Review Article

S-Nitrosylation – another biological switch like phosphorylation?

Jasmeet Kaur Abat, Pooja Saigal (Talwar) and Renu Deswal

Plant Molecular Physiology and Biochemistry Laboratory, Department of Botany, University of Delhi, Delhi - 110 007, India

ABSTRACT

Nitric oxide (NO) has emerged as a key-signaling molecule affecting plant growth and development right from seed germination to cell death. It is now being considered as a new plant hormone. NO is predominantly produced by nitric oxide synthase (NOS) in animal systems. NOS converts L-arginine (substrate) to citrulline and NO is a byproduct of the reaction. However, a similar biosynthetic mechanism is still not fully established in plants as NOS is still to be purified. First plant NOS gene (AtNOS1) was cloned from *Arabidopsis* suggesting the existence of NOS in plants. It was shown to be involved in hormonal signaling, stomatal closure, flowering, pathogen defense response, oxidative stress, senescence and salt tolerance. However, recent studies have raised critical questions/concerns about its substantial role in NO biosynthesis. Despite the ever increasing number of NO responses observed, little is known about the signal transduction pathway(s) and mechanisms by which NO interacts with different components and results in altered cellular activities. A brief overview is presented here. Proteins are one of the major bio-molecule besides DNA, RNA and lipids which are modified by NO and its derivatives. S-nitrosylation is a ubiquitous NO mediated post-translational modification that might regulate broad spectrum of proteins. In this review S-nitrosylation formation, catabolism and its biological significance is discussed to present the current scenario of this modification in plants. **[Physiol. Mol. Biol. Plants 2008; 14(1&2) : 119-130]** *E-mail : rdeswal@botany.du.ac.in; renudeswal@yahoo.co.in*

Key words : *Nitric oxide; Nitric Oxide Synthase; NO Signaling; S-nitrosylation*

Abbreviations : *NO: Nitric Oxide; NOS: Nitric oxide synthase; sGC: soluble guanylyl cyclase; cGMP: cyclic guanosine monophosphate; MAPK: mitogen activated protein kinase; GSH: glutathione; GSNO: S-nitrosoglutathione; Rubisco: Ribulose 1, 5 bisphosphate carboxylase/ oxygenase; LPS: lipopolysaccharide.*

Nitric oxide (NO) is a free radical gas having highest diffusion coefficient for any biological molecule. Earlier, NO was recognized as a component of environmentally polluting NO_x complex (NO_2 and NO) but in 1987, when endothelium derived relaxing factor (EDRF), which causes relaxation of blood vessels, was shown to display identical chemical behavior as NO, it gave birth to an idea of NO as a biologically relevant signaling molecule. From then on, intensive research on NO biological function followed worldwide. In 1992, NO was recognized by Science magazine as the "Molecule of the Year" and in 1998, three NO research pioneers (Robert F. Furchgott, Louis J Ignarro & Farid Murrad) won the noble prize in medicine for their discoveries proving NO as a signaling molecule in the cardiovascular system", since then it has become one of the most vigorously researched molecules of biological chemistry.

Correspondence and Reprint requests : Renu Deswal, Plant Mol. Physiol. and Biochem. Lab., Depart. Botany, University of Delhi, Delhi - 110 007, India. Telefax : 91-011-27662273

NO is a water and lipid soluble gaseous free radical. It contains an unpaired electron in its p_2 orbital, but remains uncharged. Its chemistry is an inter-play between the 3 redox-related species Nitric oxide radical (NO), Nitrosonium cation (NO^+) and Nitroxyl anion (NO^-). Addition of electron from NO^+ to NO^- decreases the bond order, vibrational energies and increases bond length (Fig. 1). Each of these partially reduced/reactive nitrogen species possesses its own particular reactivity (Stamler *et al*., 1994). In biological systems, NO reacts with O_2, O_2^- and transition metals ($Me^+/2^+$), generating NO_x, peroxynitrite ($ONOO^-$) and metal-NO adducts respectively. The reaction of NO with O_2 results in the generation of NO_x compounds which can either react with cellular amines or thiols or hydrolyze to form the end metabolites nitrite (NO_2^-) and nitrate (NO_3^-) (Wendehenne *et al*., 2001). NO can mediate several complex chemical reactions many of which are shown to be associated with biological effects of NO.

NO is considered as a 'do it all' molecule that plays a

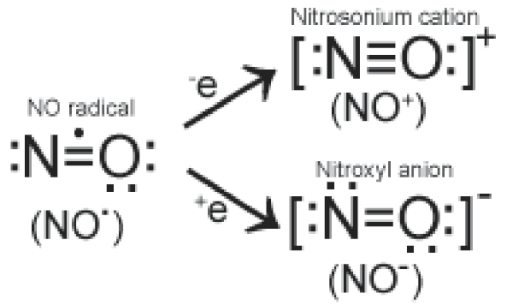

Fig. 1. Reactive forms of NO in biological systems. NO• (NO radical) is rapidly oxidized by the removal of one electron to give nitrosonium cation (NO^+), or reduced by the addition of one electron to form nitroxyl anion (NO•), which are highly reactive entities interacting with bio-molecules and manifesting the effects of NO. (Source Lamattina *et al.*, 2003).

crucial role right from seed germination to cell death. Most of the information about function of NO in plants has come from pharmacological studies using various NO donors, scavengers and NOS inhibitors. Application of NO donors and inhibitors has provided evidence for role of NO in physiological processes like wound healing (Paris *et al*., 2007), stem elongation (Qu *et al*., 2006), cell wall metabolism (Pacoda *et al*., 2004), accumulation of ferretin transcripts (Murgia *et al*., 2004), growth and reorientation of pollen tube (Prado *et al*., 2004), root organogenesis (Correa-Aragunde *et al*., 2004), stimulation of seed germination and counteracting inhibitory effects of heavy metals and salinity (Kopyra and GwoZdz, 2003), regulation of ion channels of guard cells (Garcia-Mata *et al*., 2003), improving internal Fe^{3+} availability (Graziano *et al*., 2002), senescence (Cheng *et al*., 2002), plant cell death (Beligni *et al*., 2002), defense gene induction (Durner *et al*., 1998) etc. NO is also involved in abiotic stresses. NO production was observed in response to several abiotic stressors such as high temperature, osmotic stress or UV-B in tobacco leaf cells (Beligni *et al*., 2001; Gould *et al*., 2003). Despite its involvement in these processes, NO could not be considered as a universal plant stress response as its production is not observed following mechanical or light stress up to 400 μmol $m^{-2}s^{-1}$, thus it was attributed a status of a generalized stress hormone (Gould *et al*., 2003). Due to its multifunctional activities in plants, NO is now being considered as a new candidate plant hormone (Beligine *et al*., 2001).

Biosynthesis of Nitric oxide

NO is predominantly produced by nitric oxide synthase (NOS) using L-arginine as substrate. In order to understand the status of NOS in plants it is worthwhile to point out some important facts about the animal enzyme, which is better worked out than its plant counterpart. Nitric oxide synthase was first identified and described in 1989. It has three isoforms. The first isoform to be purified was brain NOS, followed by inducible NOS and finally endothelial NOS. The physiological involvement of NO in neuronal transmission, control of vascular tone, immune response induced cytostasis as well as the deleterious effects associated with altered levels of NO synthesis has made NOS the focus of research. However, a similar biosynthesis mechanism in plants is still debatable and controversial. The NOS like activity is ubiquitously present in bacteria, fungi to higher plants like *Arabidopsis* (Guo *et al*., 2003), soybean (Modolo *et al*., 2002), tobacco (Durner *et al*., 1998), pea (Barosso *et al*., 1999), maize (Ribeiro *et al*., 1999) and *Lupinus* (Cueto *et al*., 1996). NOS-like activity was detected by utilizing the activity assays, NOS inhibitors and cross reactivity with antibodies against mammalian NOS proteins by western blotting. Although, western blotting detected many unrelated proteins also (Butt *et al*., 2003).

First plant NOS gene (AtNOS1) was cloned from *Arabidopsis* supporting the existence of NOS in plants (Guo *et al*., 2003). It encoded for a 561 amino acid protein which was similar to a protein implicated in NO synthesis in snail *Helix pometia*. It was also similar to a group of hypothetical bacterial proteins that have putative GTP binding or GTPase domains as in *H. pomatia*. The bacterial over-expressed gene product generated NO from L-arginine. NOS activity in the *Atnos1* mutant was 25% of the wild type. Wild and mutant plants were incubated in DAF- 2DA which gave strongest NO production at root tips in the wild type whereas in mutants it was greatly reduced suggesting the involvement of AtNOS1 in NO synthesis. The phenotype of mutants indicated the role of AtNOS1 in overall plant growth and development, fertility, leaf expansion, stomatal movement and hormone signaling. Guo and Crawford (2005) have demonstrated that AtNOS1 is targeted to mitochondria and is required for arginine dependent NO production in mitochondria of plant cell.

However, recent studies have raised critical questions about AtNOS1. NOS activity by citrulline and Griess reagent assays could not be detected in recombinant purified proteins obtained from cloning AtNOS1 and its orthologous genes from rice and maize. TLC analysis of the end products produced by [^{14}C] arginine also did not detect citrulline with recombinant AtNOS1 protein even though a NADPH dependent activity was present (Crawford *et al*., 2006). Also, the Griess reagent assay failed to show NO production. Although the control reactions with NO donor and with animal iNOS showed

the expected absorption shift characteristic of NO binding to heme domain of soluble guanylyl cyclase (sGC), but with AtNOS1 no such shift was obtained. The finding that AtNOS1 did not exhibit NOS activity *in vitro* suggests an indirect/ direct involvement of it in NO biosynthesis. Evidence still indicates involvement of AtNOS1 in NO synthesis as the *Atnos1* mutant had a reduced capacity to produce NO. Summing up these reports, AtNOS1 was suggested to be renamed as AtNOA1 (NO associated 1) (Crawford *et al*., 2006). However, Guo (2006) has attributed this discrepancy to low AtNOS1 activity in comparison to mammalian eNOS and iNOS respectively. An animal like NOS is still an enigmatic and controversial NO source in plants. Absence of any gene/ homolog similar to animal NOS in *Arabidopsis* genome further adds to the plant NOS controversies.

Zemojtel *et al*., 2004 found close homologs of *A. thaliana* and *H. pomatia* NOS in all sequence metazoans by PSI-BLAST searches. The alignment of these proteins with other eukaryotic sequences revealed homology and highly conserved sites. Lately, a novel family of putative NOS is described which is ubiquitous and includes AtNOS1 as a plant member. Intriguingly, these recent reports hint at the possibility of a novel arginine dependent NO source to be involved. These proteins display no homology to animal NOSs and contain a centrally positioned GTP binding domain (Zemojtel *et al*., 2006). A mammalian ortholog of AtNOS1 from mouse (mAtNOS1) was cloned and was found to be sub-cellularly localized in the mitochondria by immunofluorescence.

Regardless of all the controversies it is shown that AtNOS1 (or AtNOA1) is involved in hormonal signaling, stomatal closure, flowering, pathogen defense response, oxidative stress, senescence and salt tolerance (Guo *et al*., 2003; He *et al*., 2004; Zeidler *et al*., 2004; Guo *et al*., 2005; Zhao *et al*., 2006; Zhao *et al*., 2007). In Arabidopsis, NOS1 has a protective role against oxidative stress damage and dark induced senescence as suggested by nos1 mutant analysis. NO regulates flowering time in *Arabidopsis thaliana* by controlling the expression of flower timing genes (He *et al*., 2004). AtNOS1 is an important source of NO for signaling as well as plant defense responses (Zeidler *et al*., 2004). It is involved in providing bacterial disease resistance in *Arabidopsis*. Gene expression studies in LPS (lipopolysaccharide) treated *Arabidopsis* plants suggest a link between LPS induced NO production and defense or stress associated genes (including glutathione s-transferases, cytochrome P-450 and many genes encoding PR proteins). AtNOA1 (in previous studies it was referred to as AtNOS1) activity is associated with salt tolerance in *Arabidopsis* plants (Zhao *et al*., 2007). On treatment of plants with NaCl, the mutants showed higher Na^+ to K^+ ratio, increased inhibition of root elongation and seed germination, lower survival rates and higher accumulation of H_2O_2 compared to wild type plants.

In spite of multiple NO responses observed, little is known about the mechanisms by which NO manifests these responses resulting in altered cellular activities. The understanding about its synthesis and regulation of plant NOS still in its infancy and it remains poorly characterized in plants. This knowledge is pre- requisite to understand the role of NOS in various cellular signaling processes. The biochemical and physio-chemical characterization for nitric oxide synthase like enzyme in *Brassica juncea* seedlings provides evidence for existence of an arginine dependent enzymatic source of nitric oxide. It is activated by calcium, light and abiotic stresses. The enzyme activity was also enhanced by a phorbol ester (a protein kinase C activator) *in vitro* and *in vivo*. Further activation of the enzymatic activity by phorbol ester in presence of calcium suggests that calcium dependent protein kinase C isoform could be involved in modulating NOS like activity. Activation by sodium orthovanadate (tyrosine phosphatase inhibitor) suggests for the first time regulation of a plant NOS by phosphorylation involving key regulators protein kinase C, calcium and tyrosine phosphatase (unpublished work).

Other enzymatic source of NO apart from NOS-like enzyme is nitrate reductase, which is analyzed in many plant species like cucumber (Haba *et al*., 2001), sunflower, spinach, maize (Rockel, 2002), *Arabidopsis* (Desikan *et al*., 2002), wheat, orchid, aloe (Xu *et al*., 2003), tobacco (Planchet *et al*., 2006) and *Chlamydomonas reinhardtii* (Sakihama *et al*., 2002). Another enzyme responsible for NO production, identified only in tobacco roots till date is nitrite: NO-reductase (Ni-NOR), which is an endogenous source of NO in plants (Stõhr *et al*., 2001). Other NO producing enzymes identified in plants are xanthine oxidoreductase, peroxidase, cytochrome P450 and hemeproteins which could also contribute to NO formation (Rio *et al*., 2004). Non-enzymatically, NO may also be formed by chemical reduction of NO_2^- at acidic pH (Wendehenne *et al*., 2001). Such non-enzymatic NO formation was observed in apoplast of barley aleurone cells (Bethke *et al*., 2004). Once NO is generated in the system it reacts with biological molecules like DNA, RNA, lipids and proteins to manifest its effects.

Nitric oxide signaling

Despite the ever increasing number of NO responses observed in plants, meager is known about the signal transduction pathway(s) and mechanisms by which NO interacts with different components and results in altered cellular activities. This is due to dynamic nature of NO and the labile nature of nitrosylated species. In animals, NO stimulates signaling either through cyclic guanosine monophosphate (cGMP) dependent or independent pathways (Fig. 2). cGMP dependent signaling involves reaction of NO with transition metal (Fe^{2+}) in soluble guanylyl cyclase, which leads to production of cGMP and initiation of downstream signaling .The current scenario of cGMP dependent and independent signaling is summarized in the following sections.

cGMP dependent signaling

NO activates cGMP production via interaction with soluble guanylyl cyclase (sGC). It reacts with the iron in the heme moiety of sGC, inducing a conformational change that results in enzyme activation, which in turn leads to formation of second messenger cGMP from GTP (Hancock, 1997). Binding of NO to the heme group is reversible and GC is turned off when NO gradient is dissipated**.** Presence of cGMP in plants was confirmed by mass spectroscopy and radio-immunoassay. cGMP is degraded by phosphodiesterase (PDE). The presence of cGMP-PDE enzyme activity is shown in many plants (Newton *et al*., 1999) and several PDE genes exist in the *Arabidopsis* genome.

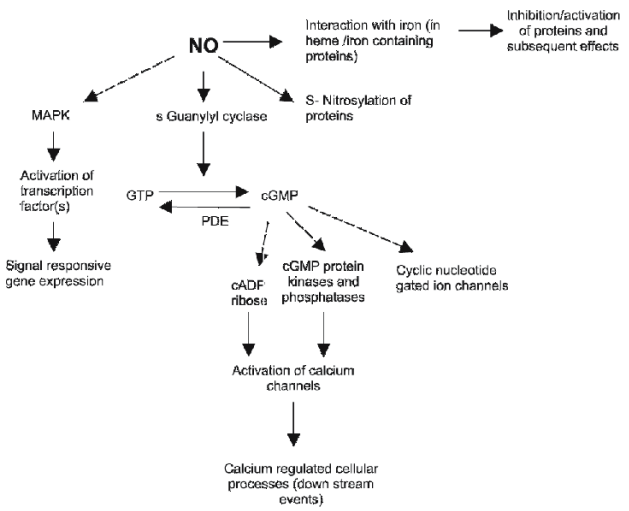

Fig. 2. Schematic representation of Nitric oxide signaling mechanism. Primary target of NO include soluble guanylyl cyclase, leading to production of cGMP and thus affecting its downstream targets. NO may also activate or inhibit proteins serving as signal transducer by promoting S-nitrosylation, tyrosine nitration and binding to metal centers in proteins. NO also causes signal responsive gene expression through MAPK.

While many of the cGMP-mediated processes in plants are reported, no plant molecule with GC activity is identified. Recently a motif search of *Arabidopsis* genome based on conserved and functionally assigned amino acids in the catalytic center of GCs one candidate is found that contains the adjacent glycine-rich domain typical for GCs. It is termed as AtGC1 and has catalytic domain in its N-terminal part. AtGC1 has arginine / lysine that participate in H-bonding with guanine and the cysteine that confers substrate specificity for GTP (Ludidi *et al*., 2003). Reports suggest that cGMP is required during ABA- and NO induced stomatal closure in pea and *Arabidopsis* (Neill *et al*., 2003). Also, NO and cGMP act as second messengers in the Indole Acetic acid – induced adventitious rooting process in cucumber (Pagnussat *et al*., 2003) and defense gene induction (PR-1, PAL) in tobacco (Durner *et al*.,1998). Therefore it is evident that cGMP dependent signaling may be relevant in plants. Existence of downstream targets and their participation in biological functions confirms this observation. Few NO mediated cGMP dependent signaling components are known in plants and animals including cyclic nucleotides, calcium ions, and protein kinases. These are briefly described here.

$ **cGMP induced Protein Kinases**

A common downstream target of cGMP in mammalian cells is cGMP- activated protein kinase (Wendehenne *et al*., 2001). Signal transduction mechanism operating during IAA and NO induced adventitious root formation shows the involvement of mitogen activated protein kinase (MAPK) cascade (Pagnussat *et al*., 2004). NO induced activation of MAPK is reported in tobacco (Kumar and Klessig, 2000) and *Arabidopsis* (Clarke *et al*., 2000). NO activated protein kinase in tobacco was identified as SIPK, a Salicylic acid (SA) -activated mitogen activated protein kinase (MAPK) and SA was required for NO activation of SIPK (Kumar and Klessig, 2000). The NO activated protein kinase in *Arabidopsis* is not yet identified, but it has characteristics of a MAPK (Clarke *et al*., 2000).

$ **Cyclic nucleotide gated ion channels (CNGCs)**

These are ion channels that are activated by binding of cyclic nucleotide to channel protein. Presence of several cyclic-nucleotide gated ion –channels are shown in plants including those that respond to cGMP (Neill *et al*., 2003) but information about role of NO in regulation of these channels through cGMP is not available.

$ cADPR and Ca^{2+}

Cyclic ADP Ribose (cADPR) is a calcium-mobilizing second messenger in animals that activates calcium release from a discrete subset of membrane vesicles. cADPR also induces Ca^{2+} release from plant endoplasmic reticulum and vacuoles (Leckie *et al*.,1998). This increase in Ca^{2+} concentration leads to the activation of many downstream Ca^{2+} regulated processes. It was shown that Ca^{2+} is a core component of ABA induced stomatal closure with NO as an essential intermediate (Neill *et al*., 2002a). Also NO and cGMP mediated IAA induced adventitious rooting involves increase in cytosolic Ca^{2+} in cucumber (Pagnussat *et al*., 2003).

It was observed that effect of NO was not completely blocked by drugs that inhibit sGC suggesting contribution of cGMP independent effects in NO signaling.

cGMP independent signaling

Acting in a cGMP independent manner NO can modulate all major macromolecules like DNA, proteins and lipids. It can mediate post-translational modifications of proteins, which is one of the mechanisms of cellular signaling. These modifications include (i) S-nitrosylation of thiol groups, (ii) nitration of tyrosine, tryptophan groups (biological nitration), (iii) oxidation of thiols and tyrosine and (iv) binding to metal centers (Gow *et al*., 2004). Fig. 3 depicts the potential protein modifications by NO. In addition to proteins, NO induces oxidative and nitrative damage to nucleic acids and lipids (Wendehenne *et al*., 2001). It is reported that as such NO itself is not very reactive with DNA but Reactive Nitrogen Species (RNS) are potent DNA-damaging agents which can cause alkylation, deamination, oxidation and nitration of nucleobases and single and double strand breaks leading to increased mutations (Sawa *et al*., 2006). NO is also a potent inhibitor of lipid peroxidation and low-density lipoprotein oxidation. It is seen that nitrated membrane lipids and lipoprotein can transduce NO-signaling reactions and mediate pathways for anti-inflammatory lipid signaling (Kalyanaraman 2004). S-nitrosylation which is redox based modification and plays a central role in NO signaling is described here, in detail.

S-nitrosylation

Many protein functions are regulated by phosphorylation and redox based signaling. Phosphorylation is a major regulatory switch in signal transduction pathways while redox based mechanisms are being described recently**.** Basic difference between phosphorylation and S-nitrosylation is that phosphorylation is enzyme driven whereas S-nitrosylation is achieved through a non-catalysed chemical modification of protein residues. In addition to its reversible nature, S-nitrosylation shares some common features with phosphorylation except that it is an enzyme independent, redox-sensitive, labile modification (Mannick and Schonhoff 2002).

S-nitrosylation is a ubiquitous NO derived posttranslational modification that serves as a major effector of NO mediated biochemistry regulating broad spectrum of proteins. Due to its redox capacity, NO is able to nitrosate many nucleophilic sites on proteins including amines, aromatic rings, alcohols and thiols. S-nitrosylation is the transfer of a NO^+ equivalent on a free SH group of a protein leading to formation of nitrosothiol (RSNO); this reaction is fully reversible (Broillet 1999). NO can be covalently incorporated into cysteine thiols, tryptophan indols and amines, but studies of free-SH groups by radioactive SH modifying reagents, ultraviolet visible spectrometry and electro spray ionization-mass spectrometry have demonstrated that cysteine residues are rapidly nitrosylated while reactions with other amino acids occur at much slower rates, therefore formation of S-nitrosothiol bond by cysteine thiol nitrosylation is considered most important due to its reactivity under

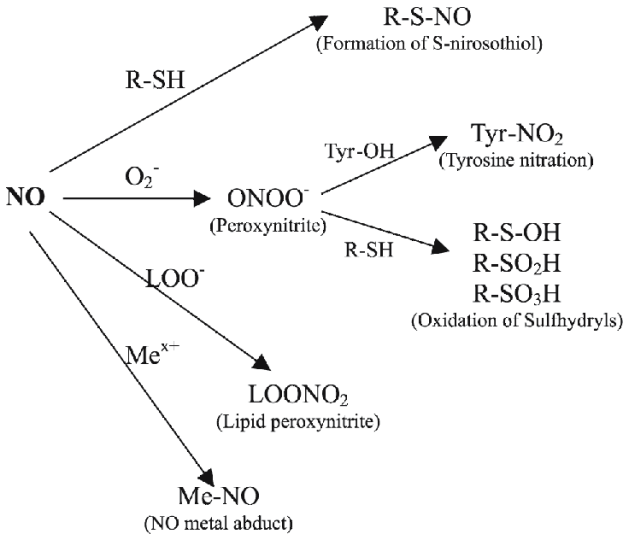

Fig. 3. Potential known targets of NO mediated cGMP independent signaling. Acting in a cGMP independent manner NO can mediate post-translational modifications of proteins, which is one of the mechanisms of cellular signaling. These modifications include (i) S-nitrosylation of thiol groups, (ii) nitration of tyrosine, tryptophan groups (biological nitration), (iii) oxidation of thiols and tyrosine and (iv) binding to metal centers. Also NO can nitrate lipids and DNA (Gow *et al*., 2004).

physiological conditions and its influence on many protein functions. S-nitrosylation solely depends on the chemical reactivity between the nitrosylating agent and the target, which in turn depends upon their localization and concentration. In the following section the formation of S-nitrosothiols, specificity motifs, catabolism and their importance would be detailed to provide insight into this modification.

$ S-nitrosothiol formation

S-nitrosothiols (SNO) are moieties on proteins in which a sulfur atom from cysteine or homocysteine reacts with NO to form S-NO bond. SNO can be formed by three pathways:

1. via formation of higher oxide of nitrogen through auto oxidation of NO.
2. via direct reaction of NOS derived NO or NO equivalents with target protein followed by electron abstraction.
3. via catalysis at metal centre.

In addition, SNO are also formed via transnitrosation reactions. During direct reaction of NO with protein thiols, SNO formation is a two step reaction, firstly NO directly reacts with reduced thiol to produce a radical intermediate RSNOH [1]. Then this reduces an electron acceptor to produce S-nitrosothiol [2]. Under aerobic condition oxygen acts as the electron acceptor and is reduced to produce superoxide (O_2^-) (Gow *et al*., 1997).

$$R\text{-}SH + NO^{\$} \leftrightarrow R\text{-}S\text{-}N^{\$}\text{-}O\text{-}H \quad \text{..... [1]}$$

$$R\text{-}S\text{-}N^{\$}\text{-}O\text{-}H + O_2 \rightarrow R\text{-}S\text{-}N{=}O + O_2^{\$-} \quad \text{..... [2]}$$

Transnitrosation is a process by which an NO is transferred from one molecule to another, this transfer could be either between thiol groups or to nitrogen or carbon containing groups although former is preferred.

Above discussion shows that SNO can be synthesized by a number of mechanisms under physiological conditions, but to participate in signaling mechanism it must have some specificity so that only required proteins can become S-nitrosylated; here S-nitrosylation sensitive motifs in proteins play a crucial role.

$ Specificity of S-nitrosylation

To be physiologically relevant any modification must follow temporal as well as spatial resolution and should be specific so that only specific subset of proteins must be either activated or inhibited. Specificity of S-nitrosylation is brought by compartmentalization of S-nitrosylation reaction and presence of S-nitrosylation sensitive motifs in proteins.

Compartmentalization

Cytosol contains reducing agents, like glutathione and ascorbic acid, which make the SNO bond unstable, therefore S-nitrosylated proteins are stored in membranes, vesicles and in interstitial spaces protecting them from cellular reductants, pH shifts, pO2 shifts, transition metals etc. This compartmentalization is beautifully depicted in animal system by caspase activation during apoptosis (Mannick *et al*., 2001). Using a combination of fluorescence labeling methods, elimination of functional mitochondria and electron transport inhibitors it was shown that S-nitrosothiol formation appears to be localized principally to mitochondria and that intact mitochondria are required for optimal S-nitrosothiol formation (Yang and Loscalzo, 2005). Also in another recent study using immuno gold electron microscopy, with anti-S-nitrosocysteine antibodies, it was shown that S-nitrosylated proteins were present in distinct cellular compartments such as endoplasmic reticulum and vesicular membrane structure near the Golgi complex (Greco *et al*., 2006). This type of sub cellular compartmentalization is yet to be deciphered in plant systems.

S-nitrosylation sensitive motifs

Although a protein can have several cysteine residues, which could undergo S-nitrosylation but only a few are susceptible to it. Inspection of known S-nitrosylated proteins revealed that the target cysteine residue lies within an acid-base or hydrophobic structure motif (Stamler *et al*., 2001). However few recent studies have raised questions about presence of such consensus motif. Study by Taldone *et al.* (2005) revealed that there is no profound effect of the primary sequence of surrounding amino acid residues on the reactivity of cysteines towards S-nitrosylation at peptide level. All the peptides independent of the amino acid surrounding the cysteine residue underwent rapid S-nitrosylation. It was suggested that rather 3D microenvironment of reactive thiol could provide enhanced nitrosative reactivity (Taldone *et al*., 2005). Also another study by Hao *et al*., 2006 supported the above view of absence of linear Cys-flanking motif. While a recent study by Greco *et al*., 2006 supports the view of presence of acid/ base motifs in enhancing cysteine reactivity towards S-nitrosylation. Sequence analysis of S-nitrosocysteine containing peptides revealed the presence of acid/ base motifs, as well as hydrophobic motifs surrounding the identified cysteine residues. Thus studies with other S-nitrosylated proteins are required to define the features that predict S-nitrosylation specificity of the reactive cysteine (s).

Similar to S-nitrosothiol synthesis through multiple pathways, their catabolism is also mediated by a number of factors (enzymatic or non-enzymatic) as discussed below.

$ S-nitrosothiol catabolism

Unlike phosphorylation, S-nitrosylation is a labile covalent modification and its effects are reversed by denitrosylation, just as dephosphorylation reverses the effects of phosphorylation. Therefore like phosphorylation/ dephosphorylation, nitrosylation/ denitrosylation act as on/off switch regulating the protein function. S-nitrosothiols are exceptionally labile as a result of their reactivity with intracellular reducing agents such as ascorbic acid and glutathione as well as reduced metal ions especially Cu (I) and have tissue half-life ranging from seconds to few minutes. Although these are stable at 37°C (pH 7.4) in the presence of transition metal ion chelator (Singh *et al*., 1996). Denitrosylation of S-nitrosolthiols could either be enzymatic or non enzymatic. Enzyme mediated decomposition of S-nitrosothiols involves participation of enzymes like xanthine oxidase, Protein disulfide isomerase, Copper and Zinc superoxide dismutase, Glutathione dependent formaldehyde dehydrogenase, Thioredoxin reductase and γ-Glutamyl transferase (Miersch and Mutus 2005).

Non enzymatic denitrosylation occurs due to cellular reductants, pH and pO2 shifts, transition metals, thiols, and light also denitrosylate proteins. Altering the redox environment of a protein or translocating a protein from a subcellular compartment that allows stable S–NO or metal–NO bond formation to a redox environment of which is unfavorable for SNO bond also causes denitrosylation. Several inorganic processes can also break the SNO bond. These include photolytic cleavage as well as cleavage caused by reactions with inorganic copper, mercury or iron. Studies have shown that in the presence of NO, Cu^{2+} ion induces a fast S-nitrosation of bovine serum albumin and human hemoglobin, and this reaction is prevented by thiol blocking reagents. During this reaction, Cu^{2+} is accumulated and causes destabilization of the S-nitrosothiols formed (Stubauer *et al*., 1999). In another study it was shown that lower concentration Fe^{2+} favored formation and stabilization of S-nitrosothiols however higher concentrations (higher than 10μM) abolished the effect. A mechanism was proposed according to which such opposite effects of iron on S-nitrosothiols were determined by the ratio between SNO, thiols, iron and NO in the reaction system (Vanin *et al*., 1997). In addition to copper and iron, calcium was also shown to regulate S-nitrosylation and denitrosylation of tissue transglutaminase (tTG). In the presence of Ca^{2+}, upto 15 cysteines were nitrosylated resulting in an inhibition of its activity. Addition of ≥1mM Ca^{2+} to nitrosylated tTG resulted in its denitrosylation. Also addition of other divalent ions, Mg^{2+} and Sr^{2+} (≥ 1mM), caused release of NO groups from nitrosylated tTG (Lai *et al*., 2001). However, these inorganic reactions are likely to be of little physiological relevance as: free copper and mercury, are nearly undetectable in cell. Thus, enzymatic processes appear to be the most important determinant of SNO concentration in cells.

Importance of S-nitrosothiols

Since the discovery of S-nitrosothiols (SNOs) in biological systems research focus has shifted to analyze specific proteins in detail. Thiol modification by reactive oxygen species such as H_2O_2 is already recognized as a potential signaling mechanism in plants (Neill *et al*., 2002b). Since thiol residues and disulphide bridges are important for tertiary protein structure, reversible modifications of thiol groups and formation of disulphide bridges may have effect on protein activity similar to protein modifications by reversible phosphorylation. S-nitrosothiols provide stabilization and transport of highly reactive agent i.e. NO in the organism. Also S-nitrosylation of thiols accelerates their oxidation as well as their reactivity.

More that 180 S-nitrosylated proteins are identified in plants and animals, belonging to versatile functional classes including various metabolic enzymes cytoskeleton proteins, stress related proteins, redox-related, signaling/regulatory proteins etc. Many proteins are identified but information about functional modification of each describing whether S-nitrosylation is inhibiting or enhancing the enzyme activity or leading to some structural modification would be important in understanding their biological roles.

NO modulates the biological function of many intracellular signaling proteins by S-nitrosylation including N-methyl D aspartate receptor, protein kinase C, caspase-3, NFκB, AP1, Ras, c-Jun N-terminal kinase / stress-activated protein kinase (JNK1) etc. In a study by Park *et al*., 2000 an increase in S-nitrosylation of JNK1 in RAW246.7 cells by NO was reported. NO inhibited JNK1 in intact cells and negatively regulated JNK/SNAP pathway. Barrelt *et al*., 2005 showed that S-nitrosylation inhibited the activity of PTPases. Both these reports suggest the role of NO in controlling many signaling cascades running in the cell.

In a recent study by Wang *et al*., 2006 regulation of endocytosis by NO via S-nitrosylation of dynamin was

shown. Dynamin is involved in vesicle trafficking including endocytosis and pathogen invasion. It was shown that S-nitrosylation of a single cysteine residue activates dynamin, suggesting that pathogenic microbes and viruses may induce S-nitrosylation of dynamin to facilitate cellular entry. In another report it was shown that function of Protein-disulphide isomerase (an important protein involved in preventing neurotoxicity) was inhibited by NO through S-nitrosylation leading to neurodegenerative disorders (Uehara *et al*., 2006). In addition to these recent reports many earlier reports have shown S-nitrosylation of several proteins in animals (see Martínez-Ruiz and Lamas, 2004; Hess *et al*., 2005).

Relatively, recent reports have also started to accumulate showing regulation of plant proteins by S-nitrosylation. Sokolovski and Blatt 2004 reported that NO can affect the gating of K^+, Ca^{2+} and Na^+ channels through S-nitrosylation of K^+ channel or a closely associated regulatory protein in *Vicia faba*. S-nitrosylation was shown to play an important role in plant disease resistance (Feechan *et al*., 2005). S-nitrosoglutathione (GSNO) is the reservoir of NO bioactivity *in vivo* and is the molecule responsible for S-nitrosylation. S-nitrosoglutathione reductase (GSNOR) metabolizes GSNO regulating both the levels of GSNO and S-nitrosylated proteins *in vivo*. In *Arabidopsis thaliana* AtGSNOR1 mutant had increased SNO levels and plant defense responses were disabled making the mutant plant susceptible to diseases. Also it was demonstrated that AtGSNOR1 positively regulates the signaling network controlled by the salicylic acid. Thus concluding that GSNOR could be an important molecule in S-nitrosothiol metabolism by controlling not only the cellular levels of GSNO but also the levels of S-nitrosylated proteins.

Lindermayr *et al*., 2006 have shown molecular mechanism for regulation of Methionine adenosyltransferase (MAT) in *Arabidopsis* via S-nitrosylation. MAT catalyses the synthesis of S-adenosylmethionine (SAM), a substrate of polyamines and ethylene. Three isoforms of MAT were cloned from *Arabidopsis* and effect of NO was tested on purified recombinant proteins. It was demonstrated that treatment with GSNO reduced the activity to 30% of MAT1 while other two isoforms (MAT2 and MAT3) were not significantly affected. Using site directed mutagenesis and mass spectrometry analysis, cysteine-114 of MAT was pointed as the critical cysteine residue undergoing S-nitrosylation and regulating the activity. Ethylene is an important plant hormone regulating important processes of maturation and senescence. Both NO and ethylene have opposite effects, NO helps in extending the shelf life of fresh horticultural products by reducing ethylene production. Present study suggests that the inhibition of MAT1 activity through S-nitrosylation results in inhibition of ethylene synthesis.

Earlier reports have shown that NO delays programmed cell death (PCD) and was speculated to be an endogenous modulator of PCD in barley aleurone cells (Beligni *et al*., 2002). Metacaspases (caspases related proteins present in plants) are involved in regulation of PCD. In a recent study it was shown that activity of metacaspases in *Arabidopsis thaliana* is suppressed by S-nitrosylation but once mature these become insensitive to S-nitrosylation (Belenghi *et al*., 2007). Increase in total S-nitrosylation content during salinity stress was observed in olive leaves (Valderrama *et al*., 2007). Subjecting olive leaves to 200mM NaCl caused an increase in NO production, total S-nitrosothiols and tyrosine nitrated proteins, suggesting role of NO during major abiotic stress i.e. salinity. All these reports suggest S-nitrosylation can act as an important post-translational modification and its reversible nature qualifies it as a candidate to act as on off switch like phosphorylation controlling activity of many proteins.

Our work on identification of S-nitrosylated proteins from *Kalanchoe pinnata* (a CAM plant) and *Brassica juncea*, using Biotin switch assay has revealed proteins including enzymes for carbon, nitrogen and sulfur metabolism, cytosketeton, stress related and proteins involved in photosynthesis as the targets of S-nitrosylation (Table 1). Out of these kinesin-like protein, glycolate oxidase, UDP glucose 4 epimerase and DNA topoisomerase II are the novel targets reported for the first time for any organism (unpublished work).

Ribulose-1,5-bisphosphate carboxylase / oxygenase (Rubisco), which is the most abundant protein and is responsible for the important process of carbon fixation, was shown to be a target of S-nitrosylationin *K. pinnata.* In addition, its activity was modulated by S-nitrosylation (unpublished). Treatment with S-nitrosoglutathione (NO donor) reduced Rubisco carboxylase activity in a dose dependent manner whereas treatment with reduced glutathione did not show much effect on the activity suggesting that free thiols are required for Rubisco carboxylase activity. This post translational modification of Rubisco might provide us with tools to modify this sluggish enzyme to enhance its carbon fixation capacity and perhaps help in efficient carbon sequestration leading to reduction in the global warming in future.

Table 1. S-nitrosylated proteins identified in *Arabidopsis*, *Kalanchoe pinnata* and *Brassica juncea*

S.No	Protein	*Arabidopsis*	*K. pinnata*[H]	*B. juncea*[H]	Functional category
1	GST, putative	+	-	+	Stress-Related proteins
2	Hsp 90 and 81-3, putative	+	+	-	
3	Cu/Zn-superoxide dismutase	+	-	-	
4	Glutathione peroxidase, putative	+	-	-	Redox-Related proteins
5	Peroxiredoxin-related	+	-	+	
6	Type 2 peroxiredoxin-related	+	-	-	Signaling proteins
7	Glutaredoxin, putative	+	-	-	
8	Elongation factor EF-2	+	-	+	
9	Elongation factor eEF-1á-chain, 1B á-subunit, 1B ã, putative	+	-	+	
10	Initiation factor eIF-4A1, 5A-4-related	+	-	-	
11	Tubulin á 6 chain, â 4 chain	+	-	-	Cytoskeleton Proteins
12	Actin depolymerizing factor 3 like	+	-	-	
12	Annexin	+	-	-	
14	Kinesin-like protein	-	+	-	
15	Fructose 1,6 bisphosphate aldolase, putative	+	+	-	Metabolic enzymes
16	Triosephosphate isomerase	+	-	+	
17	GAPDH C-subunit	+	+	-	
18	Enolase	+	-	-	
19	Phosphoglycerate kinase	+	+	-	
20	Aconitase	+	-	-	
21	SAM synthetase, putative	+	-	-	
22	Adenosylhomocysteinase	+	-	-	
23	Methionine synthase	+	+	+	
24	Cysteine synthase	+	-	-	
25	ATP synthase CF1 á-chain, â-chain	+	-	-	
26	UDP-glucose 4-epimerase	-	+	-	
27	Glutamate ammonia ligase	-	+	-	
28	Myrosinase	-	-	+	
29	Transketolase, putative	-	-	+	
30	ACC synthase	-	-	+	
31	Ascorbate peroxidase	-	-	+	
32	Rubisco large chain	+	+	+	Proteins involved in photosynthesis
33	Rubisco small chain 1a precursor	+	+	+	
34	Rubisco activase, large subunit	+	-	-	
35	PSII P680 47-KD, D2 protein, fragment and PSII oxygen-evolving complex	+	-	-	
36	Rieske Fe-S protein	+	-	-	
37	Carbonic anhydrase	-	+	-	
38	Glycolate oxidase	-	+	-	
39	PEP carboxylase	+	+	-	
40	DNA topoisomerase II	-	+	-	Proteins involved in DNA replication
41	Reverse Transcriptase	-	-	+	
42	Disease resistance protein, putative	-	+	-	Protein involved in disease resistance

[H]Unpublished work

This is an example of the functional analysis of one target of S-nitrosylation, other targets of S-nitrosylation include proteins from many important functional categories like metabolic enzymes, proteins involved in photosynthesis, signaling, cytoskeletal, stress related proteins etc, thus making S-nitrosylation a powerful mechanism to regulate activities of these in plant metabolism.

Research on S-nitrosylation in plants has initiated just a few years back. Accumulated evidences clearly suggest its significant regulatory effect on plant metabolome as newer targets are being discovered at a rapid pace. Since this area of research is in its infancy. Presence of multiple targets clearly supports it as an important post translational modification like phosphorylation. In years to come, once complete S-nitrosoproteome is deciphered and physiological significance of all the targets is established its regulatory role would be as significant as phosphorylation.

FUTURE PERSPECTIVES

Presently the studies in NO signaling are focusing on identification of entire consortium of NO responsive proteins including S-nitrosylated, tyrosine nitrated proteins etc. But still a lot of information in the field of NO research in plants is required. Detailed analysis of all the targets of NO would help us in understanding the NO signaling in a comprehensive manner. Once the targets are identified future studies should focus on analyzing the activities of most important targets under natural as well as under 'nitrosative' and other abiotic stress conditions.

ACKNOWLEDGMENT

RD is honored to contribute to this volume and would like to pay humble and warm regards to her beloved teacher, Prof. Sudhir Sopory, who shaped her to be a researcher. He is a constant source of inspiration with his untiring attitude, persistent interest and involvement in research. Thank you 'Sir', for being my guiding light. The work was partially supported by research grant to RD from University Grants Commission (F.30-122/2004(SR)) and CSIR (38[1127/06/EMR-II]), Government of India. JKA and PST thank CSIR for providing research fellowship.

REFERENCES

Barrett, D.M., Black, S.M., Todor, H., Schmidt-Ullrich., R.K., Dawson, K.S. and Mikkelsen, R.B. (2005). Inhibition of Protein-tyrosine Phosphatases by mild oxidative stresses is dependent on S-nitrosylation. *J. Biol. Chem*., 280: 14453-14461.

Barrosso, J.B., Corpas, F.J., Carreras, A., Sandalio, L.M., Valderrama, R., Palma, J.M., Lupiáòez, J.A. and Rio, L.A.D. (1999). Localization of nitric–oxide synthase in plant peroxisomes. *J .Biol. Chem*., 274: 35729-36733.

Belenghi, B., Romero-Puertas, M.C., Vercammen, D., Brackenier, A., Inzé, D., Delledonne, M. and Breusegem, F.V. (2007). Metacaspase activity of *Arabidopsis thaliana* is regulated by S-nitrosylation of a critical cysteine residue. *J. Biol. Chem*., 282: 1352-1358.

Beligni, M.V. and Lamattina, L. (2001). Nitric oxide: a non-traditional regulator of plant growth. *Trends Plant Sci*., 6: 508-509.

Beligni, M.V., Fath, A., Bethke, P.C., Lamattina, L. and Jones, R.L. (2002). Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone layers. *Plant Physiol*., 129: 1642-1650.

Bethke, P.C., Badger, M.R. and Jones, R.L. (2004). Apoplastic synthesis of nitric oxide by plant tissues. *Plant Cell,* 16: 332- 341.

Broillet, M.C. (1999). S-nitrosylation of proteins. *CMLS Cell Mol. Life Sci.,* 55: 1036-1042.

Butt, Y.K., Lum, J.H. and Lo, S.C. (2003). Proteomic identification of plant proteins probed by mammalian nitric oxide synthase antibodies. *Planta,* 216: 762-771.

Cheng, F., Hsu, S. and Kao, C.H. (2002). Nitric oxide counteracts the senescence of detached rice leaves induced by dehydration and polyethylene glycol but not by sorbitol. *Plant Growth Regulon*., 38: 265-272.

Clarke, A., Desikan, R., Hurst, R. D., Hancock, J. T. and Neill, S.J. (2000). NO way back: Nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. *Plant J*., 24: 667-677.

Correa-Aragunde, N., Graziano, M. and Lamattina, L. (2004). Nitric oxide plays a central role in determining lateral root development in tomato. *Planta*, 218: 900-905.

Crawford, N. M., Galli, M., Tischner, R. and Heimer, Y. M., Okamoto, M. and Mack, A. (2006). Response to Zemojtel et al: Plant nitric oxide synthase: Back to square one, *Trends Plant Sci*., 11: 526-527.

Cueto, M., Perera, H., Martin, R., Bentura, M.L., Rodrigo, J., Lamas, S. and Golvano, M.P. (1996). Presence of nitric oxide synthase activity in roots and nodules of *Lupinus albus*. *FEBS Lett*., 398: 159-164.

Desikan, R., Griffiths, R., Hancock, J. and Neill, S. (2002). A new role for an old enzyme: nitrate reductase- mediated nitric oxide generation is required for abscissic acid – induced stomatal closure in *Arabidopsis thaliana*, *Proc. Natl. Acad. Sci. USA*, 99: 16314 -16318.

Durner, J., Wendehenne, D. and Klessig, D. F. (1998). Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc. Natl. Acad. Sci. USA*, 95: 10328-10333.

Feechan, A., Kwon, E., Yun, B.W., Wang, Y., Pallas, J.A. and Loake, G.J. (2005). A central role for s-nitrosothiols in plant disease resistance. *Proc. Natl. Acad. Sci. USA*., 102: 8054-8059.

Garcia-Mata, C., Gay, R., Sokolovski, S., Hills, A., Lamattina, L. and Blatt, M.R. (2003). Nitric oxide regulates K^+ and Cl- channels in guard cells through a subset of abscisic acid-evoked signaling pathways. *Proc. Natl. Acad. Sci. USA*., 100: 11116-11121.

Gould, K.S., Lamotte, O., Klinguer, A., Pugin, A. and Wendehenne, D. (2003). Nitric oxide production in tobacco leaf cells: a generalized stress response? *Plant Cell Environ*., 26: 1851-1862.

Gow, A.J., Buerk, D.G. and Ischiropoulos, H. (1997). A novel reaction mechanism for the formation of S-nitrosothiol *in vivo*. *J. Biol. Chem*., 272: 2841-2845.

Gow, A.J., Farkouh, C.R., Munson, D.A., Posencheg, M.A. and Ischiropoulos, H. (2004). Biological significance of nitric oxide-mediated protein modifications. *Am. J. Physiol. Lung Cell. Mol. Physiol*., 287: L262-L268.

Graziano, M., Beligni, M.V. and Lamattina, L. (2002). Nitric oxide improves internal iron availability in plants. *Plant Physiol*., 130: 1852-1859.

Greco, T.M., Hodara, R., Parastatidis, I., Heijnen, H.F.G., Dennehy, M.K., Liebler, D.C. and Ischiropoulos, H. (2006). Identification of S-nitrosylation motifs by site-specific mapping of the S-nitrosocysteine proteome in human vascular smooth muscle cells. *Proc. Natl. Sci. USA*., 103: 7420-7425.

Guo, F.Q. (2006). Response to Zemojtel et al: Plant nitric oxide synthase: AtNOS1 is just the beginning. *Trends in Plant Sci.,* 11: 527-528.

Guo, F.Q. and Crawford, N.M. (2005). *Arabidopsis* nitric oxide synthase1 is targeted to mitochondria and protects against oxidative damage and dark induced senescence. *Plant Cell,* 17: 3436-3450.

Guo, F.Q., Okamoto, M., Crawford, N.M. (2003). Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science,* 302: 100-103.

Haba, P., Agüera, E., Benitez, L. and Maldonado, J.M. (2001). Modulation of nitrate reductase activity in cucumber (*Cucumis sativus*) roots. *Plant Sci*., 161: 231-237.

Hancock, J. T. (1997). Cell Signaling. Harlow, UK: Longman.

Hao, G., Derakhshan, B., Shi, L., Campagne, F. and Gross, S.S. (2006). SNOSID, a proteomics method for identification of cysteine S-nitrosylation sites in complex protein mixtures. *Proc. Natl. Acad. Sci. USA*., 103: 1012-1017.

He, Y., Tang, R.H., Hao, Y., Stevens, R.D., Cook, C.W. , Ahn, S.M., Jing, L., Yang, Z., Chen, L., Guo, F., Fiorani, F., Jackson, R.B., Crawford, N.M. and Pie, Z.M. (2004). Nitric oxide represses *Arabidopsis* floral transition. *Science,* 305: 1968- 1971.

Hess, D.T., Matsumoto, A., Kim, S.O., Marshall, H.E. and Stamler, J.S. (2005). Protein S-nitrosylation: Purview and Parameters. *Nat. Rev. Mol. Cell Biol.,* 6: 150-166.

Kalyanaraman, B. (2004). Nitrated lipids: a class of cell signaling molecules. *Proc. Natl. Acad. Sci. USA*., 101: 11527-11528.

Kopyra, M. and GwoZdz, E.A. (2003). Nitric oxide stimulates seed germination and counteracts the inhibitory effect of heavy metals and salinity on root growth of *Lupinus luteus*. *Plant Physiol. Biochem*., 41: 1011-1017.

Kumar, D. and Klessig, D.F. (2000). Differential induction of tobacco MAP kinases by the defense signals NO, SA, ethylene and jasmonic acid. *Mol. Plant Microbe Interact*., 13: 347-351.

Lai, T.S., Hausladen, A., Slaughter, T.F., Eu, J.P., Stamler, J.S. and Greenberg, C.S. (2001). Calcium regulates S-nitrosylation, denitrosylation and activity of tissue transglutaminase. *Biochem*., 40: 4904-4910.

Lamattina, L., Garcia-mata, C., Graziano, M. and Pagnussat, G. (2003). Nitric Oxide: The versatility of an extensive signal molecule. *Annual Rev. Plant Biol*., 54: 109-136.

Leckie, C. P., McAnish, M.R., Allen, G.J., Sanders, D. and Hetherington, A.M. (1998). ABA-induced stomatal closure is mediated by cADP-ribose. *Proc. Natl. Acad. Sci. USA*., 95: 155837-155842.

Lindermayr, C., Saalbach, G., Bahnweg, G. and Durner, J. (2006). Differential inhibition of Arabidopsis methionine adenosyltransferases by protein S-nitrosylation. *J. Biol. Chem*., 281: 4285-4291.

Ludidi, N. and Gehring, C. (2003). Identification of a novel protein with Guanylyl cyclase activity in *Arabidopsis thaliana*. *J Biol.Chem.*, 278: 6490-6494.

Mannick, J.B. and Schonhoff, C.M. (2002). Nitrosylation: the next phosphorylation? *Arch. Biochem. Biophys.,* 408: 1-6.

Mannick, J.B., Schonhoff, C., Papeta, N., Ghafourifar, P., Szibor, M., Fang, K. and Gaston, B. (2001). S-nitrosylation of mitochondrial caspases. *J. Cell Biol*., 154: 1111-1116.

Martínez-Ruiz, A. and Lamas, S. (2004). S-nitrosylation: a potential new paradigm in signal transduction. *Cardiovasc Res*., 62: 43-52.

Miersch, S. and Mutus. B. (2005). Protein S-nitrosation: Biochemistry and characterization of protein thiol-NO interactions as cellular signals. *Clinical Biochem*., 38: 777-791.

Modolo, L.V., Cunha, F.Q., Braga, M.R., Salgado, I. (2002). Nitric oxide synthase mediated phytoalexin accumulation in soybean cotyledons in response to *Diaporthe phaseolorum sp meridionolis* elicitor. *Plant Physiol*., 130: 1288-1297.

Murgia, I., de Pinto, M.C., Delledonne, M., Soave, C. and De Gara, L. (2004). Comparative effects of various nitric oxide donors on ferritin regulation, programmed cell death, and cell redox state in plant cells. J. *Plant Physiol*., 161: 777-83.

Neill, S. J., Desikan, R. and Hancock, J.T. (2003). Nitric oxide signalling in plants. *New Phytol*., 159: 11- 35.

Neill, S. J., Desikan, R., Clarke, A. and Hancock, J.T. (2002a). NO is a novel component of ABA signaling in stomatal guard cells. *Plant Physiol*., 128:13-16.

Neill, S. J., Desikan, R., Clarke, A., Hurst, R.D. and Hancock, J.T. (2002b). Hydrogen peroxide and nitric oxide as signalling molecules in plants. *J. Exp. Bot*., 53: 1237-1247.

Newton, R.P. Roef, L., Witters, E. and Onckelen, H.V. (1999). Cyclic nucleotides in higher plants: the enduring paradox. *New Phytol.,* 143: 427- 455.

Pacoda, D., Montefusco, A., Piro, G. and Dalessandro, G. (2004). Reactive oxygen species and nitric oxide affect cell wall metabolism in tobacco BY-2 cells. *J. Plant Physiol*., 161:1143-1156.

Pagnussat, G.C., Lanteri, M.L. and Lamattina, L. (2003). Nitric Oxide and Cyclic GMP are messengers in the Indole Acetic Acid-induced adventitious rooting process. *Plant Physiol*., 132: 1241-1248.

Pagnussat, G.C., Lanteri, M.L., Lombardo, M.C. and Lamattina, L. (2004). Nitric oxide mediates the indole acetic acid induction activation of a mitogen-activated protein kinase cascade involved in adventitious root development. *Plant Physiol*., 135: 279-286.

París, R., Lamattina, L. and Casalongué, C.A. (2007). Nitric oxide promotes the wound-healing response of potato leaflets. *Plant Physiol. Biochem*., 45: 80-6.

Park, H.S., Huh, S.H., Kim, M.S., Lee, S.H. and Choi, E.J. (2000). Nitric oxide negatively regulates c-Jun N-terminal kinase / stress-activated protein kinase by means of S-nitrosylation. *Proc. Natl. Acad. Sci. USA*., 97: 14382-14387.

Planchet, E., Sonoda, M. and Kaiser, W.M. (2006). Nitric oxide (NO) as an intermediate in cryptogein- induced hypersensitive responses: a critical re- evaluation. *Plant Cell Environ.*, 29: 59- 69.

Prado, A.M., Porterfield, D.M. and Feijó, J.A. (2004). Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. *Development*, 131: 2707-2714.

Qu, Y., Feng, H., Wang, Y., Zhang, M., Cheng, J., Wang, X. and An, L. (2006). Nitric oxide functions as a signal in ultraviolet-B induced inhibition of pea stems elongation. *Plant Sci*., 170: 994-1000.

Ribeiro, E.A.J, Cunha, F.Q., Tamashiro, W.M.S.C. and Martins, I.S. (1999). Growth phase- dependent subcellular localization of nitric oxide synthase in maize cells. *FEBS Lett.*, 445: 283- 286.

Rio, L.A.D., Corpas, F.J. and Barroso, J.B. (2004), Nitric oxide and nitric oxide synthase activity in plants. *Phytochemistry*, 65: 783-792.

Rockel, P., Strube, F., Rockel, A., Wildt, J. and Kaiser W.M. (2002). Regulation of nitric oxide (NO) production by plant nitrate reductase *in vivo* and *in vitro*. *J. Exp. Bot.*, 53: 103- 110.

Sakihama, Y., Nakamura, S. and Yamasaki H (2002). Nitric oxide production mediated by nitrate reductase in the green alga *Chlamydomonas reinharditii*: an alternative NO production pathway in photosynthetic organisms, *Plant Cell Physiol.,* 43: 290- 297.

Sawa, T. and Ohshima, H. (2006). Nitrative DNA damage in inflammation and its possible role in carcinogenesis. *Nitric Oxide,* 14: 91-100.

Singh, R.J., Hogg, N., Joseph, J. and Kalyanaraman, B. (1996). Mechanism of nitric oxide release from S-nitrosothiols. *J. Biol. Chem.,* 271: 18596-18603.

Sokolovski, S. and Blatt, M.R. (2004). Nitric oxide block of outward-rectifying K^+ channels indicates direct control by protein nitrosylation in guard cells. *Plant Physiol.,* 136: 4275-4284.

Stamler, J.S. (1994). Redox Signaling: Nitrosylation and related target interactions of nitric oxide. *Cell,* 78: 931-936.

Stamler, J.S., Lamas, S. and Fang, F.C. (2001). Nitrosylation: The prototypic redox-based signaling mechanism. *Cell*, 106: 675-683.

Stamler, J.S., Toone, E.J., Lipton, S.A. and Sucher, N.J. (1997). (S)NO signals: translocation, regulation, and a consensus motif. *Neuron*, 18: 691-696.

Stõhr, C., Strube, F., Marx, G., Ullrich, W.R. and Rockel, P. (2001). A plasma membrane bound enzyme of tobacco roots catalyses the formation of nitric oxide from nitrite. *Planta*, 212: 835- 841.

Stubauer, G., Giuffrè, A. and Sarti, P. (1999). Mechanism of S-nitrosothiol formation and degradation mediated by copper ions. *J. Biol. Chem*., 274: 28128-28133.

Taldone, F.S., Tummala, M., Goldstein, E.J., Ryzhov, V., Ravi, K. and Black, S.M. (2005). Studying the S-nitrosylation of model peptides and eNOS protein by mass spectrometry. *Nitric Oxide*, 13: 176-187.

Uehara, T., Nakamura, T., Yao, D., Shi, Z.Q., Gu, Z., Ma, Y., Masliah, E., Nomura, Y. and Lipton, S.A. (2006). S-nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration. *Nature Letts*., 441: 513-517.

Valderrama, R., Corpas, F.J., Carreras, A., Fernández-Ocaña, A., Chaki, M., Luque, F., Gómez-Rodríguez, M.V., Colmenero-Varea, P., Río, L.A. and Barroso, J.B. (2007). Nitrosative stress in plants. *FEBS Lett*, 581: 453-461.

Vanin, A.F., Malenkova, I.V. and Serezhenkov, V.A. (1997). Iron catalyzes both decomposition and synthesis of S-nitrosothiols: optical and electron paramagnetic resonance studies. *Nitric oxide*, 1: 191-203.

Wang, G., Moniri, N.H., Ozawa, K., Stamler, J.S. and Daaka, Y. (2006). Nitric oxide regulates endocytosis by S-nitrosylation of dynamin. *Proc. Natl. Acad. Sci. USA*., 103: 1295-1300.

Wendehenne, D., Pugin, A., Klessig, D.F. and Durner, J. (2001). Nitric oxide: comparative synthesis and signaling in animal and plant cells. *Trends Plant Sci.,* 6: 177- 183.

Xu, Y.C. and Zhao, B.L., (2003). The main organ of endogenous NO in higher nonleguminous plants. *Plant Physiol. Biochem.,* 41: 833- 838.

Yang, Y. and Loscalzo, J. (2005). S-nitrosoprotein formation and localization in endothelial cells. *Proc. Natl. Acad. Sci. USA*., 102: 117-122.

Zeidler, D., Zahringer, U., Gerber, I., Dubery, I, Hartung, T., Bors, W., Hutzler, P. and Durner, J. (2004). Innate immunity in *Arabidopsis thaliana*: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. *Proc. Natl. Sci. USA*., 101: 15811-15816.

Zemojtel, T., Frõhlich, A., Palmieri, M.C., Kolanczyk, M., Mikula, I., Wyrwicz, L.S., Wanker, E.E., Mundlos, S., Vingron, M., Martasek, P. and Durner, J. (2006). Plant nitric oxide synthase: a never ending story, *Trends Plant Sci.*, 11: 524-525.

Zemojtel, T., Penzkofer, T., Dandekar, T. and Schultz J. (2004). A novel conserved family of nitric oxide synthase? *Trends Plant Sci.,* 29: 224-226.

Zhao, M., Zhao, X., Wu, Y. and Zhang, L. (2006). Enhanced sensitivity to oxidative stress in an *Arabidopsis* nitric oxide synthase mutant. *J. Plant Physiol.*, 164: 737-745.

Zhao, M.G., Tian, Q.Y. and Zhang, W.H. (2007). Nitric oxide synthase dependent nitric oxide production is associated with salt tolerance in *Arabidopsis. Plant Physiol.,* 144: 206-217.