

Chapter 2

Biosynthesis of Nitric Oxide in Plants

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Abstract Nitric oxide (NO) regulates important events in plant physiology, disease resistance and stress tolerance. In plants, distinct enzymatic and chemical processes can generate NO from nitrite (NO_2^-), L-Arginine and possibly other *N*-compounds. Reduction of NO_2^- to NO is catalyzed by nitrate reductase and the mitochondrial electron transport chain. Deoxygenated heme-proteins also facilitate NO production from NO_2^- . NO may also be released in nonenzymatic processes from nitrous acid and *S*-nitrosogluthathione. Whether plants have a specific enzyme with primary oxidative NO synthesizing activity is an open debate. Although, NO synthase-homolog genes are present in green algae, and a protein (AtNOS1/AtNOA1) with regulatory effects on oxidative NO synthesis is known in vascular plants, integration of the multiple NO producing processes requires a complex regulatory network in the plant cell. However, our insight into the underlying molecular mechanisms is still limited. Plant hormones, stress and injury signals, modulation of intracellular Ca^{2+} levels are the potential drivers of plant NO synthesis under physiological and stress conditions.

Keywords Cell signaling • Nitrate reductase • Nitric oxide synthase • Plant hormones

2.1 Introduction

Nitric oxide (NO) is a bioactive molecule with multifaceted physiological roles in plants (Röszer 2012b). Endogenous NO synthesis has been identified in cyanobacteria (Sturms et al. 2011), green algae (Foresi et al. 2010), lichens (Catala et al.

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2010), species representing pteridophyta, gymnosperms, monocots, and eudicots (Salmi et al. 2007; Röszer 2012b; Yu et al. 2012). As a signal molecule, NO is involved in germination, root morphogenesis, pollen tube growth, chloroplast biogenesis, transpiration, cell wall synthesis, and other biosynthetic pathways (Röszer 2012b). NO is implicated in the control of oxidative phosphorylation and photosynthesis and can protect the cell organelles from oxidative damage and consequently delay senescence and cell death (Röszer 2012b). In vascular plants, NO synthesis is an important element in acquiring disease resistance as well as adapting to distinct abiotic stressors such as salinity, cold, osmotic stress, hypoxia and excess absorption of minerals and heavy metals (Camejo et al. 2012; Chun et al. 2012; Lehotai et al. 2012; Sun and Li 2012; Tan et al. 2013). NO synthesis can also initiate programmed cell death in distinct species ranging from algae to vascular plants (Lombardi et al. 2010; Ma et al. 2010; Rosales et al. 2010; Yordanova et al. 2010; Pedroso et al. 2000).

To date, eight distinct enzymatic and nonenzymatic processes have been recognized which can elaborate NO in plants (Fig. 2.1). These include NO generation by the reduction of nitrite (NO_2^-), or by the oxidation of more reduced nitrogen compounds, such as the amino acid L-Arginine or hydroxylamine (Mur et al. 2013). Major sites of NO biosynthesis are the protoplasts and the chloroplasts, the mitochondria and the peroxisomes (Röszer 2012a, b). The cytoplasm, the cell membrane, the endoplasmic reticulum, and the apoplast can also generate NO in vascular plants (Fröhlich and Durner 2011) (Fig. 2.2).

2.2 Mechanisms of Reductive NO Synthesis

The cytoplasm, the mitochondria, the chloroplasts, the peroxisomes and the apoplast are sites of reductive NO generation from NO_2^- (Röszer 2012b). The NO_2^-/NO reduction can be catalyzed by assimilatory nitrate reductase (NR; EC 1.6.6.1, transferred to EC 1.7.1.1) or the mitochondrial electron transport chain (Fig. 2.1). Deoxygenated heme-containing proteins can also facilitate the reductive NO generation from NO_2^- (Röszer 2012b). Nonenzymatic NO_2^-/NO reduction can also occur in acidic compartments of the plant tissues (Röszer 2012b).

2.2.1 Reductive NO Synthesis by Nitrate Reductase

Nitrate reductase (NR), in addition to its primary nitrate (NO_3^-) oxidoreductase activity, is capable of reducing NO_2^- to NO with low efficacy (Rockel et al. 2002). The NR-catalyzed reduction of NO_2^- to NO is apparent in green algae and vascular plants (Röszer 2012b). NR-mediated NO synthesis is involved in physiological processes, pathogen defense and stress response (Mur et al. 2013). The presence of NR-catalyzed NO synthesis in the cyanobacterium *Anabaena doliolum* (Mallick

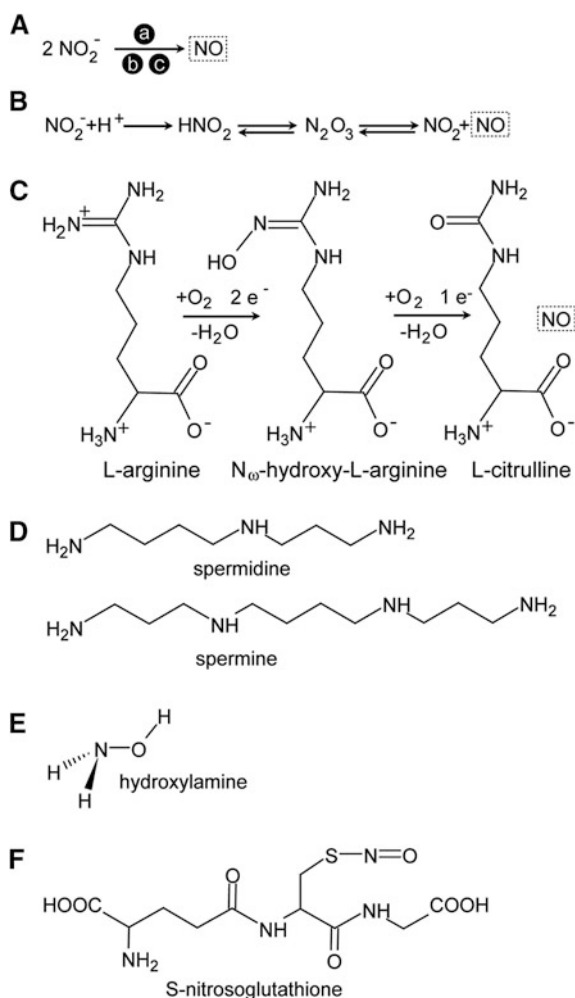


Fig. 2.1 Substrates and elicitors of NO synthesis in plants. **A** Reductive NO generation from nitrite can be catalyzed by nitrate reductase (a), the mitochondrial electron transport chain (b) or by deoxygenated heme-proteins (c). **B** Under acidotic conditions nitrous acid, the protonated form of nitrite can elaborate NO in a non-enzymatic process. **C** Oxidative NO synthesis from L-Arginine is catalyzed by a yet undefined NO-synthase in plants. To date the only plant-type NO-synthase encoding gene known is from green algae. **D**, **E** Polyamines and hydroxylamine can increase NO synthesis by unknown mechanisms. **F** NO can react with glutathione to form S-nitrosoglutathione, which can be a source of non-enzymatic NO liberation

et al. 1999) suggests that this mechanism may be one of the most ancient forms of NO generation in plants.

In green algae NR is associated with the pyrenoids and the thylakoid membranes of the chloroplasts and NR is responsible for the chloroplastic NO_2^-/NO reduction (Röszer 2012b). In vascular plants, a NO_2^-/NO reduction has also been

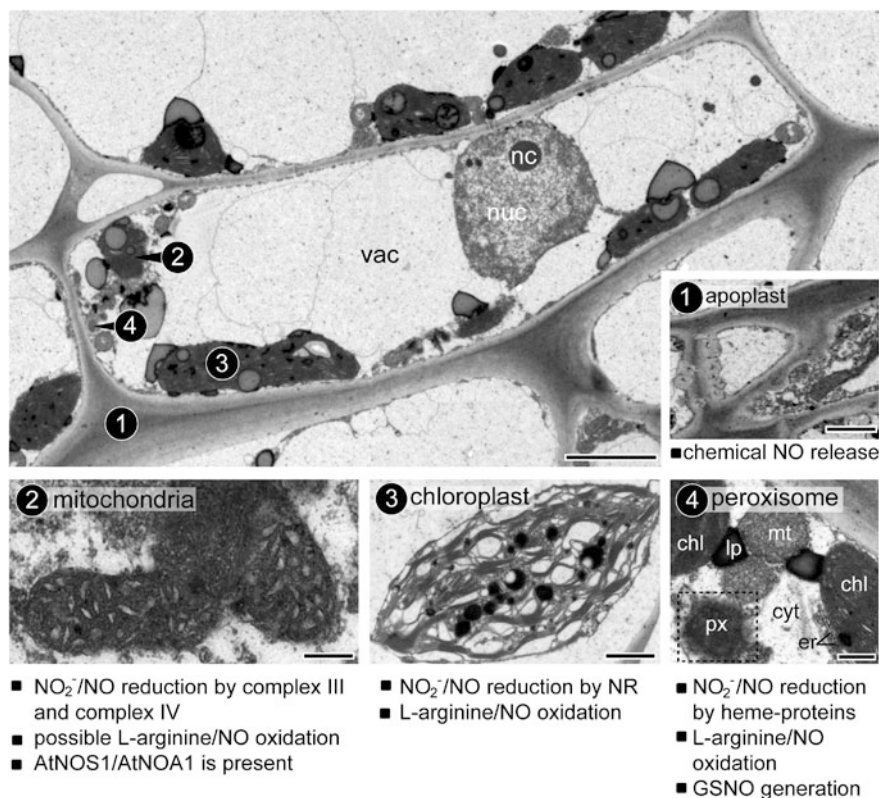


Fig. 2.2 Localization of distinct NO producing activities in the plant cell. The apoplast (1), the mitochondria (2), the chloroplasts (3) and the peroxisomes (4) are the most characterized NO-generating compartments in plants. The cytoplasm and the intracellular membranes can also be sites of enzymatic and chemical NO release. TEM images of *Scindapsus aureus* mesophyll cell. chl chloroplast, cyt cytoplasm, er endoplasmic reticulum, lp lipid droplet, mt mitochondria, nuc nucleus, nc nucleolus, px peroxisome, vac vacuole. Scale bar 2 μm (overview), 0.5 μm (inset 1), 0.2 μm (inset 2), 2 μm (inset 3), 1 μm (inset 4). Author's images

assayed in the chloroplasts, and the responsible enzyme may be a thylakoid-associated NR (Jasid et al. 2006). However, the main pool of NR is the cytoplasm and chloroplast association of NR is debated in vascular plants (Rószler 2012b).

Another possible NO_2^-/NO -reductase (NI-NOR) of vascular plants has been identified in the root plasma membrane of the tobacco, *Nicotiana tabacum* (Stohr et al. 2001). NI-NOR reduces NO_2^- to NO using reduced cytochrome c as an electron donor. NO generation of NI-NOR is comparable to the NO_3^- -reducing activity of a root-specific NR, however, NO-NOR can be a distinct protein, and it still remains to be characterized as NO producing enzyme (Stohr et al. 2001).

2.2.2 Reductive NO Synthesis by the Mitochondrial Electron Transport Chain

Mitochondria in green algae and higher plants can use NO_2^- as an alternative electron acceptor to sustain ATP synthesis under O_2 deprivation (Tischner et al. 2004; Gupta and Igamberdiev 2011). The mitochondrial respiratory chain is able to reduce NO_2^- to NO at the complex III (cytochrome *bc1*) and the complex IV (cytochrome-c oxidase, CcO) (Igamberdiev et al. 2010; Gupta and Igamberdiev 2011; Castello et al. 2006). This mechanism results in mitochondrial NO generation in plant cells experiencing hypoxia.

Hypoxia increases the activity and the transcription of NR, which converts NO_3^- to NO_2^- and leads to NO_2^- accumulation in the cytoplasm. In cells suffering from hypoxia, the further reduction of NO_2^- is limited allowing a sustained NO_2^- supply for reductive NO synthesis (Röszer 2012a, b). Due to the lack of a specific O_2 transporting system in plants, assimilating tissues in the leaf, stem or cells of rapidly growing tissues can be short of O_2 supply, making it possible that mitochondrial NO_2^-/NO reduction may be a common mechanism of NO synthesis in the plant tissues.

Mitochondrial NO_2^-/NO reduction may control the role in the initiation of seed germination. In many plants, seed dormancy is interrupted by imbibition, a process in which water penetrates the seed coat and generates a temporal hypoxic condition. Imbibition is associated with a rapid increase of NO levels (Liu and Zhang 2009), and possibly, favors the mitochondrial reductive NO synthesis (Gupta and Igamberdiev 2011). The NO generated within the mitochondria inhibits CcO, which eventually stimulates germination (Gniazdowska et al. 2010).

A recent model suggests that a recycling of $\text{NO}_2^-/\text{NO}/\text{NO}_3^-$ exists between the mitochondria and the cytoplasm experiencing hypoxia. This mechanism improves the energy status of cells suffering from O_2 limitation. Since NO inhibits electron transport to O_2 at the CcO site, mitochondrial NO production reduces further O_2 consumption when O_2 availability is already limited (Igamberdiev et al. 2010; Gupta and Igamberdiev 2011). The reductive mitochondrial NO generation also inhibits the photorespiratory cycle (Gupta and Igamberdiev 2011) and the fermentative metabolism (Oliveira et al. 2013). NO released from the mitochondria to the cytosol undergoes oxidation to nitrate (NO_3^-) by plant hemoglobin (class 1 nonsymbiotic hemoglobin), which is expressed in response to hypoxia (Igamberdiev and Hill 2004). As mentioned above, cytoplasmic NR reduces NO_3^- to NO_2^- , which recycles to the mitochondria and is being reduced to NO (Gupta and Igamberdiev 2011). This NO/NO_2^- exchange between the mitochondria and the cytoplasm maintains the NO_2^- supply for the ATP synthesis under hypoxia (Gupta and Igamberdiev 2011). The cytoplasmic $\text{NO}/\text{NO}_3^-/\text{NO}_2^-$ conversion keeps NADH/NAD⁺ and NADPH/NADP⁺ ratios low, ensuring a low redox level and helping the adaptation to O_2 limitation (Igamberdiev et al. 2010).

2.2.3 Reductive NO Generation by Heme Containing Proteins

Plant peroxisomes can also generate NO by NO_2^- reduction under hypoxic or anoxic conditions (Igamberdiev et al. 2010). The responsible mechanism may be NO_2^-/NO reducing ability of deoxygenated heme-containing proteins in the peroxisome matrix (Igamberdiev et al. 2010; Sturms et al. 2011). Similar reductive NO generation has been shown in the plant plasma membrane, cytosol and endoplasmic reticulum (Igamberdiev et al. 2010). Reduction of NO_2^- to NO by heme-proteins (e.g., hemoglobins) also occurs in cyanobacteria (Sturms et al. 2011) and mammalian tissues under O_2 limitation (Shiva et al. 2011; Tiso et al. 2011).

2.3 Mechanisms of Oxidative NO Synthesis

Oxidative NO synthesis from L-Arginine is also present in plant cells, although the responsible enzyme, the putative plant NO-synthase (NOS) has not yet been identified. In representatives of prokaryotes, unicellular eukaryotes, invertebrates, nonmammalian vertebrates and mammals, several NOS (EC 1.14.23.29) proteins and NOS-encoding genes have been identified (Röszer 2012b). Higher plants however, are lacking homolog sequences to already known NOS-encoding genes (Mur et al. 2013).

2.3.1 Oxidative NO Synthesis from L-Arginine

Enzymatic oxidation of L-Arginine to NO and L-Citrulline has been identified in the chloroplasts and the leaf peroxisomes of the vascular plants and in green algae (Röszer 2012b). The oxidation of L-Arginine to NO in the chloroplasts requires NADPH and is independent from Ca^{2+} supply (Jasid et al. 2006). In the leaf peroxisomes the L-Arginine/L-Citrulline conversion requires Ca^{2+} , calmodulin, FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide), and NADPH (Barroso et al. 1999; del Río et al. 2003; del Río 2011). Peroxisomal oxidative NO synthesis has been measured in the presence of BH_4 (tetrahydrobiopterine), although, other studies have shown that it is not required for NO synthesis in vascular plants (Röszer 2012b). It has been found recently, that oxidative NO synthesis from L-Arginine requires not only Ca^{2+} and NADPH but also BH_4 in the green algae *Ostreococcus* species (Foresi et al. 2010). Plant mitochondria may also oxidize L-Arginine to NO and the responsible enzyme may be present in the mitochondrial matrix or the intermembrane space (Guo and Crawford 2005). However, it is debatable whether plant mitochondria contain a specific enzyme which is responsible for the oxidative NO synthesis (Barroso et al. 1999).

2.3.2 The Enigmatic Plant-Type NOS

Homolog genes of mammalian NOS have been identified recently in the genome of the marine green algae *Ostreococcus tauri* and *Ostreococcus lucimarinus* (Foresi et al. 2010). The recombinant *O. tauri* NOS (OtNOS) protein shares 44 % sequence overlap with human NOS3, 45 % with human NOS1 and NOS2.

To date OtNOS is the only NOS found in plants. The enzyme responsible for the NOS-like activity in higher plants is still a subject of debate (Mur et al. 2013). A protein recognized by an antibody against mammalian NOS2 has been found in the leaf peroxisomes and the chloroplasts of *Pisum sativum* (Barroso et al. 1999; Corpas et al. 2001; del Río et al. 2003). NOS has already been characterized in many prokaryotes (bacteria and archaea). Since chloroplasts are descendants of ancient endosymbiotic cyanobacteria, one can assume that the NOS2 immunoreactive protein of the chloroplast stroma might be a cognate of a prokaryote NOS molecule (Röszer 2012b). However, the NOS molecule responsible for L-Arginine dependent NO synthesis of the chloroplasts still remains unknown (Mur et al. 2013).

One possible plant-specific NOS has been described in the mitochondria of *Arabidopsis thaliana* (Guo et al. 2003). This putative NOS molecule has been identified based on its gene sequence homology (23 % identity, 39.5 % similarity) to a putative NOS of the snail *Helix pomatia* (Huang et al. 1997). This 561-amino acid *Arabidopsis* protein has been annotated as *A. thaliana* NOS-1 (AtNOS1), later renamed as *A. thaliana* NOS-associated protein-1 (AtNOA1). It has been shown that AtNOS1 can oxidize L-Arginine to NO in a NADPH and Ca²⁺ dependent mechanism and its activity is sensitive to mammalian NOS inhibitors (Guo et al. 2003). However, AtNOS1 does not show sequence similarities to mammalian NOS isoforms (Guo et al. 2003) and further studies have concluded that AtNOS1 is a GTPase protein (Moreau et al. 2008; Sudhamsu et al. 2008). Similarly, the putative NOS in *Helix pomatia* is more likely to be an NOS-associated protein rather than a NO producing enzyme (Röszer et al. 2010).

Collectively, these data suggest that AtNOS1/AtNOA1 (*A. thaliana* NOS-associated protein 1) and its orthologs may be involved in NO synthesis only in an indirect way, by allowing proper NO synthesis of a yet undefined NO producing molecule. Since AtNOS1/AtNOA1-associated protein 1 (AtNOS1/AtNOA1) is a GTPase, it is possible that AtNOS1/AtNOA1 generates cGMP (cyclic guanosine monophosphate) and activates downstream NO signal pathways (Moreau et al. 2008). For instance, a mammalian AtNOS1-related protein is implicated in mitochondrial protein synthesis (Kolanczyk et al. 2011), thus it might have an indirect effect on the maintenance of NO production. Of note, AtNOS1/AtNOA1 is associated with the plant mitochondria, where reductive NO synthesis can overshadow a putative NOS-like activity.

2.3.3 Other Forms of Oxidative NO Synthesis

Recently it has been shown that polyamines and hydroxylamine can increase the oxidative NO synthesis in plant cells (Tun et al. 2006; Rumer et al. 2009; Wimalasekera et al. 2011). NO can mediate effects of polyamines in plants, however, the manner in which polyamines can increase NO synthesis is uncertain (Fröhlich and Durner 2011). Possible mechanisms include an interaction of polyamines with the NR-catalyzed NO production (Rosales et al. 2012) and the indirect effect of polyamine synthesis on L-Arginine metabolism (Zhang et al. 2011). Interestingly, polyamine synthesis is inhibited by NO, and *A. thaliana* plants lacking AtNOA1 accumulate polyamines, rendering a yet unexplored interplay between polyamines and NO biosynthesis in plants (Yamasaki and Cohen 2006; Majlath et al. 2011; Filippou et al. 2012). Hydroxylamine, an intermediate in the process of nitrification, can be oxidized to NO in tobacco cell cultures (Rumer et al. 2009). This mechanism may be an alternative of L-Arginine dependent oxidative NO synthesis. However, the underlying molecular mechanism is still unknown and the sufficient availability of hydroxylamine for NO synthesis is debated (Rumer et al. 2009). Other enzymes, such as xanthine oxidase, catalase) and horseradish peroxidase are able to elaborate NO under specific conditions (Huang et al. 2002; Igamberdiev et al. 2010; del Río 2011), however, their possible contribution to NO synthesis in plants is still yet to be ascertained.

2.4 Nonenzymatic NO Release

NO can be released from nitrous acid (HNO_2), a protonated form of NO_2^- . This type of chemical NO release is favored by acidic environments found, e.g., in the apoplast of germinating and thus hypoxic seeds (Yamasaki 2000; Bethke et al. 2004a). Accordingly, the NO liberation from NO_2^- has been shown in the apoplast of the aleuron layer of the barley, *Hordeum vulgare* (Bethke et al. 2004a). This nonenzymatic NO release is augmented by phenolics, compounds found in the aleuron apoplast and in the seed coat (Bethke et al. 2004a). In germinating seed, the NO release may provide an antimicrobial protection for the seeds in the soil (Bethke et al. 2004a). Moreover, seed dormancy is interrupted by NO, thus a NO generation from NO_2^- along with an enzymatic NO synthesis can contribute to the proper germination (Röszer 2012b). NO-mediated programmed cell death also occurs during germination, when the aleuron cells are being eliminated (Lombardi et al. 2010). Collectively, NO release can act synergistically with the enzymatic NO_2^-/NO reduction to evoke a NO burst during germination.

Another possible but yet unexplored mechanism of nonenzymatic NO generation is the release of NO from S-nitrosoglutathione (GSNO) (del Río 2011). This compound is formed in the oxidative environment of the peroxisomes, where both NO and the NO-derived peroxynitrite can react with glutathione to generate GSNO

(Barroso et al. 2006). GSNO behaves as a NO-donor compound and can be a transportable NO reserve distributed in the plant tissues. Genesis of NO from GSNO is facilitated by ambient light and transition metals (Floryszak-Wieczorek et al. 2006). As was described above, hydroxylamine is a possible substrate of NO synthesis (Rumer et al. 2009), however, it is uncertain that GSNOR would support NO production with hydroxylamine.

2.5 Control of NO Synthesis in the Plant Cell

Deficiencies in genes implicated in NO homeostasis lead to severe alterations in plants, underlining the importance of balanced NO production and elimination (Fröhlich and Durner 2011). However, the mechanisms which control plant NO homeostasis are largely undefined. Chemical and enzymatic NO synthesis can occur simultaneously, for example in germinating seeds (Bethke et al. 2004a; Gupta and Igamberdiev 2011). The multiplicity of NO-producing mechanisms makes plant-type NO homeostasis a complex phenomenon. As a framework for understanding the control of NO levels, we provide an overview on the potential mechanisms which can control NO synthesis. These include the regulation of substrate and cofactor availability; the chemical environment which allows non-enzymatic NO release and certain upstream signaling events that can modulate transcription and activity of NO producing enzymes.

2.5.1 Control of Reductive and Oxidative NO Synthesis

Main sources of NO in plant cells are NO_2^- and L-Arginine, thus their levels are key determinants of NO synthesis. Stress conditions, including hypoxia, inhibition of the photosynthetic electron transport or increased NO_2^- absorption from the soil lead to excessive NO_2^- accumulation in the cytoplasm, which favors NO_2^- reduction to NO (Gupta et al. 2010; Mur et al. 2013). Cytoplasmic NO_2^- can be removed through increased influx into the vacuole or efflux from the cell, however, it is yet uncertain how these mechanisms can be integrated to control NO synthesis (Mur et al. 2013). Light exposure promotes the chloroplastic reduction of NO_2^- to NH_4^+ , which impedes reductive NO generation (Sakihama et al. 2002; Röszer 2012b).

When L-Arginine is abundant and NO_2^- availability is limited, oxidative NO synthesis can be the dominant form of NO generation. Accordingly, *A. thaliana* mutants which accumulate L-Arginine in the chloroplast display increased NO synthesis (Streatfield et al. 1999; He et al. 2004). Increasing L-Arginine availability in *Arabidopsis* plants by inhibiting arginase activity also leads to an increased NO production (Flores et al. 2008; Shi et al. 2013). Importantly, the carbohydrate and ATP supply of L-Arginine synthesis is provided by the photosynthetic light

reactions, therefore L-Arginine production positively correlates with photosynthetic activity (Krueger and Kliever 1995). The light reactions of the photosynthesis also sustain the appropriate NADPH and O_2 supply for the oxidative NO synthesis (Jasid et al. 2006). Active photosynthesis also favors the consumption of NO_2^- in amino acid synthesis through reduction to NH_4^+ . Interestingly, L-Arginine inhibits chloroplastic NO_2^- uptake (Ferrario-Mery et al. 2008). These findings suggest that light exposure and photosynthesis increases the L-Arginine pool and reduces NO_2^- levels within the chloroplast, thus favoring oxidative and inhibiting reductive NO synthesis. Although a recent study proposes that a NOS-like activity may be the only source of NO in the chloroplast (Tewari et al. 2013), several others provide evidence that both reductive and oxidative NO generation takes place in the chloroplast (Röszer 2012b). Oxidative and reductive NO synthesis may be temporally separated, i.e., due to a photoperiodic change in L-Arginine and NO_2^- availability (Röszer 2012b).

2.5.2 Hormonal Control of NO Synthesis

To date, some chemical signals have already been identified as elicitors of NO synthesis (Table 2.1). The plant hormone auxin can increase both reductive and oxidative NO synthesis (Kolbert et al. 2008; Jin et al. 2011). It implies that the same signal can impact distinct forms of NO generation. NO is a downstream mediator of other plant hormones, such as cytokinins, abscisic acid and brassinosteroids (Beligni and Lamattina 2000; Tun et al. 2001; Ötvös et al. 2005; Kolbert et al. 2008; Zhang et al. 2010; Romera et al. 2011; Liu et al. 2013) but it is unknown how these hormones regulate NO synthesis. Salicylic acid, which has prominent roles in host defense against fungal and oomycete pathogens enhances NO synthesis in *A. thaliana* (Zottini et al. 2007) and tomato *Solanum lycopersicum* (Poór and Tari 2012). Expression of NR is increased in response to salicylic acid (Caamal-Chan et al. 2011). Increased NR expression can explain the elevated NO synthesis under stress conditions; however, salicylic acid induced NO synthesis can be associated with oxidative NO production (Zottini et al. 2007). In the green alga *Chlamydomonas reinhardtii* ethylene has been described as elicitor of NO synthesis under stress conditions (Yordanova et al. 2010). In vascular plants recent findings also point to the possible involvement of ethylene, a mediator produced under stress conditions and injury (Poór et al. 2013), however, the molecular link between abiotic stressors and increased NO synthesis is yet undefined.

Some mechanisms which lower NO levels have also been described in plants (Table 2.1). Oxygenated nonsymbiotic hemoglobins and glutathione are important sinks for NO (Igamberdiev and Hill 2004). A recent study shows that zeatin, a prevalent cytokinin in *Arabidopsis* can interact with NO and reduce intracellular NO levels (Liu et al. 2013). Similarly, in cadmium toxicity, gibberellic acid reduces NO accumulation (Zhu et al. 2012). GSNOR activity is also important in

Table 2.1 Signaling mechanisms affecting NO levels in the plant cell

Signal or stimulus	Effect on NO production	References
Auxin	Increases oxidative NO synthesis and NR-mediated reductive NO generation	Ötvös et al. (2005), Kolbert et al. (2008), Jin et al. (2011), Beard et al. (2012)
Abscissic acid	Increases NR-dependent NO synthesis	Bethke et al. (2004b), Bright et al. (2006)
Brassinosteroids	Increase NO levels, possibly stimulate oxidative NO synthesis	Zhang et al. (2010)
Cytokinins	Increase NO levels, possibly through oxidative NO synthesis	Tun et al. (2001)
Salicylic acid	Upregulates NR expression and increases NO synthesis, possibly stimulating oxidative NO synthesis	Zotini et al. (2007), Sun et al. (2010), Caamal-Chan et al. (2011)
Ethylene	Stimulates NO synthesis in green algae, possibly plays a similar role in vascular plants	Yordanova et al. (2010), Poór et al. (2013)
Carbon dioxide	Increases oxidative NO synthesis	Wang et al. (2013)
Ca ²⁺ , PKC	Increase oxidative NO synthesis	Zotini et al. (2007), Talwar et al. (2012)
Lipopolysaccharides	Evoke NO burst	Zeidler et al. (2004), Sun and Li (2012)
Oligogalacturonides	Induce NR-dependent NO synthesis	Rasul et al. (2012)
Hypoxia	Increases NR expression, sustains NO ₂ ⁻ supply for NO production, favors mitochondrial reductive NO production, promotes non-enzymatic NO release	Liu and Zhang (2009), Igamberdiev et al. (2010), Gupta and Igamberdiev (2011), Sturms et al. (2011)
Cold, osmotic stress	Increase NO synthesis	Wang et al. (2012), Tan et al. (2013)
Gibberellin	Can reduce NO levels under stress conditions	Fernandez-Marcos et al. (2012)
Zeatin	Scavenges NO	Liu et al. (2013)
Oleic acid	Increases AtNOAI degradation	Mandal et al. (2012)

eliminating NO in plant cells under stress conditions (Barroso et al. 2006; Lee et al. 2008). Turnover of NO generating proteins can also affect cellular NO homeostasis, however, this possibility is not analyzed in details in plants. A recent study shows that oleic acid can bind to AtNOA1 and increase its degradation in a protease-dependent manner (Mandal et al. 2012). Oleic acid is involved in pathogen defense signaling (Kachroo et al. 2008), thus increased oleic acid levels can moderate NO synthesis in infected plants (Mandal et al. 2012). It is also possible, that NO can diminish its own production, as suggested by the inhibition of NR activity by NO (Rosales et al. 2010).

2.6 Summary and Open Debates

NO plays important roles in plant physiology, disease resistance, and stress tolerance. Various enzymatic and chemical processes elaborate NO in plants; however, there are significant gaps in our understanding of plant-type NO homeostasis. A well characterized NO producing plant enzyme is NR, which generates NO as a secondary activity. Similarly, the mitochondrial electron transport chain and deoxygenated heme-proteins also facilitate NO generation from NO_2^- , although, they are not dedicated NO synthesizing enzymes. Future research should identify the enzymes responsible for oxidative NO synthesis from L-Arginine and answer the open debate whether plants have a specific enzyme with primary NO synthesizing activity. The mechanism of NO synthesis from various *N*-compounds should also be defined, as they can provide alternatives of L-Arginine dependent NO synthesis in plants. Nonenzymatic processes contribute to NO generation, however, their physiological relevance still remains elusive. Plant hormones, stress and injury signals, modulation of intracellular Ca^{2+} levels have the potential to drive NO synthesis in the plant cell. Integration of the distinct NO producing processes requires a complex regulatory network; however, our insight into the underlying molecular mechanisms is still limited.

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