

# Cross Talk between Reactive Nitrogen and Oxygen Species during the Hypersensitive Disease Resistance Response<sup>1</sup>

Federica Zaninotto, Sylvain La Camera, Annalisa Polverari, and Massimo Delledonne\*

Dipartimento Scientifico e Tecnologico, Università degli Studi di Verona, 37134 Verona, Italy

In recent years, nitric oxide (NO) has been identified as a fundamental molecule that interplays with reactive oxygen species (ROS) in a variety of ways, either as a crucial partner in determining cell fate or in signaling in response to a number of physiological and stress-related conditions. The best characterized relationship between NO with ROS refers to its role in plant defense against pathogen attack, in particular in the establishment of the hypersensitive reaction (HR; Wendehenne et al., 2004; Delledonne, 2005). HR is a form of programmed cell death that contributes to plant resistance by restricting the invading pathogen at the infection site and shows some regulatory and mechanistic features characteristic of apoptosis in animal cells, like membrane dysfunction, vacuolization of the cytoplasm, chromatin condensation, and endonucleolytic cleavage of DNA (Greenberg and Yao, 2004). Activation of the HR triggers a number of rapid cellular responses, including perturbations of ion fluxes and changes in the pattern of protein phosphorylation (Lamb and Dixon, 1997), which precede the accumulation of ROS and NO. The oxidative and nitrosative bursts are then followed by a signal cascade that mediates transcriptional activation of defense genes and finally the local and systemic expression of antimicrobial proteins, leading to the establishment of systemic acquired resistance (McDowell and Dangl, 2000).

The oxidative burst consists of a biphasic production of apoplastic ROS at the site of attempted invasion. Pharmacological, molecular, and genetic studies strongly support the idea that the primary source of ROS is an O<sub>2</sub><sup>-</sup> generating membrane-bound NADPH oxidase (Lamb and Dixon, 1997). ROS have several direct and indirect roles in plant defense: they are directly toxic to invading microorganisms, contribute to the strengthening of cell walls by cross-linking cell wall proteins, regulate the synthesis of new signals such as salicylic acid, lead to enzyme activation and gene expression

targeted toward resistance by alteration of redox status, provoke damage to DNA and proteins, and have long been considered crucial in determining cell fate during the HR (Grant and Loake, 2000). Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) has been shown to trigger cell death following either exogenous administration or genetic augmentation in transgenic plants lowered in H<sub>2</sub>O<sub>2</sub> scavenging capacity (Neill et al., 2002). However, ROS alone trigger a cell death characterized by strong oxidative cell damage that has specific morphological and biochemical features distinct from those observed in elicitor or pathogen-induced hypersensitivity (Montillet et al., 2005).

Starting from the fundamental role in the immune response that NO plays in animals in cooperation with ROS, recent studies have focused on the possible function of NO during the HR (Delledonne et al., 1998). Plants can produce NO through either two main enzymatic systems, namely NO synthase and nitrate reductase, or by several nonenzymatic reactions such as liberation of NO from nitrite under different conditions (Crawford, 2006). During the HR, a peak of NO is produced concomitant with the oxidative burst and with the increase of NO-synthase activity (Romero-Puertas et al., 2004). However, the source(s) of NO during this resistance response has yet to be unequivocally demonstrated.

Because of its chemistry and reactivity, NO can have a number of important direct functions in plant defense in parallel with ROS. It can be directly cytotoxic to microbes, affect gene expression by altering the redox status of the cell, regulate protein function through direct posttranslational modifications, and provoke damage to DNA and proteins (Stamler et al., 2001). Moreover, NO can exert important indirect signaling functions through the activation of the cGMP-dependent pathway, which mediates the expression of defense genes such as Phe ammonia lyase and chalcone synthase (Durner et al., 1998). Most importantly, a large body of pharmacological and genetic evidence has demonstrated that NO is essential, together with ROS, for triggering cell death during the HR (Romero-Puertas et al., 2004).

## NO-ROS COOPERATION DURING THE HR

Whereas in animals unregulated NO production is always lethal, NO alone does not cause cell death in

<sup>1</sup> This work was supported by the European Molecular Biology Organization Young Investigator Program (grant to M.D.).

\* Corresponding author; e-mail [massimo.delledonne@univr.it](mailto:massimo.delledonne@univr.it); fax 39-045-802-7929.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors ([www.plantphysiol.org](http://www.plantphysiol.org)) is: Massimo Delledonne ([massimo.delledonne@univr.it](mailto:massimo.delledonne@univr.it)).

[www.plantphysiol.org/cgi/doi/10.1104/pp.106.078857](http://www.plantphysiol.org/cgi/doi/10.1104/pp.106.078857).

plants. Death of host cells during the HR results from the simultaneous, balanced production of NO and ROS (Delledonne et al., 2001), although the molecular mechanism of this interplay is not yet understood. In animal models, the cytotoxic effects of NO and ROS derive from the diffusion-limited reaction of NO with  $O_2^-$  to form the peroxynitrite anion  $ONOO^-$ . Peroxynitrite causes oxidative damage and protein modifications such as Tyr nitration and oxidation of thiol residues (Radi, 2004). In animals,  $ONOO^-$  causes apoptotic or necrotic cell death, depending on its concentration (Bonfoco et al., 1995). Conversely, in plants  $ONOO^-$  does not appear to be an essential mediator of NO-ROS-induced cell death, which is triggered by the interaction of NO with  $H_2O_2$  (Delledonne et al., 2001). Genetic studies provide additional support for this model, originally based on the extensive use of pharmacology: *Arabidopsis* (*Arabidopsis thaliana*) plants lowered in thylakoidal ascorbate peroxidase show enhanced symptoms of NO-induced cell death compared to wild-type plants (Tarantino et al., 2005) and catalase-deficient tobacco (*Nicotiana tabacum*) plants, which accumulate elevated  $H_2O_2$  levels under moderate light intensity, show a dramatically augmented cell death in comparison to wild-type plants when treated with NO (Zago et al., 2006).

How the cell death program is triggered by the appropriate, balanced concentration of NO and ROS remains unknown. In animals, release of cytochrome C from mitochondria during apoptosis activates a caspase-signaling cascade that selectively cleaves and activates vital substrates in the cell, including the nucleases responsible for DNA degradation (Ueda et al., 2002). In plants, both  $H_2O_2$  and NO cause the release of cytochrome C from mitochondria and a caspase-like signaling cascade has also been identified during HR cell death (Mur et al., 2006). It is known that NO and  $H_2O_2$  can also chemically react to produce singlet oxygen or hydroxyl radicals and that these species can cause cell death. However, overexpression of a Cys protease inhibitor in *Arabidopsis* cell suspensions and tobacco plants suppresses NO-ROS-mediated cell death, providing evidence that cells die through the stimulation of an active process involving Cys proteases (Belenghi et al., 2003).

## REGULATION OF ROS AND NO LEVELS IN PLANTS

The interaction of NO with ROS is still far from being clearly elucidated; the high reactivity of these molecules gives rise to a number of possible reactions that appear to be very important in fine tuning the reciprocal concentrations, and this regulation may vary among different model systems.

The growth and reproduction of plant cells require a balance between the generation of reactive molecules and the capacity of antioxidant systems to eliminate them (Mittler, 2002). Together with the antioxidants ascorbic acid and glutathione, major ROS-scavenging

enzymes superoxide dismutase, ascorbate peroxidase, catalase, glutathione peroxidase, and peroxiredoxin provide cells with a highly efficient machinery for detoxifying  $O_2^-$  and  $H_2O_2$  (Mittler et al., 2004). Different enzymatic routes also keep NO levels under control and contribute to its detoxification. Nonsymbiotic hemoglobins metabolize NO and *S*-nitrosoglutathione (GSNO; Perazzolli et al., 2004), GSNO reductase controls intracellular levels of *S*-nitrosothiols and GSNO (Feechan et al., 2005) and peroxiredoxins are likely involved in reduction of peroxynitrite (Rhee et al., 2005).

It is interesting to note that ROS and NO exert reciprocal control on each other in several ways. A variety of NO donors have been used to demonstrate that NO can regulate ROS levels by inhibiting the activities of antioxidant enzymes (Clark et al., 2000). However, *Arabidopsis* plants unable to express At-NOS1, the only NO synthase so far identified in plants, show higher levels of ROS, suggesting that in certain conditions NO can also act as an antioxidant, reducing cell damage and senescence (Guo and Crawford, 2005). Several other lines of study have shown that the protective effect of NO against abiotic stresses is closely related to the NO-mediated reduction of ROS in plants (Lamattina et al., 2003). On the other hand, the relative rates of production of  $O_2^-$  and  $H_2O_2$  are critical in modulating the reactivity of NO. A model has been proposed in which if the NO- $O_2^-$  balance is in favor of  $O_2^-$ , NO is scavenged before it can react with  $H_2O_2$ . If the balance is in favor of NO,  $O_2^-$  is scavenged before it is dismutated to  $H_2O_2$ . Whereas the cooperation of NO and  $H_2O_2$  leads to the death of the cell, reciprocal scavenging of NO and  $O_2^-$  leads to the formation of  $ONOO^-$ , which does not appear to be particularly toxic in plants (Delledonne et al., 2001).

## MODULATION OF GENE EXPRESSION

ROS and NO induce significant transcriptional changes and can have complementary functions in regulating gene expression. For example, NO can induce the expression of Phe ammonia lyase and chalcone synthase independently of ROS, while induction of other defense-related genes by NO, such as glutathione-*S*-transferase and the pathogenesis-related protein 1 (PR-1), was shown to depend on  $H_2O_2$  and salicylic acid, respectively (Grun et al., 2006). Large-scale cDNA-AFLP and microarray analyses on cellular responses to  $H_2O_2$  in tobacco and *Arabidopsis* plants revealed up-regulation of many genes involved in the HR, oxidative stress sensing, and cell death-related signal transduction (Gechev and Hille, 2005). A similar, wide modulation of gene expression has also been observed with NO, which has been found to modulate the expression of transcription factors, receptors, and several pathogen-induced genes in addition to genes known to respond to oxidative stress, such as glutathione peroxidase and glutathione-*S*-transferase (Grun et al., 2006). The combined action of NO and

H<sub>2</sub>O<sub>2</sub> on gene expression was recently investigated in a collaborative effort between our group and that of Frank Van Breusegem by monitoring transcriptional changes during the early phases of cell death in catalase-deficient tobacco plants treated with NO and then exposed to otherwise ineffective moderate high light (Zago et al., 2006). The experimental conditions allowed the analysis of gene expression during NO-ROS-mediated cell death, identifying genes specifically induced by NO or by H<sub>2</sub>O<sub>2</sub>, as well as the set of genes regulated by both. Surprisingly, coregulated genes were the most dominant cluster, suggesting that the signal transduction pathways initiated by these two molecules consistently overlap. These findings reflect the complex interconnections between the two most important cell death signal molecules and the extensive cross talk that has been outlined in previous paragraphs.

## POSTTRANSLATIONAL MODIFICATIONS

Even though characterization of the signal transduction by ROS and NO is only at its initial stages, it is conceivable that these reactive molecules act as signals in many biological processes as a direct consequence of chemical reactions between proteins and ROS, NO, or their reaction products.

While the complete pathways induced by H<sub>2</sub>O<sub>2</sub> or NO still await identification, some evidence on their interaction with target proteins is emerging. Using a proteomic approach, Hancock and colleagues identified several proteins that might be potential targets of H<sub>2</sub>O<sub>2</sub> in *Arabidopsis* (Hancock et al., 2005), the most prominent of which is glyceraldehyde 3-phosphate dehydrogenase (cGAPDH) that is reversibly inhibited by H<sub>2</sub>O<sub>2</sub>. Besides its enzymatic activity in glycolysis, the authors suggest that GAPDH could have an additional role in mediating ROS signaling in plants as a direct target of H<sub>2</sub>O<sub>2</sub>. GAPDH is also a target of NO-mediated S-nitrosylation (see below) and is inhibited by NO (Lindermayr et al., 2005). Another direct target of H<sub>2</sub>O<sub>2</sub> action is Met adenosyltransferase, which in mammals is inactivated by H<sub>2</sub>O<sub>2</sub> through reversible and covalent oxidation of a Cys residue. Interestingly, the same Cys residue is also a target for NO, which similarly causes enzyme inactivation (Hancock et al., 2005).

Also, key components of the signaling cascade leading to the HR are known to be affected by ROS and NO reactivity. Among these are mitogen-activated protein (MAP) kinases and phosphatases (Neill et al., 2002). ROS have been shown to influence the transcription and the activity of a number of important MAP kinases, in particular AtMPK3, AtMPK6, and OX11, which acts upstream of both and that is necessary for ROS-mediated signaling (Rentel et al., 2004). MAP kinase activation by ROS induces the expression of several stress-associated genes, indicating a direct connection with the stress response, further complicated by the fact that ROS production can also be

controlled by a MAP kinase cascade (Ren et al., 2002). The MAPK signaling pathways are also potential targets for NO, which can influence the activity of at least some MAP kinases, including the salicylic acid-induced protein kinase (Neill et al., 2002). Thus, modulation of a central MAPK cascade may converge both H<sub>2</sub>O<sub>2</sub> and NO signaling pathways activated in response to various stresses (Neill et al., 2002).

Major NO-dependent protein modifications currently investigated in plants are S-nitrosylation and nitration.

### S-Nitrosylation

S-nitrosylation, the formation of S-nitrosothiols by covalent addition to Cys residues of a NO moiety (formally as NO<sup>+</sup>), has been shown to regulate the function of a broad spectrum of proteins in intact cells by switching their activities on/off (Stamler et al., 2001). Trans-nitrosylation refers to the direct transfer of an NO group from another S-nitrosylated protein or from low-molecular-weight nitrosothiols such as GSNO, which has an important role in plant resistance that may be distinct from that of NO. Whereas a reduction in NO accumulation leads to pathogen susceptibility, a decrease in the concentration of S-nitrosothiols promotes protection against microbial infection (Feechan et al., 2005).

Because of their reactivity with intracellular reducing agents such as ascorbic acid or glutathione, the half-life of S-nitrosothiols is tightly regulated by the redox state of the cell and can be very brief, making protein S-nitrosylation a highly sensitive regulation mechanism (Mannick and Schonhoff, 2004). S-nitrosylation may therefore play important regulatory roles similar to that of protein phosphorylation: both mechanisms are highly specific, rapidly reversible, and allow a prompt modification of protein functions. Many examples of protein S-nitrosylation and consequent modifications in activity have been reported in animal systems. Regulation of procaspase-3 activation is just one example of how S-nitrosylation can regulate fundamental processes such as apoptosis (Mannick and Schonhoff, 2004). The identification of S-nitrosylation substrates in plants and the elucidation of its function as a signaling mechanism are just beginning, and experimental evidence of regulation through S-nitrosylation is currently available for three proteins: glyceraldehyde 3-P dehydrogenase (Lindermayr et al., 2005), Met adenosyltransferase (Lindermayr et al., 2006), and AHB1, a nonsymbiotic hemoglobin that scavenges NO through the formation of S-nitrosohemoglobin (Perazzolli et al., 2004). The identification of many others is under way and proteomic analysis has identified more than 100 proteins in *Arabidopsis* that can be potentially S-nitrosylated (Lindermayr et al., 2005).

### Nitration

Nitration is the process by which a nitrite group is added to the *ortho*-position of Tyr residues forming 3-nitrotyrosine. Tyr nitration is mediated by reactive

nitrogen species such as ONOO<sup>-</sup> and nitrogen dioxide (NO<sub>2</sub>), formed as secondary products of NO metabolism in the presence of oxidants including O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, and transition metal centers (Radi, 2004). Because ROS and NO formation occurs under stress situations as well as under normal growth conditions, it can be hypothesized that ONOO<sup>-</sup> is continuously formed in healthy cells (Romero-Puertas et al., 2004) and, consequently, protein nitration may be physiologically relevant in plants.

The nitration of Tyr residues may alter protein conformation and structure, catalytic activity, and/or susceptibility to protease digestion (Souza et al., 2000). Proteins nitrated under pathological conditions in humans include low-density lipoprotein, Tyr hydroxylase, Mn-superoxide dismutase, Gln synthetase, and prostacyclin synthetase (Radi, 2004). Furthermore, nitration of Tyr residues may interfere with signaling processes associated with protein Tyr phosphorylation. In vitro studies have shown that nitration of a single Tyr residue in purified CDC2, a cell cycle kinase, prevents its phosphorylation on Tyr (Kong et al., 1996). Gow et al. (1996) extended these observations, showing that exposure of bovine pulmonary artery endothelial cells to ONOO<sup>-</sup> decreased the levels of Tyr-phosphorylated proteins and increased nitrotyrosine-containing protein levels. Tyr nitration may hinder the protein from performing the task of the phosphorylated form. On the other hand, it may mimic the structural changes imposed by phosphorylation and therefore imitate the consequences of phosphorylation (Monteiro, 2002).

Recent work indicates that protein nitration operates in plants: increased protein Tyr nitration has been observed in antisense nitrite reductase tobacco accumulating higher nitrate and NO levels (Morot-Gaudry-Talarmin et al., 2002), and following administration of ONOO<sup>-</sup> in vitro (Delledonne et al., 2001). A diverse group of about 20 genes encoding putative Tyr phosphatases has been identified in the Arabidopsis genome, implying that Tyr phosphorylation and dephosphorylation may serve important functions in plant biology (Luan, 2003). We are in the process of identifying the major classes of proteins that can be nitrated during the HR using a proteomic approach.

## CONCLUSION

NO and ROS have a number of complementary, synergistic, and overlapping functions in plants. This balance is achieved in a highly complicated network of reciprocal regulation, based on oxidative-nitrosative direct modification of enzymes involved in reciprocal control of their levels. The same mechanisms also affect important components of the signal transduction cascade leading to disease resistance, such as kinases and phosphatases, and expand its functions to the modulation of transcription factor activity, and thus, of gene expression. The global picture of ROS-NO

interactions is far from being complete, but it already has been revealed as a fascinating cross talk of mechanisms able to fine tune resistance responses and other plant reactions to environmental stimuli, as well as important developmental aspects in the life of the plant.

## ACKNOWLEDGMENTS

We apologize for not being able to cite many relevant original papers, replaced by reviews, due to space limitation.

Received February 7, 2006; revised February 7, 2006; accepted March 2, 2006; published June 12, 2006.

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