Author Queries

AQ1 What does the 'x' stand for here?
AQ2 Do you mean Kim et al. 2006 as listed in the References section?
AQ3 Pls define 'PTM' here.
AQ4 Explain/define this?
AQ5 Only 2009 listed for these authors; check?
AQ6 Pls check my 'key' to abbreviations and insert definition for 'PA'.
AQ7 Spelling: should be 'phytoglobin'?
AQ8 Check my expansion of ABA here.
AQ9 In what sense 'intensification'? do you mean 'upregulation'?
AQ10 Do you mean Desel & Krupinska 2005 as listed?
AQ11 Sense? Do you mean 'suggesting' or 'evidencing' maybe?
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AQ13 Do you mean Tsukagoshi et al. 2010 as listed?
AQ14 Pls define/gloss 'PIN' here.
AQ15 Sense: do you mean 'inactivating NADPH oxidase'?
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Cross-talk of Reactive Oxygen Species and Nitric Oxide in Various Processes of Plant Development: Past and Present

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Nitric Oxide (NO) and Related Intermediates: Reactive Nitrogen Species (RNS)

Nitric oxide (NO) was first characterized as a cardiovascular signal molecule in 1987 (Furchgott, 1988), some 25 years after the first demonstration of reactive oxygen species (ROS) (Iyer *et al*., 1961). Thanks to intensive research, it has been found that living organisms contain a group of NO-originated molecules, which are called reactive nitrogen species (RNS) by analogy to ROS. Both groups of intermediates are diverse in the sense that they include radical and non-radical forms with different physico-chemical properties and reactivity. Moreover, both ROS and RNS are redox active, thus contributing to the maintenance of the cellular redox homeostasis (Potters *et al*., 2010). The ROS are generated by the one-, two-, or three-electron oxidation of molecular oxygen, while RNS intermediates are nitrogenous products and derive usually from the oxidation of the nitric oxide radical (NO·). Among RNS, we can distinguish the redox forms of NO radical (nitroxyl anion, NO^-; nitrosonium cation, NO^+), the higher oxides of nitrogen – dinitrogen pentoxide (N_2O_5), dinitrogen tetroxide (N_2O_4), dinitrogen trioxide (N_2O_3) – peroxynitrite ($ONOO^-$), *S*-nitrosothiol compounds (RSNOs), and dinitrosyl iron complexes (Arasimowicz-Jelonek *et al*., 2015). Nitric oxide serves as a precursor for the other RNS molecules; at the same time NO itself is the major biologically active reactive nitrogen intermediate, therefore our chapter focuses on it.

Physico-chemical Properties of NO: A Comparison With ROS

Nitric oxide is a small diatomic gas molecule with an unpaired electron in its π orbital, which assigns a radical character for it. Moreover, because nitric oxide is a redox-active molecule it exists in three interchangeable forms in biological systems. Oxidation yields the nitrosonium cation (NO^+), while reduction of the NO radical leads to the formation of nitroxyl anion (NO^-) (Stamler *et al*., 1992). It is worth emphasizing that NO is a peculiar radical with a surprisingly low reactivity. It only shows reactivity toward molecules with a free radical character, and toward transition metals like heme iron (Beckman and Koppenol, 1996). Therefore, NO has a relatively long half-life (in the order of seconds; Table 14.1), which is, however, influenced by its

Reactive Oxygen Species in Plants: Boon or Bane? Revisiting the Role of ROS, First Edition. Edited by Vijay Pratap Singh, Samiksha Singh, Durgesh K. Tripathi, Sheo Mohan Prasad, and Devendra K. Chauhan.

Table 14.1 Some relevant physico-chemical properties of reactive oxygen and nitrogen species.

	Hydrogen peroxide (H_2O_2)	Superoxide anion ($O_2^{\cdot-}$)	Peroxynitrite ($ONOO^-$)	Nitric oxide (NO)
Radical character	No	Yes	No	Yes
Charge	Non-charged	Negatively charged	Negatively charged	Non-charged
Half-life (ms)	~1	0.002–0.004	<10	5000–15,000
Diffusion distance (μm)	~ x 10	~ x 10	4	100–200

own concentration (Wink and Mitchell, 1998). Besides its relative stability, its good diffusion capability (Lancaster, 1997), owing to its lipophilic character, helps nitric oxide to be an excellent signal molecule within and between cells.

In comparison, H_2O_2, which likewise is a non-charged signal molecule, possesses a half-life two orders of magnitude shorter (Vranova *et al*., 2002), consequently its diffusibility is less (one-tenth that of NO) (Krumova and Cosa, 2016). The other relevant reactive oxygen intermediate is superoxide radical anion ($O_2^{\cdot-}$), which has a remarkably shorter biological half-life compared to NO (Table 14.1) (Vranova *et al*., 2002) and, because of its negative charge at physiological pH, its diffusion across membranes depends on the presence of anion channels (Denicola *et al*., 1998). The fast reaction between superoxide and nitric oxide results in the formation of a non-radical anion, peroxynitrite ($ONOO^-$). The different diffusion properties of $O_2^{\cdot-}$ and NO suggest that in biological systems, peroxynitrite generates close to the sites of $O_2^{\cdot-}$ formation where NO produced at distant cellular spaces arrives (Denicola *et al*., 1998). Peroxynitrite itself has a longer half-life compared to the other discussed ROS (Siegel *et al*., 2015) (Table 14.1), but it is more reactive than NO. Regarding the diffusion distance of peroxynitrite, it is similar to that of H_2O_2 and $O_2^{\cdot-}$, but it is shorter compared to NO (Denicola *et al*., 1998).

Common Sites of ROS and NO Production Within Plant Cells

Regarding ROS–RNS cross-talk, it is important to have information about the relationships between their production in particular cellular organelles and areas. In general, ROS are formed by electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a by-product in various metabolic pathways (Bashir and Jan, 2015). In contrast, the endogenous production of NO in plant cells is more complex, since several oxidative and reductive pathways are implicated; furthermore, NO can be synthesized with or without enzymatic catalysis (Mur *et al*., 2013). The fact that there are uncertainties regarding some NO synthesis pathways further complicates the matter. Plant NO can derive from the oxidative catabolism of polyamines (Wimalasekera *et al*., 2011) or the oxidation of hydroxylamines (Rümer *et al*., 2009), although these mechanisms are barely documented.

A similarly obscure field of plant NO research is the questionable existence of nitric oxide synthase (NOS) enzymes, the well-characterized NO synthesizing enzyme family of animals. These enzymes use L-arginine as substrate to produce NO (Stamler, 1994).To this day, no enzyme protein or coding sequence for these enzymes has been identified in the genome of higher plants. Interestingly, though, NOS-like activities were detected in some green algae (e.g., *Ulva compressa*, *Chatonella marina*; Gonzalez *et al*., 2012; Kim, 2006, respectively), and

the NOS-like enzyme of *Ostreococcus tauri* was both structurally and functionally characterized (Foresi *et al*., 2010; Gupta *et al*., 2016). These results indicate that during evolution, higher plants lost their capability to generate NO via the NOS-system (Fröhlich and Durner, 2011), since during their highly nitrate-dependent metabolism, NO can be produced more thriftily via nitrate reductase activity, without maintaining the extra enzyme system. Therefore, the main enzyme for reductive NO formation is considered to be nitrate reductase (NR) (Mur *et al*., 2013). This cytosolic enzyme undergoes a regulatory switch from its preferential substrate, nitrate, to nitrite, and produces NO by the one-electron reduction of nitrite (Yamasaki and Sakihama, 2000). Another organelle-linked reductive mechanism is when nitrite acts as a terminal electron acceptor for the mitochondrial cytochrome coxidase/reductase (Castello *et al*., 2006) in case of low oxygen availability (Planchet *et al*., 2005). Mitochondria represent a major source also for intracellular ROS production. The superoxide radicals primarily generate in the electron transport chain at NADH dehydrogenase (complex I) and at ubiquinone-cytochrome *c* reductase (complex III). Furthermore, superoxide dismutase (SOD) and other antioxidants are responsible for the generation of H_2O_2 from $O_2^{\cdot-}$ in the mitochondrion (Krumova and Cosa, 2016). This means that in certain physiological conditions, NO and $O_2^{\cdot-}$ may generate at the same time in the electron transport chain, which can result in the formation of mitochondrial peroxynitrite (Noctor *et al*., 2007). The peroxisomally localized xanthine oxidoreductase (XOR) has to be considered as a reductive enzymatic source of NO, since it reduces nitrite to NO using NADH as electron donor under low-oxygen conditions (Corpas *et al*., 2008). When the oxygen tension is higher, XOR catalyzes the formation of superoxide anion, therefore it can be considered as a relevant source for ROS and RNS (del Río, 2011). Moreover, in their early work, Barroso *et al*. (1999) determined and biochemically characterized L-arginine-dependent NOS activity in isolated peroxisomes, suggesting that NO may be generated by at least two independent pathways (L-arginine-dependent and XOR-mediated) in peroxisomes. More recently, Corpas and Barroso (2013) detected the common generation of NO, $O_2^{\cdot-}$, and $ONOO^-$ in the root and stomatal peroxisomes of *Arabidopsis* expressing the CFP-PTS1 reporter gene construct.

The plasma membrane has a prominent role in intracellular ROS synthesis, since it binds the major superoxide-generating system, the NADPH oxidase enzyme complex (Torres *et al*., 2002). At the same time, plasma membrane (PM) also provides space for NO generation. The PM-localized isoform of nitrate reductase supplies the nitrite substrate for nitrite-nitric oxide reductase (Ni-NOR) (Stöhr *et al*., 2001), which reduces it and synthetizes NO. The Ni-NOR enzyme has a higher molecular weight and more acidic pH optimum than NR, it uses reduced cytochrome *c* as an *in vitro* electron donor, and it was found exclusively in root plasma membrane of tobacco. Although Ni-NOR has not been genetically characterized so far, its involvement in root development, response to anoxia, and symbiosis has been suggested (Stöhr *et al*., 2001; Vandana *et al*., 2012). Also, the plant-specific organelle, the chloroplast, provides a site for simultaneous ROS and RNS generation. Singlet oxygen ($^1O_2$) is a natural by-product of photosystem II (PSII) function, while $O_2^{\cdot-}$ and H_2O_2 are generated in the electron transport chain (Das and Roychoudhury, 2014). Regarding NO production, it was shown that the proper functionality of chloroplasts is significant for maintaining NO levels in soybean cotyledons (Galatro *et al*., 2013). Earlier, the nitrite- and L-arginine-dependent pathways of chloroplastic NO synthesis had been described (Jasid *et al*., 2006), while, according to others, NOS-like activity, but not nitrate reductase, is responsible for NO synthesis in chloroplasts of protoplasts (Tewari *et al*., 2013).Additionally, different ROS types can be generated by different cell-wall-associated enzymes such as peroxidases (Figure 14.1), oxalate oxidases, or amine oxidases (Das and Roychoudhury, 2014). The cell wall is also the site of non-enzymatic NO generation, since nitrite can be chemically reduced by ascorbic acid at pH 3–6 yielding NO and dehydroascorbic

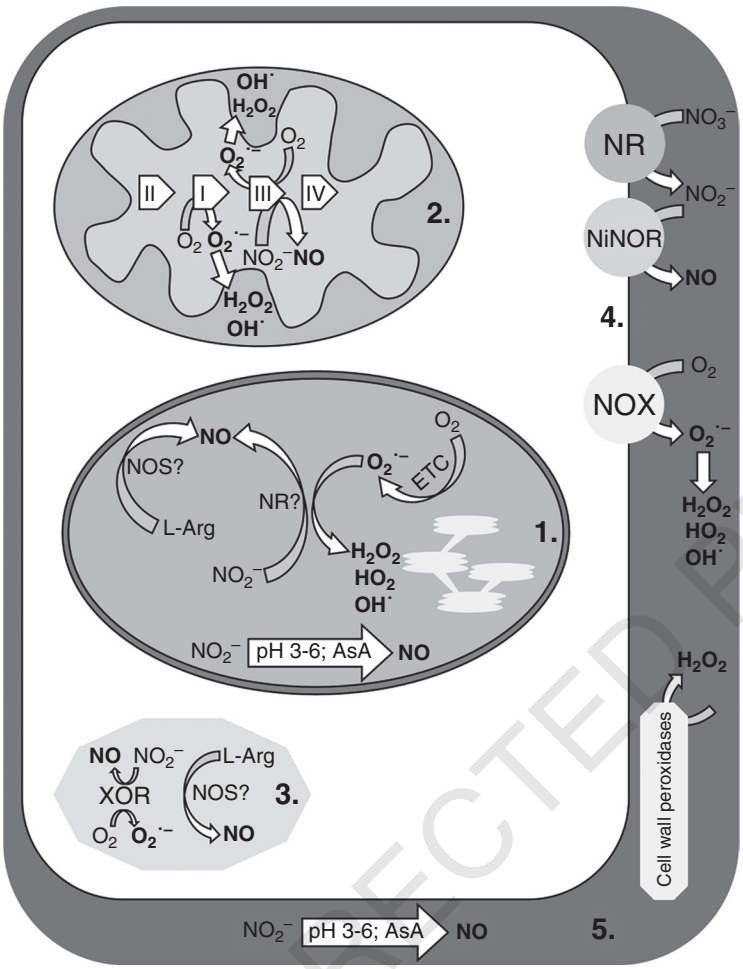

Figure 14.1 Schematic overview of the most relevant subcellular sites and mechanisms of reactive oxygen species (ROS) and nitric oxide (NO) generation in plant cells. **1.** Chloroplast: NO may originate from different sources such as nitrite or L-arginine, $O_2^{\cdot-}$ is generated in the electron transport chain (ETC), H_2O_2 and other forms are produced by enzymatic activities (e.g., superoxide dismutase, SOD). **2.** Mitochondrion: both NO and $O_2^{\cdot-}$ are produced in the ETC. SODs convert $O_2^{\cdot-}$ to H_2O_2. **3.** Peroxisome: the major source of NO is xanthine oxidoreductase (XOR), which produces also $O_2^{\cdot-}$. Another putative NO source in peroxisomes is the mammalian-like nitric oxide synthase (NOS) enzyme. **4.** Plasma membrane: NO is synthesized by nitrite-nitric oxide reductase (Ni-NOR) in the root cells, while NADPH oxidase enzyme complex (NOX) catalyzes the formation of $O_2^{\cdot-}$. Other ROS forms are produced enzymatically (e.g., SOD). **5.** Cell wall: NO is produced in a non-enzymatic reduction reaction at low pH in the presence of reductants such as ascorbic acid (AsA). ROS can be formed, *inter alia*, by the activity of cell wall-associated peroxidases.

acid (Beligni *et al*., 2002). This reaction may occur under microlocalized pH conditions in the chloroplast and apoplastic space, where ascorbic acid is known to be present (Henry *et al*., 1997).

Taken together, the mitochondrion, the chloroplast, and the peroxisome are organelles providing space for the common production of intracellular ROS and RNS. Furthermore, the plasma membrane and the cell wall contribute to the formation of both groups of reactive molecules (summarized in Figure 14.1). The simultaneous production of ROS (particularly $O_2^{\cdot-}$) and NO at the same subcellular location permits their reaction, which makes $ONOO^-$ generation probable in the above mentioned organelles.

Transfer of NO Action: NO-dependent Posttranslational Modifications

The formation of peroxynitrite represents a significant link between the actions of ROS and RNS, since this reactive species is able to perform both oxidative and nitrative modifications of macromolecules. The peroxynitrite-triggered protein tyrosine nitration (PTN) is an irreversible two-step posttranslational modification during which a nitro group ($-NO_2$) attaches to the aromatic ring of tyrosine (Tyr) in the *ortho* position yielding 3-nitrotyrosine (Souza *et al*., 2008). In plant cells, PTN mostly inhibits the activity of the particular enzyme protein (reviewed by Corpas *et al*., 2013); however, in animal systems, PTN-triggered activation or no change of activity is also conceivable (Yeo *et al*., 2015). Another exciting consequence of PTN is that it may either prevent or induce the tyrosine phosphorylation thus influencing cell signaling (Souza *et al*., 2008). In a comprehensive study, 127 nitrated proteins were identified in wild-type *Arabidopsis thaliana* grown during control conditions (Lozano-Juste *et al*., 2011).

The other amino acid modification triggered by NO/RNS ensuring the transfer of NO bioactivity, is *S*-nitrosylation. This reversible covalent chemical reaction results in the modification of the cysteine thiol group, and as a consequence generates *S*-nitrosothiol (-SNO). This PTM is catalyzed by, for example, the higher oxides of NO or nitrosonium cation (NO^+), by metal-NO complexes, by low molecular weight *S*-nitrosothiols (*S*-nitrosocysteine, CysNO) or *S*-nitrosoglutathione (GSNO) (Lamotte *et al*., 2014). *S*-nitrosylation may induce conformational changes of proteins thus affecting their activities, subcellular localization, and interactions or binding activities. In plant systems, Hu *et al*. (2015) provided the most comprehensive dataset of *S*-nitrosylated proteins so far, since they identified 1195 endogenously *S*-nitrosylated peptides in 926 proteins in the *Arabidopsis* proteome. AQ3

A further possibility for NO to modify proteins is metal nitrosylation. This NO-dependent PTM involves the attachment of NO to a transition metal center in the metalloprotein resulting in the formation of a metal-nitrosyl complex. In animal systems the best characterized enzyme candidate for metal nitrosylation is guanylate cyclase (GC) (Russwurm and Koesling, 2004). The effect of NO on a plant's GC activity as well as the nature of this interaction is still unresolved.

NO-dependent Posttranslational Modifications of ROS-related Enzymes

The above discussed posttranslational modifications accomplish the transmission of the NO signal and also ensure cross-talk with ROS. Hence, NO is able to regulate the metabolism of reactive oxygen intermediates through PTMs.

Most importantly, NO has been shown to induce the *S*-nitrosylation of AtRBOHD at Cys890 and consequently inhibit the superoxide-producing activity of this enzyme complex (Yun *et al*., 2011). Similarly to NADPH oxidase, the main enzymatic source of peroxisomal H_2O_2, glycolate oxidase (GOX), suffers *S*-nitrosylation-triggered inactivation in pea leaf (Ortega-Galisteo *et al*., 2012). Interestingly, GOX was identified among *in vivo* nitrated proteins in *Arabidopsis*as well (Lozano-Juste *et al*., 2011), which raises the possibility of dual NO-dependent regulation. Besides the producing enzymes, several ROS-scavenging antioxidants proved to be targets for NO-dependent PTMs. According to our present knowledge, catalase (CAT) can be affected by both *S*-nitrosylation (Ortega-Galisteo *et al*., 2012) and nitration (Lozano-Juste *et al*., 2011), meaning that multiple NO-related pathways may control its action. Similarly, ascorbate peroxidase (APX) is under dual, NO-mediated regulation. The *S*-nitrosylation of APX was demonstrated, *inter alia*, in tobacco, *Arabidopsis*, and pea (Correa-Aragunde *et al*., 2013; de Pinto *et al*., 2013; Begara-Morales *et al*., 2014). In case of pea APX, the nitrosylated site (Cys32) was found to be located at the ascorbate-binding site, and the modification caused functional AQ4

induction (Begara-Morales *et al*., 2014). Also in *Arabidopsis*, the *S*-nitrosylation at Cys32 in APX1 protein led to the activation of the enzyme (Yang *et al*., 2015). More interestingly, *S*-nitrosylation of APX was shown to prevent carbonylation of the protein in *Antiaris toxicaria* seeds (Bai *et al*., 2011). Moreover, Correa-Aragunde *et al.* (2013) demonstrated that denitrosylation causes partial inactivation of APX1 during root development. Regarding the nitration of APX, the modification of Tyr235 located near to the heme group resulted in functional loss (Begara-Morales *et al*., 2014). Peroxynitrite is able to catalyze the tyrosine nitration of the mitochondrial manganase superoxide dismutase (Mn-SOD, MSD1), the peroxisomal copper/zinc SOD (CSD3), and the chloroplastic iron SOD3 (FSD3) (Holzmeister *et al*., 2015). In case of the MSD1 protein, Tyr69 was identified as the site of nitration, suggesting that nitration is able to influence the substrate-binding activity of Mn-SOD. The functional consequence of the peroxynitrite-mediated nitration is the loss of Mn-SOD activity (Holzmeister *et al*., 2015). Moreover, SODs can be candidates also for NO-triggered *S*-nitrosylation, as it was revealed in different plant systems (Lindermayr *et al*., 2005; Tanou *et al*., 2009; Sehrawat *et al*., 2013), although the functional consequence has not been determined so far.

Based on the experimental evidence available so far, NO-related posttranslational modifications significantly affect relevant enzymes involved in, for example, ROS production or the ascorbate-glutathione cycle (summarized in Table 14.2). This suggests that NO keeps ROS homeostasis under tight control. However, the impact of NO goes beyond the above mentioned processes, an assertion supported by the fact that NO is able to regulate the hydrogen peroxide-reducing and peroxynitrite-detoxifying activities of peroxiredoxin II E via *S*-nitrosylation as well (Romero-Puertas *et al*., 2007).

ROS-regulated NO Production in Plant Systems

It is easy to see that the interplay between ROS and NO is not unidirectional, but that mutual relationships between them are conceivable. Accordingly, ROS are also able to influence the metabolism of endogenous NO in biological systems.

The guard cell is an excellent example to study ROS–NO signal interactions, supported by the large number of experimental data in this system. For example, Srivastava *et al.* (2009) detected fast ROS production followed by slower NO formation in stomata of chitosan-treated pea plants, which suggests that NO participates in chitosan-induced stomatal movements downstream of ROS production. Likewise, in guard cells, hydrogen-rich water-promoted NO generation was shown to be dependent on RbohF-related H_2O_2 production. In this case, nitrate reductase proved to be responsible for ROS-triggered NO generation (Xie *et al*., 2014). Similarly, a direct link between ROS and NO was observed in NO-overproducing *noe1* rice plants, where the accumulated ROS induced the activity of nitrate reductase consequently resulting in NO liberation (Lin *et al*., 2012). Another example of the signal link between ROS and NO was related to the thermotolerance of *Arabidopsis* seedlings, where NO proved to be involved in H_2O_2 signaling as a downstream factor (Wang *et al*., 2014). However, these studies often lack an explanation of the exact mechanism of the ROS–NO interaction at the molecular level. To address this shortcoming, P. Wang *et al.* (2010) reported that the H_2O_2-induced MAP kinase 6 phosphorylates NIA2 protein, whereupon the activity of nitrate reductase is promoted and NO is generated.

It is clear that the relationships between reactive oxygen and nitrogen species are mutual and diverse. At the same time, the nature of this cross-talk can also vary depending on several factors such as the plant species, tissue or cell type, and more importantly the presence or absence of stress. Regarding the interactions of ROS and NO during biotic and abiotic stress, we have extensive knowledge as evidenced by the numerous review papers and book chapters recently published on this topic (Scheler *et al*., 2013; Hebelstrup and Møller, 2015; Locato *et al*., 2016; Pucciariello and Perata, 2017). Beyond stress responses, ROS and NO are known to be involved

Table 14.2 List of antioxidant enzyme proteins modulated by *S*-nitrosylation or tyrosine nitration.

Protein	Plant species	Reference
S-nitrosylation		
RBOHD	*Arabidopsis thaliana*	Yun *et al*., 2011
GOX	*Citrus*	Tanou *et al*., 2009
	Pisum sativum	Ortega-Galisteo *et al*., 2012
APX	*Arabidopsis thaliana*	Correa-Aragunde *et al*., 2013
	Pisum sativum	Begara-Morales *et al*., 2014
	Citrus aurantium	Tanou *et al*., 2009
	Anticaris toxicaria	Bai *et al*., 2011
	Arabidopsis thaliana	Yang *et al*., 2015
	Nicotiana tabacum	de Pinto *et al*., 2013
SODs	*Arabidopsis thaliana*	Lindermayr *et al*., 2005
	Brassica juncea	Sehrawat *et al*., 2013
	Citrus aurantium	Tanou *et al*., 2009
CAT	*Pisum sativum*	Ortega-Galisteo *et al*., 2011
GR	*Antiaris toxicaria*	Bai *et al*., 2011
GPOX	*Arabidopsis thaliana*	Lindermayr *et al*., 2005
GSTs	*Arabidopsis thaliana*	Lindermayr *et al*., 2005
	Arabidopsis thaliana	Hu *et al*., 2015
	Citrus aurantium	Tanou *et al*., 2008
Thioredoxins	*Arabidopsis thaliana*	Hu *et al*., 2015
	Brassica rapa	Sehrawat *et al*., 2013
Peroxiredoxins	*Arabidopsis thaliana*	Lindermayr *et al*., 2005
	Arabidopsis thaliana	Romero-Puertas *et al*., 2007
Glutaredoxins	*Citrus aurantium*	Tanou *et al*., 2009
	Arabidopsis thaliana	Lindermayr *et al*., 2005
Tyrosine nitration		
GOX	*Arabidopsis*	Lozano-Juste *et al*., 2011
APX	*Arabidopsis thaliana*	Lozano-Juste *et al*., 2011
	guanylate cyclase; Pisum sativum	Begara-Morales *et al*., 2014
SOD (Mn)	*Arabidopsis thaliana*	Holzmeister *et al*., 2015
CAT	*Arabidopsis*	Lozano-Juste *et al*., 2011
GR	*Helianthus annuus*	Chaki *et al*., 2009
POX	*Arabidopsis thaliana*	Lozano-Juste *et al*., 2011
MDHAR	*Pisum sativum*	Begara-Morales *et al*., 2015

AQ5

APX, ascorbate peroxidase; CAT, catalase; GOX, glycolate oxidase; GPOX, glutathione peroxidase; GR, glutathione reductase; GST, glutathione *S*-transferase; MDHAR, monodehydroascorbate reductase; RBOHD, respiratory burst homolog oxidase D; SOD, superoxide dismutase.

in signal transduction regulating plant growth and development. What's more, their signaling is linked at several points and the regulation of growth responses is thought to be realized through their tight cooperation. The known elements of ROS–NO cross-talk during growth and developmental processes will now be discussed in detail.

ROS–NO Interaction During Plant Growth and Development

The seed-to-seed life cycle of seed-bearing plants includes embryogenesis, and the vegetative and reproductive phases. According to the current state of knowledge, ROS as well as NO are both relevant regulator signals involved in all three developmental periods, as discussed below.

ROS–NO Interplay During Seed Embryogenesis

Embryogenesis in higher plants involves differentiation processes resulting in the formation of a mature embryo. Moreover, somatic cells can also be induced to develop into embryos in a process called somatic embryogenesis, which is an excellent model for understanding the mechanisms of plant embryogenesis (Harada *et al*., 2010). It has been known for a while that exogenous H_2O_2 promotes embryogenesis, and elevated intracellular levels of H_2O_2 are associated with embryonic cell formation (Kairong *et al*., 1999). In an early proteomic analysis, Kairong *et al*. (2002) observed several H_2O_2-induced translated proteins and concluded that H_2O_2 is an active messenger and induces gene expression at the mRNA level during somatic embryogenesis. The involvement of nitric oxide as a signal molecule in plant embryogenesis was evidenced a couple of years later. According to Ötvös *et al*. (2005) exogenous NO (sodium nitroprusside, SNP) promoted, and the inhibitor of the mammalian NOS enzymes (L-NMMA) delayed, the exogenous auxin-dependent formation of embryogenic cells. Moreover, similarly to H_2O_2, NO is generated endogenously in the embryonic cells of different plant species as the effect of polyamine treatments (Silveira *et al*., 2006; Santa-Catarina *et al*., 2007). Nitric oxide is produced in the endosperm during the embryogenesis of *Sechium edule* and its level could be reduced by NOS inhibitor, suggesting that a NOS-like enzyme may be responsible for NO synthesis during seed embryogenesis (Lombardi *et al*., 2012). According to recent results, NO is an integral signal in glutathione-induced embryogenesis of *Araucaria angustifolia* (Vieira *et al*., 2012). Furthermore, suppression of the *Arabidopsis* hemoglobin gene (Glb1) resulted in NO overproduction, which in turn induced ethylene synthesis and altered embryogenesis. The liberated ethylene negatively affected auxin synthesis thus inhibiting somatic embryogenesis (Mira *et al*., 2015). Regarding the hormonal interactions of H_2O_2, the salicylic acid-triggered embryogenesis was accompanied by H_2O_2 production due to the inhibition of APX and CAT (Luo *et al*., 2001). In a recent proteomic study, several ROS-scavenging enzyme proteins were found to be induced in somatic embryos of cotton (Zhou *et al*., 2016). Suppressing the expression of specifically identified GhAPX proteins resulted in the inhibition of dedifferentiation, reflecting the involvement of ROS in the differentiation process. Moreover, disrupting redox homeostasis led to inhibited dedifferentiation and altered expression of genes related to auxin transport and signaling, suggesting a link between ROS and auxin during somatic embryogenesis.

Although the available literature does not reveal the exact nature of the cross-talk between ROS and NO during seed embryogenesis, similarities in their production and actions can be determined. For instance, it is apparent that both ROS (mainly H_2O_2) and NO applied exogenously are able to induce embryogenesis, and both of them are endogenously synthesized in embryonic cells (Figure 14.2). Anyway, the putative H_2O_2–NO interplay during embryogenesis could be an exciting topic for future research.

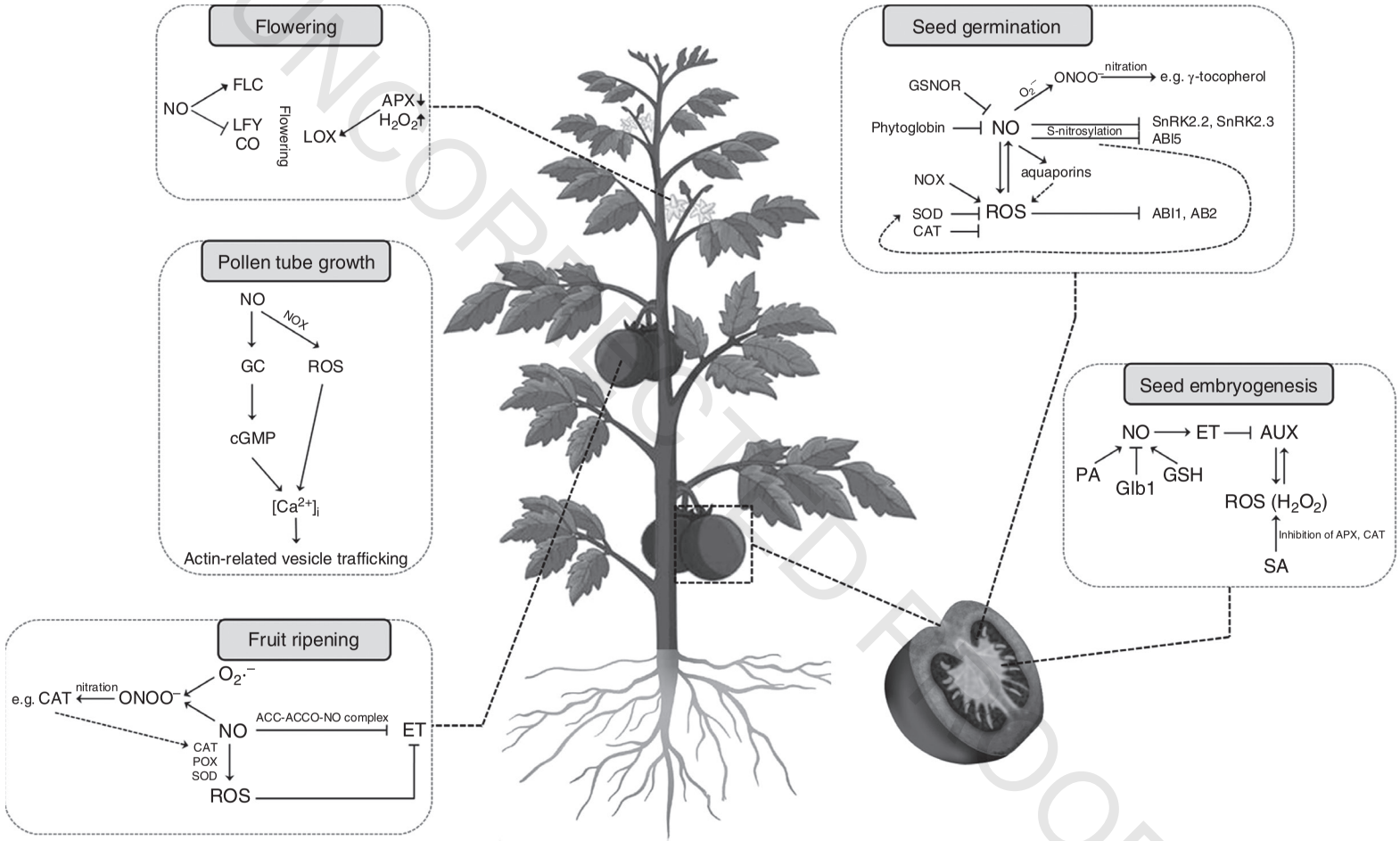

Figure 14.2 Signal interactions between reactive oxygen species (ROS) and nitric oxide (NO) during reproductive developmental processes such as flowering, pollen tube growth, and fruit ripening and during seed developmental events like embryogenesis and germination. Solid lines indicate experimentally verified links, while dashed lines represent putative interactions. ABI, ABA insensitive (transcription factor); APX, ascorbate peroxidase; AUX, auxin; CAT, catalase; CO, *CONSTANS*; ET, ethylene; FLC, *FLOWERING LOCUS* C; GC, guanylate cyclase; Gib, gibberellin; GSH, glutathione; GSNOR, *S*-nitrosoglutathione reductase; LFY, *LEAFY*; LOX, lipoxygenase; NOX, NADPH oxidase; PA; POX, peroxidase; SA, salicylic acid; SnRK, sucrose nonfermenting 1-related protein kinase; SOD, superoxide dismutase.

AQ6

ROS–NO Interplay in Mature Seeds

Within the dormant mature embryo, the shoot and root axis can be clearly recognized, and it may also contain several immature leaves. Germination is the process allowing seedling development through elongation of the embryonic axis of a seed. During the first phase (imbibition), the quiescent seed bloats as a consequence of water uptake. Then, the metabolic and molecular mechanisms are activated and germination takes place. In a germinating seed, not only does the size of the embryo increase but also internal cell differentiation occurs. Experimental results of the last 15 years have revealed that the transition of a dormant seed to a metabolically active plant involves the generation of both reactive oxygen and nitrogen species.

Regarding the formation of the main RNS, nitric oxide, in plant seeds, non-enzymatic processes seem to be primarily important. These include the spontaneous reduction of nitrite to NO at acidic pH in the apoplast of the aleurone cells, where the presence of reductants (e.g., phenolic compounds) may facilitate NO production (Bethke *et al*., 2004). Moreover, the aleurone-localized, nitrate-inducible nitrate reductase (Ferrari and Varner, 1970) may also indirectly contribute to NO production via nitrite generation. The aleurone layer is also an important source of reactive oxygen intermediates, since during germination the generation of $O_2^{\cdot-}$, H_2O_2, and $\cdot OH$ were detected in the seed coat of radish (Schopfer *et al*., 2001). The initiation of seed germination is controlled also by the mitochondrial production of NO. The seeds take up water during imbibition leading to the hypoxic conditions, during which mitochondrial nitrite reduction and the consequent production of NO can occur (Gupta and Igamberdiev, 2011). In hydrated seeds, all metabolically active compartments may be sources of ROS, such as mitochondria (Bailly *et al*., 2008), which represent another common site for ROS and NO generation. Examining the kinetics of the production of reactive species, ROS formation occurs during imbibition, while the generation of NO is induced by oxygen depletion later, upon germination. The work of Ma *et al*. (2016) gives a deeper insight into the metabolism of NO during seed germination. The germinating seed produces NO, which consequently leads to the intensification of thiol nitrosylation. The pytoglobin and *S*-nitrosoglutathione reductase (GSNOR) genes involved in the regulation of NO metabolism were highly expressed in the germinating seed, suggesting their role in controlling the NO level (Ma *et al*., 2016). At the same time, the redox state of glutathione is tightly controlled by NO via the synthesis and the GSNOR-mediated catabolism of GSNO, reflecting a link between ROS and NO metabolism during seed germination. The role of GSNOR activity in controlling both available NO and ROS pools during seed germination was supported by the delayed seed germination of AtGSNOR knockout *Arabidopsis* plants (Holzmeister *et al*., 2011). According to Oracz *et al*. (2009) the intense formation of H_2O_2 and $O_2^{\cdot-}$ during germination is associated with the inhibition of CAT and SOD and with the induction of NADPH oxidase. As discussed earlier, these ROS-producing and antioxidant enzymes can be targets of NO-dependent posttranslational modifications (Table 14.2), thus it is conceivable that germination-induced NO regulates these enzyme proteins and consequently ROS levels; however, experimental data are lacking to support such cross-talk.

The promoting effect of NO on seed germination of different plant species has been known for a while (Beligni and Lamattina, 2000; Bethke *et al*., 2004; Sarath *et al*., 2007), and the aleurone layer seems to have a pivotal role in NO-perception, NO synthesis, and NO-regulated responses (Bethke *et al*., 2007). Sarath *et al*. (2007) provided the first evidence for ROS–NO interaction in seeds, observing exogenous H_2O_2-triggered germination and concomitant NO liberation. Additionally, scavenging of NO completely inhibited the H_2O_2-induced seed germination of switchgrass. Interestingly, NO is involved in the regulation of aquaporin gene expression in germinating rice seeds, thus ensuring efficient water uptake and better germination performance (Liu *et al*., 2007). Certain isoforms of aquaporins allow the move-

ment of signal molecules (e.g., H_2O_2), and thus it is attractive to hypothesize that NO may result in intensified H_2O_2 signaling during germination.

Based on the published data, it is feasible that the signal transductions of H_2O_2 and NO in the germinating seed are linked at several points, although the molecular details about the majority of possible interactions are still obscure. One example of common targets of H_2O_2 and NO in the signaling cascade of germination is the regulation of ABI transcription factors. The NO-dependent *S*-nitrosylation of ABI5 at Cys153 results in its degradation through the activities of CULLIN4-based and KEEP ON GOING E3 ligases, inhibiting abscisic acid (ABA) signaling and promoting seed germination (Albertos *et al*., 2015). Similarly, exogenous H_2O_2 arrests ABA signaling by inactivating ABI1 and ABI2 type 2C protein phosphatases (Meinhard and Grill, 2001; Meinhard *et al*., 2002). Later, Liu *et al*. (2010) proposed that H_2O_2 mediates ABA catabolism through a NO signal and triggers gibberellic acid synthesis in germinating *Arabidopsis* seeds. However, multiple (at least two) NO-dependent pathways for the regulation of ABA signaling in germinating seed can be supposed, since *S*-nitrosylation of sucrose nonfermenting 1 (SNF1)-related protein kinases (SnRK2.2 and SnRK2.3) results in the inhibition of ABA-related signal transduction (Wang *et al*., 2015). AQ8

The antioxidant property of NO means a further possibility for ROS and NO cross-talk. In the aleurone, the endogenously generated NO regulates programmed cell death by the maintenance of antioxidant enzyme activities (Beligni *et al*., 2002). Furthermore, NO enhances the iron availability in germinating seeds, which is associated with the promotion of oxidative stress and ROS production (Jasid *et al*., 2008). As was mentioned above, the NO-associated posttranslational regulation of ROS-detoxifying enzymes in the seed has not been examined yet. In germinating durum wheat, the intensification of *S*-nitrosylation and at least 13 target proteins were observed using the biotin switch assay, although those proteins were not identified (Sen, 2010). The other NO-related macromolecule modification, nitration, requires the formation of peroxynitrite, which thus has a pivotal role in ROS–NO interactions. Similar to animal systems, the nitration of γ-tocopherol was evidenced in plant tissues (Desel *et al*., 2007), and also its negative effect on NO content and on germination was observed (Desel *et al*., 2005), although the mechanism of its action remains unknown. AQ9 AQ10

In general, both ROS and NO are formed during seed germination, and the aleurone layer represents a common site for their generation in the germinating seed.

Within the seed, the life of the endosperm is limited; it ends with germination, when it undergoes programmed cell death (PCD) (Ingram, 2010), which is a well-regulated process. In rice, a ROS burst due to mitochondrial membrane permeabilization accompanies PCD (Kobayashi *et al*., 2013). According to Fath *et al*. (2000), aleurone PCD during germination might be initiated with the invovlement of ROS and NO as second messengers.

The available data suggest that ROS and NO mutually regulate each other's metabolism and activate common downstream targets in the signal transduction of ABA-regulated germination (Figure 14.2). However, further experiments are needed to complete our knowledge of germination-associated molecules modulated by ROS and NO.

ROS–NO Interplay During Vegetative Plant Development

Shoot Developmental Processes

Cotyledon and Leaf Growth

Both groups of reactive species play a role in the growth of aerial plant parts such as cotyledon/leaf and hypocotyl. Pioneer studies showed that at low concentrations, exogenous NO increases leaf expansion, but elevated doses lead to growth inhibition of pea (Leshem and Haramaty, 1996), tomato (Anderson and Mansfield, 1979), and lettuce (Hufton *et al*., 1996). Interestingly,

similarly to NO, H_2O_2 applied exogenously had a concentration-dependent regulating effect on the growth of pea seedlings (Barba-Espin *et al*., 2010). Besides biochemical evidence, the altered morphology of NO and ROS mutants also supports the role of these reactive species in growth regulation. For instance, GSNOR-deficient plants containing elevated GSNO and NO levels have an increased number of shoots and altered shoot leaf morphology and trichome density compared to the wild-type (Holzmeister *et al*., 2011). Similarly, the NO-overproducing *nox1/cue1* mutant possesses reduced shoot size (He *et al*., 2004; Liu W-Z *et al*., 2013; Frungillo *et al*., 2014). At the same time, *nia1nia2* and *nia1nia2noa1-2 Arabidopsis* lines containing reduced NO levels also show growth defects resulting in highly reduced shoot (and root) development (Lozano-Juste and León 2010; Kolbert *et al*., 2015). These findings suggest that tight control of NO levels is needed for normal shoot growth.

Greening

A peculiar process during shoot growth is greening and plastid development. Upon seedling greening, NO intensified the light responses, and promoted the synthesis of chlorophylls and chloroplast-related proteins (Beligni and Lamattina, 2000; Zhang *et al*., 2006). Moreover, NO formation intensified in association with de-etiolation in *Arabidopsis* and wheat seedlings (Lozano-Juste and León, 2011; Liu Y *et al*., 2013) reflecting the involvement of NO in the process. The recent work of Melo *et al*. (2016) revealed that exogenous NO reduces ethylene but increases auxin content during the de-etiolation of tomato. These hormonal changes accomplish negative feedback regulation of the NO level. Furthermore, NO can mimic transcriptional changes similar to those induced during phytochrome-dependent light perception, suggesting a regulatory link also between NO and phytochromes upon greening (Melo *et al*., 2016). In greening cotyledons, the proteomic analysis of peroxisomal proteins revealed that the generated H_2O_2 is scavenged mainly by CAT3, suggesting the role of this ROS-detoxifying enzyme in regulating H_2O_2 levels during cotyledon greening (Fukao *et al*., 2002). The possible interactions are summarized in Figure 14.3.

Hypocotyl Elongation

Besides the growth processes of the cotyledons and leaves, NO also participates in the regulation of hypocotyl elongation. Exogenous application of NO negatively impacts hypocotyl growth, as reported in potato, lettuce, and *Arabidopsis* (Beligni and Lamattina, 2000). Hypocotyls also produce superoxide anions and hydrogen peroxide in the xylem tissues during their growth, and the production of these ROS is associated with the sites of distribution of CuZn-SOD enzyme and lignin (Ogawa *et al*., 1997). Cell wall stiffening, a necessary mechanism for efficient hypocotyl elongation, involves a precisely controlled ratio between ascorbate and H_2O_2 concentrations, supposing the regulatory role of ROS and the redox state in hypocotyl growth (Pedreira *et al*., 2004). In dark-grown *Arabidopsis* seedlings, extracellular ATP (eATP) caused hypocotyl elongation and the concomitant formation of NO and superoxide generation. Moreover, NO proved to be required for both eATP action and its generation is linked to that of superoxide in the *Arabidopsis* hypocotyls (Tonón *et al*., 2010).

AQ11

AQ12

Based on the available data, physiological processes during shoot development seem to be regulated in parallel by NO and ROS. However, the mechanism of their interplay is mostly unknown (Figure 14.3).

Root Developmental Processes

Development of root system architecture includes growth of the primary root, lateral or adventitious roots, and root hairs and also gravitropic responses. The accumulated literature data clearly show that NO and ROS participate in all of these processes and in most cases their signaling pathways intersect.

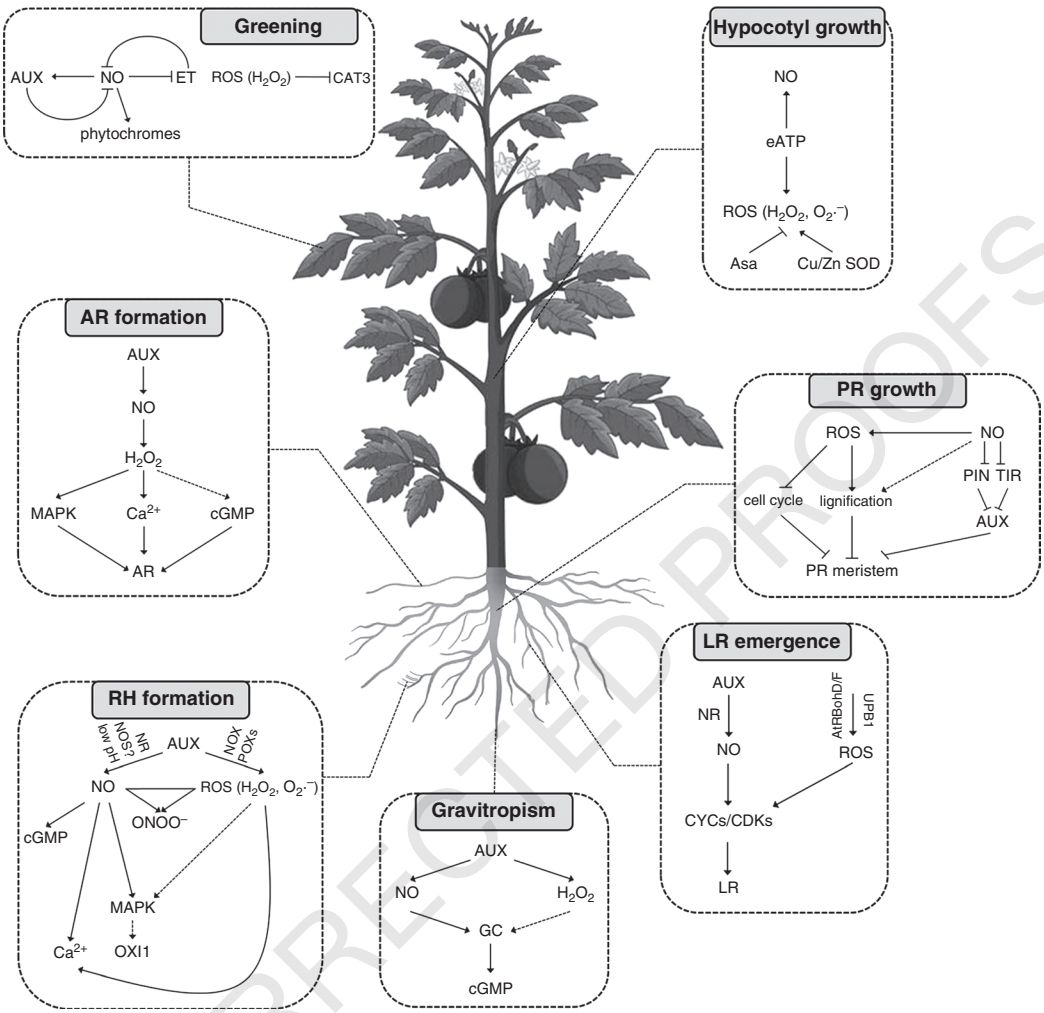

Figure 14.3 Summary of signal relationships between reactive oxygen species (ROS) and nitric oxide (NO) during different processes of vegetative plant development. Solid lines indicate experimentally verified links, while dashed lines represent hypothesized interactions. AR, adventitious root; Asa, ascorbic acid; AUX, auxin; CAT, catalase; CDK, cyclin-dependent kinase; CYC, cyclin; GC, guanylate cyclase; LR, lateral root; MAPK, mitogen-activated protein kinase; NOX, NADPH oxidase; NR, nitrate reductase; PIN, PIN-FORMED protein; POX, peroxidase; PR, primary root; RH, root hair; SOD, superoxide dismutase; TIR, TRANSPORT INHIBITOR RESPONSE protein.

Primary Root Growth

Analyzing NO and ROS mutants reveals that not only the signaling but also the metabolism of the two reactive species are connected. For instance, primary root (PR) tips of the NO-deficient *nia1nia2* and *nia1nia2noa1-2* mutants showed two-fold accumulation of superoxide anion as well as total ROS (Pető *et al*., 2013; Kolbert *et al*., 2015). In *gsnor1-3* plants with elevated NO content, the levels of superoxide and H_2O_2 were 50% lower relative to the wild-type (Pető *et al*., 2013), indicating a negative relationship between ROS and NO metabolism in the root system. Similarly, the root meristems of *noa1, nia1nia2*, *nia1nia2noa1-2*, and wild-type plants treated with L-NMMA contained increased ROS levels, which were presumed to contribute to the reduced root growth properties of these plants (Sanz *et al*., 2014).

As discussed previously, there are common sites of ROS and NO production, which in the root cells are the plasma membrane, the cell wall, the mitochondria, and the plastids. Due to the formation and the catabolism of ROS in the cell wall, the structure of the cell wall is directly affected through the induction of lignification (Francoz *et al.*, 2015). Similarly, NO and related molecules are thought to be involved in cell wall synthesis and modification (Correa-Aragunde *et al.*, 2008; Kolbert, 2016).

Interestingly, both ROS and NO were shown to decrease PR meristem size, but their modes of action seem to be different. Exogenous H_2O_2 treatment has been shown to reduce cell cycle gene expression (Tsukagoshi, 2010), while NO donor disrupted PIN-mediated auxin transport leading to the inhibition of meristem growth (Fernández-Marcos *et al.*, 2011). These results accord with those of Shi *et al.* (2015), where significantly reduced basipetal auxin transport and protein levels of PIN1 and PIN2 were measured in the *gsnor1-3* mutant. NO also modulates auxin signaling and sensitivity through the NO-dependent *S*-nitrosylation of auxin receptor TIR1 (TRANSPORT INHIBITOR RESPONSE 1) promoting its interaction with AUX/IAA (AUXIN/INDOL-3-ACETIC ACID) transcriptional co-repressor proteins (Terrile *et al.*, 2012). However, recently the degradation of AXR3NT-GUS (reporter for auxin-mediated degradation of AUX/IAA by TIR1) was found to be delayed in *gsnor1-3* plants compared with the wild-type, showing that the TIR1-mediated auxin signaling pathway was compromised in this mutant (Shi *et al.*, 2015). Other results imply the possibility also of an auxin-H_2O_2 regulatory link, since increased auxin levels were shown to mediate H_2O_2 production in the PR meristem leading to inhibition of root cell elongation and consequently root growth (Ivanchenko *et al.*, 2013). The possible interactions are summarized in Figure 14.3.

AQ13,14

Lateral Root Emergence

Both ROS and NO were shown to be involved in lateral root (LR) development (Figure 14.3), and their accumulation has been detected in LR primordia. While NO generation proved to be linked to auxin and occurs through nitrate reductase activity (Kolbert *et al.*, 2008), the production of ROS is regulated by UP BEAT 1 (UPB1) transcription factors (Tsukagoshi *et al.*, 2010) and AtrbohD and F (Li *et al.*, 2015). Nitric oxide is a downstream element of auxin-mediated signaling during LR emergence (reviewed by Kolbert, 2016), but the ROS-regulated LR formation seems to be independent from auxin (Li *et al.*, 2015). These findings suggest that although both reactive species participate in the regulatory network of LR development their signaling pathways are not linked via auxin. At the same time, ROS are known to promote auxin-mediated cell cycle entry and thus may have a positive effect on the plant cell cycle (Fehér *et al.*, 2008).

Adventitious Root Formation

NO and H_2O_2 also regulate the emergence of adventitious roots (AR), since exogenous application of them resulted in enhanced AR number in species like cucumber and mung bean (Pagnussat *et al.*, 2003; Li *et al.*, 2007, 2009). Furthermore, both molecules accumulate during the induction of AR formation (Pagnussat *et al.*, 2003; Li *et al.*, 2007). In contrast to lateral root emergence, in ARs auxin induces NO and ROS generation, and both signals seem to be downstream elements of auxin-mediated signaling (Pagnussat *et al.*, 2003, Li *et al.*, 2009). Furthermore, NO was identified as an upstream signaling molecule for H_2O_2 production in auxin-induced AR formation in marigold (Liao *et al.*, 2011). As for NO, several pathways of its action have been demonstrated such as cGMP-dependent (Pagnussat *et al.*, 2003), mitogen-activated protein kinase (MAPK) cascade-linked (Pagnussat *et al.*, 2004), calcium-associated (Lanteri *et al.*, 2006), and phosphatide acid-mediated (Lanteri *et al.*, 2008). According to Liao *et al.* (2009), cGMP is not involved in H_2O_2-induced AR formation in marigold, which suggests that signaling pathways of ROS and NO are partly independent (Figure 14.3).

Root Hair Formation

Root hairs (RHs) represent both structurally and functionally relevant parts of the root system showing characteristic tip growth primarily regulated by auxin (Lee and Cho, 2013). Within the primary root, superoxide anion was detected in the apoplast of elongation zone cells, while the formation of H_2O_2 localized in the cell walls of root hairs in the differentiation zone (Dunand *et al*., 2007) suggesting its regulatory role during root hair elongation. Peroxidases and a particular NADPH oxidase (RbohC) were held responsible for the auxin-induced generation of ROS in the root hair cell (Foreman *et al*., 2003; Dunand *et al*., 2007). Root hairs also produce NO during their normal development, but at different subcellular locations than for H_2O_2 production. In actively growing root hairs, NO was detected in the vacuole, whereas in more mature root hairs the cytoplasm proved to be the site of NO production (Lombardo and Lamattina, 2012). According to some hypotheses, auxin may promote the synthesis of NO in several ways, including nitrate reductase, NOS-like activity, and low pH-mediated nitrite reduction (Lombardo *et al*., 2006).

Scavenging of ROS ($O_2^{\cdot-}$ or H_2O_2) or NO reduced the root hair formation capability of *Arabidopsis* and lettuce, respectively (Lombardo *et al*., 2006; Dunand *et al*., 2007). The *Arabidopsis* plants carrying a mutation in RHD2/AtrbohC showed reduced ROS accumulation and defective root hair formation (Foreman *et al*., 2003). Moreover, the NO-deficient *nia1nia2* mutant had altered root hair growth (Lombardo and Lamattina, 2012), while in low SNO-containing *atgsnor1-1* mutants elongated root hairs were detected, and in SNO-overproducer *atgsnor1-3* mutants reduced root hairs were detected (Kwon *et al*., 2012). These data collectively indicate that both ROS and NO are essential for normal root hair growth. On the other hand, exogenous application of NO donor was able to induce the formation of root hairs, and the participation of NO as a downstream element of auxin action was assumed (Lombardo *et al*., 2006). More target effectors of NO signals during root cell growth are possible, such as the modulation of the redox-state via the formation of $ONOO^-$ and the activation of cGMP-, MAPK-, or calcium-dependent signaling cascades (Lombardo *et al*., 2006). The primary role of NO in root hair growth is the regulation of vesicle formation and trafficking, as proposed by Lombardo and Lamattina (2012). Some of the putative signal pathways of NO can be linked with ROS signaling. Namely, ROS was shown to facilitate Ca^{2+} influx thus contributing to the formation of the calcium gradient within the root hair cell, which in turn regulates, *inter alia*, actin dynamics (Foreman *et al*., 2003). Another downstream element of ROS signals is OXIDATIVE BURST INDUCIBLE1 (OXI1) kinase (Rentel *et al*., 2004), which is activated by ROS likely via the modulation of the MAPK cascade.

The experimental data available so far strongly indicate that both NO and ROS are essential regulators of root hair development. However, their roles seem to be different. While ROS regulate cytoskeleton dynamics, NO participates in the modulation of vesicle formation and trafficking. Although the ROS- and NO-dependent signal transductions in the root hair cell are much less known, common effectors of ROS and NO are probable (Figure 14.3).

Root Gravitropism

Root gravitropism implements adaptation to the changing environment. Despite the relevance of gravitropism in plant stress physiology, only a limited number of publications deal with it in connection with ROS and NO.

Both ROS and NO were shown to accumulate on the lower side of the gravistimulated root, and both signals proved to be auxin-inducible (Joo *et al*., 2001; Hu *et al*., 2005). As for the role of NO in gravitropism, it appears that NO signaling is mediated via cGMP (Hu *et al*., 2005); in other cell types ROS were shown to induce the GC-cGMP system (Dubovskaya *et al*., 2011), implying the possibility of similar cross-talk in gravistimulated root (Figure 14.3). Several lines

of evidence indicate that calcium and inositol trisphosphate (IP_3) as second messengers are also involved in the gravitropic response, but how ROS- and NO-mediated signals are integrated into a physiological response remains to be elucidated.

ROS–NO Interplay During Reproductive Plant Development

The survival of a plant species largely depends on sexual reproduction, which ensures the rise of new generations, and new populations. In flowering plants the adult plant produces flowers, which after fertilization develop seeds as the main method of dispersal (Arc *et al*., 2013).

Floral Transition

The switch to flowering is a crucial developmental transition in the life of a plant, with a significant effect on the reproductive success of a species (Simpson and Dean, 2002; Henderson and Dean, 2004). In this process numerous exogenous and endogenous signals have a role, for instance vernalization, photoperiod, or gibberellins were shown to have a positive effect on floral transition (Mouradov *et al*., 2002; Simpson and Dean, 2002).

During the vegetative/generative transition of the shoot apical meristem, notable NO production was detected in wheat and thus a regulatory function of this gaseous signal in the induction of flower development was supposed (Kolbert *et al*., 2011). Earlier, He *et al.* (2004) found that the application of the NO donor SNP delayed the induction of flowering. They also reported that *Arabidopsis* mutants with high endogenous NO levels have delayed flowering, while lower endogenous NO levels are associated with earlier flowering, compared to the wild-type. High NO content suppresses the *LFY* (*LEAFY*) gene in the floral meristem and *CO* (*CONSTANS*) gene responsible for floral promotion, while the expression of the *FLC* (*FLOWERING LOCUS C*) floral repressor gene is induced by NO. The *nia1nia2* mutant possessing reduced NO content produced flowers earlier than the wild-type, confirming the negative effect of NO on flowering (Seligman *et al*., 2008).

The effect of ROS on floral transition is mostly unknown. Similarly to NO, H_2O_2 also is generated in the shoot meristem during floral transition (Zimmermann *et al*., 2006). The H_2O_2 formation was accompanied by reduced APX activity, but was not associated with ROS-related senescence; thus it might act as a signal for leaves for flower induction or for preparation of the development of reproductive structures (Bañuelos *et al*., 2008). Moreover, the accumulated H_2O_2 proved to be associated with transient increase in leaf lipoxygenase (LOX) activity before/at the floral transition. These findings suggest that H_2O_2 activates plastid LOX, which plays a role in oxylipin synthesis, an important factor in the transient lipid peroxidation observed at floral transition (Bañuelos *et al*., 2008). The possible mechanisms are visualized in Figure 14.2.

Pollination

For the success of the entire fertilization process, many factors have to match in time. For successful pollen germination, pollen tube development, and fertilization, the male pollen and the female pistil must continuously exchange several different signals, to assure the compatibility of the two partners (Heslop-Harrison, 1978, 2000; Herrero, 2003).

Several studies have considered the involvement of ROS and NO signaling in pollen germinaton and pollen tube growth (Prado *et al*., 2004; Cárdenas *et al*., 2006; Potocký *et al*., 2007) or pollen–stigma interactions (McInnis *et al*., 2006).

An interesting connection between ROS and NO in the process of pollination/pollen germination is hypothesized by Hiscock and Allen (2008). They observed that H_2O_2/ROS levels are high in stigmas, as a defense against pathogens, while pollens with high NO content may reduce

the levels of ROS locally, making pollen germination possible. The interaction between ROS and NO might have a potential role in species recognition, to avoid non-compatible pollination; however, little is known regarding this (Hiscock *et al*., 2007; Swanson *et al*., 2004).

Another prospect is that both NO and ROS may be part of a basic recognition system that can distinguish pollen and microbes. Despite the constant secretion of sugars and lipids, receptive stigmas are highly resistant to microbial attack, because of the high concentrations of ROS here and in the nectar, which might be sufficiently toxic against pathogens (Heslop-Harrison and Shivanna, 1977; Carter and Thornburg, 2000, 2004). The source of the ROS in the stigma is mostly unknown; only one stigma-specific peroxidase has been detected so far (McInnis *et al*., 2005).

It is now known that ROS and NO coordinate the self-incompatibility (SI) response in incompatible pollen (Serrano *et al*., 2015). The ultimate result of the SI response is PCD, in which both reactive species have important roles (Bosch *et al*., 2010; Wilkins *et al*., 2011; Serrano *et al*., 2012a,b; Jiang *et al*., 2014). In the growing pollen tube tip of incompatible pollen of *Pyrus pyrifolia*, the inactivation NAPDH oxidase is responsible for the S-RNase-dependent disruption of the ROS content (Wang *et al*., 2010). The lack of high ROS levels in the tip alters intracellular Ca^{2+} levels, leading to disruption of the actin cytoskeleton and to DNA degradation in the nucleus (Obara *et al*., 2001; Thomas *et al*., 2006). In *Papaver rhoeas* SI pollen tube, ROS and later NO accumulation lead to the reorganization of the actin cytoskeleton (Geitmann *et al*., 2000; Bosch and Franklin-Tong, 2007; Wilkins *et al*., 2011) through posttranscriptional modifications (carbonylation, *S*-nitrosylation) causing interference with actin polymerization, leading to PCD (Rodríguez-Serrano *et al*., 2014). In *Olea europaea* SI pollens, NO and $O_2^{\cdot-}$ are generated and react to produce peroxynitrite, which promotes nitration in both papillar cells of the stigma and pollen undergoing PCD. It raises the possibility that tyrosine nitration triggered by ROS–NO interaction might have a role in SI PCD (Serrano *et al*., 2012a). AQ15

If the ROS content of the stigma is deadly to microbes, why is it not toxic to pollen? It is now known that in stigmatic papillae in contact with pollen grains the ROS level is reduced (Hiscock *et al*., 2007). Considering the high amounts of NO in pollen, it seems likely that NO acts as a signal by which the pollen "turns off" the ROS barrier (Hiscock *et al*., 2007, McInnis *et al*., 2006). AQ16

Numerous ROS-associated activities (catalase, superoxide dismutase, ascorbate peroxidase, monodehydroascorbate reductase, or GSH-dependent processes) were characterized in pollen grains (Acevedo and Scandalios, 1990; Dai *et al*., 2006). Also, NADPH oxidase (NOX)-dependent ROS production is found at the tip of the growing pollen tube, which is thought to be important in maintaining polarized tip growth (Potocký *et al*., 2007), just like in the tip-localized growth of root hairs.

Unlike ROS, NO is excluded from the tip of the growing pollen tube, but is generated in peroxisomes of subapical regions, and it is proposed to play an important role in directional growth of the pollen tube. This spatial distribution might allow the apical growth of the pollen tube, while on the other hand it might act as a positive feedback for elongation when the surrounding environment lacks an exogenous NO source. Also, external NO is able to cause the reduction and reorientation of pollen tube growth (Prado *et al*., 2004). The effect of NO seems to be mediated by at least two pathways. Endogenous NO generated by the germinating pollen tube activates cGMP synthesis, which in turn increases Ca^{2+} uptake leading to the accumulation of intracellular calcium. At the same time NO can promote NOX activation and consequently ROS formation, also leading to calcium accumulation. As a signal, calcium regulates actin-related vesicle transport to the tip of the pollen tube. Nitric oxide also might have a role (as a "guide") in the further orientation of the growing pollen tube until it reaches the micopyle; however, the exact mechanisms are yet to be established (Hiscock and Allen, 2008).

When the pollen tube reaches the micropyle, several events happen close to each other in time. Accessory cells, like antipodial cells and synergids, undergo PCD around fertilization (Heydlauff and Groß-Hardt, 2014). The receptive synergid attracts the pollen tube to the female gametophyte, and then degenerates rapidly (Sandaklie-Nikolova *et al*., 2007). In this PCD peroxisome functions hereby ROS and NO as signal molecules are suspected to play an important role in the communication between gametophytes before sperm release (Boisson-Dernier *et al*., 2008).

As detailed above NO and ROS contribute to pollen–microbe distinction, to the self-incompatibility response, as well as to pollen germination. During the SI response, the importance of NO- and ROS-dependent posttranslational modifications have been demonstrated, while in the growing pollen tube NO-induced ROS production and a common downstream signal (Ca^{2+}) have been described (Figure 14.2).

Fruit Ripening

Fruit set, development, maturation, and ripening are controlled by several phytohormones such as ethylene, auxin, cytokinin, and gibberellin (McAtee *et al*., 2013). The involvement of ROS- or NO-dependent signaling in the early stages after pollination, such as fruit set and development, is mainly unexplored. In contrast, the ripening of fruits is a well-characterized process in regard to NO and ROS actions.

Ripening of climacteric fruits, such as mango or papaya, is an oxidative phenomenon, in which ROS play an important role (Mondal *et al*., 2004). Furthermore, it is known that ROS have a role in fruit development, but the available information is limited. In peach, two peaks of ROS content were observed by Huan *et al*. (2016) in the middle of fruit development, which proved to be important in the further development of the fruit, suggesting that ROS might act as positive signals in ripening. Hydrogen peroxide formation occurs during the ripening of tomato (Kumar *et al*., 2016), grape (Pilati *et al*., 2007), or guava (Mondal *et al*., 2009).

As for NO, it has anti-ripening and senescence effects, and is able to extend postharvest shelf-life of crops (Manjunatha *et al*., 2010). It is known that the NO level in unripe fruits is much higher than in ripe ones (Leshem *et al*., 1998). Principally, NO inhibits the autocatalytic biosynthesis of ethylene through its binding to 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase, resulting in the formation of the ACC-ACC oxidase-NO complex, which leads to the reduction of ethylene production (Rudell and Mattheis, 2006). Alternatively, NO is able to trigger ROS-associated redox changes, which may affect ethylene biosynthesis, but further research is needed to support these putative interactions (Manjunatha *et al*., 2012). Moreover, the major antioxidant enzymes like CAT, peroxidase (POX), and SOD are regulated by NO in peaches (Flores *et al*., 2008) or in kiwi fruit (Zhu *et al*., 2008). Also, polyphenolase, phenylalanine ammonia lyase, or lipoxygenase activities were shown to be affected by NO in fruit tissues (Duan *et al*., 2007; Zhu *et al*., 2009). Another link between ROS and NO can be the accumulation of salicylic acid triggered by NO and suppressing the level of superoxide radical and other ROS (Zhang *et al*., 2003), resulting in the maintenance of membrane integrity. During the ripening of pepper, protein nitration intensified and six proteins were identified to be nitrated (Chaki *et al*., 2015). Among them catalase was the most abundant in both green and ripped fruits. In red pepper fruits, the decrease of catalase activity suggests diminished H_2O_2 detoxification, which presumably promotes lipid peroxidation (Chaki *et al*., 2015).

The results available so far indicate that NO exerts its anti-ripening effect both through the modulation of ET biosynthesis and signaling, and through the modification of ROS metabolism. This also reveals an antagonistic relationship between NO and ROS during fruit ripening (Figure 14.2).

Conclusion

It is established that both ROS and NO are involved in the regulatory networks of all three phases of plant development from seed. However, in certain processes (e.g., seed or pollen tube germination, root system growth) their role and interplay are better known, whereas in others (e.g., shoot system development, flowering, fruit set) they are poorly characterized. Nevertheless, ROS–NO interaction may occur by direct chemical reaction between NO and $O_2^{\cdot-}$ (e.g., during seed germination, fruit ripening) or by the modifying effect of NO on ROS-detoxifying enzymes (e.g., during seed germination, pollen tube growth). Furthermore, there are common effector elements in their signal transduction, such as MAPK and cGMP during root hair or adventitious root formation, for instance, representing an additional level of ROS–NO cross-talk. So, thanks to intensive research, a lot of information is now available regarding the relevance of ROS and NO in plant growth and development, but many exciting questions remain unanswered.

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