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REVIEW PAPER

# Nitric oxide (NO) and phytohormones crosstalk during early plant development

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## Abstract

**During the past two decades, nitric oxide (NO) has evolved from a mere gaseous free radical to become a new messenger in plant biology with an important role in a plethora of physiological processes. This molecule is involved in the regulation of plant growth and development, pathogen defence and abiotic stress responses, and in most cases this is achieved through its interaction with phytohormones. Understanding the role of plant growth regulators is essential to elucidate how plants activate the appropriate set of responses to a particular developmental stage or a particular stress. The first task to achieve this goal is the identification of molecular targets, especially those involved in the regulation of the crosstalk. The nature of NO targets in these growth and development processes and stress responses remains poorly described. Currently, the molecular mechanisms underlying the effects of NO in these processes and their interaction with other plant hormones are beginning to unravel. In this review, we made a compilation of the described interactions between NO and phytohormones during early plant developmental processes (i.e. seed dormancy and germination, hypocotyl elongation and root development).**

**Key words:** Dormancy, germination, hypocotyl elongation, reactive nitrogen species, root development, seeds.

## Introduction

### *Impact of nitric oxide (NO) in early plant development*

Early plant development includes the formation of a complete embryo from a zygote, seed germination and seedling growth as the main biological processes. Early stages of germination involve the enlargement of the root, hypocotyl and cotyledons that were preformed in the embryo. Environmental factors are important regulators of these processes. For instance, germination in the dark results in hypocotyl elongation while cotyledon expansion is suppressed. In contrast, if seeds germinate in light, the hypocotyl hardly elongates, while the cotyledons quickly expand.

Both plant development and environmental responses activate phytohormonal signals. Mutations in genes involved in phytohormone metabolism reveal events that are controlled by their action. Thus, phytohormones auxin and cytokinin (CK) control major cell specification events, stimulate growth and are present during embryogenesis and seedling establishment (Bennett and Scheres, 2010; Perilli *et al.*, 2010). As the embryo matures, abscisic acid (ABA) is synthesized by the embryo, providing a developmental signal to initiate the synthesis of storage compounds and to undergo desiccation. ABA is present in dormant seeds and plays an important role in maintaining seed dormancy (Finkelstein *et al.*, 2008). Gibberellic acid (GA) induces the synthesis of enzymes required for the metabolism of stored nutrient, thus providing energy for seedling growth. GA also induces cell division and cell expansion in dark-grown hypocotyls, maintaining their rapid growth through the soil (Claeys *et al.*, 2014). In addition to GA and ABA, ethylene (ET) also plays a key role in dormancy release in numerous plant species (Corbineau *et al.*, 2014).

Research over the last decades has identified nitric oxide (NO) as a rapidly induced and potent plant growth regulator. This low molecular weight gaseous compound not only increases during stress responses, but also during specific plant developmental processes (Delledonne, 2005). Thus, NO is involved in the promotion of seed germination, photomorphogenesis, mitochondrial activity, leaf expansion, root growth, stomatal closure, fruit maturation, senescence and iron metabolism (Leshem *et al.*, 1998; Beligni and Lamattina, 2000; Wendehenne *et al.*, 2001; Graziano *et al.*, 2002; Neill *et al.*, 2002*a*, *b*; Neill, 2007) (Table 1). NO as a signalling

**Table 1.** *Summary of the NO involvement in plant growth and development processes*

| **NO role in plant developmental processes** | | | | |
|---|---|---|---|---|
| **Developmental Map** | **Growth and development** | | **NO role** | **References** |
|  | Aleurone | | Lead to aleurone cells vacuolation and dormancy loss. | Bethke *et al*., 2004a, b, 2007 |
| | Seed dormancy | | Break dormancy | Batak *et al*., 2002<br>Bethke *et al*., 2004b, 2006a, b, 2007 |
| | Germination | | Promote germination | Beligni and Lamattina 2000 |
|  | Root organogenesis | Primary Root | Inhibit root elongation | Correa-Aragunde *et al*., 2004 |
| | | Secondary Root | Induce lateral root growth | Correa-Aragunde *et al*., 2004 |
| | | Adventitious Root | Promote adventitious roots | Pagnussat *et al*., 2002 |
| | | Root hair | Induce root hair formation | Lombardo *et al*., 2006 |
| | Etiolation | | Promote De-etiolation | Beligni and Lamattina 2000 |
| | Hypocotyl | | Reduce hypocotyl elongation | Beligni and Lamattina 2000 |
|  | Stomatal movement | | Induce stomatal closure | García-Mata and Lamattina, 2001, 2002, 2007, García-Mata *et al.,* 2003, Desikan *et al.,* 2004 |
| | Growth of pollen tubes | | Inhibit pollen tubes growth | Prado *et al*., 2004 |
| | Flowering | | Inhibit floral transition | He *et al*., 2004 |
| | Cell wall lignification | | Lignine increasing | Gabaldón *et al*., 2005 |
| | Xylem differentiation | | Induce tracheary element differentiation | Barceló *et al*., 2002, Gabaldón *et al*., 2005 |
| | Cellulose biosynthesis | | Affect cellulose content in roots in a dose-dependent manner | Correa-Aragunde *et al*., 2008 |
| | Chlorophyll biosynthesis/Photosynthesis | | Increase chlorophyll content | Zhang *et al*., 2006 |
| | Iron availability | | Increase Fe availability | Graziano *et al*., 2002 |
| | Nodulation | | Control nodule formation | Pii *et al*, 2007 |
| | Gravitrophism | | Lead gravitropic bending | Hu *et al*., 2005 |
| | Cell polarity | | Control gravity stimulus and polarized growth | Bushart *et al*., 2007, Salmi *et al*., 2007 |
| | Maturation | | Delay fruits maduration | Leshem and Pinchasov, 2000 |
| | Senescence | | Delay leaves senescence | Leshem and Pinchasov, 2000, Mishina *et al.,* 2007 |

molecule usually interacts with plant hormones and other endogenous molecules during early growth and development of plants (Freschi, 2013) (Fig. 1).

### *NO production in plants*

NO is the most abundant reactive nitrogen species (RNS) in plants. In fact, plants are able to accumulate and metabolize atmospheric NO (Nishimura *et al.* 1986) and, by measuring the emitted gases, it has been shown that NO can be synthesized in plants (Leshem and Haramaty 1996; Yamasaki, 2000).

The identification of the enzymes involved in NO synthesis is of great interest to our current concept of the functions that NO plays in plant growth and development. There are two main pathways to produce NO in plant tissues: the enzymatic and the non-enzymatic (García-Mata and Lamattina, 2003; Wendehenne *et al.*, 2004). Enzymatic pathways of NO production have been thoroughly studied, and much information about the type and subcellular localization of the enzymes involved is now available. Different enzymes have been identified to catalyse the synthesis of NO mainly from two different substrates, nitrate and arginine. Nitrate reductase (NR; Wilkinson and Crawford, 1993) was the first identified NO biosynthetic enzyme. It usually reduces nitrate to nitrite, but is also able to reduce nitrite to NO using NADPH as a cofactor (Desikan *et al.*, 2002). AtNOS1 (NO synthase1, later renamed AtNOA1) is another enzyme that contributes to the synthesis of NO. Although it was originally identified as a NOS-like enzyme that produces NO and L-citrulline from L-arginine (Guo *et al.*, 2003*a*, Guo and Crawford, 2005; Crawford, 2006), further studies confirmed that it lacks its originally reported NOS activity (Crawford *et al.*, 2006; Zemojtel *et al.*, 2006). ATNOA1 is a GTPase that binds to ribosomes and plays a role in their proper assembly and stability (Flores-Pérez *et al.*, 2008; Moreau *et al.*, 2008). Interestingly, although the mechanism underlying reduced NO levels in *atnoa1* plants is still not well characterized, this mutant is widely used as an experimental tool in NO research. Other enzymes like xanthine oxidase/dehydrogenase and cytochrome P450 have occasionally been suggested as sources for NO (Planchet and Kaiser, 2006). Evidence also shows a non-enzymatic pathway to produce NO based on the reduction of nitrite to NO at acid pH, mainly in the apoplast of the aleurone cell layer during seed germination (Bethke *et al.*, 2004*b*).

NO accumulation in seeds, hypocotyls and roots can be identified using the fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2DA) (Correa-Aragunde *et al.*, 2004; Illés *et al.*, 2006; Liu *et al.*, 2009; Fernández-Marcos *et al.*, 2011; Sanz *et al.*, 2014) (Fig. 2). DAF-2DA is a permeable compound hydrolysed inside the cells and able to emit fluorescence when it reacts with $N_2O_3$, a by-product of NO oxidation. Three local centres of NO production were detected in roots, at the root cap statocytes, at the quiescent centre and distal portion of the meristem, and the most prominent one, at the distal part of the transition zone (Illés *et al.*, 2006; Fernández-Marcos *et al.*, 2011; Sanz *et al.*, 2014). Thus, the specific site of NO synthesis may be important to exert the different physiological functions during plant growth and development.

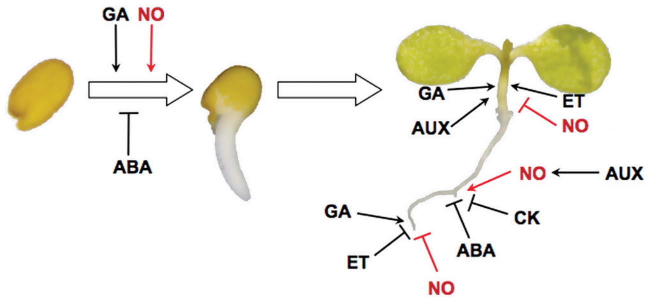

**Fig. 1.** Schematic representation of the physiological NO role in *Arabidopsis* seed germination and early seedling development together with the different phytohormones. AUX, auxin; ABA, abscisic acid; GA, gibberellins; CK, cytokinin; ET, ethylene. Arrows and bars indicate positive and inhibitory effects, respectively.

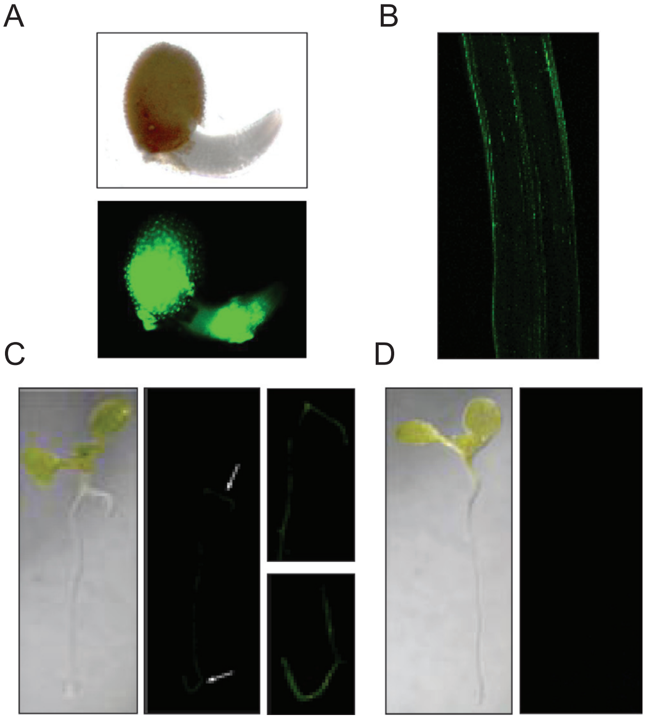

**Fig. 2.** Nitric oxide (NO) tissue accumulation at different stages of early plant development using DAF-2DA. (A) Endogenous NO detection in two-day-old *Arabidopsis* seedlings. (B) External layers of hypocotyls. (C) Seedling roots. (D) DAF-2DA fluorescence of seedlings treated with the NO scavenger cPTIO (1mM). Plants were grown for 7 d on agar plates and then subjected to DAF 2DA incubation. Arrows indicate places of high NO production. NO accumulation was detected as described in Fernández-Marcos *et al.* (2011) and Sanz *et al.* (2014).

### *Mechanism of NO action in plant tissues*

A key feature in the biology of NO in plant tissues is the post-translational modification of target proteins through *S*-nitrosylation and nitration. *S*-nitrosylation consists in the modification of the thiol group present in cysteines to form nitrosothiols (Lindermayr *et al.*, 2005; Belenghi *et al.*, 2007; Serpa *et al.*, 2007). Recent evidence indicates that

*S*-nitrosylation is emerging as a typical redox signalling mechanism. Proteomic profiling in plants has identified a short number of *S*-nitrosylated proteins (Lindermayr *et al.*, 2005, 2006; Ortega-Galisteo *et al.*, 2012; Camejo *et al.*, 2013; Puyaubert *et al.*, 2014). Nitration of tyrosine residues has also been described as another post-translational modification, due to the high reactivity of peroxynitrite ($ONOO^-$), generated by NO and $O_2$, with amino acids. This protein modification, recently proposed to be reversible, commonly leads to loss of protein function and is able to modulate signalling processes relying on tyrosine phosphorylation and dephosphorylation (Monteiro *et al.*, 2008; Vandelle and Delledonne, 2011).

The nature of NO targets in plant growth and developmental processes still remains unclear. In spite of its relevance as a plant growth and stress regulator, the current knowledge about the NO signalling pathway is limited. Thus, the identification and characterization of new components at the molecular level is essential to get a deeper insight into this network. Here we report a compilation of the described NO roles in seeds and early plant developmental processes through its interaction with phytohormones. For a better understanding of this crosstalk, the different developmental cues along the early plant life cycle are followed in sequence, beginning with plant seeds and following with the development of hypocotyls and roots (Table 1).

## Crosstalk between NO and phytohormones in seed dormancy and germination

Dormancy is defined as the developmental state in which a viable seed fails to germinate under favourable environmental conditions (Bewley, 1997). Once dormancy is released, seeds can germinate. Thus, seed germination is considered the initiation of the first developmental step in the life cycle of higher plants. The hormonal balance, mainly between ABA and GAs, acts as an integrator of environmental cues to maintain dormancy or activate germination (Arc *et al.*, 2013*b*). Pioneer reports highlighted the role of NO as a dormancy-relieving molecule. NO can break seed dormancy in *Arabidopsis* and barley while the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) effectively promotes the maintenance of seed dormancy (Bethke *et al.*, 2004*b*, 2006*a*, *b*; Libourel *et al.*, 2006). These findings suggested that NO is an endogenous regulator of seed germination in these species. Sarath *et al.* (2005) extended this study to other species and confirmed that NO is also a potential regulator of seed germination in warm-season $C_4$-grasses. Accurate experiments examined the contribution of the embryo, aleurone layer and testa to seed dormancy and determined exactly where in the seed NO is perceived. By seed dissection, Bethke *et al.* (2007) revealed that the aleurone layer perceives and responds to NO during the process of dormancy release. In agreement with this, Vitecek *et al.* (2008) quantified production of NO in barley aleurone layers after addition of nitrite or nitrate to the incubation medium. NO production requires an acid apoplast and is accompanied by a loss of nitrite from the medium via a non-enzymatic reaction (Bethke *et al.* 2004*a*). Another means for NO accumulation in seeds seems to rely on localized NO synthesis in the endosperm. A rapid increase in NO levels appears in the endosperm of *Arabidopsis* seeds after imbibition during seed germination (Liu *et al.*, 2009). Furthermore, NO produced by the endosperm during *Sechium edule* Sw. seed embryogenesis, is abolished after using L-NAME, the mammalian NOS inhibitor, positioning a putative NOS-like enzyme responsible for NO synthesis during seed embryogenesis (Lombardi *et al.*, 2012).

### *NO and ABA*

ABA plays a key role during the induction and maintenance of seed dormancy, the inhibition of seed germination and later post-germination developmental checkpoints (Finch-Savage and Leubner-Metzger, 2006; Finkelstein, 2013). Conversely, NO acts as a dormancy-relieving molecule and promoter of seed germination (Bethke *et al.*, 2004*b*). An extensive crosstalk between both molecules at the synthesis level has been reported. Endogenous NO content increases after exogenous ABA application in *Arabidopsis* and tobacco plant tissues (Guo *et al.*, 2003*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.*, 2004*a*, *b*). This accumulation has been proposed to be the underlying mechanism for ameliorating the repressor effect of ABA. Thus, dormant *Arabidopsis* seeds treated at the same time with ABA and NO donor sodium nitroprusside (SNP) display a reduction in ABA sensitivity and therefore germination rates are increased (Bethke *et al.*, 2006*b*). In agreement with this, Liu *et al.* (2009) observed that a rapid accumulation of NO induces an equally rapid decrease of ABA that is required for NO action in *Arabidopsis* (Fig. 3). The NO-induced ABA decrease correlates with CYP707A2 protein accumulation, an enzyme involved in ABA catabolism (Liu *et al.*, 2009). Furthermore, SNP addition induces *CYP707A2* transcription while the cPTIO represses it. Such response precedes the enhancement of ABA catabolism, which is required for subsequent seed germination (Liu *et al.*, 2009). In addition, it has been demonstrated that dry seeds from $NO_3^-$-treated mother plant, as well as imbibed dormant seeds in the presence of $NO_3^-$, show the up-regulation of *CYP707A2* expression which results in a decrease in ABA levels and seed dormancy (Alboresi *et al.*, 2005; Matakiadis *et al.*, 2009). There is clearly a close relationship between $NO_3^-$, NO biosynthesis and the corresponding physiological effects in seeds. Although it has been suggested that dormancy breaking in *Arabidopsis* seeds produced by $NO_3^-$ can take place in a NO-dependent manner, it is unclear whether the up-regulation of *CYP707A2* caused by $NO_3^-$ is dependent or independent of NO, or if it takes place in parallel or is complementary to NO at the biosynthesis level (Bethke *et al.*, 2006*a*).

Remarkably, proteomic analyses of *S*-nitrosylated proteins in *Arabidopsis* have allowed the identification of only a few endogenously *S*-nitrosylated proteins (Fares *et al.*, 2011; Puyaubert *et al.*, 2014) and none yet involved in ABA biosynthesis or signalling. However, a recent discovery of the *S*-nitrosylation of OST1/SnRK2.6 in guard cells impairing

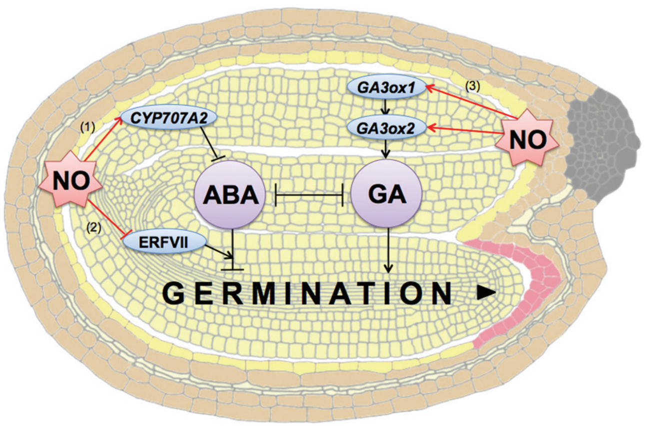

**Fig. 3.** Crosstalk between NO, ABA and GAs during seed dormancy release and germination in *Arabidopsis*. During germination, NO induces ABA catabolism through the transcriptional up-regulation of *CYP707A2* (*Cytochrome P450 ABA 8′-hydroxylase*) (1: Liu *et al.*, 2009). ERFVII (ET Response Factor group VII) sense NO production during seed imbibition prior to germination and are degraded through the N-end rule pathway, avoiding *ABI5* transcriptional induction (2: Gibbs *et al.*, 2014). NO also promotes GA biosynthesis by inducing *GA3ox1* and *GAox2* transcription (*Gibberellic acid oxidase1 and 2*) (3: Bethke *et al.*, 2007). Arrows and bars indicate positive and inhibitory effects, respectively. (adapted from the illustration by Meryl Hashimoto from John Harada lab at UC Davis, with permission).

its function in the ABA signalling pathway, leaves the door open for new connections between ABA and NO signalling crosstalk during seed germination (Wang *et al.*, 2015). Additionally, proteomic identification of nitrated proteins identified molybdenum cofactor (MoCo) sulfurase ABA3 as a target of protein nitration (Lozano-Juste *et al.*, 2011). ABA3 is involved in the last step of ABA synthesis (Mendel, 2007). The inactivation of this protein could inhibit ABA biosynthesis and contribute to dormancy release and germination promotion (Arc *et al.*, 2013*a*). Genetic evidence also supports this proposed role for NO. Thus, seeds from NO deficient mutants, *nia1nia2* and *nia1nia2noa1-2* (Lozano-Juste and León, 2010) are more dormant and show increased sensitivity to ABA-mediated inhibition of germination than wild-type seeds (Lozano-Juste and León, 2010). Nevertheless, in order to have a deeper understanding of the effects caused by the balance ABA/NO, a more complete study in mutants that have altered NO content with respect to their sensitivity to ABA should be necessary.

Recently, group VII ET response transcription factors (ERFVII), degraded by the N-end rule pathway, have been identified as new NO sensors during seed germination, among other processes (Gibbs *et al.*, 2014) (Fig. 3). Protein stability of this group of transcription factors increased after treatment with cPTIO and in NO deficient mutant backgrounds (Gibbs *et al.*, 2014). Nevertheless, it is still unclear whether the critical Cys residue of ERFVII could be *S*-nitrosylated prior to degradation (Gibbs *et al.*, 2014). Mutants for genes involved in N-end rule pathway machinery as *PRT6 (PROTEOLYSIS 6)* or *ATE1ATE2 (ARGININE-TRNA PROTEIN TRANSFERASE 1 and 2)* and *ERFVII* overexpressor lines showed ABA hypersensitivity during seed germination. This is due to non-degradation or over-accumulation of ERFVII proteins, respectively (Holman *et al.*, 2009; Gibbs *et al.*, 2014). ERFVII induces *ABI5* expression, especially in the endosperm, which triggers ABA repressor responses during seed germination (Gibbs *et al.*, 2014). In summary, ERFVII transcription factors would be able to detect the increase in NO production during seed imbibition prior to germination, leading to their entry into the N-end rule degradation pathway, avoiding *ABI5* transcriptional induction, thus inhibiting ABA responses and contributing to seed germination.

### *NO and GAs, ET and polyamines (PAs)*

Seed germination is promoted by GAs and ET. A relationship between GAs biosynthesis and NO has been established. Thus, NO is required for the transcription of *GA3ox1* and *GA3ox2*, two key biosynthetic enzymes for active GA (Fig. 3). Furthermore, GA is required for cell vacuolation in isolated aleurone layers in the absence of NO (Bethke *et al.*, 2007). This data suggests that NO may coordinate a reduction in ABA-imposed dormancy with the onset of GA-stimulated germination. Similarly, NO induces dormancy breakage and stimulates germination of apple embryos by induction of ET biosynthesis (Gniazdowska *et al.* 2007). Short-term pre-treatment of the embryos with NO modified activity of both key enzymes of ET biosynthetic pathway: 1-aminocyclopropane-1-carboxilic acid (ACC) synthase and ACC oxidase (ACO). The results indicate that NO may alleviate dormancy of apple embryos via transient accumulation of ROS, leading to enhanced ET emission which is required to terminate germination *sensu stricto* (Gniazdowska *et al.*, 2010). It is still unclear how NO modifies the activity of these proteins during germination, although *S*-nitrosylation of ACS and ACO might be determining their biological function (Hebelstrup *et al.* 2012). Polyamines (PAs) are polycationic nitrogenous growth regulators ubiquitous in all living cells. Inhibition of PA synthesis accelerates germination (Gallardo *et al.*, 1994). PA catabolism stimulates the biosynthesis of NO and it is a potential intermediate of their action (Tun *et al.*, 2006; Wimalasekera *et al.*, 2011). Since S-adenosylmethionine (SAM) is the common precursor of ET and PA, a negative feedback regulation has been suggested between ET and PA-dependent NO biosynthesis (Arc *et al.*, 2013*b*). According to this idea, an enzyme involved in polyamine catabolism (copper amine oxidase, CuAO1) regulates NO biosynthesis and participates in ABA signalling (Wimalasekera *et al.*, 2011). *cuao* knockout mutants show lower NO production in response to exogenous PAs and are less insensitive to exogenous ABA supplementation during germination, seedling establishment and root growth inhibition as compared to the wild-type (Wimalasekera *et al.*, 2011).

## Crosstalk between NO and phytohormones in hypocotyl elongation and root development

### *NO and GAs*

In *Arabidopsis thaliana* seedlings, the two cotyledons and the root are connected by the embryonic stem called the hypocotyl. Most hypocotyl cells are formed in the embryo (Gendreau *et al.*, 1997). Therefore, after germination only a few divisions occur and only cell longitudinal expansion processes take place during hypocotyl growth. There are many factors regulating this process, such as light, hormones, gravity and temperature.

GAs are endogenous regulators of hypocotyl growth via cellular elongation through degradation of DELLA proteins in *Arabidopsis* (de Lucas *et al.*, 2008). In addition to GAs, light and temperature also control hypocotyl growth through the regulation of PIF transcription factors (de Lucas *et al.*, 2008). Previous reports highlighted the role of NO as an inhibitor of hypocotyl elongation. Increased NO levels in *Arabidopsis thaliana* and lettuce seedlings grown in dark conditions prevented hypocotyl growth (Beligni and Lamattina, 2000). In addition to NO, a balanced pool of reductants/oxidants and ATP concentration are essential for hypocotyl elongation in etiolated *Arabidopsis thaliana* seedlings (Tonón *et al.*, 2010). A possible mechanism underlying the crosstalk between NO, light and GAs seems to involve PIF and DELLA proteins. NO coordinates the repression of growth-promoting *PIF* genes and the increase in the content of DELLA proteins (Lozano-Juste and León, 2011).

### *NO and auxins*

Roots are the plant organs that ensure nutrient and water supply for the whole organism. Many lines of evidence highlight the central role of auxins in modulating root architecture. Well-known examples of auxin-dependent phenotypes are the dose-dependent increase in the length of epidermal-derived root hairs, the bimodal effect of auxin concentration on primary root length, the dose-dependent increase in number of lateral root (LR) primordia and the response to gravity (Overvoorde *et al.*, 2010). NO is a central signalling molecule with several effects on control of root architecture. Most evidence suggests that NO acts downstream of auxin *in planta* (Pagnussat *et al.*, 2002; Correa-Aragunde *et al.*, 2004; Hu *et al.*, 2005; Lombardo *et al.*, 2006; Chen *et al.*, 2010), although auxin seems not to be effective in stimulating NO release in plant cell culture (Tun *et al.*, 2001). NO is involved in primary root growth and LR formation (Correa-Aragunde *et al.*, 2004; Fernández-Marcos *et al.*, 2011), in root hair development (Lombardo *et al.*, 2006) and in gravitropic response. An extensive crosstalk between both molecules at all levels (synthesis, transport and perception) has been reported (Fig. 4).

At the level of synthesis, NR and NOS-dependent NO production seems to be involved in auxin-induced LR development. NR was postulated to be important due to exogenous auxin failure to induce NO in the NR-deficient mutant (Kolbertz *et al.*, 2008). NR-dependent NO production shows a complex regulation. NR activity is modulated by the function of mitogen-activated protein kinase 6

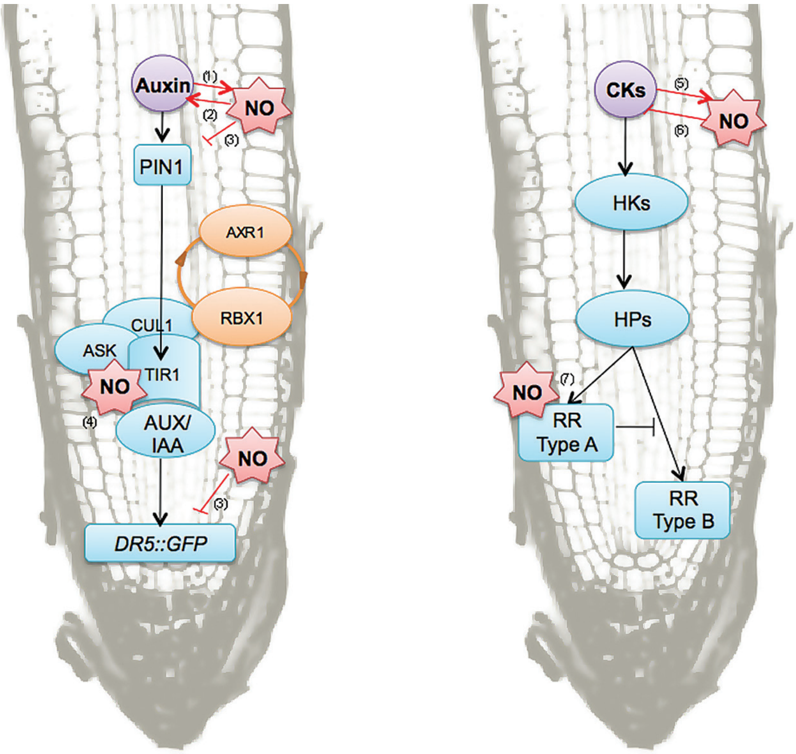

**Fig. 4.** Crosstalk NO-auxins (left) and NO-CKs (right) during root development. Auxin increases NO production under certain stresses such as iron deficiency (1: Chen *et al.*, 2010). At the same time, NO reduces auxin degradation by inhibiting IAA oxidase activity (2: Xu *et al.*, 2010). On the other hand, enhanced NO levels interfere with acropetal auxin transport through PIN1 auxin efflux carrier, which correlates with a reduction in auxin response (3: Fernández-Marcos *et al.*, 2011). However, NO also acts positively on auxin signalling through *S*-nitrosylation of the auxin receptor F-box protein TIR1 (4: Terrile *et al.*, 2012). CKs induce NO biosynthesis depending on plant cell status (5: Yu *et al.*, 1998), while NO-derived peroxynitrite (ONOO$^-$) reacts with certain CKs such as zeatin rendering them less active (6: Liu *et al.*, 2013). In addition, NO regulates CK signalling through *S*-nitrosylation of type-A response regulators (7: Feng *et al.*, 2013). Arrows and bars indicate positive and inhibitory effects, respectively.

(MPK6) (Wang *et al.*, 2010, 2011). Notably, NIA2 interacts physically with MPK6 and serves as a substrate of MPK6. Phosphorylation of NIA2 by MPK6 led to an increase in NR activity and NO production. Furthermore, Wu and Wu (2008) indicated the involvement of ATP in activating the NR-dependent NO biosynthesis in plant hairy roots. NOS-dependent NO production also seems to be involved in auxin-induced LR development. Thus, arginine (Arg) or an Arg derivative (spermine) could be a potential NO source in root development, while L-NAME, an analogue of Arg that inhibits NO production, would inhibit auxin-induced LR formation (Flores *et al.*, 2008). Additional genetic studies with arginase mutants support previous results. Arginase mutants have increased LR formation in response to auxin due to a greater conversion rate of Arg to NO. In contrast to NR-activity, Arg-dependent NOS activity is CaM/$Ca^{2+}$ dependent (Bogdan, 2001) and both of them contribute to the ATP-induced NO biosynthesis (Wu and Wu, 2008). Remarkably, NOS activity is also required during cytokinin induction (Tun *et al.*, 2008) or osmotic stress (Kolbertz *et al.*, 2008), although time-courses of NO-production curves are different (Kolbertz *et al.*, 2008). After auxin addition, LR number increased in parallel with an intensified NO generation. However, under osmotic stress, the onset of LR initials was preceded by a transient burst of NO. It is therefore feasible that NO signalling pathways act differently in these situations.

On the other hand, NO can indirectly increase auxin levels (Hunt *et al.*, 2002; Xu *et al.*, 2010; Elhiti *et al.*, 2013). In *Medicago truncatula* Gaertn. seedlings under cadmium stress, exogenous NO application improves stress tolerance by reducing oxidative damage and indole-3-acetic acid (IAA) oxidase-driven auxin degradation, thus maintaining auxin equilibrium (Xu *et al.*, 2010). According to Hunt *et al.* (2002), non-symbiotic haemoglobins (nsHbs), which reduce endogenous NO levels, inhibit auxin metabolism, resulting in a drastic modification of root morphology and development.

Interactions between NO and auxin transport and perception are well documented (Fig. 4). NO promotes primary root growth at low concentrations (Gouvêa *et al.*, 1997; Hunt *et al.*, 2002; Pagnussat *et al.*, 2002) 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). Perilli *et al.* (2012) identified PIN1 (PIN-FORMED 1)-mediated polar auxin transport as a key regulatory element of meristem size. Cytoskeleton and vesicular transport are essential to the polar localization of auxin transport proteins and the resulting asymmetric distribution of this phytohormone. Pharmacological studies have shown that internalization and recycling of auxin transport proteins PIN1, PIN2 and AUX1 are dependent on actin (Grunewald and Friml, 2010). Recently, it has been demonstrated that increased NO levels promote disturbances on the actin cytoskeleton and actin-dependent endocytosis in the maize root apex (Kasprowicz *et al.*, 2009; Lombardo and Lamattina, 2012) and *Arabidopsis* plants (Rodríguez-Serrano *et al.*, 2014). Remarkably, these alterations could be generated as a consequence of post-translational modification of actin by oxidation and *S*-nitrosylation (Rodríguez-Serrano *et al.*, 2014).

Recent studies have shown that NO can also modify auxin transport and signalling (Fernández-Marcos *et al.*, 2011, 2012, 2013; Terrile *et al.*, 2012; Sanz *et al.*, 2014) (Fig. 4). Enhanced NO levels inhibit acropetal auxin transport in *Arabidopsis* roots through the alteration of PIN1 levels in a mechanism independent of proteasome-mediated protein degradation. This is correlated with a reduction in auxin response in NO-overproducer *cue1/nox1* mutant, as shown by the diminished expression of *DR5pro:GFP* marker (Fernández-Marcos *et al.*, 2011). Furthermore, *noa1* and *nia1nia2noa1* NO-deficient mutant roots display small root meristems with abnormal divisions. Concomitantly, auxin biosynthesis, transport and signalling are perturbed (Sanz *et al.*, 2014). However, Terrile *et al.* (2012) have observed that NO positively regulates auxin signalling through *S*-nitrosylation of the auxin receptor protein TIR1 (TRANSPORT INHIBITOR RESPONSE 1). This post-translational modification improves TIR1 binding to auxin response repressor proteins Aux/IAAs (AUXIN/INDOLE-3-ACETIC ACID), resulting in their degradation, thus promoting transcription of auxin-responsive genes.

In addition, NO plays an important role during LR formation in response to biotic and abiotic stresses (Creus *et al.*, 2005; Chen *et al.* 2010; Jin *et al.* 2011; Liao *et al.*, 2012; Wang *et al.*, 2013). In higher plants, root hairs are specialized cell types with an important role in root anchoring and in the absorption of water and nutrients. NO is involved in *Arabidopsis* root hair formation in both the initiation and elongation phases (Lombardo *et al.*, 2006). It was found that exogenous treatment with SNP enhances initiation of root hairs in the elongation zone through the reorientation of cortical microtubules (Yemets *et al.*, 2009). Additionally, NO is necessary for the proper dynamics of the endocytosis and vesicle formation routes in root hairs (Lombardo *et al.*, 2012). Root hairs secrete ATP as they grow, and these extracellular nucleotides trigger signalling pathways functioning as plant cell growth regulators. NO plays a role in this process, mediating the effects of extracellular nucleotides on root hair growth (Clark *et al.*, 2010; Terrile *et al.*, 2010). Taken together, all these results show the key role of NO at different levels in a great number of developmental processes related to root architecture.

### *NO and cytokinins*

CKs are key hormones that regulate root development, its vascular differentiation and root gravitropism. CK addition reduces primary root growth and meristem length by stimulating the expression of *SHY2* through ARR CK-responsive transcription factors, which are also stimulated by the RGA DELLA protein. The levels of *RGA* expression are suppressed by GAs. Synergistic and antagonistic effects of NO on CK metabolism have been observed (Fig. 4). Therefore, NO might act downstream mediating CKs responses (Tun *et al.*, 2008) or independently (Romanov *et al.*, 2008), probably depending on plant cell status. Remarkably, most of the synergistic effects between both molecules have been established by combining CKs with molecular tools that reduce NO levels (NO-scavengers, arginine-based inhibitors of NOS or NO-deficient mutants). Thus, NO deficient mutant *noa1* exhibits reduced sensitivity to the inhibition of root growth when treated with CKs. Furthermore, NO mediates CK-induced activation of *CYCD3;1* during cell proliferation and overexpression of *CYCD3;1* complements meristematic defects of the *noa1* mutant in root tissues (Shen *et al.*, 2013).

However, antagonistic effects of NO and CKs have been established by combining CKs with molecular tools which increase NO levels (NO donors or overaccumulator mutants). CKs rescue the minor shoot growth phenotype resulting from high levels of endogenous NO in the *nox1/cue1* mutant (Liu *et al.*, 2013). Conversely, treatment of plants with NO brings a net decrease in CK activity, while exogenous CKs inhibit primary root growth. A possible mechanism underlying this regulation might involve the interaction between both molecules since peroxinitrite can react with zeatin (Liu *et al.*, 2013). In agreement with this, high levels of endogenous NO (SNP, GSNO, *gsnor1, nox1*) repress CK signalling. When treated with CK, *gsnor1* and *nox1* mutants showed a decreased sensitivity to CK in the inhibition of root growth. Recently, Feng *et al.* (2013) demonstrated that NO represses CK signalling by inhibiting the phosphorelay activity through *S*-nitrosylation of the histidine phosphotransfer protein AHP1.

## Future prospects

NO is a versatile free radical that mediates numerous biological functions within early plant development. It is an excellent signalling molecule as its toxicity requires NO to be kept at low levels in cells. This allows subtle changes in its synthesis to lead to large differences of magnitude in its levels, like other important signalling molecules, such as calcium and protons. As a result, there is a precise balance between synthesis and scavenging of NO that allows its level to be carefully modulated. In its interaction with phytohormones, NO is one of the first signalling cues being produced after auxin, ABA, GAs or CKs addition. It is still an open question whether NO roles in response to phytohormones could be varied enough, because in addition to its different levels of accumulation, NO meets specific cellular functions within the routes of hormonal regulation in plant cells. Recent findings have allowed the identification of key components in NO homeostasis. Targeted and inducible systems will allow for a temporal and spatial controlled activation of those components involved in NO homeostasis. Therefore, future experiments will test whether or not local NO production in specific cell types is sufficient to mimic NO-related phenotypes. Future studies should deepen more in direct targets of NO biosynthesis, perception and signalling to help us understand the precise regulatory mechanisms that occur during germination and subsequent early growth and development.

In our group, a phenotypic, molecular, and genetic characterization in *Arabidopsis* is carried out in order to further understand the mechanisms by which NO acts during growth and development, studying NO involvement in hormonal signal transduction pathways and gene expression regulation during these processes. In this sense, we have implemented several genetic screenings of cPTIO- and ABA-insensitive mutants during the process of dormancy release and germination promotion in *Arabidopsis thaliana* seeds (authors own unpublished data) and proper NO in the process of hypocotyl elongation during etiolation and root development (Fig. 5).

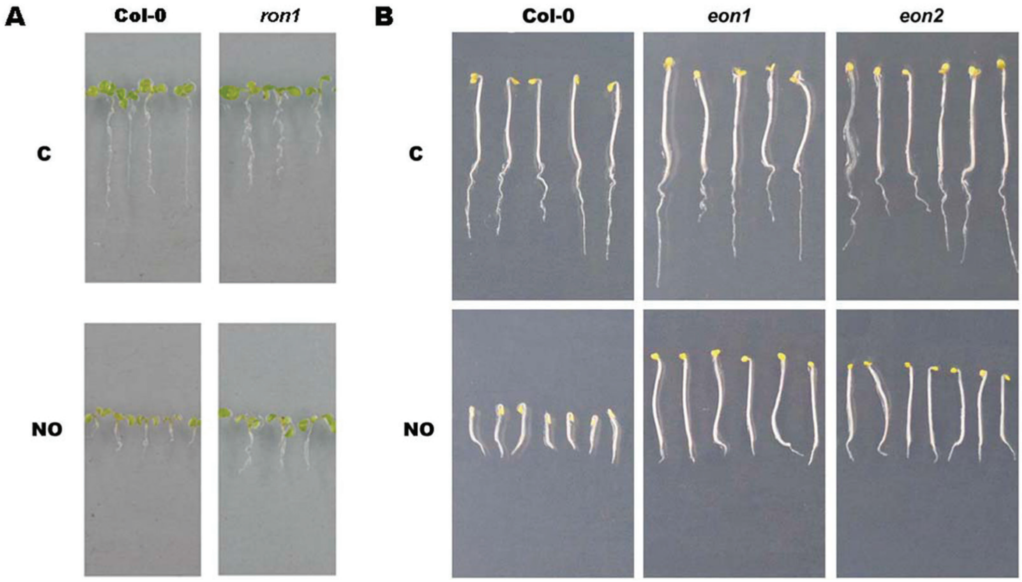

**Fig. 5.** Screening approaches to identify nitric oxide (NO) insensitive mutants during primary root growth and hypocotyl elongation (*ron* and *eon*, respectively). (A) Phenotype of *ron* (*roots on NO*) mutants impaired in primary root growth. (B) Phenotype of *eon* (*elongated on NO*) mutants impaired in hypocotyl elongation under dark growth. We screened 32 M1 ethylmethane sulfonate (EMS)-mutagenized Col-0 parental seed batches (Lehle seeds) using NO donors (SNP, SNAP). The screening yielded new putative mutants (M2) showing NO-insensitive root growth and hypocotyl elongation phenotypes compared to that of Col-0 and control conditions. M3 seeds were obtained from the putative mutants, which confirmed reduced NO sensitivity.

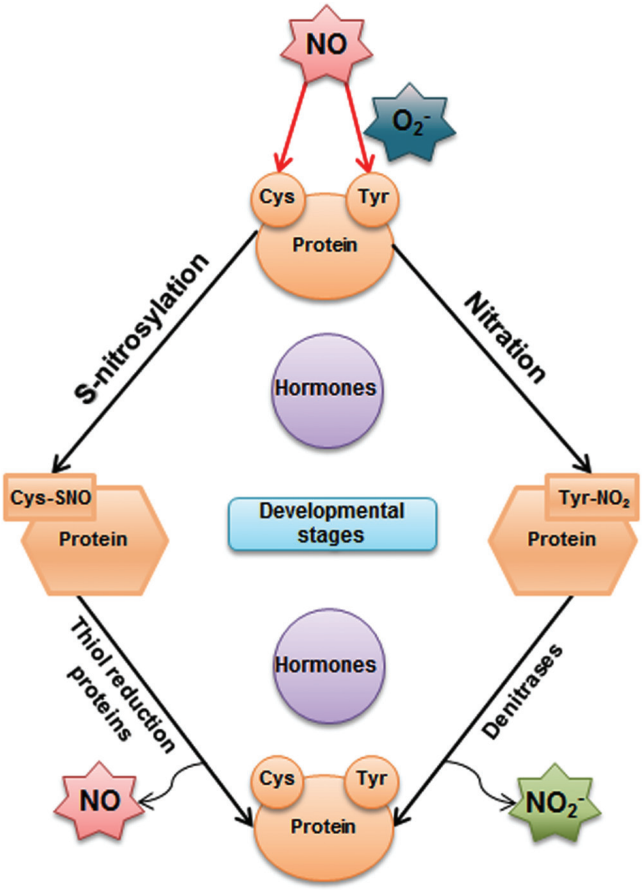

**Fig. 6.** Outline of the post-translational modifications of proteins by NO in a hormone- and developmental stage-based context. The putative role of Cys *S*-nitrosylation (Cys-SNO), Tyr nitration (Tyr-$NO_2$), thiol reduction proteins (denitrosylases) and denitrases is included.

Several candidate genes have been cloned and are under study to determine key targets that regulate NO and various plant hormones during early development. The inhibition of root growth by NO has already been used as a phenotype to screen NO-hypersensitive mutants (He *et al.*, 2004). This screening resulted in the isolation of the NO overproducer mutant *nox1* and the identification of *chlorophyll alb binding protein (CAB) underexpressed 1* (*CUE1*) as the mutated gene, evidencing the role of NO as a repressor of the floral transition in *Arabidopsis*.

NO directly modulates the activity of proteins through post-translational modifications (PTM). PTMs mediated by NO, such as cysteine *S*-nitrosylation or tyrosine nitration can result in an alteration of diverse protein functions. Protein de-nitrosylation, the removal of NO groups primarily from Cys thiol side chains in proteins, is an important but less studied aspect of NO-based signalling. Given the reversible nature of this PTM, *S*-nitrosylation and de-nitrosylation could be an efficient and rapid mechanism of response to redox status. Thus, de-nitrosylation through the thioredoxin/thioredoxin reductase system seems to be a part of signal transduction mechanisms (Lindermayr and Durner, 2009). In fact, some proteins belonging to Trx and Grx families change their expression patterns differently during seed germination, early plant development and in response to phytohormones (Belin *et al.*, 2014). It still remains to be established how these proteins act in these processes. Our hypothesis is that NO acts in plant developmental processes through a complex signalling pathway that includes the cellular redox levels, the PTMs of specific proteins by *S*-nitrosylation and the interaction with other plant growth regulators (i.e. phytohormones) using similar molecular components. This conceptual change in our current view of the NO pathway is also essential to understand the crosstalk between different plant growth regulators, and thus, to decipher the plant molecular mechanisms that select the correct set of responses to different developmental cues.

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