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Nitric oxide signalling in plants: interplays with Ca^{2+} and protein kinases

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Abstract

Much attention has been paid to nitric oxide (NO) research since its discovery as a physiological mediator of plant defence responses. In recent years, newer roles have been attributed to NO, ranging from root development to stomatal closure. The molecular mechanisms underlying NO action in plants are just begun to emerge. The currently available data illustrate that NO can directly influence the activity of target proteins through nitrosylation and has the capacity to act as a Ca^{2+}-mobilizing intracellular messenger. The interplay between NO and Ca^{2+} has important functional implications, expanding and enriching the possibilities for modulating transduction processes. Furthermore, protein kinases regulated through NO-dependent mechanisms are being discovered, offering fresh perspective on processes such as stress tolerance.

Key words: Ca^{2+}, cADPR, nitric oxide, protein kinases, signalling, SnRK2.

Introduction

If one lists the number of cellular processes under the control of NO in animals, the physiological importance of NO becomes immediately apparent. NO was reported to play pivotal roles in synaptic transmission, vasodilatation, erection, egg fertilization, defence against pathogenic micro-organisms, and apoptosis (Schmidt and Walter, 1994). Exactly how NO exerts such diversity of functions is the subject of intense study. An increasing body of evidence indicates that NO, and derived species such as peroxynitrite ($ONOO^-$), exert part of their biological activities by chemical modification of protein targets (Bogdan, 2001). These include the nitrosylation, nitrosation, nitration, and oxidation of proteins (Bogdan *et al.*, 2000). In particular, nitrosylation, that is the direct binding of an NO group to a transition metal or cysteine residues (Mannick and Schonhoff, 2002), is emerging as an important post-translational modification of proteins. More than 100 proteins have been found to undergo reversible regulation by nitrosylation *in vitro* and/or *in vivo* (Hanafy *et al.*, 2001; Stamler *et al.*, 2001). Remarkably, the broad spectrum of functions ascribed to proteins found to be nitrosylated affects essentially all major cellular activities, highlighting the multifunctional roles of NO (Stamler *et al.*, 2001). Furthermore, nitrosylation is one of the molecular strategies used for signalling (Hess *et al.*, 2005). Accordingly, through nitrosylation, NO was shown to regulate key signalling-related proteins including the soluble guanylate cyclase (sGC), the small GTP-binding protein p21ras, and a number of Ca^{2+}-permeable channels (Mannick and Schonhoff, 2002). Furthermore, NO-dependent regulation of protein kinase activities occurs by nitrosylation of the kinases themselves or by modulation of interacting/upstream factors such as cGMP, Ca^{2+}, p21ras or protein phosphatases (Beck *et al.*, 1999). It is worth realizing that the analysis of the cross-talk operating between NO, protein kinases, and the second messengers Ca^{2+} and cGMP provided a framework for understanding the molecular bases of major physiological processes, such as egg fertilization or modulation of neuronal excitability (Willmott *et al.*, 1996; Ahern *et al.*, 2002).

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The past few years have seen an increasing number of studies dedicated to NO functions in plants. NO appears to be involved in plant developmental processes and participates in a number of physiological processes such as stomatal closure, flowering, response to environmental stresses, and cell death (for reviews see Lamattina *et al.*, 2003; Wendehenne *et al.*, 2004; Delledonne, 2005; Lamotte *et al.*, 2005). Understanding of the mechanisms by which NO contributes to these processes is still in its infancy, but promising results are being obtained. Notably, the identification of NO target proteins and genes whose expression is regulated by NO has already borne fruit, indicating that NO can directly influence the activity of plant proteins as well as the signalling cascade leading to gene expression (Lindermayr *et al.*, 2005, 2006; Grun *et al.*, 2006; Belenghi *et al.*, 2007). These observations also paved the way for research into NO involvement in plant signalling. This article focuses on advances in the characterization of NO signalling activities in plants with respect to its cross-talk with Ca^{2+} and protein kinases.

Interplay of NO and Ca^{2+}

Ca^{2+} is well established as a universal intracellular second messenger (Sanders *et al.*, 2002; Petersen *et al.*, 2005). Notably, increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) have been detected in response to a wide range of environmental, developmental, and growth stimuli (Lecourieux *et al.*, 2006). According to the 'Ca^{2+} signature hypothesis', each stimulus induces Ca^{2+} transients which have unique temporal and spatial arrangements determining the specificity of the physiological response (Scrase-Field and Knight, 2003). The changes in $[Ca^{2+}]_{cyt}$ are decoded and relayed through Ca^{2+} sensors such as calmodulins (CaMs), CDPKs (Ca^{2+}-Dependent Protein Kinases) or annexins (Berridge *et al.*, 2003). Interestingly, recent progress on animal cells have highlighted that, similarly to Ca^{2+} signalling, NO signalling might display spatial and temporal organization (Stamler *et al.*, 2001). Accordingly, several NO-signalling components such as Ca^{2+}-permeable channels, p21ras, sGC, and MAPKs (Mitogen-Activated Protein Kinases) are organized into macromolecular complexes in which NO signalling functions within highly localized environments (Kone *et al.*, 2003). The complementary aspect of NO and Ca^{2+} signalling is reinforced by the occurrence of transduction networks in which Ca^{2+} acts both as a promoter and a sensor of NO signalling (Clementi, 1998). In plants, concomitant changes in $[Ca^{2+}]_{cyt}$ and NO levels are apparent during the transduction of biotic and abiotic signals (Garcia-Mata *et al.*, 2003; Gould *et al.*, 2003; Lamotte *et al.*, 2004; Vandelle *et al.*, 2006). As reported in animal cells, growing evidence suggest that these two messengers may interact in subtle ways.

Ca^{2+}-dependence of NO synthesis

Current reports show that there are at least two main enzymatic routes for NO synthesis in plants: a nitrite-dependent pathway and a L-arginine-dependent pathway. The nitrite pathway mainly involves nitrate reductase (NR) which catalyses the NAD(P)H reduction of nitrite to NO (Yamasaki *et al.*, 1999), and a root-specific plasma membrane-bound nitrite reductase not identified yet (Stöhr *et al.*, 2001). NR is the only enzyme whose NO-producing activity has been rigorously confirmed both *in vivo* and *in vitro*. A question that still arises is whether the NR-derived NO can act as a signal or whether it is just the product of an enzyme side reaction (Desikan *et al.*, 2002; Meyer *et al.*, 2005; Bright *et al.*, 2006; Crawford, 2006; Modolo *et al.*, 2006). Whatever scenario, no functional link between Ca^{2+} and NR-mediated NO synthesis has been reported so far. The L-arginine-dependent pathway is based principally on the assumption that plants do possess nitric oxide synthase (NOS)-like enzyme(s). In animals, NOSs catalyse the conversion of L-arginine to L-citrulline and NO. Activities of the constitutive isoforms of mammalian NOSs (cNOSs) are strictly CaM/Ca^{2+}-dependent (Bogdan, 2001). During the past decade, there has been an increasing number of reports showing the presence of NOS activity in plants and a candidate catalysing a L-arginine-dependent NO synthesis has been identified (Guo *et al.*, 2003). However, its ability to catalyse NO synthesis has been questioned and a mechanism for L-arginine-dependent NO synthesis in plants is still unknown (Crawford *et al.*, 2006; Zemojtel *et al.*, 2006). Interestingly, plant NOS activity measured in several plant species and various tissues requires Ca^{2+} and CaM as cofactors, suggesting that Ca^{2+} or Ca^{2+}-bound CaM might directly interact with the plant NOS-like enzyme (Delledonne *et al.*, 1998; Modolo *et al.*, 2002; Corpas *et al.*, 2004, 2006; del Rio *et al.*, 2004). The importance of Ca^{2+} in L-arginine-dependent NO synthesis is further supported by experiments showing that elicitor-induced NO synthesis in tobacco and grapevine cells was suppressed by pharmacological agents that inhibit mammalian NOS activities and increases in $[Ca^{2+}]_{cyt}$ (Lamotte *et al.*, 2004; Vandelle *et al.*, 2006). In agreement with these findings, in a recent study by Ali *et al.* (2007) genetic evidence has been provided that lipopolysaccharide-induced NO synthesis, which was found to be suppressed by mammalian NOS inhibitors, is controlled by an upstream Ca^{2+} influx mediated by the plasma membrane Ca^{2+}-permeable channel cyclic-nucleotide-gated channel (CNGC)2. In addition, NO synthesis catalysed by NOS-like-enzyme(s) was shown to be up-regulated by H_2O_2-, SA- (salicylic acid), and aldehyde-induced elevations of $[Ca^{2+}]_{cyt}$ in various plant species and phytoplankton populations (Lum *et al.*, 2002; Allen *et al.*, 2006; Zottini *et al.*, 2007). While these studies did

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not ascertain a direct interacting role for Ca^{2+} with plant NOS-like enzyme, they provided physiological evidence that changes of $[Ca^{2+}]_{cyt}$ might be involved intimately in mediating NO synthesis in plant cells and that NO appears as a step in the signalling cascade initiated by the cation.

NO as a Ca^{2+}-mobilizing agent

In animals, NO regulates Ca^{2+} channel activities through different mechanisms: direct S-nitrosylation or indirect modulation via cGMP-dependent cascades (Fig. 1). S-nitrosylated Ca^{2+} channels include voltage-gated Ca^{2+} channels (P/Q- and L-type), CNGC, ryanodine receptors (RyR), and the *N*-methyl D-aspartate receptor (Clementi, 1998; Ahern *et al.*, 2002). For example, NO was reported to enhance the activity of type 1 RYR by S-nitrosylation of a single cysteine (Sun *et al.*, 2001). The NO-cGMP cascade modulates the activity of L-, T-, and N-type voltage-gated Ca^{2+} channels, CNGC, inositol (1,4,5)-triphosphate receptor, and RyR (Clementi, 1998; Ahern *et al.*, 2002). Cyclic GMP targets Ca^{2+} channels by virtue of their cyclic nucleotide binding sites (particularly in the case of CNGC) or mediates its effects through serine/threonine cGMP-dependent protein kinases (PKGs). It is especially noteworthy that protein sequences from practically every type of ion channel contain PKG consensus phosphorylation sites (Ahern *et al.*, 2002). To add further to the complexity of this picture, in various cell types PKGs trigger the activation of RYR by promoting the synthesis of the nicotinamide adenine dinucleotide (NAD^+) metabolite cyclic ADP ribose (Fig. 1). Furthermore, NO also decreases $[Ca^{2+}]_{cyt}$ by activating plasma membrane and endomembrane Ca^{2+} transporters (Clementi, 1998; Fig. 1). The detailed mechanism of this action is still unclear and might involve both nitrosylation and cGMP-dependent processes (see, for instance, Yao and Huang, 2003). Because cNOSs activity is strictly Ca^{2+}-dependent, the finding discussed above also indicates that NO exerts a positive or a negative feedback control of its own production by promoting activation or inhibition of Ca^{2+}-channels and Ca^{2+} transporters, respectively.

The first arguments that Ca^{2+} might participate downstream of NO in plant signal transduction pathways were provided by Durner *et al.* (1998) and Klessig *et al.* (2000). These authors showed that the cADPR antagonist 8-bromo-cADPR reduces and delays NO-induced accumulation of *PR* (*Pathogenesis-Related*)*-1* transcripts in tobacco leaves. Accordingly, cADPR itself caused substantial *PR-1* expression through a mechanism sensitive to RyR inhibitors. This set of observations provided evidence that the dynamic regulation of plant gene expression by NO could take place via a NO-cADPR-Ca^{2+}

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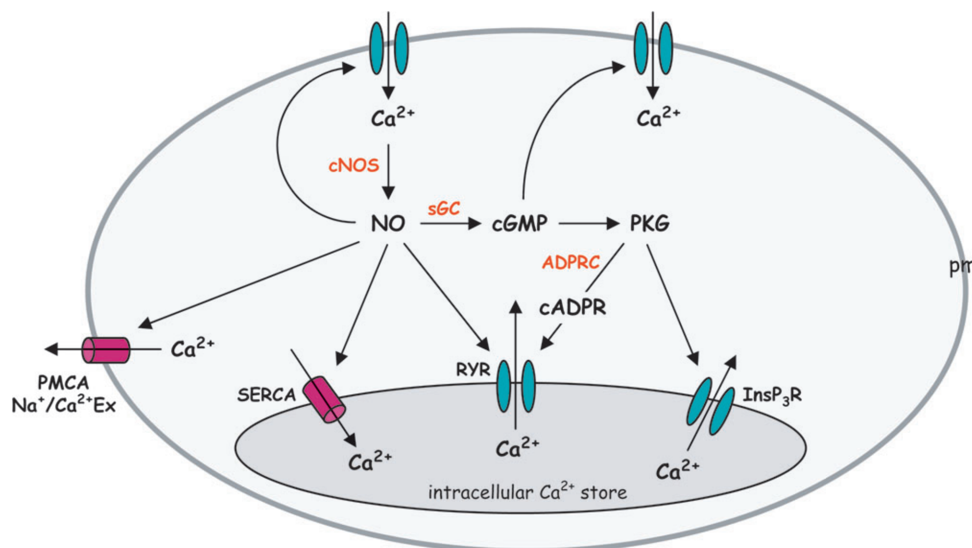

Fig. 1. NO-based Ca^{2+} signalling in animal cells. The Ca^{2+} channels shown in the plasma membrane (pm) are representative of voltage-gated Ca^{2+} channels, cyclic-nucleotide-gated channels (CNGCs) and of the N-methyl D-aspartate (NMDA) receptor. NO, produced by the Ca^{2+}/CaM-dependent constitutive NOSs (cNOSs), generates Ca^{2+} signals via several routes (not all pathways occur necessarily in the same cell). First, NO regulates plasma membrane and endomembrane Ca^{2+} channels through direct *S*-nitrosylation of critical cysteines, leading to activation (P/Q- and L-type voltage-gated channels, CNGCs, RYRs) or inhibition (NMDA receptor, L-type voltage-gated channels) of the channels. Second, NO promotes cGMP synthesis by nitrosylation of the soluble guanylate cyclase (sGC). In turn, cGMP activates cGMP-dependent protein kinases (PKGs) and CNGCs through its binding to cyclic-nucleotide binding sites. cGMP might also regulate L-, T- and N-type voltage-gated channels indirectly (both activation and/or inhibition have been reported). Depending on the cell type and the initial stimulus, PKGs inhibit (most frequent situation) or activate the inositol (1,4,5)-triphosphate receptor ($InsP_3R$) via phosphorylation. PKGs might also activate ADP-ribosyl cyclase (ADPRC) by phosphorylation. ADPRC catalyses the cyclization of NAD to cADPR, an endogenous ligand for RYR. Finally, NO contributes to the overall cytosolic Ca^{2+} homeostasis through the positive regulation of Ca^{2+} transporters including Na^+/Ca^{2+} exchanger (Na^+/Ca^{2+} Ex) and plasma membrane and sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPases (PMCA and SERCA, respectively).

cascade. Subsequent studies have partly reinforced this conclusion. First, artificially generated NO has been reported to raise $[Ca^{2+}]_{cyt}$ of *Vicia faba* guard cells by promoting Ca^{2+} release from intracellular stores (Garcia-Mata *et al.*, 2003). The rise in $[Ca^{2+}]_{cyt}$ was blocked by sGC and RYR inhibitors, establishing cGMP as a putative mediator for NO-induced activation of cADPR-dependent endomembrane Ca^{2+} channels. A similar situation was found by analysing the effects of the non-thiol NO donor DEA-NONOate in tobacco cell suspensions expressing the Ca^{2+} reporter apoaequorin in the cytosol (Lamotte *et al.*, 2004, 2006). Here too, NO markedly enhanced the $[Ca^{2+}]_{cyt}$ through a process sensitive to 8-bromo-cADPR, indicating that NO-induced Ca^{2+} mobilization operates predominantly via a cADPR-mediated Ca^{2+}-release mechanism. Interestingly, these latter studies also pointed to a role for NO in modulating plasma membrane Ca^{2+}-permeable channels. Indeed, NO released by DEA-NONOate was found to trigger a fast and transient influx of extracellular Ca^{2+} in tobacco cells. Because the NO-evoked Ca^{2+} influx occurred concomitantly with a Ca^{2+}-independent plasma membrane depolarization, it was assumed that NO may promote the opening of voltage-gated Ca^{2+} channels subsequent to membrane depolarization (Lamotte *et al.*, 2006). At present, however, the exact mechanisms of NO-induced plasma membrane depolarization remain to be elucidated. Recent work has suggested that NO might modulate plasma membrane Ca^{2+}-permeable channels by showing that NO negatively regulates Ca^{2+} entry in grapevine cells challenged by the elicitor endopolygalacturonase 1 (Vandelle *et al.*, 2006). This finding is of particular interest because it expands the role of NO in plant signalling as a more general regulator of Ca^{2+} homeostasis, promoting both activation and/or inhibition of Ca^{2+} fluxes. As reported in endothelial cells or neurons, a negative feedback could serve to protect cells from the detrimental effects of excessive NO and Ca^{2+} (see, for instance, Yao and Huang, 2003). Finally, it should be mentioned that artificially generated NO did not evoke rises in nuclear free Ca^{2+} concentration in tobacco cell suspensions expressing apoaequorin in the nucleus (Lecourieux *et al.*, 2005). Therefore, NO action on Ca^{2+} homeostasis might be restricted to specific cellular compartments.

Although these studies support a possible link between NO, cADPR, cGMP, and Ca^{2+}, a note of caution is required. First of all, it remains to be seen whether the level of cADPR changes in response to NO-dependent processes. Second, while the ability of NO to induce cGMP synthesis in plant cells is well established (Durner *et al.*, 1998; Hu *et al.*, 2005), the plant NO-sensitive sGC-like enzyme is unknown. Finally, although several investigators have reported the requirement of phosphorylation-dependent events in the mediation of NO-induced Ca^{2+} mobilization (Sokolovski *et al.*, 2005; Lamotte *et al.*, 2006), plant PKGs have not yet been identified. Therefore, at this stage of knowledge, the conclusion that the NO signalling pathway leading to Ca^{2+} mobilization in plants is similar to that defined in animals remains speculative.

Several studies supportive of a potential role for NO as an endogenous regulator of Ca^{2+} mobilization in physiological contexts have been reported. Notably, it has been shown that NO contributes to $[Ca^{2+}]_{cyt}$ increases in plant cells exposed to biotic and abiotic stresses including hyper-osmotic stresses and elicitors of defence responses (Gould *et al.*, 2003; Lamotte *et al.*, 2004, 2006; Vandelle *et al.*, 2006). For example, a specific role for NO in activating intracellular Ca^{2+} channels was assumed through pharmacological and biochemical approaches in tobacco and grapevine cells exposed to the elicitors cryptogein and endopolygalacturonase 1, respectively (Lamotte *et al.*, 2004; Vandelle *et al.*, 2006). Furthermore, pharmacological experiments suggested that NO is active upstream of $[Ca^{2+}]_{cyt}$ transients during the processes of ABA-induced stomatal closure and auxin–induced adventitious root formation (Desikan *et al.*, 2002; Lanteri *et al.*, 2006).

These studies also raise the question of how the NO-mediated Ca^{2+} fluxes are propagated downstream into cellular responses. Clearly, this aspect has been poorly investigated. However, it is likely that protein kinases might represent an important pathway by which NO-dependent Ca^{2+} signals are decoded. Recently, a 50 kDa CDPK, the activity of which was induced by NO through a Ca^{2+}-dependent process, was characterized in cucumber explants (Lanteri *et al.*, 2006). The 50 kDa CDPK might contribute to NO-induced adventious root formation. Likewise, in our laboratory, evidence was obtained that the activation of the tobacco MAPK SIPK (Salicylic acid-Induced Protein Kinase) by NO donors (Klessig *et al.*, 2000) requires a transient influx of extracellular Ca^{2+} in tobacco cells (C Courtois and D Wendehenne, unpublished data).

Another puzzling aspect to discuss here is the cellular impact of the NO/Ca^{2+} pathway. Here too, the first hypotheses have only just begun to emerge. First, a rise in $[Ca^{2+}]_{cyt}$ may serve to initiate but also to amplify and/or maintain NO production. Accordingly, Lamotte *et al.* (2004) showed that addition of inhibitors of plasma membrane Ca^{2+}-permeable channels in the mid-course of cryptogein-induced NO synthesis in tobacco cell suspensions suppressed NO production within minutes. Second, by elevating $[Ca^{2+}]_{cyt}$, NO might influence indirectly the activity of proteins including protein kinases (see above) and Ca^{2+}-sensitive K^+ and Cl^- channels as described in guard cells (Garcia-Mata *et al.*, 2003). Third, based on animal studies (see, for instance, Peunova and Enikolopov, 1993) and the data discussed above, it is reasonable to speculate that NO/Ca^{2+} pathways, as well as the combined action of NO and Ca^{2+}, might modulate the transcriptional regulation of specific set of genes involved, for

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instance, in disease resistance or developmental processes. Finally, it seems plausible that interplays of NO and Ca^{2+} might be implicated in cell death. This idea is supported by the fact that both NO and Ca^{2+} are triggers and modulators of cell death (Lam, 2004; Delledonne, 2005). This hypothesis is further reinforced by the finding that H_2O_2, which acts in concert with NO in triggering cell death (Delledonne *et al.*, 2001; Zago *et al.*, 2006), also contributes to stimulus-induced $[Ca^{2+}]_{cyt}$ changes (Garcia-Brugger *et al.*, 2006; Lecourieux *et al.*, 2006). Mechanistically, Ca^{2+} might represent a signalling carrier of NO and/or H_2O_2-triggered cell death pathways or, as described in animal cells, cell death could also be related to the cellular Ca^{2+} overload or perturbation of intracellular compartmentalization resulting from NO- and/or H_2O_2-induced Ca^{2+} fluxes (Orrenius *et al.*, 2003). Therefore, understanding of the mechanisms underlying cell death should also include experiments designed to delineate the cross-talk between Ca^{2+}, NO, and H_2O_2 in further detail.

Protein kinases as targets for NO action

In animal cells, NO modulates the activity of distinct classes of protein kinases that play a key role in signal transduction, including MAPK cascades, protein kinase C, and Janus kinases (Beck *et al.*, 1999). Furthermore, kinases related to primary metabolism such as pyruvate kinase were reported to be activated or inhibited by S-nitrosylation (Gao *et al.*, 2005). In plants, the possibility that NO might influence protein kinase activities has been poorly explored and most of the available data come from studies based on artificially generated NO. NO-dependent activation of protein kinases exhibiting, for instance, MAPK or CDPK properties were reported in *A. thaliana* suspension cell cultures and roots (Clarke *et al.*, 2000; Capone *et al.*, 2004), cucumber explants (Pagnussat *et al.*, 2004; Lanteri *et al.*, 2006) and tobacco leaves and suspension cell cultures (Klessig *et al.*, 2000; Yamamoto *et al.*, 2004). With the exception of SIPK (Klessig *et al.*, 2000), none of these protein kinases has been identified. Furthermore, the NO donor SNP was shown to increase the amount and histone H1 phosphorylating activity of the $p34^{cdc2}$ cyclin-dependent kinase in auxin-treated alfalfa protoplasts (Ötvös *et al.*, 2005). The NO-induced activation of these kinases has been thought to be part of processes related to defence responses and/or cell death (Clarke *et al.*, 2000; Klessig *et al.*, 2000; Yamamoto *et al.*, 2004) and to auxin-mediated adventitious root formation and cell division (Pagnussat *et al.*, 2004; Ötvös *et al.*, 2005; Lanteri *et al.*, 2006). Finally, it should be mentioned that several kinases including phosphoglycerate kinase, nucleoside diphosphate kinase, and adenosine kinase have been shown to be S-nitrosylated *in vitro* (Lindermayr *et al.*, 2005). The biological relevance of the S-nitrosylation of these kinases has not yet been defined.

The first evidence that NO modulates the activation of a member of the plant SNF1-related protein kinase 2 (SnRK2) subfamily has been reported recently (Lamotte *et al.*, 2006). Plant SnRKs are classified into three subfamilies: SnRK1, SnRK2, and SnRK3, the SnRK2 and SnRK3 subfamilies being specific to plants (Harmon, 2003). Members of the SnRK2 subfamily function in abiotic stress signalling and include the tobacco 42 kDa protein kinase NtOSAK (*Nicotiana tabacum* Osmotic Stress-Activated Protein Kinase; Mikolajczyk *et al.*, 2000). NtOSAK is activated very rapidly in response to osmotic stress through phosphorylation of two serine residues (154 and 158) located within the enzyme activation loop (Burza *et al.*, 2006). In our laboratory, it has been demonstrated that the NO donor DEA/NONOate induced a fast and transient activation of NtOSAK in tobacco suspension cell cultures (Lamotte *et al.*, 2006). Furthermore, evidence has been provided that NO might be a key component of the hyperosmotic stress-induced signalling cascade leading to NtOSAK activation. An attempt was also made to clarify the NO-dependent upstream pathway of NtOSAK activation. Initial data established that neither NO-mediated Ca^{2+} influx nor Ca^{2+} release from internal stores were required for NtOSAK activation (Lamotte *et al.*, 2006). Among the other possibilities, NtOSAK activity might be up-regulated through phosphorylation by an upstream NO-dependent protein kinase, by auto-phosphorylation, and/or through direct S-nitrosylation or nitration by NO-derived species. Preliminary experiments are not in favour of the last possibility.

The question that further comes to mind is the incidence of the NO/NtOSAK pathway on the cell response. Protein kinases of the SnRK2 subfamily are activated by osmolytes and some of them by ABA as well, highlighting a role for these enzymes in a general response to osmotic stress (Boudsocq *et al.,* 2004; Kobayashi *et al.,* 2004). The SnRK2 kinases present in guard cells, AAPK (ABA-Activated Protein Kinase) from *Vicia faba* and its *Arabidopsis* orthologue SnRK2.6/OST1/SRK2E play an important role in ABA signalling in response to drought and regulate stomata closure under low humidity stress (Li *et al.*, 2000; Mustilli *et al.*, 2002; Yoshida *et al.*, 2002). It has been shown that the other *Arabidopsis* ABA-dependent SnRK2 kinase, SRK2C/SnRK2.8, improves plant drought tolerance, probably by promoting the up-regulation of stress-responsive genes expression, including *DREB1A/CBF3* encoding a transcription factor that broadly regulates stress-responsive genes (Umezawa *et al.*, 2004). Several lines of evidence indicate that SnRK2 kinases can also phosphorylate and, in this way, activate transcription activators AREB1 and TRAB1 in *Arabidopsis* and rice, respectively (Kobayashi *et al.*, 2005; Furihata *et al.*, 2006). These data strongly suggest that

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SnRK2 protein kinases are involved in the regulation of expression of ABA-responsive genes. Based on these studies, it is plausible that plant cells challenged by osmotic stress might use NO as an early signalling compound acting upstream of SnRK2-induced pathways. This could be true for other plant responses in which both changes in osmotic pressure and NO production are observed, such as responses to pathogens or elicitors of defence responses (Lamotte *et al*., 2004; Gauthier *et al*., 2007). Regarding this aspect, it has recently been shown that *Arabidopsis* OST1 kinase and NO production are required in the plant innate immunity against bacterial invasion (Melotto *et al*., 2006). However, the involvement of NO in OST1 activation was not investigated. Here again, future work will have to clarify this possibility, but the findings so far are certainly promising.

Conclusion

The field of nitric oxide in plant biology was born almost ten years ago, when it was first revealed that this free radical gas is involved in defence responses (Delledonne *et al*., 1998; Durner *et al*., 1998). Since that time, NO has been shown to function as an ubiquitous molecule with diverse physiological roles. Although much has been learnt about NO, several issues concerning its action remain outstanding. As discussed above, mechanisms through which it might affect transduction processes imply the regulation of key signalling proteins such as protein kinases and Ca^{2+}-permeable channels as well as the mobilization of second messengers including Ca^{2+}, cGMP, and cADPR (Fig. 2). However, little is known at the molecular level concerning these signalling proteins and important goals are to identify them and to investigate how NO modulates their activities. Furthermore, it is necessary to define the physiological relevance of these modulations and to understand how interplays between NO and Ca^{2+} guide the cell toward a specific response. Such tasks will require functional analysis of the molecular mechanisms that relay NO-dependent Ca^{2+} signals. Finally, it should be kept in mind that pharmacological evidence for cADPR involvement in mediating NO-induced Ca^{2+} mobilization has been obtained, but the direct measurement of cellular cADPR levels is urgently

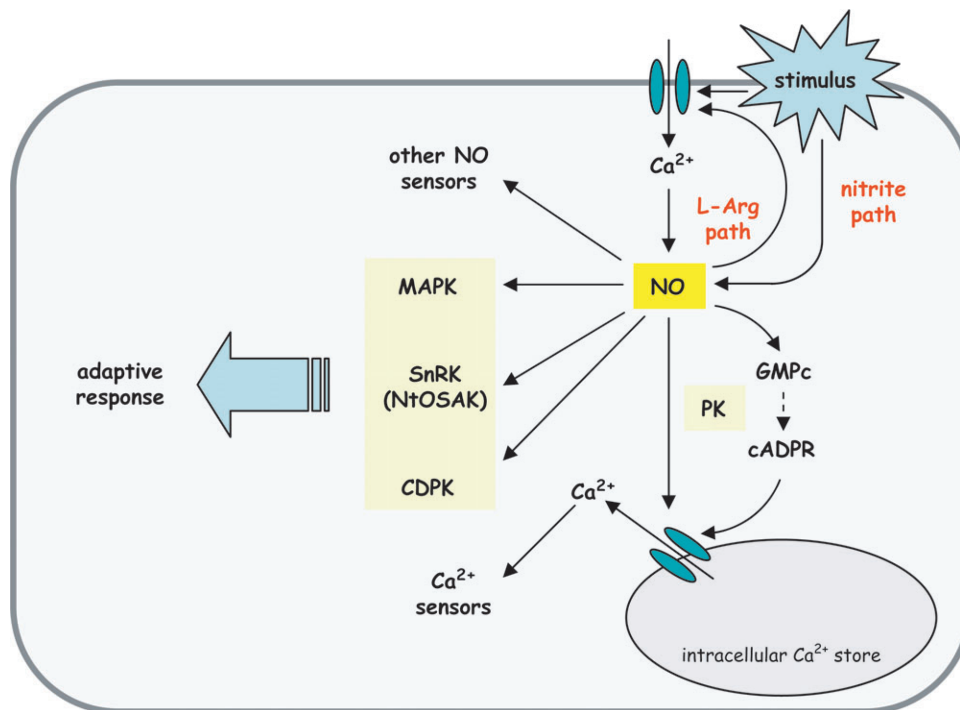

Fig. 2. Interplays between NO, Ca^{2+} and protein kinases in plant cells. NO production occurs through two enzymatic routes: a nitrite-dependent and a L-arginine (L-arg) route. The L-arginine-dependent pathway is up-regulated by upstream Ca^{2+} fluxes which might be partly mediated by CNGCs. The increases in $[Ca^{2+}]_{cyt}$ caused by NO are due to extracellular Ca^{2+} uptakes and/or mobilization of intracellular Ca^{2+}. Mechanisms through which NO mobilizes intracellular Ca^{2+} might involve cADPR, cGMP, and phosphorylation-dependent processes. The NO/Ca^{2+} information seem to be partly processed by CDPKs and MAPKs. In tobacco, NO-sensor protein kinases include NtOSAK, a member of the SnRK2 subfamily activated in response to osmotic stress. Other NO sensors correspond to nitrosylated proteins. The cross-talks operating between NO, Ca^{2+}, and protein kinases in plant cells exposed to biotic and abiotic stimuli (including auxin, ABA, osmotic stress, pathogens, and elicitors of defence responses) might have important functional implications such as the dynamic regulation of gene expression. As described in animal cells, NO might also display a dual role in controlling $[Ca^{2+}]_{cyt}$ concentration since both NO-dependent activation and inhibition of extracellular Ca^{2+} uptakes are reported. PK: protein kinases.

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required. Although in its infancy, research into the signalling functions of NO in plants is advancing rapidly and there should soon be a much better understanding of this most unusual signalling agent.

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