

Chapter 7

Regulation and Function of Protein S-Nitrosylation in Plant Stress

Gitto Thomas Kuruthukulangarakoola and Christian Lindermayr

Introduction

Physiological processes in plants are regulated by a complex network of signaling processes and the role of biological molecules that can mediate signals across this network is very vital. Nitric oxide (NO) is one such signaling molecule known to coordinate many physiological processes in almost all the organisms studied. Following the discovery of NO as a signaling molecule in animals (Ignarro et al. 1987), it was first identified in plants as a mediator of defense responses during disease resistance (Delledonne et al. 1998; Durner et al. 1998). Since then, studies have revealed the ubiquitous signaling nature of NO in regulating plethora of physiological processes in plants like germination (Bethke et al. 2006; Belenghi et al. 2007), stomatal closure (Neill et al. 2002a; Garcia-Mata et al. 2003; Sokolovski et al. 2005), flowering (He et al. 2004), senescence (Corpas et al. 2004; Guo and Crawford 2005), wounding responses (Huang et al. 2004), and abiotic stresses (Grun et al. 2006; Corpas et al. 2011). Ubiquitous behavior of NO in signaling processes puzzled the researchers to find an answer on how this sensitive and highly diffusible gaseous free radical can be regulated spatially and temporally. It is now known that plants scrutinize this regulation by controlling the NO bioactivity at different levels ranging from NO production to site-specific reactivity and finally, the NO turnover. Cellular redox status, a primary means of coordinating many signaling pathways (Spoel and Loake 2011), is also a crucial regulator of NO bioactivity.

Various upstream signaling pathways like extracellular adenosine triphosphate, phosphatidic acid, cyclic nucleotide phosphate, calcium and mitogen-activated protein kinases coordinates plant stress responses to induce NO production in plants

G.T. Kuruthukulangarakoola • C. Lindermayr (✉)
Institute of Biochemical Plant Pathology, Helmholtz Zentrum München,
Ingolstädter Landstrasse 1, 85764 Neuherberg, Germany
e-mail: lindermayr@helmholtz-muenchen.de

(Sueldo et al. 2010; Gaupels et al. 2011a; Ma and Berkowitz 2011). However, efforts to identify the mechanism through which these upstream signaling events regulate NO production are hampered due to the fact that an exact enzymatic source of NO production is yet to be revealed in plants. Nevertheless, NO accumulation in plants has witnessed to induce downstream signaling events (Besson-Bard et al. 2008; Aboul-Soud et al. 2009). More importantly, effectiveness of NO-signaling strongly relies on its spatial regulation that is conferred by the specificity of NO to react with selective targets and on its temporal regulation that is achieved by the reversibility of NO-induced target modification.

Mechanisms to regulate biological processes in eukaryotes are multilayered and interconnected. They range from transcriptional, post-transcriptional, and translational to post-translational regulation. Post-translational modifications (PTMs) change the properties of proteins by addition of a modifying chemical group (biomolecules or other small agents) to an amino acid residue. More than 200 different types of PTMs are known that can regulate protein properties like affecting the catalytic activity, changing the ligand binding affinity, altering protein structure and/or protein–protein interactions (Mann and Jensen 2003; Kho et al. 2004). NO can mediate several PTMs, such as tyrosine nitration or metal nitrosylation or protein S-nitrosylation. The nitration of free tyrosine or protein tyrosine residues generates 3-nitrotyrosine. This reaction has been utilized as a footprint for the *in vivo* formation of peroxynitrite and other reactive nitrogen species. Metal nitrosyls are formed by the reaction of NO with transition metals. The predominant mode of action of NO seems to be protein S-nitrosylation, a PTM that involves covalent attachment of NO moiety to the thiol side chain of redox sensitive cysteine residue. This covalent attachment of NO to the thiol group is reversible and is determined by the redox status of its micro-environment. Fluctuations in the cellular redox status are a typical phenomenon associated with stress-related response. Also redox-sensitive cysteine residues are often key regulators of protein function (Spoel and Loake 2011) and are present in all major classes of proteins. Thus, ubiquitous signaling behavior of NO and its ability to sense the changes in cellular redox status and reversibly modify functionally important redox-sensitive cysteine residue (Stamler et al. 2001) make protein S-nitrosylation an illustrative example for redox-regulated PTM.

Protein S-nitrosylation is the most studied NO-mediated signaling mechanism in plants. Several proteins prone to S-nitrosylation have been identified in plants (Lindermayr et al. 2005; Romero-Puertas et al. 2008; Abat and Deswal 2009; Palmieri et al. 2010; Maldonado-Alconada et al. 2011; Lin et al. 2012). These proteins are involved in different physiological and stress-related processes highlighting the ubiquitous regulatory role of S-nitrosylation. In the model plant *Arabidopsis thaliana* non-symbiotic hemoglobin (Perazzolli et al. 2004), S-adenosylmethionine synthetase (SAMS1) (Lindermayr et al. 2006), metacaspase 9 (Mc9) (Belenghi et al. 2007), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Holtgreffe et al. 2008; Wawer et al. 2010), salicylic acid-binding protein 3 (SABP3) (Wang et al. 2009), non-expressor of pathogenesis-related gene1 (NPR1) (Lindermayr et al. 2010), glycine decarboxylase complex (GDC) (Palmieri et al. 2010) and NADPH oxidases (Yun et al.

Table 7.1 List of proteins regulated by S-nitrosylation

| Name of the protein | Function of the protein | Effect of S-nitrosylation |
|---|---|---|
| Nonsymbiotic hemoglobin1 (Hb1) | NO dioxygenase activity | NO detoxification during hypoxia (Perazzolli et al. 2004) |
| S-adenosylmethionine synthetase (SAMS1) | Catalyzes the synthesis of the ethylene precursor S-adenosylmethionine | Inhibits the catalytic activity of SAMS1 (Lindermayr et al. 2006) |
| Metacaspase 9 (Mc9) | Proteolytic caspases activity | Inhibition of the proteolytic activity (Belenghi et al. 2007) |
| Peroxiredoxin II E (PrxII E) | Reduces H ₂ O ₂ and alkyl hydroperoxides to H ₂ O and the corresponding alcohol. Also functions in detoxifying peroxynitrite (ONOO ⁻) | Inhibits hydroperoxide-reducing peroxidase activity and ONOO ⁻ detoxification activity (Romero-Puertas et al. 2007) |
| R2R3-MYB transcription factors | Upon DNA binding, R2R3-MYB induce many physiological and stress related pathways | Inhibits the DNA binding of R2R3-MYB transcription factor (Serpa et al. 2007) |
| Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) | Oxidative catalysis during glycolysis | Inhibit glycolytic activity. Initiates nuclear localization and induce programmed cell death (putative function) (Holtgreffe et al. 2008) |
| Salicylic acid-binding protein 3 (SABP3) | SA binding to SABP3 activates its carbonic anhydrase (CA) activity and modulates plant defense response | Inhibits SA binding and CA activity (Wang et al. 2009) |
| Non-expressor of pathogenesis-related gene1 (NPR1) | Conversion of polymeric NPR1 (oxidized form) to monomeric NPR1 isomers (reduced form) helps the nuclear localization of monomers and binding to TGA1 transcription factor that leads to DNA binding and PR1 gene expression | Helps in achieving redox equilibrium between oxidized and reduced forms of NPR1 (Tada et al. 2008; Lindermayr et al. 2010) |
| Glycine decarboxylase complex (GDC) | Involved in the mitochondrial photorespiratory C2 cycle of C3 plants | Inhibited the photorespiratory function of GDC and induced ROI accumulation and cell death (Palmieri et al. 2010) |
| NADPH-dependent oxidases (NADPH-oxidase) | Synthesis of pathogen-induced ROI production and mediates fully developed hypersensitive response | Inhibits pathogen-induced ROI production (Yun et al. 2011) |

2011) are the proteins reported to be regulated by S-nitrosylation. Proteins that are known to be S-nitrosylated in plants are listed in Table 7.1 along with their function and inhibitory effect by S-nitrosylation.

Regulation of Protein S-Nitrosylation

Multiple pathways of regulation and technical limitations to find these pathways have considerably slowed down the progress of understanding the regulatory mechanisms that govern S-nitrosylation. Despite these obstacles, considerable progress has been made over the last decade in unveiling its basics that has formed the foundation for a promising field of cellular signaling ahead. Regulation of protein S-nitrosylation has a hand-in-hand association with NO-bioactivity. Essential steps during S-nitrosylation regulation are i) cellular S-nitrosothiol formation ii) transnitrosylation and iii) denitrosylation. Cellular S-nitrosothiol formation is closely associated with NO production. In animals, the enzyme nitric oxide synthase (NOS) is the main source of NO production (Bredt and Snyder 1990; Jaffrey et al. 2001). Gupta et al. has recently reviewed about various sources of NO production in plants (Gupta et al. 2011). Unique chemical nature of NO that makes it physiologically stable, but with high target specific reversible reactivity is the prime basis of S-nitrosylation signaling event. Cellular redox status utilizes these unique features of NO and co-ordinates the spatio-temporal regulation through the controlled S-nitrosylation/denitrosylation mechanisms.

Mechanism of Protein S-Nitrosothiol Formation

Stress related responses in plants are often associated with increase in the steady-state levels of cellular NO in plants that induce protein S-nitrosylation. However, an exact *in vivo* reaction mechanism describing the formation of S-nitrosothiols from cellular NO is not known yet. The intrinsic biochemistry of NO suggests multiple reaction pathways for S-nitrosylation mechanisms with evidences supported by various *in vitro* studies. Most of these studies have used thiol-containing molecules like cysteine (CySH) and glutathione (GSH) as model compounds as the reaction targets of NO, which upon S-nitrosylation yield low molecular weight (LMW) S-nitrosothiols such as S-nitrosocysteine (CySNO) and S-nitrosogluthathione (GSNO) (Gow et al. 1997; Keszler et al. 2010). They form the integral part of total cellular nitrosothiol (RSNO) pool along with S-nitrosylated peptides and proteins.

Unique chemistry of NO allows it to exist in three redox-related forms, all with different biochemical properties; the reduced nitroxyl anion (NO^-), the NO radical ($\cdot\text{NO}$) and the oxidized nitrosonium cation (NO^+) each with different oxidation state for the nitrogen atom, +1, +2 and +3, respectively (Arnelle and Stamler 1995). NO^- can further exist in two chemical forms; high energy singlet form and low energy triplet form, with zero or two unpaired electrons respectively (Lipton et al. 1998). In mammals, neuronal nitric oxide synthase produces singlet NO^- that reacts with thiols to form S-nitrosothiols (Schmidt et al. 1996). However, this does not exclude the involvement of NO^- as a source for other S-nitrosylation pathways. Low energy triplet NO^- may react with dioxygen to form peroxyxynitrite (Lipton et al. 1998) that

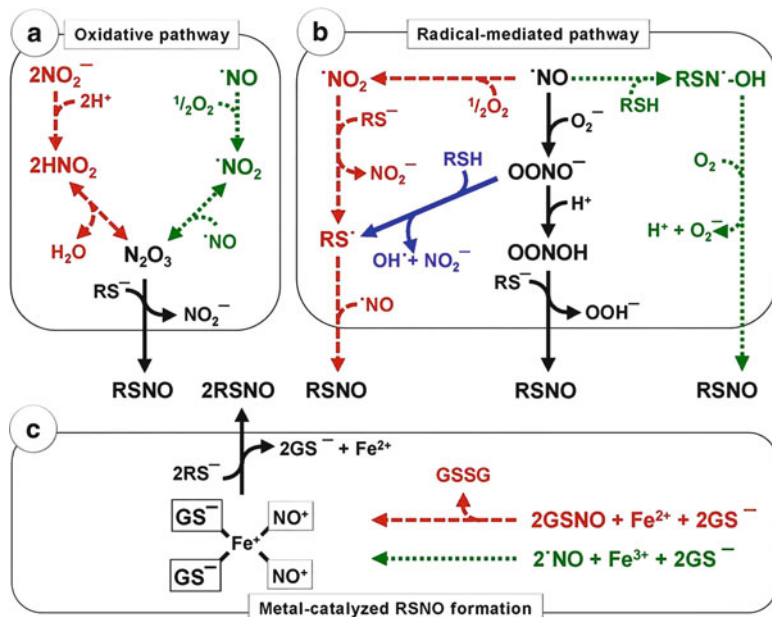


Fig. 7.1 Pathways leading to S-nitrosothiol (RSNO) formation. (a) N_2O_3 can be formed from protonated nitrite at very low pH (dashed arrows) and by the auto-oxidation of $\cdot\text{NO}$ in an O_2 rich environment (dotted arrows). N_2O_3 provides NO^+ equivalence to nucleophilic thiols to form RSNO (undashed arrows). (b) RS^\cdot radicals produced either by peroxynitrite radical (undashed bold arrows) or by the auto-oxidation products of $\cdot\text{NO}$ can directly react with $\cdot\text{NO}$ radical to form RSNO (dashed arrows). In the presence of thiolate anions (RS^-) protonation of peroxynitrite can also result in the formation of RSNO (undashed arrows). Furthermore, $\cdot\text{NO}$ can form an intermediate radical with thiols which then oxidizes to form RSNO (dotted arrows). (c) Chelatable iron pool can mediate the formation of dinitrosyliron complexes (dashed and dotted arrows) that yields NO^+ equivalence to form RSNO (undashed arrows)

in-turn may influence S-nitrosylation (Balazy et al. 1998; van der Vliet et al. 1998). Even though, the free radical $\cdot\text{NO}$ has reported to interact with cysteine thiols to form S-nitrosothiols in the presence of a suitable electron acceptor (Gow et al. 1997) this interaction did not happen with glutathione and is therefore doubtful to happen in physiological conditions (Keszler et al. 2010). Thiyl radicals (RS^\cdot) that is a byproduct of stress-related redox chemical pathways also (see Fig. 7.1b) can react with $\cdot\text{NO}$ to form S-nitrosothiols (Jourdeuil et al. 2003). The existence of free NO^+ is favored only at very high pH values and is therefore biologically non-viable. But oxidation products of $\cdot\text{NO}$ that are functionally equivalent to NO^+ exist under physiological conditions and can donate NO^+ to more nucleophilic thiols resulting in S-nitrosylation (Hughes 1999). In general, none of the three redox-related forms of NO is known to mediate S-nitrosothiol formation independently in its free form. Alternatively, various reaction mechanisms that lead to the formation of S-nitrosothiols from NO have been proposed. Their possible physiological relevance in the context of plant stress responses are discussed in the following section.

Oxidative S-Nitrosylation by Higher Oxides of NO: Formation of N₂O₃

Dinitrogen trioxide (N₂O₃) is generally considered as a nitrosylating agent that can directly mediate S-nitrosylation (Wink et al. 1994). N₂O₃ often donates NO⁺ to the reduced (nucleophilic) thiolate anion (RS⁻) to yield S-nitrosylated product (RSNO) (Fig. 7.1a). In biological systems N₂O₃ can be formed in two ways (Fig. 7.1a). In a pH-dependent pathway N₂O₃ is formed by the reversible dehydration of nitrous acid (HNO₂) (Guikema et al. 2005) (Fig. 7.1a). Since the pK_a of HNO₂ is approximately 3.4, at higher pH values HNO₂ dissociates into nitrite (NO₂⁻). Hence N₂O₃ formation from HNO₂ occurs only at low acidic pH. The apoplast of plants is acidic nature (Yu et al. 2000) and might be mediating pH-dependent synthesis of N₂O₃. Furthermore, changes of the pH value in various plant compartments are associated with signaling in plants and regulate both physiological processes (Gibbon and Kropf 1994; Feijo et al. 1999) and stress related defense responses (Mathieu et al. 1996; Roos et al. 2006; Kader et al. 2007). In an acidified apoplast NO can be produced from nitrite (Bethke et al. 2004). Moreover, acidification of cytoplasm in tobacco suspension cultures induced defense related genes and interestingly NO responsive genes (Lapous et al. 1998). Therefore, combining all these factors argue for the possibility of a pH-dependent formation of S-nitrosothiol in plants.

Another mechanism to produce N₂O₃ is the direct oxidation of the radical [•]NO by O₂ (Fig. 7.1a) (Wink et al. 1994; Goldstein and Czapski 1996). This aerobic formation of N₂O₃ depends on the concentration of available [•]NO and O₂ because of the second order dependence of the reaction with respect to [•]NO concentration and first order dependence of the reaction with respect to O₂ concentration (Goldstein and Czapski 1996). Even though an enzymatic source for the production of [•]NO is not known until now, [•]NO burst is a typical stress-associated phenomenon in plants (Desikan et al. 2002; Zeidler et al. 2004). It is possible that under these conditions oxidation of [•]NO to [•]NO₂ occurs to counteract exceeding levels of cellular [•]NO. N₂O₃ is formed by the reversible reaction of [•]NO₂ with another molecule of [•]NO (Fig. 7.1a). Due to the hydrophobic nature of [•]NO and O₂ the reaction rate increases 300-fold in a hydrophobic environment (Liu et al. 1998). Thus, cellular hydrophobic milieu like lipid membranes and protein interiors can accelerate N₂O₃ formation. Consequently, increased S-nitrosylation of proteins has been reported under these conditions (Rafikova et al. 2002). However, some recent studies have contradicted this finding (Zhang et al. 2009; Keszler et al. 2010). They support the assumption that hydrophobic environment protonate thiol (RSH) and thereby hindering it from reducing to thiolate anion (RS⁻) for accepting NO⁺ from N₂O₃.

Radical Mediated S-Nitrosylation

Radicals play an important role in mediating cellular signaling processes during stress responses. Although there are many radicals proposed to mediate RSNO formation, their influence in the *in vivo* formation of RSNO is not known. [•]NO can

react directly with cysteine thiols to form free radical intermediate $\text{RSN}^{\cdot}\text{OH}$ (Fig. 7.1b) that in the presence of an electron acceptor like O_2 or NAD^+ get oxidized to S-nitrosocysteine (Gow et al. 1997). However, kinetic stimulation studies involving glutathione rather than single cysteine thiols have suggested this pathway either as a negligible pathway for RSNO formation or a pathway that might play only a role at low steady-state levels of NO (Keszler et al. 2010). Other molecules that mediate the formation of RSNO via radical interactions are $\cdot\text{NO}/\text{O}_2$, peroxynitrite (OONO^-) radical and $\cdot\text{NO}/\text{superoxide anion} (\text{O}_2^{\cdot-})$. In the $\cdot\text{NO}/\text{O}_2$ pathway, auto-oxidation of $\cdot\text{NO}$ results in the formation of $\cdot\text{NO}_2$, which can oxidize thiols (RSH) to thiyl radicals (RS^{\cdot}) (Jourdeuil et al. 2003). These thiyl radicals can directly react with $\cdot\text{NO}$ to form RSNO (Fig. 7.1b) (Jourdeuil et al. 2003; Schrammel et al. 2003; Madej et al. 2008; Keszler et al. 2010). Peroxynitrite-dependent RSNO formation can occur in two different ways, either by a direct electrophilic attack of OONO^- on the thiolate anion (van der Vliet et al. 1998) or through an intermediate thiyl radical formation (Goldstein and Czapski 1996; Keszler et al. 2010) (Fig. 7.1b). Peroxynitrite itself can be produced by the reaction of $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$. Interestingly, co-production of $\cdot\text{NO}/\text{O}_2^{\cdot-}$ can mediate S-nitrosylation in a peroxynitrite-independent manner (Schrammel et al. 2003). In plants, many stress related responses are associated with rapid production of reactive oxygen and nitrogen species (Neill et al. 2002b; Modolo et al. 2005; Torres and Dangel 2005). Since, both reactive oxygen and nitrogen species have signaling function as well as toxic effects effective regulatory mechanisms are necessary. Plants possess antioxidants like glutathione and ascorbate that is shown to detoxify and regulate the free radical cellular levels (Dahm et al. 2006; Foyer and Noctor 2011). While $\text{O}_2^{\cdot-}$ influences radical mediated S-nitrosylation formation, $\text{O}_2^{\cdot-}$ production is under the influence of S-nitrosylation during stress (Yun et al. 2011) that highlights the mutual regulatory roles of these two signaling mechanisms in a balanced cellular homeostasis of free radicals. Thus, it is possible that radicals and their regulatory systems together might be playing a crucial role in RSNO formation in plants during stress conditions.

S-Nitrosylation Catalyzed by Metals

Transition metals, especially iron, are important elements for the proper regulation of physiological functions in plants. Both iron and NO are redox related species and can take part in reversible electron transfer processes depending on the redox environment. While, ferric iron (Fe^{3+}) can accept electrons from radical $\cdot\text{NO}$ resulting in the formation of ferrous (Fe^{2+}) and NO^+ ions, Fe^{2+} can donate electron to radical $\cdot\text{NO}$ to form Fe^{3+} and NO^- (Graziano and Lamattina 2005). Due to high affinity of iron for NO they form coordinate complexes named iron-nitrosyl complexes. Changes in the iron pool have shown to influence signaling processes mediated by S-nitrosylation in mammalian cell lines (Kim et al. 2000). Fe^{2+} , NO and low molecular weight thiols can form *in vivo* metal containing S-nitrosothiols called as dinitrosyl iron complexes

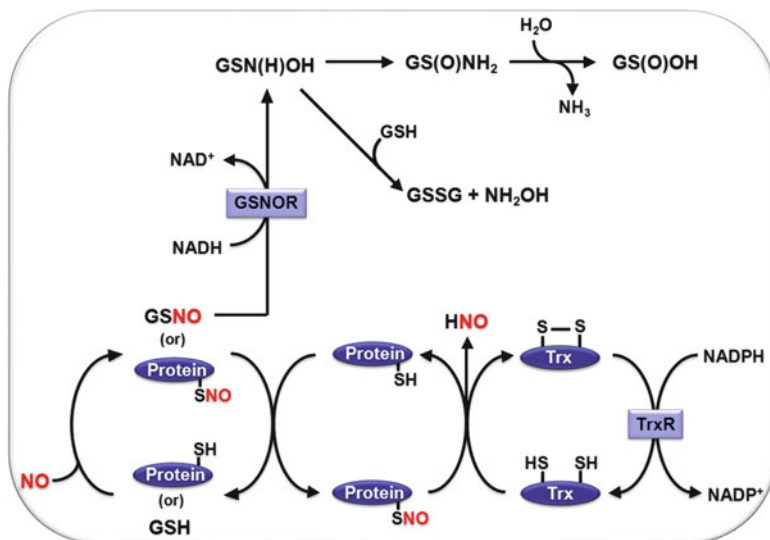


Fig. 7.2 Schematic illustration of different mechanisms used to regulate protein S-nitrosylation. RSNO comprises of low (e.g. GSNO) and high (S-nitrosylated proteins) molecular weight molecules. GSNO and S-nitrosylated proteins can mediate S-nitrosylation of specific free thiol groups of other proteins through transnitrosylation. Trx/TrxR mediates denitrosylation by reducing S-nitrosylated thiol group of the proteins. Regulation of protein-S-nitrosylation by GSNOR is indirect. It metabolizes the transnitrosylation mediator GSNO in an NADH-dependent pathway to an intermediate S-(N-hydroxyamino)glutathione (GSNHOH). This intermediate is converted to glutathione sulphinamide (GSONH₂), which is then spontaneously hydrolyzed to glutathione sulphinic acid (GSOH) and ammonia (NH₃). However, in the presence of GSH, GSNHOH is converted oxidized glutathione (GSSG) and hydroxylamine (NH₂OH) (Jensen et al. 1998; Liu et al. 2001; Hedberg et al. 2003; Staab et al. 2008)

(DNICs) (Fig. 7.1c) (Musch et al. 1993). DNICs are considered as endogenous NO carriers like LMW nitrosothiols. They have shown to transfer NO to the metal-centers of metalloproteins (Ueno et al. 2002) and/or can donate NO⁺ equivalents to thiol groups to form RSNO (Bosworth et al. 2009) (Fig. 7.1c). Increased NO levels in plants elevate the levels of nitrosyl-iron complexes (Simontacchi et al. 2012). Moreover, under oxidative conditions, in NO-binding hemeproteins NO can be transferred from the heme group to intramolecular cysteine thiol residues (Luchsinger et al. 2003).

Transnitrosylation

Apart from the direct modification of thiol group by NO equivalents, both low molecular weight S-nitrosocysteine thiols and S-nitrosylated proteins can directly transfer the nitrosyl moiety (NO group) to non-S-nitrosylated cysteine thiols through a process termed transnitrosylation (Fig. 7.2) (Zhang and Means 1996; Pawloski et al. 2001; Dahm et al. 2006; Mitchell et al. 2007; Kornberg et al. 2010;

Nakamura et al. 2010). Transnitrosylation is largely responsible for *in vivo* S-nitrosocysteine thiol activity and NO signaling. Not all, but specific thiol containing cysteine residues of proteins are the targets of S-nitrosylation. Low molecular weight S-nitrosocysteine thiols like GSNO mediate transnitrosylation of specific cysteine thiols on multiple proteins. In contrast, transnitrosylation mediated by S-nitrosylated proteins (protein-protein transnitrosylation) occurs only with their binding partners, thus showing additional selectivity along with specificity (Kornberg et al. 2010). In the fly *Drosophila melanogaster*, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that is physiologically S-nitrosylated at a specific cysteine residue can transfer its NO moiety to the protein sirtuin, as soon as they interact. Abolishing their interaction eliminates the transnitrosylation ability (Kornberg et al. 2010).

Among the low molecular weight S-nitrosocysteine thiols, GSNO is the major physiological NO-donor and is known for its ability to mediate transnitrosylation (Dahm et al. 2006). Treatment of proteins and cell cultures with GSNO significantly increased the S-nitrosylation levels of proteins in plants (Lindermayr et al. 2005; Palmieri et al. 2010). Cellular GSH pool is the key in regulating the levels of S-nitrosylated thiols, which under the oxidized conditions favors an increase in the levels of S-nitrosylated thiols (Dahm et al. 2006). Plant defense responses are often associated with rapid changes in the cellular redox environment that induce oxidation of GSH pool (Vanacker et al. 2000). Stress-related responses are accompanied by an apparent increase in the levels of S-nitrosylated thiols (Feechan et al. 2005; Lee et al. 2008; Chaki et al. 2011a, b), which also constitute higher GSNO levels. Increased GSNO levels can mediate S-nitrosylation of specific cysteine residues of proteins.

Several attempts have been made to reveal the factors that influence the S-nitrosylation specificity of cysteine residue. These studies did not reveal a linear sequence motif on the proteins that could mediate S-nitrosylation of specific cysteine residues. Rather they showed various factors that can enhance the chance for the cysteine residue to be S-nitrosylated like:

- (a) Presence of an acid–base motif flanking the cysteine residue either in the primary sequence or in the tertiary structure (Stamler et al. 1997; Greco et al. 2006; Doulias et al. 2010).
- (b) Cysteine residue in the hydrophobic vicinity due to enhanced NO autooxidation to form S-nitrosylating species N_2O_3 (Nedospasov et al. 2000; Greco et al. 2006)
- (c) Cysteine residues skewed towards accessible surface areas in the α -helices with charged amino acids within a 6°Å distance (Doulias et al. 2010)
- (d) Low pK_a values of cysteine residue (Foster et al. 2009) and
- (e) Cysteine thiol in the proximity of the amino acid interacting with transnitrosylating protein (Kornberg et al. 2010).

But, none of these factors were conclusive to define a general mechanism of S-nitrosylation specificity for cysteine residue (Doulias et al. 2010; Marino and Gladyshev 2010). Each factor has shown to be significant in one or the other situation of protein S-nitrosylation (Greco et al. 2006; Foster et al. 2009; Doulias

et al. 2010; Kornberg et al. 2010). This suggest that each factor might be playing different role in mediating protein S-nitrosylation under different circumstances of nitrosothiol formation like N_2O_3 -mediated S-nitrosylation, metal-catalyzed S-nitrosylation, transnitrosylation by GSNO and protein-protein transnitrosylation (Foster et al. 2009).

Protein Denitrosylation: A Regulator of S-Nitrosylation Signaling

Removing NO moiety from the S-nitrosylated cysteine residue of the proteins, known as denitrosylation, is very important for proper regulation of protein S-nitrosylation. While S-nitrosylation of proteins is generally considered to initiate stress-induced signaling pathways, denitrosylation is responsible for maintaining cellular S-nitrosylated levels of this protein during the response and finally to switch-off the same pathway to reconstitute the normal situation after stress response. However, denitrosylation can also function as a switch to induce pathways during stress response. Mitochondrial caspase-3 zymogens apoptotic activity is inhibited by S-nitrosylation in resting cells. Stress-induced denitrosylation of caspase-3 activates the protein (Mannick et al. 1999; Kim and Tannenbaum 2004; Reynaert et al. 2004; Erwin et al. 2005). Thus, denitrosylation can have dual roles in regulating signaling pathways. Even though several enzymes have been proposed to mediate denitrosylation, S-nitrosoglutathione reductase (GSNOR) and thioredoxin/thioredoxin reductase are the two systems that are characterized to have significant role in mediating this process in animals (Fig. 7.2) (Benhar et al. 2009; Lopez-Sanchez et al. 2010). A similar role of their counterparts in plants, especially of that of GSNOR is emerging and is of considerable interest.

GSNOR is Crucial in Regulating S-Nitrosothiol Levels

Search for an enzyme that can mediate metabolism of physiological NO molecule GSNO has led to the identification of GSNOR that is conserved in almost all living systems including plants (Liu et al. 2001; Sakamoto et al. 2002; Diaz et al. 2003). GSNOR was classified to class III alcohol dehydrogenase (ADH) and was originally found to function as glutathione dependent-formaldehyde dehydrogenase (FALDH) in plants. FALDH has been a well characterized enzyme in several plant species (Uotila and Koivusalo 1979; Martinez et al. 1996) before its GSNOR activity was discovered (Sakamoto et al. 2002; Achkor et al. 2003; Diaz et al. 2003). GSNOR metabolizes GSNO with NADH as an electron donor (Fig. 7.2) (Wilson et al. 2008). While NOS-like activity, nitrate reductase activity and other non-enzymatic sources for NO are associated with NO accumulation in plants (Gupta et al. 2011), GSNOR is associated with the removal of NO through

GSNO metabolism. This is evident in the *gsnor* knock-out and overexpression lines of *Arabidopsis* plant that showed increased and reduced nitrosothiol levels respectively (Feechan et al. 2005). Since oxidized GSH (GSSG) is a product of GSNO metabolism (Fig. 7.2), it is possible that the redox status of the glutathione pool has a great influence on protein S-nitrosylation. Under oxidizing conditions, GSNOR mediated metabolism might be less favored. Thus GSH and GSNOR indirectly mediate protein denitrosylation through GSNO metabolism. GSNOR, however, cannot metabolize S-nitrosylated moiety of proteins or peptides (Liu et al. 2001). There is an equilibrium that exists between low molecular weight S-nitrosothiols like GSNO and S-nitrosylated proteins and peptides (Seth and Stamler 2011). This equilibrium allows regulation of GSNO metabolism by GSNOR to indirectly regulate S-nitrosylated proteins (Fig. 7.2). In *gsnor* knock-out mutant plants, an increase in low molecular weight nitrosothiols resulted in a corresponding increase in the levels of high molecular weight S-nitrosothiols that is assumed to include proteins which is a clear indication of indirect effect of GSNOR regulation of protein S-nitrosylation (Liu et al. 2001; Liu et al. 2004; Yun et al. 2011).

GSNOR is receiving increasing attention for its role in plant stress responses. Physiological role of GSNOR is evident from the *Arabidopsis gsnor* knock-out mutant plants that showed delayed and stunted growth phenotype and altered flower development (Lee et al. 2008; Holzmeister et al. 2011). *Gsnor* mutant plants showed a reduced cell death phenotype after treatment with paraquat, a herbicide that is known to induce cell death phenotype in wild type plants via generation of reactive oxygen intermediates (ROI) (Chen et al. 2009). Interestingly, both wild type and *gsnor* mutant plants showed same levels of ROI accumulation after paraquat treatment. (Chen et al. 2009). Lack of sensitivity of the *gsnor* knock-out plants to increased ROI can be due to altered cellular ROI/NO homeostasis, which is very important for plant defense responses (Delledonne et al. 2001).

Arabidopsis gsnor knock-out mutants, challenged with avirulent *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000, showed low levels of salicylic acid accumulation that resulted in a compromised disease resistance (Feechan et al. 2005; Yun et al. 2011). However, these plants with high cellular RSNO levels showed an increased cell death induced by hypersensitive response (CDHR) through a pathway independent of SA and ROI production (Yun et al. 2011). On the other hand, even though SA-induced defense is compromised, increased CDHR rate prevented avirulent oomycete pathogens to complete its life cycle (Yun et al. 2011). These evidences highlight two different roles of GSNOR during defense response; positive regulator of SA-induced defense and negative regulator of CDHR-induced defense responses. Conversely, GSNOR transcript levels and GSNOR activity in *Arabidopsis* and tobacco respectively, were shown to be up regulated when treated with SA (Diaz et al. 2003). These studies indicate the possibility of a mutual regulation between GSNOR and SA during plant defense.

Interestingly, in another study on *gsnor* knock-out plants, there was no difference in the level of disease resistance against *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 with respect to the wild type plants (Holzmeister et al. 2011). However, here the knock-out plants used were from different background ecotype

of *Arabidopsis thaliana* plants and also procedures to inoculate them were also different. These contrary results have raised the questions on how GSNOR regulates disease resistance in various ecotypes. On the contrary, plants with reduced *gsnor* expression levels (antisense technology) have affirmed the negative regulatory role of GSNOR during disease resistance against oomycetes (Rusterucci et al. 2007). Further studies are required to show how this enzyme is regulated at transcript and protein levels during attempted pathogen invasions.

Transcripts of GSNOR, however, were down regulated transiently and systemically during wound-induced responses in *Arabidopsis* plants (Diaz et al. 2003). In tobacco plants, wound-induced down-regulation of GSNOR is mediated by jasmonic acid (JA) signaling pathway (Diaz et al. 2003). In *Arabidopsis*, GSNO accumulation is required to activate the JA-dependent wound responses, whereas the alternative JA-independent wound-signaling pathway did not involve GSNO. Furthermore, it was shown that GSNO acts synergistically with salicylic acid in systemic acquired resistance activation (Espunya et al. 2012). Plant stress responses induced by wounding are often associated with nitrosative stress and tyrosine-nitration (Chaki et al. 2011b). Stress experiments in sunflower plants have demonstrated that wound-induced nitrosative stress is mediated by down-regulation of GSNOR expression levels resulting in decreased activity and in considerable increase in cellular RSNO levels (Chaki et al. 2011b). In pea plants wounding enhanced RSNO levels, but surprisingly GSNOR activity too is also increased (Corpas et al. 2008). The same phenomenon was observed during cold stress (Corpas et al. 2008). The reason for this unexpected co-relation between RSNO levels and GSNOR activity is not clear. Furthermore, GSNOR is regulated in pea plants during cadmium stress, both on activity and transcript level (Barroso et al. 2006). However, a pathway that regulates GSNOR under cadmium stress is not known. Cadmium treatment also induced SA, JA and ethylene levels in pea plants (Rodriguez-Serrano et al. 2006) accompanied by a decrease in the glutathione content (Barroso et al. 2006).

Gene silencing studies in tobacco plants have demonstrated the significant role of GSNOR in plant-herbivore interaction (Wunsche et al. 2011). Silencing *GSNOR* compromised plant defense against herbivore with a decrease in the accumulation of JA and ethylene (Wunsche et al. 2011). However, this silencing did not affect transcriptional regulation of all the secondary metabolites that are regulated by JA signaling (Wunsche et al. 2011) implying the specificity of GSNOR in mediating defense response against the herbivore *Manduca sexta*. GSNOR is also required for thermo tolerance. It has been observed that *Arabidopsis* knock-out mutants of GSNOR were highly sensitive to hot temperatures (Lee et al. 2008). This heat sensitivity was associated with increased NO species in these knock-out plants. *NO-overproducing* mutants and wild-type plants treated with NO donors were also sensitive to high temperatures (Lee et al. 2008). Consequently, thermo tolerance was restored in *gsnor* mutants when treated with chemicals that scavenge NO. Furthermore, expression of heat-shock-proteins that are essential for thermo tolerance was not affected in *gsnor* mutant plants (Lee et al. 2008). Interestingly, neither expression nor activity of GSNOR was altered in wild-type plants due to heat stress

(Lee et al. 2008). This study suggests that though GSNOR do not regulate heat stress response in plants, its activity to regulate cellular RSNO levels is essential for thermo tolerance.

Denitrosylation Mediated by Trx/TrxR System

The thioredoxin/thioredoxin reductase (Trx/TrxR) system, present in almost all organisms, consists of oxidized and reduced forms of Trx, TrxR and NADPH/NADP⁺ (Fig. 7.2) (Lillig and Holmgren 2007). In animals, Trx/TrxR system was recently proved to mediate denitrosylation (Benhar et al. 2008; Benhar et al. 2010). Unlike GSNOR, Trx/TrxR system is proposed to mediate denitrosylation of S-nitrosylated proteins directly (Fig. 7.2). In a recent review, Trx from plants is mentioned to possess *in vitro* denitrosylation activity with reference to an unpublished data (Spoel and Loake 2011). Also, thioredoxin (TRX-5h) is a positive regulator of SA-induced defense response in plants (Tada et al. 2008), probably by denitrosylation.

Physiological Functions of Protein S-Nitrosylation in Plants During Stress Response

To get insight into the physiological function of protein S-nitrosylation the target proteins for this type of modification have to be identified. In plants, potential candidates for S-nitrosylation have been identified from GSNO-treated cell culture extracts, NO-treated plants, infected plants and plants undergoing HR (Fig. 7.3) (Lindermayr et al. 2005; Romero-Puertas et al. 2008; Maldonado-Alconada et al. 2011; Yun et al. 2011). Until now, considerable progress has been made to demonstrate the physiological role of S-nitrosylation for distinct proteins.

Regulation of Pathogen-Induced ROI Production by S-Nitrosylation

Defense related CDHR can be mediated by pathogen-induced accumulation of ROI, which was inhibited by high RSNO levels (Fig. 7.3) (Yun et al. 2011). In *Arabidopsis*, AtRBOHD (NADPH-oxidase) activity is required for the pathogen-induced ROI production (Torres et al. 2002). Interestingly, during hypersensitive response AtRBOHD activity is inhibited by S-nitrosylation of its cysteine residue (Cys890) (Yun et al. 2011). Cys890 is an evolutionary conserved amino acid residue in humans and *Drosophila* and is positioned closely behind the binding

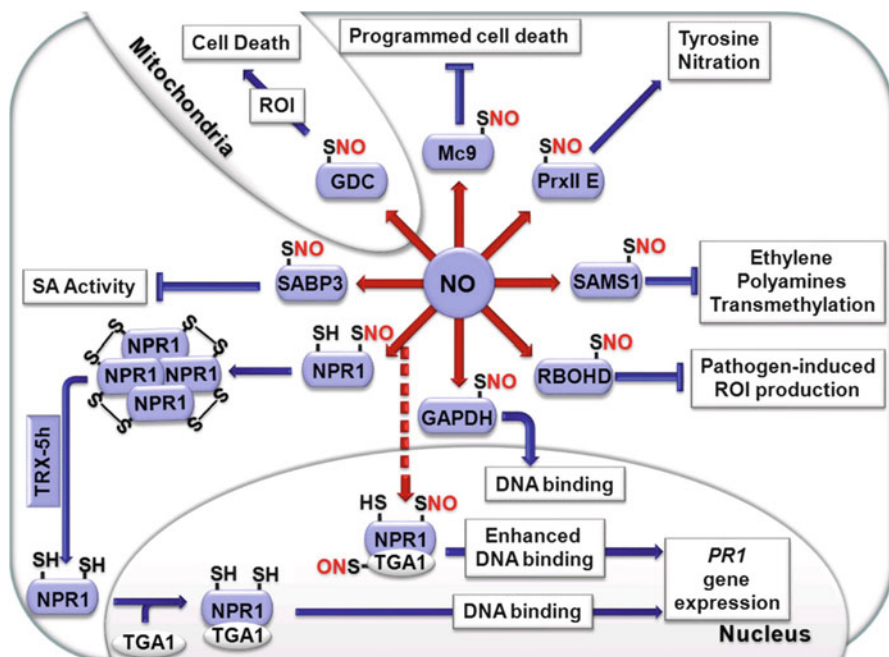


Fig. 7.3 Function of protein S-nitrosylation in plant stress response. Stress-induced accumulation of nitric oxide species can inhibit, activate or alter the function of proteins through S-nitrosylation. The activity of SABP3 (important mediator of SA signaling), Mc9 (cysteine protease activity), PrxII E (detoxifying peroxynitrite—regulate tyrosine nitration), SAMS1 (enzyme involved in ethylene and polyamine synthesis and transmethylation reactions) and RBOHD (synthesis of pathogen-induced ROI) is inhibited by S-nitrosylation. Furthermore, inhibition of GDC induces mitochondrial ROI production and cell death. S-Nitrosylation of mammalian GAPDH mediates its nuclear localization and induces cell death. Plant GAPDH can also be S-nitrosylated, but its role in cell death is not yet known. Monomer to oligomer transition of NPR1 is proposed to be mediated by S-nitrosylation and reversible transition by thioredoxin and induce PR1 gene expression. Moreover, NO-treatment enhances the DNA binding activity of the NPR1/TGA1 complex

site of flavin adenine dinucleotide (FAD). FAD mediates the electron transfer from NADPH through heme to O_2 to produce O_2^- (Sumimoto et al. 2004). S-nitrosylation of Cys890 prevented FAD binding and ROI production of AtRBOHD (Yun et al. 2011). Mutation of Cys890 however abolished S-nitrosylation ability without disturbing FAD binding ability to its active site that in turn enhanced the pathogen-induced ROI production and corresponding CDHR in *Arabidopsis* plants (Yun et al. 2011). These data demonstrate that plants regulate ROI production by AtRBOHD through S-nitrosylation which might help restricting cell-death to the site of infection. It is, however, not clear whether pathogens utilize this negative regulatory role on ROI production as a strategy to overcome the plant defense. In sunflower, biotrophic oomycete *Plasmopara halstedii* induce accumulation of RSNO in susceptible but not in resistant cultivars (Chaki et al. 2009). Moreover,

CDHR is the key reaction in restricting the pathogen spreading in resistant cultivar (Radwan et al. 2005). Thus, it is possible that pathogen-induced RSNO accumulation in the susceptible cultivar might inhibit pathogen-induced CDHR through S-nitrosylation and inhibition of NADPH-oxidase. Analysis to see the difference in the activity of NADPH-oxidase in the two sunflower cultivars upon pathogen challenge and their role in regulating CDHR are required to verify these arguments.

S-Nitrosylation is a Regulator of SA-Dependent Signaling in Plant Defense Response

SA is an important signaling molecule in plant defense response. SA regulates the function of NPR1 (non-expressor of PR1), a co-activator of transcription of the pathogenesis-related gene 1 (*PR1*). Endogenous NPR1 is located in the cytoplasm in an oligomeric status. Upon SA-dependent activation NPR1 dissociates into its monomers, which are translocated into the nucleus (Mou et al. 2003; Pieterse and Van Loon 2004). Nitric oxide plays a crucial role in regulating oligomer/monomer transition. S-nitrosylation of NPR1 facilitates its oligomerization, which keeps it in the cytosol and is essential for NPR1 homeostasis upon SA induction (Tada et al. 2008). The monomerization of NPR1 is catalyzed by thioredoxin TRX-5h, which reduce NPR1 and allow the translocation into the nucleus. But surprisingly, in *Arabidopsis* mesophyll protoplasts nuclear localization of NPR1 is promoted by GSNO (Lindermayr et al. 2010). However, the S-nitrosylation-mediated oligomerization of NPR1 is not seen as an inhibitory effect of NPR1 signaling but rather as a step prior to monomer accumulation. From this point of view, the observed NO-mediated nuclear translocation of NPR1 is not contradictory to the results described by Tada et al. (2008). During defense responses, GSNOR plays a crucial role in regulating the cellular RSNO and SA levels which are essential for mediating oligomer to monomer transition of NPR1. Inside the nucleus NPR1 interacts with the transcription factor TGA1 (TGACG motif binding factor) and activates PR1 gene expression (Despres et al. 2003). Both NPR1 and TGA1 were S-nitrosylated when treated with GSNO resulting in enhanced DNA binding of the NPR1/TGA1 complex (Lindermayr et al. 2010). But S-nitrosylation can also serve as a negative regulator of SA-signaling. Binding of SA to SA-binding protein 3 (SABP3) can activate its carbonic anhydrase activity and thereby positively regulate the plant defense response. S-nitrosylation of SABP3 reduced its SA binding ability resulting in reduction of the CA activity of the enzyme (Wang et al. 2009). S-nitrosylation of SABP3 might be used either by the plant as a negative feedback loop to modulate SA signaling or by the pathogen as a strategy to suppress the plant defense response.

S-Nitrosylation Might be Crucial in Mitochondrial-CDHR

A role of S-nitrosylation in mitochondria mediated CDHR has also been demonstrated. Inhibition of the activity of glycine decarboxylase complex (GDC), a key enzyme involved in the mitochondrial photorespiratory C2 cycle of C3 plants, resulted in ROI accumulation and cell death (Palmieri et al. 2010). Interestingly, GDC is S-nitrosylated/S-glutathionylated at specific cysteine residues when incubated with physiological concentrations of GSNO resulting in inhibition of its activity (Palmieri et al. 2010). Moreover, this inhibition is part of the stress-related response of Arabidopsis to the bacterial elicitor hairpin. In sum, these data reinforce the model of cross talk between NO/ROS and mitochondria in the activation of stress-related responses in plants (Palmieri et al. 2010).

S-Nitrosylation Positively Regulates Tyrosine Nitration

Interestingly, protein S-nitrosylation regulates nitration of tyrosine residues. Stress related processes are often associated with the accumulation of $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$ radicals. Diffusion-limited reaction of $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$ radicals results in the formation of peroxynitrite (OONO^-), an effective tyrosine nitrating compound. Defense related responses in plants are accompanied by OONO^- accumulation (Saito et al. 2006; Gaupels et al. 2011b). In plants however, detoxification of OONO^- is carried out by Peroxyredoxin II E (PrxII E). During HR responses PrxII E gets S-nitrosylated and its activity is inhibited (Romero-Puertas et al. 2008). This allows the accumulation of peroxynitrite which can mediate tyrosine nitration. Consequently, higher tyrosine nitrate levels can be found in plants undergoing biotic stress (Saito et al. 2006). In sunflower-mildew interaction, susceptible cultivars with increased levels of RSNO showed increased tyrosine nitrate levels whereas resistant cultivars did not (Chaki et al. 2009). Moreover, enhanced RSNO levels are accompanied by accumulation of nitrated tyrosine residues in sunflower after mechanical wounding (Chaki et al. 2011b). This correlation between RSNO levels and tyrosine nitration is again seen in sunflower plants stressed with high temperature (Chaki et al. 2011a). All these evidences point out the regulatory role of S-nitrosylation over other NO-related mechanisms.

Stress Induced Function of GAPDH Is Regulated by S-Nitrosylation

S-Nitrosylation of a protein can also alter its function. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), for instance, is an enzyme of the glycolytic pathway and is present in almost all species. In plants, however, GAPDH plays also an important

role in regulating cellular metabolic process during stress conditions to remove toxic ROI accumulation (Sweetlove et al. 2002; Graham et al. 2007; Rius et al. 2008). Interestingly, GAPDH has been identified as a candidate prone to S-nitrosylation and treatment of the enzyme with GSNO inhibited its enzymatic activity (Lindermayr et al. 2005). Surprisingly, plant GAPDH was observed to move into the nucleus and it was shown that GAPDH can bind to a partial gene sequence of the NADP-dependent malate dehydrogenase (Holtgreffe et al. 2008).

In rat cells S-nitrosylated GAPDH interacts with the E3-ubiquitin-ligase Siah1, translocates into the nucleus and mediates cell death (Sen et al. 2008). If there is a similar function in plants have to be proven. In *Nicotiana tabacum*, NtOSAK is a protein kinase that is induced partially by NO during salt stress. GAPDH, a binding partner of NtOSAK has found to be S-nitrosylated and the level of S-nitrosylation was proportional to the increase in the kinase activity of NtOSAK. However, S-nitrosylation of GAPDH did not have any impact on the activity of NtOSAK and hence might not be the mediator of NO signaling in regulating NtOSAK (Wawer et al. 2010). Additionally, in *Drosophila melanogaster*, S-nitrosylated GAPDH can transfer its NO moiety to other proteins through transnitrosylation (Kornberg et al. 2010).

Regulation of Ethylene Biosynthesis by S-Nitrosylation

Ethylene is a natural regulator of growth, development and stress-related processes in plants. Ethylene emission from *Arabidopsis* cell culture was significantly reduced when treated with NO donors indicating an opposite effect of NO on ethylene production (Lindermayr et al. 2006). Such a negative correlation has been also observed in plant foliage and fruits when they are switching from growth stage (low ethylene, high NO) to ripening stage (high ethylene, low NO) (Leshem and Haramaty 1996; Leshem et al. 1998). S-adenosylmethionine synthetase (SAMS) is an enzyme that catalyzes the biosynthesis of S-adenosylmethionine (SAM), a precursor of ethylene. Among three known isoforms of SAMS, one isoform SAMS1 can be regulated by S-nitrosylation (Lindermayr et al. 2006). This regulatory check-point of SAMS1 through S-nitrosylation might link NO signaling with ethylene signaling. But, NO can also inhibit ethylene production in a pathway independent of S-nitrosylation (Leshem et al. 1998; Lindermayr et al. 2005; 2006).

Inhibition of Metacaspase Activity by S-Nitrosylation

Plant metacaspases are cysteine-dependent proteases, which contain a specific cysteine residue that can serve as a nucleophile for the substrate to mediate peptide bond hydrolysis. They are related to animal caspases, a family of proteins involved

in the execution of programmed cell death. In plants, fungi, and protozoa metacaspases are homologs of caspases that belong to the D cysteine protease superfamily. *Arabidopsis* has nine metacaspases groups that are classified into two types based on their difference in the N-terminal region (Coll et al. 2010). For type II metacaspase 9 (MC9) has been demonstrated that S-nitrosylation of their active site cysteine residue results in suppression of its autoprocessing and proteolytic activity (Belenghi et al. 2007). A similar NO-dependent regulation has been described for animal caspase3 (Mitchell and Marletta 2005). While S-nitrosylated caspase3 is inactive under normal physiological conditions, denitrosylation activates its proteolytic activity to trigger programmed cell death (Mannick et al. 1999). The regulatory role of S-nitrosylation of MC9 is still unknown. Probably inhibition of MC9 by NO is responsible for avoiding its inappropriate activation.

Conclusions

NO plays important role virtually in all physiological, patho-physiological and stress related responses in plants. Recent advances in NO research have identified S-nitrosylation as a key regulatory mechanism for its mediation. Here we discussed the regulatory function of S-nitrosylation for different stress-related signaling pathways. However, we are still at the beginning of understanding the function of S-nitrosylation in plants and also great effort has to be done to understand how S-nitrosylation is regulated. A combination of proteomics and bioinformatics approach will boost the identification of potential S-nitrosylation targets. Furthermore, biochemical and genetic studies will provide insight into the physiological function of S-nitrosylated proteins/enzymes. Moreover, identification of the exact sources of NO for S-nitrosylation as well as characterization of the denitrosylation process will help to understand the S-nitrosylation mechanism.

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