Emerging functions of nitric oxide in plant immunity

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Chapter 11

NO Signalling in Plant Immunity

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Abstract The importance of nitric oxide (NO) in innate and adaptive immunity in mammals is well recognised. NO exerts antimicrobial properties against invaders but also displays immunoregulatory functions in which S-nitrosylation represents a signalling process of major importance. Over the last two decades, a growing body of evidence suggests that NO is also a major component of plant immunity. Our understanding of its role in plant defence has been enriched by the identification and functional analysis of S-nitrosylated proteins. The recent identification of new S-nitrosylated proteins including the chaperone-like enzyme cell division cycle 48 (CDC48), histone deacetylases (HDACs) and calmodulin (CaM) reveals that NO could act as a modulator of epigenetic changes and targeting of ubiquitinated proteins for degradation. These findings also expand our understanding of the mechanisms controlling NO synthesis and its crosstalks with calcium signalling in plant immunity.

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1 Introduction

Nitric oxide (NO) is a simple diatomic molecule with one unpaired electron in an anti-bonding π molecular orbital. Primarily known as an environmental pollutant, its role in mammalian physiology was recognised in the 1980s (Schmidt and Walter 1994). It is now well established that NO is a major physiological mediator playing key functions in, amongst others, the cardiovascular, the nervous and the immune systems. This diversity of its function is related to the variety of chemical reactions associated with it. Notably, NO undergoes reduction and oxidation and reacts with molecular oxygen (O_2), superoxide anion ($O_2^{\bullet -}$), low molecular thiols, thus leading to numerous derivatives such as nitrosonium cation (NO^+), nitroxyl (HNO), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3) and the major physiological NO donor nitrosoglutathione (GSNO). All these compounds, referred as reactive nitrogen species (RNS), possess their own biological properties and react with cellular components including proteins, lipids and nucleic acids (Stamler et al. 1992; Hill et al. 2010; Thomas et al. 2008). The production of NO is mainly catalysed by nitric oxide synthases (NOSs) which use L-arginine or N-hydroxy-L-arginine as substrates. Mammals express three isoforms of NOSs, namely, inducible NOS (iNOS), neuronal NOS (nNOS) and endothelial NOS (eNOS), the latter two being constitutive (Campbell et al. 2014).

A great deal of research has also unravelled the roles of NO in plants. Again, NO displays wide-ranging biological and physiological actions. It contributes to the regulation of various processes such as seed dormancy, pollen tube and root growth, flowering, stomatal closure, iron homeostasis, hormone signalling, immunity and adaptive responses to stresses (Besson-Bard et al. 2008b; Domingos et al. 2014; Yu et al. 2014; Mur et al. 2013a). Plant NO sources include nitrate reductase (NR) catalysing NO synthesis from nitrite (NO_2^-) and still unidentified enzymatic and/or chemical processes using polyamines or L-arginine as substrates (Corpas et al. 2009; Gupta et al. 2011; Bellin et al. 2013). In addition, NO is produced via non-enzymatic routes through NO_2^- reduction. So far, although NOS-like activities have been measured in plant tissues (Corpas et al. 2009), only one functional NOS has been identified in the green alga *Ostreococcus tauri* (Foresi et al. 2010). Furthermore, a recent investigation provided clear evidences that land plants do not possess animal NOS-like enzymes (Jeandroz et al. 2016).

The exact processes underlying the signalling functions of NO in plants remain relatively unknown. However, the search of its direct or indirect molecular targets provides steadily growing insights into possible mechanisms of NO signalling. All these efforts lead to the preliminary conclusion that the basic concepts of nitric oxide signalling in animals are largely conserved in plants (Besson-Bard et al. 2008b). In this regard, the analysis of the mode of action of NO in plant immunity was particularly relevant.

In this article, we provide an overview of the mode of action of NO in plant immunity with special emphasis on recently identified proteins regulated by NO at the post-translational level, namely, cell division cycle 48 (CDC48), histone deacetylases (HDACs) and calmodulin (CaM). Identification of these proteins suggests that plant cells undergoing an immune response use NO as a signal for

modulating the targeting of ubiquitinated proteins for degradation and the remodelling of chromatin and provides new insights in the understanding of the interplays between NO and Ca^{2+} signalling.

2 Brief Overview of NO Functions in Immunity

The importance of NO in innate and adaptive immunity in mammals is well recognised. NO is produced by NOSs in immune system cells including macrophages, neutrophils, dendritic or natural killer cells (Bogdan 2001). The iNOS is induced at the transcriptional level in macrophages stimulated by cytokines, pathogen-associated molecular patterns (PAMPs) and other microbial-derived products. Once produced by macrophages, NO displays antimicrobial properties killing or reducing the replication of infectious agents. In addition, NO produced by all isoforms of NOSs plays immunoregulatory functions including the control of the differentiation of immune system cells (for instance B and T lymphocytes) and the modulation of the production and function of cytokines (Bogdan 2015).

The signalling functions of NO in innate and adaptive immunity involve the post-translational modification of proteins. The process of S-nitrosylation has been particularly investigated. S-nitrosylation is defined as the covalent addition of an NO group to the thiol of a cysteine (Cys) residue, leading to a nitrosothiol (S-NO) (Gould et al. 2013; Hess et al. 2005; Martinez-Ruiz et al. 2011). S-nitrosylation is mediated by RNS, notably NO^+ , N_2O_3 and GSNO. Therefore, S-nitrosylation is a chemical-based mechanism although the contribution of S-NO proteins displaying a nitrosylase activity has recently been reported (Stamler and Hess 2010). This activity, also named trans-nitrosylation, corresponds to the transfer of an NO group from an S-nitrosylated protein to the Cys residue of a target protein through protein–protein interaction. S-nitrosylation is reversible, and here too, the removal of the NO group is a chemical-based mechanism but could be also catalysed by enzymes such as thioredoxins and GSNO reductase (GSNOR) which reduces GSNO and, consequently, decreases protein S-nitrosylation levels (Benhar et al. 2009). Not all reduced Cys residues and not all Cys-containing proteins are modified. Indeed, although strict consensus motifs for S-nitrosylation have not been identified, Cys residues targeted by NO seem to be localised in solvent-accessible surfaces of proteins and positioned in local environments favouring thiol ionisation or attracting NO (Lamotte et al. 2015; Cheng et al. 2014). The occurrence of such motifs within proteins and the involvement of nitrosylases acting on specific targets through protein–protein interaction as well as those of denitrosylases are key parameters explaining how the specificity of S-nitrosylation is determined and how NO nitrosylation-based signals are transduced. In addition, this specificity is provided by the co-localisation of NOS and NO targets (Hess et al. 2005).

Depending on the proteins, S-nitrosylation impacts their enzymatic activities, their structure and their subcellular localisation and could also facilitate or impair their oligomerisation and interaction with partners. In the mammalian immune

system, several proteins appear to be regulated through S-nitrosylation such as the surfactant protein D (SP-D), the transcription factor NF- κ B and the non-receptor protein tyrosine kinase p60^{Src} (Src) or CD40. Their S-nitrosylation might drive the switch between anti- and pro-inflammatory activities (Hernansanz-Agustin et al. 2013). Furthermore, activated macrophages protect themselves against their own production of NO through denitrosylases (Benhar et al. 2009).

Although plants lack specialised immune cells, they have also evolved a sophisticated immune system which has been and is still particularly well researched (Spoel and Dong 2012; Zipfel 2009). Over almost 20 years now, it has been recognised that NO operates in plant immunity. Many investigations reported that plant cells challenged by PAMPs, effectors or pathogens produce NO (Besson-Bard et al. 2008b). The corresponding sources include NR and the possibility that a NOS-like enzyme could contribute to this production has been recently minimized by the demonstration that land plants do not possess animal NOS-like enzymes (Corpas et al. 2009; Gupta et al. 2011; Wendehenne et al. 2004; Bellin et al. 2013; Jeandroz et al. 2016). Similarly to animals, the possibility that NO displays antimicrobial activities in plants facing pathogen attack has been demonstrated (Boccardo et al. 2005). Furthermore, the signalling capacities of NO in host cells have been widely studied. Results from these analyses demonstrated that, as a cellular messenger, NO contributes to the activation of the phenylpropanoid pathway (Zeier et al. 2004; Durner et al. 1998), to the modulation of hormonal signalling (Mur et al. 2013b) and defence-related gene expression (Huang et al. 2002; Palmieri et al. 2008), to the hypersensitive response (HR) (Leitner et al. 2009) and to the systemic acquired resistance (SAR) (Wendehenne et al. 2014). Therefore, there is no simple picture and NO appears to work at multiple levels in plant immune responses. This complexity is reinforced by the diversity of the molecular mechanisms underlying its effects. Indeed, NO was shown to act in conjunction with reactive oxygen species (ROS) (Delledonne et al. 2003; Skelly and Loake 2013; Gross et al. 2013); with other second messengers including Ca^{2+} , cyclic GMP (cGMP) and cyclic ADP ribose (cADPR; see below) (Jeandroz et al. 2013; Gaupels et al. 2011); and also with hormones, notably salicylic acid, jasmonate and ethylene (Durner and Klessig 1999; Mur et al. 2013b). In addition, NO modulates the activity of protein kinases, notably of mitogen-activated protein kinases (MAPK). The tight cross-regulations occurring between NO and these mediators of defence responses make it difficult to provide an overview of NO functions.

Recently, the search for proteins regulated through S-nitrosylation has expended our understanding of its function in plant immunity. In particular, main actors of the plant immune response have been shown to undergo S-nitrosylation. Peroxiredoxin II E (PrxII E), nonexpressor of pathogenesis-related gene 1 (NPR1) and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase AtrbohD rank amongst the most relevant examples. By promoting their S-nitrosylation, NO was shown to control the level of ROS during HR (Yun et al. 2011), to fine-tune the damaging and signalling effects of ONOO^- (Romero-Puertas et al. 2007) and to promote NPR1 inactivation by oligomerisation (Tada et al. 2008) or its translocation to the nucleus, thus favouring defence gene expression (Lindermayr

et al. 2010). For a detailed description of these processes and a general overview of S-nitrosylation in plant immunity, the reader is referred to previous reviews (Skelly and Loake 2013; Astier et al. 2012b). Recently, new candidates for S-nitrosylation have been identified, notably CDC48, HDACs and CaM. The incidence of their S-nitrosylation, which could provide novel insights into the role of NO in the plant immune response, is discussed below.

3 CDC48, HDACs and CaM: New Insights into NO Functions in Plant Immunity

3.1 CDC48

CDC48, also named p97 or valosin-containing protein (VCP) in animals, is a member of the AAA+ ATPase (ATPase associated with various cellular activities) which assembles as a homohexameric complex. This chaperone-like enzyme contains two ATPase domains (D1 and D2), each consisting of Walker A and Walker B motifs contributing to the binding of ATP and hydrolysis of its β - γ phosphate bond, respectively. In mammals and in yeast, CDC48/VCP plays a crucial role in protein turnover and degradation and helps to protect cells against the toxic effects of protein aggregates. More precisely, CDC48/VCP converts the energy of ATP hydrolysis to structurally remodel ubiquitinated client proteins, thus segregating them from cellular structures or binding partners. Then, CDC48/VCP delivers the client proteins to the proteasome for degradation (Schrader et al. 2009; Madsen et al. 2009; Meyer et al. 2012). Furthermore, CDC48/VCP has been linked to the endoplasmic reticulum (ER)-associated protein degradation (ERAD) where it extracts unfold proteins from the ER membrane after their ubiquitination and before their degradation by the cytosolic proteasome (Baek et al. 2013). CDC48/VCP also edits ubiquitin modification of target proteins with the help of associated cofactors, thus providing an additional level of regulation to ubiquitin-mediated processes (Meyer et al. 2012). These functions contribute to the regulation of many cellular processes, including immune responses, and are also relevant to pathological disorders (Braun and Zischka 2008; Hauler et al. 2012). Notably, inactivations/mutations of VCP elicit the unfold protein response (UPR) which triggers ER-stress-induced apoptosis and are associated with degenerative diseases and other pathologies (Meyer and Wehl 2014).

In plants, the role of CDC48 remains poorly investigated. Pioneer studies demonstrated that it contributes to development, cell division, protein degradation, low-temperature-induced freezing tolerance, ERAD and centromere disassembly (Chamberlain et al. 2008; Feiler et al. 1995; Gallois et al. 2013; Marshall et al. 2008; Merai et al. 2014; Park et al. 2008; Wang et al. 2012). Furthermore, an involvement of CDC48 in plant immunity has emerged recently (Niehl et al. 2012). These authors reported that in *A. thaliana*, the isoform B of CDC48 (AtCDC48B) is induced upon infection by the tobamovirus *oilseed rape mosaic*

virus and interacts with virus-encoded movement proteins (MPs). Through this interaction, AtCDC48B extracts MPs from the ER-associated inclusions where they are produced and promotes their degradation. Importantly, the possibility that CDC48 could be regulated through S-nitrosylation in plant cells expressing an immune response has been highlighted in a parallel study (Astier et al. 2012a). In the latter, a search for S-nitrosylated proteins has been conducted in tobacco cell suspensions elicited by cryptogein, an elicitor produced by the oomycete *Phytophthora cryptogea* triggering an HR and an SAR in tobacco. A dozen of proteins undergoing S-nitrosylation, including CDC48 (NtCDC48), were identified during the first 2 h of cryptogein treatment, a time window in which NO production occurs (Lamotte et al. 2004; Besson-Bard et al. 2008a; Kulik et al. 2015). A detailed analysis of features of NtCDC48 S-nitrosylation indicated that this post-translational modification targets the Cys residue 526 (Cys-526) located in the Walker A motif of the D2 domain involved in ATP binding, a highly ordered region of the protein. The S-nitrosylation of Cys-526 impacts the local structure of the protein, suggesting that this process interferes with ATP binding through a mechanism of steric hindrance. Accordingly, NO was shown to inhibit the ATPase activity of the enzyme in vitro. CDC48 has been also identified in three other studies screening for S-nitrosylated proteins in *A. thaliana*. In the first one, the isoform A of CDC48 was found to be constitutively S-nitrosylated on residue Cys-109 in cultured cell suspensions (Fares et al. 2011). This residue was also S-nitrosylated in vitro in a recombinant isoform of NtCDC48 (Astier et al. 2012a). In the second study, the CDC48B and CDC48C isoforms were identified together with about 900 other protein candidates in a large-scale, site-specific proteomic analysis of endogenously S-nitrosylated proteins (Hu et al. 2015). Again, Cys-109 and Cys-526 were targeted by NO. In the third one, a search for S-nitrosylated proteins was carried out in *A. thaliana* cell cultures infected with the avirulent strain of *Pseudomonas syringae* DC3000 (Chaki et al. 2015). The authors identified a hundred of nuclear proteins including isoforms of CDC48. Highlighting the possibility that CDC48 could represent a general cellular redox target, several oxidants including H₂O₂ were also able to impair the activity of the drosophila CDC48 isoform (Noguchi et al. 2005). Here too, the authors identified Cys-522 (corresponding to Cys-526 in NtCDC48) as being the site of H₂O₂-induced oxidation of the protein. Further strengthening the importance of this residue in CDC48 regulation, a recent investigation demonstrated that in mammalian CDC48 orthologues, Cys-522 is covalently and specifically modified by the pharmacological VCP inhibitor NMS-859 (Magnaghi et al. 2013). Interestingly, this particular Cys residue is evolutionally conserved in multicellular organisms but not in unicellular ones (Noguchi et al. 2005). As a functional consequence, CDC48 isoforms from unicellular organisms such as the *Saccharomyces cerevisiae* or the archaeobacterium *Thermoplasma acidophilum*, in which Cys-526 is replaced by an Ala or a Thr residue, were poorly sensitive to oxidative stresses. More generally, according to several authors (Noguchi et al. 2005; Marino and Gladyshev 2012), the presence of Cys residues in several protein families of multicellular organisms versus their unicellular counterparts is under strict evolution. Thus, the percentage of Cys in

proteomes is substantially greater in complex eukaryotes than in archaeobacteria and eubacteria (2.2 % versus 0.5 %) (Go et al. 2015). Because this residue provides a considerable range of chemical reactivity and structural flexibility, its occurrence could confer an additional and advantageous level of regulation.

This discussion raises the question on the physiological incidence of CDC48 S-nitrosylation. As specified above, in animals and yeast, loss of function of VCP/CDC48 due to genetic manipulation or pharmacological treatments leads to the accumulation of poly-ubiquitinated proteins, activates UPR and also affects mitochondrial function, thus leading to apoptotic cell death (Meyer and Weihl 2014; Meyer et al. 2012). Thanks to the use of the VCP inhibitor NMS-859, a crucial role for Cys-522 in such processes has been demonstrated. Indeed, by establishing a covalent bond with Cys-522, this pharmacological compound was shown to induce an accumulation of poly-ubiquitinated proteins in human colon cancer cell lines that ultimately resulted in cell death (Magnaghi et al. 2013). Therefore, VCP and, more specifically, Cys-522 emerge as a druggable target in human therapy strategies. In regard to these studies, it is tempting to speculate that the inhibition of CDC48/VCP ATPase activity due to S-nitrosylation or oxidation could impact cellular proteostasis. This hypothesis is particularly relevant in the context of plant immunity. Indeed, an increasing body of evidence emphasises that the ubiquitin-26 proteasome system (UPS) is mobilised in plant cells undergoing defence responses and plays key functions such as the regulation of hormone signalling and HR (Marino et al. 2012; Trujillo and Shirasu 2010; Dielen et al. 2010). The importance of UPS in plant defence is also underlined by the findings that several of its components are manipulated by pathogens in order to suppress immunity (Alcaide-Loridan and Jupin 2012; Spallek et al. 2009). Furthermore, several virulence factors display ubiquitin ligase activity such as the bacterial effector AvrPtoB from *P. syringae* pv. *tomato* (*Pst*) which catalyses the ubiquitination and subsequent degradation of the host kinase Fen required for resistance in *A. thaliana* (Janjusevic et al. 2006; Banfield 2015). How CDC48 S-nitrosylation could enter the scene? At least two main scenarios are possible here:

First, the inhibition of CDC48 caused by S-nitrosylation induces the accumulation of ubiquitinated proteins. As widely reported in animals and yeast (see above), such accumulation could be part of mechanisms underlying cell death. However, a recent investigation analysing the role of NtCDC48 in tobacco cells elicited by cryptogin did not support this hypothesis (Rosnoblet et al. 2015). Indeed, only a few percent of the cellular NtCDC48 population appeared to undergo S-nitrosylation, minimising the impact of NO on the overall cellular NtCDC48 activity. In accordance with this statement, the NtCDC48-dependent protein quality control machinery was shown to be induced and functional in cryptogin-elicited tobacco cells. Notably, the search of NtCDC48 partners led to the identification of numerous client proteins related to primary and secondary metabolisms and to several components of UPS. Furthermore, a transgenic tobacco line overexpressing a functional NtCDC48 displayed a premature and exacerbated cell death, pointing out a role for the functional and not the inhibited

NtCDC48 as a positive regulator of HR. How NtCDC48 contribute to HR remains to be investigated. In other organisms such as drosophila (Higashiyama et al. 2002), CDC48 orthologues were reported to trigger cell death via an undefined pathway distinct from the known cell death pathway, suggesting that CDC48 is a cell death effector by itself.

Second, the members for the cellular NtCDC48 population undergoing S-nitrosylation could act as NO sensors. This hypothesis is based on the concept proposing that particular Cys residues such as Cys-526 evolved with the specific purpose of detecting and responding to NO or ROS (see above; Marino and Gladyshev 2012; Derakhshan et al. 2007). If so, for what purpose? One possibility is that NtCDC48 S-nitrosylation could lead to its inhibition and, subsequently, to a dysfunction of the process of ubiquitinated protein degradation. We assume that this event could constitute an alert signal promoting the upregulation of UPS (Fig. 11.1). Consistent with an involvement for NO as an inducer of UPS, in tobacco cell suspensions exposed to cryptogein, NO was shown to trigger the expression of the tobacco orthologues of the *A. thaliana* ubiquitin-ligases PUB26, RHC2A and DUF1 (Kulik et al. 2015). This idea remains to be verified.

Fig. 11.1 Model for the incidence of CDC48 S-nitrosylation in plant immunity. *Upper panel:* Functional CDC48 binds to a poly-ubiquitinated substrate protein (yellow) interacting with partners (green). CDC48 converts the energy of ATP hydrolysis to structurally remodel the substrate, thus segregating it from binding partners and allowing its degradation by the proteasome. *Lower panel:* The S-nitrosylation of CDC48 impairs its ATPase activity. To reduce the risk of toxicity due to the accumulation of ubiquitinated client proteins, S-nitrosylated CDC48 acts as a sensor of nitrosative stress and initiates a signalling cascade leading to the upregulation of genes encoding functional components of UPS including CDC48. This process contributes to the hypersensitive response through a mechanism that remains to be discovered. *HR* hypersensitive response, *Ub* ubiquitin, *UPS* ubiquitin-26 proteasome system

Collectively, these data shed light on the involvement of NO in the regulation of UPS and design CDC48 as a NO-regulated component. Further molecular and physiological analyses are still needed to provide some clues for the understanding of the incidence of CDC48 S-nitrosylation in plant immunity.

3.2 Histone Deacetylases of Type-2

HDACs are conserved enzymes catalysing the removing of acetyl groups from the side chain of Lysine (Lys) residues in histones as well as in other proteins such as transcription factors, DNA repair enzymes, nuclear import regulators or chaperone (Sengupta and Seto 2004). By controlling the acetylation state of the chromatin, both HDACs and histone acetyltransferases provide the enzymatic basis for transcriptional activation and repression (Legube and Trouche 2003). Plants share the reduced potassium dependency protein 3—HDAC1 (RPD3-HDAC1) superfamily and Silent information regulator 2 (Sir2) family of HDACs with other eukaryotes but also express a specific family named type-2 HDACs (HD2s) (Pandey et al. 2002). Plant HDACs contribute to various physiological processes including seed dormancy, leaf morphology, flowering, senescence, abscisic acid and salt stress signalling as well as defence (Grandperret et al. 2014). Analysis of the involvement of members of the HD2 family in plant immunity brings a new perspective on the function of these proteins. Specifically, upon treatment of tobacco cell suspensions with cryptogein, Bourque et al. (2011) showed that two isoforms of HD2 (NtHD2a and b) are rapidly phosphorylated in the nucleus and further downregulated both at the gene and protein levels. Furthermore, impairment of their expression led to an exacerbated and systemic HR in response to cryptogein but not in response to non-necrotising elicitors, indicating that these proteins negatively regulate the kinetic and intensity of cell death. Reinforcing these data, silencing of the HD2 isoform HDT701 in rice, caused an enhanced resistance to *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *Oryzae*. This process was accompanied by increased levels of histone H4 acetylation and transcription of defence-related genes (Ding et al. 2012). Therefore, HDT701 appeared to act as negative regulator of plant immunity in rice. Collectively, these examples highlight that epigenetic regulation due to changes in the acetylation levels of nuclear proteins plays important roles in the host defence.

Recent studies provided first arguments suggesting that HD2s might operate as positive regulators of NO synthesis in plant immunity. Indeed, impairment of the expression of the NtHD2a and b HD2 isoforms in tobacco cell suspensions strongly reduced the rate of NO production triggered by cryptogein (Kulik et al. 2015). This reduction was not specific to NO as the synthesis of ROS and, consequently, of ONOO⁻ also decreased substantially. This observation was expected as in cryptogein signalling NO and ROS modulate their respective productions/levels and interact by forming ONOO⁻. Interestingly, a similar observation was reported in mice macrophages activated by PAMPs such as lipopolysaccharides (LPS)

(Serrat et al. 2014). In these cells, the LPS-dependent *iNOS* expression and subsequent NO production were blocked by the HDAC inhibitor trichostatin A. Further analysis indicated that this inhibitor prevents the recruitment of the RNA polymerase II on the *iNOS* gene promoter while it facilitates the recruitment of the protein kinase CDK8 acting as a transcriptional repressor. How NtHD2a/b positively regulate NO production in tobacco cells undergoing an immune response is currently unknown. As reported in macrophages, NtHD2a/b deacetylase activities might be required for the transcriptional activation of the gene encoding the enzyme catalysing NO synthesis or controlling its regulation. Alternatively, via Lys deacetylation, NtHD2a/b could directly regulate the activity of the NO source, or of its regulators, at the post-translational level.

The occurrence of a functional link between NO and HD2s was further supported by the finding that members of these family in *A. thaliana* are putative targets for S-nitrosylation (Chaki et al. 2015). In this study, *A. thaliana* cell suspensions were first treated with a virulent or an avirulent strain of *Pst* DC3000. Next, the resulting nuclear-enriched fractions were exposed to GSNO before the identification of the resulting S-nitrosylated proteins. The identified HD2s, namely, AtHD2B and AtHD2C (also named AtHDT2 and AtHDT3), underwent S-nitrosylation in response to the virulent or the avirulent strain of *Pst* DC3000, respectively. Complementary experiments indicated that all the isoforms of *A. thaliana* (AtHD2A, B, C and D) could be S-nitrosylated in vitro with varying efficiency, depending on the protein. Here too, there is a parallel with mechanisms encountered in animal cells. In human neurons, the HDAC2 isoform belonging to the RPD3-HDA1 superfamily was shown to be S-nitrosylated in response to the brain-derived neurotrophic factor (BDNF) (Nott et al. 2008). The S-nitrosylation occurred at two Cys residues (Cys-262 and Cys-274) which are not located in the zinc-binding catalytic domain. This post-translational modification did not affect the enzyme deacetylase activity but promoted its release from chromatin and, therefore, histone acetylation at specific promoter regions and transcription of genes related to neuronal development. Regulation of deacetylase function by NO in animals is not restricted to HDAC2 as sirtuin-1 (SIRT1), which belongs to the Sir2 family, and is also prone to S-nitrosylation (Kornberg et al. 2010). In situ, S-nitrosylation of SIRT1 inhibits its activity and its downstream effects on transcription. Importantly, the mechanism underlying SIRT1 and HDAC2 S-nitrosylation in nucleus involved glyceraldehyde-3-phosphate dehydrogenase (GAPDH) acting as a nitrosylase. In plant, GAPDH was also found to be regulated through S-nitrosylation and to translocate into the nucleus (Lindermayr et al. 2005; Wawer et al. 2010; Zaffagnini et al. 2013; Chaki et al. 2014). Whether plant GAPDH acts as a nitrosylase has not been reported so far.

The process by which plant HD2s become S-nitrosylated as well as the incidence of this process on their activities and cell functions are unknown. Notably, the residues found to be S-nitrosylated in mammalian HDAC2 and SIRT1 are not conserved in plant HD2s. An in silico analysis on the *A. thaliana* AtHD2C and AtHD2B isoforms, found to be S-nitrosylated in cell suspensions challenged by *Pst* DC3000 strains (Chaki et al. 2015), provides a first insight:

- The AtHD2C (AtHDT3, At5G03740) isoform contains only two Cys residues (Cys-269 and Cys-272) in the C-terminal part of the protein which, together with two histidine (His) residues (His-285 and His-190), form a zinc finger domain of C2H2-type. This domain might be involved in protein–protein interaction (Grandperret et al. 2014). Thus, the S-nitrosylation of one or both of these Cys residues could affect zinc binding to the protein and its ability to interact with partners.
- The AtHD2B (AtHDT2, At5G22650) isoform does not contain a zinc finger domain and possesses only one Cys residue (Cys-33). This latter is located in the deacetylase domain, closed to the conserved His-25 residue, thought to be required for deacetylase activity (Zhou et al. 2004). It is therefore plausible that Cys-33 S-nitrosylation could modulate, for instance, through steric hindrance, the deacetylase activity of AtHD2B and thus impact transcriptional regulation of defence genes.

Clearly, this *in silico* analysis indicates that NO does not target the same Cys residues on AtHD2B and AtHD2C. As a consequence, the cellular incidence of NO on these proteins might differ. As HD2s positively regulate NO synthesis (Kulik et al. 2015), an original process would be that their S-nitrosylation inhibits their activity and, subsequently, downregulates NO synthesis once the defence responses are initiated. This feedback mechanism could provide an additional level of regulation of NO synthesis (Fig. 11.2). More generally, as noticed by Mengel et al. (2013), the possibility that HDACs belonging to the RPD3-HDA1 superfamily and Sir2 family also constitutes NO targets in plants should be also considered. The recent identification of AtHDA5 (At5G61060), belonging to the RPD3-HDA1 superfamily, in a large-scale proteomic search for S-nitrosylated proteins in *A. thaliana* further supports this assumption (Hu et al. 2015).

In summary, data from these studies highlight that members of the HD2 family of HDACs might constitute key components of NO signalling in plant immunity by acting as both regulators of NO synthesis and putative mediators of its effects on gene expression. NO could therefore constitute a regulator of epigenetic changes associated with plant immunity.

3.3 Calmodulin

The first hint that NO could potentially modulate Ca^{2+} signalling in plant immunity emerged from the pioneer study of Durner et al. (1998) demonstrating that cADPR could mediate NO effects on defence gene expression. In animals, cADPR is a major Ca^{2+} -mobilising second messenger which activates the Ca^{2+} channels ryanodine receptors (RYRs) and acts downstream of cGMP in several cellular processes such as fertilisation (Berridge 1993; Willmott et al. 1996). The role of NO in controlling Ca^{2+} homeostasis in plant was investigated more thoroughly in the following years. This led to the findings that NO is a key component of the

Fig. 11.2 Interplays between NO and HD2s in plant immunity.

(1) Induction phase: NO and ROS modulate their respective productions/levels. By catalysing deacetylation of protein substrates, HD2s contribute to the production of NO and/or ROS. This process is modulated at the transcriptional level or, alternatively, could involve upstream actors which activities are positively modulated by deacetylation. (2) Feedback phase: The S-nitrosylation of HD2s impairs their deacetylase activities and, consequently, negatively regulates NO and/or ROS production. Ac acetyl group

signalling cascades leading to increases in the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) in cells undergoing an immune response as well as in guard cells exposed to abscisic acid (Courtois et al. 2008; Garcia-Mata and Lamattina 2003). Pharmacological and electrophysiological studies provided evidences that the NO-dependent enrichment in $[\text{Ca}^{2+}]_{\text{cyt}}$ involves the activation of plasma membrane and/or intracellular Ca^{2+} -permeable channels sensitive to RYR inhibitors. Reinforcing the concept that NO and Ca^{2+} work together, NO appears as a step in the Ca^{2+} signalling cascades mobilised in plant immunity. As a matter of fact, several studies underlined that the production of NO observed in cells exposed to PAMPs and pathogens are under the control of upstream Ca^{2+} fluxes and require CaMs (Lamotte et al. 2004; Rasul et al. 2012; Ma et al. 2008). In *A. thaliana*, the CaM-like protein (CML) CML24, the cyclic nucleotide-gated Ca^{2+} -permeable channel CNGC2 and the glutamate receptor (GLR) GLR3.3 have been proposed to be critical mediators of this process (Manzoor et al. 2013; Ali et al. 2007; Rasul et al. 2012; Ma et al. 2013). Furthermore, once produced NO was shown to upregulate the expression of genes encoding proteins related to Ca^{2+} signalling including CML, GLRs and Ca^{2+} -dependent protein kinases (Jeandroz et al. 2013). Taken together, these investigations point out the existence of complex interplays between NO and Ca^{2+} signalling in plant immunity.

The molecular mechanism by which NO modulates Ca^{2+} signalling is poorly known. The second messengers cADPR, cGMP as well as changes in the plasma

membrane electrical potential might account for the effects of NO on Ca^{2+} fluxes, although their role remains unclear (Garcia-Mata et al. 2003; Lamotte et al. 2004, 2006). The contribution of protein kinases has also been proposed. This assumption was based on pharmacological studies showing that the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ triggered by NO donors in distinct cell types is reduced by protein kinase inhibitors (Lamotte et al. 2006; Sokolovski et al. 2005). So far, the identity of these protein kinases has not been reported, although the putative involvement of members of the SnRK2 family has been suggested (Courtois et al. 2008). A clearer picture has recently emerged with the finding that plant CaMs could be regulated through S-nitrosylation. A tobacco CaM isoform was indeed found in the screening for S-nitrosylated proteins in tobacco cell suspensions elicited by cryptogein (Astier et al. 2012a). Tobacco contains 13 CaM genes encoding three distinct protein isoforms (named type I to III) (Yamakawa et al. 2001). The peptide identified in the mass spectrometry analysis by Astier et al. (2012a) is conserved between the type I and type II NtCaM and corresponds to the first EF-hand Ca^{2+} -binding motif. It contains the sole Cys residue (Cys-27) of the protein. An *in silico* structural analysis further indicated that Cys-27 may participate in Ca^{2+} coordination through its carbonyl oxygen and displays typical features of residues regulated through S-nitrosylation (Jeandroz et al. 2013). The propensity of Cys-27 to be S-nitrosylated was confirmed *in vitro* (O. Lamotte, personal communication). Importantly, this particular Cys residue is conserved in the first EF hand of plant CaMs while it is rare in non-plant CaMs (McCormack et al. 2005). It is therefore not surprising that CaMs have never been reported as NO targets in animals. Instead, these organisms express CaM-dependent protein kinases in which Cys residues of the protein kinase partner are subjected to S-nitrosylation (Coultrap and Bayer 2014; Erickson et al. 2015).

The structural as well as the physiological incidence of type I/II NtCaMs S-nitrosylation are currently unknown. Jeandroz et al. (2013) hypothesised that the S-nitrosylation of Cys-27 might impact their interactions with partners and/or the binding properties of Ca^{2+} to the EF-1 motif. Investigating these processes is highly attractive as they could lead to the discovery of unprecedented redox-based mechanisms explaining how NO modulate Ca^{2+} signalling in plants.

In sum, the results we have summarised here document the complexity of the interactions between Ca^{2+} and NO signalling in plant immunity. NO production requires upstream Ca^{2+} -dependent events and is active at multiple sites in the regulation of Ca^{2+} signalling. Understanding of how NO contributes to this regulation represents a major challenge.

4 Conclusion

With the identification of new S-nitrosylated proteins such as CDC48, HDACs and CaM, our appreciation of the diverse roles of NO in plant immunity continues to grow. Specifically, data summarised here designate NO as a putative modulator of

epigenetic changes and of targeting of ubiquitinated proteins for degradation, thus leading to promising areas of research. Furthermore, they confirm the occurrence of tight interplays between NO and Ca²⁺ signalling. Clearly, these concepts are nascent, and work will be definitively required to study these issues in more detail, both from the molecular and pathological points of view. In particular, it is essential to confirm the functionality, as physiological NO sensors, of the S-nitrosylated Cys residues of CDC48, HDACs and CaM. Furthermore, as these proteins operate through protein–protein interactions, investigating the incidence of their S-nitrosylation on these interactions could be of help in the next future to better understand the role of NO in plant immunity.

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