Diverse functional interactions between nitric oxide and abscisic acid in plant development and responses to stress

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Abstract

The extensive support for abscisic acid (ABA) involvement in the complex regulatory networks controlling stress responses and development in plants contrasts with the relatively recent role assigned to nitric oxide (NO). Because treatment with exogenous ABA leads to enhanced production of NO, it has been widely considered that NO participates downstream of ABA in controlling processes such as stomata movement, seed dormancy, and germination. However, data on leaf senescence and responses to stress suggest that the functional interaction between ABA and NO is more complex than previously thought, including not only cooperation but also antagonism. The functional relationship is probably determined by several factors including the time- and place-dependent pattern of accumulation of both molecules, the threshold levels, and the regulatory factors important for perception. These factors will determine the actions exerted by each regulator. Here, several examples of well-documented functional interactions between NO and ABA are analysed in light of the most recent reported data on seed dormancy and germination, stomata movements, leaf senescence, and responses to abiotic and biotic stresses.

Key words: ABA, abiotic stress, defence, leaf senescence, nitric oxide, seed germination, stomatal closure.

Introduction

Plant growth is the successful consequence of a finely regulated network of hormone-controlled metabolic processes. Endogenous metabolic cues and exogenous environmental factors are transmitted through intricate signalling pathways that involve the perception of different stimuli and the downstream alteration of multiple processes. Changes in ion channels, enzyme activities, levels of reactive molecules, post-translational modification, and localization of proteins are often linked to modification of gene expression. The proper execution of such a complex scenario of regulatory events allows the plant to complete developmental transitions throughout its life cycle. However, plants do not perceive only the effects of positive environmental stimuli. Some environmental stress conditions of both biotic and abiotic origin affect plants negatively. Because plants cannot travel and their capacity for movement is limited, they rely on the plasticity of metabolism and the versatility of hormonal regulation to avoid detrimental effects of stress.

The small molecule nitric oxide (NO) has recently been characterized as a co-regulator of many plant processes. This small molecule has the peculiarity of being a gas and also of being a free radical. These physicochemical features determine NO regulatory functions as a result of the balance between diffusivity from the biosynthesis site, and reactivity...
with components of the cellular microenvironment surrounding its production site. Its regulatory role is often exerted in connection with the classical hormones auxins, cytokinins, gibberellins (GAs), ethylene, and abscisic acid (ABA), as well as the more recently characterized jasmonates, salicylates, brassinosteroids, and strigolactones. Functional interactions between most of the hormones and regulators mentioned above have been reported in plant development and responses to stress (Durbak et al., 2012; Freschi 2013; Simontacchi et al., 2013). Because some of the NO effects on plant physiology have been particularly well studied in processes such as seed germination and guard-cell movement controlling stomatal closure, both being critically regulated by ABA, this review will focus on the functional connections between ABA and NO. Although both molecules have mostly been considered as functioning in the same direction, with NO acting as a second messenger of ABA (Hancock et al., 2011), there is much evidence suggesting that NO and ABA do not always function in this way. In fact, NO may exert a general negative regulation on ABA perception that may function as a feedback-loop mechanism to fine tune the magnitude or intensity of ABA-triggered responses (Lozano-Juste and León, 2010b). Special emphasis will be placed on these cases in this review, with the final goal of discussing how the diverse functional interactions between NO and ABA fit with our current knowledge of different plant physiological processes ranging from seed germination to different responses to stress.

Controversy surrounding NO biosynthesis and its connection to ABA

Pharmacological and genetic approaches have been used to elucidate the existence of different NO sources in plants. It has been reported that ABA induces NO production in plants (Guo et al., 2003), and also that NO synthesis requires enhanced production of \( \text{H}_2\text{O}_2 \) (Bright et al., 2006). The use of mammalian NO synthase (NOS) inhibitors, such as L-NG-nitroarginine methyl ester (L-NAME), has been reported to reduce NO levels (Corpas et al., 2006), thus suggesting the existence or arginine-dependent production of NO in plants. In Arabidopsis thaliana, there are contradictory published data, either describing no inhibition (Desikan et al., 2002) or L-NAME-inhibited NO production by ABA (Guo et al., 2003; Bright et al., 2006). Alternatively, by using nitrate reductase (NR) inhibitors, the role of this enzyme in ABA-induced NO production has also been probed (Neill et al., 2003). In parallel, several genetic studies have been carried out to uncover the source of ABA-induced NO production. First, the nia1nia2 double mutant, with less than 1% of NR activity, showed a reduced accumulation of NO in guard cells (Desikan et al., 2002). Later work suggested that NIA1 but not NIA2 is required for NO production in response to ABA (Ribeiro et al., 2009). Secondly, Guo et al. (2003) showed that the Atnola1 mutant has reduced accumulation of NO in response to ABA in both the root tip and the guard cells, thus pointing to cell-type-independent impaired responses to NO in this mutant. Here, it should nevertheless be highlighted that either pharmacological or genetic approaches used for elucidating NO biosynthesis in plants have also reported weaknesses that should be borne in mind when analysing NO production. Interestingly, L-NAME has recently been reported to inhibit the oligogalacturonide-induced NR activity and consequent nitrite-dependent NO production (Rasul et al., 2012b). However, NO production was still detected in L-NAME-treated nia1nia2 mutant plants (Rasul et al., 2012b), indicating complex interactions between NO derived from arginine or from nitrite. Despite L-NAME has being reported to inhibit NR activity only in the context of oligogalacturonide-activated defence responses, this effect might also be functional in other circumstances. On the other hand, it has been reported that the nia1nia2 plants contained low endogenous levels or arginine, thus also suggesting the possible defective arginine-dependent production of NO in NR mutant plants (Modolo et al., 2006). The NOA1 gene codes for a cGTPase (Moreau et al., 2008) and not for a NOS-like enzyme as considered previously. It has been proposed that the reduced NO content in the AtnOa1 mutant is the result of indirect effects derived from altered protein synthesis in chloroplasts (Liu H et al., 2010a). Finally, the generation of a triple nia1nia2noa1-2 mutant affected in both NR activity and NOA1 function revealed that both pathways are independent and contribute to NO production in response to ABA in Arabidopsis (Lozano-Juste and León, 2010a). Therefore, despite the possible interaction between both genetic pathways, other pathways have also been proposed to be involved in NO production. Several reviews published recently have addressed in detail the different origins and biosynthetic pathways of NO in plants (Moreau et al., 2010; Gupta et al., 2011; Mur et al., 2013). Consequently, emphasis should be placed on the assessment of advantages/weaknesses of pharmacological and genetic approaches when studying NO function in plants. Moreover, additional caution should be taken when the analysis of NO production is performed in different plant organs or under different physiological conditions, where the contribution of different components and pathways to NO synthesis might also be different. As an example, ABA-triggered production of NO might be different in roots and shoots of the same plant, or even when comparing mesophyll with guard cells on the same leaf.

Role of NO in ABA-inhibited seed germination

The germination of seeds occurs only when dormancy, which negatively controls germination, is released. It is widely assumed that dormancy is directly promoted by endogenous levels of ABA and also that germination is enhanced by GAs (Finch-Savage and Leubner-Metzger, 2006). Both hormones interplay with other components such as ethylene to control seed germination (Finkelstein et al., 2008; Holdsworth et al., 2008; Gruaer et al., 2012). Although dormancy can be seen as a negative input for plant development, it is, in turn, an
essential quality of seeds of many species that allow them to keep quiescent until environmental conditions allow a future successful development.

In many plant species, endogenous cues as well as environmental factors favour seed germination through the mobilization and use of endosperm carbohydrate and lipid reserves. These catabolic processes allow embryo root to grow and emerge through the seed coat. Besides the positive and negative effects exerted by GAs and ABA on these events, several other regulatory components have been identified that influence seed germination. Nitrate, a major nitrogen source for plants, as well as nitrite have been reported to promote seed dormancy release (Bethke et al., 2006b). Exogenously supplied nitrate seems to promote seed germination (Alboresi et al., 2005) through the regulation of phytochrome signalling (Batak et al., 2002). Because seed levels of nitrate and ABA are negatively correlated, and the expression of ABA catabolic genes is positively regulated by NO (Matakiadis et al., 2009), nitrate effects on seed dormancy and germination are probably exerted through the stimulation of NO-mediated ABA catabolism. A more complex regulatory model including nitrogen and reactive oxygen species (ROS), such as NO and H₂O₂, respectively, has emerged to better explain the transition from dormancy to germination. These types of reactive species would act as synergistic effectors in releasing dormancy by acting upstream of ABA. Despite the fact that ABA induces NO production, it remains controversial which reductive or oxidative pathway for NO biosynthesis is functional in seeds (recently reviewed by Arc et al., 2013). Nevertheless, it is well known that NO does not cooperate with ABA in inhibiting seed germination but, in contrast, promotes it (Beligni and Lamattina, 2000; Libourel et al., 2006; Bethke et al., 2007). Because treatment with NO scavengers reduces germination in dormant but not in non-dormant Arabidopsis seeds, the positive effect exerted by NO seems to be due mainly to released dormancy (Bethke et al., 2006a, b). NO scavengers have been also reported to prevent germination of imbibed tomato seeds treated with the ABA biosynthesis inhibitor fluridone (Piterková et al., 2012). In turn, NO donors potentiates the germination induced by the ABA biosynthesis inhibitor norflurazon and reduced the sensitivity to ABA in Arabidopsis seeds (Bethke et al., 2006a). In agreement with this pharmacological approach, seeds of NO-deficient Arabidopsis mutants are hypersensitive to ABA and have enhanced dormancy and reduced germination potential (Lozano-Juste and León, 2010a). Moreover, increased production of NO due to both NR- and NOS-like activities has been described in embryos upon imbibition of seeds (Simontacchi et al., 2007). NO derived from the storage form nitrosoglutathione (GSNO) also seems to be relevant to control seed germination, as demonstrated by the reduced germination of atgsnor1-3 mutant seeds with loss of GSNO reductase function (Kwon et al., 2012). Although still far from being completely known, the mechanism underlying the releasing effect exerted by NO on dormancy seems to involve the production and perception of NO in aleurone cells (Bethke, 2009), the promotion of GA synthesis and downstream signalling in the embryo, and a decrease in ABA content by upregulating the metabolic CYP707A2 gene encoding an enzyme with (+)-abscisic acid 8’-hydroxylase activity (Liu et al., 2009). Both negative and positive effects exerted on ABA and GA accumulation and signalling, respectively, are the result of H₂O₂-triggered events that are mediated by NO (Liu Y et al., 2010b). Despite the existence of extensive experimental data supporting the role of NO in promoting ABA catabolism, it has also been proposed that NO might function by decreasing the sensitivity of seeds to ABA (Bethke et al., 2006a, b; Lozano-Juste and León, 2010b). On the other hand, aquaporin-encoding genes have been characterized as NO-inducible targets promoting seed germination in rice (Liu et al., 2007), thus supporting the importance of NO-regulated water transport during seed germination. An extensive review describing the current knowledge on how NO and ethylene counteract ABA action, thus releasing dormancy and promoting seed germination, was published recently (Arc et al., 2013).

NO-mediated post-translational modification is a field of growing interest because both cysteine S-nitrosylation (Lindermayr et al., 2005) and tyrosine nitration (Lozano-Juste et al., 2011) of proteins have been identified and characterized as an important level of regulation in NO-modulated processes. Interestingly, a functional connection between NO and ABA has been deduced from the proteomic identification of several nitrated proteins including the E3 SUMO-protein ligase SIZ1, and the molybdenum co-factor (Moco) sulfatase encoded by ABA3 gene (Lozano-Juste et al., 2011), both reported to be related to ABA signalling and biosynthesis. The SUMO E3 ligase SIZ1 has been reported to control ABA-related responses through sumoylation of the transcription factors ABI5 and MYB30 (Miura et al., 2009; Zheng et al., 2012). Moreover, the ABA3/Moco sulfatase is involved in catalysing the conversion between the sulfo- and desulfo-forms of Moco, which are the co-factors of aldehyde oxidase and NRs (Mendel and Hänsch, 2002), involved in ABA and NO biosynthesis, respectively. The identification of nitrated forms of the S-adenosylmethionine synthases METK1 and METK2 (Lozano-Juste et al., 2011) involved in the biosynthesis of both ethylene and polyamines opens another interesting functional link between polyamine-triggered NO production, ethylene biosynthesis, and ABA-related signalling (Wimalasekera et al., 2011).

All data available on NO-mediated promotion of seed germination fit with a model (Fig. 1) where NO produced and perceived in the aleurone/endosperm cell layer is followed by increased ABA catabolism in the aleurone and by the synthesis of active GAs in the embryo. The signalling cascade is then directed back to the aleurone where GA-inducible cell-wall degrading enzymes facilitate weakening of the physical barrier that encloses the root embryo, allowing root emergence and thus germination. In this series of events, the above-presented data strongly suggest that NO has a negative role in ABA signalling exerted at least at the ABA homeostasis checkpoint. How NO controls the expression of ABA catabolic genes is still unknown, although the post-translational modification of key proteins is certainly a possibility. The same situation is valid to explain the NO regulation of ABA
sensitivity, where nitration of regulatory proteins could affect transcription factors required for ABA signalling.

**NO–ABA interactions in the control of stomata movements**

Guard cells are unique specialized epidermal plant cells that, in pairs, form the stomata, which are involved in the precise control of gas exchange in leaves. Stomatal closure is the result of water-content-driven changes in the turgor of guard cells. ABA has been considered as a major component controlling guard cell signalling acting in close connection with other hormones, and NO has emerged lately as an important signalling molecule in the control of stomata movements (Daszkowska-Golec and Szarejko, 2013). It has been reported that ABA induces an increase in NO that is necessary for ABA-triggered stomatal closure or inhibition of opening (Desikan et al., 2002). An extensive body of evidences has supported an established model assigning a positive role for NO in ABA signalling controlling stomata movements. However, there are some recently published data that contradict this assumption. It is important for NO researchers to be aware of such controversy and to keep it in mind for a careful interpretation of future work. We will highlight these controversial issues in this review.

A pharmacological approach led to the finding that NO, released by the application of NO donors, such as sodium nitroprusside (SNP), S-nitroso-N-acetyl-DL-penicillamine and GSNO, induced stomatal closure in different plant species. Concomitantly, treatment with the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) prevented ABA-induced stomatal closure (Garcia-Mata and Lamattina, 2001, 2002; Desikan et al., 2002; Neill et al., 2002a). Although NO scavenging by cPTIO attenuated stomatal closure induced by ABA, this scavenger did not block stomatal closure completely. These data suggest the existence of ABA-dependent NO-independent stomatal closure pathways, although it cannot be ruled out that incomplete NO scavenging may be the cause of partial stomata responses. Supporting a NO regulatory role in stomata movements, pharmacological and genetic approaches have demonstrated that NO is synthesized in guard cells. However, there is still some controversy regarding data derived from pharmacological approaches that deserves more attention. By using NR inhibitors such as tungstate, the role of this enzyme in ABA-induced NO production as well as its effect on stomatal closure has been demonstrated (Neill et al., 2003). The use of mammalian NOS inhibitors, such as L-NAME, was also reported to reduce NO levels and stomatal closure in plants (Neill et al., 2002a, b). These data support the contribution of NO biosynthetic pathways to stomata movements. However, it has been reported that NO donors such SNP or GSNO also inhibited NR activity in wheat leaves (Rosales et al., 2011). Both inhibition and activation of NR activity by L-NAME treatment have been reported in Arabidopsis (Rasul et al., 2012b) and wheat (Rosales et al., 2011), respectively, providing concern as to how this compound alters NO synthesis in plants, and also about the usefulness of these pharmacological approaches. Fortunately, the genetic approach serves as an alternative strategy to overcome doubts generated by the pharmacological characterization. The double nia1nia2 and the noa1-1 mutants showed reduced accumulation of NO in guard cells accompanied by impaired stomatal closure in response to ABA (Desikan et al., 2002; Guo et al., 2003). Moreover, the generation of a triple mutant affected in NR activity and NOA1 function revealed that both pathways are independent and contribute to NO production (Lozano-Juste and León, 2010a). The accumulation of NO in the guard cells of this triple mutant was undetectable even in the presence of ABA (Lozano-Juste and León, 2010a). Therefore, genetic analysis of NO production in guard cells clearly shows that NO is produced in guard cells through both NO biosynthetic pathways. These studies also proved that genetic analysis might be a determinant, especially when pharmacological approaches are not clear enough.

The transport of osmotically active ions and malate across membranes and subsequent membrane depolarization are essential processes in controlling stomatal closure (Pandey et al., 2007). The slow (S)-type anion channel SLAC1 and the potassium inward rectifier channel KAT1 have been characterized as key components in regulating membrane depolarization and stomatal closure, and both are targets of the ABA-related OST1/SnRK2.6 kinase (Sato et al., 2009; Vahisalu et al., 2010). Interestingly, the slac1 mutant displayed reduced stomatal closure in response to factors different to ABA, including CO2, ozone, transition from light to darkness, Ca2+, H2O2, and NO (Vahisalu et al., 2008). It has also been reported that NO participates in ABA-induced
stomatal closure through the regulation of inward-rectifying K\(^+\) and anion channels (García-Mata et al., 2003). In this context, NO negatively regulates inward-rectifying K\(^+\) channels, while it exerts a positive effect on anion channels (García-Mata et al., 2003). This regulation over inward K\(^+\) channels promotes an increase in cytoplasmic Ca\(^{2+}\) (García-Mata et al., 2003). However, because Ca\(^{2+}\) is required for NO production but not for NO-induced inhibition of stomatal opening, it seems that Ca\(^{2+}\) acts upstream of NO production for the inhibition of stomatal opening by ABA but downstream during ABA-induced stomatal closure (García-Mata and Lamattina, 2007).

It is noteworthy that NO also regulates the activity of K\(^+\) efflux channels perhaps through the nitrosylation of cysteine sulphhydril groups either of the K\(^+\) channel or of a closely associated regulatory protein (Sokolovski and Blatt, 2004).

Several molecules including H\(_2\)O\(_2\), cyclic GMP (cGMP), cyclic ADP ribose (cADRP), and Ca\(^{2+}\) have been characterized as working together with NO as part of ABA signalling in guard cells. Whereas in wild-type guard cells ABA induces a burst of H\(_2\)O\(_2\) and NO, in the rbohD/F double mutant affected in NADPH oxidases D and F, H\(_2\)O\(_2\) and NO production as well as ABA-induced stomatal closure were impaired (Bright et al., 2006). This points to an ABA-dependent and rbohD/rbohF-mediated production of NO in guard cells. Moreover, the nia1nia2 double mutant, but not the noa1 mutant, is severely affected in NO production in response to H\(_2\)O\(_2\) in guard cells (Bright et al., 2006), suggesting that H\(_2\)O\(_2\) directly or indirectly affects NR-dependent but not NOA1-related NO production in guard cells. In root cells, H\(_2\)O\(_2\) regulation of NR activity involves mitogen-activated protein kinase (MAPK) signalling, in such a way that MAPK6 phosphorylates NIA2 to induce NO production (Wang et al., 2010b). However, in guard cells, nia2-5 but not nia1-2 mutant retained wild-type levels of NO production after H\(_2\)O\(_2\) treatment (He et al., 2013), suggesting that NIA1 seems to be the isoform required for NO production in response to H\(_2\)O\(_2\) in stomata. Whether differences in the NR isoform required for H\(_2\)O\(_2\)-induced NO production between guard and root cells respond to a tissue-specific H\(_2\)O\(_2\)-NO signalling is an important and unexplored question. It is noteworthy that crosstalk between these two molecules is likely to be more complex than described above. NO seems to regulate H\(_2\)O\(_2\) content by inducing its metabolism (Hasanuzzaman and Fujita, 2013) or by inhibiting its production through NAPDH oxidase S-nitrosylation (Yun et al., 2011). This process would represent a negative-feedback loop mechanism by which ABA could induce H\(_2\)O\(_2\) production that activates NO biosynthesis that then reduces H\(_2\)O\(_2\) levels. Taking into account that H\(_2\)O\(_2\) is a positive regulator of ABA-induced stomatal closure, NO could have a dual role in this process, exerting a positive effect probably in fast responses, but acting later as a negative modulator of protein function through post-translational modifications of enzymes or proteins with signalling potential. Such an expanding panorama on NO–ABA interactions weakens the current established model considering NO just as a downstream intermediate in ABA signalling.

As mentioned above, Ca\(^{2+}\), cADPR and cGMP are also involved in NO signalling in guard cells. Even though stomatal closure in the absence of calcium has also been described (Roelfsema and Hedrich, 2010), the entry of Ca\(^{2+}\) from the extracellular space as well as its release from intracellular reservoirs are required for a correct response to ABA in guard cells (MacRobbie, 2000). Extracellular Ca\(^{2+}\) could be perceived by a membrane receptor in guard cells (Han et al., 2003) or by extracellular calmodulin activating a signalling pathway that includes the α-subunit of G protein (Li et al., 2009). Perception by calmodulin activates AtRbohF-dependent H\(_2\)O\(_2\) production and, subsequently, NOA1-related NO production (Li et al., 2009). On the other hand, it has also been reported that ABA-induced cytoplasmic Ca\(^{2+}\) release might be mediated either by cGMP production (Dubovskaya et al., 2011) or by changes in cADPR levels (Meimoun et al., 2009). Treatment with the cADPR synthesis inhibitor nicotinamide reduces ABA-induced stomatal closure and ion flux through the plasma membrane (Leckie et al., 1998; MacRobbie, 2000) and inhibited ABA and NO-induced stomatal closure in pea (Neill et al., 2002a). NO promotes intracellular Ca\(^{2+}\) release and thereby regulates guard cell ion channels via a subset of signalling events provoked by ABA that involve cGMP function (García-Mata et al., 2003). A recent report has described that, in the presence of ROS, ABA and NO trigger the nitration of cGMP to produce 8-nitro-cGMP, which acts as a NO-derived second messenger that promotes stomatal closure in light (Joudoi et al., 2013). On the other hand, the NO-dependent guanylate cyclase mutant (nogc) that is impaired in cGMP production induced by NO is also impaired in ABA- and NO-induced stomatal closure (Joudoi et al., 2013). Because 8-bromo-cGMP, a membrane-permeating analogue of cGMP, did not trigger stomatal closure in light and instead promoted stomatal opening in darkness, cGMP and its nitrated derivative probably play different roles in the control of stomatal opening and closure (Joudoi et al., 2013). Thus, both agonistic and antagonistic effects can be depicted in the NO–ABA functional interaction in controlling stomatal closure through secondary metabolites such as cGMP and its derivatives. Interestingly enough, it has been reported that mutant plants severely compromised in NO production are hypersensitive to ABA in stomatal closure and also display a non-wilted phenotype under water shortage and a strong resistance to drought (Lozano-Juste and León, 2010a). Because NO-dependent guanylate cyclase-mediated production of cGMP will be severely reduced in NO-deficient plants, its proposed role in stomatal opening under darkness would also be diminished, thus also leading to closed stomata during the dark periods. Based on these data, the assumption of NO as a simple positive regulator of stomata movements acting downstream of ABA needs to be reassessed. A detailed study of cGMP’s role on stomatal closure and the NO-deficient mutant phenotypes points to a new role of NO negatively regulating stomatal closure (Lozano-Juste and León, 2010a; Joudoi et al., 2013). However, more work is needed to fully understand the complex functional interaction between NO and ABA in the stomatal context.
Despite a large amount of information supporting an involvement of NO in the regulation of stomatal closure, only a few reports describe this regulation at the whole-plant level. The majority of papers analysing this response are based on studies with epidermal peeling of leaves. If ABA-induced stomatal closure is mediated by NO, could we tune stomatal closure and therefore drought stress tolerance by regulating NO levels? Desikan et al (2002) reported that they could not observe a wilting phenotype in the nia1nia2 mutant as expected for a mutant totally blocked in ABA-induced stomatal closure. Furthermore, NO generation seems to be required for the ABA-induced closure of stomata in turgid leaves but not in dehydrated leaves (Ribeiro et al., 2009). Besides, neither wild-type plants treated with NO donors or scavengers nor NO-deficient nia mutants showed a significant alteration in the ABA-induced stomatal closure of leaves undergoing water deficit (Ribeiro et al., 2009). Again, this data clearly suggests that NO is not a mere positive regulator of ABA-induced stomatal closure. Actually, these studies described a role of NO on stomatal closure in epidermal peels that did not fit well with the behaviour of whole plants deficient in NO production (Desikan et al., 2002). Additionally it seems that this is related to the plant water content and to the drought conditions (Ribeiro et al., 2009). Taken together, we propose that NO synthesis and action are not required for ABA-mediated stomatal closure during drought responses in whole plants. Moreover, because NO-deficient plants are hypersensitive to ABA (Lozano-Juste and León, 2010a), it is logical to propose that, under certain conditions, NO might actually act as an early negative modulator of ABA action, maybe by altering hormone perception (Lozano-Juste and León, 2010b).

To summarize, the role that NO has in ABA-induced stomatal closure is still far from being clearly defined. Figure 2 provides a schematic diagram including some, but not all, the regulatory components and their functional interactions in promoting stomatal closure or opening depending on the environmental conditions. The complexity of the NO–ABA interactions is shown by both positive and negative effects exerted by NO on different ABA-regulated targets. The difficulty in matching data from epidermal peels and whole plants makes it difficult to provide conclusive evidence for the role of NO–ABA interaction in regulating stomatal closure. Based on pharmacological approaches in a decontextualized experimental system such as epidermal peels, a large body of data suggest that NO acts downstream of ABA in promoting stomatal closure. However, genetic approaches applied on whole plants under different watering conditions suggest that the role of NO interacting with ABA in guard-cell signalling is primarily devoted to regulating stomatal closure during the light-to-dark transition under non-stressed conditions. In fact, some recent reports suggest, as mentioned above, a negative role of NO in ABA-induced stomatal closure during the night. This contradictory scenario will certainly be clarified when exhaustive molecular and cellular studies are performed on previously identified direct targets of NO.

**NO and ABA in senescence**

Senescence is an active genetically controlled process that affects cells, tissues, organs, and even entire plants during the last developmental stages. It is characterized by a sharp decline in photosynthetic capacity, chlorophyll degradation, visible leaf yellowing, and a decrease in total RNA and protein content. A large number of factors influencing senescence have been described including age, developmental stage, nutrient supply, light, environmental interactions, and classical and newly characterized hormones and growth regulators, as well as varied metabolites (Fischer, 2012). Li et al. (2012) recently built gene networks with A. thaliana genes promoting or delaying senescence to identify common regulators. Their results demonstrated that cytokinin, auxin, and NO delay leaf senescence, whereas ethylene, ABA, salicylic acid (SA) and jasmonic acid (JA) promote leaf senescence. Many of these regulators might control leaf senescence in coordination with environmental and developmental cues, showing divergence in early senescence initiation-related gene expression but convergence in senescence execution-related molecular processes (Guo and Gan, 2012). Although the putative
connection of NO with ethylene, SA, and JA is very interesting, we are going to focus the next section on the NO–ABA interaction during the senescence process.

Several studies support a negative regulatory role of NO in plant senescence (Guo and Crawford, 2005; de Michele et al., 2009; Procházková and Wilhelmová, 2011). It has been reported that NO production decreases during maturation of plant organs (Leshem et al., 1998) and also that peroxisomes isolated from senescent pea leaves contain lower NO content than that of young leaves (Corpas et al., 2004). Accordingly, NO application has been demonstrated to delay yellowing and to retard the onset of chlorophyll degradation (Laxalt et al., 1997). By contrast, leaf senescence is promoted by reducing NO production in either loss-of-function mutants or upon heterologous inducible expression in Arabidopsis of a NO-degrading dioxygenase from Escherichia coli (Guo and Crawford, 2005; Mishina et al., 2007; Liu and Guo, 2013). Despite the experimentally supported role for NO in regulating senescence in plants, our knowledge about the dynamics of changes in NO and reactive nitrogen species as well as relevant enzymes during ageing and senescence is still deficient (Procházková and Wilhelmová, 2011). The contradictory effects observed for NO in regulating senescence at different concentrations might be, at least in part, explained by the complex interaction of NO with ROS that has also been characterized as important in regulating cell-death-related senescence symptoms (Li et al., 2012; Wang et al., 2013).

In contrast to the negative regulation exerted by NO on senescence, ABA is one of the most efficient promoters of leaf senescence. Accordingly, increased endogenous ABA levels have been detected in senescent plants, and exogenously applied ABA promotes leaf senescence and induces expression of senescence-related genes in several plant species (Even-Chen and Itai, 1975; Gepstein and Thimann, 1980; Smart, 1994; Yang et al., 2002, 2003). Moreover, several genes involved in ABA synthesis, metabolism, and signalling are upregulated during senescence (Buchanan-Wollaston et al., 2005). As mentioned before for NO, ABA also displays an extensive interaction with ROS in promoting leaf senescence. Elevated ABA levels cause an increased generation of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) and the enhanced expression of antioxidant genes and their corresponding enzyme activities, thus protecting the cellular functions required for the onset of senescence (Hung and Kao, 2003). The balance between cellular protection and senescence activities regulated by ABA seems to be determinant in controlling progression of leaf senescence, probably in connection with other senescence-affecting factors such as age (Lim et al., 2007; Khanna-Chopra, 2012). Despite the fact that ABA and NO are thought to act together in multiple plant responses, the scenario is opposite in senescence (Fig. 3A). NO prevents increases in \( \text{H}_2\text{O}_2 \) levels and lipid peroxidation and has a protective effect against ABA-, methyl jasmonate- and \( \text{H}_2\text{O}_2 \)-promoted senescence of rice leaves (Hung and Kao, 2003, 2004, 2005). This effect of NO is presumably due to its scavenging activity on \( \text{H}_2\text{O}_2 \) and other ROS, thus suggesting that antioxidant properties of NO are operating to counteract oxidative stress in rice leaves (Hung and Kao, 2003). It remains unclear whether the increased ABA levels in senescent leaves does not lead to increased NO accumulation, as occurs in non-senescent leaves, because of the antioxidant activity of NO, thus quickly reacting with ROS, or whether it is due to defective production in senescent tissues. Nevertheless, these data represent another example of how the environment and endogenous plant cues might alter the functional interaction between ABA and NO in different processes. Additionally, as the senescence programme is activated at the whole-plant level, the tissues responsible for NO signalling in this case are different from those on seed germination or stomatal movement pointing to a tissue-dependent mode of action of NO in plants.

### NO and ABA in plant responses to abiotic stress

Extensive experimental support accumulated during the last decade suggesting that NO plays an important role in a
broad spectrum of plant responses to abiotic stress together with ABA and other hormones. Under adverse environmental conditions, altered balance of NO production and metabolism causes nitrosative stress that influences both the responses to oxidative cues and the development and stress responses of plants (Corpas et al., 2011). The antioxidant properties of NO as well as its potential in activating ROS-scavenging enzyme activities under abiotic stress may explain many of their regulatory functions (Siddiqui et al., 2011; Fig. 3B). However, the mechanism(s) by which NO, in coordination with other hormones such as ABA, modulates the impact of abiotic stress often remains unclear.

Drought

Drought is one of the major constraints in limiting crop yield. ABA has been characterized extensively as a principal regulator of plant response to drought by inducing stomatal closure, therefore reducing water loss via transpiration (Davies and Zhang, 1991; Zhu, 2002). It has been received wisdom that NO cooperates in ABA-induced stomatal closure (Neill et al., 2003). Accordingly, the application of the NO donor SNP enhanced plant tolerance to drought stress by reducing water stress, ion leakage, transpiration rate and by inducing stomatal closure (Garcia-Mata and Lamattina, 2001). The activated antioxidant systems also contribute to a better performance of plant under water stress by removing excess ROS and by inhibiting lipid peroxidation. It has been proposed that NO and ROS enhance plant tolerance to water stress by regulating ABA biosynthesis (Zhao et al., 2001; Xing et al., 2004). Moreover, it has been also reported that the brassinosteroid (BR)-induced increase in ABA biosynthesis and tolerance to oxidative damage caused by water stress in maize leaves is, at least in part, due to the production of NO induced by BR (Zhang et al., 2011). The complexity of the functional interaction between NO and ABA in regulating responses to water stress is evident by the fact that, reciprocally, ABA induces the accumulation of NO, which, in turn, activates MAPK and upregulates the expression of genes coding for antioxidant enzymes (Lamattina et al., 2003; Zhang et al., 2007; Lu et al., 2009). NO, but not ABA or H$_2$O$_2$, has recently been reported to be essential in the cold- and dehydration-induced expression of a gene coding for a hybrid proline- and cysteine-rich protein from Medicago falcata that confers resistance to abiotic stress (Tan et al., 2013a). Another gene coding for myo-inositol phosphate synthase, which also confers enhanced resistance to chilling, drought, and salt stresses, was induced by H$_2$O$_2$ and NO but was not responsive to ABA (Tan et al., 2013b). These data point to the existence of different signalling pathways in drought responses, depending either on the coordinated action of NO, H$_2$O$_2$, and ABA, or, by contrast, only on subsets.

High salt

Nearly half of irrigated land and around 20% of cultivated land is currently affected by salinity (Misra et al., 2001). A high salt concentration causes osmotic and ionic stress in plants. It limits the growth and development of plants by affecting several key metabolic processes (Hasegawa et al., 2000). Much of the injury at the cellular level caused by salinity stress is associated with oxidative damage due to ROS. Plants appear to possess a wide array of defence strategies to protect themselves from oxidative damage. However, less is known about NO involvement in salt stress tolerance in plants. The application of NO stimulated the expression of the plasma membrane H$^+$-ATPase, a well-characterized target in ABA signalling and high-salt-triggered responses (Cerana et al., 2006; Janicka-Russak and Klobus, 2007). It has been proposed that NO serves as a signal, inducing salt resistance by increasing the K$^+$/Na$^+$ ratio (Zhao et al., 2004). Tolerant plants typically maintain high potassium (K$^+$) and low sodium (Na$^+$) in the cytosol of cells under salinity. This mechanism is mediated by H$^+$-ATPase, carriers (sympoters and antiporters), and channels associated with plasma membranes and tonoplasts. It has been recently reported that energization of plasma membranes is a determinant for K$^+$ uptake (Haruta and Sussman, 2012). Also, the key role exerted by the tonoplast in controlling K$^+$ uptake has been documented extensively (Zhao et al., 2004; Zhang et al., 2006). Guo et al. (2009) suggested that NO might confer salt tolerance to plants by preventing both oxidative membrane damage and translocation of Na$^+$ from root to shoots (Fig. 3B). It has been also proposed that plants possess priming-like mechanisms that allow them to memorize previous NO exposure events and generate defence responses following salt stress (Molassiotis et al., 2010). The increased drought and salt tolerance in transgenic tobacco plants overexpressing the ABA-biosynthetic enzyme 9-cis-epoxy-carotenoid dioxygenase is associated with the ABA-induced production of H$_2$O$_2$, via NADPH oxidase and NO, via NOS-like activity, and the subsequent induction of antioxidant enzymes (Zhang et al., 2009).

UV radiation stress

UV-B radiation has been shown to trigger an increase in ABA concentration in plants (Tossi et al., 2009). This increase activates NADPH oxidase and H$_2$O$_2$ generation, and also the arginine-dependent production of NO that maintains cell homeostasis and attenuates UV-B-derived cell damage (Mackerness et al., 1999; Tossi et al., 2009). Moreover, NO generated from NOS-like activity appears to act synergistically with ROS in inducing ethylene synthesis in the defence response under UV-B radiation in maize leaves (Wang et al., 2006). Shi et al. (2005) reported that addition of SNP can partially alleviate the UV-B-induced decrease in chlorophyll content, and the oxidative damage to the thylakoid membrane in bean leaves. It has been recently proposed that high doses of UV-B light seem to induce common signalling components such as ABA, NO, and Ca$^{2+}$ in plants and other multicellular organisms (Tossi et al., 2012), thus suggesting that ABA and NO function cooperatively in UV-B-triggered responses (Fig. 3B).
NO and ABA in PAMP-triggered immunity (PTI), effector-triggered immunity (ETI) and defence responses against necrotrophs

SA-related or JA/ethylene-related signalling events have traditionally been described as the major pathways in response to pathogen attack in plants (Glazebrook, 2005). However, the final response is mediated by a complex interconnected network comprising different phytohormones and other regulators (Robert-Seilaniantz et al., 2011). The regulatory roles exerted by NO and ABA in plant defence against pathogens have been the focus of recent work (reviewed by Asselbergh et al., 2008; Cao et al., 2011; Gaupe1 et al., 2011; Arasimowicz-Jelonek and Floryszak-Wiezorek, 2013; Bellin et al., 2013). NO and ABA contribute to defence against pathogens both as alternative pathways to those centrally regulated through SA-related or JA/ethylene-related signalling events (Robert-Seilaniantz et al., 2011), or in coordination with these classical pathways.

Lipopolysaccharide-triggered NO production contributes to Arabidopsis resistance to Pseudomonas syringae pv. Tomato strain DC3000 as demonstrated by the increased susceptibility observed in the NO-deficient noa1 mutant (Zeidler et al., 2004). Moreover, upon perception of pathogen-associated molecular patterns (PAMPs), plant leaves close their stomata (Fig. 2). This process leading to enhanced resistance to P. syringae is mediated by the FLS2 receptor and requires ABA and NO production and the function of the ABA-related OST1 kinase (Melotto et al., 2006). These data established an important role for stomata as a defensive structure of plants linking ABA- and NO-related signalling to PTI against phytopathogenic bacteria. Both NO and ABA seem to play positive roles in activating PTI.

By contrast, antagonistic roles for NO and ABA have been reported in the activation of ETI. ETI is a subclass of plant immunity that is activated in response to pathogen effectors recognized by R proteins in the plant. NO functions as a positive regulator of ETI, as demonstrated by the enhanced susceptibility to P. syringae avrRpm1 and avrRps4 observed in wild-type Arabidopsis plants treated with inhibitors of NO production (Delledonne et al., 1998), and in NO-deficient Arabidopsis mutants (Mandal et al., 2012). In turn, ABA treatment leads to increased susceptibility of Arabidopsis plants to avirulent P. syringae, suggesting that ABA is a negative regulator of ETI (Mohr and Cahill, 2003). Arabidopsis aba1-1 and aba2-1 mutants, which are insensitive to ABA, and the era1 hypersensitive mutant, were more resistant and susceptible, respectively, to virulent P. syringae (de Torres-Zabala et al., 2007). ABA accumulation also compromised resistance to the biotrophic oomycete Hyaloperonospora arabidopsidis (Fan et al., 2009). A large body of evidence supports the hypothesis that pathogen-modulated ABA signalling rapidly antagonizes SA-mediated defence (de Torres-Zabala et al., 2009; Kim et al., 2011). However, it has been reported recently that constitutive expression of a mammalian NOS in tobacco triggers NO accumulation, resistance to a wide array of pathogens and activation of the expression of SA-related genes (Chun et al., 2012), pointing to NO as an agonist of SA-related responses. The role of NO in plant immunity has been associated with changes in intracellular Ca\(^{2+}\) and cyclic nucleotide levels that exert a signalling function both upstream and downstream of NO (Ma, 2011). NO and ABA have also been reported to modulate cell death typical of the hypersensitive response in incompatible plant–pathogen interactions. ABA-RESPONSIVE1 (ABR1) has been characterized as a negative regulator of ABA signalling and its nuclear pool seems to be essential for cell death induction associated with ABA–SA antagonism (Choi and Hwang, 2011). On the other hand, treatment of Arabidopsis plants with either an inhibitor of NO synthesis or with NO scavengers impaired the proper activation of hypersensitive responses (Delledonne et al., 2001), pointing to NO together with H\(_2\)O\(_2\) as essential components in regulating cell death in pathogen-triggered responses. However, NO seems to have both promoting and suppressing effects on cell death, depending on cell type, cellular redox status, and dose (Wang et al., 2010a).

Necrotrophic pathogens display a wide range of aggressive virulence strategies that promote host cell death to enable feeding from dead cells. This lifestyle strongly contrasts with immunity responses raised against biotrophic pathogens. As no resistance R gene has been associated with resistance to necrotrophs, ETI and its accompanying immune responses are primarily not effective regulators of resistance to necrotrophs. In turn, PTI has been reported to be efficient in triggering resistance to necrotrophs through a sequential signalling process involving MAPKs, camalexin biosynthesis, and ethylene that ends with suppression of SA signalling (Mengiste, 2012). Necrotrophic pathogens commonly trigger JA and ethylene signalling to activate plant defence responses (Glazebrook, 2005), although they can alter SA signalling to promote disease symptoms in the plant (Rahman et al., 2012). It has been reported recently that ABA signalling modulates basal resistance in Arabidopsis through negative regulation of SA/JA/ethylene-mediated resistance to necrotrophic fungi (Sánchez-Vallet et al., 2012). Moreover, resistance to Botrytis cinerea is enhanced by the increased permeability of the leaf cuticle, and is strongly linked to ROS formation through a process negatively regulated by ABA (L’Haridon et al., 2011). On the other hand, infection with Botrytis caused a rapid increase in NO levels, and NO seems to cooperate in activating resistance, as treatment of plants with either biosynthesis inhibitors or scavengers of NO or mutant plants impaired in NO biosynthetic genes led to enhanced susceptibility to this necrotrophic fungi (Asai and Yoshioka, 2009; Rasul et al., 2012a).

Taken together, current knowledge on plant immunity proposes that ABA and NO can act positively in promoting PTI (Fig. 3C). It seems that ABA can be very efficient in impeding pathogen entrance through stomata, although it is unclear whether NO cooperates in this process or whether it plays an additional role in defence mechanisms. By contrast, the role of ABA and NO in ETI or in defence against necrotrophic pathogens indicates an antagonism mode of action between these molecules (Fig. 3C). While ABA negatively regulates resistance, NO positively contributes to plant defence during ETI and necrotrophs attack.

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**Robert-Seilaniantz et al.**. ABA accumulation also compromised resistance to a wide array of pathogens and activation of the expression of SA-related genes (Chun et al., 2012), pointing to NO as an agonist of SA-related responses. The role of NO in plant immunity has been associated with changes in intracellular Ca\(^{2+}\) and cyclic nucleotide levels that exert a signalling function both upstream and downstream of NO (Ma, 2011). NO and ABA have also been reported to modulate cell death typical of the hypersensitive response in incompatible plant–pathogen interactions. ABA-RESPONSIVE1 (ABR1) has been characterized as a negative regulator of ABA signalling and its nuclear pool seems to be essential for cell death induction associated with ABA–SA antagonism (Choi and Hwang, 2011). On the other hand, treatment of Arabidopsis plants with either an inhibitor of NO synthesis or with NO scavengers impaired the proper activation of hypersensitive responses (Delledonne et al., 2001), pointing to NO together with H\(_2\)O\(_2\) as essential components in regulating cell death in pathogen-triggered responses. However, NO seems to have both promoting and suppressing effects on cell death, depending on cell type, cellular redox status, and dose (Wang et al., 2010a).

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Perspectives

Although during the last two decades an extensive experimental support has been conducted on multiple aspects of plant biology involving NO and ABA, many unsolved processes remain. Some topics, such as stomatal closure, would largely benefit from better contextualized experimental systems. Regarding this, work on deconstructed systems should be backed up by whole-plant studies. A large number of these processes would also benefit from better analytical tools allowing the quantification of NO and ABA as well as other hormones and growth regulators at tissue or even cellular levels. These tools should be developed in the next few years to allow more precise approaches. Pharmacologically based studies should be performed carefully to avoid misinterpretations due to unknown side effects of chemicals. An extensive characterization of chemicals used in pharmacological strategies and the use of only those displaying better specificity will help in clarifying our understanding of how functional interactions between plant regulators occur. Moreover, this sort of approach must be complemented with double experimental support. First, genetic studies using well-characterized mutant or transgenic plants will allow us to define crosstalk signalling by identifying epistatic or additive effects. Secondly, the identification of direct NO targets and their characterization, at both gene and protein levels, will be the main challenges for the future.

Future work will need to pay special attention to the characterization of protein modifications altering the function of proteins involved in the perception and downstream signalling of NO and ABA. All these considerations will help us to define whether NO exerts mainly a co-regulatory effect on other regulatory molecules, as extensively documented to date, or whether it might perform specific roles in controlling some aspects of plant physiology.

Another important issue to solve that is derived from the above-described data is the importance of the tissue-dependent mode of action of NO. It is becoming evident that NO can have a different regulatory role depending on the tissue or cellular type where it is generated. It is therefore feasible that its interaction with other regulators (i.e. ABA) will also be different. The tissue- and/or cell-type-specific NO role should be deciphered, and its interaction with specific regulatory networks within this cellular space will help to address current controversy and move forward for a better understanding of this fascinating molecule in plant physiology and the response to stress.

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