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Reactive Nitrogen Species and Nitrosative Stress in Plants†

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ABSTRACT

Nitric oxide ($^{\bullet}$NO) is a gaseous free radical which can have two opposite physiological roles in higher plants. While a high cellular production of $^{\bullet}$NO can bring about extensive cellular damage, at low levels this compound is involved as a signal molecule in many important physiological processes. $^{\bullet}$NO and a family of related molecules, including peroxynitrite ($ONOO^-$), *S*-nitrosoglutathione (GSNO) and nitrotyrosine (nTyr), among others, are designated with the term reactive nitrogen species (RNS). Under stress conditions, plants can undergo a de-regulated synthesis or overproduction of $^{\bullet}$NO and NO-derived products that can have toxic physiological consequences. This situation is known as nitrosative stress, due to its similarities with animal systems, and can produce important changes in plant cells. In this article the current knowledge of these effects of RNS on the physiology of plants under stress conditions is briefly reviewed.

† This article is dedicated to Mrs. María Luisa Aguirre-Romero and Mr. José Corpas-Martínez with love.

Keywords: nitric oxide, *S*-nitrosoglutathione, *S*-nitrosothiols, nitrotyrosine, nitric oxide synthase

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INTRODUCTION

At present nitric oxide ($^{\bullet}$NO) is one of the most studied bioactive gas molecules, due to its involvement in a wide spectrum of physiological processes (Pacher *et al*. 2007). In higher plants, $^{\bullet}$NO has an important function in plant growth and development, in processes including seed germination, primary and lateral root growth, flowering, pollen tube growth regulation, fruit ripening, senescence, defence response and abiotic stress, and $^{\bullet}$NO is also a key signalling molecule in different intracellular processes (Lamattina *et al*. 2003; del Río *et al*. 2004; Shapiro 2005). However, although intensive work is being done on the physiological function of $^{\bullet}$NO under normal and stress conditions, much less information is available on other reactive nitrogen species (RNS), like *S*-nitrosoglutathione, peroxynitrite, and nitrotyrosine. This review will focus on the description of the elements necessary to establish relationships between adverse environmental conditions, $^{\bullet}$NO metabolism and nitrosative stress.

METABOLISM OF NO IN PLANT CELLS

Enzymatic and not enzymatic sources of $^{\bullet}$NO

A key point in the metabolism of $^{\bullet}$NO in plant cells is the identification of the endogenous sources of $^{\bullet}$NO. It is known that plants can generate $^{\bullet}$NO by non-enzymatic and enzymatic mechanisms (Neill *et al*. 2003; del Río *et al*. 2004) but the contribution of each $^{\bullet}$NO source in the different physiological processes is not clear. Thus, nitrification/denitrification cycles provide $^{\bullet}$NO as a by-product of N_2O oxidation into the atmosphere. It is known that the non-enzymatic reduction of nitrite can lead to the formation of $^{\bullet}$NO, and this reaction is favoured at acidic pH when nitrite can dismutate to $^{\bullet}$NO and nitrate. Nitrite can also be chemically reduced by ascorbic acid at pH 3-6 to yield $^{\bullet}$NO and dehydroascorbic acid. This reaction could occur at microlocalized pH conditions in barley aleurone layers and in the chloroplast and apoplastic space where ascorbic acid is known to be present. Another non-enzymatic mechanism proposed for $^{\bullet}$NO formation is the light-mediated reduction of NO_2 by carotenoids (see del Río *et al*. 2004).

Nitrate reductase (NR) is a well-established generator of

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Table 1 Some plant species where the presence of L-arginine-dependent nitric oxide synthase (NOS) activity has been reported.

Species/Tissue or cell type	NOS activity ($pmol \cdot min^{-1} \cdot mg^{-1}$ protein)	Reference
White lupine (*Lupinus albus*)/Roots and nodules	296 [(a)]	Cueto *et al*. 1996
Cowitch or velvetbean (*Mucuna hassjoo*)/Green husks	2.7 [(a)]	Ninnemann and Maier 1996
TMV-infected tobacco (*Nicotiana tabacum*)/Leaves	6 [(a)]	Durner *et al*. 1998
Maize (*Zea mays*)/Root tips and young leaves	0.18 [(a)]	Ribeiro *et al*. 1999
Pea (*Pisum sativum*)/Leaf peroxisomes	5.0×10^3 [(a,b)]	Barroso *et al*. 1999; Corpas *et al*. 2004
Roots, stems, leaves	240, 630, 120 [(b)]	Corpas *et al*. 2006
Soybean (*Glycine max*)/Cotyledons	7.7 [(a)]	Modolo *et al*. 2002
Leaf chloroplasts	760 [(c)]	Jasid *et al*. 2006
Arabidopsis thaliana/Leaves	4.5 [(a)]	Zhao *et al*. 2007
Hibiscus moscheutos/Roots	0.7 [(a)]	Tian *et al*. 2007
Olive (*Olea europaea*)/Leaves	294 [(b)]	Valderrama *et al*. 2007
Sunflower (*Helianthus annuus*)/Hypocotyls	280 [(b)]	Chaki *et al*. 2007

[(a)] Arginine-citrulline assay
[(b)] Ozone chemiluminiscence assay
[(c)] Spin trapping EPR assay

$^{\bullet}NO$, using NO_2^- and NADH as substrates, and it has been proposed that it is involved in $^{\bullet}NO$ production in some processes such as stomata closure (Neill *et al*. 2003). However, there is little information on the involvement of NR-derived $^{\bullet}NO$ in plant stress. In the past decade many plant biologists intensively searched for an NO-generating enzyme similar to the nitric oxide synthase (NOS) identified in mammalian systems (del Río *et al*. 2004). Several reports claimed the identification of two plant NOSs and the characterization of their genes, but further research demonstrated that both the P protein variant of the glycine decarboxylase and the AtNOS1 protein did not produce $^{\bullet}NO$, and therefore they were not characteristic NOSs (Klessig *et al*. 2004; Zemojtel *et al*. 2006). Therefore, at this moment the search for the protein and gene of the plant NOS still continues. In any case, it must be taken into account that there are many biochemical evidence showing the presence of L-arginine dependent NOS activity in different plant species under physiological and stress conditions (**Table 1**).

S-Nitrosothiols (RSNOs)

In the last few years, interest in the study of *S*-nitrosothiols has increased considerably. The main reason is that the half-life of $^{\bullet}NO$ *in vivo* is very short whereas RSNO species are generally more stable in solution. RSNOs can participate in the transport, storage, and delivery of $^{\bullet}NO$, as well as in post-translational modifications in cell signalling and stress conditions (Foster *et al*. 2003; Benhar *et al*. 2006).

In plants cells, there is little information on the metabolism of RSNOs and still less is known on its modulation under stress conditions. It has been shown that under biotic stress the RSNO formation and turnover regulate multiple modes of plant disease resistance (Feechan *et al*. 2005). Similar results have been obtained in sunflower where the content of RSNO in hypocotyls increased after the infection by *Plasmopara halstedii* (M. Chaki *et al.*, unpublished results). On the other hand, in olive plants under salinity a significant increase of the total RSNO of leaves was found (Valderrama *et al*. 2007). Using a new commercial fluorescent probe, Alexa Fluor 488 Hg-link (Molecular Probes), the cellular localization of RSNOs by confocal laser scanning microscopy has been reported. In olive leaf sections, the green fluorescence attributable to RSNO was present mainly in phloem whereas in leaves of NaCl-treated plants, the distribution of RSNOs was diminished in vascular tissue but was stronger in spongy mesophyll and epidermal cells (Valderrama *et al*. 2007). Furthermore, in pea plants subjected to six abiotic stress conditions (low and high temperature, high light intensity, mechanical wounding and continuous dark), the total content of *S*-nitrosothiols was increased between 3 to 5 times (FJ Corpas *et al*. unpublished results). Therefore, the available data indicate that under biotic/abiotic stress conditions plant respond with an increase in the RSNO content.

Function of *S*-nitrosoglutathione (GSNO) and *S*-nitrosoglutathione reductase (GSNOR)

Among the different RSNOs, *S*-nitrosoglutathione (GSNO) which is formed by the *S*-nitrosylation reaction of $^{\bullet}NO$ with GSH, could have a significant physiological relevance in plants since GSNO is thought to function as a mobile reservoir of $^{\bullet}NO$ bioactivity (Durner *et al*. 1999; Díaz *et al*. 2003), as it has been reported in animals (Stamler *et al*. 2001). However, the experimental evidence available on the presence of GSNO in plant cells is scarce. In pea leaf sections, immunohistochemical analysis has shown the presence of GSNO in collenchyma cells which was drastically reduced when plants were grown in a toxic Cd concentration (50 μM) (Barroso *et al*. 2006). However, in olive leaves by confocal laser scanning microscopy GSNO was localized in vascular tissues and spongy mesophyll cells, and under salt stress its intensity was diminished in vascular tissues and was stronger in spongy mesophyll cells (Valderrama *et al*. 2007).

In recent years, the glutathione-dependent enzyme formaldehyde dehydrogenase (FALDH; EC 1.2.1.1) has been demonstrated to have GSNO reductase activity in bacteria, yeast and mammals and to be involved in the mechanism of protein *S*-nitrosylation in mammalian cells (Haqqani *et al*. 2003). The enzyme GSNO reductase (GSNOR) catalyzes the NADH-dependent reduction of GSNO to GSSG and NH_3 (Liu *et al*. 2001; Lamotte *et al*. 2005). Therefore, this activity can control the intracellular level of GSNO and consequently the $^{\bullet}NO$ effects. In plants, the presence of GSNOR activity has been found in *Arabidopsis* (Sakamoto *et al*. 2002; Achkor *et al*. 2003), tobacco (Díaz *et al*. 2003), and pea plants (Barroso *et al*. 2006). In tobacco, the gene coding for this enzyme is modulated in response to wounding, jasmonic acid and salicylic acid (Díaz *et al*. 2003). In pea plants grown with cadmium, the analysis of *S*-nitrosoglutathione reductase (GSNOR) activity and its transcript expression showed a reduction of 31% by cadmium (Barroso *et al*. 2006), but the GSNOR activity was induced by low temperature (Corpas *et al*. 2006).

NO–PROTEIN INTERACTIONS AND POST-TRANSLATIONAL MODIFICATIONS

It is well known that $^{\bullet}NO$ can react with metal centres present in many metalloproteins, and in animal cells good examples are guanylate cyclase or haemoglobin (Williams 2003). Moreover, $^{\bullet}NO$ or NO-derived molecules can react with proteins, forming covalent post-translational modifications which can modulate the protein functions. Among these modifications nitrosylation and *S*-nitrotyrosination are the most studied ones.

Protein *S*-nitrosylation

Protein *S*-nitrosylation is a post-translational modification

of cysteine residues produced by nitric oxide (•NO) which can modify the function of a broad spectrum of proteins (Stamler *et al*. 2001). In animal systems, there is evidence that dysregulated, diminished, or excessive *S*-nitrosylation may be implicated in a wide range of pathophysiological conditions (Foster *et al*. 2003; Bernhar *et al*. 2006) which can contribute to the generation of nitrosative stress. On the contrary, in plant cells, much less is known on these processes. Several potential candidates for protein *S*-nitrosylation have been identified, including stress-related, redox-related, signalling/regulating, cytoskeleton, and metabolic proteins (Lindermayr *et al*. 2005). Moreover, there is experimental evidence showing that only few proteins are regulated by this post-translational modification (Stamler *et al*. 2001). In *Arabidopsis*, non-symbiotic hemoglobin (AHb1) scavenges •NO with production of *S*-nitrosohemoglobin and reduces •NO emission under hypoxic stress (Perazzolli *et al*. 2004). Glyceraldehyde-3-phosphate dehydrogenase suffers a reversible inhibition by •NO (Lindermayr *et al*. 2005), and methionine adenosyltransferase is also inhibited by *S*-nitrosylation (Lindermayr *et al*. 2006). The *Arabidopsis* type-II metacaspase AtMC9 blocks autoprocessing and activation of the AtMC9 zymogen through *S*-nitrosylation of its catalytic cysteine residue (Belenghi *et al*. 2006).

Protein nitrotyrosination (nTyr)

Tyrosine nitration is a covalent modification of proteins resulting from the addition of a nitro (-NO_2) group to one of the two equivalent ortho carbons of the aromatic ring of tyrosine residues (Gow *et al*. 2004). The reaction of superoxide radicals ($O_2^{\bullet-}$) with nitric oxide generates a powerful oxidant, peroxynitrite ($ONOO^-$), which has been shown to participate in the nitration of tyrosine residues of proteins (Radi 2004). In animal cells, this protein modification is starting to be used as marker of pathological disease and nitrosative stress (Ischiropoulos 2003; Bartesaghi *et al*. 2007). However, *in vivo* examples of molecular targets for tyrosine nitration involved in specific pathophysiologic phenomena are still few. For example, it has been described the inactivation of MnSOD, actin in sickle cell disease, prostacyclin synthase in vascular dysfunction, tyrosine hydroxylase in Parkinson's disease, and prostaglandin endoperoxide synthase-2 in vascular inflammation (Bartesaghi *et al*. 2007).

In plant cells, much less information is available on protein tyrosine nitration. In suspension cultures of *Taxus cuspidate* an increase of 31% in the free 3-nitrotyrosine content during shear stress has been reported (Gong and Yuan 2005). In leaves from olive plants under salt stress conditions an increase in the number of proteins of 44-60 kDa that experimented tyrosine nitration has been described (Valderrama *et al*. 2007). In nitrite reductase antisense tobacco leaves, the induction of several tyrosine-nitrated polypeptides with molecular masses between 10- and 50-kDa has been reported (Morot-Gaudry-Talarmain *et al*. 2002). Moreover, in tobacco BY-2 suspension cells treated with a fungal elicitin, the induction of tyrosine nitration in proteins with molecular masses in the range 20-50 kDa has been demonstrated (Saito *et al*. 2006). Conversely, in tobacco transgenic plants with genetically increased cytokinin levels the content of tyrosine nitrated proteins decreased (Wilhelmova *et al*. 2006). In leaves of pea seedlings, using an antibody against nitrotyrosine a single band of 89-kDa has been identified, and when plants were exposed to low temperature (8ºC for 48 h), the expression of this protein was increased (FJ Corpas *et al*. unpublished results). Although many of these proteins have not been identified yet, these data indicate that an increase in the number of proteins or an intensification of specific proteins that experimented tyrosine nitration could be considered as a footprint of nitrosative stress, such as has been demonstrated in animal cells (Ischiropoulos 2003) (**Fig. 1**).

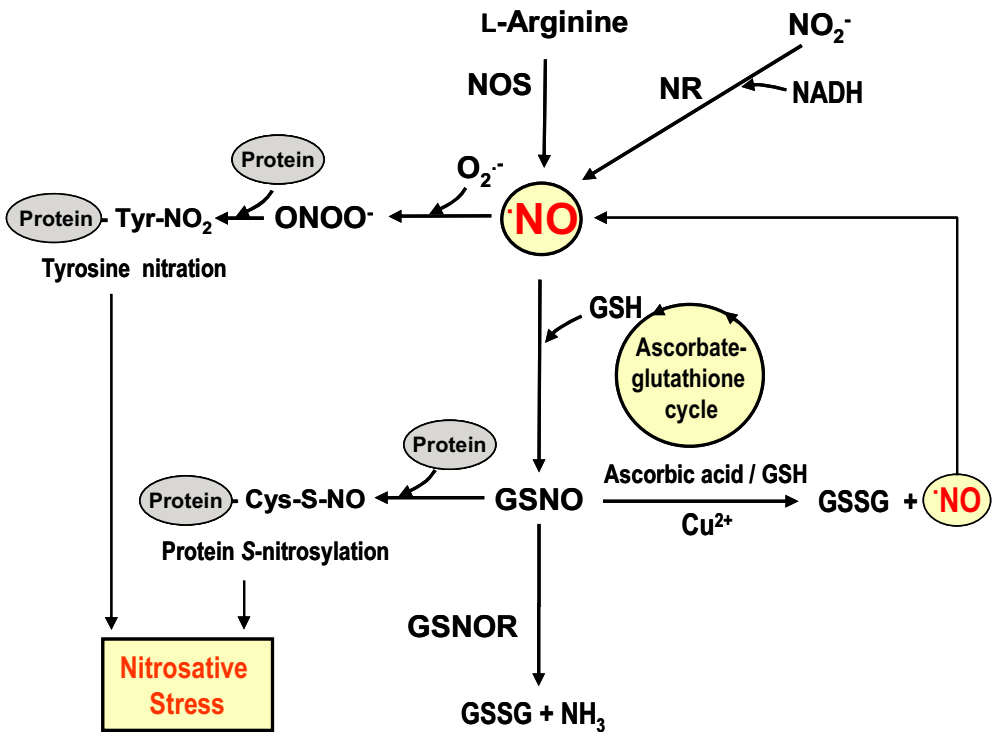

Fig. 1 Schematic model of the metabolism of reactive nitrogen species in plant cells. L-Arginine-dependent nitric oxide syntase (NOS) and nitrate reductase (NR) generate •NO (Neill *et al*. 2003; Corpas *et al*. 2004) which can react with reduced glutathione (GSH) in the presence of O_2 to form *S*-nitrosoglutathione (GSNO). This metabolite can be converted by the enzyme GSNO reductase (GSNOR) into oxidized glutathione (GSSG) and NH_3 (Liu *et al*. 2001). But alternatively, GSNO in the presence of reductants, such as glutathione (GSH) and ascorbate, and Cu^{2+}, can be decomposed to produce •NO and GSSG (Gorren *et al*. 1996; Holmes and Williams 2000; Smith and Dasgupta 2000). In this competitive mechanism, the reduced forms of ascorbate and glutathione could be recycled by the ascorbate-glutathione cycle. NO and GSNO could mediate protein *S*-nitrosylation. On the other hand, nitric oxide can react with superoxide radicals ($O_2^{\bullet-}$) to generate peroxynitrite (ONOO-), a powerful oxidant molecule that can mediate the tyrosine nitration of proteins (Radi 2004). In animals, this protein modification can be considered as a marker of nitrosative stress (Ischiropoulos 2003).

RNS METABOLISM AND ABIOTIC STRESS

Most of the studies carried out in plants so far have been focused on the modulation of •NO and less attention has been paid to other RNS (Corpas *et al*. 2006). A role for •NO has been reported in different plant species in response to certain abiotic stresses, including xenobiotics (paraquat, diquat, methylviologen), heavy metals, salinity, mechanical stress, ozone, and environmental conditions (high light intensity, UV-B radiation, low and high temperature). However, many of the data available have been obtained by indirect studies using exogenous •NO donors and some of the results are contradictory depending of the type of •NO donors (Murgia *et al*. 2004). Furthermore, in many cases it has not been clearly determined which is the specific endogenous source of •NO, either non-enzymatic or enzymatic, and therefore it is difficult to establish a clear relationship between stress conditions and activation/ inhibiton of a specific •NO source.

It has been shown that •NO produced by NR is required for abscisic acid-induced stomatal closure in *Arabidopsis* guard cells and this suggests the involvement of •NO in signalling processes leading to tolerance to drought and osmotic stress (Neill *et al*. 2003). On the other hand, using olive plants as model it has been described that salt stress (200 mM NaCl) caused an increase of the L-arginine-dependent production of nitric oxide (•NO), total *S*-nitrosothiols (RSNO) and number of proteins that underwent tyrosine nitration (Valderrama *et al*. 2007). Confocal laser scanning microscopy analysis of olive leaf sections using either specific fluorescent probes for •NO and RSNO or antibodies to *S*-nitrosoglutathione and 3-nitrotyrosine, also showed a general increase of these RNS mainly in the vascular tissue (Valderrama *et al*. 2007). In this case, a previous study in olive plants showed that NaCl caused oxidative stress, indicating that salinity induced both oxidative and nitrosative stress (Valderrama *et al*. 2006). However, this does not mean that in plants under other types of abiotic conditions oxidative and nitrosative stress necessarily overlap.

CROSSTALK BETWEEN NITRIC OXIDE AND OTHER SIGNALLING PATHWAYS

Nitric oxide is a chemical messenger which can interact with different hormones such as cytokinins, abscisic acid (ABA), salicylic acid (SA) and ethylene, as well with signal molecules like hydrogen peroxide, polyamines, phosphatidic acid and mitogen-activated protein kinases (MAPKs) (Lamattina *et al*. 2003; Lamotte *et al*. 2005; Yamasaki and Cohen 2006). There are different physiological processes where these interactions have been shown. In cucumber, an NO-dependent MAPK signalling cascade is activated during the adventitious rooting process induced by indole acetic acid, confirming that MAPKs are targets of •NO action in this physiological process (Pagnussat *et al*. 2004). In *Arabidopsis*, there is a strong inter-relationship between ABA, endogenous H_2O_2 and NO-induced stomatal closure (Bright *et al*. 2006), and it has been reported that •NO is a key player in reducing seed dormancy and promoting germination (Bethke *et al*. 2007). On the other hand, in *Arabidopsis* roots SA induced •NO synthesis through a nitric oxide synthase activity and calcium and casein kinase 2 activity were essential components of this signalling cascade (Zottini *et al*. 2007). However, in plants under abiotic stress conditions there is a considerable lack of information on the interactions between RNS and other signalling pathways in abiotic stress.

CONCLUSIONS AND FUTURE PROSPECTS

In plants, our knowledge of the metabolism of endogenous •NO and other RNS is still rudimentary. There are several key challenges that must be solved including: i) the identification of the enzymatic sources of endogenous •NO and their subcellular localization and regulation under stress conditions; ii) the identification of nitrosatively modified proteins in plants under optimal and stress conditions; and iii) to substantiate if the increase of the basal levels of protein 3-nitrotyrosine could serve as a footprint or marker of nitrosative damage *in vivo*. On the other hand, nitration processes are not limited to proteins and can also occur in DNA and lipids, being this a new aspect that must be explored. Further research is necessary to know the interconnecting pathways existing between reactive oxygen species (ROS) and reactive nitrogen species (RNS) in abiotic stresses, and establish biotechnological strategies against these stresses which are responsible for important annual losses in plant yield and world crop productions.

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