"Nitric Oxide in Plants - A Molecule with Dual Roles"

Chapter 4. Nitric oxide and ROS interaction for stress signaling

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ABSTRACT

Both reactive nitrogen species (RNS) and reactive oxygen species (ROS), among which nitric oxide (NO), can behave as key signals in plants able to regulate several aspects of plant metabolism, among which development, growth, reactivity and tolerance to environmental stress, the transport of solutes and apoptotic processes. After its formation in plant tissues, nitric oxide will bring about signaling together with several other reactive molecules having a similar chemical behavior, among which ROS, H_2S, glutathione, and other antioxidants, depending on the redox state of the reaction milieu. By reacting with such reaction partners, nitric oxide will form new molecules prone to behave as signals, among which peroxynitrite and nitrosothiols, giving rise to chemical competitions which will set the final sign and extent of the signaling pathway. In plants, responses to environmental stresses (biotic or abiotic) triggered by ROS and NO may give raise to general outcomes, such as an increase in the antioxidant status, or may consist of stress-specific adaptations,

according to the stress type, and require the crosstalk with other signaling molecular entities, among which protein kinases, phytohormones, and second messengers such as calcium. The activity of ROS and NO *in vivo* often stems from their ability to bring about proteins modifications at the post-translational level, especially through *S*-glutathionylation and *S*-nitrosylation respectively. Such changes will affect proteins in their activity, stability, cellular localization as well as reaction with other molecules, influencing the dynamics of the entire cell and helping in maintaining homeostasis. Despite the recent progress in understanding the signaling actions of reactive oxygen species and of nitric oxide, several open questions remain, so that further research is required, especially as far as their molecular crosstalk is concerned.

KEYWORDS:

Reactive nitrogen species; nitric oxide; reactive oxygen species; hydrogen sulfide; nitrosative stress; oxidative stress; antioxidants; redox signaling; crosstalk in signaling; stress responses; environmental stress tolerance; S-glutathionylation; S-nitrosation; S-nitrosylation; S-nitrosothiols.

INTRODUCTION

Plants' interactions with the environment involve the participation and reciprocal crosstalk among several molecular routes. Among the most common plant response to environmental challenges there is the deploying of reactive nitrogen species (RNS) and of reactive oxygen species (ROS) and, which are important in signaling and, by activating signaling chains, regulate several different developmental processes, through both transcriptional gene activation and influencing enzyme activity at the post-transcriptional level (Farnese et Al. 2016; Patel et Al. 2019).

Reactive oxygen species derive from the univalent reduction of molecular oxygen. They comprise the superoxide radical anion ($^{\cdot}O_2^-$), peroxide of hydrogen (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$). Reactive oxygen species have distinct effects on biological systems, having in common an elevated chemical reactivity and a much reduced half-life: the hydroxyl radical is amongst the most chemically reactive and indiscriminate species known, whereas $^{\cdot}O_2^-$ and hydrogen peroxide have a more selective and specific chemical reactivity (del Rio, 2015).

Besides ROS, the H_2S and its derivatives, denoted as reactive sulfur species (RSS) is an important signaling molecule alike, coming into play not only in plants' responses to environmental stress. However, either alone or together with NO, H2S regulates the main events even during the normal growth and development, but also during the plant growth developmental program,

including photosynthesis, seed germination, root development, stomatal behaviour and fruit ripening (Chen et Al. 2015a, 2015b; Mukherjee and Corpas 2020).

The term reactive nitrogen species describes both short-lived free radicals and non-radical species, which are able to either oxidize or reduce appropriate reaction partners or undergo transformation into radicals. Apart from nitric oxide, and mainly derived from it, other reactive nitrogen species encompass: *S*-nitrosothiols (SNOs), peroxynitrite (ONOO−), dinitrosyl–iron complexes, and higher oxides of nitrogen (NOx; Umbreen et Al. 2018). Nitric oxide is a gaseous, small, readily diffusible molecule reacting with several different compounds, including other radicals, such as alkyl- and lipid peroxyl radicals. It is also able to behave as an antioxidant, by interrupting spontaneous, self-fueling radical chain reactions leading to the peroxidation of lipids, so acting as a defensive agent against the cytotoxic effects caused by ROS in plants (Correa-Aragunde et Al., 2015; Arora et Al. 2016).

Because they can impair structures and functions in the plant cell whenever redox homoeostasis is overcome, ROS and RNS generation was regarded at first only as an undesirable and unavoidable consequence of life in the presence of O_2 (Demidchik, 2015; Lushchak, 2015). Further studies in the last decades, however, made it clear that these molecules are also mostly important as signal transducers in various plant processes, providing they are kept under control by the antioxidant machinery of the aerobic cell. (del Rio 2015; Farnese et Al. 2016).

ROS and nitric oxide, either *per se* or in combination, and ROS are important players in many different processes of the plant cell, such as reaction to physical/chemical stress and plant immunity (Joudoi et Al., 2013; Montilla-Bascon et Al. 2017; Sahay and Gupta 2017; Begara-Morales *et Al.*, 2018; Hasanuzzaman et Al. 2018; Sami et Al. 2018; Sun et Al. 2018; Corpas et Al. 2019; Kaya et Al. 2019; Kushwaha et Al. 2019; Liu et Al. 2019; Munawar et Al. 2019; Saddhe et Al. 2019; Sharma et Al. 2019; Jedelska et Al. 2020), symbiotic associations (Fukudome et Al. 2016; Hichri et Al. 2016; Martinez-Medina et Al. 2019), promotion of high microbial diversity in the rhizospheric environment (Ma et Al. 2020), organogenesis, ion homoeostasis, hormonal regulation, germination, flowering and fruit ripening/long term storage (Freschi 2013; Mur et Al. 2013b; Parí et Al. 2013; Zhou and Zhang 2014; Sanz et Al. 2015; Astier *et Al.* (2018); Corpas *et Al.* (2018; Moni et Al. 2018; Del Castello et Al. 2019; Kolbert et Al. 2019; Steelheart et Al. 2019; Raya-Gonzalez et Al.2019; Jafari and Daneshvar 2020; Khan et Al. 2020; Ren et Al. 2020).

It is mostly the environmental context that sets the route and the extent of the changes triggered by RNS and ROS. Because of the high complexity of these processes, much remains to do to unravel the signaling mechanisms triggered by ROS and NO, either per se or in combination and their interaction with the plethora of other signaling molecules in plants, as affected by the overall

balance among pro-oxidants and antioxidants. Many studies in the last years have been focused on these issues, whose interest for plant scientists is still steadily growing. In the present chapter, the most recent of such studies are sketchily summarized, concentrating on the adaptive responses plants to those environmental changes able to affect the generation ROS and RNS and their reciprocal interactions.

PLANT METABOLISM OF ROS AND NITRIC OXIDE: A SUMMARY

In plants, many sources of environmental stress are able to rapidly alter the cell redox balance, both by increasing the production of ROS and nitric oxide and by inactivating the enzymes involved in antioxidant defense (del Rio, 2015). This increases the formation of reactive species, which is needed to activate defense/compensation responses, leading to the upregulation of antioxidant systems (Shi et Al., 2014) or to the launching of a cell death program (PCD), which is important for adaptation to oxidative stress as well as for deploying resistance mechanisms (Yun et Al., 2011).

Reactive oxygen species and nitric oxide syntheses take place at different locations in the plant cell, and their localization in time and space is important for signals generation and their intracellular spread (Groß et Al., 2013). Depending on the compartment of origin, reactive oxygen species and nitric have distinct roles, deriving from local interactions with specific reaction partners in each organelle (Shapiguzov et Al., 2012; Mur et Al., 2013a); in the presence of hypoxia, for example, an important role is assigned to NO generated at the plasma membrane level, whereas stress due to heavy metals evokes NO generation in the chloroplasts and mitochondria (Kumar and Trivedi, 2016).

Reactions and dynamics governing ROS production have been investigated in plant cells in response to a variety of environmental circumstances and developmental processes (see reviews by Krasensky-Wrzaczek and Kangasjärvi 2018, Ozgur *et Al.* 2018, and Turkan *et Al.* (2018). The plasmalemma has been found to be among the most important sites for ROS production, because of the activity of proteins belonging the the respiratory burst oxidase homolog family (RBOH), commonly denoted as NADPH oxidases. These enzymes are integral to the membrane and catalyze the transfer electrons taken from NADPH in the cytoplasm to molecular oxygen in the apoplast, forming the superoxide radical anion and causing extracellular ROS accumulation (Das and Roychoudhury, 2014). Many different stresses activate the transcription and activity of NADPH oxidases, producing a so called oxidative burst, i.e. localized ROS overproduction (Jajic et Al., 2015; Wang X. et Al., 2015). Other cell wall-associated oxidases and peroxidases also participate in apoplastic ROS overproduction, even if their participation in the stress response is not fully understood (Das and Roychoudhury, 2014). Apart from triggering specific signaling events, in

cooperation with other local signals, the oxidative burst mediated by RBOH can spread in surrounding cells, causing a far reaching signaling cascade denoted as the ROS wave. Each cell receiving the ROS wave will activate their own ROS-generating RBOH, so propagating the wave systemically, from the primary source to the whole plants, a picture referred to as systemic acquired acclimation (Mittler and Blumwald, 2015). So that, not just in the plant cells which were the first to perceive the stressor, but in principle in all the remaining cells in the plant, gene expression and metabolism is altered to counteract the adverse stimulus. Although the ROS wave is needed for inducing acclimation to stress, the resulting response is not always stress-specific, therefore the main functional significance of the wave could be to alert the plant cells to produce stress-specific signals and responses (Gilroy et Al., 2014).

Apart from the apoplast, mitochondria and chloroplasts can also generate reactive oxygen species, as a result of the possible leakage of electrons during the flow along the electron transport chains and the consequent partial reduction of molecular oxygen (Gupta and Igamberdiev, 2015). Also peroxisomes can generate ROS as a consequence of their metabolic activity, e.g. during photorespiration, the β-oxidation of fatty acids and the glyoxylate cycle (del Río 2015). Reactive oxygen species pools produced in different compartments not necessarily remain isolated from each other, but can instead communicate with each other, contributing to metabolic regulation. An an example, it has been shown that the ROS signal produced by the apoplastic oxidative burst can be communicated to the chloroplasts, generating there a secondary ROS wave (Shapiguzov et Al., 2012).

Such signal communication probably requires components from the cytosol, and implies that ROS have to cross membrane systems - for example by converting $^{\cdot}O_2^-$ to H_2O_2 in the apoplast, by the action of an apoplast-localized superoxide dismutase (SOD), which would allow H_2O_2 to enter the cell via aquaporins - or ROS signals may be detected by proteins in apoplast or receptors associated to membrane systems (de Dios Barajas-Lopez et Al., 2013). In addition, ROS originating from the chloroplasts or from the peroxisome can bring the signal to the nucleus, where they can regulate the expression of many defense-related genes, (de Dios Barajas-Lopez et Al., 2013; Sandalio and Romero-Puertas, 2015; Locato *et Al.* 2018).

The equilibrium among ROS formation and removal primarily depend from the action of antioxidants, which are involved the elimination of such reactive species through both non-enzymatic and enzymatic mechanisms. Among the enzymes involved in ROS-scavenging, superoxide dismutase is particularly important in that it removes the ROS which is formed first upon exposure to several stressors, namely $^{\cdot}O_2^-$. Other ROS-scavenging enzymes include glutathione peroxidase (GPX), ascorbate peroxidase (APX), and catalase (CAT), which converts

hydrogen peroxide to H_2O (Lazaro et Al., 2013). Either alone or as the electron-donating substrates of some of the above enzymes, ascorbate, glutathione, and tocopherol, i.e. non-enzymatic antioxidants, also give a decisive contribution in keeping the balance among ROS formation and removal in plant cells. To allow the occurrence of the oxidative burst and the consequent generation of the ROS signal(s), the cellular levels of such non-enzymatic antioxidant molecules might be kept low at first, (del Rio, 2015), but once the ROS signal has been generated, their levels become upregulated and act in a cooperative manner, e.g. through the so called ascorbate-glutathione cycle (Viehweger, 2014).

Unlike ROS, the generation of nitric oxid in plant cells is less well understood, and this still constitutes one of the major obstacle in understanding its roles as a signal (Farnese et Al. 2016; Kolbert et Al. 2019). Nitric oxide is intrinsically produced in the plant cells as a result of several catabolic transformations starting from of nitrogen-containing molecules. Besides, it can be taken up by plants from external environment, including air and soil.

Nitric oxide has two biosynthetic pathways, one of which is oxidative in nature, *via* the oxidation of polyamines (PAs) or L–arginine, whereas the other is a reductive one, in which the activity of nitrate reductase (NR) is deeply involved.

Nitric oxide synthesis by the oxidative pathway usually involves polyamines or L-arginine as well as hydroxylamine. L-arginine is oxidized to produce NO plus citrulline by the action of peroxidase (POD), starting from N-omega hydroxy-L-arginine (NOHA) and H_2O_2. In addition, cytochrome P450 and hemoglobin can oxidize NOHA leading to NO formation, in the presence of NADPH and by using the oxidizing power of oxygen (Astier et Al. 2016; Bhuyan et Al. 2020). Moreover, arginase can convert L-arginine to urea and L-ornithine and urea by the enzyme arginase, which leads to PAs and hence to NO, after PAs oxidation (Peng et Al. 2016; da Silva et Al. 2018). If hydroxylamine is provided exogenously, NO can be generated via the the oxidative pathway, but the enzyme responsible for such conversion is not known (Hasanuzzaman et Al. 2018). In the presence of hypoxic conditions, cytochrome-c oxidase and/or reductase are reputed to oxidize NADH/NADPH to produce NO. In addition, NO can be formed from the oxidation of salicylhydroxamate, whereas POD can produce NO by using hydroxyurea and H_2O_2 as the substrates (Hasanuzzaman et Al. 2018).

It is thought that nitrate reductase (NR) in the cytosol, whose primary role is the reductive N assimilation, is essential for the reductive NO biosynthesis in plants (Horchani et Al., 2011) and this has been observed under a variety of physiological circumstances, either in the presence of adversities, such as bacterial infection (Mur et Al., 2013a), hypoxia, cold, drought (reviewed by Farnese et Al. 2016), or during normal development, as occurs when the lateral roots are formed

and during floral transition (Mur et Al., 2013a). In ordinary growth circumstances, however, NR usually plays its inherent role, i.e. reducing nitrate to nitrite, and only in the presence of specific circumstances, such as anaerobiosis or high concentrations of nitrite, significant amounts of NR-derived nitric oxide can be measured (Gupta et Al., 2011; Mur et Al., 2013a).

Apart from NR, also enzyme participating in the mitochondrial electron transport chain can be involved in the reductive pathway of NO (Leon and Costa-Broseta 2020), as do xanthine oxidoreductase and NO_2-dependent NO reductase (Sahay and Gupta 2017). The reductive pathway of NO synthesis normally prevails under normal partial pressure of O_2, whereas under conditions of microaerobiosis or anoxia the oxidative pathway is stimulated (Leon and Costa-Broseta 2020; Zhou et Al. 2021a, 2021b).

Among those putatively involved in NO synthesis, one enzyme which conceivably has been among the most intensively studied is nitric oxide synthase (NOS). Previous literature survey concerning the presence of a typical NOS enzyme in plants gave negative results (Hancock and Neill 2014; Foresi et al. 2017**).** More convincingly, Jeandroz et Al. (2016) reported about a genomic search of canonical NOS sequences within a large data sets of sequenced transcriptomes from land plants, which gave no result. This led those authors to conclude that molecular mechanism for producing nitric oxide are probably different among plants and animals. As a consequence, it has been recommended to avoid using terms such as "NOS" nor "NOS-like" referred to higher plants, for which instead the expression "nitric oxide generating" enzymes and proteins should be used (Santolini et Al. 2017; Astier et Al. 2018; Hancock and Neill 2019).

Aside the enzymatic pathways presented above, non-enzymatic routes also exist for the production of NO in the plant cells. During atmospheric N_2 fixation by nitrification and denitrification, nitric oxide can be released. The reaction among two molecules of HNO_2 can produce NO and NO_2 which is further converted to NO and superoxide radical anion. Acidic pH can favor non-enzymatic NO production, while NO_2 is converted to NO and NO_3. Upon exposure to light β-carotene reduces NO_2 to NO. Reducing electrons for converting NO_2 to NO can be also donated by ascorbic acid, which becomes oxidized to dehydroascorbic acd (DHA). In addition, NO is produced non-enzymatically from *S*-nitrosoglutathione (GSNO), and by by deoxygenated heme-proteins too, which under anoxic conditions convert NO_2 to NO (Sami et Al. 2018; Bhuyan et Al. 2020).

As seen above for ROS, also the formation of nitric oxide occurs in various organelles in the plant cell, such as peroxisomes, chloroplast, the endoplasmic reticulum, mitochondria, plasma membrane, apoplast, as well as in the cytosol (Sahay and Gupta 2017). The mechanism of NO formation in each cell compartment depends on the local predisposition to carry out reductive or

oxidative NO synthesis (see above). At the plasma membrane level, the reductive synthesis is preferred, because a comparatively lower pH favors there the conversion of NO_2 to NO and O_2; the reductive NO biosynthesis is also favored in the apoplast, from which the root-specific NO is released by using the local abundance of nitrite substrates. In chloroplasts, mitochondria, peroxisomes, either of the two NO formation routes is favored, and a switch among the two can also occur, dictated by the local availability of substrates and by the environment. In the cytosol, a NADPH-dependent conversion of nitrate to nitrite occurs, which is further reduced to NO at the expense of polyamine oxidation. Always in the cytoplasm, NO is also produced as an intermediate during the conversion of reduced glutathione (GSH) to GSNO (Nabi et Al. 2019; Zhou et Al.2021a).

Nitric oxide given exogenously is absorbed by the plant cell as nitrate, by the action of nitrate transporters, and then nitrate is regenerated as NO by NR, at the expense of NADPH (Zhou et Al. 2021a).

As many other metabolites, not to say all of them, cellular concentration of nitric oxide has to be maintained at definite values in space and time, which is accomplished by scavenging and metabolic activities set in turn by specific enzymatic activities and the associated substrates. Similarly to other organisms, plants have their own NO scavengers, namely non-symbiotic hemoglobins known as phytoglobins (Hill et al. 2016), which, in the presence of hypoxia, are particularly efficient in converting nitric oxide to nitrate, being oxidized to metphytoglobin (Gupta and Igamberdiev 2011). These are then reduced back to phytoglobins at the expense of ascorbate, by the action of a specific reductase (Gupta and Igamberdiev 2011; Gupta et Al. 2020b; Zhou et Al.2021a).

Endogenous levels of NO are also controlled by its reaction with GSH, to form GSNO, which is possibly used to supply back nitric oxide employed in proteins nitrosylation, thus contributing to cellular the cellular homoeostasis of nitric oxide (Leon and Costa-Broseta 2020; Kalinina and Novichkova 2021). S-nitrosoglutathione can be transported in the phloem, thus acting as a stable and soluble source for the transport of the NO signal over long distances, which may be important for plants acclimation to stress (Arasimowicz-Jelonek et Al., 2014). S-nitrosoglutathione also contribute to setting the cellular level of NO by inhibiting the pathways of N_2 assimilation (Fungillo et Al., 2014). The turnover of GSNO also depends on the activity of GSNO reductase (GSNOR), which deaminates S-nitrosoglutathione to glutathione disulfide (GSSG) and ammonia. As a consequence, GSNOR can control the cellular levels of GSNO, which in turn has important consequences for maintaining NO homeostasis and its transitory signaling activity in cells (Corpas 2011; Malik et Al., 2011; Lindermayr 2018). The expression of GSNOR is responsive to

environmental stress and are influenced by the ROS/NO ratio (Cheng et Al. 2015; Wang D. et Al., 2015; Yang et Al., 2015; Farnese et Al. 2016) thus constituting a pivotal point in the crosstalk among the two signaling routes. A further oxidation of GSNO produces ammonia and GSSG by the action of a specific reductase (Leon and Costa-Broseta 2020). Upon de-nitrosylation of previously nitrosylated proteins, NO is released back and this affects NO-cellular homoeostasis (Leon and Costa-Broseta 2020). This occurs when the S–nitrosylation-dependent signaling is altered upon attack of pathogens, which activate responses at the immunological level. Proteins S–nitrosylation is thought to be regulated by thioredoxins, another interaction giving its own contribute to NO homeostasis and participating in plant immunity (Mata-Perez and Spoel 2019; Leon and Costa-Broseta 2020).

Dehydrogenases of the NAD(P)H dehydrogenases-type are seen as additional regulators of NO degradation. They convert NO into peroxynitrite via reaction with superoxide anion, being their activity stimulated by calcium and hindered under anoxia by SOD (Gupta et Al. 2020a).

Nitric oxide can be also scavenged when it reacts with O_2, yielding nitrate and nitrite, or it reacts with lipid peroxyl radicals, forming nitro fatty acids (Hancock 2012; Rubbo 2013; Leon and Costa-Broseta 2020). Haemoglobins can contribute to scavenge nitric oxide by reducing themselves to Fe(II)-hemoglobin, and the regaining oxidation at the expense of NO (Chamizo-Ampudia et Al. 2017). In the presence of O_2, nitric oxide is metabolized to nitrous anhydride (N_2O_3) and NO_2. In an aqueous milieu, they are then transformed to nitrite and nitrate, taking part to the response to nitrooxidative stress (Corpas and Palma 2018).

ROS AND NO INTO ACTION AT THE MOLECULAR LEVEL

The action molecular mechanisms of ROS and NO have been widely studied in many different organisms, including plants (Green et Al., 2014; Lamotte et Al., 2015; Morales et Al., 2015). The effects of NO and related chemical species depend on its ability to chemically modify proteins, according to three possible distinct mechanisms: the nitrosylation of metals, the nitration of tyrosinyl residues in proteins, and *S*-nitrosylation (Lamotte et Al., 2015). When nitrosylation of metals occurs, nitric oxide binds to transition metals in metalloproteins; NO binds to the iron at the center of heme, the prosthetic group of several enzymes involved in ROS-scavenging enzymes, like POD, CAT, and SOD, not to say guanylate cyclase, among the others, and this results in their inhibition or activation. The possibly opposite effects of NO on proteins bearing a heme group, namely inhibition or activation, are thought to depend on their oxidation state. Because nitric oxide resembles O_2, as far as paramagnetic properties, hydrophobicity, charge, and size are concerned, it can be predicted that any metalloenzyme containing a heme group reactive towards O_2 will be potentially prone to become inhibited by nitric oxide (Arora et Al. 2016).

During tyrosine nitration, a nitro group is added to tyrosinyl residues in proteins. This is mainly brought about by peroxynitrite, which is formed from the reaction between nitric oxide and the superoxide radical anion. After that for much time it has been regarded as a consequence of nitrosative stress, tyrosine nitration is assumed nowadays to be also behave as a signal (Mengel et Al., 2013).

Last, *S*-nitrosylation, or more rigorously S-nitrosation, during which nitric oxide binds to the cysteinyl residue of a given proteins, appears to be the main avenue for transducing the nitric oxide signal. *Trans*-nitrosation is an alternative manner for S-nitrosation to occur, in which there is an exchange of NO from one *S*-nitrosylated residue to another thiol group, mediated by nitrosothiols, among which GSNO (Lamotte et Al., 2015). In both cases, S-nitrosation causes nitric oxide to become reversibly bound to a S atom, resulting in the production of S-nitrosothiol (-SNO). Since S-nitrosation does not depend on enzymatic catalysis, its extent is dictated by the reactivity among the nitrosating agent and the local redox potential. S-nitrosation is selectively brought about on specific cysteinyl residues and targeted proteins become post-translationally modified (PTM), which increases specificity and flexibility in the adaptive responses to a changing cellular environment (Gong et Al. 2014; Arora and Bhatla 2015; Arora et Al. 2016; Hancock **2020**). So far, S-nitrosation-dependent reversible PTMs have been found in more than 200 proteins, allowing to identify possible new functions for many proteins during ad hoc proteomic studies (Minguez et Al., 2012, Sevilla et Al., 2015).

S-nitrosation remarkably contributes in plant adaptation to abiotic stress. The stress caused by excess salinity, for example, appears to be able to induce *S*-nitrosation of respiratory-, photorespiratory, as well as antioxidant enzymes (Camejo et Al., 2013), whereas upon exposure to cold a preferential *S*-nitrosation of the enzymes involved in carbon metabolism was observed (Puyaubert et Al., 2014), which could suggest a major role for *S*-nitrosation in maintaining the balance among energy-capturing and energy-processing reactions under stress conditions.

Apart from influencing the post-transcriptional expression of plant enzymes, *S*-nitrosation may also impact the transcriptional level, deriving *S*-nitrosation of transcription factors (TFs), which affects their binding to *cis*-acting elements on DNA and/or their cellular localization. The myeloblastosis (MYB) family of TF, playing important roles in th onset of tolerance to both abiotic and biotic stress, can be inactivated by *S*-nitrosation, which could suggest that the they would need to be downregulated after the initial stress response (Tavares et Al., 2014).

S-nitrosation can also impact nuclear proteins, including histone deacetylases, deeply involved in the histone-acetylation/deacetylation cycle, which contribute to the reversible transitiong from the more condensed and less accessible status of chromatin (heterochromatin) and the its less

compact and more accessible counterpart (euchromatin; Mengel et Al., 2013; Chaki et Al., 2015; Ageeva-Kieferle et Al 2021). Upon S-nitrosation, at least in animal systems, histone deacetylase detaches from chromatin, and this promotes acetylation, making DNA more accessible for replication/transcription (Farnese et Al. 2016). By the way, this suggests the intriguing possibility that NO can have regulatory roles in the epigenetic control of plants' processes (Floryszak-Wieczorek et Al., 2012; Chaki et Al., 2015; Ageeva-Kieferle et Al. 2021).

Both *S*-nitrosation and denitrosation, involving the removal of NO from cysteinyl residues, have to occur, in oder to allow the reversibility of the subsequent effects in terms of enzyme activities, protein–protein interactions, as well as signaling (Sevilla et Al., 2015). Nitric oxide-dependent S-nitrosation of the cysteinyl residue of GSH leads to the synthesis of GSNO, which, as mentioned before, can reversibly store NO, and thus participate in the control of its biological activity, e.g. by donating NO for S-nitrosation-dependent proteins PTM, apart from acting as a long-distance signal on its own (see above). The aforementioned nitrosoglutathione reductase (GSNOR), firstly described in plants by Sakamoto et Al. (2002), by regulating the endogenous levels of S-nitrosoglutathione and subsequently the global extent of *in planta* S-nitrosation, is now recognized as a central regulator of NO signaling in plants (Leterrier et Al. 2011, Jahnová et Al. 2019). A direct reversion mechanism leading to S-denitrosation, thought to be specific or a subset of plant proteins, has been envisaged for thioredoxin (Trx) h5. (Kneeshaw et Al. 2014). Therefore, two different strategies at least might exist in plants to arrest nitric-oxide mediated signaling through S-nitrosation: one indirect, mediated by the action of GSNOR on GSNO (Malik et Al. 2011; ,Kolbert et Al. 2019) and the other direct, in which Trxh5 selectively de-nitrosate proteins (Kneeshaw et Al. 2014).

Similar to nitric oxide, ROS signals are also transmitted via PTM of proteins (see above), cysteinyl residues being again involved. However, unlike via *S*-nitrosation operated by NO, ROS appear to bring about several different types of oxidative PTM (Ox-PTM), reversible or not, which include disulfide bond formation, sulfhydration, and *S*-glutathionylation (Akter et Al., 2015). Each interested protein can be subjected to different modes of Ox-PTMs, and it has been proposed that each of these OxPTM types may have a different role (Couturier et Al., 2013). The initial reaction of ROS-induced PTM is the reversible oxidation of a cysteinyl residue, which yields sulfenic acid (R-SOH). Being remarkably unstable, sulfenic acid will readily react with free protein thiols, forming –S-S- bonds or causing the covalent attachment of glutathione, increasing the extent of *S*-glutathionylation; such a reaction plays a remarkable role important in signaling and protects the protein from the deleterious action of $^{\cdot}O_2^-$. De-glutathionylation and reduction of disulfide bonds, respectively regulated by thioredoxin and glutaredoxins, terminate the *ab initio* Ox-PTM signal

(Waszczak et Al., 2014, 2015).

Although mostly studied in animals and in bacteria, one Ox-PTMs in particular, namely *S*-glutathionylation, is reputed to be a key player in the abiotic stress responses also in plants, being able to impact the functional state of enzymatic proteins and TFs, as well as the chromatin structure. (Zagorchev et Al., 2013; Dietz, 2014).

Even if much experimental evidence has been accumulating over the recent years concerning the impact of Ox-PTMs and *S*-nitrosation on metabolism and genes expression in plants, a unifying picture concerning signals mediated by ROS, NO and their crosstalk is still far from being proposed.

CROSSTALK BETWEEN REACTIVE NITROGEN, OXYGEN AND SULFUR SPECIES

Every time exposure to a stress causes the production of nitric oxide, it is never produced alone, and this implies that the accumulation of other signals will occur in parallel, including reactive oxygen species and hydrogen sulfide. In such respect, the case of plants glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is paradigmatic: this enzyme can be S-nitrosated, becoming able to interact in a specific manner with partial gene sequence coding NADP-dependent malate dehydrogenase in nuclear DNA. On the other side, GAPDH modification can also occur via oxidation brought about by H_2O_2 (Hancock and Neill 2019) and moreover it can be S-persulfidated by hydrogen sulfide (Aroca et Al. 2017), which causes its translocation to the nucleus. This exemplifies that signals generated by reactive species can compete for the same protein target (Hancock et Al. 2017), and therefore that NO-dependent signal will be the dominant one is not guaranteed. In such context, methods for identifying thiol modifications could be helpful for understanding signals overlapping and crosstalk (Williams et Al. 2015, 2019). Every time nitric oxide and $^{\cdot}O_2^-$ are formed together in the plant cell, the synthesis of peroxynitrite can result, having signaling capacity on its own (Speckmann et Al. 2016).

S-nitrosation can mediate the crosstalk among nitric oxide and other signaling routes, included those in which ROS are involved. This is exemplified by the case of NADPH-oxidase, a fundamental ROS-generating enzyme (see above), which is subjected to modification caused by nitric oxide (Yu et Al. 2012a). As seen above, tyrosine nitration is another PTM in which nitric oxide is involved (Kolbert et Al. 2017; Jiménez at al. 2019), again possibly resulting in functional changes. By nitrating the tyrosinyl residues of target proteins, peroxynitrite can regulate their function. For example, $ONOO^-$ can influence the activities of SOD enzymes, thus indirectly regulating the formation of other signaling molecules, such as reactive oxygen species

(Holzmeister et Al. 2015)). As in the case of S-nitrosation, tyrosine nitration brought about by nitric oxide can cause PTM of the target proteins, leading to either decreased or increased activities when the target proteins are enzymes.

Because nitric oxide and reactive oxygen species are formed in parallel in the cell, their interactions and the complexity of their chemistry have to be considered. A mostly well studied interaction among nitric oxide and reactive oxygen species, in particular $^{\cdot}O_2^-$, leads to the formation of $ONOO^-$. This has two potentially remarkable consequences. First, such reaction scavenges both $^{\cdot}O_2^-$ and nitric oxide from the cell milieu, lowering in this manner the effective concentrations and hence the signaling capacity of both of them. Furthermore, a different signaling molecule is generated, namely $ONOO^-$ (Speckmann et Al. 2016), able to trigger an array of effects potentially different from those which would have been resulted from reactive oxygen species or nitric oxide per se (Hancock and Neill 2019).

It has also to be considered that the formation of nitric oxide will often occur in an antioxidant-rich cellular environment. As mentioned above, NO may influence the expression of antioxidant enzymes, as occurs when SOD activity is altered in the presence of $ONOO^-$ (Holzmeister et Al. 2015; Romero-Puertas and Sandalio 2016), thereby reducing the removal of $^{\cdot}O_2^-$ and the production of a key signaling molecule such hydrogen peroxide. Likewise, nitric oxide can influence CAT activity (Bauer 2015), thereby reducing the removal of H_2O_2 and possibly stabilizing and sustaining the signal brought about by ROS. On the other side, there are many reports suggesting that NO can activate ROS-scavenging enzymes, such SOD and CAT (reviewed in Arora et Al. 2016; Klein et Al. 2018), also with the participation of H_2S signaling (da Silva et Al. 2018). Such apparent contradiction could stem from the fact that interaction between different ROS-scavenging enzymes (and their different isoenzymes) and nitric oxide takes place at different levels, so that the intracellular equilibrium among reactive oxygen species and nitric oxide is key in establishing the sign and the extent of modulation of the enzyme activities: as discussed above, in fact, on one side NO-dependent nitrosylation can modulate, not only post-translationally but even at the genetic level, the activities of ROS-scavenging enzymes, all of which are metalloproteins; on the other side, and reciprocally, endogenous NO availability can be regulated by ROS-scavenging enzymes. In this respect, the case of SOD is paradigmatic: SOD isoforms can not only be in competition with nitric oxide for the common substrate/reaction partner, namely superoxide radical anion, but also catalyze the release of NO from GSNO: indeed, providing hydrogen peroxide is present, GSNO is rapidly acted upon by Cu/ZnSOD to yield oxidized glutathione and nitric oxide (Arora et Al. 2016). Hence, NO-based PTMs may modulate the activities of enzymatic antioxidants (Yang et Al., 2015; Kolbert & Feigl, 2017, Gupta et Al. 2020**)**. In general, nitric oxide has been proposed to control at a

remarkable degree the cellular levels of reactive oxygen species, and, as a consequence, their downstream signaling potential, by strengthening antioxidant defense, reducing lipid peroxidation and balancing the redox status of the cell to mitigate concentration peaks of ROS production following oxidative stress (Fancy et Al. 2017; Hasanuzzaman et Al. 2018; Su et Al. 2018; Jiménez et al. 2019; Sharma et Al. 2019).

A considerable part of the burden of antioxidant defense in the plant cell is born by non-enzymatic antioxidants. Significantly, nitric oxide and GSH can generate GSNO, and this can lower the GSH/GSSG ratio in the plant cell, thereby altering the intracellular redox balance, and the more so if the reaction is restricted within the boundaries of a given cell compartment. Furthermore, glutathione reductase, re-reducing back one molecule of GSSG to two molecule of GSH at the expense of reducing equivalents such as NADPH, possesses two catalytically important thiols which can undergo S-nitrosation, thereby leading to enzyme inhibition causing in turn a decrease in cellular levels or reduced glutathione. On the other hand, nitric oxide has been shown to upregulate glutathione synthesis, which could be beneficial in the presence of oxidative stress (Xiong et Al. 2011; Arora et Al. 2016). Formation of GSNO will scavenge nitric oxide, preventing it from further participation in signaling events. S-nitrosoglutathione, on the other side, can act not only as a molecular sink of nitric oxide, but also as a source of it, and also to act as a mediator of certain effects of nitric oxide (Yun et Al. 2016), showing distinct and overlapping molecular targets in comparison to NO itself.

S-nitrosoglutathione has also been appreciated as a physiologically significant mechanism for the long distance transport of the NO signal, possibly via the plant vascular system. To stop the S-nitrosoglutathione-mediated signaling, its removal by means of GSNOR is needed (Hancock and Neill 2019), which would lower the bioavailability of NO. A number of different stress, either biotic or abiotic, also were shown to influence GSNOR activity and modulate its expression (Chaki et Al. 2011; Corpas 2011; Gong et Al. 2015; Arora et Al. 2016). It has been proposed (Lindermayr 2018) that ROS can finely tune GSNOR activity, thereby affecting the cell concentrations of NO/SNO, which activate protection towards oxidative stress.

Nitric oxide-induced PTM appears to increase the activities of the antioxidant-regenerating enzymes involved in the ascorbate-GSH cycle, but not in response to nitric oxide or drying, at least in seeds, where only slight changes in the activity of above enzymes are seen (Bai et Al. 2011). By enhancing the activities of mono DHA- and DHA reductase, nitrogen-oxide has also claimed to be involved in the regeneration of ascorbate in response to salinity (Hasanuzzaman et Al. 2011; Arora et Al. 2016)).

By summarizing, the available evidence points to a role for nitric oxide-induced PTMs, by targeting the enzymes involved, in modulating both ROS formation and removal, which implies that NO can strictly control ROS homeostasis. Aside from acting as a direct agent of PTM, nitric oxide can also been involved in a competition for target proteins with molecules able to do the same, among which reactive oxygen species and hydrogen sulfide, suggesting the existence of multiple regulatory levels.

Nitro-fatty acids behave on their own as signaling molecules whose formation follows the reaction among unsaturated fatty acids, often oleic- or linolenic acids, and nitric oxide and related reactive species (Corpas et Al., 2013; Gupta et Al. 2020). Nitro-oleic acid has been recently reported to increase the activity of NADPH-oxidase, thus affecting ROS formation (Arruebarrena et Al., 2020), which points to a previously uncovered level of crosstalk among the signals generated by nitric oxide and reactive oxygen species.

An additional source of interference and crosstalk with nitric oxide-generated signals could arise from H_2S, which has been recently claimed to be an important signal-generating molecule on its own in both plant and animal systems (Lisjak et Al. 2013; Olas 2015; Kimura 2017; Zhou et Al. 2021b). Hydrogen sulfide is formed following plants' exposure to to a variety of stresses, among which cadmium (Mostofa et Al. 2015). Hydrogen sulfide can participate in a direct reaction with nitric oxide, from which nitrosothiols are formed. As previously seen in the case of the reaction among NO and GSH, the direct chemical interaction among hydrogen sulfide and nitric oxide could decrease the availability of both in terms of direct signaling capacity, but on the other side it would form a new signal, with its own signaling potential (Hancock and Neill 2019). Hydrogen sulfide may also enhance the cellular contents of glutathione (Hancock and Neill 2019), thereby potentially altering the cellular levels of S-nitrosoglulathione. Hydrogen sulphide and H_2O_2 can also cause PTM of proteins by interacting with catalytically important thiols on cysteinyl residues. Hydrogen peroxide is able to oxidize the –SH group of cysteinyl residues in proteins to frm sulfenic acid, a reaction denoted as S-sulfenylation (Huang et Al., 2019), whilst hydrogen sulfide can bring about persulfidation (Hancock, 2019; Corpas et Al., 2019; Zhou et Al. 2021b). Interestingly, several of the enzymes targeted by the above reaction actively participate in ROS formation or removal.

Finally, hydrogen gas has recently been claimed to act as a signal in both plants and animals (Wilson et Al. 2017; Hancock and Neill 2019). Molecular hydrogen can influence the cellular levels of antioxidants (Ohta 2015), thereby affecting the metabolism of nitric oxide in an indirect manner, although it has also been proposed to directly interact with certain compounds of nitrogen (Hancock and Hancock 2018). For sure, hydrogen gas can affect plants' metabolism (Lin et Al. 2014; Wu et

Al. 2015; Su et Al. 2018), to the point which it has been envisaged as a future protective/biostimulating agent for treating plants (Zeng et Al. 2014). Nitric oxide has been shown to be required for the occurrence of certain effects attributable to hydrogen gas (Zhu et Al. 2016a, 2016b) and therefore it is highly probable that further studies will reveal more about interaction and crosstalk in signaling between nitric oxide and H_2.

Crosstalk in abiotic stress

Heavy metals

It has been proposed that the interaction between nitric oxide and reactive oxygen species is an important determinant of the tolerance against heavy metals (HMs; Wang et Al., 2014; Feigl et Al., 2015; Silveira et Al., 2015; Thao et Al., 2015; Farnese et Al. 2016; Rizwan et Al. 2018; Okant and Kaya 2019; Singh and Prasad 2019), although relevant the mechanisms according to which such interactions take place have not been elucidated yet. An increases in ROS production is believed to be among the first cellular responses to excess heavy metals, which have been shown to be able to stimulate apoplastic NADPH oxidases, but also to promote oxidative stress in mitochondria and chloroplasts (Chmielowska-Bak et Al., 2014). As far as the synthesis of nitric oside is concerned, heavy metals are known to enhance it, although the mechanisms are thought to be metal-specific. Lead, for example, has been shown to increase NR activity in the cytosol (Yu et Al., 2012b), whereas cadmium-induced enhancement of cellular NO levels appear to be associated to iron deficiency (Farnese et Al.2016). Instead, in response to certain metalloids, the operating level of the signal will be set by the balance among NO synthesis and GSNOR activity, both of which were found to be stimulated (Leterrier et Al., 2012). Furthermore, an increased synthesis of NO might be fueled by ROS-mediated signaling following the ROS overproduction unleashed by heavy metal stress (Yun et Al., 2011).

As discussed above, ROS and NO can behave as stressing agents or signals, depending on their concentration in the cellular milieu. At low concentrations, NO can promote tolerance to heavy metals by favoring the metal-binding capacity of the cell wall, so reducing their net influx into the cell (Singh et Al., 2011) or increasing the HMs sequestration into the vacuole, either via stimulation of phytochelatin biosynthesis or by influencing the electrochemical gradient of vacuolar proton pumps in order to favor the vacuolar uptake of heavy metals (see Farnese et Al. 2016 and references therein). Furthermore, nitric oxide can ameliorate the plants' antioxidant capacity, thereby limiting the heavy HM-induced oxidative stress (Cheng et Al., 2015; Andrade et Al., 2016). Last but not least, NO-dependent post-translational protein modification (PTM, see below), can down-regulate the activity/level of ROS-generating enzymes, such as NADPH oxidase

and glycolate oxidase, thus supporting the plant cell in maintaining or recovering its homeostasis in redox chemistry (Yun et Al., 2011; Quiang et Al., 2012).

Water shortage

To tolerate drought, plants must have a precise and timely control of stomatal behavior and a finely-tuned balance in cellular metabolism, in both of which nitric oxide and reactive oxygen species are deeply involved (Osakabe et Al., 2014). That nitric oxide is strictly required for generating the signal following exposure to water shortage can be argued by the fact that drought-dependent NO stimulation has been seen in a wide variety of both higher and lower plant species (Santisree et Al., 2015; Nabi et Al. 2019; Shams et Al. 2019). The metabolic pathways participating in drought-dependent NO stimulation, however, are still mostly uncovered, even if roles for nitrate reductase (Farnese et Al. 2016) and for xanthine oxidoreductase (Yu et Al., 2014) have been suggested. On the other hand, is well known that drought stimulates ROS generation and this can depend on the over-reduction of the photosynthetic electron transport chain, resulting as a backward effect from the stress-dependent impairment of Rubisco activity and the regeneration of $NADP^+$ operated by the Calvin cycle. Apart from the ROS overproduction originating from the photosynthetic machinery, water stress has been shown to activate an oxidative burst via NADPH oxidases, which is key for the deployment of defense/signaling in response to water shortage (Wang X. et Al., 2015; Farnese et Al. 2016).

A reduction in stomatal opening is the most important and easily predictable effect of water shortage; stomatal behavior appears to respond to the complex interplay reactive oxygen species, nitric oxide and abscisic acid (ABA). By acting as an the first element of such regulatory chain, ABA induces the synthesis of nitric oxide, which, in synergy with ROS, acts in turn upon stomatal closure by producing 8-nitro-cyclic guanosine monophosphate (cGMP; Pasqualini et Al. 2009). Reactive oxygen species overproduction and the resulting alteration of the cellular redox state of the cell are thought to be essential for transducing the *ab initio* ABA signal (Joudoi et Al., 2013).

On its own, and likewise ABA and ROS, under conditions of water shortage nitric oxide is thought to influence transcriptional activation, with beneficial effects in terms of biomass production and survival, which suggests a protective role for such molecule (Shi et Al., 2014). DNA methylation, and its consequences in terms of epigenetic changes, is another of the effects attributed to NO in plants exposed to water stress: as shown by Fan and Co-workers (2012), a NO donor such as sodium nitroprusside supplied exogenously caused an overall decrease in the levels of DNA-methylation in *Dendrobium huoshanens*e, which resulted in an increased transcription of genes coding for antioxidant enzymes.

Flooding

In the mechanisms underlying the perception of reduced oxygen availability in plants, changes in the balances between ROS/RNS formation and removal may participate, via the inhibitory effect of hypoxia on the mitochondrial electron transport chain (complex III) and mediated by a transient MAPK (mitogen-activated protein kinase) signaling cascade (Chang et Al., 2012). Furthermore, the ROS-dependent response regulation under conditions of micro-aerobiosis or anaerobiosis might imply, at least partially, the participation of ethylene, since both has been found to stimulate the formation of adventitious roots in rice plants exposed to oxygen deprivation (Steffens et Al., 2012). On the other side, exogenous NO application can alleviate flooding stress in maize plants by upregulating the activities of antioxidant enzymes and of alcohol dehydrogenase, and the levels of phenols and other non-enzymatic antioxidants (ascorbate) as well (Jaiswal and Srivastava 2018).

The mechanisms of nitric oxide synthesis under oxygen deprivation are still unclear, even if there are evidences of involvement of nitrite and ascorbate, within the context of reactions occurring in the mitochondria (Wang and Hargrove, 2013). Nitrite could accept electrons from mitochondrial complex IV and complex III, and from alternative oxidase as well, with the purpose of limiting ATP production under hypoxic conditions and so keep in balance the energy status of the cell (Gupta et Al., 2011). The nitric oxide produced in the mitochondria could diffuse into the cytosol, being converted to nitrate by phytoglobins. Nitrate could be then reduced to nitrite by the action of nitrate reductase (Dordas, 2015) and, nitrite, in turn, could undergo mitochondrial conversion again to NO, so dissipating a potentially dangerous overflow of mitochondrial electron under conditions of scarcity of the terminal electron acceptor (Gupta et Al., 2011).

Salinity stress

Arabidopsis mutants deficient in NO synthesis show a salt-hypersensitive phenotype, and this points to an essential role for nitric oxide in the tolerance of plants to salinity stress. Nitric oxide could ameliorate tolerance to salinity by increasing the activities of ATPase and of H^+-pyrophosphatase at the plasma membrane and the tonoplast levels, which could contribute to keep in balance the Na^+/K^+ ratio (see Farnese et Al. 2016 and references therein). Furthermore, NO appears to be able to activate the transcription of stress-related genes and to ostensibly increase the activity of antioxidant enzymes, both the ROS-scavenging- and the antioxidant-recycling ones (Hasanuzzaman et Al. 2011;, Arora et Al. 2016); Khoshbakht et Al. 2018). Likewise, hydrogen peroxide also modulates the transcription of stress-related genes in plants exposed to salinity, especially in the root cells, and stimulates the activity of specific enzymes. It is noteworthy that the

compensatory changes promoted by reactive oxygen species and nitric oxide do not remain confined within the tissues where these molecules are primarily produced, and this is reminiscent of the ROS/RNS "wave" propagating mechanisms discussed before: in this respect, as a matter of fact, both NO and GSNO, whose role in NO transport has been described above, have been shown to be present in the vasculature of plants undergoing salt stress (see Farnese et Al. 2016 and references therein).

Nitric oxide and H_2O_2 could also participate in the development of acquisition of plant immunity against salt stress. In such process, denoted as priming or hardening, the previous exposure of a plant to sublethal doses of a given abiotic stress accelerates and potentiates its defense capacity upon re-exposure to that same stressor (see Farnese et Al. 2016 and references therein). As a matter of fact, plants given low levels of nitric oxide and hydrogen peroxide showed an upregulation of their physiological and metabolic responses upon exposure to salinity (Molassiotis et Al. 2016). Consistently, in primed wheat roots a stimulation in the production of H_2O_2 and nitric oxide was observed, which was associated to a priming-dependent induction of tolerance to drought (Wang et Al. 2020). This study also pointed the a direct interaction among H_2O_2 biosynthesis deriving from the stress-induced activation of NADPH-oxidase and the downstream signaling operated by nitric oxide during the H_2O_2-mediated accumulation of proline and glycine betaine. In this same vein, halophytes and glycophytes showed an increase in their tolerance to salt stress when previously treated with nitric oxide donors. The above evidence suggests that nitric oxide and hydrogen peroxide are able to prime for increased tolerance to salt stress, protecting the plant from undesirable effects upon subsequent exposure to such stressing agent (see Farnese et Al. 2016 and references therein.

Dystermia

Dystermia, often in combined with drought, mostly determines the plant biodiversity worldwide, and even the more so under the present era of climate change. Even if exposure to high or low temperatures evoke quite distinct metabolic patterns, a commonalty exists among the two opposite circumstances, i.e. ROS overproduction and the derived challenge to the plant cell structure and function (Zhou et Al., 2012; Bita and Gerats, 2013; Farnese et Al. 2016). As in the cases of other abiotic stresses in plants, ROS overproduction arising from dystermia appears to be a deleterious consequence of the loss of capacity to keep pace among energy-capturing and energy-processing reactions (Hasanuzzaman et Al., 2013). In contrast, experimental evidence has been increasingly demonstrating that ROS, RNS and their crosstalk, as well as their capacity of inducing post-transcriptional modification of proteins (see above), are essential components of acclimatization

(Habibi, 2014; Yu et Al., 2014; Hossain et Al., 2015; Zhou et Al. 2021a). As a matter of fact, treating plants with low levels of nitric oxide and hydrogen peroxide allows a better performance of them when challenged by thermal stress (Cantrel et Al., 2011; Dong et Al. 2018).

High temperatures usually cause an increase in the production of endogenous nitric oxide, a response thought to be important for plant acclimation (Yu et Al., 2014). Treating plants with an exogenous source of nitric oxide during their exposure to high temperature stress stimulates the level/activity of both non-enzymatic and enzymatic antioxidants, which reduces the extent of stress-induced cellular damage (Hasanuzzaman et Al., 2013; Rai et Al. (2018). Symmetrically, giving to plants a NO scavenger, such as 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), suppresses the aforementioned protective effects of nitric oxide against high temperature stress, which further support the idea that this molecule could be an important determinant for the onset of tolerance (see Farnese et Al. 2016 and references therein). Likewise, accumulation of reactive oxygen species, and in particular of hydrogen peroxide, is reputed to bring about the signal transduction leading to the transcriptional activation of heat shock genes, encoding proteins reputed to have key roles in the preservation of homeostasis at the cellular level under stressing circumstances (see Farnese et Al. 2016 and references therein). Rai et Al. (2020) proposed that in plants the heat stress-induced shock response is brought about via synthesis and distribution of nitric oxide and salicylate (SA), through calcium signaling.

As in the case of elevated temperatures, cold stress can also lead to a quick increase in the cellular levels of nitric oxide and reactive oxygen species (Cantrel et Al., 2011). Working with plant mutants defective in NR expression suggested that NO most likely participates in the onset of tolerance to cold, since mutant lines showed reduced NO levels if compared to the wild type and a concomitantly higher incidence of damage from cold stress (Cantrel et Al., 2011). The promotive action of NO towards tolerance to low temperatures may imply effects at the gene expression level, seen in the transcriptional regulation of both biosynthetic (*P5CS1)* and catabolic (*ProDH*) proline genes, resulting in the accumulation of such compatible solute and osmoprotectant.

Aside of the effects attributable to nitric oxide, the role of hydrogen peroxide has been the subject on many studies, showing that exogenous application of H_2O_2 attenuates the damaging effects of cold stress and increases the post-stress survival rates (Farnese et Al. 2016; Dong et Al. 2018). Such beneficial impact of H_2O_2 appears to rely again on the promotion of antioxidant defense, both enzymatic, through the activation of CAT, GPX and APX, and non-enzymatic, through the increase of glutathione levels (Iseri et Al., 2013; Wang et Al., 2013). In this case, the signaling pathway leading to the mitigation of cold stress effects might involve, at least in part, transitory “waves” of Ca^{2+} ions in the cytosol deriving from the hydrogen peroxide-mediated

opening of Ca^{2+} channels at the plasma membrane level (Farnese et Al. 2016). Although the studies suggesting that reactive oxygen species and nitric oxide are central players in raising plant tolerance to dystermia have been many, a comparatively scanty amount of work has been dedicated to unraveling the molecular mechanisms underpinning the signaling cascades involved. In such respect, it has been reported that in some plants cold stress could evoke nitric oxide formation by activating ion channels causing the downstream upregulation of TFs such as the inducer of CBF (ICE) proteins and the C-repeat binding factor/Dehydration-responsive element binding-1 (CBF /DREB1). These transcription factors are known to be under the regulation brought about by several miRNA candidates, among which miR172, miR166, and miR397, and by certain regulators of ABA signaling, such as miR168 (Kumar 2014). The CBF/ DREB TFs are known to work by binding to the C-repeat elements /low-temperature response *cis*-acting elements (CRT/LTRE) in the promoter regions of cold-responsive genes assumed to confer tolerance to cold stress in plants (Kumar 2014). In several plant species, the activities of the aforementioned miRNAs were found to be affected by nitric oxide and are thought to be involved in the development of cold tolerance (Kumar 2014; Singh et Al. 2017; Prakash et Al. 2019).

Crosstalk in biotic stress

A landmark paper about the crosstalk among ROS and NO in plant-host interactions was published at the very end of the previous century, in which NO was found to participate in the ROS-induced PCD resulting from the HR raised by *Arabidopsis thaliana* plants infected by virulent *Pseudomonas syringae* (Delledonne et Al. 1998). Already at that time, it was apparent that GSNO behaves as ta long-distance signal in the development of systemic acquired resistance in plants. It became then apparent that, unlike in animal cells, HR-induced PCD in plants includes the interaction of nitric oxide with H_2O_2 generated by SOD, and not a $NO\text{-}^{\cdot}O_2^{-}$ interaction. However, a ROS-independent PCD pathway during compatible plant-host interaction was also proposed, in which a cGMP-dependent pathway involving MAPK was envisaged (see Kolbert et Al. 2019 and references therein).

A new pathway concerning the crosstalk among nitric oxide and reactive oxygen species during plant-pathogen interaction was also proposed, based on the inhibition of the two major H_2O_2-scavenging enzymes, namely CAT an APX, exerted by NO and $ONOO^{-}$. in tobacco BY-2 cells, it was also shown that the ascorbate and glutathione redox couples fuel the NO- and ROS-dependent transduction signaling leading to PCD and plant defence responses. In some cases, however, nitric oxide and ROS were found to not participate as signals in the early stage of HR initiation, being

involved instead in the subsequent stage of HR spreading to the cells surrounding the infection entry point (see Kolbert et Al. 2019 and references therein).

Mitochondrial nitrite reduction (see above), together with NOG (see above) and NR activities, were found to cooperate for generating nitric oxide following in fection of A. thaliana leaves brought about by *P. syringae*. Likewise, NR was shown to originate the nitric oxide produced under the induction by infestin, the main eliciting molecule produced by *Phytophthora infestans* when attacking *Nicotiana benthamiana*. In this same pathosystem, MAPK were found to be involved in the signaling cascade initiated by nitric oxide- and RBOH-dependent oxidative burst. Furthermore, a prosthetic group in common among NOG, NR and NADPH-oxidase, namely flavin, was found to be required for both nitric oxide and reactive oxygen species waves leading to HR-induced PCD (see Kolbert et Al. 2019 and references therein).

A crosstalk among NO and GSH was proposed concerning the S-nitrosation of regulatory elements, such as NPR1 (non-expressor of pathogenesis-related gene 1), participating in the salicylic acid (SA)-induced activation of pathogenesis-related genes, and nitrosative PTMs were also shown to target ROS-producing enzymes and components of the SA-dependent signaling (Sun et Al. 2012; Kovacs et Al. 2015). Oligogalacturonides eliciting defense responses in Arabidopsis may promote NR-dependent nitric oxide production. This can negatively affect the capacity of RBOH to initiate the HR-dependent oxidative burst, because, under high S-nitrosothiol levels, nitric oxide can bring about S-nitrosation of the enzyme at a specific and conserved cysteinyl residue, resulting in RBOH inhibition ((Yun et Al. 2011); Rasul et Al. 2012).

In summary, a considerable amount of literature testifies that both nitric oxide and ROS, both *per se* and through a crosstalk, participate in the molecular mechanisms following plant-host interaction, from the early pathogen recognition leading to HR, to the later signaling cascades resulting in the reprogramming of gene expression aimed at local as well as systemic defense and immunity. Mechanisms and pathways, however, may strongly depend on the phytopathogen considered, its plant host(s), including its genotype, and the resistance strategies deployed upon plant-pathogen interaction. As the majority of available evidence has been derived from herbaceous plant models, such as *Arabidopsis* and tobacco and their available mutant lines, a considerable amount of further work is needed on one side for transferring this into practical applications focused on agriculturally important crops, and on the other side for understanding NO/ROS crosstalk during plaant-pathogen interactions in non-model species, including those belonging to natural or semi-natural plant communities, e.g. forest trees.

CONCLUSION AND PERSPECTIVES

The recent literature clearly support the notion that redox signaling play key roles in almost all the aspects concerning normal plant growth and development, as well as those associated to beneficial, such as symbiosis, or potentially detrimental plant-environment interactions, as under biotic or abiotic stress. The most relevant redox molecules are ROS - such as the superoxide anion, singlet oxygen, hydrogen peroxide, and the hydroxyl radical - and nitric oxide. In plants, they play key signaling functions and participate in the regulation of gaseous exchange, defense, cell death, seed germination, and the transition from the vegetative to the reproductive stage, among the others.

The diversity, as well as the outreach, of the above functions becomes explainable if one considers that reactive oxygen species and nitric oxide interact rapidly to form a number of RNS. Besides the direct interactions of these redox molecules, both ROS and nitric oxide can modify the activity or function of proteins involved in their own generation and signaling, as well as in their metabolism and homeostasis.

The studies reported above suggest the importance of the interplay and crosstalk between NO, ROS and H_2S. They recall that the signaling brought about by nitric oxide and reactive oxygen can be influenced by either synergistic or antagonistic interactions, or instead to function in parallel. In addition, the available evidence supports the notion that the nitric oxide-dependent influence on the activity of ROS-scavenging and antioxidant-recycling enzymes plays a key role in the regulation of oxidative stress and ROS homeostasis.

Nitric oxide is produced in the plant cell in response to the same stimuli able to promote ROS and H_2S overproduction, so that NO will never work alone. The reaction of nitric oxide with reactive oxygen species and/or with hydrogen sulfide will decrease the cellular NO level, but also give rise to new signaling molecules, such as $ONOO^-$ and SNOs, able to act themselves as signal, with possibly distinct signaling outcomes. The impact of nitric oxide on the cellular redox balance, especially within the plant cell compartments, needs to be also considered, as well as, and reciprocally, the effects of the inherent redox environment on the nitric oxide metabolism that may derive. The way(s) in which nitric oxide is interconnected with the signaling behavior of other key reactive chemicals need to be deciphered.

Much more information is also needed to understand how and to which extent these signaling molecules regulate and modulate gene transcription, by unraveling, for example, their influence on epigenetic processes. Along with present-day and future progresses in the field of redox regulation in plants, a better understanding as well as new practical applications will become attainable in relation to plant-environment interactions, protection of biodiversity, agriculture, and plant biotechnology.

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