerance Responses

Plants possess an array of defense system to regulate ROS level that includes the antioxidative enzymes superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1), glutathione reductase (GR; EC 1.6.4.2), catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GPX; EC 1.11.1.9), glutathione *S*-transferase (GST; EC 2.5.1.18), and water soluble

compounds such as ascorbic acid (AsA) and GSH (Mobin and Khan 2007). In the presence of greater than the threshold level of the transition metals, Fe, Cu, and Zn initiate the increased production of highly reactive substances such as O_2^- and HO^- (Casano et al. 1997). So, the alleviation of the transition metal stress in the plants will involve the upregulation of the series of events that may counteract the deleterious impacts of ROS.

NO is a small, diffusible, ubiquitous, gaseous molecule which plays a significant part in several mechanisms of abiotic stress tolerance in plants (Lamattina et al. 2003). Unequivocal evidences have indicated that NO protects the plant cells against oxidative damages by scavenging the Fenton reaction Fe and regulating antioxidant enzymes (Laspina et al. 2005; Floryszak-Wieczorek et al. 2006).

Iron as the most abundant redox active element may bind to heme groups, iron–sulfur clusters, or directly reacts with proteins and is essential for many cellular functions. However, in the free ionic form, iron can catalyze the formation of ROS through the Haber-Weiss reaction. These ROS in turn damage cell membranes, DNA, and proteins (Guerinot and Yi 1994; Noctor and Foyer 1998). Iron homeostasis heavily relies on iron-storage proteins, ferritins, which are present in bacteria (Andrews et al. 1991), plant (Lobreaux et al. 1992; Prouhdon et al. 1996), and animal cells (Harrison and Arosio 1996). Since the plant ferritin expression and NO application were related, a role for NO in Fe homeostasis in plants has been proposed (Murgia et al. 2002). Graziano et al. (2002) observed NO-induced greening in Fe-deficient maize plants without changes in total Fe content per gram of fresh matter while Graziano and Lamattina (2007) have suggested NO in Fe uptake. Zhang et al. (2012) and Kong et al. (2014) showed that exogenous application of NO under Fe deprivation stress significantly increased the activities of H^+-ATPase and Fe^{3+} reductase and total Fe concentration in the leaves of peanut (Table 10.1). It also enhanced the actual photochemical efficiency (ΦPSII) and photochemical maximum efficiency of PSII (Fv/Fm). The indicator of oxidative stress malondialdehyde (MDA) was lower in NO-treated peanut leaves while the activities of antioxidative enzymes such as SOD, POD, and CAT were enhanced. Analogous to these observation, Kumar et al. (2010) also recorded enhanced activities of enzymes involved in antioxidative process and depletion of H_2O_2 and non-protein thiol content in NO-treated maize plants when iron was deficient (Table 10.1).

Supplementation of NO (10 μM) in *Arabidopsis* resulted in the depletion of O_2^- and H_2O_2 levels (Peto et al. 2013). Mostofa et al. (2014) found that exogenous NO diminished a Cu enabling the adventitious root of *Panax ginseng* to cope with Cu toxicity (Table 10.1). Furthermore, Hu et al. (2007) studied the effect of exogenous copper application on Cu toxicity in wheat. They observed that NO donor SNP sustained a lower level of MDA, stimulated the activities of SOD and CAT, and decreased the activity of lipoxygenases. In accordance with this, Cui et al. (2010) noted that exogenous application of NO alleviates the effect of excess Cu through the activation of some antioxidative enzymes, decreased accumulation of H_2O_2 and adjustment of H^+-ATPase and H^+-PPase in the plasma membrane or tonoplast of tomato plants. Exogenous NO counteracted a Cu-induced

Table 10.1 Supplementation of exogenous NO and alleviation of transition metal induced oxidative stress

Transition metal	Plant	NO concentration (μM)	Mechanism of stress alleviation	References
Cu	Tomato	100	Promoted antioxidant enzymes	Cui et al. (2010)
			Reduced accumulation of H_2O_2	
			Adjusted the activity of H^+-ATPase and H^+-PPase in plasma membrane or tonoplast	
	Ryegrass	100	Improved antioxidant enzyme activities	Dong et al. (2014)
			Reduced oxidative damages	
			Increased Cu concentration in roots and restriction of Cu accumulation in leaves	
	Arabidopsis	10	Modulation of O_2^- and H_2O_2 levels	Peto et al. (2013)
	Wheat	100	Stimulation of the activities of SOD and CAT	Hu et al. (2007)
			Decreased activities of lipoxygenases, sustained lower level of MDA	
	Rice	200	Lowered the LOX activity, O_2^-, H_2O_2 MDA, and proline content	Mostofa et al. (2014)
			Decreased SOD, APX, GR, MDHAR and glyoxalase I and glyoxalase II activities	
			Enhancement of the activities of CAT, GPX, DHAR, and GST	
			Increased level of AsA and PC	
Ni	Wheat	100	Enhanced activities of GPX, APX, SOD, GR, and GST	Wang et al. (2010)
	Tomato	100	Enhanced activities of CAT, GPX, and APX in leaves	Kazemi (2012)
			Increased sequestration of Ni in roots	
	Bean	300	Decreased SOD activity	Mihailovic and Drazic (2011)
			Increased POX and CAT activity	
	Brassica napus	200	Reduction of Ni translocation from root to shoot	Kazemi et al. (2010)
			Increased activity of antioxidative enzymes	
Fe deficiency	Maize	100	Decreased SOD activity	Kumar et al. (2010)
			Increased activities of APX, CAT, and POD	
			Reduction in the contents of H_2O_2 and non-protein thiol	
	Peanut	1000	Increased activities of APX, CAT, and POD	Zhang et al. (2012)
			Reduced MDA accumulation	

(continued)

Table 10.1 (continued)

Transition metal	Plant	NO concentration (μM)	Mechanism of stress alleviation	References
	Peanut	100	Enhanced actual photochemical efficiency (ΦPSII) and photochemical maximum efficiency of PSII (Fv/Fm)	Kong et al. (2014)
			Significant increase in H^+-ATPase and Fe^{3+} reductase activities	
			Enhanced leaf Fe concentration	
			Enhanced activity of SOD, POD, and CAT	
Zn deficiency	Wheat	100 (GSNO)	Reduced form of ascorbate in roots increased	Buet et al. (2014)
			Total glutathione remains unchanged	
			Enhanced Zn translocation from root to shoot	
Zn deficiency/ toxicity	Wheat and Bean	100	NO alters the total and free/total SH, GSH, and SOD activity	Abdel-Kader (2007)
			Maintain the sufficient level of Zn	
Mn toxicity	Rice	100	Reduced levels of H_2O_2 and lipid peroxides	Srivastava and Dubey (2012)
			Increased activities of SOD, GPX, CAT, and GR	
	Chamomile	500	Increased activity of GPX and GR	Kováčika et al. (2014)
			Decreased the superoxide and H_2O_2 accumulation	

increase in SOD, APX, GR, MDHAR, glyoxalase I, and glyoxalase II activities in rice (Mostofa et al. 2014). In ryegrass, the addition of NO to Cu stressed plants improved the antioxidant enzyme activities, reduced the oxidative damages, and restricted the accumulation of Cu in leaves (Dong et al. 2014) (Table 10.1).

Ni, essential element for the urease activity, stimulates the growth at low concentrations, but at higher concentrations, it can retard the growth and metabolism of plants (Seregin and Kozhevnikova 2006). Although the mechanism of Ni toxicity is still about to be unraveled but analogous to other transition metals, Ni also triggers oxidative stress (Gajewska et al. 2006; Yan et al. 2008). Ni-induced oxidative stress was shown to be alleviated by the exogenous application of nitric oxide in *Brassica juncea* (Kazemi et al. 2010), tomato (Kazemi 2012), and wheat (Wang et al. 2010) (Table 10.1). They noticed that the application of SNP enhanced the activities of GPX, APX, SOD, CAT, POX, GR, and GST and reduced the translocation of Ni from root to the shoot. Similar to this, Mihailovic and Drazic (2011) observed that Ni toxicity in bean could be counteracted by the application of NO as it decreased the SOD activity and increased the POX and CAT activity.

Zn as one of the essential element for plant is intimately involved with the growth, photosynthesis, nitrogen metabolism, and protein synthesis (Broadley et al. 2007; Wang et al. 2009). As constituent of copper/zinc superoxide dismutase (Cu/Zn SOD), the Zn deficiency may also affect the antioxidative enzyme activity and accumulation of ROS (Sharma et al. 2004). Apart from deficiency, the higher concentration of Zn may disrupt the photosynthesis, transpiration, and antioxidant defense system. It may also disturb the balance of uptake and redistribution of mineral nutrient in the plants. Wo'jcik et al. (2006) have found that excess Zn affect the activities of SOD, CAT, and APX. Exposure of plants to higher concentrations of Zn could result in the deficiency of Fe that may lead to ROS accumulation and oxidative stress (Tewari et al. 2008). It has been demonstrated that NO plays a significant role in Zn homeostasis. Buet et al. (2014) have shown that the addition of exogenous NO (in the form of GSNO) in Zn-deficient plants enhanced the translocation of Zn from root to shoot and also increased the ascorbate content in wheat. Moreover, Abdel-Kader (2007) reported that at both the deficient and toxic level of Zn, application of exogenous NO alters the total and free/total SH, GSH, and SOD activity in wheat and bean. He also noted that NO helps to maintain the sufficient level of Zn in the plant tissues (Table 10.1).

The deleterious effects of Mn toxicity in plants include stunted growth, chlorosis, crinkled leaves, and brown lesions. The presence of Mn is necessary for the water-splitting system that furnishes electrons to PSII. Mn is involved in redox reactions as a cofactor for Mn-containing isozyme of Mn-SOD, which is one of the essential mechanisms in protection against oxidative stress in plants (Bowler et al. 1994). In relation to alleviation of toxic effects of Mn by exogenous NO, Srivastava and Dubey (2012) have observed that the level of H_2O_2 and lipid peroxides decreased and SOD, GPX, CAT, and GR activities increased when NO was present along with the toxic Mn concentrations (Table 10.1). Similar results were noted in chamomile by Kováčika et al. (2014), who found that exogenous NO

(500 μM SNP) increased the activities of GPX and GR and depressed the accumulation of superoxides and H_2O_2.

10.7 Conclusions

Nitric oxide targets the transition metals, especially haem iron and iron–sulfur centers of proteins (Stamler et al. 1992; Wojtaszek 2000). So understanding the basic mechanism involved in NO-bolstered protection against transition metal stress condition by eliminating the O_2^- and reducing toxicity caused by ROS together with its physiological effects is necessary for the analyzing the antitoxicity mechanism of NO. These approaches may include the identification of the endogenous sources of nitric oxide and NO biosynthetic pathway in the plants.

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Chapter 11
Nitric Oxide Action in the Improvement of Plant Tolerance to Nutritional Stress

Vasileios Ziogas and Athanassios Molassiotis

Abstract Nitric oxide (NO) is a chemical messenger that actively operates in the plant kingdom. In recent years, NO has been shown to be involved in many growth, developmental, and physiological processes in plants. NO has been also shown to be involved in the metabolic reactions evoked by abiotic stresses, including mineral nutrients-associated stressful conditions. This chapter presents an overview of the present knowledge on the involvement of NO in plant response to nutritional stress, with special emphasis to salinity, calcium and iron homeostasis, and heavy metal stress.

Keywords Heavy metals · Iron · Nitric oxide · Salinity

11.1 Introduction

Plant nutrition is one of the fundamental aspects of plant growth and development under stressful and physiological conditions. The overall processes involved in the application of nutrient to the soil, the movement of the nutrients to the rhizosphere, the uptake by the roots, and finally the translocation and utilization of nutrient by the plant are all the elements of plant nutrition (Epstein and Bloom 2004). Plant survival under those adverse environmental conditions is crucial and is depended upon the ability of plants to perceive the deleterious stimulus, generate and translocate specific messenger molecules (signals) to target molecules, and finally orchestrate the biochemical pathways toward stress tolerance (Misra et al. 2011).

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During the last decade, several studies have established the crucial signaling role of NO in both animals and plants (Wendehenne et al. 2001; Neill et al. 2003; Corpas et al. 2004, 2006; Delledonne 2005). NO is an electroneutral lipophilic gas with moderate solubility in water considered a highly reactive membrane-permeable free radical, initially characterized as highly toxic compound (Krasylenko et al. 2010). NO molecule exist within the cell in three interconnecting forms, as the free radical nitric oxide ($NO^{\cdot}$), as nitrosonium cation (NO^{+}) and as nitroxyl anion (NO^{-}) (Hong et al. 2008). Within the cell, the molecule *S*-nitrosoglutathione (GSNO) is considered the long distance carrier and reservoir of NO in plant cells, which is achieved via S-to-N- and S-to-S-trans-nitrosylation of protein thiol groups (Besson-Bard et al. 2008; Krasylenko et al. 2010). The chemistry of NO and the interaction with other molecules are based upon a series of reactions, namely, nitration (addition of nitryl), nitrosylation (addition of nitrosyl residue from NO^{+} to amide, thiol or aromatic hydroxyl group), nitrosation (addition of $NO^{\cdot}$ free radical) causing the formation of C–N, N–N, S–N, and N–O bonds, and finally oxidation (self oxidation and production of peroxynitrite $ONOO^{-}$) (Arasimowicz and Floryszak-Wieczorek 2007; Krasylenko et al. 2010). NO can be produced either by enzymatic or non-enzymatic pathways, and its ability to exert a protective or a toxic role in different cell compartments is depended upon the concentration or situation occurred (Crawford and Guo 2005; Delledonne 2005). During the last few years, many research groups have attributed to NO the role of a key signaling molecule in different intercellular processes in plants. In this chapter, aspects of the interactive role of NO with mineral nutrients under some common stress factors are presented.

11.2 Modulation of K^{+}/Na^{+} Homeostasis by NO Under Salinity Stress

Salinity is one of the major environmental factors that limit plant growth and productivity by disrupting physiological processes leading to ionic toxicity and nutritional disorders (Zhu 2003; Zhao et al. 2007). Under salinity stress, plants developed an array of strategies in order to achieve ion homeostasis and overall survival, including selective accumulation or exclusion of Na^{+} to the external medium, control of ion uptake by roots, sequester excess salt into the vacuoles, and/or compartmentalize it in different tissues, maintain K^{+} uptake and high K^{+}/Na^{+} ratio into the cytosol (Zhu 2003). Under salinity, K^{+} uptake by the roots is blocked as a result of Na^{+} uptake and the overall levels of Na^{+} and Cl^{-} are increased with a parallel decrease of K^{+}, Ca^{2+}, and Mg^{2+} (Aziz and Khan 2001). In order to avoid cellular damage and nutrient deficiency, plants under salinity stress, try to maintain high K^{+} and low Na^{+} concentrations into the cytosol (Serrano et al. 1999; Misra et al. 2011). Research has proven the role of plasma membrane (PM) H^{+}-ATPase in the adaptation of plants to salinity, as well as the involvement of symporters and antiporters and channels associated with PM and

tonoplasts (Morsomme and Boutry 2000). Active Na^+ extrusion from the cytosol is facilitated by transmembrane transport proteins, such as PM-located Na^+/H^+ antiporters and vacuolar membrane-located Na^+/H^+ antiporters (Chen et al. 2010; Oh et al. 2010) which are energy demanding and driven by the electrochemical gradient created by PM H^+-ATPase (Serrano 1996) and by vacuolar membrane H^+-ATPase (V-H^+-ATPase) and H^+-pyrophosphatase (V-H^+-PPase) (Chen et al. 2007). PM H^+-ATPase belongs to a family of P-type ATPase, which has a catalytic subunit of approximately 100 kDa. This specific enzyme is a proton pump, whose major task is to couple ATP hydrolysis to proton transport resulting in the creation of electrochemical gradient across the PM, used by the secondary transporters (Serrano 1989). Previous reports indicated that regulation of the PM H^+-ATPase and Na^+/H^+ antiporters are key elements in salt tolerance (Zhao et al. 2004; Zhang et al. 2007).

Nitric oxide, produced under salinity could act as a second messenger that reprogram potential plant strategies, such as the induction of PM H^+-ATPase expression (Zhao et al. 2004), the depression of oxidative membrane damage, the translocation of Na^+ from roots to shoots (Misra et al. 2011), and the stimulation of K^+ uptake (Zhao et al. 2004; Khan et al. 2012). There are several reports indicated the interplay of NO with nutrient uptake and translocation under salinity. Zhao et al. (2004) reported a significant improvement in salinity resistance in NO treated calluses of two *Phragmites communis* ecotypes, with different salt tolerance ability, via the decrease in Ca^{2+} concentration and the shift of K^+/Na^+ ratio. Also in the work of Zhang et al. (2004) in *Zea mays* seedlings and Khan et al. (2012) in excised mustard leaves, treatment with the NO donor sodium nitroprusside (SNP) resulted in the stimulation of K^+ uptake and translocation to the shoots with parallel decrease in the accumulation and transport of Na^+. Evidence suggests that NO resulted in salt tolerance in calluses of *Populus euphratica* by increasing the K^+/Na^+ ratio through the augmentation of the PM H^+-ATPase activity, demonstrating an interaction of NO with nutrient uptake (Zhang et al. 2007). NO produced under salt stress may serve as a second messenger molecule leading to the induction of PM H^+-ATPase expression, maintaining high K^+ to Na^+ uptake and increased Ca^{2+} uptake, confirming NO-associated salt tolerance mechanism (Zhao et al. 2004). In the work of Ruan et al. (2004) even though NO did not affect the Na^+ content, it significantly increased the K^+ level, thus leading to an augmentation of the K^+/Na^+ ratio in roots of wheat seedlings under salinity stress. The beneficial role of NO toward salinity stress tolerance was also indicated in the work of Wang et al. (2009) where treatment with *SNP* alleviated the salt-derived injury in callus of wild-type Arabidopsis through the maintaining of high K^+/Na^+ ratio and increased PM H^+-ATPase activity. Similar effect was recorded in *Arabidopsis* mutants (*AtNOA1*) treated with 100 μM SNP, suggesting an interplay of NO and salinity tolerance via nutrient regulation (Zhao et al. 2007). Furthermore, Chen et al. (2010) suggested that NO functions as a signal in salt resistance of *Avicennia marina* by enhancing salt secretion and Na^+ sequestration coupled with the induction of the expression of H^+-ATPase and Na^+/H^+ antiporters. In this sense, Zhang et al. (2006) investigated the mode of action through

which NO regulates Na^+ and K^+ homeostasis under salinity in maize plants. The authors suggested that NO acts as a signal molecule resulting in increased activities of vacuolar H^+-ATPase and H-PPase, providing the necessary driving force for Na^+/H^+ exchange.

11.3 The Interplay of NO with Calcium Under Abiotic Stress Conditions

Calcium (Ca^{2+}) is one of the essential plant macronutrients which is absorbed by the roots and translocated, through the xylem, to the shoots where orchestrates many physiological processes mainly involved in cell wall structure and membrane permeability (Tuteja 2007; Tuteja and Sopory 2008). In various plant systems, Ca^{2+} has long been established as an intercellular second messenger leading to plant stress tolerance responses (Zhao et al. 2004; Courtois et al. 2008; Tuteja and Sopory 2008). Research progress upon animal cells has indicated similarities between Ca^{2+} and NO signaling, considering their spatial and temporal organization (Stamler et al. 2001). An interconnected role of NO and Ca^{2+} is highlighted by the existence of transduction networks where Ca^{2+} possess the dual role of promoter and sensor of NO signaling (Clementi 1998). Also, the suppression of the elicitor-induced NO synthesis in tobacco and grapevines cells, by the usage of pharmacological agents that inhibit mammalian NOS activity and increase of $[Ca^{2+}]_{cyt}$, strengthens the necessity of Ca^{2+} in L-arginine-dependent NO synthesis (Vandelle et al. 2006; Courtois et al. 2008). Courtois et al. (2008) proposed that changes in cytosolic Ca^{2+} might participate in the regulation of NO biosynthesis in plants, and NO might be a key element in the signaling cascade initiated by Ca^{2+}.

In plants exposed to abiotic stress conditions, parallel changes in $[Ca^{2+}]_{cyt}$ and NO levels have been reported (Garcia-Mata et al. 2003; Gould et al. 2003), and recent reports attribute to NO the role of cytosolic regulator of Ca^{2+} homeostasis in plant cells (Vandelle et al. 2006; Aboul-Soud et al. 2009). Notably, it has been shown that in tobacco plants (*Nicotiana plumbaginifolia)* under the effect of hyper-osmotic stress by sorbitol, NO contributes to an increased level of $[Ca^{2+}]_{cyt}$ concentration by facilitating the mobilization of free cytosolic Ca^{2+} (Gould et al. 2003). Also a correlation between the concentration of cytosolic Ca^{2+} and NO was found in the ABA-induced stomatal closure of guard cells (García-Matta and Lamattina 2003). Emerging evidence suggested that both Ca^{2+} and NO-mediated signaling pathways participate in the ABA inhibition light-induced stomatal opening in *Vicia faba* cells (Garcia-Matta and Lamattina 2007). In the work of Lamotte et al. (2006), experimental evidence links NO with the regulation of Ca^{2+} homeostasis in plant cells. In this work, by using *N. plumbaginifolia* cells subjected to hyper-osmotic stress showed that NO was able to activate both PM and intercellular Ca^{2+}-permeable channels through signaling cascades involving PM depolarization, cyclic adenine dinucleotide phosphate ribose (cADPR) and protein

kinases. Lamotte et al. (2005) suggested that NO participates in the mobilization of intercellular Ca^{2+} through the manipulation of Ca^{2+} channels by NO. NO also influenced the absorption of Ca^{2+} in an ecotype of reed (*Phragmites communis* Trin.) by demonstrating elevated levels of Ca^{2+}, in the dune reed callus, proposing a potent protection against salinity due to increased Ca^{2+} levels via NO regulation (Zhao et al. 2004). It has been also proposed that NO regulates the signaling cascade via Ca^{2+} mobilization in plant cells (Palavan-Unsal and Arisan 2009). Arasimowicz and Floryszak-Wieczorek (2007) suggested that NO may act through cyclic guanosine monophosphate (cGMP) and cADPR in order to modulate intercellular Ca^{2+}-permeable channels with the outer goal the increased levels of free cytosolic Ca^{2+} levels. Even though the induction of cADPR synthesis by NO has not been established in plants (Lamotte et al. 2005), the addition of cADPR in tobacco cells induced the expression of *PAL* and *PR-1* genes, demonstrating a resemblance of the NO-induced expression (Durner et al. 1998).

11.4 Role of NO in Plant Iron Homeostasis Under Nutritional Stress

Iron is an essential mineral nutrient for plant growth and development (Curie and Briat 2003). The tight control of Fe homeostasis is of vital importance in plants to cope with the abiotic stress induced by Fe starvation and Fe toxicity (Sun et al. 2007; Ramirez et al. 2010). Plants have adopted two distinct series of morphological and physiological reactions for iron acquisition, namely the Strategy I, for the non-graminaceous plants and the Strategy II, for graminaceous ones (Graziano et al. 2002). Under Fe deficiency, dicot and non-grass monocot roots apply the Strategy I reactions in order to achieve Fe solubility and uptake, which are characterized by: (i) induction of rhizosphere acidification mediated by the induction of H^+-ATPase, (ii) enhanced activity and expression of Fe^{+3}-chelate reductase (FRO), (iii) increased expression of Fe^{+2}-transporter (IRT), and (iv) root hair proliferation (Molassiotis et al. 2006; Ramirez et al. 2008). In contrast, grasses (graminaceous plants) correspond to Fe starvation by implying Strategy II array of reactions, which are characterized by the secretion of phytosiderophores to facilitate Fe uptake from the soil.

Recent studies have established NO as a new key player in plant Fe homeostasis, metabolism, and nutrition, based upon strong interactions of NO with Fe, the Fe-storage protein ferritin, and cellular redox state (Murgia et al. 2002; Graziano and Lamattina 2005, 2007; Ramirez et al. 2010). NO-derived molecules can interact with Fe are based upon its chemical properties. The non-charged form of nitric oxide ($NO^{\cdot}$) reacts with metals, forming nitrosonium cation (NO^+), which reacts with thiol groups resulting in the formation of *S*-nitrosothiols (Watts and Richardson 2002). Also, NO is capable of forming complexes with transition metal present in aqueous solutions or in the nucleophylic moieties of metalloproteins, justifying the high affinity of NO toward Fe (Stamler et al. 1992). Another

interaction of NO with Fe is the formation of mononitrosyl-iron complexes (MNICs) or dinitrosyl-iron complexes (DNICs) (Vanin 1998). Furthermore, there is evidence indicated that NO is a key component in the regulation of Fe uptake and homeostasis in plants. Tomato plant (Strategy I) responded to Fe-deficient conditions by controlling the transcription factor FER, a helix-loop-helix (bHLH) protein (Brumbarova and Bauer 2005). Under Fe-deprived conditions, exogenous application of NO enhanced the accumulation of *FER*, *FRO*1, and iron responsive transporter 1 (*IRT*1) mRNAs in the root of tomato plants, increased the activity of FRO, and improved root hair proliferation along with overall plant fitness. Also, the prevention of the up-regulation of *FER*, *FRO1,* and *IRT*1 genes along with the inability of NO to induce Fe-deficient responses in the *fer* mutant plant indicates primarily that NO is required for the expression of genes involved in Fe uptake under Fe-starvation stress, and secondly that the FER protein is necessary for the induction of NO-mediated responses under Fe starvation (Ramirez et al. 2010; Graziano and Lamattina 2007). In another work by Graziano et al. (2002), the addition of NO to maize plants grown under low Fe concentrations resulted in the amelioration of Fe starvation by inducing the augmentation of the chlorophyll content. NO was also able to revert the chlorotic phenotype of two Fe-inefficient maize mutants (*ys*1 and *ys*3), both impaired Fe-uptake mechanisms. Addition of the 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl imidazoline-1-oxyl-3-oxide (*cPTIO*), a *NO scavenger*, to Fe-sufficient plants resulted in Fe-originated chlorosis in younger leaves, demonstrating a potent physiological regulatory role of NO in Fe nutrition. The authors suggested that NO exhibits its biological action by facilitating the availability of Fe inside the plant, since there are no changes in the total Fe content in maize mutants. The latter assumption is strengthened by the fact that plants grown under Fe-deficient conditions do not demonstrate increased total Fe content after supplementation with NO due to remobilization of Fe within the plant by NO, as already been reported in animal cells (Vanin et al. 2004). This hypothesis was also stated by Jasid et al. (2008) who demonstrated that SNP improves internal Fe availability with a parallel increase of the labile Fe pool in sorghum embryonic axis, without analogous increase of total Fe concentration. Iron homeostasis is also strongly regulated by ferritins, a Fe-storage proteins formed by 24 subunits arranged to form a protein coat able to sequester up to 4500 Fe atoms in a non-noxious form (Murgia et al. 2002). In the work of Murgia et al. (2002), NO was identified to be involved in the regulation of Fe-induced ferritin expression in plants. Also, they showed that the NO-mediated induction of ferritin is regulated in a dose-depended manner and is still active even under the effect of different Fe scavengers (Murgia et al. 2002). The group showed that NO mediates plant ferritin regulation via the iron dependent regulatory sequence (IDRS) of the *AtFer*1 promoter, which is also an element involved in the transcriptional repression of *AtFer*1 under Fe-starvation conditions (Murgia et al. 2002; Ramirez et al. 2010). Arnaud et al. (2006) also showed that Fe overload is capable of producing rapid NO burst in cell plastids. Taking the above into consideration, Xiong et al. (2010) stated that exogenous addition of NO possible alleviates Fe-toxicity stress by inducing the expression of ferritin and regulating the homeostasis of excessive

Fe. Ramirez et al. (2010) gave a profile of the downstream signals that are induced by NO under either Fe starvation or toxicity. In Fe-deficient conditions, NO is acting like a key molecule that orchestrates the Strategy I responses, while under Fe-toxic levels (overload), NO is generated and demonstrates a crucial role in the induction of ferritin gene expression, diminishing excess Fe level and finally control the oxidative stress.

11.5 The Interplay of NO with Mineral Nutrients Under Heavy Metal Stress

In the recent decades, many anthropogenic activities have attributed to the heavy metal contamination of natural ecosystems, causing heavy metal stress. Plants, in order to cope with the deleterious effects of heavy metal stress, developed an array of defense strategies, such as metal exclusion, active excretion, restricted distribution of the metal to sensitive tissues, metal binding to the cell wall via metal immobilizing pectic sites and histidinyl groups, chelation by specific peptides called phytochelatin, compartmentalization to the vacuole, enrichment in leaf trichomes, and induction of the antioxidant machinery (Benavides et al. 2005; Verbruggen et al. 2009).

Among the physiological processes mediated by NO, the regulatory effect under heavy metal stress is gaining the attention of many research groups. It has been proposed a protective role for NO, via the NO donors, against heavy metal stress and particularly Cd toxicity (Gill et al. 2013), firstly due to the ability of NO to act as an antioxidant and secondly by provoking the activation of an array of antioxidant enzymes and genes (Xiong et al. 2010; Arasimowicz-Jelonek et al. 2011; Gallego et al. 2012; Gill et al. 2013). Recent scientific reports try to elucidate aspects of the interplay among NO and the uptake of nutrient elements under heavy metal stress. For example, it has been observed that NO, by affecting root wall components, might lead to Cd accumulation in root cell wall, causing a reduction of Cd concentration in the arial part of the plant (Xiong et al. 2009). Furthermore, it has been indicated that NO contributes to the deleterious effect of Cd toxicity by facilitating Cd uptake and subsequent Cd-provoked root growth inhibition, an effect that reverted by the NO scavenger cPTIO in Arabidopsis (Besson-Bard and Wendehenne 2009) and wheat (Groppa et al. 2008). Meanwhile, Cd possesses the ability to enter root cells via IRT1. The antagonism between Cd and Fe leads to the reduction of the intercellular Fe pool, favoring NO biosynthesis. The resulting NO, accumulates and initiates the activation of the Fe-starvation pathway, causing the up-regulation of the Fe acquisition-related genes *IRT*1, Ferric Reductase Oxidase 2 (*FRO*2) and Fe-deficiency Induced Transcription Factor (*FIT*), facilitating Cd uptake. It has also been hypothesized that the accumulation of NO in root cells favors Cd toxicity by promoting Cd ions versus Ca^{2+} uptake and/or Ca^{2+} extrusion, partly by regulating the activity of Ca^{2+}-permeable channels and/or Ca^{2+} transporters (Besson-Bard et al. 2009). A cross-talk among

Ca^{2+}, Cd, ROS and NO has been established in plants, where exogenous addition of Ca^{2+} under Cd stress, caused a reduction of the Cd-depended ROS formation and restored the NO levels to that of the control plants (Rodríguez-Serrano et al. 2009). In addition, Ma et al. (2010) showed that NO regulates Cd uptake and promotes the overall Cd accumulation in BY-2 cells, having a positive impact in $CdCl_2$-induced Programmed Cell Death (PCD) in tobacco. In this work, the research group observed that the addition of SNP accelerated the PCD, while the usage of NO scavengers, like c-PTIO or L-NAME, alleviated the toxicity. In another work of Wang et al. (2013) it has been found that NO influenced absorption of Ca^{2+}, Mg^{2+} and Zn^{2+} under Cd toxicity in ryegrass. Under Cd toxicity the concentrations of Ca^{2+}, Mg^{2+} and Zn^{2+} were significant decreased, indicating that Cd participates in the disturbance of ion homeostasis, and the addition of NO stimulated the mobilization of nutrients to the ryegrass seedlings. Based on this finding, it has been proposed that the latter stimulation of nutrient uptake under Cd stress could be attributed to the stimulation of PM H^+-ATPase by NO, causing the absorption of Ca^{2+}, Mg^{2+} and Zn^{2+} (Wang et al. 2013). It has been well established that PM H^+-ATPase is implicated in the mobilization of multiple ions and that NO facilitates the activity of PM H^+-ATPase (Cui et al. 2010). Under heavy metal stress, such as Cd, Cu or Al, the activity of H^+-ATPase is inhibited (Burzynski and Kolano 2003). It has been also proposed that the ability of plants to transport heavy metal ions, such as Cu, Zn or Cd from the cytoplasm into the

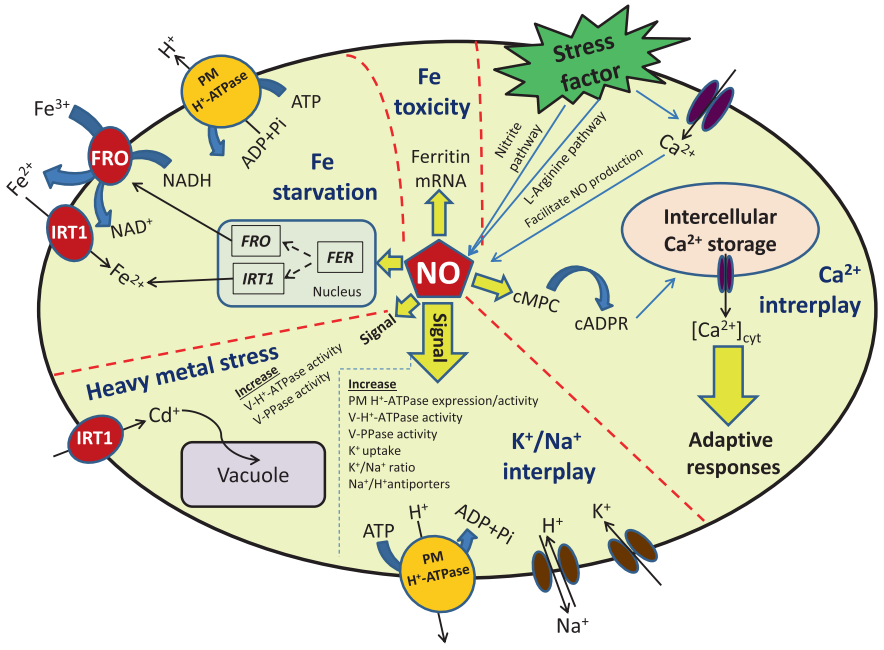

Fig. 11.1 Hypothetical model of the active interaction between NO and *ionic homeostasis* in plants (see text for details)

vacuole via the tonoplast is considered a potent survival strategy toward heavy metal stress (Cui et al. 2010). Exogenous addition of NO significantly elevated the activities of PM H^+-ATPase, V-H^+-ATPase and H^+-PPase under the effect of Cu stress, attributing to NO a potent interactive role in ion homeostasis under heavy metal stress (Cui et al. 2010).

11.6 Conclusion

In conclusion, multiple lines of evidence presented above suggest that there is a biologically active interplay between NO and nutrient homeostasis (Fig. 11.1). Therefore, there is a need of greater understanding of the impact of NO in plant nutrition. Powerful molecular tools, including metabolomictranscriptome and proteome analyses, sequencing of entire genomes in plants, bioinformatic analyses and functional studies, will enable the dissection of the cross-talk between NO and ion balance in plants.

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Chapter 12
Role of Nitric Oxide in Heavy Metal Stress

R. Cerana and M. Malerba

Abstract Nitric oxide (NO) is a diffusible gaseous molecule first identified in mammalian systems as the endothelium-derived relaxing factor, a potent endogenous vasodilatator. NO is also synthesized and released by plants, where it is involved in several physiological processes and in adaptative response against different stresses; hence, NO is considered to be a general signal molecule in plant and animal cells. In recent years, heavy metals (HMs), naturally present or added to the soil through diverse anthropogenic activities, have become a problem of agricultural and environmental significance. Although the mechanism by which HMs affect plants integrity is not fully understood, there is increasing evidence suggesting that an important component of the responses against HM stress is NO. This chapter presents an overview on the effects of HMs on endogenous NO content. In addition, the role of exogenous-applied NO in alleviating HM toxicity is summarized and discussed.

Keywords Heavy metals · Nitric oxide · Reactive oxygen species · Reactive nitrogen species · Stress

12.1 Introduction

In recent years, heavy metals (HMs), naturally present in soils or added through diverse anthropogenic activities, have become a problem of agricultural and environmental significance. Unlike many organic compounds that can be metabolically

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degraded or transformed, the non-biodegradability of HMs leads to prolonged persistence in the environment. This leads to the bio-enrichment of food chain endangering people's health (Sharma and Dietz 2008). Although the mechanism by which HMs affect plants integrity is not fully understood but, there is increasing evidence suggesting that an important component of HM toxicity is oxidative damage (Laspina et al. 2005). Many reports undoubtedly demonstrate that HMs cause oxidative stress by altering both the activities of antioxidative enzymes and the content of soluble antioxidants (Sharma and Dietz 2008). Among about 40 HMs, they can be broadly grouped into non-redox-active (e.g., zinc and cadmium) and redox-active elements (e.g., iron and copper). The redox-active metal ions can facilitate the fast generation of membrane damaging reactive oxygen species (ROS) during the Fenton reaction. The non-redox-active metals instead cannot generate free radicals directly in single-electron reactions, but they could indirectly trigger oxidative stress by destroying the balance of production and scavenging of ROS. Thus, in general, all HMs are able to induce the production of ROS. In addition, HMs can directly interact with proteins due to their affinities for thioyl-, histidyl-, and carboxyl-groups, causing the metals to target structural, catalytic, and transport sites of the cell and can displace essential cations from specific binding sites, causing functions to collapse. For example, Cd^{2+} replaces Ca^{2+} in the photosystem II reaction center, causing the inhibition of PSII photoactivation (Faller et al. 2005). Among many effects of HMs, the accumulation of ROS is one of the earliest and best demonstrated. The most ROS is hydroxyl radical (OH·), which is able to react directly with biological membranes causing lipid peroxidation. Superoxide radical ($O_2^{\cdot -}$) does not penetrate membranes, but it is able to reduce transition metal complexes of Fe^{3+} and Cu^{2+}, thus affecting the activity of metal-containing enzymes. Hydrogen peroxide has properties such as biological membrane permeability and relative long half-life which enable it to act as a signal molecule. In addition, it may inactivate enzymes by oxidizing their thiol groups (Mahalingam and Fedoroff 2003). Thus, apart from their toxic effects, ROS are involved in signaling events during different normal and stress conditions (Apel and Hirt 2004).

Heavy metal-induced accumulation of reactive nitrogen species (RNS) also appears to be responsible for HMs toxicity. RNS such as nitric oxide (NO), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), and S-nitrosoglutathione (GSNO) can be produced under different stress conditions. Excessive amount of RNS induces nitrosative stress that can damage DNA, lipids, proteins, and carbohydrates leading to impaired cellular functions (Corpas et al. 2007). Similarly to ROS, RNS especially NO can trigger signal transduction pathways leading to defense gene expression in plants (Leitner et al. 2009). NO is a diffusible gaseous molecule first identified in mammalian systems as the endothelium-derived relaxing factor, a potent endogenous vasodilatator (Schmidt and Walter 1994). Further investigations have demonstrated that NO is a multifunctional effector involved in several physiological processes, including relaxation of smooth muscle, inhibition of platelet aggregation, neuronal communication, immune regulation, apoptosis, and cancer (Brune et al. 1998 and references therein). Animal cells

mainly produce NO by the enzyme nitric oxide synthase (NOS, EC 1.14.13.39), which catalyzes the conversion of L-arginine to L-citrulline and NO in the presence of NADPH and O_2 (Griffith and Stuehr 1995). NO is also synthesized and released by plants, where it is involved in several processes, including growth and development, hypersensitive response, programmed cell death, and adaptative response against different stresses; hence, NO is considered to be a general signal molecule in plant cells (Delledonne 2005). The activity of NOS-like enzymes has been reported in different plant materials using inhibitors of mammalian NOS and antibodies raised against mammalian NOS cross-react with plant proteins (Wendehenne et al. 2003 and references therein). The suitability of this experimental approach is still very controversial and, using a proteomic approach, Butt et al. (2003) has shown that several maize proteins are recognized by antibodies against mammalian NOS, but these proteins have no homologous regions to the animal counterparts. However, an active NOS enzyme was purified and characterized in *Ostreococcus tauri* (Foresi et al. 2010) and recently Shi et al. (2012) reported that *Arabidopsis* expressing rat neuronal NOS showed increased endogenous NO content. Nitric oxide can also be generated through the NAD(P)H-dependent reduction of NO_2 in a reaction catalyzed by the enzyme nitrate reductase (NR, EC 1.6.6.1) and reduction of nitrite by NR was considered to be the major NO source in plants (Xu and Zhao 2003). In fact, the NR-deficient *Arabidopsis* mutant (*nia1nia2*) shows only 1 % NR activity of the wild type and has significantly reduced NO levels which reflects the involvement of NR in endogenous NO synthesis (Wilkinson and Crawford 1993). NO can also be produced non-enzymatically from nitrite under acidic conditions in the presence of reductant or antioxidant compounds or as a by-product of chemical reactions between N oxides and plant metabolites (Bethke et al. 2004). Whatever the source of NO, it is clear that its synthesis is rapidly regulated in response to different stresses and its importance as signaling molecule in plants is unquestionable (Leitner et al. 2009 and references therein).

12.2 NO Generation Under HMs Stress

Even though the molecular details remain to be resolved, continuous researches clearly suggest a causal relationship between HMs toxicity and endogenous NO content in plants and algae. A lot of published data describe the effects of different HMs on endogenous NO content in several plant species and tissues; however, many reports are conflicting regarding the HMs impact on NO level (Table 12.1). Cell suspensions represent a useful system to study the responses to exogenous compounds as the administration of compounds and the reproducibility of the experimental conditions are easy in this more controlled system. *Glycine max* cell suspensions treated with 4 or 7 μM Cd^{2+} for 72 h exhibit a dose-dependent and rapid production of NO, which may suggest that NO functions as a signal molecule (Kopyra et al. 2006). Similarly, application of 300 μM $FeSO_4$ to

Table 12.1 Effects of heavy metals on nitric oxide content in different plants and algae

Plant/Algae species	Organ/Tissue	Heavy metals	Metal concentration (μM)	Treatment time	NO content	References
Glycine max	Cell suspension	Cd	4, 7	72 h	Increase	Kopyra et al. (2006)
Arabidopsis thaliana	Cell suspension	Cd	50, 100, 150	72 h	Increase	De Michele et al. (2009)
Nicotiana tabacum	Cell suspension	Cd	150	12 h	Increase	Ma et al. (2010)
Arabidopsis thaliana	Cell suspension	Fe	300	30 min	Increase	Arnaud et al. (2006)
Clamydomonas reinhardtii	Cell suspension	Cu	1, 2.5, 5, 7.5, 10	240 h	Increase	Zhang et al. (2008)
Brassica juncea, Pisum sativum	Root	Cd, Cu, Zn	100	7 days	Increase	Bartha et al. (2005)
Triticum aestivum	Root	Cd	100	5 days	Increase	Groppa et al. (2008)
Triticum aestivum	Root	Cd	1–10	3 h, 4 weeks	Increase	Mahmood et al. (2009)
Panax ginseng	Root	Cu	50	24 h	Increase	Tewari et al. (2008)
Arabidopsis thaliana	Leaf	Cd	50	96 h	Increase	Besson-Bard et al. (2009)
Arabidopsis thaliana	Root	Cd	200	7 h	Increase	Besson-Bard et al. (2009)
Hordeum vulgare	Root	Cd	1000	24 h	Increase	Valentovicová et al. (2010)
Solanum nigrum	Root	Zn, Zn + Fe	200, 400	0–10 days	Initial increase (up to 2 days) then decrease	Xu et al. (2010b)
Hibiscus moscheutus	Root	Al	100	20 min	Decrease	Tian et al. (2007)
Arabidopsis thaliana	Root	Al	100	60 min	Decrease	Illéš et al. (2006)
Medicago truncatula	Root	Cd	50	48 h	Decrease	Xu et al. (2010a)
Pisum sativum	Root	Cd	50	14 days	Decrease	Rodríguez-Serrano et al. (2009)
Pisum sativum	Leaf	Cd	50	14 days	Decrease	Rodríguez-Serrano et al. (2009)
Oryza sativa	Root	Cd	100	24 h	Decrease	Xiong et al. (2009b)

Arabidopsis cell suspension cultures leads to a rapid NO burst in the plastids of the cells (Arnaud et al. 2006). Interestingly, by using NOS inhibitors and mutants-lacking NR, the authors show that this burst does not involve NOS-like nor NR activities (Arnaud et al. 2006). More recently, while treatment of *Arabidopsis* cell suspensions with 50 μM $CdCl_2$ has no effect on the vitality of the cells, 100 and 150 μM of $CdCl_2$ induces cell death accompanied by a rapid increase in NO production. Treatment with NO scavengers shows that this NO production is required for Cd^{2+}-induced cell death (De Michele et al. 2009). Similar results have been obtained in tobacco BY-2 cells where a significant increase in NO production is observed after exposure to 150 μM $CdCl_2$. Inhibition of NOS activity and scavenging of NO alleviates Cd^{2+} toxicity in this experimental system too, indicating that increased NO concentration is required for Cd^{2+} toxicity (Ma et al. 2010).

The root system of the immobile plant is the primary contact site of the metal ions in the metal-contaminated environment, hence the root system has often been chosen for "in vivo" studies. In the roots of the metal accumulator *Brassica juncea* and of the crop plant *Pisum sativum*, an increase in NO content is measurable after treatment with either 100 μM Cd, Cu, or Zn. Interestingly, different NO levels have been obtained with the different metal ions, the most effective ones being copper and cadmium (Bartha et al. 2005). An increase in NO content, both in long- (4 weeks under 1 μM Cd^{2+}) and in short-time (10 μM Cd^{2+} for 3 h) experiments, has been also reported in wheat roots grown in hydroponics (Mahmood et al. 2009). At least in the short-time experiments, the NO production is due to NR activity (Mahmood et al. 2009). NO increases in the adventitious roots of *Panax ginseng* after exposure to 50 μM $CuSO_4$ for 24 h and in the root tips of barley treated with 1 mM Cd^{2+} for 24 h, where the effect of NOS inhibitors suggests an involvement of NOS-like activities in the NO production (Tewari et al. 2008; Valentovicová et al. 2010). NO content significantly increases also in the roots of wheat plants exposed to 100 μM Cd^{2+} for 5 days (Groppa et al. 2008). A strong increase in NO accumulation contributing to Cd^{2+} toxicity by promoting Cd^{2+} accumulation in roots and by up-regulating genes related to iron uptake has been observed both in roots and leaves of *Arabidopsis thaliana* treated with 200 μM Cd^{2+} for 7 h and with 50 μM Cd^{2+} for 96 h, respectively (Besson-Bard et al. 2009). In this paper, the source of NO production was well characterized by using mutants and inhibitors. The Cd^{2+}-induced NO production was not suppressed in the *atnoa1* mutant impaired in the expression of the AtNOA1 gene encoding an enzyme initially thought to catalyze NO synthesis from L-Arg nor in the *nia1 nia2* NR-null mutant impaired in both NR1 and NR2 gene activities indicating that neither AtNOA1 nor NR catalyze this NO synthesis. On the other hand, Cd^{2+}-induced NO production in roots was significantly reduced by mammalian NOS inhibitors suggesting the possible occurrence of a NOS-like enzyme in this process (Besson-Bard et al. 2009). A time- and concentration-dependent accumulation of NO, associated with synthesis of proline, can also be observed in the test alga *Chlamydomonas reinhardtii* treated with various $CuSO_4$ concentrations (Zhang et al. 2008).

In contrast to these observations, several authors report a negative effect of HMs stress on NO production. For example, in the two-week-old seedlings of Zn-hyperaccumulator plant, *Solanum nigrum* exposed to Zn^{2+} or Zn^{2+} plus Fe^{2+} a NO accumulation, almost completely abolished by NOS inhibitors, has been also observed for up 2 days after HMs supply. However, this initial stimulation of NO production was followed by a marked decrease in the next 8 days of HMs treatment (Xu et al. 2010b). In addition, a 60-min treatment with 90 μM Al is sufficient to substantially reduce NO production in *Arabidopsis* roots and a treatment with 100 μM Al for 20 min is sufficient to induce a strong decrease of NO content in the roots of 4-day-old *Hibiscus moscheutos* seedlings, possibly through inhibition of a NOS-like activity (Illéš et al. 2006; Tian et al. 2007). More recently, it has been shown that treatment with 50 μM Cd^{2+} for 48 h significantly decreases NO in *Medicago truncatula* roots and treatment with 50 μM Cd^{2+} for 14 days also produces a significant reduction of NO content in the roots and leaves of *P. sativum* (Rodríguez-Serrano et al. 2009; Xu et al. 2010a). In addition, a 24-h treatment with 100 μM Cd^{2+} significantly decreases the NO content in the crown roots of 7-day-old rice seedlings, and in crown roots of 4-week-old plantlets a 0.2 mM Cd^{2+} stress induces, after a rapid increase in the first half-hour, a strong and progressive decrease in the NO level (Xiong et al. 2009b).

This contradictory effect of HMs on NO production in plants is likely to be associated with the observation that the effect of NO may be beneficial or protective, but may also be toxic for plants depending on the concentration of NO and other cell components (Arasimowicz and Floryszak-Wieczorek 2007). Anyway, the reasons of these discrepant reports on the effects of HMs on endogenous NO content in different plant species and tissues is still matter of debate, and some possible reasons for this discrepancy have been proposed. For example, it was attributed to differences in the cell responses to short and long periods of metal treatment, a short-time HM treatment leading to a NO burst, and a long-time treatment directly or indirectly decreasing NO generation (Rodríguez-Serrano et al. 2009). Another explanation could be the use of different HM concentrations, the age of the plants, and the variety of plant tissues used (Groppa et al. 2008; Xiong et al. 2009b). In addition, it should also be noted that NO can rapidly react with oxygen to produce a variety of nitrogen oxides, so the local concentration of NO depends on a complex balance among its concentration, system redox state of the cells, and the utilization of NO by its target molecules and metals (Lamattina et al. 2003). Finally, it should be considered that an important reason of these controversial observations and opinions on the effects of HMs on endogenous NO content in plants can reside in the technical difficulties in detection and quantification of NO. Until now, NO production by organs of plants, plant cells, purified peroxisomes, and mitochondria, was measured with a remarkably wide variety of methods, including fluorescence imaging, chemiluminescence, electron-spin resonance (ESR), oxyhemoglobin/methemoglobin, quantum cascade laser-based spectroscopic detection, laser-based photoacoustic detection, NO electrodes, and membrane inlet mass spectrometry (MIMS) (Gupta and Igamberdiev 2013 and references therein). All these techniques present advantages and disadvantages

and can give significantly different results. In summary, the variability in NO concentration and the large discrepancies that are sometimes observed when NO is measured by different methods in the same system warrant improvements of the available methods and support the indication of using at least two different methods for measuring NO in the same experimental material and condition (Gupta and Igamberdiev 2013).

12.3 Effects of NO in the Protection Against HMs Stress

NO is a diffusible gas signal molecule having a wide range of physiological roles in plants. Besides regulating normal development, it plays a role also in different biotic and abiotic stress responses. Nitric oxide may act as an antioxidant by elimination of superoxide radical and by formation of the less-toxic peroxynitrite (Vandelle and Delledonne 2011). On the other hand, high concentrations of NO may have toxic effect which has most widely been attributed to its ability to damage cell membranes, cause DNA fragmentation, and inhibit some potential antioxidative enzyme activities such as catalase (CAT, EC 1.11.1.6) leading to accumulation of ROS (Leitner et al. 2009). In the last two decades, an increasing number of studies employing NO donors indicated that exogenously applied NO can provide protection against HM toxicity, and extensive reviews devoted to a specific HM such as aluminum or cadmium have been published recently on this topic (He et al. 2012; Gill et al. 2013). Now it has been widely accepted that NO protects plant cell against stress induced by different HMs by decreasing (or compartmenting) metal accumulation or attenuating metal-induced oxidative stress. For example (Table 12.2), NO applied exogenously as 50 μM sodium nitroprusside (SNP) protects roots of rice (*Oryza sativa*) against As (as sodium arsenate) toxicity by scavenging of ROS, thus reducing oxidative damage (Singh et al. 2009). Pretreatment of lupin (*Lupinus luteus)* seedlings for 24 h with 10 μM SNP resulted in efficient reduction of the detrimental effect of Pb^{2+} and Cd^{2+} on root growth and morphology. SNP counteracts the inhibitory effect of HMs on root growth of lupin by stimulation of superoxide dismutase (SOD, EC 1. 1.5.1.1) activity and/or direct scavenging of the superoxide anion (Kopyra and Gwóźdź 2003). Pretreatment with 100 μM SNP also efficiently alleviates the Cu^{2+} toxicity in tomato (*Lycopersicon esculentum*) seedlings through induction of the transcription and increment in the activities of antioxidant enzymes, including CAT, SOD, and ascorbate peroxidase (APX, EC 1.11.1.11), and accumulation of metallothionein (Wang et al. 2010a). 10 μM SNP alleviates Al-induced inhibition of growth and impairment of the whole photosynthetic electron transport chain in sour pummelo (*Citrus grandis*) seedlings through increasing Al immobilization in roots and Al-induced secretion of malate and citrate from roots, and decreasing Al accumulation in shoots (Yang et al. 2012). SNP, in a dose-dependent manner, significantly reverses the inhibitory effect of Ni on the growth of wheat (*Triticum aestivum* L.) seedlings through modulation of antioxidant enzymes (Wang et al. 2010b). In particular, SNP enhances the activities of guaiacol peroxidase (POD,

Table 12.2 Effects of exogenous nitric oxide on heavy metals stress in different plants

Plant species	Organ/tissue	Dose and duration of heavy metal exposure	NO treatment	Plant responses	References
Oryza sativa	Root/ coleoptile	25, 50 μM As 4, 8, 24 h	50 μM SNP	SNP ameliorates the As-induced decrease in root and coleoptile length by reduction of oxidative stress through ROS scavenging	Singh et al. (2009)
Lupinus luteus	Root	700, 1500 μM Pb^{2+}; 50–100 μM Cd^{2+} 0–48 h	10 μM SNP 24 h before HMs	SNP prevents the inhibitory effect of HMs on root growth and morphology by the stimulation of SOD activity and ROS scavenging	Kopyra and Gwóźdź (2003)
Lycopersicon esculentum	Seedlings	0–5 μM Cu^{2+} 0–6 d	100 μM SNP 48 h before Cu^{2+}	SNP prevents the Cu-induced decrease in biomass and chlorophyll content through induction of the transcription and increment in the activities of antioxidant enzymes	Wang et al. (2010a)
Citrus grandis	Seedlings	1200 μM Al^{3+} 18 weeks	10 μM SNP	SNP alleviates Al-induced inhibition of growth and impairment of the whole photosynthetic electron transport chain through increasing Al immobilization in roots and stimulation of Al-induced secretion of malate and citrate from roots and prevention of Al-induced oxidative stress	Yang et al. (2012)
Triticum aestivum	Seedlings	100 μM Ni^{2+} 0–96 h	100 μM SNP	SNP prevents from the Ni^{2+}-induced oxidative damage through modulation of antioxidant enzymes	Wang et al. (2010b)
Triticum aestivum, Phaseolus vulgaris	Seedlings	0–21.6 μM Zn^{2+} 21 d	100 μM SNP	SNP alleviates the adverse effect of deficient and toxic level of Zn^{2+} by adjusting the levels of total and free sulfhydryl groups and increasing the level of reduced glutathione and the activity of SOD	Abdel-Kader (2007)

EC 1.11.1.7), APX, SOD, glutathione reductase (GR, EC 1.6.4.2), and glutathione S-transferase (GST, EC 2.5.1.18) in wheat seedling roots under nickel stress, while no significant difference in the activity of CAT has been observed with SNP with or without Ni (Wang et al. 2010b). Interestingly, 100 μM SNP alleviates the adverse effect of deficient and toxic levels of Zn^{2+} in both wheat and bean (*Phaseolus vulgaris*) seedlings by adjusting the levels of total and free sulfhydryl groups and increasing the level of reduced glutathione and the activity of SOD (Abdel-Kader 2007). Finally, many papers describe the protective effect of exogenously supplied NO in several plant species exposed to Cd^{2+} stress by modulating the activities of antioxidant enzymes (Gill et al. 2013 and references therein). Even though some authors report that high concentrations of exogenous NO even enhance the toxicity of HMs and single treatment with a high concentration of exogenous NO also inhibits the growth of plants (Groppa et al. 2008; Rodríguez-Serrano et al. 2009; Xu et al. 2010a), the large majority of reports reveals the importance of exogenous NO in protection against the deleterious effects of HMs and suggests possible mechanisms by which NO can help plants to counteract HM stress. First of all, by scavenging Fenton-reaction Fe and regulating antioxidant enzymes, NO might increase the antioxidant content and antioxidative enzyme activity of plant cells (Leitner et al. 2009 and references therein). Second, by affecting cell wall components, primarily pectin, and hemicellulose, but also cellulose, NO might increase HMs accumulation in root cell walls, subsequently decreasing it in the aerial parts of the plant (Xiong et al. 2009a). Third, NO can regulate cellular responses through transcriptional activation of tolerance-related genes and post-transcriptional modification of target proteins such as S-nitrosylation of specific Cys residues (Grün et al. 2006 and references therein). Furthermore, NO may alleviate HMs toxicity by maintaining auxin equilibrium through reduction in the activity of 3-indoleacetic acid (IAA) oxidase (Xu et al. 2010a), by modulating the activities of protein kinases, and by increasing cytosolic Ca^{2+} concentration through the regulation of Ca^{2+} channels and transporters and mobilizating other second messangers, including cGMP and cADPR that may be involved in the signaling cascade that regulates gene expression (Besson-Bard et al. 2009).

12.4 Conclusions and Future Prospects

NO, a diffusible gas signal molecule of plants and animals, has a wide range of physiological roles. Besides regulating normal growth and development, it also plays a significant role in different biotic and abiotic stress responses. There are conflicting results regarding the effect of different HMs on NO accumulation in different plant species and tissues and these conflicting data have been explained by the different HM concentrations utilized, the different plant species and tissues used, the ages of the plants, and the duration of the experimental treatment.

Recently, an increasing number of articles have reported the effects of exogenous NO supply in protecting plants against the deleterious effects of HMs and suggest that this protective effect of NO largely can be assigned to its capacity to increase the antioxidant content and the activity of antioxidative enzyme as well as in its capacity to regulate the expression of stress-related genes. On the other hand, HM-induced accumulation of NO also appears to be involved in HM toxicity. These conflicting results on the relationships between NO and HM toxicity are attributed to the different effects of different HMs on NO content in different plant species and tissue reflecting the different sources of NO production in plants. In addition, it should be considered that the application of NO donors probably does not exactly reflect the spatiotemporal aspects of NO signaling in plants (Arasimowicz and Floryszak-Wieczorek 2007).

With oxidative and antioxidative properties, deleterious and protective, repressive and inductive: The conflicting data reported in the large number of papers published in recent years dealing with HM stress and NO strongly strengthen the chameleon character of this signal molecule. Further studies and the development of better experimental systems to monitor the NO content in plants will be necessary to understand the complex network(s) involved in plant defenses against HM stress and the role of NO in regulating the cellular responses against this stress.

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Chapter 13
Role of Nitric Oxide in Salt Stress-induced Programmed Cell Death and Defense Mechanisms

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Abstract During the last decade, it has been shown by several authors and in several plant species that nitric oxide (NO) accumulates in tissues exposed to high salinity. This gaseous-free radical and signaling compound can attenuate the ionic component of salt stress by enhancing Na^+ extrusion from the cells via Na^+/H^+ exchange through the activation of plasma membrane and vacuolar H^+-ATPases and H^+-pyrophosphatase. NO alleviates the osmotic stress caused by high salt concentrations by stimulating the biosynthesis of compatible osmolytes such as proline, glycine betaine, and soluble sugars, and it also protects the cells from the oxidative damage by enhancing non-enzymatic and enzymatic antioxidants. However, NO may promote programmed cell death (PCD) depending on the NO scavenging capacity of the cells and on the cellular redox status as well as on the flux and dose of local reactive nitrogen and oxygen forms generated in various cell compartments. Particular attention is paid to the role of NO and NO-induced protein modifications in the activation of specific steps of PCD during salt stress.

Keywords High salinity · Nitric oxide · Programmed cell death · Reactive oxygen species · Salt stress

13.1 Introduction

It is estimated that more than 6 % of the total land area and about 30 % of the world's irrigated area are salt affected and the increasing secondary salinization of soil and groundwater is one of the most important environmental factors that decrease the productivity of crop plants (Munns 2002; Lambers 2003).

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High salinity imposes osmotic stress on plants by preventing water uptake of root tissues and causes a rapid reduction in stomatal closure, transpiration, photosynthesis, and eventually in shoot growth rate. The salt-specific ionic effects appear later and the excessive amount of NaCl in leaf tissues results in premature senescence of older leaves, decreases the biomass production, and finally it may induce programmed cell death (PCD) in root or leaf tissues of salt-sensitive species or genotypes (Munns and Tester 2008; Shabala 2009). Understanding the mechanisms that regulate salt tolerance and the salt-induced PCD at the molecular, cellular, tissue, or whole-plant levels is an important problem in plant biology and molecular biological technics speeded up the elucidation of the distinct and relevant responses in salt-tolerant and sensitive plants. Nitric oxide (NO) is a biologically active gaseous-free radical that transmits the environmental or hormonal signals during different biotic and abiotic stresses including salt stress (Corpas et al. 2011). Salt-induced NO generation in parallel with hydrogen peroxide (H_2O_2) accumulation can act both independently and synergistically, and they are involved in several downstream signal transduction pathways determining cell fate (Siddiqui et al. 2011). In the past ten years, the role of NO in salt tolerance and salt toxicity has been studied using exogenously applied NO generators, NO scavengers, and mutant plants with higher or lower activities in NO production. These plants have generally but not exclusively mutations in the genes encoding the most important enzymes participating in NO biosynthesis or NO levels may be controlled by specific inhibitors.

In this review, we highlight the current state of the art of the physiological and molecular aspects of NaCl-induced NO signaling in plants and discuss the roles of endogenous and exogenous NO in NaCl toxicity and salt tolerance mechanisms.

13.2 NaCl Tolerance in Plants

Plant responses to salt stress can be divided into two phases: A rapid, osmotic phase that inhibits the growth of young leaves, and a slower, ionic phase that elicits ion-specific effects and accelerates senescence of mature leaves (Munns and Tester 2008). It can be concluded, however, that the effects of salt stress depend not only on the concentration of NaCl but also on the duration of the stress and on the age and sensitivity of the plants. Salt stress-induced osmotic, ionic, and oxidative stresses cause strong toxicity and finally induce PCD in sensitive species (Zhu 2002) while salt-tolerant plants, especially the halophytes, might be growing at their optimum rate at the same salt concentration.

Munns and Tester (2008) defined three distinct types of plant adaptation to highsalinity: osmotic stress tolerance, the exclusion of Na^+ or Cl^- ions from the cells, and tissue tolerance that is the tolerance of Na^+ after vacuolar sequestration inside the cells.

Salt stress reduces water uptake by the roots leading to a reduction in relative water content (RWC) of the tissues and to decreased water potential which results

in growth inhibition, stomatal closure, and a decrease in photosynthesis (Munns and Tester 2008). These short-term changes are solely due to osmotic stress evoked by high salinity. The balance of osmotic potential between the soil solution and cell compartments can be achieved by the accumulation of compatible solutes such as proline (Pro), glycine betaine, mannitol, or sugars. They can also act as osmoprotectants because their high concentration in the cytosol and organelles is beneficial for the maintenance of the tertiary structure of proteins (Rhodes et al. 2004). Halophytes or salt-tolerant glycophytes with high tissue tolerance can use Na^+ for osmotic adjustment after its sequestration into the vacuole (Munns and Tester 2008).

Plants have multiple Na^+ transport systems to circumvent salt toxicity. The maintenance of the optimal K^+/Na^+ ratio, the high K^+, and low Na^+ concentrations in the cytoplasm are of primary importance to protect cellular functions (Zhu 2003). In barley high-affinity Na^+ uptake trough, a uniporter was inhibited by external K^+ in long-term experiments (Haro et al. 2005), suggesting that the uptake of sodium depends on the K^+/Na^+ discrimination of the transporter system. The removal of the excess Na^+ from the cytoplasm occurs by secondary active transport through Na^+/H^+ antiporters which can be localized to the plasma membrane (PM) and tonoplast, the former is known as SOS1 in *Arabidopsis* and the latter is the member of NHX vacuolar exchanger family (Blumwald 2000, Hasegawa et al. 2000). The importance of these antiporters to improve salt tolerance was confirmed by the overexpression of vacuolar Na^+/H^+ (AtNHX1) or PM (SOS1) antiporter genes in *Arabidopsis* (Zhu 2002; Pardo et al. 2006). SOS1 is preferentially expressed in the xylem parenchyma cells, suggesting a role of this antiporter in loading Na^+ into the xylem (Pardo et al. 2006). Na^+/H^+ antiporter activity is driven by the electrochemical proton gradient generated by H^+ pumps, such as PM and vacuolar H^+-ATPases and H^+-pyrophosphatase (H^+-PPase) (Hasegawa et al. 2000). Zhang et al. (2006) found that the salt stress-induced increase in vacuolar H^+-ATPase and H^+-PPase activities resulted in an increased H^+ translocation, Na^+/H^+ exchange, and enhanced salt tolerance. Recent research has demonstrated that the members of HKT transporter family play an essential role in Na^+ exclusion from the leaves under salt stress. *Arabidopsis* HKT1;1 was found to localize to the plasma membrane of xylem parenchyma cells and this transporter mediates the removal of Na^+ from the xylem sap via Na^+ uptake into xylem parenchyma cell (Horie et al. 2009). Comparing the salt stress-induced gene expression of the salt-sensitive *Arabidopsis thaliana* with its salt-tolerant relative *Thellungiella salsuginea* (previously *Thellungiella halophyla*), the salt-tolerant species showed stress-specific and stress intensity-dependent responses and a fundamental difference was found in the steady-state amount of SOS1 and other salt stress-related mRNAs and in the level of their induction during salt stress (Dassanayake et al. 2011).

Various sub-cellular organelles or cell compartments such as chloroplasts, mitochondria, peroxisomes, and the apoplast are common sites of reactive oxygen species (ROS) production under high salinity (Ashraf 2009). The survival of plants under salinity stress depends on the balance between the generation and detoxification of

ROS by various antioxidant systems (Foyer and Noctor 2005; Miller et al. 2010). These enzymes act as ROS scavengers to alleviate the salt-induced oxidative damage in distinct cell compartments. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide radical into molecular oxygen and hydrogen peroxide (H_2O_2). H_2O_2 can be scavenged by peroxidases (POX), especially by ascorbate- or glutathione peroxidase (APX or GPX) and by catalase (CAT). Glutathione reductase (GR) recycles oxidized glutathione (GSSG) to glutathione (GSH) using NADPH to re-establish the reduced GSH pool (Miller et al. 2010). These enzymes, in accordance with non-enzymatic antioxidants such as ascorbate (ASA), GSH, tocopherol, and flavonoids (Foyer and Noctor 2003), maintain appropriate H_2O_2 levels required for signal transduction processes under salt stress. The association of high levels of non-enzymatic antioxidants and the high activity of ascorbate–glutathione cycle with salt tolerance can be observed in several species (Ashraf 2009), but the activities of antioxidant enzymes were not a reliable criterion for salt tolerance in some species from the Brassicaceae family (Siegal et al. 1982).

Similarly, the accumulation of polyamines, putrescine (Put), spermidine (Spd), and spermine (Spm) is associated with salt stress (Gill and Tuteja 2010) because they have a positive effect on the maintenance of ion balance, membrane stabilization, and ROS scavenging in plants and inhibit both lipid peroxidation and stress-induced oxidative reactions (Ashraf and Harris 2004; Groppa and Benavides 2008). Alternatively, polyamines can act as prooxidants because their catabolism by the activities of diamine oxidase (DAO) and polyamine oxidases (PAO) produces H_2O_2, which can induce PCD (Cona et al. 2006; Yoda et al. 2006; Moschou et al. 2008; Wimalasekera et al. 2011).

Photosynthesis is among the primary processes affected by salinity. The osmotic component of salt stress restricts CO_2 availability by limitation of its diffusion through stomata and mesophyll. Ionic effects can seriously inhibit the photosynthetic metabolism; excessive Na^+ or Cl^- accumulation in chloroplasts leads to the degradation of chlorophyll, damage to light-harvesting complexes, suppression of PS II activity (Mehta et al. 2010), and reduced efficiency of ribulose-1,5-bisphosphate carboxylase (Rubisco) (Abdel-kader et al. 2007; Chaves et al. 2009). Genes or proteins associated with photosynthesis are not among the most altered genes in plants exposed to salt stress. It was found that larger alterations can be observed at transcriptomic level (5–10 %) than at protein level (<1 %) (Chaves et al. 2009). Several genes encoding proteins of the photosynthetic apparatus (subunits of ATP synthase, proteins from PS II and PS I) or enzymes of the Calvin cycle and photorespiration such as fructose-*bis*phosphatase, aldolase, phosphoribulokinase, glycine hydroxymethyltrasferase, or transketolase were downregulated by salt stress; however, the members of multigene families may be differently affected (Kilian et al. 2007).

The biomass production of plants is largely determined by the balance between photosynthesis and respiration. The respiration of tissues exposed to salt stress may increase or decrease or there are no consistent changes in respiratory rate in about one-third of the studies. Depending on salt tolerance strategy, a higher respiration rate may be either beneficial or detrimental to growth rate. Respiration

determines the carbon allocation from shoot to roots and thus the growth capacity of plant parts and it fuels the ATP requirement for the ion exclusion mechanisms in root tissues (Jakoby et al. 2011).

Respiratory rate of isolated plant mitochondria may be reduced by high salt concentrations. Mitochondrial electron transport produces ATP by utilizing the H^+ electrochemical gradient generated by Complex I, Complex III, and Complex IV localized in the inner mitochondrial membrane. The activity of electron transport chain complexes, the NADH dehydrogenases (Complex I), and succinate dehydrogenase (Complex II) is inhibited at toxic Na^+ concentrations owing to protein denaturation and complex disassembly (Flowers 1974; Hamilton and Heckathorn 2001). Salt stress decreased the capacity of the cytochrome pathway, whereas the capacity of alternative oxidase (AOX) increased, which prevented the high reduction level of the ubiquinone pool. Since the major site of superoxide production along the respiratory chain is the ubiquinone pool, higher AOX activity plays a pivotal role in lowering mitochondrial ROS formation in plant cells (Jolivet et al. 1990; Smith et al. 2009). Mitochondria generate a large portion of cellular ROS, and a strong link exists between the mitochondrial antioxidant capacity and the salt tolerance of plants. ROS signals emanating from plant mitochondria determine the survival or death signals under salt stress (Dutilleul et al. 2003). Although nuclear-encoded mitochondrial proteins were over-represented among salinity responsive proteins, there was no strict correlation between transcript level and protein abundance. In contrast, they showed an inverse relationship (reduced mRNS/increased protein abundance) in the cases of NADH dehydrogenase 39-kDa subunit and malate dehydrogenase while there was a decrease in both transcript and protein abundances of cytochrome *c* oxidase subunit 6b and fumarase in short-term experiments. Glutathione peroxidase and mitochondrial heat shock protein 70-2 were up-regulated at mRNS level while salt stress enhanced the protein abundance of Mn-SOD (Jiang et al. 2007; Taylor et al. 2009). This suggests that the protection of mitochondrial function under abiotic stress takes place by fast changes at transcriptional and protein levels.

13.3 NaCl Toxicity and Salt-induced Cell Death in Plants

PCD is an organized and controlled active cell suicide during the normal life cycle of plants and in response to changing environment. It can be triggered by the developmental program or by abiotic and biotic stressors and it is associated with specific biochemical and molecular hallmarks (van Doorn 2005; van Doorn and Woltering 2005; Gunawardena 2008; van Doorn 2011). Although the three main forms of animal PCD, the apoptosis, autophagy, and necrosis-like cell death, were compared with various types of plant PCD, there is no clear correspondence between these main categories due to the presence of cell wall and the lack of phagocytes in plants. Cytoplasmic shrinkage, nuclear chromatin condensation, and finally the fragmentation of DNA, the hallmarks of animal apoptosis, are found in several types of plant cell death (Huh et al. 2002; Lin et al. 2006).

In animal cells, DNA fragmentation during apoptosis is catalyzed by a cysteinyl-aspartate specific proteinase, "caspase-"activated DNase which cleaves DNA into internucleosomal fragments. These cysteine proteases and caspases are central players of animal apoptosis. They exist as inactive proenzymes that are activated by proteolytic processing catalyzed by initiator caspases. They work in a cascade and cleave the inactive proenzyme of the down-stream effector caspases to form active heterodimers (Enari et al. 1998) which then degrade specific protein substrates. However, up to now, no homolog of animal caspases has been found in plants, but there are other enzymes, such as vacuolar processing enzyme (VPE) which exhibit caspase-like activities (Hatsugai et al. 2004). In plants, other cell-death-specific cysteine proteases and metacaspases show structural similarities to caspases, but they have proteolytic activity adjacent to arginine and lysine residues of the substrates (Woltering 2010). Metacaspases are involved in various types of cell death induced by abiotic stressors, e.g., oxidative stress (He et al. 2008). However, it was found that type I metacaspases suppress and type II metacaspases stimulate cell death program in plants (Woltering 2010).

Release of apoptogenic factors such as cytochrome *c* (cyt *c*) from the intermembrane space of mithocondria is a common feature of animal and plant PCD which drives the assembly of the apoptosome, a caspase-activating complex in the cytoplasm of animal cells (Kroemer et al. 2007). The permeabilization of mitochondrial membrane and cyt *c* release was associated with plant PCD as well, following death stimuli, such as heat shock, menadione, or ceramide treatments (reviewed by Reape and McCabe 2010).

In plants, the generation of excess amount of ROS was proposed as a key inducer of various types of PCD (De Pinto et al. 2012). Increased ROS, especially H_2O_2, may activate PM-Ca^{2+} channels and increase $[Ca^{2+}]_{cyt}$ which is an inducer of the permeabilization of mitochondrial membranes. In animal cells, the integrity of the outer mitochondrial membrane is maintained through the balance of pro-apoptotic (Bax, Bak, Bad, Bid) and anti-apoptotic (Bcl-2 and Bcl-xL) proteins. These proteins can control the release of proteinaceous factors from the intermembrane space by controlling the formation of permeability transition pore (PTP) on the mitochondrial envelope through the interaction of voltage-dependent anion channel, the adenine nucleotide transporter, and cyclophilin D (Jones 2000). Although the pro-apoptotic Bax protein operates in PTP formation in animal systems and Bcl-2 and Bcl-xL antiapoptotic factors inhibit Bax action and can confer resistance to death, their plant homologs have not been identified yet. However, a cell death suppressor, Bax-inhibitor-1, has been reported in *Arabidopsis* plants that localizes to the endoplasmatic reticulum and exhibits a pH-dependent Ca^{2+} channel-regulating activity (Ihara-Ohori et al. 2007; Kim et al. 2008).

The formation of PTP following stress-induced increase in $[Ca^{2+}]_{cyt}$ and ROS production leads to the loss of mitochondrial membrane potential ($\Delta\psi_m$) and to the release of cyt *c* into the cytoplasm during PCD of plant cells, too. Although cyt *c* does not appear to directly activate PCD in plants (Balk et al. 2003), its absence from the mitochondrial electron transport chain may lead to enhanced generation of ROS (Vianello et al. 2007). However, in rice, an increased ROS production

preceded the cyt *c* release from root mitochondria under salt stress. The analysis of the rice salt proteome by two-dimensional IEF/SDS-PAGE revealed that four mitochondrial proteins, glycoside hydrolase, mitochondrial heat shock protein 70, 20S proteasome subunit, and Cu/Zn-SOD, were up-regulated during PCD induction (Chen et al. 2009).

Autophagic cell death, also known as type II cell death in animals, was characterized by a lack of chromatin condensation and by the appearance of autophagosomes, the double membrane-possessing structures. Autophagosomes contain hydrolytic enzymes and digest the engulfed cellular components, but their role as executioners of PCD has been debated in plants (Cacas and Diamond 2009). During micro-, macro-, and megaautophagy, the disappearance of organelles and cytoplasmic constituents occurs through the activity of lytic compartments (authophygosomes or vacuole) (van Doorn and Woltering 2005), which contributes to the recycling of cellular components, thus the outcome of authophagy is dependent on the plant fitness and may have a pro-life and/or pro-death function.

Salt stress-induced PCD is also accompanied by the retraction of the plasma membrane from the cell wall, condensation of the cytoplasm and the nucleus, DNA laddering, loss of membrane integrity, release of cytochrome *c* from mitochondria, increase in caspase 3-like protease activity, and changes in the ion homeostasis and K^+ efflux (Fig. 13.1) (Wang et al. 2010a; Poór et al. 2013).

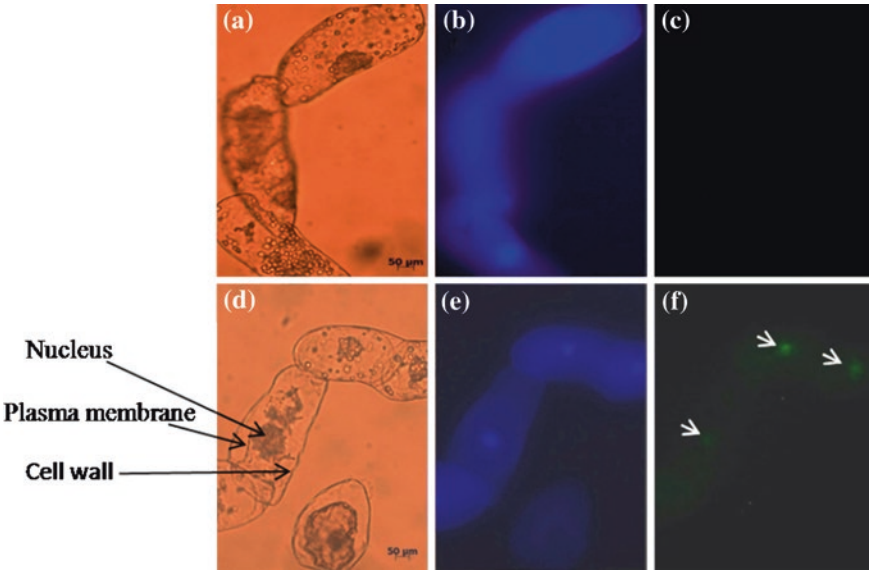

Fig. 13.1 Detection of DNA fragmentation by TUNEL staining in tomato cell suspension treated with 250 mM NaCl for 6 h. *Part A* Control (**a**, **b**, **c**), 250 mM NaCl (**d**, **e**, **f**), bright field microscopy (**a**, **d**); Hoechst 33258 staining (**b**, **e**); TUNEL-positive nuclei (*f*) (reproduced from Poór et al. 2013)

NaCl has multiple ways to induce toxicity and cell death in plants. First, the NaCl-induced osmotic stress reduces cell expansion in root tips and young leaves; however, the osmotic component of salt stress does not lead to the activation of the endonuclease involved in DNA degradation and it is not enough to induce PCD in plant cells. Affenzeller et al. (2009) reported that the exogenous treatment with sorbitol (an agent which induces only osmotic but not ionic stress) did not result in DNA laddering on agarose gel, which is one of the hallmarks of the apoptosis-like PCD.

Secondly, NaCl treatment induces ion disequilibrium in plant cells (Huh et al. 2002). Under saline conditions, Na^+ enters the cell cytoplasm through the non-selective cation channels (NSCC) causing membrane depolarization and resulting in K^+ leakage from the cell through depolarization-activated outward-rectifying K^+ (KOR) channels (Shabala 2009). The accumulation of toxic Na^+ can induce a loss of enzyme activities in the cells. The release of K^+ from the cytoplasm causes K^+ deficiency, which can activate cysteine proteases, the effectors of PCD (Shabala 2009; Demidchik et al. 2010). Potassium is required in many physiological processes of plants, including protein and nucleic acid synthesis. It can be concluded that the decrease in the cytoplasmic K^+/Na^+ ratio is crucial for triggering PCD in living cells (Joseph and Jini 2010).

Finally, salt stress also causes strong oxidative stress-generating ROS such as singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydroxyl radical (OH), and H_2O_2, which can react with lipids, proteins, and nucleic acids, inactivate enzymes, and enhance lipid peroxidation, membrane leakage, and DNA breakdown; thus, they can induce PCD. ROS are also involved in various signaling pathways such as those of plant hormones, e.g., abscisic acid, jasmonic acid, or ethylene during the plant stress responses (Van Breusegem and Dat 2006; De Pinto et al. 2012). Salt stress increases cytosolic $[Ca^{2+}]$ and activates PM-bound NADPH oxidase, which generates superoxide in the apoplast. At the same time, ROS production causes K^+ efflux via ROS-activated NSCC channels, which also induces PCD (Shabala 2009). Other sources of ROS are the photosynthetic and respiratory electron transport chains or the peroxisomes (Foyer and Noctor 2003). In addition, the interaction of ROS and NO and the balance between antioxidants and hormone levels can adjust the induction of PCD or tolerance mechanisms in plants under high salinity.

13.4 NO Production in Plants Exposed to NaCl

NO is a gaseous signaling molecule, which regulates a wide range of physiological and biochemical processes, and the growth and development of plants as well as their responses to biotic and abiotic stresses (Neill et al. 2003; Wendehenne et al. 2004; Delledone 2005; Corpas et al. 2011; Siddiqui et al. 2011).

In plant kingdom, NO can be generated non-enzymatically via reduction of nitrite to NO by the mitochondrial electron transport chain and by the reduction of apoplastic nitrite under acidic conditions. The enzymatic reduction of nitrite to NO is a function of nitrate reductase (NR) in plant tissues (Yamasaki and Sakihama 2000).

Arabidopsis NR is encoded by two nitrate reductase genes, *NIA1* and *NIA2*, and *nia1/nia2* double mutant plants show low NO accumulation (Desikan et al. 2002; Bright et al. 2006). In this case, NO production can be reduced by specific NR inhibitors such as sodium azide (NaN_3) or tungstate (Bright et al. 2006; Sang et al. 2008).

Arginine (Arg)-dependent NO production by nitric oxide synthase (NOS)-like activities suggests the presence of oxidative pathway of NO synthesis in plants (del Rio et al. 2004; Corpas et al. 2006). Plant NOS activity can be inhibited by animal NOS inhibitors that act as Arg analogs, such as NG-nitro-L-arginine-methyl ester (L-NAME) and NG-monomethyl-L-arginine (L-NMMA) (Guo and Crawford 2005). Xanthin oxidoreductase and polyamine/hydroxylamine-dependent NO production has also been reported in plants (reviewed by Mur et al. 2013). NO content of tissues can be elevated by the application of exogenous NO donors (e.g., diethylamine nitric oxide, DEANO; 2,2'-(hydroxynitrosohydrazono)*bis* ethane DETA/NO; *S*-nitrosoglutathione, GSNO; sodium nitroprusside, SNP) which cause different concentration and time-dependent increases in tissue NO level (Mur et al. 2013).

Various salt treatments enhanced NO production in many plant species and plant organs (Fig. 13.2). NO accumulation was observed in roots (Xie et al. 2008, Xu et al. 2011a), leaves (Tanou et al. 2012), calli (Zhao et al. 2004, 2007; Vital

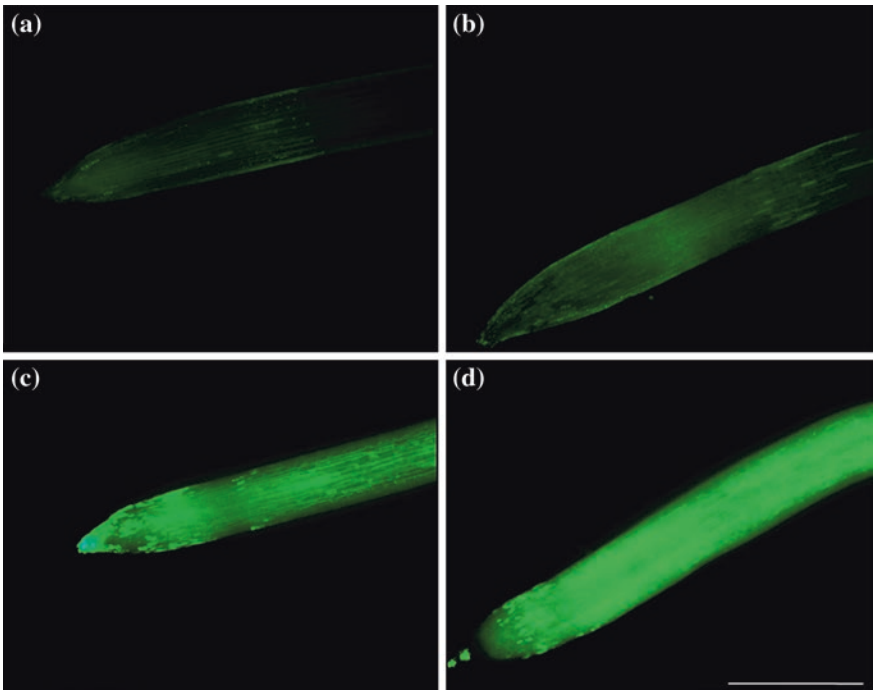

Fig. 13.2 Changes in NO levels in the apical segments of tomato roots treated with 250 mM NaCl for 0 (**a**), 1 (**b**), 3 (**c**), and 6 (**d**) hours. NO was visualized using 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA). White bars represent 1 mm (Poór et al. unpublished results)

et al. 2008; Wang et al. 2009; Sun et al. 2010; Yang et al. 2010), cell suspension cultures (Banu et al. 2010; Poór and Tari 2011), and protoplasts (Gémes et al. 2011) after NaCl treatment. Zhang et al. (2006) observed time-dependent changes in NO production in *Zea mays* leaves after treatment with 100 mM NaCl. NO accumulation displayed a maximum after 2 h of NaCl exposure and after 8 h it decreased back to the control level. A similar tendency was observed in *Arabidopsis thaliana* calli (Wang et al. 2009) and *Solanum lycopersicum* cell suspension culture (Poór et al. 2013). Rapid NO and ROS productions were detected in calli of a highly resistant species, *Populus euphratica* during salt stress(Sun et al. 2010). These results suggested that the NaCl-induced NO production was transient and NO levels increased significantly in parallel with ROS production in the first minutes and hours of salt treatment. Unfortunately, in most of the studies, the production of NO was determined only at one time-point after the salt exposure and there was no time-dependent analysis of NO and other reactive nitrogen species (RNS) such as peroxinitrite ($ONOO^-$) during salt stress in parallel with the determination of various types of reactive oxygen forms, which can determine the fate of the cells later.

Numerous studies reported that NO could be synthesized by NOS-like activity as well as by NR after salt treatment. NaCl-induced NO synthesis was inhibited by the NOS inhibitor L-NAME in *Atriplex centralasiatica* (Xu et al. 2011a), *Olea europaea* (Valderrama et al. 2007), *Phragmites communis* (Zhao et al. 2004), and *Populus euphratica* (Zhang et al. 2007). In contrast, Liu et al. (2007) reported that NO production, induced by 100 mM NaCl, was not affected by L-NAME in *Phaseolus vulgaris*. In *Arabidopsis thaliana*, Zhao et al. (2007a) observed a reduced quantity of the NOA1 protein and decreased NO levels after being exposed to 100 mM NaCl for 2 h. *AtNOA1* (*AtNOS1*) was identified as a putative *Arabidopsis* NOS gene, because these *Arabidopsis* mutants were defective in NO accumulation in the roots (Guo et al. 2003), but later it was found that *AtNOS1* had no NOS activity in vitro. Thus, *AtNOS1* was renamed as NO-associated protein 1 (*AtNOA1*), which is a chloroplast-targeted GTPase essential for ribosome assembly (Moreau et al. 2008). On the other hand, the NR-mediated NO production was involved in salt stress response of *Nicotiana tabacum* (Charrier et al. 2013) and *Olea europaea* (Valderrama et al. 2007). These discrepancies could be explained by the differences in the salt concentrations applied, the duration of salt exposure, the plant species and tissues, and the age of plants in the experiments. It can be concluded that the origin of NO may vary from species to species and from tissue to tissue that show different sensitivities to increased Na^+ concentrations.

13.5 NO in Signal Transduction

The role of NO in the regulation of gene expression was revealed first in large-scale transcriptional analysis of *Arabidopsis thaliana* by DNA-microarrays (Grün et al. 2006). Using NO donor, SNP 342 genes were up-regulated and 80 were

down-regulated in *Arabidopsis* roots (Parani et al. 2004). These genes are involved in signal transduction, cellular transport, photosynthesis, abiotic stress response, and disease resistance. The transcript levels of signal transduction components such as defence-related *MAP* kinases, *WRKY* transcription factors, and *ERE* (ethylene response element) binding proteins, dehydration responsive element binding proteins (*DREB*1 and *DREB2*), oxidative stress-related proteins (glutathione transferases, *ABC* transporters), mitochondrial (*AOX1*) or chloroplastic proteins, e.g., *SOD* and *APX*, and genes participating in iron homeostasis (ferritin) were enhanced by SNP (reviewed by Grün et al. 2006).

NO participates in multiple signaling pathways in plants (Lamattina et al. 2003; Corpas et al. 2004; Lamotte et al. 2005; Besson-Bard et al. 2008; Distefano et al. 2008; Moreau et al. 2010; del Rio 2011). It interacts with the iron atom of the hem moiety in guanylate cyclase (GC), which, in this activated form, produces the second messenger cyclic GMP (cGMP) and interferes with the activity of protein kinases (PKs). A novel soluble GC that binds NO and generates cGMP has been recently described in *Arabidopsis* (Mulaudzi et al. 2011). NO increases cytosolic Ca^{2+} levels, which modulate the activity of Ca^{2+}-dependent protein kinases (CDPKs) or mitogen-activated protein kinases (MAPKs). Phosphatidic acid (PA), a lipid-derived second messenger is also involved in NO signaling. NO interferes with various steps of ROS-induced signaling and acts as an important mediator of H_2O_2-induced cell death (Wang et al. 2013).

NO can modulate protein structure or activity through *S*-nitrosylation of specific cysteine residues, through nitration of specific tyrosines or through binding to metal cofactors of the enzymes. *S*-nitrosylation of proteins, a reversible post-translational modification, occurs when a cysteine thiol in special position of the protein reacts with NO in the presence of an electron acceptor. The formation of *S*-NO bonds affects the function of a wide range of proteins, e.g., of those participating in signal transduction, such as tyrosine phosphatase 1B (Li and Whorton 2003), nuclear factor-κB kinase (Reynaert et al. 2004), or R2R3-MYB class transcription factors (Palmieri et al. 2008). *S*-nitrosylation of proteins participating in auxin and salicylic acid signaling has been excellently reviewed recently by Astier et al. (2012). NO in the presence of O_2 can react with GSH to form *S*-nitrosoglutathione (GSNO), a reactive nitrogen form, which is a long-distance signaling molecule and a natural reservoir of NO.

NO is able to react with various forms of ROS, e.g., with superoxide generating $ONOO^-$ and thus can regulate the redox status of the cell. Peroxynitrite is a strong oxidant, thus may target and inhibit cysteine-containing thiols, such as tyrosine phosphatases (Spoel et al. 2010). Peroxynitrite can react with tyrosine and tryptophan residues yielding 3-nitrotyrozine and nitrotryptophane (Vandelle and Delledonne 2011). Tyrosine nitration can change the function of proteins, and it may promote or inhibit the activity of enzymes. Tyrosine nitration may also interfere with signal transduction because the nitration of specific tyrosines prevents the phosphorylation of the regulatory proteins (Corpas et al. 2013).

Salt stress disrupts the equilibrium between NO production and elimination resulting in changes in NO signaling and in physiological responses. NO

as a messenger and effector molecule can induce both cell death and salt stress tolerance, which depends on a variety of factors, such as cell type, cellular redox status, and the flux and dose of the local NO concentration (Wang et al. 2010b).

13.6 NO and Salt Tolerance

The NO donor SNP enhanced the salt tolerance in many plant species. SNP increased germination, and root- and shoot growth under salinity stress as compared to the NaCl-treated controls in various plant species.

Osmotic stress tolerance induced by the application of an NO donor is associated with an enhanced RWC in several species under high salinity (Sheokand et al. 2010; Zeng et al. 2011; Khan et al. 2012).

NO application significantly enhanced the NaCl-induced osmotic stress tolerance via the accumulation of osmoprotectants such as glycine betaine, soluble sugars, or proline (Pro). Khan et al. (2012) found that SNP treatment increased the glycine betaine content in *Brassica juncea* leaves under salt stress. Enhanced soluble sugar content was found in *Triticum aestivum* seedlings (Zheng et al. 2009) and *Solanum lycopersicum* (Wu et al. 2011) after pre-treatment with the NO donor SNP under salt stress, which ameliorated the NaCl-induced osmotic stress component.

The NO donor induced the accumulation of Pro by enhancing the activities of Δ^1-pyrroline-5-carboxylate synthetase (P5CS), the enzyme catalyzing the rate limiting step of Pro biosynthesis, and by inhibiting the activity of the catabolic enzyme, Pro dehydrogenase (ProDH) in *Triticum aestivum* (Ruan et al. 2004b). Similarly, Pro was found to accumulate after SNP treatment in *Arabidopsis thaliana* (Zhang et al. 2010), *Brassica juncea* (Khan et al. 2012), *Kosteletzkya virginica* (Guo et al. 2009), and *Solanum lycopersicum* (Wu et al. 2011) under salt stress that enhanced tolerance to high salinity. In contrast, SNP decreased the Pro content in *Brassica juncea* (Zeng et al. 2011) and in *Brassica rapa* (López-Carrión et al. 2008). In the latter case, the NO donor did not change the activity of the biosynthetic enzyme, Δ-1-pyrroline-5-carboxylate reductase (P5CR), but increased the activity of ornithine-δ-aminotransferase (δ-OAT) implicated also in Pro biosynthesis under salt stress. SNP together with 100 mM NaCl induced a significant rise in the activity of ProDH as compared to salt treatment suggesting that an increased Pro degradation contributed to low proline level in this species.

Salt stress increased the permeability of membranes, which can be partially prevented by the use of an NO donor leading to increased viability of cells. Accordingly, SNP decreased the membrane leakage in *Cicer arietinum* (Sheokand et al. 2010), *Phragmites communis* (Zhao et al. 2004), *Triticum aestivum* (Ruan et al. 2002), *Zea mays* (Zhang et al. 2006), and *Brassica juncea* (Khan et al. 2012).

The ionic effect of salt stress and the decrease in optimal K^+/Na^+ ratio can also be mitigated by the use of NO donors. Higher NO levels correlated with higher K^+/Na^+ ratios in *Arabidopsis thaliana* (Wang et al. 2009), *Brassica juncea* (Khan

et al. 2012), *Brassica rapa* (López-Carrión et al. 2008), *Kosteletzkya virginica* (Guo et al. 2009), *Phragmites communis* (Zhao et al. 2004), *Populus euphratica* (Zhang et al. 2007), and *Triticum aestivum* (Ruan et al. 2004a; Zheng et al. 2009). Zhao et al. (2007a, b) found that due to the reduced NO levels, *Atnoa*1 mutants displayed lower K^+/Na^+ ratios in their shoots than wild-type plants under salt stress. These results confirm the positive effect of NO in maintaining the optimal K^+/Na^+ ratios. In contrast, SNP had no effect on the osmotic potential and on the concentrations of Na^+, K^+, Cl^-, and NO_3^- in the halophyte *Suaeda salsa* shoots (Song et al. 2009). The NO donor SNP enhanced the activities of PM and vacuolar H^+-ATPases as well as that of vacuolar H^+-PPase in *Arabidopsis thaliana* calli (Wang et al. 2009), *cucumber* (Shi et al. 2007), reed (Zhao et al. 2004), wheat (Ruan et al. 2004a; Xie et al. 2008), and maize tissues (Zhang et al. 2006) under salt stress. SNP treatment also enhanced the contents of other ions such as Ca^{2+} in *Brassica juncea* (Khan et al. 2012) and *Phragmites communis* (Zhao et al. 2004) under salinity. In wheat leaves, Ca^{2+} accumulation has been associated with increased Pro content on NO donor treatment (Ruan et al. 2004b).

Application of exogenous NO donor can also enhance salt tolerance by alleviating oxidative damage. SNP decreased the salt stress-induced harmful lipid peroxidation, malondialdehyde (MDA) content or the level of thiobarbituric acid reactive substances (TBARS) in *Atriplex centralasiatica* (Xu et al. 2011a), *Brassica juncea* (Zeng et al. 2011; Khan et al. 2012), *Brassica rapa* (López-Carrión et al. 2008), *Cicer arietinum* (Sheokand et al. 2008, 2010), *Cucumis sativus* (Shi et al. 2007; Lin et al. 2012a, b), *Glycine max* (Simaei et al. 2011), *Hordeum vulgare* (Li et al. 2008), *Kosteletzkya virginica* (Guo et al. 2009), *Solanum lycopersicum* (Wu et al. 2011), and *Triticum aestivum* (Ruan et al. 2002; Zheng et al. 2009; Hasanuzzaman et al. 2011; Xu et al. 2011b).

NO can scavenge H_2O_2 and can protect plant cells from oxidative damage under salt stress by increasing the activity of antioxidative enzymes although their activities can be affected differently. NO donors decreased the salt-induced ROS production and activated SOD, peroxidases such as APX and GPX, CAT, and GR in various plant species. SNP promoted SOD-, POD-, and APX activities in *Brassica juncea* (Zeng et al. 2011; Khan et al. 2012), *Populus euphratica* (Sun et al. 2010), and *Cicer arietinum* plants (Sheokand et al. 2010). In contrast, higher activities of POX, APX, and GR were observed in the NO-deficient *Atnoa1* plants, and these mutants also showed lower activities of SOD and CAT than wild-type plants under NaCl stress (Zhang et al. 2010). 0.05 mM of SNP increased the activity of APX, whereas it decreased that of SOD and POD, and did not affect the activity of CAT in the roots of *Phaseolus vulgaris* plants exposed to high salinity (Liu et al. 2007). The NO donor SNP can also control the level of non-enzymatic antioxidants, such as those of ascorbate or glutathione. SNP treatments increased the reduced form of both metabolites and elevated the ratios of GSH/GSSG and ASC/DHA (Sheokand et al. 2010; Hasanuzzaman et al. 2011; Wu et al. 2011; Lin et al. 2012a). In other species such as *Gossypium hirsutum*, the NO donor decreased APX- and GR activity under salt stress, but CAT activity was not different from the salt-treated controls (Vital et al. 2008).

The positive effect of exogenous NO on photosynthesis is well demonstrated in rice (Uchida et al. 2002). The maximal photochemical efficiency of photosystem II (Fv/Fm) decreased in WT and *Atnoa1* mutant of *Arabidopsis* under salt stress, but the extent was higher in the mutant (Zhao et al. 2007a, b). Wu et al. (2010) observed that in salt-stressed plants, the NO donor attenuated the decrease in stomatal conductance (g_s), transpiration rate (E), leaf chlorophyll content, net CO_2 fixation rate (P_N), the ratio of variable to maximum fluorescence (Fv/Fm), relative electron transport rate (ETR), the effective quantum efficiency of photosystem II (PS II) reaction centers (Fv′/Fm′), the photochemical quenching coefficient (qP), and counteracted the increase in non-photochemical quenching coefficient (qN) in tomato plants. SNP decreased the salt-induced loss of chlorophyll content in *Arabidopsis thaliana* (Zhang et al. 2010), *Brassica juncea* (Khan et al. 2012), *Citrus aurantium* (Tanou et al. 2012), *Triticum aestivum* (Ruan et al. 2002, 2004a) and *Zea mays* (Zhang et al. 2006).

Moreover, exogenous NO treatment affects the stress hormone levels under salt stress. The concentration of the stress hormone abscisic acid (ABA) increased after SNP treatment in the leaves of wheat seedlings (Ruan et al. 2004b) but ethylene production decreased in the wild type but not in ethylene receptor mutant, *etr1-3 Arabidopsis* callus cultures (Wang et al. 2010c).

Protein nitration and *S*-nitrosylation are also involved in acclimation to salinity stress (Valderrama et al. 2007; Fares et al. 2011; Tanou et al. 2012). Tanou et al. (2009) reported that some proteins undergo a post-translational regulation through either oxidation and/or *S*-nitrosylation in citrus plants exposed to salinity. These authors found that both H_2O_2 and SNP pretreatments before salt stress alleviated the salinity-induced protein carbonylation and caused the accumulation of *S*-nitrosylated proteins in the leaves. These results indicate an overlap between H_2O_2- and NO-mediated protein modifications in citrus plants during the acclimation to salinity.

Not only Pro but also polyamine contents changed significantly under salt stress. NO donor caused an increase in Spm content, (Spd + Spm)/Put ratio, and PAO activity in cucumber plants (Fan et al. 2010). This increase in Spm content may play a role in the protection against high salinity-induced damage (Yamaguchi et al. 2006).

13.7 NO- and Salt-induced Programmed Cell Death

NO is involved in plant PCD induction as a messenger and an effector molecule (Wang et al. 2010b) but in contrast to the H_2O_2-triggered PCD (Dat et al. 2003; Gechev et al. 2006), NO alone is not able to kill the cells (Zago et al. 2006). NO also plays a fundamental role in senescence and HR (Lin et al. 2012a, b), and it acts in strong partnership with H_2O_2 during the induction of PCD (Delledonne et al. 2001; De Pinto et al. 2002). Moreover, it can react with superoxide generating toxic $ONOO^-$ (Delledonne et al. 2001), which at high concentration initiates

cell death (Gupta and Igamberdiev 2011). Time-course experiments indicated that O_2^- rather than H_2O_2 functions in synergism with NO to trigger the PCD program (Blokhina and Fagerstedt 2010; Arasimowicz-Jelonek et al. 2012).

In salt-tolerant *Populus euphratica* callus cells, the antioxidant enzymes were induced by the rapid increase in NO and ROS production after salt treatment, thus the cells could maintain high K^+/Na^+ ratios. NO and ROS productions exhibited only small changes in salt-sensitive *Populus popularis,* therefore the cells displayed low K^+/Na^+ ratio and cell viability (Sun et al. 2010). Zhao et al. (2007a, b) demonstrated that a NOS inhibitor or a NO scavenger reduced endogenous NO levels and enhanced NaCl-induced decrease in K^+/Na^+ ratio in *Arabidopsis*. Moreover, the decrease in the K^+ concentration and the increase in that of Na^+ were greater in *Atnoa1* mutant than in wild-type plants under salt stress due to the reduced endogenous NO levels. *Atnoa1* plants exhibited a much higher increase in H_2O_2 contents than wild types in response to NaCl treatments, which indicates that endogenous NO can effectively attenuate oxidative stress. The ion disequilibrium was the primary cause of cell death in the apical region of primary roots in wild-type and *sos1 Arabidopsis* mutant but the effect was much more pronounced in the mutant plants. Salt-induced PCD displayed the hallmarks of both apoptosis- and lysigenous-type PCD, the nuclear fragmentation, and DNA ladder as well as high vacuolation of cells in the elongation/differentiation zones. The authors suggested that the elimination of primary roots and the differentiation of the secondary roots in plants exposed to salt shock appear to be an adaptive mechanism (Huh et al. 2002).

Both enhanced and reduced Ca^{2+} levels were reported in plants exposed to high salinity (Halperin et al. 1997; Rabie and Almadini 2005; Yang et al. 2007), which can alleviate salt injury or may be involved in signal transduction. Gao et al. (2004) found an elevated steady-state level of the cytoplasmic Ca^{2+} and an increased apoplastic Ca^{2+} concentration in *Arabidopsis* roots during salt stress. Other authors found a significantly increased Ca^{2+} content on dry mass basis in tomato suspension cells when exposed to lethal concentrations of NaCl (Poór et al. 2012).

Overproduction of NO or the other RNS can cause toxic symptoms, called "nitrosative stress." This, in combination with oxidative stress due to overproduction of ROS, can lead to a "point of no return" in PCD initiation. The generation of RNS and ROS takes place in various cell compartments and depending on the half-life, transport properties, and concentrations of molecular species, they can act synergistically or can scavenge one another.

PM NADPH oxidase, the enzyme that catalyzes one electron reduction of O_2 to O_2^-, has been described as an important source of apoplastic H_2O_2 after dismutation of superoxide during salt stress (Yang et al. 2007). Treatment with 150 mM NaCl or the NO donor SNP increased the activity of the PM NADPH oxidase and the generation of H_2O_2 in *Populus euphratica* callus cultures. Moreover, it was observed that the application of NMMA, a NOS inhibitor, strongly blocked the plasma membrane NADPH oxidase activity and the production of H_2O_2 under salt stress in these tissues (Zhang et al. 2007). It was found by other authors that

S-nitrosylation of the Cys-890 of the protein reduced the activity of NADPH oxidase in the leaves of *Arabidopsis* in response to an avirulent pathogen (Yun et al. 2011), suggesting that NO might regulate ROS production by this enzyme in various ways.

Chloroplasts, the important sources of ROS under salt stress, are capable of both Arg- and nitrite-dependent NO generation (Jasid et al. 2006) suggesting that they contribute to nitrosative and oxidative stresses very effectively. Chloroplastic proteins such as oxygen-evolving enhancer protein 1 and 2, small and large subunits of Rubisco, Rubisco activase, plastocyanin, and phosphoribulokinase are targets of *S*-nitrosylation in potato (Kato et al. 2012). Photosynthesis-related proteins involved in the Calvin–Benson cycle were largely targeted by carbonylation, nitration, and nitrosylation in citrus plants primed with H_2O_2 or SNP prior to salt stress (Tanou et al. 2012). The authors discussed the importance of these post-translational modifications in the acclimation to high salinity. In contrast, nitration of specific tyrosine of D1 protein (PSBA) of photosystem II leads to the dissociation of the PS II dimers and PS II-LHCII supercomplexes during high light stress (Galetskiy et al. 2011). Carbonic anhydrase in sunflower (Chaki et al. 2013) and chloroplastic glycerinaldehyde-3-phosphate dehydrogenase in *Arabidopsis* (Lozano-Juste et al. 2011) were also among the Tyr-nitrated proteins under stress conditions that showed reduced activity.

Peroxisomes are sites of the L-Arg-dependent NO production and contain CAT- and H_2O_2-producing flavin oxidase that participates in photorespiration (del Río 2011). Peroxisomes in *Arabidopsis* were also shown to be required for the accumulation of NO in the cytosol during salt stress (Corpas et al. 2009).

The peroxisomal enzyme, CAT, and APX activities were inhibited by NO (Clark et al. 2000). Since glycine hydroxymethyltrasferase, a mitochondrial enzyme participating in photorespiration, was also down-regulated by salt stress and NO (Kilian et al. 2007), it can be assumed that nitrosative stress contributes to salt stress-induced inhibition of photorespiration. The catabolism of PAs by DAO or PAO enhanced H_2O_2 production which was suggested to be a signal leading to PCD in salt-stressed tobacco tissues. In these experiments, the authors demonstrated that spermidine was secreted and oxidized by PAO in the apoplast upon salt stress (Moschou et al. 2008), but other PAO isoenzymes (AtPAO2, AtPAO3, and AtPAO4) were localized to peroxisomes (Kamada-Nobusada et al. 2008) or to the vacuole (Cervelli et al. 2004).

Salt stress-induced PCD is mediated by the status of the mitochondrial PTP and ROS production in tobacco protoplasts. The cells displayed several morphological hallmarks of apoptosis, such as nuclear DNA degradation, formation of PTP on mitochondrial membrane, and a decrease in the mitochondrial membrane potential ($\Delta\psi_m$). Externally applied ascorbic acid prevented the decrease in $\Delta\psi_m$ and the increase in ROS production, suggesting that ROS formation plays a key regulatory role in PCD initiation. ATP synthesis was reduced in plant mitochondria by the inhibition of cytochrome oxidase activity (Yamasaki et al. 2001). In *Arabidopsis* plants, NO induced the de-polarization of ψ_m and the release of cyt *c* from the intermembrane space and resulted in increased ROS production, suggesting that the

NO effect depends on the physiological status of plant cells (Zottini et al. 2002). Since the role of cyt *c* may be different in plant and animal PCDs, other apoptosis-inducing factors can also be released from the mitochondrial intermembrane space (reviewed by Reape and McCabe 2010).

Rice *nitric oxide excess* (*noe1*) mutant was identified due to high NO and *S*-nitrosothiol contents of plants. The map-based cloning revealed that *NOE1* encoded *OsCATC*, so the plants were catalase deficient (Lin et al. 2012b). These plants had higher H_2O_2 levels under high light, which consequently promoted NO production via the activation of NR. The plants exhibited a cell death phenotype under high light intensity and the accumulation of NO promoted the *S*-nitrosylaton of glyceraldehyde-3-phosphate (GAPDH) and thioredoxin (TRX), the two enzymes, which are involved in *S*-nitrosylation-regulated cell death in animal cells (Wang et al. 2013). TRX is presumed to be *S*-nitrosylated in the active site, which inhibits its oxido-reductase function (Sumbayev 2003). Since TRX is a key modulator of cell redox status, this post-translational modification promotes apoptosis. GAPDH has also been shown to be *S*-nitrosylated, which decreases its enzymatic activity (reviewed by Astier et al. 2012). The NtGAPCb isoform of this protein constitutively interacts with NtOSAK (*Nicotiana tabacum* osmotic stress-activated protein kinase) both in the cytosol and the nucleus. This kinase was found to be phosphorylated and thus activated in plant tissues during salt stress. The *S*-nitrosylation of NtGAPCb had no influence on the interaction with NtOSAK or on its kinase activity but prevented the nuclear localization of the complex (Burza et al. 2006). In *Arabidopsis* peroxiredoxin IIE (PerIIE), an enzyme involved in the maintenance of redox status of the cells possesses a peroxynitrite reductase activity, thus it can control $ONOO^-$ levels. PerIIE activity is inhibited by *S*-nitrosylation, suggesting a regulatory effect of NO on the strength of nitrosative stress (Romero-Puertas et al. 2007).

It has been shown recently that short-term salt stress induces apoptosis-like, whereas long-term salt stress non-apoptotic PCD (Andronis and Roubelakis-Angelakis 2010). In both cases, the execution of cell death is mediated by the induction of cysteine proteases, some of them showing caspase-like activities. Caspase-like activity seems to be activated by K^+ deficiency in plant cells (Demidchik et al. 2010), which is in good correlation with the induction of cysteine protease activity in salt-stressed plants (Kovács et al. 2012).

Long-term salt stress increased H_2O_2 and NO productions, resulted in elevated lipid peroxidation, caspase-like activity, and cell death in maize roots. However, an exogenously applied NO donor, DETA/NO, reversed the detrimental effect of salt stress and reduced the salt-induced oxidative stress and caspase-like activity in maize tissues (Keyster et al. 2012). Plant metacaspases are considered to be ancestors of metazoan caspases, their maturation involves an autocatalytic processing of the zymogen form and their active participation in PCD. The zymogen form of AtMC9, a member of *Arabidopsis* metacaspase family, was constitutively *S*-nitrosylated in vivo which kept the enzyme in an inactive form. However, the active, processed enzyme was no longer a target of *S*-nitrosylation (Belenghi et al. 2007).

Poór et al. (2013) found that NO production by tomato cell suspension increased in the first hour, but did not change later at the lethal concentration of NaCl. In contrast, ROS production increased significantly for 6 h during the experimental period. Salt-induced ROS production was controlled by ethylene, which enhanced the percentage of dead cells. The salt stress caused a loss of the membrane semipermeability, chromatin condensation, cell shrinkage, and DNA fragmentation in the cells. The effect of specific inhibitors confirmed that the Ca^{2+} signaling, MAPKs, and cysteine proteases are involved in salt stress-induced PCD in tomato suspension cultures.

13.8 Conclusion and Perspectives

Despite the fact that the knowledge concerning the role of NO in higher plants under salt stress has increased considerably in the recent ten years, there are still many gaps in comprehending the mode of NO action that controls the salt stress-induced cell death or the acclimation of plants to saline environments.

Salt stress enhanced the NO production of tissues in many plant species. Unfortunately, most of the authors determined NO accumulation only at one time-point after salt exposure although the rise is transient and may vary from some minutes to few hours or days (Keyster et al. 2012). This NaCl-generated NO can be inhibited by NOS and NR inhibitors, suggesting that several NO-producing mechanisms can be activated in a time- and tissue-specific manner.

It can be concluded from several reports that the treatment with exogenous NO donors could alleviate the toxic effect of salt stress. The application of SNP increased the biomass, the contents of osmoprotectants such as glycine betaine, proline, and soluble sugars, and increased RWC and K^+/Na^+ ratios under salt stress. SNP decreased the salt stress-induced lipid peroxidation, ROS production, activated the antioxidant enzymes such as SOD, POX, GPX, APX, CAT, GR, and DHAR, and enhanced the non-enzymatic antioxidant pools (ASA and GSH). The NO donor has also a positive effect on photosynthesis under high salinity. Unfortunately, in most of the experiments, the NO levels of the tissues have not been determined during the acclimation period after SNP treatment. Moreover, SNP can generate not only NO but highly toxic cyanide thus treatment with 0.5 mM SNP caused PCD within 12 h in tobacco BY-2 cell suspensions (Vitecek et al. 2007).

NO-mediated *S*-nitrosylation and nitration of proteins may be involved in acclimation to salinity stress, but the enhancement of peroxynitrite-dependent Tyr nitration may shift the cells to the initiation of PCD (Corpas et al. 2013).

In plant tissues, NO and ROS produced during salt stress cooperate to trigger PCD (Fig. 13.3). The reaction of NO with superoxide radical leads to peroxynitrite, which is not essential in NO-mediated PCD. Moreover, NO and H_2O_2 can chemically react with each other producing singlet oxygen and hydroxyl radicals. These very reactive radicals can induce fast cell death (Wang et al. 2013).

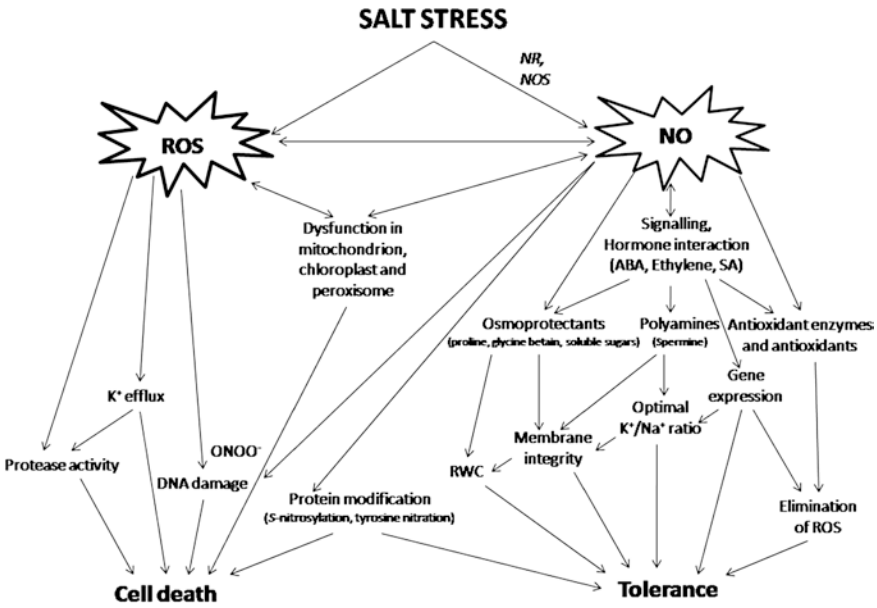

Fig. 13.3 The role of NO in salt-induced cell death and salt salt tolerance

Although there are a number of experimental data on the physiological aspects of NO-mediated alleviation of NaCl-induced stress, our knowledge is imperfect concerning the changes in NO contents in parallel with those of individual species of RNS and ROS.

The NO-mediated signal transduction, the detailed investigation of NO-mediated post-translational protein modifications, and gene expression changes have been performed only in few species under lethal salt concentrations. To see whether these changes were due to the NO-dependent changes in the activity of the related enzymes or due to the NO-induced control of gene expression, further analyses have to be done.

The description of the specificity of the individual reactive nitrogen forms in the signal transduction of plant hormones accumulating under salt stress would also provide new insights into converging and diverging signaling pathways.

Finally, the comparison of salt stress-dependent changes in the transcriptome, proteome, and metabolome and the evaluation of the data by systems biology tools would be particularly useful in defining differences between salt-sensitive and salt-tolerant genotypes or between glycophytes and halophytes.

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Chapter 14
Nitric Oxide and Postharvest Stress of Fruits, Vegetables and Ornamentals

R.B.H. Wills, P. Pristijono and J.B. Golding

Abstract Horticultural produce is impacted by a range of abiotic stresses during storage and marketing that accelerate ripening and senescence and thus reduce shelf life. Exogenous application of nitric oxide (NO) by gas fumigation or dipping in a solution of NO-donor compound has been demonstrated to alleviate some of the effects of abiotic stress on a wide range of produce. A key reported beneficial effect of NO treatment is to reduce the production of ethylene. Other reported beneficial effects of NO treatment include a reduced rate of respiration and reduced ion leakage resulting from better maintenance of cellular integrity; reduction in oxidative stress through reduced lipid oxidation and enhanced activity of a range of antioxidant enzymes which have been implicated in defence mechanisms; inhibition of polyphenol oxidase activity associated with reduced internal and surface browning; and alleviation of chilling injury potentially through enhancing the natural antioxidant defence systems which could include endogenous NO. Postharvest application of NO is a potential new technology to reduce losses of horticultural produce during handling and marketing.

Keywords Chilling injury · Ethylene · Polyphenols · Ripening · Senescence

14.1 Introduction

Horticultural commodities such as fruits, vegetables and ornamentals can be considered as being under continuous abiotic stresses as they are still biologically active. Even after harvest, they maintain a full range of metabolic sequences in

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order to maintain cellular integrity. However, the physical act of harvesting has removed the ability of fruits and vegetables to obtain water and nutrients from the parent plant. Thus, fruits and vegetables must utilise the components present at harvest for all subsequent postharvest metabolism, and therefore, all fruit and vegetables are under abiotic stress. A similar situation of continuous abiotic stress also applies to ornamentals although in parts of the marketing chain, water and nutrients can be supplied by standing stems in a vase solution.

The depletion of metabolic reserves inevitably leads to loss of cellular integrity and the onset of various forms of deterioration that a consumer recognises as a loss in quality which may be either physiological or pathological in nature. The rate and nature of this deterioration varies greatly between individual produce and physical conditions of the postharvest environment. However, the increasing distance between production areas and markets, the growing complexity of marketing systems to support international trading and extended domestic marketing to capture better out-of-season prices result in considerable abiotic stress being imposed on produce.

Postharvest research has developed various technologies to minimise deterioration after harvest. These technologies range from environment management (principally of temperature and humidity but also of ethylene which is an important ripening- and senescence-inducing compound produced by all horticultural commodities) to chemical intervention to inhibit respiration and ethylene action or growth of invading pathogens. While considerable advances have been made in extending the postharvest life of horticultural produce, research continues to find new technologies to better maintain product integrity and quality, particularly if they can utilise naturally occurring compounds.

14.2 Relationship Between Endogenous Nitric Oxide and Ethylene

Research into the possibility of using nitric oxide (NO) to minimise the effects of abiotic stress on postharvest horticultural commodities was instigated in the mid-1990s by the late Ya'acovLeshem from Bar-Ilan University in Israel. Leshem was aware of the growing medical interest in the potential therapeutic benefits of NO arising from its involvement in numerous mammalian metabolic sequences and sought to uncover if NO had a parallel role in plants. His initial publication (Leshem and Haramaty 1996) examined a role for NO in plant signalling and reported that adding the NO-donor compound *S*-nitroso-*N*-acetylpenicillamine (SNAP) to pea leaves resulted in a greater emission of NO than ethylene and that both NO and ethylene increased with the addition of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). They suggested that NO may regulate the ethylene production in growing plants. Subsequent studies reported an inverse relationship between endogenous NO and ethylene released from four unripe and ripe fruits and two cut flowers (Leshem et al. 1998), and increased ethylene

production during ripening of banana and strawberry was found to coincide with reduced NO emission (Leshem 2000; Leshem and Pinchasov 2000). Leshem et al. (1998) also reported that alfalfa sprouts, heat stressed at 37 °C, showed increasing production of NO and decreasing production of ethylene with time suggesting a stress coping role for NO. The documentation of an inverse link between endogenous NO and ethylene was the trigger that spawned research over the last 15 years to ascertain whether exogenous application of NO to both normal intact produce and the rapidly growing retail market segment of fresh-cut fruits and vegetables would extend postharvest life.

14.3 Postharvest Application of NO

14.3.1 Fumigation with NO Gas

The commercial availability of NO gas cylinders and industry experience of fumigation with 1-methylcyclopropene (1-MCP) to inhibit ethylene action on a range of horticultural produce (Blankenship and Dole 2003) makes NO gas fumigation an obvious potential method of application. However, treatment with the free radical NO gas needs to take into account the rapid oxidation of NO by atmospheric oxygen; e.g., Snyder (1992) reported a half-life of NO in air of 5 s. In addition, the rate of oxidation of NO increases with decreasing temperature due to increased intermolecular interaction between NO and oxygen molecules (Tsukahara et al. 1999). This led early studies (e.g. Leshem et al. 1998) to treat horticultural produce with gaseous NO in a nitrogen atmosphere. However, oxygen (O_2) is required for the maintenance of normal aerobic metabolism, and while produce can tolerate some reduction below the normal atmospheric concentration (about 21 % O_2), extended exposure below threshold levels results in anaerobic metabolism and leads to the subsequent development of tissue damage, off-flavours and off-odours (Wills et al. 2007a). This would limit the fumigation time to relatively short periods and imposes additional logistical and equipment requirements on any commercial treatment.

Soegiarto et al. (2003) examined the rate of loss of 30 μl l^{-1} NO gas at 20 °C from atmospheres containing 0.3 % O_2 to 21 % O_2 and found that the rate of NO loss was much lower than expected with the half-life of NO ranging from about 16 h in 0.3 % O_2 to 3.5 h in 21 % O_2. They also showed that the rate of loss of NO from the atmosphere was much greater in the presence of horticultural produce than from air only, indicating a rapid uptake of NO by produce, but the rate of uptake varied considerably between different types of horticultural produce. Thus, NO seems to be sufficiently stable at the concentrations and fumigation times utilised to allow produce to be treated in normal air.

Wills et al. (2007b) subsequently developed a solid mixture that released NO gas in the presence of horticultural produce. They found that the solid NO-donor compound, 2,2′-(hydroxynitrosohydrazino)-bisethanamine (diethylenetriamine nitric

oxide, DETANO) (Fitzhugh and Keefer 2000) quantitatively liberated NO gas in the presence of a range of acidic substances including citric acid. A solid mixture of DETANO, citric acid and wheat starch (added as a filler and moisture absorbent) in the ratio of 1:10:20 was found to be stable when stored in dry air. However, in humid air, the absorption of moisture from the atmosphere led to reaction of DETANO with citric acid and the evolution of NO gas. When the dry mixture was placed in a container with strawberry fruit and mushrooms, the moisture given off by produce activated the mixture and resulted in a similar extension in postharvest life as achieved by direct fumigation with NO gas. The industrial use of such a solid mixture could be through tablets or sachets which are more manageable in a farm or packing house than gas fumigation.

14.3.2 Dipping in Aqueous Solution of NO-Donor Compounds

Postharvest treatment by dipping in aqueous solutions (e.g. fungicides) is a common practice in the horticultural industry and is therefore of interest for research into the application of NO. The requirements for any commercial treatment are for the NO-generating compound to be quite stable as a solid, relatively stable in solution but to quantitatively release NO when absorbed by produce. There are now a wide range of NO-donor compounds that are relatively stable in solution with the list being considerably expanded due to medical interest in using NO donors (Hou et al. 1999). However, only a few NO-donor compounds have been used in postharvest horticulture research.

The most commonly examined NO-donor compound is sodium nitroprusside (SNP) ($Na_2[Fe(CN)_5NO]2H_2O$). SNP is a transition metal NO complex class of donor compound that releases the NO^+ cation and has found clinical use (Pitkanen et al. 1999; Wang et al. 2002). SNP is soluble in water and relatively stable in solution, resisting oxidation at a neutral or slightly acidic pH (Verner 1974), and is widely available and relatively inexpensive.

DETANO was first utilised in postharvest research by Bowyer et al. (2003) on carnation cut flowers although Noritake et al. (1996) had applied DETANO to growing potato plants and found an effect on potato tubers (see Sect. 14.4.2). DETANO was selected as a candidate for NO treatment as it was claimed to be the most stable diazeniumdiolate with a half-life in solution of 20 h at 37 °C and pH 7.4 and followed first-order kinetics in degrading to two molar equivalents of NO^* free radical with the reaction being dependent on solution temperature and pH (Lemairè et al. 1999; Fitzhugh and Keefer 2000). The half-life of 0.1 mM DETANO solution markedly decreases at low pH with Davies et al. (2001) reporting it to be 24 s in solutions buffered to pH 2.

Only three other NO-donor compounds have been utilised and each in just one postharvest study. These compounds are as follows: Piloty's acid (*N*-hydroxybenzenesulfonamide), N-tert-butyl-α-phenylnitrone (PBN) and 3-morpholinosyl-nonimine (Sin-1).

14.4 Effects of NO on Intact Produce

The postharvest behaviour of horticultural commodities traditionally classifies individual produce into two groups—climacteric and non-climacteric produce. Climacteric produce that can be fruits (such as banana and tomato) or ornamentals (such as carnation) has a definite ripening phase which is characterised by a marked increase in respiration and ethylene production, and the produce is transformed into the desirable colour, texture and flavour of edible produce. They are generally harvested at a mature but unripe stage with the aim of preventing ripening occurring until produce reaches the wholesale or retail market. Non-climacteric produce that can be fruits, vegetables or ornamentals does not undergo a marked physiological change after harvest but follows a general senescence pathway which can be accelerated by exposure to enhanced ethylene concentrations. Research with climacteric produce often aims to inhibit the initiation of the ripening process, while studies with non-climacteric produce aim to inhibit general senescence (Wills et al. 2007a).

14.4.1 Effects of NO Gas

The first reported postharvest study with exogenous application of NO was conducted by Leshem et al. (1998) who treated strawberry, broccoli, cucumber, Chinese broccoli, kiwi fruit and mushroom with 0.05–0.25 μmol l^{-1} NO gas in a nitrogen atmosphere for 2–16 h followed by storage in air at 20 °C and found a 70–180 % extension of postharvest life compared to untreated produce. Ku et al. (2000) then reported that rate of water loss was reduced by about 20 % from eleven fruits and vegetables and five cut flowers following a 24-h exposure to NO in nitrogen but did not measure the effect beyond this period.

Studies on the effects on NO on climacteric fruit would seek inhibition of ripening as the desired outcome. Indeed, numerous studies on a range of climacteric fruits have shown that fumigation with NO gas can regulate ethylene biosynthesis which is the trigger for initiation of ripening (Wills et al. 2007a). In mango fruit, Zaharah and Singh (2011b) showed that NO fumigation (20 μl l^{-1} NO in air for 2 h at 21 °C) inhibited ethylene biosynthesis through inhibition of the activity of the ethylene biosynthetic enzymes 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO), leading to reduced level of the immediate precursor of ethylene (ACC) in the fruit pulp. These two key enzymes (ACS and ACO) effectively regulate ethylene production (Alexander and Grierson 2002). The consequent lower levels of ethylene reduced the activity of fruit-softening enzymes (polygalacturonase and pectinesterase) but increased the level of endo-1,4-D-glucanase in pulp tissue during ripening and cool storage. In tomato fruit, Eum et al. (2009b) showed that fruit at different ripening stages was affected by different concentrations of NO fumigation as mediated through different ripening genes.

The regulation of ethylene has significant physiological effects in fruit which significantly affect ripening and senescence. For example, in peaches, Zhu et al. (2006) found that fruit treated with 5 and 10 $\mu l\, l^{-1}$ NO gas in air for 3 h delayed ripening, as measured by fruit firmness, through reduced ethylene biosynthesis. They also reported that NO inhibited lipoxygenase (LOX) activity and suggested the decrease in LOX activity might be a collateral process with the inhibition of ethylene biosynthesis. Flores et al. (2008) also found that peaches fumigated with 5 $\mu l\, l^{-1}$ NO gas in nitrogen for 4 h at 20 °C resulted in a reduced respiration rate. They further showed NO-treated fruit remained firmer after storage and the degree of disintegration of cell membranes, as assessed as the percentage of electrolyte leakage, was also lower in fruits treated with NO. Singh et al. (2009) reported that Japanese plums fumigated with NO in air had a reduced rate of respiration and ethylene production rates during ripening at 21 °C. They further showed that NO fumigation caused a 3- to 4-day delay in ripening as evidenced by restricted skin colour changes and retarded softening. In pears, Sozzi et al. (2003) fumigated mature fruit with 10 $\mu l\, l^{-1}$ NO gas in air for 2 h and showed decreased ethylene production, while fumigation with 10 and 50 $\mu l\, l^{-1}$ NO for 12 h delayed skin yellowing but did not affect fruit softening. They concluded that the NO treatments had differential effects on pear ripening and suggested a time × concentration effect existed for applied NO.

Chilling injury is a major storage problem for some horticultural crops (particularly of crops of tropical and subtropical origin) when they are stored at low temperatures (Wills et al. 2007a). NO has been implicated in improving chilling tolerance. Zaharah and Singh (2011a) fumigated mangoes with 10, 20 and 40 $\mu l\, l^{-1}$ NO gas in air for 2 h and showed that NO treatment not only delayed ripening (as defined by softening and colour changes) during storage at 5 °C for 2 and 4 weeks but also reduced the incidence of chilling injury. Singh et al. (2009) also reported that Japanese plums fumigated with 10 $\mu l\, l^{-1}$ NO in air showed reduced chilling injury symptoms during storage at 0 °C for 6 weeks. Zhu et al. (2010) further found that fumigation with 15 $\mu l\, l^{-1}$ NO gas with intermittent warming cycles of 1 day at 25 °C after 14 and 28 days at 5 °C was effective in preventing mealiness in peaches during storage at 5 °C. Xu et al. (2012) demonstrated that endogenous NO plays a role in chilling injury development. They showed that loquat fruit stored at 1 °C induced the accumulation of endogenous NO, but pretreatment with the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) not only abolished endogenous NO accumulation but also enhanced chilling injury symptoms. The action of NO in alleviating chilling injury symptoms was ascribed to stimulating the antioxidant defence systems in the fruit.

Wu et al. (2012) found that fumigation of the climacteric Chinese bayberry (*Myrica rubra*) fruits with 20 $\mu l\, l^{-1}$ NO gas in nitrogen for 2 h inhibited ethylene production and disease incidence and delayed the decrease in firmness, total phenolics and DPPH radical scavenging activity. They also suggested that NO might maintain the balance between the formation and detoxification of reactive oxygen species (ROS) and enhance the resistance of tissues to decay. Indeed, NO

seems to play a regulatory role in the antioxidant balance within the fruit. Flores et al. (2008) also measured a range of antioxidant enzymes and systems and showed that fumigation with NO seemed to have a beneficial effect on the oxidation equilibrium and the antioxidant capacity of peach fruit. Zhu et al. (2008) also found NO in solution significantly reduced the accumulation of malondialdehyde (MDA), superoxide and hydrogen peroxide, delayed the decrease in vitamins C and E, maintained the content of soluble solids, inhibited the activity of LOX and peroxidase (POD) and increased the activity of superoxide dismutase (SOD) and catalase (CAT) in kiwi fruit during storage.

Research on the effect of NO gas on non-climacteric produce was reported by Wills et al. (2000) who examined strawberries stored at 20 and 5 °C in humidified air containing 0.1 $\mu l\, l^{-1}$ ethylene and found that fumigation with NO gas in nitrogen resulted in an increase in postharvest life with the most effective concentration being 5–10 $\mu l\, l^{-1}$ NO at both temperatures. Soegiarto and Wills (2004) fumigated broccoli, green beans and bok choy (*Brassica chinensis*) with NO gas in air for 2 h and then stored produce at 20 °C in air containing 0.1 $\mu l\, l^{-1}$ ethylene. All produce showed an extension in postharvest life through inhibition of yellowing, but the optimum concentration of NO differed markedly, being about 50 $\mu l\, l^{-1}$ for green bean and bok choy and 4000 $\mu l\, l^{-1}$ for broccoli. Eum et al. (2009a) later reported that NO fumigation with 1000 $\mu l\, l^{-1}$ in nitrogen for 5 h delayed yellowing and retarded the onset of chlorophyll degradation in broccoli florets.

Soegiarto and Wills (2006) examined the interaction of NO fumigation and modified storage atmospheres with strawberries and iceberg lettuce. The effect of low O_2 was examined by fumigating with 10 and 100 $\mu l\, l^{-1}$ NO gas in air and 2 % and 5 % O_2 in air followed by storage at 10 °C for strawberries and 5 °C for lettuce in the same atmosphere. They found an increase in postharvest life due to NO and to storage in low O_2, but when both treatments were applied concurrently, there was no additional increase in postharvest life. The effect of elevated carbon dioxide (CO_2) and reduced O_2 was also examined by placing produce in a sealed polyethylene bag and injecting NO gas at 10 and 20 $\mu l\, l^{-1}$ into the bag. The atmosphere stabilised after 7 days at about 20 % CO_2 and 3 % O_2 for strawberries and 5 % CO_2 and 13 % O_2 for lettuce. The results showed that the postharvest life was enhanced by NO fumigation and was further enhanced by co-application of the modified atmosphere (Soegiarto and Wills 2006).

The role of NO in improving the tolerance of non-climacteric fruit against chilling injury was examined by Yang et al. (2011). They treated cucumber fruit with 25 $\mu l\, l^{-1}$ NO for 12 h at 20 °C and then stored the treated fruit at the chilling temperature of 2 °C for 15 days. The application of NO reduced the increases in membrane permeability and lipid peroxidation associated with chilling injury and delayed the increases in both the superoxide anion $\left(O_2^{\cdot -}\right)$ production rate and hydrogen peroxide content. The NO-treated fruit exhibited significantly higher activities of SOD, CAT, ascorbate peroxidase (APX), POD and higher DPPH radical (2,2-diphenyl-1-picrylhydrazyl) scavenging activity than control fruit during the storage. The overall results suggest that NO enhanced chilling tolerance in cucumber fruit by improving the antioxidative defence system.

Zhu et al. (2009) found that Chinese winter jujube fumigated with 20 μl l^{-1} NO gas in nitrogen at 22 °C for 3 h inhibited polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) activities. Indeed, they also showed that treatment with different NO concentrations from a saturated solution diluted to less than 1 μmol l^{-1} exhibited inhibitory effects on in vitro PPO and PAL activities in a dose-dependent manner.

Dong et al. (2012) found that mushrooms fumigated with 10, 20 and 30 μl l^{-1} NO gas in air for 2 h had increased antioxidant activities as determined by assays of reducing power, chelating effect on ferrous ions, scavenging effect on hydroxyl free radicals and DPPH radical scavenging activity. Furthermore, they found that NO fumigation significantly enhanced phenolic and flavonoid contents and stimulated the activities of PAL and chalcone synthase.

14.4.2 Effects of NO-Donor Compounds

The most studied NO-donor compound with climacteric fruit has been SNP. Sis et al. (2012) showed that a 1 mM SNP aqueous dip treatment for 5 min could prolong the postharvest life of peaches with a reduced ethylene production rate and increasing firmness and antioxidant enzymes activity. Zhang et al. (2008) found reduced flesh browning in plum during storage at 2 °C following dipping in 1 mM SNP for 3 min, but no treatment had any effect on PPO activity. Lai et al. (2011) showed that a 1 mM SNP dip applied to tomatoes delayed ripening and enhanced resistance to fungal pathogens such as *Botrytis* spp. They further showed that the activity of antioxidant enzymes in NO-treated fruit was higher late in the storage period compared to the control. They concluded that NO suppressed ethylene biosynthesis, stimulated antioxidant enzymes activity and regulated the expression of age-related genes.

The use of SNP has been found beneficial in the elucidation of chilling injury. Zhao et al. (2011) showed that a 0.02 mM SNP dip protected tomatoes from cold injury by inducing NO accumulation and expression of a C-repeat/dehydration-responsive element (CRT/DRE)-binding factors (CBFs) which play a prominent role in cold response regulation. They showed that SNP treatment protects tomatoes from cold injury by inducing expression of LeCBF1 and that nitric oxide synthase (NOS) activity may play a role in NO accumulation associated with cold tolerance in tomato fruit.

For non-climacteric produce, Zhu and Zhou (2007) dipped strawberries in 5 μmol l^{-1} SNP in water for 2 h at 25 °C and found that it inhibited ethylene production, respiration rate, activity of ACS and reduced the level of ACC. Unfortunately, the storage quality of the fruit in this trial was not quantified. Cheng et al. (2009) showed that mature green banana slices treated with 5 mM SNP solution had reduced ethylene production which was associated with reduced ACO activity and expression of the *MA-ACO1* gene. Duan et al. (2007) reported that dipping longan fruit in 1 mM SNP for 5 min inhibited pericarp browning and

pulp breakdown and reduced PPO activity and the level of MDA, a marker of lipid peroxidation (Mittler 2002), but SNP treatment maintained higher levels of total soluble solids and ascorbic acid during storage. Liu et al. (2012) found that Glorious oranges dipped in 30, 50 and 100 μmol l^{-1} SNP for 10 min maintained a higher level of titratable acidity, soluble protein, ascorbic acid and reducing sugar, and a lower weight loss and soluble solids concentration as well as retarded ripening.

The initial study with DETANO was by Noritake et al. (1996) who applied DETANO to growing potato plants and found that it induced accumulation of the phytoalexin rishitin in potato tubers. The result suggests a key role for NO in the synthesis of the defence mechanism in plants possibly in response to abiotic stress. The only postharvest study with DETANO on intact fruit and vegetables was by Jiang et al. (2011) who dipped button mushrooms for 10 min in DETANO and stored produce in sealed modified atmosphere biorientated polypropylene bags at 4 °C for up to 16 days at 4 °C. They found that treatment with 1 mM DETANO maintained a higher level of firmness, delayed browning and cap opening, promoted the accumulation of phenols and ascorbic acid and reduced the increases in both respiration and hydrogen peroxide content. Furthermore, the DETANO dip inhibited PPO activity and increased the activity of the antioxidant enzymes CAT, SOD and APX throughout storage period. Unfortunately, Jiang et al. (2011) did not measure the effect of the treatments without MA packaging, and hence, it is not known whether the effects of the NO and MAP treatments were additive.

Postharvest application of DETANO has also been reported for cut flowers (see Sect. 15.6) and fresh-cut produce (see Sect. 15.5).

14.5 Effects of NO on Fresh-Cut Produce

Due to increased consumer interest, fresh-cut produce is a growing sector of the food service industry; however, fresh-cuts are more perishable than intact produce arising from significant abiotic stresses during preparation which involves removal of protective epidermal cells. This leads to reduced shelf life due to an increase in respiration, ethylene evolution, water loss, alterations in flavour and aroma and enhanced surface browning (Watada et al. 1996; Artes et al. 1998).

The development of browning on the cut surfaces of apple slices was examined by Pristijono et al. (2006). They fumigated Granny Smith apple slices with NO gas in air and showed a delay in the onset of browning on the apple surface during storage at 0 °C with the most effective treatment being fumigation with 10 μl l^{-1} NO for 1 h. They also reported that Royal Gala, Golden Delicious, Sundowner, Fuji and Red Delicious apple slices stored at 10 °C and fumigated with 10 μl l^{-1} NO for 1 h were effective in inhibiting surface browning. Pristijono et al. (2008) further examined the effect of DETANO on browning of apple slices and found that dipping in 10 mg l^{-1} DETANO in pH 6.5 phosphate buffer for 1 min was the most effective treatment in inhibiting surface browning of apple slices stored

at 0 °C with the postharvest life more than double that of untreated slices. They showed that it was necessary to buffer the DETANO solution to be slightly acidic, probably due to the apple vacuolar acids leaking into the dip solution and degrading the DETANO before it was absorbed into the apple slice. Pristijono et al. (2008) also compared the effect of dipping in DETANO solution and fumigating with NO gas and found that DETANO was more effective than NO gas in inhibiting browning development. Huque et al. (2013) confirmed that dipping in a buffered DETANO solution was more effective than fumigation with NO gas in inhibiting surface browning at 5 °C. They also reported that DETANO and NO gas resulted in a lower level of total phenols, inhibition of PPO activity, reduced ion leakage and reduced rate of respiration but had no significant effects on ethylene production or lipid peroxide level as measured by MDA and hydrogen peroxide levels with DETANO having a greater effect than NO gas. In addition, they found that apple slices dipped in aqueous chlorogenic acid solution enhanced browning development, but subsequent dipping in DETANO solution negated the effect of chlorogenic acid while fumigation with NO gas gave partial relief. Huque et al. (2013) suggested that an increase in phenols occurs on the apple surface soon after cutting, possibly as a defensive mechanism of the apple to limit damage to surface cells, and the effectiveness of NO to inhibit surface browning may relate to minimising the level of phenols active on the cut surface possibly in conjunction with a reduced PPO activity.

Huque et al. (2013) also found that the NO-donor compound, Piloty's acid, inhibited browning with the optimum aqueous dip solution concentration being 100 mg l^{-1}. They compared the optimum concentrations of 500 mg l^{-1} SNP and 100 mg l^{-1} Piloty's acid dissolved in water with 10 mg l^{-1} DETANO in phosphate buffer and fumigation with NO gas to inhibit browning. While all forms of NO extended the postharvest life of apple slices, the order of effectiveness was DETANO > SNP > NO gas = Piloty's acid.

The effect of NO gas and DETANO on fresh-cut iceberg lettuce was examined by Wills et al. (2008). They found that development of browning on the cut surface during storage at 0 °C was inhibited with the optimal treatments being dipping in 500 mg l^{-1} DETANO in water for 5 min and fumigation with 500 μl l^{-1} NO for 1 h with DETANO more effective than NO gas. The use of a water dip compared to the need for a buffered dip with apple slices was attributed to the higher pH of lettuce tissue not degrading DETANO in solution. Huque et al. (2011) extended the findings to four other types of fresh-cut lettuce (*Lactucasativa*), Green Oak, Green Coral, Baby Cos and Butter lettuces, during storage at 5 °C. They reported that fumigation with NO gas for 2 h, or dipping in a solution of DETANO dissolved in 0.01 M phosphate buffer pH 6.5 or dipping in an aqueous solution of SNP all inhibited the development of browning. The optimum treatments to suppress surface browning were dipping in 500 mg l^{-1} DETANO or SNP and fumigation with 100 μl l^{-1} NO gas, with the donor compounds more effective than NO gas. Dipping lettuce slices in 500 mg l^{-1} SNP was considered to be the most feasible commercial option due to the stability of SNP in unbuffered water.

Zhu et al. (2009) fumigated pre-climacteric peach slices with a 5 μM NO solution for 10 min followed by storage at 10 °C for up to 10 days. Treatment with NO suppressed the increased rate of leakage and thus maintained compartmentalisation between the enzymes and their substrates. They also reported that NO increased PAL activity and total phenol content and inhibited PPO and POD activity. PPO is a complex enzyme with two copper ions in its active centre, and they speculated that NO could react with the copper of PPO to form copper–nitrosyl complexes which could change the normal structure of the active site in PPO and thus reduce PPO activity.

NO may also be a promising method for maintaining the quality of peeled bamboo shoots. Yang et al. (2010) showed that bamboo shoots dipped in 0.5 mM SNP for 1 h had delayed onset of external browning during storage for 10 days at 10 °C. SNP also inhibited PPO, POD and PAL activity and maintained total phenol content during storage. Furthermore, SNP treatment inhibited the synthesis of lignin and cellulose and delayed tissue lignification.

14.6 Effects of NO on Ornamentals

Leshem et al. (1998) assessed postharvest senescence of Pink Sim and White Sim carnation flowers when continually exposed to a solution containing the NO-donor compounds *N*-*t*-butyl-α-phenylnitrone (PBN) and 3-morpholinosyl-nonimine (Sin-1) in the presence of the senescence promoting ethylene precursor, ACC. They found that the NO-releasing compounds inhibited the action of ACC as observed by petal infolding and wilting.

Bowyer et al. (2003) treated White Sim carnation cut flowers with NO by fumigation with gas in nitrogen for 1 h and by standing flower stems in an aqueous DETANO solution for 24 h. All flowers were then stored at 20 °C in the presence of 0.1 μl l^{-1} ethylene. They found that both NO gas and DETANO increased vase life with the optimum concentrations being 1 μl l^{-1} for NO gas and 10 mg l^{-1} for DETANO. Buffering the solution to pH 6.5 gave a further small but significant increase in vase life. While no direct comparison was made of their relative effectiveness, the data suggest that DETANO may give a greater extension in vase life than NO gas. Badiyan et al. (2004) further explored the potential of DETANO with eight flower types with a span in the range of sensitivity to ethylene. Chitchat snapdragon, Bellisimo delphinium, Regan chrysanthemum, Golden Brush tulip, Manovale gerbera, Specisiom Simplon-oriental lily, Carnavale rose and Blue Magic iris cut flower stems were placed in a solution of 10 and 100 mg l^{-1} DETANO buffered to pH 6.5, and after 24 h, they were placed at 20 °C in air containing 0.1 μl l^{-1} ethylene. All flowers showed an extension in vase life over control flowers but with a wide range in level of extension. The greatest benefit of NO treatment was obtained with gerbera and the least was with chrysanthemum and rose. Zeng et al. (2011) studied the effect of SNP on Monte carnation cut flowers and found that dipping in 0.1 mmol l^{-1}

SNP extended the vase life and maintained fresh weight during storage. They concluded that NO could delay petal wilting in carnation cut flowers, maintain water metabolism, cell membrane stability and the activity of antioxidative enzymes (SOD, POD, CAT and APX) and eliminate ROS.

14.7 Mode of Action of NO on Postharvest Produce

The initiation of postharvest research with NO was based on the findings by Leshem and Haramaty (1996) on pea leaves that NO and ethylene had an antagonistic relationship. The ability of NO to inhibit ethylene production through inhibition of the ethylene biosynthetic enzymes, ACS and ACO, resulting in a reduced level of ACC, has now been demonstrated for a range of climacteric and non-climacteric produce and can be promoted as one of the key effects of NO that inhibit ripening and delay senescence. However, it is noted that Huque et al. (2013) did not find any reduction in ethylene production in the NO-induced inhibition of browning of apple slices. They attributed the lack of effect of NO as due to the apples being postclimacteric and thus having a substantial production of ethylene. Nonetheless, the data would suggest that either ethylene is not a direct causative factor in surface browning of apples or NO can inhibit browning through other modes of action.

A common finding of many studies with applied NO is a reduced rate of respiration. This is consistent with findings of Millar and Day (1996) and Zottini et al. (2002) that NO affects the function of mitochondria in plant cells and reduces cell respiration by inhibiting the cytochrome pathway. NO can then be ascribed an anti-senescent action which could arise from a general reduction in the rate of cellular metabolism. Whether this occurs via inhibition of ethylene action or independently needs to be determined. Other effects such as reduced ion leakage could be ascribed as a side benefit of better maintenance of cellular integrity through reduced general metabolism.

The role of NO in reducing oxidative stress and its involvement in plant signalling (Besson-Bard et al. 2008) would seem to also apply to postharvest tissue. Reduced lipid oxidation caused by ROS is purported to be a major cause of membrane deterioration in plant tissues (Mittler 2002). Reduced levels of biomarkers for lipid peroxidation, MDA and hydrogen peroxide, have been found for various postharvest produce, while NO has also been found to stimulate the activity of a range of antioxidant enzymes and enhance PAL activity which has been implicated in defence mechanisms. Indeed, the stimulation of the phytoalexin rishitin in growing potato tubers by NO (Noritake et al. 1996) may be a NO-moderated response to either abiotic stress or pathogenic invasion. Work is needed to examine whether postharvest NO application can stimulate phytoalexins in other tissues and thereby reduce the need for chemical biocides to combat microbial infections. It is noted that Lazar et al. (2008) in an in vitro study found that NO gas inhibited spore germination, sporulation and mycelial growth of three common postharvest

fungi, *Aspergillus niger*, *Penicillium italicum* and *Monilinia fructicola*. NO may thus have a dual role in reducing the spread of postharvest diseases.

Inhibition of PPO activity by NO has been demonstrated in many intact and fresh-cut produce and was associated with reduced internal and surface browning. Zhu et al. (2009) speculated that NO could react with the copper to produce a copper–nitrosyl complex that was metabolically inactive. There may be applications with processed fruits and vegetables where browning of juices and purees during storage is a common problem. Pre-treatment with NO to reduce PPO activity may enhance the storage life of the processed product. The action of NO on the phenolic substrates for PPO action is not clear as the various studies give conflicting data with some showing a reduced level of phenols while others show an enhanced level.

Alleviation of chilling injury in a range of produce by NO is a recent expansion of the mode of action of NO. Current suggestions are that the beneficial effect of NO is due to enhancing the natural antioxidant defence systems. In addition, the data on loquat fruit by Xu et al. (2012) indicate that endogenous NO production is a natural defence mechanism triggered when produce is subject to abiotic chilling stress. Application of exogenous NO would therefore seem to be enhancing a natural response.

The findings of the only study by Soegiarto and Wills (2006) where NO and a modified atmosphere were simultaneously applied (with appropriate air controls) showed that exposure to both NO and elevated CO_2 gave an added extension in postharvest life of strawberries and lettuce over the extension due to the individual component, whereas exposure to NO and reduced O_2 gave no added benefit. The findings suggest that NO and CO_2 have a different mode of action while that of O_2 and NO is similar and hence not additive. The benefits of modified atmospheres are generally considered to be through an inhibition of respiration via a feedback signal from the respiratory gases.

Despite the use of NO as a gas and various donor compounds in the reported studies, all forms gave a beneficial effect for inhibiting postharvest changes and therefore extending shelf life. However, the different forms of NO degrade to release different NO moieties. DETANO, Piloty's acid and NO gas release the $NO^{\bullet}$ free radical, and SNP releases the NO^{+} cation (Zamora et al. 1995; Hou et al. 1999; Saavedra and Keefer 2002). It might be expected that each NO moiety has different reactivity on produce metabolism with the moiety more directly linked to the key metabolic action(s) being more effective in inhibiting postharvest change. Since all NO compounds produced some beneficial effect, it would seem likely that the released NO moieties are interconvertible to some extent.

Direct comparison of the effectiveness of different forms of NO has only been conducted with fresh-cut apples and lettuces and then only in relation to development of surface browning. The applicability of conclusions drawn from these studies to ripening and general senescence of intact produce must therefore be made with some caution. For apple slices, Huque et al. (2013) found all four NO forms inhibited development of browning with DETANO > SNP > NO gas = Piloty's acid, while for the four lettuces evaluated, Huque et al. (2011) found that all three

forms of NO examined inhibited browning with SNP = DETANO > NO gas. As an applied treatment, fumigation with NO gas is therefore less effective than dipping in DETANO and SNP solutions.

However, in determining the relative effectiveness of each NO form, the effect of the solvent must also be considered. In the above studies, DETANO was dissolved in phosphate buffer, and SNP and Piloty's acid in water, while NO gas has no solvent. Since both phosphate buffer and water alone inhibit browning, the relative effectiveness of NO gas and DETANO would be, respectively, higher and lower than that found for the actual applied treatments. For the lettuces, Huque et al. (2011) calculated the effectiveness of NO as a percentage of the respective control treatments as SNP > NO gas > DETANO.

While a range of effects of NO on metabolism of horticultural produce has been identified, it is not clear whether the various actions of NO are at the chemical enzymic or genetic level and which are the key reactions that may trigger a cascade of supporting reactions. However, a growing number of studies are beginning to examine the effects of NO at the genetic level. Zhao et al. (2011) demonstrated that an elevated NO content up-regulated the expression of a cold regulation gene (LeCBF1) and also induced antioxidative processes to scavenge ROS resulting in treatment improved cold tolerance in tomatoes in cold storage, while Eum et al. (2009b) showed that NO has the potential to regulate ethylene biosynthesis. It is conceivable that NO may be acting on a range of systems which have different levels of importance in different produce and in response to differing abiotic stresses.

Additional studies would also seem to be required to determine the level and activity of endogenous NO at the stage of commercial maturity of individual horticultural produce and therefore whether exogenous NO is accentuating an active system. The early studies by Leshem (2000) indicated that the level of endogenous NO in mature produce is relatively low. Studies that utilise NO scavengers as used by Xu et al. (2012) would seem to be useful. Examination of the interaction between applied ethylene and NO would appear worthwhile given the central role of ethylene in promotion of ripening and general senescence. It could be reasoned that NO has a major role in maintaining cellular integrity and organisation during produce development and maturation but is replaced by the deteriorative effect of ethylene in mature produce which have reached the end of their developmental life. Application of exogenous NO could then be analogous to applying a juvenile hormone to prevent ageing.

14.8 Commercial Usage

Commercial postharvest usage of NO on fruit and vegetables would require approval from the regulatory agencies in the respective countries. Since NO is synthesised by plants, it can be considered as a naturally occurring material which would assist in gaining regulatory approval. The human synthesis of NO and

the use of various forms of NO in therapeutic applications should also be positive factors. However, plants and mammals do not have totally identical pathways for metabolism of NO. Of particular interest is the presence in higher plants of nitrate reductase which is a key enzyme that catalyses the conversion of nitrate to nitrite (Yamasaki and Sakihama 2000). Nitrites have the potential to be converted to nitrosamines which are potent carcinogens (Walker 1990). The addition of NO gas or individual donor compounds would need to be shown to not generate nitrosamines. The use of NO with ornamentals does not pose such a regulatory hurdle and should be easier to obtain.

Use of NO gas would require gas-tight fumigation chambers, but this is not an insurmountable issue particularly if added to air. A more convenient delivery system could be along the lines proposed by Wills et al. (2007b) of solid mixture that releases NO gas when placed with horticultural produce (see Sect. 14.3.1). Tablets or sachets of the mixture would be placed in packages in a packing house. The package would not need to be sealed as the steady release of NO gas over a few days would be rapidly absorbed by the surrounding produce.

Of the NO donors presently evaluated, SNP would seem to offer the most potential due to its stability in water which eliminates the need for addition of a buffer. Water dipping is a common practice in many packing houses to clean and/or move produce around the packing house. DETANO has been shown to be more effective than SNP for some produce but is unstable at even mildly acidic pH (Hrabie et al. 1993). The quality of water used in commercial operations is variable and would require the use of a buffered solution to ensure that a near-neutral pH is maintained throughout dipping. For ornamentals, the addition of DETANO or some other solid NO-donor compound to vase water would seem to offer a commercial opportunity.

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Chapter 15
Insights into the Participation of Nitric Oxide and Extra Cellular ATP in Wounding

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Abstract In natural environments, wounding represents an abiotic stress in plants that is closely linked to biotic factors such as herbivory and insect feeding. Wounding also regularly occurs during harvesting, storage, and handling of fresh crop products. A proper wound-healing response is critical to restore optimal health and physiological conditions aimed to facilitate plants to overcome the adverse condition-associated challenges. Wounding and wound-healing responses have been characterized in mammals as well as in plants, and the role of some key molecules has been established during the last few years. Extracellular ATP (eATP) coming from other injured cells has been described as a wound signal also in animals and plants. At the same time, oxidative and nitrosative signals contribute to the mentioned pathway; the small gaseous and free radical reactive nature of nitric oxide (NO) allows it to participate in a plethora of relevant molecular and cellular reactions even in eATP-mediated responses. In this chapter, we shortly reviewed the participation of eATP and NO as players of an orchestrated complex net of events occurring downstream wounding in plants. A brief discussion on the interplay between ROS/RNS and Ca^{2+} as counterpart signal molecules is also presented.

Keywords Extracellular · ATP · Nitric oxide · Signaling · S-nitrosylation · Wounding

15.1 Introduction

In plants, wounding is a common stress as a result of different types of challenges. Very frequently, biotic factors such as herbivory from plant predators and insect feeding are the cause of wound tissues which in turn results in biotic infections.

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Under human influences, wounding also occurs regularly during harvesting, storage, and handling of fresh crop products. After wounding, plants are at elevated risk of water loss. Therefore, a proper wound-healing response is the key to restore optimal physiological conditions that may enable the plants to overcome the adverse condition-associated challenges. Recently, a huge number of genes, proteins, and phytochemicals have been shown to be wound induced, giving a comprehensive view of the functional response to mechanical injury. The nature of the sets of genes and compounds and their activation timing have led to the recognition of two different phases: (i) a mechanical barrier formation and tissue sealing and (ii) the activation of the signaling net that triggers a complex plant defense mechanism. Both phases are tightly coordinated in timing and space; however, superimposition between them does also exist in order to keep metabolic integrity at the wound site.

In the last years, the availability of genome sequence data has considerably advanced our understanding about the molecular basis of regulatory networks that underline different forms of environmental stress. Particularly, transcriptomic research studies have brought completely new insights about the involvement of ancient and highly conserved protein families during wounding. For instance, heat-shock proteins (HSPs), which functions mainly as molecular chaperones, have been proved to be essential for wound healing in mammals (Atalay et al. 2009). In plants, emerging evidences point to the up-regulation of HSPs upon wounding. Using a transcriptomic analysis approach, Swindell et al. (2007) discovered that all Arabidopsis HSPs gene families (*HSP20*, *HSP70*, *HSP90* and *HSP100*) are early up-regulated upon wounding. Further, similar examples are showed in different plant species; *StHSP90* from potato, as well as other two HSPs transcripts *Ma-HSP90* and *Ma-HSP101* from banana fruit is accumulated rapidly after the onset of wounding treatments (Chen et al. 2009; D'Ippólito et al. 2011). In rice, a class II small heat-shock protein, Oshsp18.0-CII is also induced by mechanical injury (Chang et al. 2007). Interestingly, in Arabidopsis, HSF21 a member of the heat-shock transcription factor family that mediates *HSP* gene activation is specifically induced after wounding (Cheong et al. 2002). Together, these findings open a novel window to study the role of HSP, as a gene family common to different biological systems, during wounding. Nevertheless, their functional roles remain unexplored, and further investigation is necessary to shed light on this query.

In addition, several signal transduction nodes are activated upon wounding in plants. In Arabidopsis leaves, jasmonic acid accumulates immediately after injury both proximal and distal to the wound site and works as a long-distance signal for wounding (Glauser et al. 2009). The phytohormones ethylene, salicylic acid, abscisic acid, and auxin also contribute to the integration of wounding signal (Kazan and Manners 2009). In sum, all these events must be finely synchronized at both spatial and temporal levels in order to ensure a successful healing response.

In the last years, nitric oxide (NO) has been described to function as a signal molecule coordinating multiple plant responses, including mechanical injury. Here, we raise a discussion about the participation of NO as a specific signal in the wounding and wound-healing processes in plants. Moreover, the putative involvement of extracellular ATP (eATP) as small molecule acting in coordination with NO for wounding signaling is discussed.

15.2 Wounding-Mediated Downstream Events and NO

The very early events after wounding are of paramount importance for the proper recognition and deciphering of the incoming challenge; these conduce to trigger the adequate signal transduction pathways. The first measurable event occurring after wounding is the change in plasma transmembrane potential (*V*m) (Shabala et al. 2006). Wound-induced *V*m changes can be attributed to different signal molecules that affect it either directly or via specific receptors. Elicitors such as peptides (Nürnberger et al. 1994), β-glucans (Mithofer et al. 2005), oligosaccharides (Shibuya and Minami 2001), and eATP (Chivasa et al. 2009; Tanaka et al. 2010) from leaking cells also induce *V*m depolarization. In addition, the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is a common response to stresses in plants; at low concentration, they act as second messengers involved in cell signaling and at high concentration, they can be part of the direct defense against stress (Chaki et al. 2009; Miller et al. 2010). Particularly, H_2O_2 is a strong depolarizing molecule occurring immediately after wounding (Orozco-Cardenas and Ryan 1999).

Within RNS, NO is the most studied molecule (Mur et al. 2013). In injured plants, NO production is transiently induced, increasing from minutes to a few hours locally at wound site, returning to basal levels afterward (Arasimowicz et al. 2009; París et al. 2007). In plants, two major sources for NO production were identified, an enzymatic and a non-enzymatic mechanisms which depend on L-arginine and nitrite, respectively (Corpas et al. 2009; Wilson et al. 2009). Even when the relevance of NO in plants is evident, the precise source of endogenous NO involved in a given physiological process is still under revision (Corpas et al. 2011). NO has been extensively reported in a multiplicity of paths for downstream cellular processes. In animals, several studies revealed that NO controls the activity of plasma membrane as well as intracellular Ca^{2+} permeable channels (Clementi 1998). Interestingly, extracellular Ca^{2+} is one of the common factors acting at wound zones; it could increase as consequence of damaged cell-content leakage or by specific channel activation. NO induces a transient rise in cytoplasmatic Ca^{2+} concentration in *Vicia faba* guard cells and tobacco cell suspensions (Garcia-Mata et al. 2003; Lamotte et al. 2004). The use of transgenic *Nicotiana* cells expressing the Ca^{2+} reporter aequorin introduced in the cytosol evidenced that NO might activate plasma membrane Ca^{2+} channels by inducing a rapid and transient plasma membrane depolarization (Lamotte et al. 2006). Further, it has been shown that NO could also perform its effects into Ca^{2+} channels through both cyclic ADP-ribose or protein kinases activation (Sokolovski et al. 2005).

Other layer of NO action upon wounding could be pointed out at cell wall components structure and dynamic remodeling. In plants, cell walls play a pivotal role in mediating responses to external stimuli; perturbation of wall components often leads to responses associated with biotic and abiotic stresses (Ellis et al. 2002). Cellulose is the main load-bearing component of primary cell walls. Pathogen attacks, wounding, and other environmental cues that damage or alter the cellulose in some way lead to consequences in either compensatory or integrity responses.

For instance, Arabidopsis mutants in the cellulose synthase gene *CESA3*, with consequent reduction in cell wall cellulose contents exhibit increased production of lignin (Cano-Delgado et al. 2003). *CESA3* mutants and plant treatments with inhibitors of cellulose synthesis, such as isoxaben, also invoke plant defense responses induced by the wounding-related jasmonic acid- and ethylene-dependent gene expression (Cano-Delgado et al. 2003; Ellis et al. 2002). These data suggest that alterations in cell wall integrity can activate lignification and wound responses. Moreover, it has been shown that wounding induced both NO burst and deposition of the cell wall glucan callose (París et al. 2007). Interestingly, low concentrations of NO donor sodium nitropruside increased cellulose content in root cell walls, while higher concentrations had the opposite effect (Correa-Aragunde et al. 2008). The authors demonstrated that the NO outcome, measured as ^{14}C-glucose incorporation to cell walls, is transient and reversible. In summary, these results lead us to speculate that NO could work locally as a modulating molecule for the remodeling of plant cell walls in response to incoming stresses such as wounding.

15.3 Extracellular ATP (eATP) and NO Are Co-players in Plant and Animal Systems

At the wound site, spontaneous eATP leakage contributed by injured cells results in an external signal to nearby healthy cells. eATP has been described as a wound signal in different organisms including mammals, algae, and plants (Jeter et al. 2004; Sen 2009; Torres et al. 2008). In mammals, eATP activates surrounding cells by binding to purinogenic and adenosine receptors and meanwhile in plants, the existence of eATP binding receptors has not been conclusively demonstrated. The absence of genes encoding for purinogenic receptors in some animal groups such as insects and roundworms, as well as in plants, contrast to the pharmacological-mediated eATP pathway characterized in these same organisms, suggesting that novel receptors are still to be discovered (Burnstock and Verkhratsky 2009; Tanaka et al. 2010). In human monocytes, eATP binding to purinogenic receptors activates NADPH oxidases with the consequent induction of Ca^{2+} waves and ROS production (Kaufmann et al. 2005). Notably, in plants, eATP also activates Ca^{2+} influx and ROS production resulting in gene expression changes as a consequence of wounds and other stresses (Chivasa et al. 2009; Demidchik et al. 2009; Song et al. 2006). Foresi et al. (2007) demonstrated that exogenous ATP induces NO production in tomato cell suspensions. Similar to animals, a non-hydrolyzable ATP analog (known agonist of P2-like receptors) also stimulates NO production to regulate physiological processes (Tonón et al. 2010). At this point, it is becoming evident that, as signals, eATP and NO integrate different transduction pathways; nevertheless, more knowledge on the accompanying actors is required. It is known that heterotrimeric G-proteins could be responsible for translation of a myriad of extracellular signals, including light, ions, hormones, and even NO and eATP. In Arabidopsis plants, null mutants of the heterotrimeric G-protein α subunit showed

suppression of several physiological and cellular events as ATP-promoted stomatal opening, cytoplasmic ROS generation, Ca^{2+} influx, and H^{+} efflux (Hao et al. 2012). This indicates that heterotrimeric G-proteins may also link eATP signal with stomatal opening by activating ROS-related ion transport. Therefore, we propose that both heterotrimeric G-proteins and ROS may also act as important signal transducers in the eATP pathway occurring in wounded cells. In addition, a role for G-protein action in cell wall biogenesis/metabolism has been demonstrated (Klopffleisch et al. 2011), suggesting that during wounding, G-protein mediation may take part on cell wall reinforcement. Finally, observations of phenotypes for the loss and gain of function mutants of G-protein components, their regulators, and proposed effectors allow to hypothesize that G-protein plant signaling has followed a conservative route rather different to others eukaryotic systems (Urano et al. 2013).

15.4 Participation of S-Nitrosylation in Wounding

As we mentioned (see model on Fig. 15.1), an NO burst takes place rapid and locally after plant wounding and both the production source/s and direct effector/s upon wounding remain elusive. Besides, NO is the most studied RNS and the role of intracellular S-nitrosothiols (SNO) species is emerging as a key transducer and regulator for the NO signal. S-nitrosoglutathione (GSNO) is an endogenous SNO that plays a role as NO reservoir and carrier. It is formed by the S-nitrosylation reaction of NO with glutathione and has been demonstrated to have important physiological functions both in animals and in plants (Wang et al. 2006). GSNO reductase (GSNOR) is the main enzyme responsible for the in vivo control of GSNO intracellular levels (Ng and Kubes 2003). Control of NO availability through regulation of GSNOR at different levels was observed as a consequence of different stresses including wounding (Leterrier et al. 2011). In general,

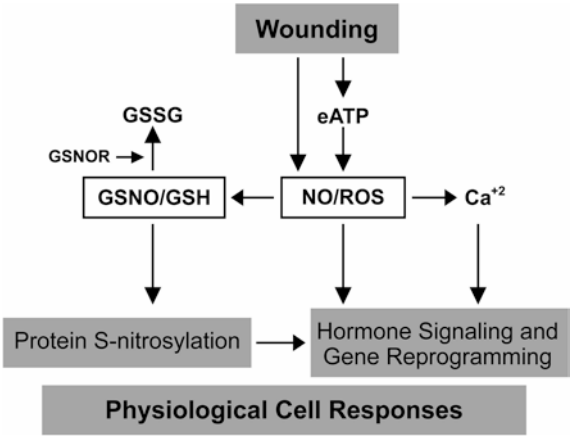

Fig. 15.1 Plant wound signaling through NO and eATP. Model shows the main cellular pathways operating upon wounding to the final physiological responses. *White boxes* indicate fine tuning of cellular balance between NO and ROS or GSNO and GSH

GSNOR results repressed after wounding and this down-regulation correlates with increments in GSNO contents. In tobacco leaves, a decrease in both GSNOR mRNA and protein levels was observed 2 h after wounding (Díaz et al. 2003). Meanwhile, in sunflower seedlings, GSNOR activity and protein levels decreased after mechanical wounding along with an increment in SNOs content; however, L-arginine-dependent NOS activity and NO levels were both reduced (Chaki et al. 2011). Besides, GSNO levels increased early after wounding, and it was proposed the participation of GSNOR in the regulation of systemic wound responses and in the transmission of a systemic signal through vascular tissue in Arabidopsis leaves (Espunya et al. 2012). NO accumulation and increase in SNO content were also observed after 4 h of mechanical wounding in pea leaves, but in this case, increases in GSNOR and NOS activities were detected (Corpas et al. 2008).

The thioredoxin system, comprising thioredoxin, thioredoxin reductase, and NADPH, has been found to participate in the regulation of a variety of cellular processes by S-nitrosylation and thioredoxin-mediated cellular homeostasis of SNOs in animals (Sengupta and Holmgren 2012). Mammalian thioredoxins play a major role in the denitrosylation of S-nitrosylated proteins (Sengupta et al. 2007), but also they were proved to be involved in the S-nitrosylation of proteins, as caspase 3 (Mitchell and Marletta 2005). Plants have a much higher number of thioredoxin genes as compared to prokaryotes, yeast, and vertebrates showing a more complex scenario (Meyer et al. 2012; Reichheld et al. 2002). In plants, participation of thioredoxin system in the control of nitrosylation/denitrosylation has not been yet investigated in deep. However, the involvement of two thioredoxins, TRX-h3 and TRX-h5, has been proved in the regulatory denitrosylation of NPR1, the master regulator of defense gene expression in Arabidopsis (Tada et al. 2008). In a similar fashion, a previous report by Laloi et al. (2004) showed an increase of *AtTRXh5* transcript level in plants challenged to wounding and other abiotic and biotic stresses. Taking together, these data support a role for the thioredoxin system in nitrosylation/denitrosylation in plants and suggest the specific participation of thioredoxin-mediated S-nitrosylation modifications in response to wounding. The central-hub character of SNOs may explain the difficulties in the generation of viable mutants in the operating enzymatic systems that regulate the dynamic of SNOs in plants. Therefore, the identification via genomic and transcriptomic studies could be of greater applicability to shed light on this topic.

15.5 Concluding Remarks

In summary, the molecular basis of eATP- and NO-coordinated wounding signaling pathways seems to have an early origin and conservative traits during the biological evolution. Nonetheless, the scenario remains to be fully understood, since in plants, eATP binding receptors have not been identified yet. For instance, in mammals, macrophages are a major cellular source of NO at the wound site; meanwhile, it is still unknown whether specific tissue- and/or cell-type NO

producers may exist in plants. Additionally, alternative sources of NO production as well as the mechanism of action of NO, NO-derived molecules as S-nitrosothiols and their regulating enzymes GSNOR and thioredoxins are still to be clarified. In plants, much more fundamental knowledge and mechanistic studies are still required in order to understand how the wounding responses counteract the impact of wounding itself and the indirect consequences as plant biotic infections in biotic stress. Finally, the development of applied strategies to manipulate response signals at wound site is of outstanding significance in both plant phytopathology and crop production fields.

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