Chapter 5
Gasotransmission of Nitric Oxide (NO) at Early Plant Developmental Stages

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Abstract The versatility of nitric oxide (NO) as a free radical that mediates numerous biological functions within early plant development is widely accepted. NO action in seed germination and root developmental processes involves a complex signaling pathway that includes the cellular redox levels, the posttranslational modification of specific proteins by S-nitrosylation, and the interaction with other plant growth regulators (i.e., phytohormones) using similar molecular components. Recent evidence indicates that changing levels of this reactive nitrogen species (NO) may also fine-tune the molecular mechanisms by which NO leads to changes in seed germination and root growth. This chapter briefly introduces the key processes for the NO transmission during seed germination and root development and focuses on the sensing mechanisms underlying the effects of NO and its interaction with other plant hormones linking these changes.

1 Introduction

Nitric oxide (NO) is a gaseous lipophilic free radical with a very short life (Lamattina et al. 2003). Due to its chemical nature, it can react with different molecules, as a key signaling element (Fig. 5.1). NO has been described to possess a high reactivity with other radical and transition metal ions (Wink and Mitchell 1998; Thomas et al. 2008), influencing the cellular redox status. NO release is related to reactive nitrogen species (RNS) production, including nitrosonium (NO^+)

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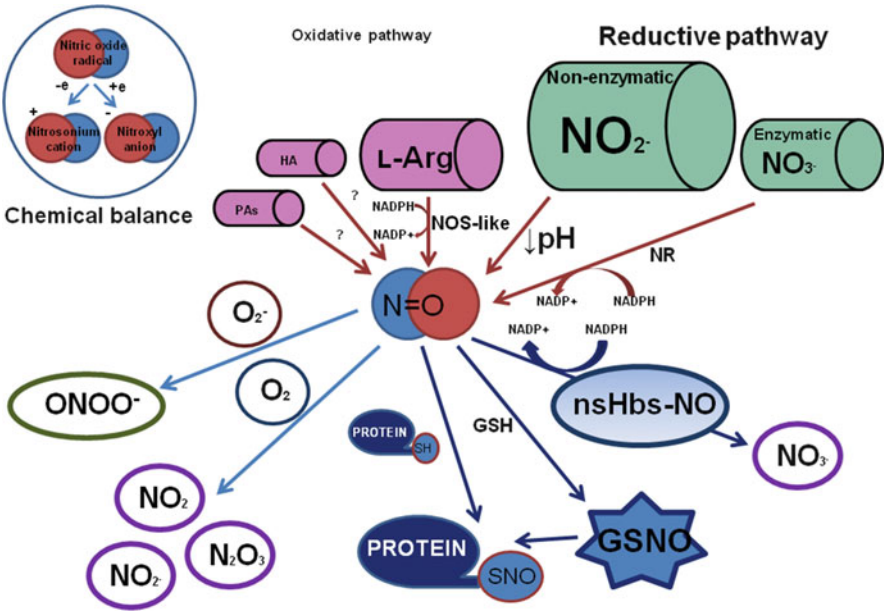

Fig. 5.1 Formation of reactive nitrogen species (NO) with emphasis on the diverse enzymatic or nonenzymatic reactions potentially involved in NO synthesis in seeds. Box sizes correspond to the relevance of the pathway. *AtNOA Arabidopsis thaliana* nitric oxide associated, *NR* nitrate reductase, *HA* hydroxylamine, *PAs* polyamines, *L-Arg* L-arginine, *GSNO* S-nitrosoglutathione, *GSH* S-nitrosoglutathione reduced, *nsHbs* nonsymbiotic hemoglobins, *NO* nitric oxide, NO_3^- nitrate, NO_2^- nitrite, N_2O_3 nitrogen trioxide, $NO_2^{\bullet}$ nitrogen dioxide radical, $O_2^{\bullet -}$ superoxide, $ONOO^-$ peroxynitrite. Adapted from Moreau et al. (2010)

and nitroxyl anion (NO^-), resulting from a gain or loss of one electron by NO, and peroxynitrite ($ONOO^-$) product of the reaction with superoxide anion radical (O_2^-) (Stamler et al. 1992b). The reaction between NO and molecular oxygen (O_2) leads to the formation of nitrogen dioxide (NO_2), nitrous anhydride (N_2O_3), nitrite (NO^-_2), and nitrate (NO^-_3). In the cell environment, a strict NO and RNS spatiotemporal control is fundamental to maintain the proper conditions for the signaling events inside the plant cell (Moreau et al. 2010; Baudouin 2011). In addition, NO homeostasis is an important physiological event in controlling the development along all the plant life cycle. Specifically, this tight line is fundamental for seed germination and the proper seedling establishment.

Plants have developed sophisticated mechanisms for NO sensing to trigger a variety of specific responses in their life cycle. Among them, S-nitrosylation has been proposed as the most relevant posttranslational modification directed by NO through which plants are able to sense this gaseous redox signal (Stamler et al. 1992a, b; Hess et al. 2001; Astier and Lindermayr 2012; Kovacs and Lindermayr 2013). After the covalent union of NO groups to free thiols of cysteine residues in proteins, some conformational changes may occur in the protein structure and consequently affect to their biological function and stability (Stamler

et al. 2001; Hess et al. 2005). By this signaling mechanism, NO is involved in multiple hormonal regulatory processes, acquiring an essential role to achieve the success throughout early plant physiological stages (i.e., seed germination and root growth).

2 Gasotransmitter Function of NO in Seeds

Seeds are the dispersal organs of higher plants, being of key relevance to the establishment and survival of a new plant generation. Additionally, seeds constitute one of the most important features in global feeding, representing a basic component in the whole world diet.

Germination starts with the uptake of water (triphasic event) by the dry seed in a quiescent state, finishing with the protrusion of the embryonic radicle through the seed coat layers, the endosperm, and the testa (Bewley 1997; Weitbrecht et al. 2011). To optimize germination, seeds show an adaptive mechanism known as dormancy, which can be defined as a failure of an intact viable seed to complete germination under favorable conditions (Bewley 1997; Finch-Savage and Leubner-Metzger 2006; Graeber et al. 2012).

Dormancy and germination are closely related, which makes it difficult to treat them like two separate events. At the end, the research related to one or the other comprises a balance between the germination promotion and the dormancy degree, a very tight genetic and physiological line. Both correlated processes are regulated by internal (complex transcriptional and hormonal crosstalk) and external (environmental) factors. At the hormonal level, germination depends on abscisic acid (ABA), gibberellins (GAs), auxins, cytokinins (CKs), salicylic acid, brassinosteroids, and ethylene (ET) (Bentsink and Koornneef 2008; Lee and Park 2010; Finkelstein 2013). Among them, GA and ABA crosstalk balance has proved to control the main part of this network (Finkelstein et al. 2008; Nambara et al. 2010). Several environmental factors affect dormancy and germination, such as after-ripening, light, water soil content, cold treatment and nitrate compounds (Alboresi et al. 2005; Penfield et al. 2005; Holdsworth et al. 2008; Arc et al. 2012). The production of seeds is also influenced by the conditions of the mother plant (Munir et al. 2001). Anyhow, the need for one of these factors requires the interplay of the rest, being of key importance to the global interaction map for proper seedling establishment (Seo et al. 2009; Footitt et al. 2011).

Recent research has shown the fundamental role of redox state in governing the central processes during seed germination (Marx et al. 2003; Bykova et al. 2011a, b; Diaz-Vivancos et al. 2013). Thus, reactive oxygen and nitrogen species (ROS/RNS) are the main players related with stress and considered to be detrimental to seed viability so far. Recent discoveries have change the known paradigm regarding these species—from toxic by-products of oxidative metabolism to key regulators of cellular functions (Bailly et al. 2008; Oracz et al. 2009; Leymarie et al. 2012; Kumar et al. 2015). The balance of ROS/RNS determines their role in seed

germination. Understanding the mechanisms implicated in this tight regulation is of key relevance for the improvement and maintenance of seed longevity, vigor, and quality, which are the basis of crop yield. One of the main molecules involved in these processes is NO. This gaseous free radical constitutes a key player during the regulation of seed dormancy and germination.

2.1 Physiological Role of NO in Dormancy and Germination

NO is able to release seed dormancy and promote seed germination in different species (Bethke et al. 2007). In the last years, pharmacological approaches using NO donors or scavengers modified the NO levels in seeds to mimic the effect of this gas during dormancy breaking and seed germination (Bethke et al. 2011). According to this idea, the application of NO donors (i.e., SNAP, SNP, and GSNO) breaks seed dormancy and promotes seed germination in *Arabidopsis* and barley, while the NO scavenger cPTIO induces the maintenance of seed dormancy (Bethke et al. 2004b, 2006a, b; Libourel et al. 2006). Furthermore, this kind of studies was extended to other species and confirmed that NO is also a potential regulator of seed germination in warm-season C4-grasses (Sarath et al. 2006). In order to understand the contribution of the embryo, aleurone layer, and testa to seed dormancy and determine where NO is sensed in the seed, several experiments were carried out. Seeds lacking a testa responded to NO, indicating that the site of NO sensing was in the aleurone cells or in the embryo (Bethke et al. 2007). Growing isolated embryos were insensitive to NO scavenger cPTIO, and NO did not increase significantly the growth potential of embryos in dormant seeds, which indicates that embryos do not sense NO (Bethke et al. 2007). Moreover, experiments with the NO scavenger in isolated aleurone layers demonstrated that this tissue senses and responds to NO because vacuolation of isolated aleurone layers was inhibited by the NO scavenger (Bethke et al. 2007). Furthermore, barley aleurone layers produce NO upon addition of nitrite or nitrate to the incubation medium (Vitecek et al. 2008). All these results indicate that the aleurone layer senses and responds to NO during dormancy release.

2.2 NO and Phytohormones During Germination

Hormonal balance acts as an integrator of environmental cues to maintain dormancy or activate germination in seeds (Arc et al. 2013a). The interaction between different hormones, such as ABA, GA, ET, CK, and polyamines (PAs) and NO in these processes, has been thoroughly described (Fig. 5.2; Arc et al. 2013a; Sanz et al. 2015). ABA and NO play opposite roles: while ABA induces and maintains seed dormancy, inhibits seed germination, and controls post-germination developmental checkpoints (Finch-Savage and Leubner-Metzger 2006; Finkelstein 2013),

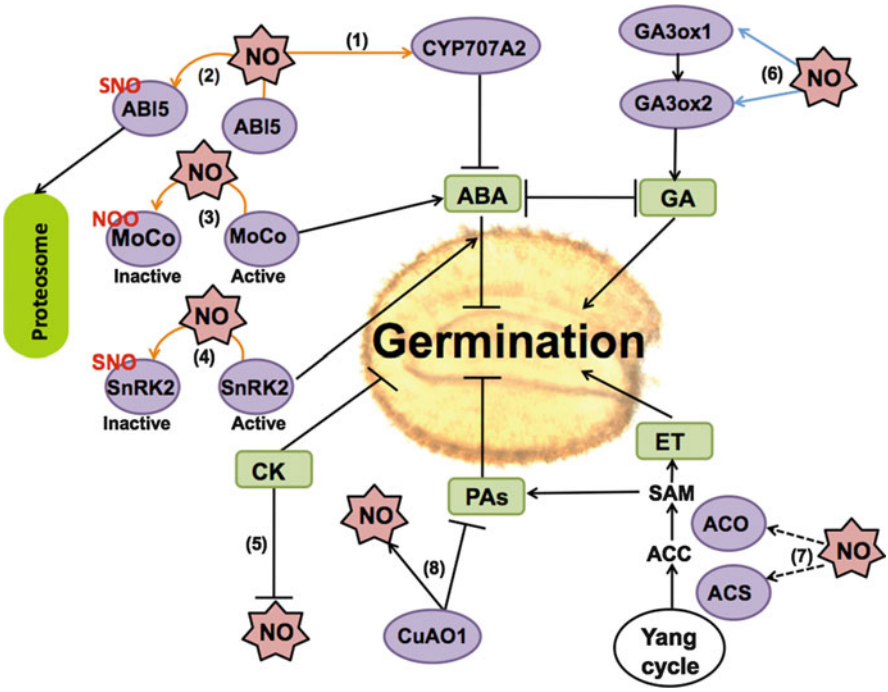

Fig. 5.2 Crosstalk between NO and key phytohormones (ABA, GAs, ET, PAs, and CKs) in *Arabidopsis* seed germination. During germination, NO induces ABA catabolism through the transcriptional upregulation of CYP707A2 (cytochrome P450 ABA 8′-hydrolase) (1: Liu et al. 2009). ABI5 is S-nitrosylated and degraded by the proteasome (2: Albertos et al. 2015). Molybdenum cofactor (MoCo) sulfurase ABA3 is inactivated by nitration and thus could inactivate ABA synthesis (3: Arc et al. 2013a). NO can inactivate SnRK2.2 by S-nitrosylation and negatively regulates ABA signaling (4: Wang et al. 2015). CKs suppress the action of NO leading to the reduction of endogenous NO levels (5: Liu et al. 2013). NO promotes GA biosynthesis by upregulating *GA3ox1* and *GAox2* transcription (gibberellic acid oxidases 1 and 2) (6: Bethke et al. 2007). ET biosynthesis enzymes may be S-nitrosylated although it is unknown how this posttranslational modification can modify their activity during germination (7: Freschi 2013). CuAo1 (copper amine oxidase) induces NO biosynthesis and participates in ABA signaling (8: Wimalasekera et al. 2011). *Arrows* and *bars* indicate positive and inhibitory effects. *Blue arrows* represent transcriptional upregulation, *orange arrows* posttranslational modifications, and *dashed arrows* putative processes of S-nitrosylation

NO acts as a dormancy-relieving molecule and promotes seed germination (Bethke et al. 2004b). The first point of crosstalk between both molecules has been established at the synthesis level. Thereby endogenous NO content increases after exogenous ABA application in *Arabidopsis* and tobacco plant tissues (Guo et al. 2003a, b; Bright et al. 2006; Liu et al. 2009) and in the apoplast of the aleurone cell layer during barley seed germination (Bethke et al. 2004a, b). Furthermore, the treatment with both ABA and NO donor together on dormant seeds triggers a reduction in hormone sensitivity and therefore germination rates are increased (Bethke et al. 2006b). This accumulation of NO is related with a decrease

of ABA (Liu et al. 2009). ABA inactivation by CYP707A during seed maturation also regulates dry seed ABA levels and dormancy depth, as concluded from *cyp707a* mutant analysis (Okamoto et al. 2006). Additionally, SNP treatment significantly enhances CYP707A protein levels, while cPTIO decreases it. This increase in protein levels is correlated to a NO-induced ABA decrease (Liu et al. 2009). Moreover, NO modifies the activity of proteins implicated in ABA signal transduction and biosynthesis through S-nitrosylation and nitration (Sanz et al. 2015). *SnRK2.2* and *SnRK2.3* are *sucrose non-fermenting 1 (SNF1)-related protein kinases* that are mainly expressed in seeds and young seedlings. They play redundant roles in ABA inhibition of seed germination (García-Mata et al. 2003). SnRK2.2 can be inactivated by GSNO treatment through S-nitrosylation (Wang et al. 2015). Furthermore, S-nitrosylated abscisic acid insensitive 5 (ABI5) transcriptional activator is degraded by the proteasome during germination (Albertos et al. 2015). The physiological meaning of this modification will be discussed below. Proteomic analyses of nitrated proteins identified the molybdenum cofactor (MoCo) sulfurase ABA3, a protein involved in ABA synthesis (Mendel 2007), as a target of protein nitration (Lozano-Juste et al. 2011). These posttranslational modifications contribute to decreased ABA content and therefore dormancy release and germination promotion (Arc et al. 2013a). CK and NO have antagonistic effects in the control of germination (Bethke et al. 2006a, b; Riefler et al. 2006). Under physiological conditions, CK acts as a negative regulator of seed germination. Loss-of-function mutants in three *Arabidopsis thaliana* sensor histidine kinases, *AHK2*, *AHK3*, and *CRE1*/*AHK4*, showed faster germination in the dark (Riefler et al. 2006). The interaction between the hormone and the gas molecules during germination is unclear, but studies performed in seedlings indicated that NO could be catalyzed by the action of CK. NO-insensitive mutants *cnu1* (*continuous NO-unstressed 1*) have elevated levels of CK (Liu et al. 2013). A double mutant of *cnu1-2* and *nitric oxide overexpression 1* (*nox1*) showed reduced severity of the phenotypes related to increased NO levels, similarly to the treatment of the *nox1* line with *trans*-zeatin. Also peroxynitrite, an active NO derivative, can react with zeatin in vitro. All these results together could indicate that cytokinins might suppress the action of NO leading to the reduction of endogenous NO levels (Liu et al. 2013). On the other hand, seed germination is promoted by GA and ET. NO is required for the transcription of two active GA biosynthetic enzymes (*GA3ox1* and *GA3ox2*). Moreover, GA is required for cell vacuolation in isolated aleurone layers in absence of NO (Bethke et al. 2007). Therefore, NO could coordinate a reduction in ABA-imposed dormancy with the onset of GA-stimulated germination (Sanz et al. 2015). It is well known that ET plays a crucial role in the control of seed dormancy removal and early germination (Kucera et al. 2005; Matilla and Matilla-Vazquez 2008). A crosstalk between NO and ET has been described in the regulation of dormancy release and germination onset (Gniazdowska et al. 2010). NO stimulated embryo germination in an ET-dependent manner in apple embryos by inducing ET biosynthesis (Gniazdowska et al. 2010). Thus, the interaction could be established at the biosynthesis level. Treatments with NO donors induce the

activity of two enzymes of ET biosynthetic pathway, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, and ACC oxidase (ACO). Proteomic analysis of *Arabidopsis* plants revealed that several enzymes that participate in ET biosynthesis may be S-nitrosylated (reviewed in Freschi 2013), although it is unknown how this posttranslational modification can modify their activity in the germination process. PAs negatively regulate germination (Gallardo et al. 1994). They are accumulated in relatively high quantities in mature dry seeds of some species, and their concentration can be altered by the process of stratification (Matilla 1996). NO production increases when PAs are catabolized and it is a potential intermediate of their action (Tun et al. 2006; Wimalasekera et al. 2011). According to this idea, an enzyme implicated in PA catabolism (copper amine oxidase, CuAo1) induces NO biosynthesis and participates in ABA signaling, based on the ABA-hyposensitive phenotype of *cuao1* mutants (Wimalasekera et al. 2011). Loss-of-function mutants for *CuAO1* show lower NO production in response to exogenous PAs and are less insensitive to exogenous ABA addition during germination (Wimalasekera et al. 2011).

2.3 NO Homeostasis in the Seed

Whereas in animal systems the synthesis of NO is widely described, in plants the mechanisms leading to changes in NO levels are poorly understood. To date, different possible pathways have been described, classified as reductive and oxidative (Gupta et al. 2011a). We will summarize the main mechanisms taking place in seeds as illustrated in Fig. 5.1 (Sirova et al. 2011; Arc et al. 2013a, b). A nonenzymatic pathway in which nitrite is reduced to NO is of key relevance in the seed, because of the acidic conditions in the apoplast (Bethke et al. 2004a, 2007a) and in hypoxic mitochondria (Igamberdiev et al. 2010; Gupta and Igamberdiev 2011). Nitrate reductase (NR) and NO synthase-like activity (NOA) contribute as enzymatic NO sources (Simontacchi et al. 2004, 2006). NR (NIA1, NIA2) catalyzes the reduction of nitrate to nitrite, but it is also able to reduce nitrite to NO using NADPH as a cofactor (Bethke et al. 2004a). NOA1 is a GTPase that plays a role in ribosomal assembly and stability, but the *atnoa1* mutant lacking this gene function presents lower NO levels (Guo et al. 2003a). The triple *nia1nia2noa1-2* and the *atnoa1 Arabidopsis* mutants display increased dormancy and decreased germination rate (Lozano-Juste and Leon 2010). Other alternative oxidative synthesis pathways could produce NO from hydroxylamine, PAs, or L-arginine.

There are distinct mechanisms in which NO balance is regulated in the seed. This molecule can react with reduced GSH (or another thiol protein) to produce *S*-nitrosothiols (GSNO and S-nitrosylated proteins). In fact, GSNO is suggested to be a storage/transporter NO form (Sakamoto et al. 2002). Another NO scavenging pathway is composed by nonsymbiotic hemoglobins (nsHb) due to its heme binding group (Gupta et al. 2011b; Hill 2012). In plants, nsHbs are divided into two main

forms depending on their oxygen affinity properties. nsHb1 constitutes a key player because of its implication in the hemoglobin-NO cycle (Igamberdiev et al. 2010). This hemoglobin can metabolize NO into nitrate acting as an NADPH-dependent dioxygenase (Perazzolli et al. 2004). Under hypoxic conditions in the mitochondria, deoxyhemeproteins can produce NO from nitrite, whereas in the cytosol, nsHb1 together with the NR can oxidize NO into nitrite in a NADPH-dependent pathway. This dynamic cycle adjusts the cellular redox and energy state (Hebelstrup et al. 2007; Igamberdiev et al. 2010). At the same time, it has been described that *nsHb1* overexpression can reduce the NO levels in seeds (Thiel et al. 2011), modulating the mentioned homeostasis for proper seedling establishment.

2.4 Group VII ERFs and ABI5 Are Key NO Sensors in Seeds

As previously discussed in this chapter, NO has a crucial significance in seed biology. During the first hours of seed imbibition, in the transition from seed dormancy to germination, a burst of NO has been detected in the endosperm of *Arabidopsis* seeds (Liu et al. 2009). Additionally, treatments with NO gas or NO donors break seed dormancy and promote seed germination (Beligni and Lamattina 2000; Bethke et al. 2004a). Conversely, NO depletion during seed germination by NO scavengers maintains seed dormancy preventing germination (Bethke et al. 2004a, 2006a, b). Furthermore, NO counteracts the inhibitory effects of ABA on seed germination by affecting a key transcription factor (TF) involved in seed dormancy and germination (Bethke et al. 2004a, b; Albertos et al. 2015).

Recently, two different molecular mechanisms related to NO sensing in seeds have been discovered (Gibbs et al. 2014; Albertos et al. 2015). In these new insights, the ABA central repressor of germination ABI5 (ABSCISIC ACID INSENSITIVE 5) is modulated not only at the expression but also at the protein level by the NO produced after imbibition during seed germination (Fig. 5.3). During the last stages of seed maturation, in dormant and after-ripened seeds, the group VII ERFs of TFs induces the expression of *ABI5* by direct binding to the *cis*-elements (GCCGCC EBP box) present in its promoter (Büttner and Singh 1997; Yang et al. 2009; Gibbs et al. 2014). However, after seed imbibition when germination starts, the increase in endogenous NO levels is sensed by this group of ERFs VII TFs compromising their stability (Gibbs et al. 2014). The effect of this NO together with the presence of oxygen in the seed provokes the proteolytic degradation of group VII ERFs through the N-end rule pathway, inhibiting *ABI5* expression and, therefore, promoting seed germination (Gibbs et al. 2011, 2014). Although the destabilization of group VII ERFs requires oxygen and NO, the specific effect of NO on critical cysteine residues of these TFs for the N-end rule pathway functioning still remains unknown.

Besides the transcriptional regulation exerted by NO on *ABI5* expression through group VII ERFs proteolytic control, the gaseous redox signal also acts at the posttranslational level of this master regulator of seed germination (Albertos

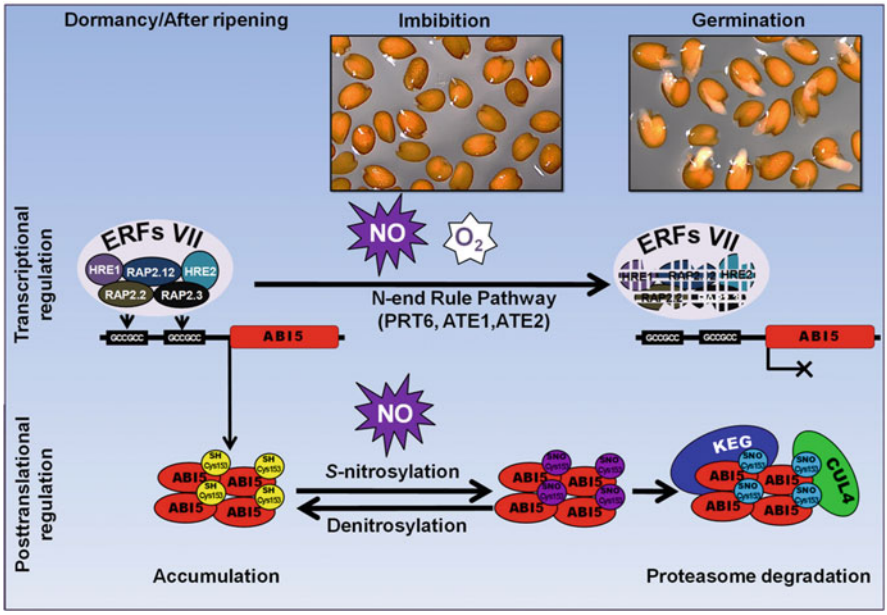

Fig. 5.3 Model for NO-mediated regulation of seed dormancy and germination through ABI5 transcriptional and posttranslational regulation. The burst of NO early produced after seed imbibition degrades group VII ERFs via the N-end rule proteolytic pathway avoiding ABI5 expression. On the other hand, this NO induces ABI5 S-nitrosylation facilitating the interaction with CUL4-based and KEG E3 ligases and promoting ABI5 protein degradation via the proteasome. Thus, not only the avoidance of ABI5 expression but the degradation of ABI5 protein enables seed germination and seedling growth (adapted from Albertos et al. 2015)

et al. 2015). In a novel mechanism recently discovered, ABI5 central repressor is S-nitrosylated by the NO produced after seed imbibition, promoting the interaction with CUL4-based and KEG E3 ligases, and consequently, ABI5 is rapidly degraded by the proteasome during seed germination (Albertos et al. 2015). The destabilization of ABI5 caused by NO depends on the critical cysteine residue 153 of the ABI5 protein sequence. Mutations on the ABI5 Cys153 residue abolish protein S-nitrosylation and disrupt ABI5 degradation and seed germination promotion mediated by NO. Similarly, group VII ERFs and ABI5 protein accumulation rates are affected by alterations in NO levels in the seed (Gibbs et al. 2014; Albertos et al. 2015). Treatments with NO donors, or using genetic backgrounds with increased NO levels in the case of ABI5, enhance ERFs and ABI5 protein destabilization and germination promotion. On the other hand, NO depletion by NO scavengers, and using NO-deficient genetic backgrounds in the case of ABI5, stabilizes these TFs and inhibits seed germination.

Nevertheless, these two essential mechanisms controlled by NO affecting *ABI5* gene expression and protein stabilization are independent in their mode of action (Albertos et al. 2015). Seeds impaired in the degradation of group VII ERFs by the N-end rule pathway, such as mutations in *PRT6* (*PROTEOLYSIS 6*), the key E3

ligase of this proteolytic pathway, display higher *ABI5* expression levels, even after exogenous NO treatments, and ABA hypersensitive phenotype due to the greater extent of ABI5 protein after ABA treatments (Holman et al. 2009; Gibbs et al. 2014; Albertos et al. 2015). Conversely, NO destabilizes ABI5 protein by the proteasome in *prt6* mutant genetic background even if the VII ERFs group degradation by N-end rule is impaired, revealing the independence of both NO sensing processes (Albertos et al. 2015).

These insights have shed light on the new NO sensing pathways and the NO counteraction on the ABA inhibitory effect during seed germination and post-germination processes of the plant life cycle. The transcriptional and posttranslational regulation of ABI5 via a dual NO-responsive mechanism highlights the essential process of ABI5 removal from the seed to allow the establishment of a new plant.

3 Gasotransmitter Function of NO in Roots

Roots perform various essential functions in plant growth and development, among which anchorage, storage, nutrient absorption, and water supply are the most notable. NO is an important metabolite and signal molecule in plants (Sanz et al. 2014), and in particular, it takes a key role in root structure and growth (Stöhr and Stremlau 2006; Bai et al. 2014). In this way, the principal function attributed to NO in primary root development is its synergic effect with auxins (Pagnussat et al. 2002, 2003, 2004; Lanteri et al. 2006). It has been observed that NO acts downstream of auxin (Pagnussat et al. 2002). Depending on their concentration, both NO and auxin can exert either a positive or a negative effect on primary root growth (Hu et al. 2005; Fu and Harberd 2003). Thus, NO promotes primary root growth at low concentrations (Pagnussat et al. 2002; Hunt et al. 2002) by inducing cell elongation in a similar way to auxin (Gouvêa et al. 1997) and represses it at higher levels (He et al. 2004; Chen et al. 2013) by reducing cell division and the overall root meristem size (Fernández-Marcos et al. 2011). Recently, it has been demonstrated that exogenous NO application via NO donor compounds modulates the organization of actin cytoskeleton and actin-dependent endocytosis in maize root apex (Kasprowicz et al. 2009; Lombardo and Lamattina 2012). In agreement with Elhiti et al. (2013), nonsymbiotic hemoglobins (nsHbs), which decrease endogenous NO levels, inhibit auxin metabolism, resulting in an important alteration of root morphology and development (Hunt et al. 2002).

In a similar sense, there have been conflicting studies about effects of NO and CK during primary root growth (Shen et al. 2013; Liu et al. 2013). Other functions related to root development have been attributed to NO. Hu et al. (2005) suggested the participation of NO in gravitropic bending in soybean roots. In this case, gravistimulation produces asymmetric accumulation of NO necessary for the gravitropic response.

3.1 Importance of NO in Root Mitochondria

Mitochondria play an essential regulatory role during root growth. Active cell division in the root meristem may require additional energy input from the mitochondria (Zhou et al. 2011). In addition to ATP production, this organelle has other important functions and participates in diverse processes such as synthesis of coenzymes (folic acid), metabolism of organic acids, amino acids, lipids, photorespiration, maintenance of ROS homeostasis, involvement in programmed cell death (PCD) (Rébeillé et al. 1997; Zorov et al. 1997; Bartoli et al. 2000; Kowaltowski 2000; Balk and Leaver 2001; Bauwe et al. 2010), and participation in retrograde signaling via ROS generation (Rhoads and Subbaiah 2007). Current studies propose that mitochondria are one of the major producers of NO in plants (Tischner et al. 2004; Planchet et al. 2005; Gupta et al. 2005, 2010; Stoimenova et al. 2007). Since NO has achieved special relevance, these mitochondrial features have stimulated interest in NO signaling research. However, there are few data in the literature about how much do mitochondria really contribute to NO production in roots. In tobacco root segments, 2/3 of NO emission was inhibited by myxothiazol (an inhibitor of the mitochondrial cytochrome bc1 complex) suggesting that mitochondria are the main NO producers in roots (Gupta et al. 2011c).

3.2 NO Production in the Root

Roots, in contrast to leaves, are in an environment where NO concentrations can be higher than in aboveground tissues, at least during soil flooding periods. Plant tissues under anoxia reduce nitrate to nitrite at a high rate and therefore accumulate nitrite (Gupta et al. 2005). Stöhr et al. (2001) reported a plasma membrane-bound enzyme is able to reduce nitrite to NO in tobacco roots, undetectable in leaves. This enzyme, designated as NI-NOR (nitrite-NO oxidoreductase), requires cytochrome c as an electron donor. Previous findings allowed the identification of a mitochondrial protein, prohibitin (PHB3), as a key component in NO homeostasis and NO-mediated responses (Wang et al. 2010). Stress-mediated NO generation is dependent on PHB3 activity, and *phb3* knockout mutants show less primary root growth inhibition from stress than wild-type seedlings.

Two predominant pathways are known for NO production in eukaryotic mitochondria. One is an oxidative pathway, which uses L-arginine as a substrate and produces NO and citrulline catalyzed by an NO synthase (NOS) (Giulivi et al. 1998; Daff 2010), and the other is a reductive pathway which uses nitrite as a substrate and produces NO at low oxygen conditions (Fig. 5.1) (Kozlov et al. 1999; Kaiser et al. 2007). In higher plants, it has not been possible to identify any enzyme with NOS activity yet. However, AtNOA1, a GTPase present in the chloroplast (Moreau et al. 2008), is another key enzyme involved in the oxidative pathway, although no

evidence of NOS activity has been found in other species, like barley (Gupta et al. 2010). Planchet et al. (2005) were the first to point out that root tissues, as well as mitochondria isolated from roots, were able to reduce nitrite to NO via mitochondrial electron transport under anoxia, but not in air. Nitrite reduction to NO is associated with the mitochondrial membrane fraction but not with the matrix (Gupta et al. 2010) and is only present in root tissues (Gupta et al. 2005). Indeed, mitochondrial NO emission under anoxia was strongly blocked by mixothiazol, strengthening the evidence for the involvement of electron transport proteins (Gupta et al. 2005). This was later confirmed in barley and rice roots, where externally fed NADH and NADPH were oxidized in the presence of nitrite, giving NO as a product (Stoimenova et al. 2007).

3.3 Effect of NO on Mitochondrial Metabolism

However, higher levels of NO over long periods of time inhibit mitochondrial respiration, via nitrosylation of complex I and inhibition of other enzymes involved in mitochondrial electron transport (cytochrome oxidase, ATP synthase, creatine kinase, and aconitase), ultimately causing cell death (Fig. 5.4) (Brown and Borutaite 2002). Hence, any excess of NO is potentially toxic and can cause nitrosative stress (Corpas et al. 2007; Brown and Borutaite 2002). Plants make use of a scavenging system to control NO levels and preserve NO at very low concentrations by forming other less toxic nitrogen oxides, NO_2, N_2O, and N_2O_3 (Gupta et al. 2010), or via peroxynitrite ($ONOO^-$) formation (Radi et al. 2002). The degradation of NO by mitochondria was also evidenced to be stimulated by Ca^{2+} supply suggesting that Ca^{2+}-dependent external NAD(P)H dehydrogenases are implicated in NO scavenging (Oliveira et al. 2008). Plants also employ an NO-resistant alternative oxidase (AOX1) in mitochondria and can maintain non-ATP-coupled electron transport even in the presence of NO (Millar and Day 1996). In view of these data, it is possible to say that there is a regulation of NO homeostasis in the mitochondria.

Hypoxic and anoxic conditions trigger changes at organelle level. Igamberdiev and Hill (2009) defined anoxia as a condition whereby the oxygen concentrations in the cytoplasm are such that cytochrome oxidase (COX) cannot effectively donate electrons to oxygen, while hypoxia is a condition whereby COX has at least a limited capacity to use oxygen but several other oxidases such as AOX1 are inhibited. Gupta et al. (2011b) assume that the mitochondrial compartment senses oxygen depletion much faster than the rest of the cell because mitochondria are the primary consumers of oxygen in the cell. These organelles do not develop correctly without oxygen within a few hours (Vartapetian et al. 2003). Nitrite to NO reduction of root mitochondria may improve the survival of cells during hypoxic or anoxic periods, and it has been previously shown that root mitochondria are able to synthesize small amounts of ATP under anoxia by nitrite reduction to NO (Stoimenova et al. 2007) and recycling of nitrite via the so-called hemoglobin

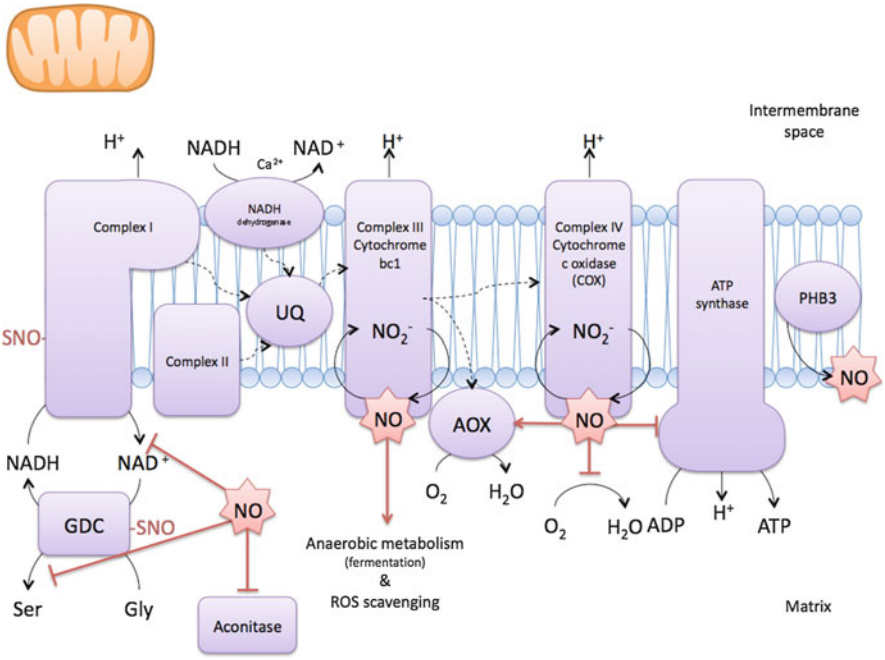

Fig. 5.4 NO regulates mitochondrial metabolism in the root. Root mitochondria are important centers of NO homeostasis in the cell. Stress-mediated NO generation is dependent on the inner mitochondrial membrane prohibitin (PHB3) complex through an unknown mechanism. Under hypoxia, mitochondria reduce nitrite (NO_2^-) to nitric oxide (NO) through complex III and complex IV of electron transport chain. In view of the oxygen (O_2) scarcity, these complexes use NO_2^- as an electron acceptor, which aids the cell in keeping NADH/NAD^+ levels constant and uncouples electron transport from ATP synthesis, increasing O_2 availability. In turn, NO inhibits O_2 reduction by complex IV and ATP synthesis via ATP synthase, while promoting the activity of alternative oxidases (AOX). This results in a reduction of superoxide formation and along with NO-promoted ROS scavenging reduces the risk of cell death under hypoxia. NO also promotes a shift toward anaerobic metabolism as a means of obtaining ATP, reinforcing it by inhibiting enzymes like aconitase or glycine decarboxylase (GDC), and reducing the energy requirements of the cell by inhibiting actin polymerization and cytoskeleton-dependent processes like vesicle recycling and cell division. Once normoxia is reached again, NO is oxidized to nitrite and nitrate

cycle (Igamberdiev and Hill 2004). In this manner, mitochondria maintain the electron transport chain functional, which is necessary for plant survival. NO homeostasis depends on concentrations of oxygen: when oxygen is below a certain threshold, there is NO production; when it is above, there is NO scavenging. NO regulates oxygen consumption by acting as an inhibitor of electron transport to oxygen at the site of COX (terminal electron acceptor site) (Benamar et al. 2008; Gladwin and Shiva 2009). Using pea (*Pisum sativum*) seeds, Borisjuk et al. (2007) showed that oxygen deprivation triggers a nitrite-dependent increase in the levels of NO in the mitochondrial matrix. Higher levels of NO inhibit COX. This mitochondrial inhibition conserves oxygen and tends to increase oxygen availability.

Increasing oxygen availability decreases NO levels, which suspends mitochondrial inhibition and enhances oxygen consumption and ATP availability. NO produced in the mitochondrial matrix goes to the cytosol where it is oxidized to nitrite, which can go back to the mitochondria and restart the cycle. This autoregulatory and reversible oxygen balancing, via NO, prevents complete depletion of oxygen (Gupta et al. 2011b).

While optimal NO concentrations are required for a signaling function in oxygenated plant tissues, in oxygen-reduced or oxygen-deprived tissues, NO turns into a dominant metabolite synthesized primarily by mitochondria, playing a role in the survival strategy of cells.

3.4 Mitochondria-Dependent NO Production and Root Growth

Roots must have adequate oxygen supply. Soil compaction or waterlogged soil situations reduce soil oxygen levels and can deeply affect the root system. Under these hypoxic and anoxic conditions, the resultant oxygen shortage restricts aerobic respiration and hence ATP synthesis in root cells (Gibbs and Greenway 2003), causing a rapid change in the intracellular energy status (Juntawong et al. 2014; Pu et al. 2015). Nitric oxide (NO)-NR dependent is one of the primary signals released by plants in response to hypoxia and other stress conditions, including drought, temperature, pathogen attack, nutrient deficiency, and salt stress (Gupta et al. 2011a; Chen et al. 2010; Xie et al. 2013; Royo et al. 2015). During hypoxic conditions, NO is required for inhibiting aconitase and causing a shift of metabolism toward amino acid biosynthesis (Gupta et al. 2012). Previous data revealed that the oxygen deprivation of roots induces local NO release, particularly in the transition zone (Mugnai et al. 2012). Proposed model suggests that TZ-dependent NO emission functions to protect the root apex cells and also to lower the oxygen demand via the inhibition of actin polymerization and endocytic vesicle recycling. This lowered oxygen demand then induces, through an as yet unknown process, systemic signaling throughout the root, allowing the hypoxic acclimation of the entire root.

Supporting a putative role of the root TZ in NO metabolism, several articles have demonstrated that the control of NO homeostasis occurring in maize root after nitrate perception takes preferentially place at the level of the TZ and that this mechanism could be involved in the regulation of root growth by nitrate (Manoli et al. 2014; Trevisan et al. 2015). Recently, Alemayehu et al. (2015) have also concluded that enhanced NO generation in root TZ during the early stage of cadmium stress is required for maintaining root growth in barley. In conclusion, it could be speculated that the local production of NO in the TZ may be a result of plant roots exposed to abiotic stresses (such as hypoxia or metal toxicity) to preserve the integrity of root cells.

In addition to the TZ, NO also accumulates in root stem cells (Sanz et al. 2014). Within these cells, NO acts redundantly with the central regulator of stem cell maintenance WOX5 (Haecker et al. 2004). Although no direct link between root stem cells and hypoxia has been designated yet, there are other plant stem cells which thrive in hypoxic conditions, which parallel the situation of other eukaryotic stem cells (Beltran-Povea et al. 2015). Hypoxia, arising naturally within growing tissue, acts as a positional cue to set germ cell fate (Kelliher and Walbot 2012). This state seems to be accommodated by diverting carbon away from mitochondrial respiration into alternative pathways that prevent the production of ROS (Kelliher and Walbot 2014). Remarkably, expression of several hypoxia-inducible genes is enriched in cortex and endodermis initial cells (Sozzani et al. 2010). Since mammalian stem cells also exist in a hypoxic niche (Morrison et al. 2000), it could be speculated that reducing ROS via NO accumulation in root stem cells may be of great value to protect genomic integrity. Deciphering oxygen distribution in the root stem cell niche in response to NO will provide useful information to unravel the importance of NO in maintaining the hypoxic state of initial cells.

4 Future Perspectives

In the plant life cycle, seed germination and meristem control and maintenance events are of great significance since the development of a new plant depends on both. The identification of new components involved in the regulation of these processes will allow, in the near future, to understand and manipulate seed germination and plant development. One of the biggest challenges about the role of NO in plant signal transduction networks is the finding of real targets of NO. The nature of these NO targets in plant growth and developmental processes start to be uncovered, being master regulators of phytohormones in most cases.

Also, the identification of the elements that participate in NO homeostasis is, thus, essential to understand the NO production, sensing, and signaling within the seed and the plant, which is a prerequisite for its genetic improvement. The use of biochemical techniques (i.e., TAP-tagging) to map the interactome of master regulators will be powerful approaches to identify these key components in NO homeostasis.

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