PROTEIN S-NITROSYLATION IN PHOTOSYNTHETIC ORGANISMS: A COMPREHENSIVE OVERVIEW WITH FUTURE PERSPECTIVES

Zaffagnini[a,1] M., De Mia[b,1] M., Morisse[b] S., Di Giacinto[a] N., Marchand[b] CH., Maes[b] A., Lemaire[b,2] SD., and Trost[a,2] P.

Affiliation:

[a]Laboratory of Plant Redox Biology, Department of Pharmacy and Biotechnology, University of Bologna, 40126 Bologna, Italy.

[b]Sorbonne Universités, UPMC Univ Paris 06, Centre National de la Recherche Scientifique, UMR8226, Laboratoire de Biologie Moléculaire et Cellulaire and des Eucaryotes, Institut de Biologie Physico-Chimique, 75005 Paris, France.

[1]equal contribution

[2]corresponding authors: Prof. Paolo Trost (email: paolo.trost@unibo.it) and Dr. Stéphane D. Lemaire (email: stephane.lemaire@ibpc.fr)

ABSTRACT

Background: The free radical nitric oxide (NO) and derivative reactive nitrogen species (RNS) play essential roles in cellular redox regulation mainly through protein S-nitrosylation, a redox post-translational modification in which specific cysteines are converted to nitrosothiols.

Scope of view: This review aims to discuss the current state of knowledge, as well as future perspectives, regarding protein S-nitrosylation in photosynthetic organisms.

Major conclusions: NO, synthesized by plants from different sources (nitrite, arginine), provides directly or indirectly the nitroso moiety of nitrosothiols. Biosynthesis, reactivity and scavenging systems of NO/RNS, determine the NO-based signaling including the rate of protein nitrosylation. Denitrosylation reactions compete with nitrosylation in setting the levels of nitrosylated proteins *in vivo*.

General significance: Based on a combination of proteomic, biochemical and genetic approaches, protein nitrosylation is emerging as a pervasive player in cell signaling networks. Specificity of protein nitrosylation and integration among different post-translational modifications are among the major challenges for future experimental studies in the redox biology field.

KEYWORDS: cysteine, denitrosylation, nitric oxide, nitrosothiols, redox signaling, S-nitrosylation

1. INTRODUCTION

Nitric oxide (NO) is a gaseous radical molecule produced in both animals and plants by different biosynthetic systems. Several biological effects are attributed to NO, but in many cases NO itself is not the only responsible of such effects, because NO can react inside the cell with transition metals and other radicals like superoxide and triplet oxygen, giving rise to further nitrogen oxides that are more reactive than NO and are collectively named reactive nitrogen species (RNS). Like reactive oxygen species (ROS), also RNS may be toxic for cells. Toxicity may simply derive from the strong oxidizing character of some RNS such as nitrogen dioxide (N_2O) or peroxynitrite ($ONOO^-$), or it may derive from the capability of other RNS or even metal-NO complexes to indiscriminately transfer an NO moiety to different types of biological molecules that contain a suitable nucleophile. The primary amine of a nucleobase is an example of such suitable nucleophile and DNA can actually be damaged by RNS. In Arabidopsis, NO and derivative RNS have been proposed to restrain root growth via DNA damage-induced cell cycle arrest [1]. Besides DNA, numerous amino acid residues are susceptible to be modified by RNS. Tyrosine, for example, can be converted to 3-nitrotyrosine upon reaction with peroxynitrite. Tyrosine nitration is irreversible in a biological context and is often associated to stress conditions [2-4]. On the other hand, cysteines are the most nucleophilic amino acids and different mechanisms exist by which thiols can be converted into nitrosothiols. Depending on the protein microenvironment, different cysteines show dramatically different reactivity toward RNS or NO, making nitrosylation a specific post-translational modification. Moreover, the nitroso group of nitrosylated cysteines can be removed by common reductants of the cell such as reduced glutathione (GSH) or thioredoxins (TRXs). Thanks to its specificity and reversibility, protein nitrosylation is a post-translational modification with a fundamental role in signaling networks and pervasive biological relevance. Whether NO and RNS trigger signaling cascades through S-nitrosylation of specific cysteines, or they rather damage the cell via an indiscriminate modification of biological molecules, will largely depend on the local concentration of NO that dynamically vary inside the cells as a result of different NO biosynthetic pathways, intricate NO chemistry and a plethora of NO scavenging systems.

Therefore, RNS alone or in combination with ROS play a dual role as toxic and signaling molecules in aerobic organisms. A fundamental concept of redox biology that seems to be even more important in oxygen photosynthetic organisms. In this review, we first present the major mechanisms of NO production/removal and the most representative reactions producing derivative RNS. Subsequently, we discuss the interactions between NO/RNS and protein cysteine residues with special emphasis on the mechanisms of protein nitrosylation/denitrosylation, the methods

allowing quantification of protein nitrosothiols and identification of S-nitrosylated targets. Then, we provide some examples of established targets of S-nitrosylation and insights into nitrosylomes in photosynthetic organisms. Finally, future perspectives are presented with special emphasis on the major topics that still remain to be investigated.

2. REGULATING REACTIVE NITROGEN SPECIES LEVELS *IN VIVO*

2.1 Major Systems of NO production

In animal systems the production of nitric oxide (NO) has been extensively characterized over the last decades. Nowadays it is universally accepted that the main pathway goes through nitric oxide synthase (NOS), a ubiquitous enzyme existing in three isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). NOSs are homodimers that catalyze the NADPH-dependent five-electron oxidation of L-arginine to L-citrulline and NO [5, 6]. Beside NOSs, animal cells can also produce NO from nitrite via nitrate reductase (NR), an enzyme that, besides its main reaction in which nitrate is reduced to nitrate by two electrons derived from NADPH, can also catalyze the single-electron reduction of nitrite to NO under anaerobic conditions [7, 8].

In photosynthetic organisms, despite the increasing number of studies on NO since its discovery [9, 10], the sources of NO production are still controversial. Up to now, NO is considered to be mainly produced by two distinct routes, the arginine-dependent and the nitrite-dependent pathways. Paradoxically, the arginine-dependent pathway, involving NOS enzymes, is the most controversial one. Indeed, the direct molecular proof of the existence of NOS in plants is still missing although several studies provided some indirect evidence of the presence of NOS activity [11-14]. For instance, a NOS-like activity was observed in plant extracts [15] and an arginase-deficient mutant with higher levels of arginine was reported to accumulate more NO compared to WT plants [16]. Nevertheless, genetic analyses revealed that the genomes of several photosynthetic organisms do not contain any gene showing significant homology with mammalian NOS, with the notable exception of the alga *Ostreococcus tauri* [17]. In addition, Tun and colleagues [18] showed that an exogenous supply of polyamines (spermine and spermidine) to Arabidopsis seedlings induce immediate release of NO, suggesting that polyamines may be involved in the generation of NO through an Arg-dependent pathway [19]. However, a direct proof of the existence of this pathway is still missing.

Considering the debate around the existence of NOS, the nitrite-dependent pathway is emerging as the main source of NO production in plants [20, 21]. Nitrate reductase (NR), which in plants often prefers NADH as the coenzyme, is the key player of this pathway [22-24]. NR has been first evidenced as a source of NO in Arabidopsis guard cells. Stomata movements are regulated by

abscisic acid (ABA) and this hormone is known to interact with NO in many processes and to stimulate its accumulation during stomata regulation [25]. In Arabidopsis mutants lacking the two NR isoforms, neither ABA-induced NO production nor ABA-induced stomata closure were observed, providing a first evidence for a role of NR in NO production in plants [22]. At the same time, it has been shown that NR is able to convert, both *in vivo* and *in vitro*, nitrite into NO using saturating NADH as a cofactor [26]. Notably, this enzyme undergoes a regulatory switch based on substrate competition that allows NO production. Nitrate (NO_3^-), which is the high affinity substrate, competes with the low affinity substrate nitrite (NO_2^-). Thus, when NO_2^- accumulates due to external signals or stress conditions, nitrate reduction is inhibited and NR preferentially produces NO from nitrite [27]. Nevertheless, nitrite-dependent NO production is not strictly dependent on the activity of NR. In roots, a plasma membrane-bound nitrite/NO reductase (Ni-NOR), distinct from cytosolic NR, has been shown to produce NO in tobacco roots under hypoxic conditions, even though the detailed mechanism is still lacking [28]. Recently, Wei and colleagues observed that Chlamydomonas cells deficient for nitrate reductase were able to produce NO from nitrite but the underlying mechanism remains to be elucidated [29].

In addition to these major pathways, several studies described other sources of NO, but their physiological significance is still under debate. For instance, the enzyme xanthine oxidoreductase (XOR) was proposed to constitute a possible additional source of NO [30]. Moreover, other groups suggested non-enzymatic NO sources involving chemical reactions that might occur in specific subcellualr compartments such as the apoplast [31] and mitochondria [32] [33].

2.2 NO chemistry and production of reactive nitrogen species in a biological context

NO is a free radical with an unpaired electron shared between the nitrogen and the oxygen atom. As a radical, NO is relatively unreactive. It does not react with itself nor it reacts with most biological molecules that have no unpaired electrons, but it tends to combine with other radicals and transition metals [34]. NO is also a small and lipohilic molecule that can easily cross lipid membranes and, thanks to its limited reactivity, is considered relatively long living *in vivo* [35]. With a half life of up to 2 seconds in animal cells, NO may eventually travel over hundreds of microns within and between cells [36, 37]. Under stress conditions, however, NO half life is much shorter because of the prompt reaction with oxygen radicals like superoxide [38] (Figure 1). Although H_2O_2 may accumulate in plant cells at much higher concentrations than superoxide, the reaction between NO and H_2O_2 is instead prevented by spin forbidness [34]. The reactions involving NO inside the cell are extremely important because they give rise to more reactive molecules, particularly nitrogen oxides (*e.g.* NO_2, N_2O_3) and peroxynitrite ($ONOO^-$), collectively named reactive nitrogen species (RNS).

These molecules have much higher reactivity than NO and may play a dual role in biological systems, both as signalling molecules and toxic compounds [34, 35, 38-40]

Upon reaction with superoxide, NO forms peroxynitrite ($ONOO^-$) (Figure 1; reaction 1) which is not a radical species but nevertheless a strong oxidizing agent and a quite reactive molecule. For instance, tyrosines may react with peroxynitrite and receive a nitrated adduct (NO_2) in a reaction named tyrosine nitration (Figure 1; reaction 2). Alternatively, peroxynitrite may react with NO generating nitrogen dioxide (NO_2) and nitrite (NO_2^-) (Figure 1; reaction 3). Nitrogen dioxide is, like NO, a radical, and can be also produced by the reaction between NO and triplet oxigen (a biradical). However, the reaction: $2NO + O_2 \rightarrow NO_2$ (Figure 1; reaction 4) may be very slow, just because its rate depends on the square of NO concentration (rate = $k[NO]^2[O_2]$). In practical terms, the reaction would proceed very slowly at low NO concentrations and increase exponentially at high NO concentrations. Since both NO and O_2 are lipophilic, this reaction would however proceed much faster [41] within membranes where the substrates may be sequestered at higher concentrations [42].

Whichever the route by which NO_2 is formed, NO_2 may undergo a further radical reaction with NO ending up with dinitrogen trioxide (N_2O_3) (Figure 1; reaction 5). Since NO_2 is a good one-electron oxidant, N_2O_3 has some character of an ionic compound (NO^+-NO_2^-). In the presence of a nucleophile, like a thiolate, N_2O_3 can thus transfer its nitrosonium group (NO^+) and generate a nitrosothiol (Figure 1; reaction 6).

In spite of the common belief, NO does not react with thiolates which in fact are not radicals. However, nitrosothiols (-SNOs) may readily derive from NO if the thiol is previously oxidized to a thiyl radical. Reactive oxygen species like superoxide or hydroxyl radicals, or RNS like peroxynitrite or NO_2, may all serve as acceptors of single electrons in thiolate oxidation. Then, thiyl radicals and NO readily combine their unpaired electrons giving rise to nitrosothiols (Figure 1; reaction 7). At difference with the addition of an NO^+ to a thiolate, which is named S-nitrosation (Figure 1; reaction 6), the formation of a nitrosothiol by addition of an NO to a thiyl radical is called S-nitrosylation (Figure 1; reaction 7).

NO may also nitrosylate transition metals including iron centers of proteins such as hemes and, in some cases, iron-sulfur clusters. In these reactions, NO may form irreversible complexes with ferrous iron (Fe^{II}) or reversible complexes with ferric iron (Fe^{III}). Though unstable, ferric nitrosyl [$Fe^{III}(NO)$] complexes can donate a nitrosonium group to a thiolate ($-S^-$) resulting in its S-nitrosation:

$Fe^{III} + NO \rightarrow [Fe^{III}(NO) \leftrightarrow Fe^{II}(NO^+)]$, followed by
$Fe^{II}(NO^+) + -S^- \rightarrow Fe^{II} + -SNO$

In the special case of hemoglobins (Hb), NO may react with the $Fe^{II}(O_2)$ complex and be rapidly converted into met-hemoglobin ($HbFe^{III}$) and nitrate:

$$HbFe^{II}(O_2) + NO \rightarrow HbFe^{III} + NO_3^-$$

This reaction can be part of a scavenging cycle for NO, once the hemoglobin is reduced back to the $HbFe^{II}$ form for further oxygen binding [40].

2.3 RNS reactivity and scavenging

The biological activity of NO/RNS is dependent on both spatio-temporal regulation of NO production, NO reactions and NO removal by scavenging systems. Consequently, the systems that control the level of NO and derivative RNS play an important role in regulating the NO-based signaling events.

Oxygen – As described in the previous section, NO reacts with oxygen and superoxide ($O_2^{-\bullet}$) leading to the formation of nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$), respectively. These reactions, besides contributing to modulate NO levels, generate additional RNS that are more reactive than NO itself. While decreasing NO levels, the reaction with oxygen stimulates the formation of protein nitrosothiols via S-nitrosation, and the reaction of NO with superoxide may end up with tyrosine nitration. While reactions of NO with oxygen or superoxide cannot be easily controlled inside the cell, they are predicted to be particularly important in subcellular compartments where the production of oxygen (chloroplasts) and ROS (both chloroplasts and mitochondria) may be massive [43].

Hemoglobins – It is well established that NO can form complexes with plant metal-containing proteins and several studies have focused their attention on the interactions between NO and hemoglobin (Hb). In plants, there are three main types of Hb: symbiotic Hb, also named leghemoglobin (Lb), localized in nitrogen-fixing root nodules of leguminous plants; non-symbiotic Hb (nsHb), consisting of two classes based on oxygen affinity (*i.e.* class I has higher affinity for oxygen than class II); and truncated Hb (tHb) [44]. In 1998, Davies and colleagues reported the existence of a complex between ferrous leghemoglobin ($LbFe^{II}$) and NO in intact root nodules or extracts of root nodules [45]. Additional evidence showed that other Lb forms that are produced *in vivo* (*i.e.* oxyLb, $LbFeO_2$; and ferrylLb, $LbFe^{IV}$=O), are able to scavenge *in vitro* NO and/or peroxynitrite [46], with concomitant formation of nitrate. The interaction of NO with Hb is not exclusive to Lb. In fact, several

studies reported that nsHb from distinct plant species can convert NO into nitrate via an NAD(P)H-dependent reaction [47-49]. The analysis of transgenic plants with altered expression of class-I nsHb sustained the functional role of these proteins in the control of NO bioavailability especially under stress conditions such as hypoxia [48, 50, 51] and pathogen attack [52]. Accordingly, overexpression of class-I nsHb improved plant fitness during hypoxic stress while mutant plants with defective expression of class I nsHb accumulated NO with concomitant decrease of plant growth and enhanced resistance to the hemibiotrophic *P. syringae* and the necrotrophic fungus *Botrytis cinerea*. Altogether, these studies evidenced a strong relation between nsHb proteins and NO suggesting that nsHb can control NO levels with consequent adaptation to stress conditions. Besides Lb and nsHb, also truncated Hb was shown to play a role in NO modulation. In the green alga *Chlamydomonas reinhardtii*, tHb1 was recently shown to bind NO [53] and to modulate NO levels and NR activity [54] while tHb8 was proposed to participate in an NO-dependent signaling pathway required for anaerobic survival [55].

Glutathione – An important molecule involved in the control of NO levels *in vivo* is glutathione (γ-L-glutamyl-L-cysteinyl-L-glycine). This molecule is a small tripeptide of 307 Da present in almost all living organisms and widely spread in all cellular compartments [56]. Relatively abundant (1-10 mM), glutathione is considered as the major low molecular weight thiol in cells [57]. It exists in two different redox states: reduced (GSH) and oxidized, the latter bearing a disulfide bridge between two glutathionyl moieties (GSSG). In plant cells, GSH has several antioxidant functions (*e.g.* regeneration of reduced ascorbate during the ascorbate/glutathione cycle [43]; detoxification of xenobiotics [58] or heavy metals [59], and also participates in NO scavenging by forming nitrosoglutathione (GSNO). The exact mechanism of GSNO formation is still unclear: GSH might be either nitrosated by N_2O_3 or, alternatively, an NO molecule might nitrosylate a glutathionyl radical [60] (see Figure 1). GSNO can be reduced to GSSG and ammonia by the zinc-containing enzyme nitrosoglutathione reductase (GSNOR, for further details see below and paragraph 3.2). Besides modulating NO levels, GSNO is considered as the major reservoir of NO in cells and serves as an important vehicle for NO throughout the cell, enabling NO biological activity to be expanded [61].

Nitrosoglutathione reductase – Originally considered as a glutathione-dependent formaldehyde dehydrogenase, GSNO reductase (GSNOR) belongs to the alcohol dehydrogenase protein family III (ADH3 in human) and it was shown to be involved in GSNO catabolism [60, 62-64]. The enzyme catalyzes the NADH-dependent transformation of GSNO into GSSG and ammonia in the presence of GSH [62, 65, 66]. More precisely, GSNO reduction by GSNOR produces an intermediate

(glutathione sulfonamide, GSNHOH) that can either react with GSH to produce GSSG and ethanolamine (NH_2OH) or rearrange and then spontaneously hydrolyze to produce glutathione sulfinate (GSO_2H) and ammonia [67]. GSNOR is highly conserved between kingdoms and is present in most photosynthetic organisms. In these organisms, GSNOR is generally cytoplasmic and encoded by a single gene, except for few cases known, such as poplar that contains two GSNOR genes [63, 68]. GSNO can act as an NO donor to protein cysteine thiolates to form protein nitrosothiols, and since GSNOR can regulate GSNO cellular homeostasis, it indirectly controls the levels of protein nitrosothiols [62, 69-72]. These functions will be presented with more details in the section dedicated to protein S-nitrosylation and S-nitrosation (see paragraph 3.2).

Peroxiredoxins – Peroxiredoxins (PRXs) are small non-heme peroxidases present in diverse compartments and divided into four classes: 2-cys PRX, 1-cys PRX, PRX-Q and type-II PRX [73-75]. Peroxiredoxins catalyze the reduction of H_2O_2 to water or alkylhydroperoxides (ROOH) to the corresponding alcohol (ROH). PRXs are mainly reduced by thioredoxins but alternative electron donors such as glutathione are possible. Alternatively, PRXs can also scavenge RNS by reducing peroxynitrite into nitrite [76, 77]. PRXs were demonstrated to act as peroxynitrite scavengers under conditions of RNS production in Arabidopsis [78] and bacteria [79].

Additional RNS scavenging systems – In addition to the best known RNS-scavenging systems described above, other mechanisms might have a role in the modulation of NO levels in plants. A most likely mechanism involves thioredoxins (TRXs) that are small oxidoreductases known to control the redox state of target proteins through dithiol/disulfide exchange reactions [80, 81]. Besides reducing selected disulfides, dithiolic TRXs can reduce GSNO to GSH and concomitantly release a nitroxyl anion (NO^-) that immediately gets protonated (HNO). While this reaction is catalyzed by human and plant TRXs *in vitro* [82], there is no clear evidence that TRXs may be involved in GSNO catabolism *in vivo*. Another intriguing mechanism potentially implicated in NO scavenging is protein S-nitrosylation (or S-nitrosation). This thiol-based redox modification involves specific target cysteines on proteins and mainly serves as a signaling mechanism. Being reversible, nitrosylation/nitrosation reactions might contribute to NO/RNS scavenging as long as nitrosothiols formation decreases the intracellular levels of NO and its derivatives. However, this putative function is strictly dependent on the stability of nitrosothiols and may become particularly effective when nitrosylated proteins are subject to denitrosylation reactions that end up with compounds with no nitrosylating activity. This is the case of the GSH-mediated denitrosylation, which forms GSNO which can be reduced (and inactivated) by GSNOR.

3. RNS-PROTEINS INTERACTIONS: METAL LIGATION, TYROSINE NITRATION AND FORMATION OF CYSTEINE NITROSOTHIOLS

In plants, NO and derived RNS are involved in the regulation of several physiological processes [83, 84], and their cellular effects are mediated by three major mechanisms: (1) metal-nitrosylation; (2) tyrosine nitration, and (3) formation of cysteine nitrosothiols, which may occur either by S-nitrosylation or S-nitrosation mechanisms.

NO can directly bind transition metals generating NO-metal complexes [35]. When these metals are located in the prosthetic group of metalloproteins, this reaction can lead to the metal-catalyzed (or metal-assisted) nitrosylation of protein thiols (see 2.2 and 3.1). Among the NO-targeted metalloproteins of animal systems, soluble guanylate cyclase (sGC) is a well-known example [85, 86] together with aconitase [87], hemoglobin and myoglobin [88], NOS and cytochrome P450 [89, 90]. Apart from aconitase, which contains an iron-sulfur cluster, the others are all heme-proteins. Also plants contain heme-proteins that are targeted by NO, including soluble guanylate cyclase [91] besides the already mentioned Hbs [92].

NO and its derivatives also modify specific amino acid residues. Tyrosine is a possible target and the reaction, called nitration, consists in the binding of an NO_2 moiety to the aromatic ring of a tyrosine generally resulting in the modulation of the function of the target protein [93, 94]. The main agent inducing Tyr nitration is peroxynitrite, suggesting that this modification mainly occurs under severe stress conditions (*i.e.* concomitant production of NO and $O_2^{-\bullet}$) (Figure 1) [84, 95]. Tyr nitration is implicated in neurodegenerative diseases and cell death pathways and has therefore been extensively studied in mammals [35]. Nevertheless, several studies suggested that this modification may also have important roles in plants [94, 96-101]. At the moment, the number of studies on nitration in plants is limited compared to other post-translational modifications and its role in cell regulation remains unclear because of its irreversibility and limited knowledge on possible underlying mechanisms [35, 84]. Conversely, the formation of cysteine nitrosothiols has been deeply studied in the last years and is considered as the main redox post-translational modification triggered by NO and its derivatives. In animals, it is well-established that it plays a critical role in the control of numerous fundamental processes, regulating the activity, stability and subcellular localization of a large number of target proteins as well as their interactions with DNA/RNA and other protein partners [102-104]. In photosynthetic organisms, formation of protein nitrosothiols has recently emerged as an important mechanism of redox regulation and redox signaling, although much remains to be done [83].

3.1 Protein S-nitrosothiols: biochemical features and mechanisms of formation

Protein S-nitrosylation consists in the formation of a nitrosothiol (-SNO) after the reversible and covalent linkage of NO to a protein cysteine thiol. The term S-nitrosylation is commonly used as a general term for the formation of S-nitrosothiols, but actually S-nitrosylation should only be applied to the reaction between NO and a thiyl radical, while S-nitrosation describes the reaction between nitrosonium (NO^+) and a thiolate (Figure 1). However, taking into account the widely accepted use of the term S-nitrosylation as a synonym of "S-nitrosothiol formation", here we will refer to S-nitrosylation whenever the underlying mechanism of S-nitrosothiol formation is not considered, or unknown.

To be suitable for a nitrosylation reaction, cysteine residues must be "reactive", namely in the form of thiyl radicals ($-S^{\bullet}$) or thiolate anions ($-S^-$) [105]. The thiyl radical is typically formed under stress conditions (for details see paragraph 2.2) while the thiolate requires a specific protein microenvironment. The thiolate form of a cysteine depends on several factors such as solvent accessibility, the pK_a of the thiol and the microenvironment surrounding the residue or a combination of these factors [106-109]. The cysteine microenvironment has been also proposed to have an important impact on the sensitivity of a given cysteine to nitrosylation. Notably, the presence of acidic and basic amino acid residues flanking the cysteine is important to favor thiol deprotonation but at the same time they may be involved in the stabilization of the major nitrosylating agent in the cell, GSNO [110]. Based on this property, Stamler and colleagues defined a consensus SNO motif for S-nitrosylation [111]. However, since acidic and basic residues that are in proximity of the reactive Cys in the three-dimensional structure of the protein might not be close in the amino acid sequence, a confident prediction of nitrosylation sites from amino acid sequences was precluded [112].

The pathways leading to the formation of a protein nitrosothiol in a cell are mainly three: direct nitrosylation, metal-mediated nitrosylation and trans-nitrosylation, depending on whether the nitroso group of the SNO derives directly from NO/RNS or is donated by a metal-NO complex or by another nitrosothiol (Figure 2).

Direct S-nitrosylation – As mentioned above, direct reactions are represented by two major mechanisms involving the reaction between NO and thiyl radicals (S-nitrosylation *stricto sensu*), and the reaction between dinitrogen trioxide and thiolate anions, where N_2O_3 acts as a donor of NO^+ (S-nitrosation) (Figure 2, reaction 1). While the former occurs under severe stress conditions that allow the formation of thiyl radicals, the latter typically befalls under physiological conditions, when the redox potential of the cell is not perturbed [113, 114].

Metal-mediated S-nitrosylation – As already mentioned, metal-mediated nitrosylation involves metal-nitrosyl complexes formed by NO reacting with transition metals of metalloproteins [115] (Figure 2, reaction 2). Once formed, these metal-nitrosyl complexes are responsible for the transfer of NO to a cysteine residue belonging to the same protein (auto-nitrosylation), possibly following an S-nitrosation mechanism as described in part 2.2. To date, the most extensively studied example of an auto-nitrosylated protein is mammalian hemoglobin (Hb) [88] in which NO binding to the heme center allows the protein to auto-catalyze the S-nitrosylation of its Cys93, thereby modulating the enzymatic activity in response to oxygen tension and maximizing oxygen delivery to peripheral tissues.

Trans-nitrosylation – Trans-nitrosylation can involve low molecular weight S-nitrosothiols such as GSNO, or nitrosocysteine (CysNO), or a protein-mediated mechanism where an S-nitrosylated protein transfers its NO moiety to another protein [105] (Figure 2, reaction 3). Among the diverse mechanisms of S-nitrosylation, GSNO-mediated trans-nitrosylation is considered the most physiologically relevant. In fact, GSNO is the main NO reservoir and acts as signal transducer, transferring its NO moiety to numerous targets within the cell. Its importance has been firstly clarified in the mouse model, where knocking out GSNOR, an enzyme specifically involved in GSNO breakdown (for further details see paragraph 3.2), results in a high level of nitrosylated proteins, indicating that GSNO is in equilibrium with a pool of SNO-proteins [62, 116]. Similarly, Arabidopsis plants deficient for GSNOR [69, 70] exhibited increased levels of nitrosylated proteins. Conversely, GSNOR overexpression was found to decrease protein nitrosylation levels [71]. Although GSNO-dependent trans-nitrosylation is considered as the main mechanism, a growing interest has recently emerged on protein-mediated trans-nitrosylation. Notably, proteins containing a nitrosylated cysteine may be able to transfer their NO moiety to other proteins. Theoretically, this mechanism may lead to the nitrosylation of proteins that are not themselves target of direct nitrosylation, thereby amplifying the signal. At the moment, there are few examples of trans-nitrosylases in the literature: SNO-hemoglobin, SNO-glyceraldehyde-3-phosphate dehydrogenase, SNO-caspase 3 and SNO–Thioredoxin1 [117-119]. To date, nothing is known in plant systems.

3.2 Major mechanisms of denitrosylation

The extent of S-nitrosylation of any given protein depends on the ratio between the rates of nitrosylation and denitrosylation. S-nitrosothiols are generally considered labile entities because of their light- and redox-sensitivity. For example, S-nitrosothiols are often easily reduced by low

molecular weight thiols such as reduced glutathione (GSH) [120] (and references therein). However, other S-nitrosothiols may not be easily reverted *in vivo* despite the presence of a high concentrations of reductants in the cell, and seem to be tightly regulated [121]. Proteomic analyses identified a large set of nitrosylated proteins that are relatively stable under normal physiological conditions and are not readily denitrosylated by GSH [122]. Accumulating evidence indicate that specific redox proteins can catalyze the denitrosylation of a subset of nitrosylated proteins thereby contributing to the specificity of S-nitrosylation.

Non-enzymatic denitrosylation – The S–NO bond can be broken by the action of intracellular reducing agents such as low molecular weight thiols. Since GSH is present at millimolar concentrations (1-5 mM) in plant cells [123], it is considered to play a major role in the control of SNO homeostasis (Figure 3, reaction 1). However, the reduction of SNOs by GSH is a trans-nitrosylation reaction leading to the formation of GSNO. Consequently, GSH denitrosylates a target protein but the GSNO released could serve as a nitrosylating agent for the same protein or other potential targets. To be effective, the denitrosylating activity of GSH requires the intervention of the enzyme GSNOR, which is the only enzyme able to reduce GSNO without producing NO or RNS (Figure 3, for further details see below). Recently, we provided a clear demonstration of the denitrosylating activity of GSH [124]. In this study, the cytoplasmic GAPDH isoform from *A. thaliana* (AtGAPC1) was effectively denitrosylated by GSH *in vitro.* The nitrosylation state of AtGAPC1 was found under the control of the GSH/GSNO ratio but not affected by GSSG. As a consequence, the nitrosylation state of AtGAPC1 is set by the balance between the GSNO-dependent nitrosylation and GSH-dependent denitrosylation, whichever the redox state of the glutathione pool (GSH/GSSG).

Enzymatic denitrosylation – To date, the foremost enzyme that has been clearly demonstrated to play a role in controlling nitrosylation in both animal and plant systems is nitrosoglutathione reductase (GSNOR) [68, 121]. Indeed, alteration of GSNOR expression strongly impacts on the level of protein nitrosylation [69-71, 116, 125]. However, this effect is indirect since GSNOR does not display a direct protein-SNO denitrosylase activity but indirectly impact protein nitrosylation by controlling the intracellular GSNO levels [66, 69]. GSNOR catalyzes the reduction of GSNO to GSSG and NH_3 in the presence of GSH (Figure 3, reaction 2). An interesting feature of this enzyme relies in its specificity for NADH unlike all other antioxidant enzymes that use NADPH as the electron donor. GSNOR from different plant sources is totally unable to catalyze GSNO reduction in the presence of NADPH ([66]; authors' personal communication).

Another enzymatic pathway involved in SNOs reduction is the TRX system [121, 126]. This system is composed of TRX, thioredoxin reductase (TR) and NADPH and is best known for its role in catalyzing dithiol/disulfide exchange reactions. Different from GSNOR, TRXs can also directly interact with protein SNOs and catalyze protein denitrosylation by means of their active site redox cysteines [120] (Figure 3, reaction 3). Holmgren and colleagues showed that TRX can also reduce GSNO *in vitro*, suggesting that this oxidoreductase might be involved in the control of cell nitrosothiols [127]. The role of TRX as denitrosylase has been clearly demonstrated in human cells, where reduced TRX denitrosylates a subpopulation of mitochondrial caspase-3 constitutively nitrosylated, promoting their activation and consequent apoptosis [128]. The involvement of TRXs as denitrosylating enzymes is not restricted to animal cells and has been recently suggested in the context of the regulation of plant immunity [129]. Two molecular mechanisms have been proposed. In the first [128], the most nucleophilic cysteine of the TRX active site attacks the nitrosylated cysteine of the target protein to form a mixed-disulfide intermediate in which TRX and target are covalently linked and a nitroxyl (NO^-) is released. The mixed-disulfide can then be resolved by the nucleophilic attack of the second TRX cysteine with formation an internal disulfide bond and release of the target protein in the reduced state. In the other possible mechanism [130], the initial nucleophilic attack leads to the nitrosylation of TRX and release of the reduced target. The nitrosylated state of TRX is then resolved by the vicinal cysteine through disulfide bond formation and NO^- release (Figure 3). Recently, Spoel and colleagues demonstrated that a plant TRX belonging to the h-type (TRXh5) catalyzes selective NPR1 denitrosylation following the second mechanism in which a nitrosylated TRX intermediate is formed [131]. Both cysteines of the TRXh5 active site were found able to perform the first nucleophilic attack on nitrosylated NPR1. In addition, this study nicely demonstrated that TRXh5 cannot restore the immunity response in the *gsnor1* genetic background, suggesting that the denitrosylation activity of the GSH/GSNOR and the TRXh5 systems are selective and not overlapped.

In summary, denitrosylation reactions are dynamic processes involving reducing agents and/or redox-related enzymes. This process appears to be mainly controlled by two redox systems (*i.e.* GSH and TRX and their associated proteins) that show selectivity towards protein-SNO substrates, allowing specificity of SNOs-dependent signaling.

4. DETECTION OF S-NITROSYLATED PROTEINS AND PROTEOMICS

The quantification of nitrosothiols (-SNOs) and the identification of nitrosylated proteins in biological samples remain a challenge due to the lability of nitrosylated proteins [132]. Indeed, the

stability of SNOs is strongly influenced by several factors including light, metals and reducing agents (*e.g.* GSH, TRXs, etc., see paragraph 3.2).

4.1 Methods for quantification of protein nitrosothiols

The multiplicity of effects of SNOs has prompted the development of reliable methods to detect these adducts. SNO detection methods can be direct (by which the SNO group is detected as such) or indirect (by which the unstable S–N bond of SNO is broken and either the sulfur or the nitrogen-containing product is captured for detection) [133-135]. Readers interested on direct methods such as mass spectrometry, gold nanoparticles, immuno-histochemical approaches, may find excellent reviews on the subject [133-136]. This review will focus on widely used indirect methods.

The most common indirect method is the Saville method, which is based on the heterolytic cleavage of SNOs by mercury(II) to mercurythiolate and nitrous acid (HNO_2) [137, 138]. Reaction samples containing protein SNOs are first treated with $HgCl_2$ in the presence of sulfanilamide (SUF) under acidic conditions. The resulting diazonium salt then reacts with N-(1-naphthyl) ethylenediamine to give an azo dye that can be detected colorimetrically at 540 nm. A correct estimation of SNOs content requires a standard curve generated with low-mass SNOs such as GSNO [139]. While the Saville method is easy to perform and reproducible, the sensitivity of this method is low being in the µM range (0.5-100 µM) [132, 139].

Another mercury-based method consists in the use of the fluorescent probe 2,3-diaminonaphthalene (DAN) or analogous compounds [138-140]. DAN, upon reaction with RNS forms the fluorescent 2,3-naphtotriazole [139]. In this assay, SNOs are cleaved by mercury(II) at neutral pH following the addition of DAN under acidic condition and subsequent neutralization, since the DAN triazole fluorescence is highly pH sensitive. The fluorescence is measured with excitation light of 375 nm and emission at 415 nm [139]. Different from the Saville method, the DAN assay exhibits a higher sensitivity, being in the nM range (50 nM-5000 nM).

The combination of these two methods provides a large detection range (*i.e.* from 50 nM to 100 µM SNOs), allowing the determination of the overall concentration of SNO moieties in biological samples [61].

Another representative indirect method is based on chemiluminescence. In this method, NO reacts with ozone to give an excited state of nitrogen dioxide. Upon return to the ground state, nitrogen dioxide emits light, which is then detected by a photomultiplier tube [136, 138].

4.2. Methods of detection of S-nitrosylated proteins and peptides

The lability of S-nitrosothiols precludes their direct detection by MALDI-TOF or ESI mass spectrometry since most peptides lose the NO moiety during MS analysis [141, 142]. High throughput proteomic detection of nitrosylated proteins therefore relies on indirect methods mostly based on the replacement of the NO moiety by a more stable tag, such as biotin, followed by an enrichment step. Most studies rely on the Biotin Switch Technique (BST), initially developed in 2001 by Snyder and colleagues [143]. It was the first strategy designed for the detection and identification of S-nitrosylated proteins in a total proteome and it is, by far, the most popular (Figure 4). This method relies on the replacement of the NO moiety of S-nitrosylated cysteine residues by a disulfide-bonded biotin tag in a three step process:

(1) initial blocking of unmodified cysteine thiols by methyl methanethiosulfonate (MMTS) under denaturing conditions;

(2) selective conversion of S-nitrosothiols into free thiols by treatment with ascorbate;

(3) labeling of the nascent thiol groups by thiol/disulfide exchange with the biotinylating reagent N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide (Biotin-HPDP).

Steps 2 and 3 are performed simultaneously to allow immediate biotin labeling of nascent thiols. After the biotinylation step, previously S-nitrosylated proteins can be easily detected by immunoblotting with anti-biotin antibodies or purified by avidin-based affinity chromatography and identified by MS after elution with dithiothreitol (DTT) to reduce the disulfide that links the target protein to biotin [144]. Since its invention in 2001, the BST was applied in many biological systems including photosynthetic organisms (see section 5 below). Many variants of the original BST have been proposed. The SNO site identification (SNOSID) approach includes a trypsin digestion step before affinity purification allowing the selective isolation of nitrosylated peptides (Figure 4). Therefore SNOSID allows not only identification of the modified protein but also of the modified cysteine [145]. In the SNO resin-assisted capture (SNO-RAC) strategy, proteins denitrosylated by ascorbate are retained on a Thiopropyl Sepharose 6B thiol-reactive resin and can be eluted by thiol-reducing agents either directly or after on-resin trypsinization for identification of nitrosylated proteins and cysteines respectively [146]. SNORAC appears more sensitive than the BST for high molecular weight proteins and the use of solid phase cysteinyl peptide enrichment, which is well suited for on-resin chemical reactions, may be particularly useful to introduce quantitative labels [146, 147]. Alternate approaches have also been designed based on the direct capture of S-nitrosocysteine residues using organomercury-based chemistry [148], that allows conversion of thiols of formerly S-nitrosylated cysteines to sulfonic acids (+48 Da), using a direct reaction of S-nitrosocysteine residues with gold nanoparticles [149] or using phosphine based direct reaction [134]. Despite promising preliminary results, the methods based on direct capture need further confirmation of their potential

for large-scale studies. The BST remains the most reliable approach for large scale proteomic analysis of protein nitrosylation.

Several quantitative BST approaches allowing quantification of nitrosylation levels have also been developed. Quantification is generally performed by combining BST with chemical labeling strategies based on isotope coded probes. Isotope-coded affinity tag (ICAT) allowed monitoring changes in nitrosylation levels in response to cold or salt stress in Arabidopsis [150, 151]. Synthetic isotopic S-nitrosothiol capture reagents (SNOCAP) was used to identify stable nitrosylated proteins that are not reduced in the presence of GSH and are therefore likely reduced through specific denitrosylation pathways [122]. The new isotope-coded cysteine thiol-reactive multiplex reagents cys-TMT [152] and iodo-TMT [153] have been used in combination with BST to investigate nitrosylation in human and murine cells, respectively. Isobaric tag for relative and absolute quantification (iTRAQ) has been used in conjunction with the SNO-RAC method to examine kinetics of intracellular nitrosylation and revealed that denitrosylation is a major determinant of steady-state SNO levels [146]. Besides chemical labeling, quantitative analysis of nitrosylation can be achieved by combining BST with metabolic labeling strategies such as stable isotope labeling with amino acids in cell culture (SILAC) [154, 155].

5. ROLES AND TARGETS OF NITROSYLATION IN PLANT PHYSIOLOGY

5.1 Targeted studies

Although protein nitrosylation is by far more documented in mammals where it participates in numerous physiological and pathological cellular processes [115, 156], our understanding of the biological importance of nitrosylation in photosynthetic organisms is progressing, as recently extensively reviewed [102, 105, 157]. The major nitrosylated proteins characterized to date in photosynthetic organisms are listed in Table 1 and briefly presented below in their biological context.

Many studies pointed out the importance of nitrosylation in plant immune responses [158-161]. The best studied mechanism implicates the NPR1 (non-expressor of pathogenesis related protein 1) transcription factor and constitutes the most documented example of GSNO-mediated trans-nitrosylation in plants. NPR1 is a key regulator in the signal transduction pathway that leads to systemic acquired resistance in plants [162]. In non-infected cells, NPR1 is located in the cytoplasm, inactive in its oligomeric form stabilized by intermolecular disulfide bonds. Upon redox changes triggered by salicylic acid (SA) produced in response to pathogen infection, NPR1 active monomers are released and translocated into the nucleus where their stimulate the transcriptional activation of defense-related genes through binding to TGA transcription factors, including TGA1 [163, 164]. The

reduction of the oligomeric form of NPR1 is catalyzed by TRXs while nitrosylation of Cys156 triggers conformational changes that facilitate oligomerization via disulfide bonding [129]. TRXh5 was recently shown to catalyze the selective denitrosylation of NPR1 ([131]; see 3.2). Moreover, TGA1 was also reported to be nitrosylated by GSNO. Under oxidative conditions, four cysteines contained in TGA1 form intramolecular disulfide bonds locking the protein in an inactive conformation unable to interact with NPR1 [165, 166]. GSNO was found to trigger nitrosylation and glutathionylation of several cysteine residues of TGA1 and to enhance, in the presence of NPR1, the DNA binding activity of TGA1 to the activation-sequence-1 (as-1) element found in the promoter of several defense-related genes [166].

Many studies have reported that a strong interplay between ROS- and NO- (or RNS-) dependent signaling pathways is required to regulate numerous processes, especially responses to biotic and abiotic stresses [49, 167-170]. Modulation of this interplay involves the reciprocal regulation of intracellular ROS and NO (or RNS) levels [169, 171, 172]. During plant immune responses, NO and ROS are considered to act concertedly [171-173]. The regulation of NADPH oxidase constitutes a key example of the control of ROS by NO through nitrosylation [172]. Indeed, in plants, NADPH oxidase is responsible for the generation of ROS during the hypersensitive response [174]. In addition, pathogen infection has been reported to trigger rapid NO synthesis [9, 10]. During the initial response, in the absence of SA, the accumulation of NO facilitates the hypersensitive response [HR]. Then, as the concentration of NO arises, it governs a negative feedback leading to the inactivation of the AtRBOHD NADPH oxidase, the major source of ROS during HR, by nitrosylation of Cys890 [172]. This mechanism was proposed to allow a tight regulation of programmed cell death during the hypersensitive response [102].

Several ROS detoxifying enzymes were also found to be regulated by nitrosylation. For example, Arabidopsis chloroplastic PRXIIE was found to undergo nitrosylation upon pathogen exposure [175, 176]. Both peroxide and peroxynitrite reductase activities of PRXIIE were found to be inhibited by nitrosylation of Cys121. This regulation of PRXIIE was proposed to control the damaging and signaling effects of $ONOO^-$. Another good example of ROS/NO crosstalk is cytoplasmic ascorbate peroxidase (APX) that appears to be controlled by NO at multiple levels although contrasting results have been reported in different species. Nitrosylation of tobacco APX was proposed to trigger its ubiquitination and subsequent proteasomal degradation during programmed cell death [177]. By contrast, pea APX1 was activated by nitrosylation of Cys32 and inhibited by tyrosine nitration at Tyr235 [178]. Recently, nitrosylation of Arabidopsis APX1 at Cys32 was shown to increase its activity with hydrogen peroxide and to play an important role during immune responses and abiotic stresses [179]. APX activity was also found to be activated by NO

donors in soybean root nodules [180] and auxin control of root growth may act, in part, through denitrosylation of APX1 [181]. While potato dehydroascorbate reductase is inhibited by nitrosylation [182], pea monodehydroascorbate reductase is inhibited by tyrosine nitration [183] and the inhibition of several superoxide dismutases by $ONOO^-$ was proposed to participate in the control of cell death [184]. Moreover, the nitrosylation of GSNO reductase, that appears to inhibit the enzyme, was suggested to participate in the control of SNO levels [185].

Nitrosylation appears to play an important role in the signaling of many hormones. NO and ROS act in concert together with ABA during stomatal closure [186]. Indeed, NO production induced by ABA, was shown to regulate ABA signaling by nitrosylation of Cys137 of the kinase OST1/SnrK2.6 resulting in inhibition of activity [187]. Nitrosylation of the TIR1 auxin receptor on Cys140 was reported to enhance TIR1 interaction with Aux/IAA proteins, facilitating their degradation and subsequently promoting activation of gene expression [188]. Nitrosylation of the AHP1 at Cys115, a histidine phosphotransfer protein involved in cytokine signaling, represses its phosphorylation and subsequent transfer of the phosphoryl group to downstream ARR response regulators [189].

Other enzymes involved in immune responses have been shown to be regulated by nitrosylation. The carbonic anhydrase SABP3 (salicylic acid binding protein-3), playing an important role in defense responses, was found to be inhibited by nitrosylation of Cys280 [190]. In tobacco, NO is responsible for the inactivation of CDC48, a protein that has an activity associated with the ERAD (ER-associated degradation) pathway, disease resistance, cytokines, cell expansion and differentiation [191-193]. Nitrosylation of the Chaperone like AAA+ ATPase CDC48 occurs on three cysteines (Cys110, Cys526, Cys664) and Cys526, located in the ATP binding site, was found to be responsible for structural changes leading to drastic loss of activity upon nitrosylation [194]. The Arabidopsis Metacaspase-9 (AtMC9) was found to be nitrosylated *in vivo* at catalytic Cys147 and this process was proposed to regulate AtMC9 function in the regulation of PCD [195].

Besides plant immune responses, a number of metabolic enzymes participating in diverse pathways have been also reported to be regulated by nitrosylation in photosynthetic organisms. In most cases nitrosylation was found to inhibit the activity of the modified enzyme. As its mammalian counterpart, Arabidopsis glycolytic GAPDH is inhibited by nitrosylation of its catalytic cysteine [124, 196]. In tobacco, salt stress induced nitrosylation of cytoplasmic GAPDH and activation of the GAPDH-interacting protein NtOSAK (*Nicotiana tabacum* osmotic stress activated protein kinase) [197]. In Arabidopsis, Myb2 DNA binding activity is inhibited by nitrosylation at Cys53 [198, 199] and methionine adenosyltransferase is inhibited by nitrosylation at Cys114 [200]. In Arabidopsis mitochondria, GSNO was found to trigger nitrosylation and glutathionylation of several cysteines of glycine decarboxylase, an enzyme important for glycine catabolism during photorespiration [201]. In

addition, ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) also appears to be inhibited by nitrosylation [202, 203].

5.2. Proteomic analysis of protein nitrosylation in photosynthetic organisms

In addition to targeted studies, numerous proteomic analyses allowed identification of hundreds of proteins undergoing nitrosylation in a wide range of photosynthetic organisms (reviewed in [114, 204-206]). Different organs and subcellular compartments were analyzed such as peroxisomes [207], mitochondria [201], the nucleus [208] or the apoplast [205]. Several nitrosylomes were also performed on mutants including lines deficient for GSNOR [209] or catalase [71]. While most studies were initially performed adding exogenous NO donors [144, 201, 202], numerous physiological biotic and abiotic stresses have also been investigated including cold stress [151, 203, 205, 210], high light [71], ozone fumigation [211], salinity stress [150, 212, 213], polyamines [214] or in response to diverse pathogens or elicitors [176, 194]. The most recent studies identified 492 proteins and 392 sites in *Chlamydomonas reinhardtii* cells submitted to a 15 minutes GSNO treatment [215] and 926 proteins and 1195 sites in Arabidopsis Col-0 and *gsnor1-3* lines [209]. These studies illustrate the progress made in this type of proteomic analyses. While initial studies focused only on the identification of nitrosylated proteins, nowadays, the nitrosylated cysteines can be identified concomitantly, providing further insights into the exact site of modification. Moreover, the number of identified proteins has increased very significantly lately, mainly because modern mass spectrometers are more sensitive and allow a higher coverage of the proteome. This suggests that low abundant nitrosylated proteins can also be identified. This low level can be either due to a low level of nitrosylation or to the low abundance of the protein. In order to distinguish between these two possibilities and provide further insights into the impact of nitrosylation on the proteome, future studies should include quantitative proteomic approaches to monitor protein abundance and nitrosylation extent (see section 6).

Altogether, these studies suggested the implication of nitrosylation in a myriad of cellular processes and pathways. However, all these proteins identified as nitrosylated by large scale proteomic studies remain putative targets that require further confirmation. Indeed, the nitrosothiols are labile and BST is a very difficult technique with inherent limitations and flaws. A major drawback of BST relies in the identification of false positives due to incomplete blocking or denitrosylation during sample handling that cannot be fully taken into account. The reproducibility of the BST-based proteomic analyses is also unexplored but is expected to be variable due to differences in biological material, growth conditions, experimental design, sample handling, instrument setup and bioinformatic data analysis. Nevertheless despite these considerations, proteomic studies have

made an important contribution to the field by revealing that, in photosynthetic organisms, nitrosylation is a widespread post-translational modification that potentially regulates hundreds of proteins involved in a multitude of biological processes. Moreover, one can anticipate that future quantitative proteomic approaches will improve the confidence into data generated by BST-based studies by shedding light on outliers.

6. PERSPECTIVES AND FUTURE DIRECTIONS

Considerable progress has been achieved in our understanding of the importance of nitrosylation in photosynthetic organisms. However, although the diversity of the targets is emerging with large scale proteomic analyses, our understanding of the underlying mechanisms and functional roles of protein nitrosylation remains limited. Particular attention should be devoted in the future to explore the determinants of cysteine nitrosylation, the molecular mechanisms controlling nitrosylation levels and the mechanisms of signal integration at the interface between the nitrosylation network and other post-translational modifications and signaling molecules.

6.1. Understanding the determinants of cysteine nitrosylation

The ability of a given cysteine to undergo a specific type of redox PTM is governed by multiple factors. Proteomic and biochemical approaches revealed that some cysteines undergo multiple redox modifications, as demonstrated for Cys178 of Chlamydomonas isocitrate lyase [216] and Cys149 of Arabidopsis GAPC1 [217] that both undergo nitrosylation and glutathionylation. In the case of other enzymes, such as the Calvin-Benson cycle enzymes fructose-1,6-bisphosphatase and transketolase, the two modifications occur on distinct cysteine residues [218]. The specificity is primarily linked to the biochemical properties of the cysteine residue that are largely dependent on its microenvironment within the folded protein. This microenvironment can influence the accessibility, the acidity and the nucleophilicity of the residue [107, 108, 219]. The microenvironment of the cysteine is also crucial for the stability of the modification since the additional moieties may trigger local structural changes that will allow accommodating and stabilizing the modification. For example, a cysteine residue can possess the reactivity to undergo nitrosylation but once modified, the presence of a nearby cysteine will favor formation of a disulfide bond that substitutes the initial modification, as recently demonstrated for the yeast Atg4 cysteine protease [220]. Hence, the sites of modification are hardly predictable from the primary structure of proteins as recently underscored [221]. In the future, identification and quantification of numerous sites of modification by proteomics will generate large datasets that should contribute, together with improvements of the 3D-modeling

of protein structures, to the development of new algorithms aimed at predicting more accurately redox modification sites.

6.2. Molecular mechanisms controlling nitrosylation levels

Besides the properties of the target cysteines, nitrosylation can be influenced by multiple external factors including small molecules and enzymes. Since the level of nitrosylation is dependent on both the forward reactions of nitrosylation and the reverse reactions of denitrosylation, any molecule affecting these reactions will influence the modification state of cysteine residues. Numerous small molecules influence the forward (*e.g.* NO and its multiple derived species including GSNO) and the reverse (*e.g.* GSH, cysteine, ascorbate) reactions and alteration of their local concentration or redox state can significantly impact the level of nitrosylation. For example, denitrosylation of Arabidopsis GAPC1 was dependent on the GSH/GSNO ratio [124]. Future studies should be aimed at measuring the *in vivo* dynamics of these small molecules affecting nitrosylation.

Numerous enzymes can also control the level of nitrosylation for a specific set of proteins. Human TRX1 was found to denitrosylate only a subset of nitrosylated proteins [222]. In addition, several enzymes, including TRX and GAPDH, were found to trans-nitrosylate specific target proteins [113, 115]. The specificity of these trans-nitrosylases may allow modification of specific cysteines, even when target cysteines do not appear to be candidates for modification based on their reactivity [125]. These specific sites of modification under the control of diverse enzymes will hardly be predictable and will have to be determined experimentally. Only a few trans-nitrosylating enzymes have been identified recently in mammals. Numerous other activities may remain to be discovered and could constitute an important network of redox signal transduction pathways also in photosynthetic organisms.

6.3. Functional role of nitrosylation

Despite the identification of hundreds of putative nitrosylated proteins, the functional role of the modification and its impact on the protein function have only been unraveled for a few of them. Targeted studies will be required to fully understand the functional consequence of nitrosylation for a given protein. However, it should be kept in mind that the nitrosylation of an enzyme involved in a given pathway does not necessarily imply that this pathway is regulated by nitrosylation. Indeed, nitrosylation may have no effect on the activity of the protein, it may only affect a minor pool of the total protein content and nitrosylation of this pool may not be limiting for the pathway flux. Moreover, some nitrosylated proteins may represent moonlighting proteins diverted to new functions unrelated to their known function as shown for GAPDH in mammals [103]. As its mammalian counterpart, plant

GAPDH is nitrosylated and re-localizes to the nucleus under stress conditions but the exact role of nitrosylation remains to be established [196, 223, 224].

6.4 Signal integration: a complex interplay of signaling pathways and molecules

Despite the complexity of nitrosylation signaling networks and associated regulatory pathways, their contribution to signal transduction and environmental adaptation clearly requires signal integration taking into account multiple other signaling pathways and molecules. In addition to nitrosylation, cysteine residues undergo many other types of post-translational modifications, among which glutathionylation and oxidoreduction of disulfide bonds have been shown to play a major role in redox signaling [225]. These redox modifications are interconnected and influence each other. For example, in *Chlamydomonas reinhardtii*, the targets of glutathionylation, nitrosylation [215] and thioredoxin [226] form a highly complex and interconnected redox network with numerous proteins regulated by several modifications (Figure 5). This complexity is likely required to allow a limited number of simple molecules such as ROS and RNS to play a signaling role. The redox signaling network, which remains largely unexplored, is likely a major component of signal integration and constitutes the molecular signature of the ROS/RNS crosstalk whose importance in cell signaling has been recognized [49, 161, 170]. However, this view (Figure 5) is rather static while the redox network probably involves spatial and temporal regulation of several redox post-translational modifications on hundreds of proteins in a highly dynamic manner. The dynamic nature of the network is most likely playing a central role in the mechanisms of cell signaling.

Although most studies on redox PTMs have been focused on nitrosylation, glutathionylation and oxidoreduction of disulfide bonds, other redox modifications exist and should be considered. These modifications include reversible and non-reversible modifications such as S-sulfhydration, tyrosine nitration, protein carbonylation, sulfenic/sulfonic/sulfinic acids, methionine oxidation and diverse types of S-thiolation like cysteinylation [2, 108, 225, 227-231]. The importance of these additional redox modifications is emerging and future studies should provide new insights into their functional role and their integration within the redox signaling network. Therefore, understanding the redox network will require quantitative and temporal analysis of multiple redox PTMs. Despite a growing interest during recent years in the analysis of redox PTMs and especially nitrosylation and glutathionylation, these modifications are most exclusively studied independently and qualitatively. Time-resolved quantitative analysis of redox PTMs may also provide insights into the importance of redox PTMs *in vivo* under diverse physiological conditions or in different genetic backgrounds. For example, analysis of mutant lines devoid of key proteins involved directly or indirectly in the regulation of nitrosylation and glutathionylation, such as GSNOR, GR, GRXs TRXs, GAPDH or NO-

generating enzymes, will be necessary to understand their role and the importance of the redox PTM they control under basal and stress conditions [72, 158, 232, 233]. Moreover, to date, proteomic analyses were mainly focused on soluble proteins. One can imagine that signal integration can implicate membrane proteins. To unravel these type of proteins, adaptation of current methods targeting redox PTM is necessary and will constitute a challenge for future studies.

In addition, redox modifications are also tightly linked to other signaling networks mediated by non-redox post-translational modifications. For example, in mammals, nitrosylation was reported to interfere with signaling processes mediated by phosphorylation, ubiquitylation, sumoylation, acetylation or palmitoylation [234, 235]. The redox PTM often affects the activity of enzymes controlling these modifications such as kinases and phosphatases in the case of phosphorylation. These observations led to propose that among post-translational modifications that convey cell signals, the breadth of the influence of S-nitrosylation may be comparable with that of phosphorylation and ubiquitylation, where signal crosstalk is established as a central operating principle [234, 236]. Very few studies have addressed this type of crosstalk in plants [227] but it likely plays, as in mammals, an important role in cellular signaling. Besides redox PTMs, the integration of the signal implicates a myriad of other molecules and processes acting at the translational, transcriptional and post transcriptional levels [237]. For example, nitrosylation is clearly linked to signaling pathways controlled by hormones [187, 189] or calcium [238]. A strong effort is therefore required to integrate redox signaling with other signaling pathways and to analyze the cellular responses at multiple levels. The emergence of systems biology approaches will certainly be crucial to rapidly increase our understanding of cell signaling pathways and the mechanisms of adaptation to environmental changes.

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PROTEIN	ORGANISM	TARGET	EFFECT	REFERENCE
NPR1	*Arabidopsis thaliana*	Cys156	Conformational changes	164, 165
TGA1	*Arabidopsis thaliana*	Cys260, Cys266	Activation	166, 167
RBOHD	*Arabidopsis thaliana*	Cys890	Inhibition	172
PRXIIE	*Arabidopsis thaliana*	Cys121	Inhibition	176
APX1	*Nicotiana tabacum* *Pisum sativum* *Arabidopsis thaliana*	— Cys32 Cys32	Ubiquitination Activation Activation	177 178 179
DHAR	*Solanum tuberosum*	Cys20, Cys147	Inhibition	183
GSNOR	*Arabidopsis thaliana*	—	Inhibition	186
OST1/SnRK2.1	*Arabidopsis thaliana*	Cys137	Inhibition	188
TIR1	*Arabidopsis thaliana*	Cys140	Facilitation of protein-protein Interaction	189
AHP1	*Arabidopsis thaliana*	Cys115	Inhibition	190
SABP3	*Arabidopsis thaliana*	Cys280	Inhibition	191
CDC48	*Nicotiana Tabacum*	Cys110, Cys526, Cys664	Inhibition	195
Metacaspase-9 (MC9)	*Arabidopsis thaliana*	Cys147	Inhibition	196
GAPDH	*Arabidopsis thaliana* *Nicotiana tabacum*	Cys149	Inhibition	125, 197 198
Myb2	*Arabidopsis thaliana*	Cys53	Inhibition	199, 200
MAT	*Arabidopsis thaliana*	Cys1145	Inhibition	201
RubisCO	*Arabidopsis thaliana*	—	Inhibition	203, 204

Table 1. Established nitrosylated proteins in photosynthetic organisms

Protein: abbreviated protein name (see text for further details). Organism: source organism of the protein. Target: Cysteine target residue. Effect: effect of nitrosylation on the protein activity and/or function. Reference: corresponding reference(s) for each protein.

FIGURE LEGENDS

Figure 1. Most relevant reactions in which NO may be involved in a biological context. All reactions are numbered and explained in the text. Reactions involving only inorganic molecules are indicated by full arrows. Broken arrows represent reactions between a RNS and an amino acid.

Figure 2. Major mechanisms of protein nitrosothiols formation. The formation of protein nitrosothiols can occur via three major mechanisms: direct nitrosylation (reaction 1), metal-mediated nitrosylation (reaction 2) or trans-nitrosylation (reaction 3). All reactions are numbered and explained in the text. Abbreviations: GSH, reduced glutathione; GSNO, nitrosoglutathione; Met, metal; Met-NO, metal-nitrosyl complex; PSH, reduced protein; PSNO, nitrosylated protein.

Figure 3. Major mechanisms of protein denitrosylation. The reduction of protein nitrosothiols can occur via non-enzymatic mechanisms involving GSH (reaction 1) or via enzymatic mechanisms involving GSNOR (reaction 2, specific reduction of GSNO) or TRX (reaction 3). The reduction of oxidized TRX requires the intervention of NADPH and NTR (reaction 4). Abbreviations: GSH, reduced glutathione; GSNO, nitrosoglutathione; GSNOR, nitrosoglutathione reductase; GSSG, oxidized glutathione; HNO, nitroxyl; NH_3, ammonia; NTR, NADPH-Thioredoxin reductase; TRX_{rd}, reduced thioredoxin; TRX_{ox}, oxidized thioredoxin

Figure 4. Workflow for the analysis of nitrosylated proteins with the BST and SNOSID approaches

This workflow presents the main steps of the Biotin Switch Technique (BST) [143] and of the SNO Site IDentification approach [145]. In the version of BST presented, the first step consists of blocking with iodoacetamide while MMTS was employed in the original version.

Figure 5. A complex redox network

Each ball represents a protein. Data were extracted from large scale studies performed in *Chlamydomonas reinhardtii* on S-nitrosylation [215] S-thiolation/S-glutathionylation [239, 240] and TRX targets [226].

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