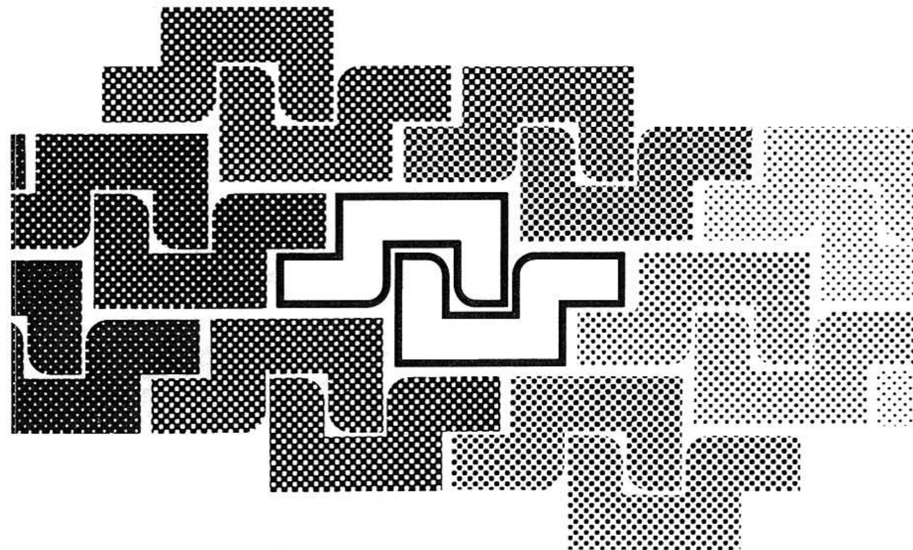

Cell Biology and Instrumentation: UV Radiation, Nitric Oxide and Cell Death in Plants

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NO Signaling Fonctions in the Biotic and Abiotic Stress Responses

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Abstract. In the lace 1990s, NO became an increasingly popular target of investigation in plants. As in mammals, NO fulfils a broad spectrum of signaling functions in pathophysiological processes in plants. Here, we summerize studies published in recent years that provide novel insights into the Signaling functions of NO produced by plant cells exposed to abiotic stresses and biotic stress (pathogen-derived elicitors). Particularly, we report chat NO emerges as a key messenger governing the overall control of Ca^{2+} homeostasis. Although the precise signaling functions of NO are poorly understood, its capacity to modulate Ca^{2+} homeostasis provides an extraordinary and remarkably effective way of conveying information.

Introduction

Over the past two decades, it has been recognized that nitric oxide (NO) plays important roles in diverse mammalian physiological processes. NO impinges on almost all areas of biology, including the regulation of blood vessels, immunological defense against invading organisms, apoptosis and neurotransmission. The production of NO is primarily catalysed by nitric oxide synthase (NOS) that converts L-arginine to L-citrulline and NO [1]. Because of its high reactivity, NO synthesis is under tight and complex control. Nitric oxide regulates physiological processes by modulating the activity of proteins principally by nitrosylation, a process referring to the binding of NO to a transition metal centre or cysteine residues [2] Nitrosylation is a reversible, post-translational modification that plays a central role in NO-mediated signaling. An important class of proteins that constitutes key targets of NO is that of Ca^{2+} channels including cardiac and skeletal ryanodine receptors (RYR), voltage-gated Ca^{2+} channels, store-operated Ca^{2+} channels, cyclic nucleotide-gated Ca^{2+} channels, the N-methyl-D-aspartate receptor and the inositol triphosphate receptor (IP_3R). NO modulates these channels directly by nitrosylation, but also indirectly *via* the second messenger cyclic GbIP (cGMP) and/or cyclic ADP ribose (cADPR), a direct triggering molecule for RYR-mediated Ca^{2+} release [2,3]. Therefore, NO, which may be produced intracellularly or may originate from neighbouring cells, emerges as a key messenger governing the overall control of Ca^{2+} homeostasis [3].

In the late 1990s, NO also became an increasingly popular target of investigation in plants. As in mammals, NO fulfils a broad spectrum of Signaling functions in (patho)physiological processes in plants [4-6]. Furthermore, two enzymes capable of

producing NO in plants have been recently identified , p.p nitrate reductase [7] and AtNOS1 [8], a NOS-like enzyme showing sequence identity with a protein implicated in NO synthesis in the mail *Helix pomatia*. This review summarizes studies published in recent years that provide novel insights into the signaling functions of NO produced by plant cells exposed to abiotic stresses and biotic stress (pathogen-derived elicitors). It focuses particularly on the cross-talk operating between NO and Ca^{2+}.

1. Nitric oxide signalling activities in response to abiotic and abiotic stressors

Analysis of the involvement of NO in plant immune responses is a fruitful area of research. Such studies have not only enhanced our understanding of the role of NO in plant defense, they also serve to illustrate the potential benefits of using plant-pathogen interactions as a model to investigate NO signaling. Over the past few years, we have studied the functions of NO in plant cells challenged with elicitors of defense responses. Two elicitors have been used primarily, p.p cryptogein, a 10 kDa elicitor produced by the oomycete *Phytophthora cryptogea* [9], and endopolygalacturonase (BcPG1) purified from the culture filtrates of *Botrytis Cinerea* [10].

Upon application to tobacco, cryptogein causes a hypersensitive-like response (HR), elicits the accumulation of transcripts encoded by defense-related genes and induces acquired systemic resistance (SAR) to diverse pathogens [9]. Using tobacco cell suspensions, it has been possible to characterize early events implicated as transduction components in the cryptogein induction of defense responses. These include cryptogein-binding to high affinity binding sites in the plasma membrane [11], ion channel-mediated changes in plasma membrane permeability [12], intracellular Ca^{2+} release through activation of RYR- and IP_3-R-like receptors [13], increase of nuclear free Ca^{2+} concentration (D Lecourieux, personal communication, 2004), modulation of protein kinases including mitogen activated protein kinases (MAPK) [14], production of NADPH oxidase-dependent reactive oxygen species (ROS) [15] and disruption of the microtubular cytoskeleton [16]. Cryptogein-induced elevation of cytosolic free Ca^{2+} concentration is required for the elicitor-triggered early and late reactions including phytoalexin synthesis and cell death [13].

Using the NO sensitive fluorophore 4,5-diaminofluorescein diacetate (DAF-2DA), we reported the real-time imaging of NO production in epidermal tobacco cells treated with cryptogein [17]. After elicitation with the elicitor, the earliest burst of NO was in the chloroplasts, where NO production occurred within 3 minutes. The level of fluorescence increased with time, and after 6 minutes NO was also found along the plasma membrane, in the nucleus and most probably in peroxisomes. In mammals, NO-dependent responses are governed by the subcellular compartment of NO production and by the frequency/duration of its synthesis [1,2]. Therefore, because the extent and timing of NO synthesis observed in response to cryptogein seems tightly regulated, we would anticipate that NO operates over a spatial and temporal range both for specificity of targeting and for propagation of the elicitor signal.

To investigate the signaling events that mediate NO production, and to analyse NO signaling activities in the cryptogein transduction pathway, a spectrofluorometric assay using DAF-2DA was developed to follow NO production in tobacco cultured cells. As observed in tobacco epidermal tissue, cryptogein induced a fast and transient NO production in tobacco cell suspensions [18]. This production was completely suppressed in the presence of the NO scavenger cPTIO, and was reduced by 55 to 85% by mammalian NOS inhibitors. By contrast, inhibitors of NR had no effect on cryptogein-induced NO production. Interestingly, the NO burst was also sensitive to carboxymethoxylamine and aminoacetonitrile, two inhibitors of the

variant of the P protein of glycine decarboxylase complex (iNOS), an enzyme originally reported as being a plant NOS enzyme [19]. These inhibitors also suppressed NO production induced by other elicitors of defense responses in tobacco, grapevine and *Arabidopsis* suspension cells (unpublished data). Although we can not gave a conclusive answer, we suggest that the inhibitory effect of these compounds is caused by their ability to act on plant NOS or on proteins acting upstream of NOS.

Calcium signals are thought to play an important role in the tobacco cells response to cryptogein [12-16]. To investigate whether NO was active in this process, the recombinant aequorin technology was used. Aequorin is a photoprotein from *Aequora victoria* which undergoes a conformational change and emits luminescence when occupied by Ca^{2+} [20]. Using transgenic *Nicotiana plumbaginifolia* cell suspensions that constitutively express aequorin in the cytosol, it was shown that cryptogein triggers a first increase of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) resulting from an influx of extracellular Ca^{2+} and Ca^{2+} release from internal stores [13]. This first Ca^{2+} peak was followed immediately by a second, sustained $[Ca^{2+}]_{cyt}$ mainly due to the influx of extracellular Ca^{2+}. When cryptogem-triggered NO production was suppressed by cPTIO or inhibitors of mammalian NOS, the intensity of the first $[Ca^{2+}]_{cyt}$ increase was reduced by almost 50% whereas the second $[Ca^{2+}]_{cyt}$ peak was unaffected [18]. Because similar pharmacological treatments did not affect cryptogein-induced entry of extracellular Ca^{2+} (as measured by quantifying uptake of extracellular $^{45}Ca^{2+}$), we assumed that NO participates in the elevation of cryptogein-mediated $[Ca^{2+}]_{cyt}$ through the mobilization of Ca^{2+} from intracellular stores. We also examined whether NO has any role in initiating the increase of nuclear free Ca^{2+} concentration ($[Ca^{2+}]_{nuc}$) observed in cryptogein-treated cells. Result from this study indicates that NO generation did not lead to the rise of nuclear free Ca^{2+}.

The ability of endogenous NO to promote Ca^{2+} changes has been also analysed in grapevine suspension cells treated with BcPG1 (E Vandelle, personal communication, 2004). This elicitor mediates a rapid NO production, which is sensitive to mammalian NOS inhibitors and strictly dependent on extracellular Ca^{2+} influx. Here too, NO appears to contribute to the elicitor-induced $[Ca^{2+}]_{cyt}$ elevation by promoting the release of Ca^{2+} from intracellular Ca^{2+} stores into the cytosol. Moreover, surprisingly, when the BcPG1-triggered NO burst was suppressed, the elicitor-induced influx of extracellular Ca^{2+} was clearly increased, indicating that NO also negatively regulates Ca^{2+} entry. The physiological significance of this inhibition remains unknown. From what is known of the NO-dependent transduction mechanisms in animals [2], we postulate that this inhibition in plants could constitute a negative feedback loop which assists the cells to reduce extracellular Ca^{2+} influx and therefore regulates Ca^{2+}-dependent processes including NO synthesis and NO-derived deleterious effects.

To expand our understanding of NO signaling, its putative involvement in cryptogein-induced defense gene expression was investigated. The selected genes included those that encode pathogenesis-related (PR) proteins, glutathione-S-transferase (GST), lipoxygenase 1 (LOX), the ethylene-forming enzyme cEFE-26, a low-molecular-mass heat-shock protein (TLHS-1), the hypersensitive-related proteins hsr515 and hsr203J, sesquiterpene cyclase, phenylalanine ammonia-lyase and SAR 8.2, a small highly basic protein of unknown function expressed during the HR and SAR. Amongst these genes, only *cEFE-26* and *TLHS-1* transcripts, were shown to accumulate under regulatory control by NO, suggesting that NO might contribute to ethylene and HSP synthesis [18]. Accordingly, the levels of both mRNAs were elevated in tobacco cells that had been treated with NO donors (O Lamotte, personal communication, 2004). In plants, low-molecular-mass heat-shock protein (sHsps) are synthesised rapidly in response to a wide range of environmental stressors, including heat, cold, drought and salinity. These proteins are thought to play an important role in the

acquisition of stress tolerance by binding and stabilizing denatured proteins, facilitating their refolding by Hsp70/Hsp100 complexes [21]. Furthermore, in mammals, sHsps are known to be involved in cellular functions such as apoptosis by regulating cellular redox state. However, the involvement of TLHS-1 as regulator of NO-induced stress response remains to be established. In contrast to cryptogein, NO produced in response to BcPG1 was shown to alter the expression of genes associated with phytoalexins synthesis, namely *PAL* and *VST1* encoding stilbene synthase (E Vandelle, personal communication, 2004). Therefore, NO may confer to grapevine cells a selective advantage against *Botrytis Cinerea* by contributing to phytoalexin synthesis. In support of this hypothesis, NO released by NO donors was shown to activate phytoalexin accumulation in potato tuber tissue and soybean cotyledon [22, 23].

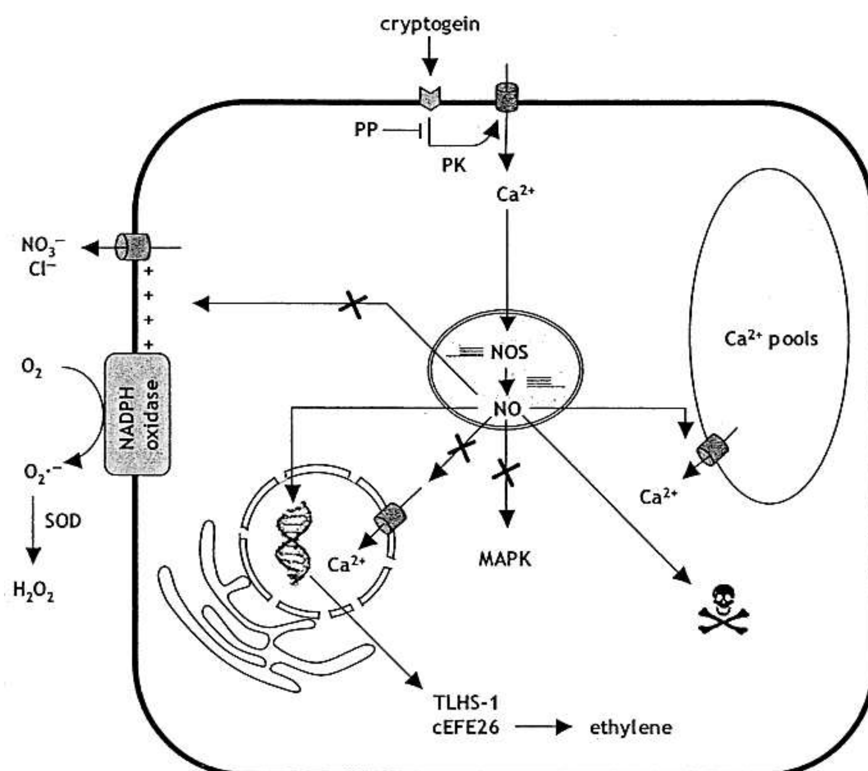

Figure 1. Figure Caption , p.p Hypothetical model of NO functions in cryptogein signaling in tobacco cells. Cryptogein binding to high affinity binding sites is followed by protein kinase-mediated phosphorylation events leading to a large and sustained Ca^{2+} influx. Both protein phosphorylation and Ca^{2+} influx are required for NOS-dependent NO synthesis which may occur in plastids. Once produced, NO activates intracellular Ca^{2+} permeable channels contributing to the elevation of cytosolic but not nuclear free Ca^{2+} concentrations. Furthermore, NO production is an upstream step in the elicitor transduction pathway leading to cell death and *TLHS-1* and *cEFE-26* transcripts accumulation. In contrast to its fonction in BcPG1 signaling (see text for details), NO produced in response to cryptogein does not regulate ROS synthesis. In addition, MAPK, anion channels activation and the subsequent plasma membrane depolarisation are NO-independent processes. All the cryptogein cascade seems negatively regulated by at least one phosphatase. PK, p.p protein kinase, PP, p.p protein phosphatase, SOD, p.p superoxide dismutase.

In animals, superoxide collaborates with NO *via* the formation of peroxynitrite ($ONOO^-$) to induce apoptosis and execute invading pathogens. In plants, the NO and ROS burst observed in response to elicitors and avirulent pathogens are usually synchronized, suggesting a concerted action of both compounds. Accordingly, NO was shown to act synergistically with ROS to promote cell death in soybean cells [24]. Interestingly, HR seems to be activated when NO interacts with H_20_2 generated from superoxide by superoxide dismutase, rather than with superoxide per se [25]. Similarly, in our own investigations we identified NO but not $ONOO^-$ as an effective inducer of cryptogein-triggered HR [18]. In o model system, however, HR seems to occur independently of H_20_2, because the inhibition of NADPH oxidase had no effect on the elicitor-mediated cell death [12,16]. Because the overall time course of NO and ROS are almost identical in elicitor-treated cells, it is also conceivable that NO might regulate ROS production and *vice versa* in particular contexts. Supporting this hypothesis, BcPG1-induced H_20_2 production in grapevine cells was shown to be tightly dependent on NO. The question of whether NO produced in response to BcPG1 (in)directly activates NADPH oxidase, or whether it inhibits H_20_2 scavenging enzyme as suggested by Clark *et al.* [26] will require further investigation.

Our data, along with those from other studies, highlight the crucial role of NO in protecting plants against pathogens by promoting Ca^{2+} mobilization, ROS synthesis, defense- and stress-related gene expression and HR (Figure 1). This concept was recently confirmed at the genetic level by Zeidler *et al.* [27] who reported that *Atnos1* mutant plants showed dramatic susceptibility to the pathogen *Pseudomonas syringae* pv. tomato DC3000. Besides pathogen attack, abiotic stressors, such as drought, salinity and extreme temperature are serious threats to agriculture. In the recent years, a significant amount of work has gone into investigating NO synthesis and functions in plants exposed to abiotic stressors. For example, it was shown both in tobacco leaf peels and tobacco suspension cells that high temperature, osmotic stress, or salinity, generate a rapid and significant surge in NO levels [28]. In contrast, light stress and mechanical injury had no apparent effect on NO production in tobacco and/or tomato [28, 29]. Thus, although NO synthesis can be triggered by several, disparate abiotic stressors, it cannot be considered a universal plant stress response.

Experiments using NO donors suggest that NO may exert beneficial effect on stress tolerance. For instance, it was shown that NO released by sodium nitropruside (SNP) enhanced the tolerance of wheat seedlings to drought stress [30]. A key question which remains mostly unresolved is how NO contributes to plant adaptation to abiotic stress ? As commonly suggested, by scavenging the superoxide radicals commonly produced by plants challenged by abiotic stressors, NO would prevent superoxide-induced deleterious effects such as the formation of the highly toxic hydroxyl radicals. It is also reasonable to expect that NO synthesized by plant cells in response to abiotic stressors encodes and conveys information leading to stress tolerance. Supporting this hypothesis, recent evidence shows that NO participate in the elevation of $[Ca^{2+}]_{cyt}$ triggered by hyperosmotic stress in tobacco cell suspensions [28]. Exploring these pathways will undoubtedly aid our understanding of plants acquired stress tolerance.

2. The NO/Ca^{2+} cross-talk

The hypothesis that NO might play a key role in controlling free Ca^{2+} mobilization in plant cells was first postulated by Durner *et al.* [31] and Klessig *et al.* [32]. Both studies provided pharmacological arguments suggesting the occurrence of a NO/cGMP/cADPR/Ca^{2+} signaling cascade in plant cells. The results discussed here confirm this tight interaction between the

Ca^{2+} and the NO signaling systems. A similar NO/Ca^{2+} connection has also been found to be active in abscisic acid signaling leading to stomatal closure [33].

Recently, the role of NO in controlling Ca^{2+} homeostasis was investigated more thoroughly. Its has been shown that NO, released by the sulphur-free NO donor DEA-NONOate, elicits within minutes a transient influx of extracellular Ca^{2+} and a synchronized increase of $[Ca^{2+}]_{cyt}$ in aequorin-transformed tobacco cells [18]. As predicted from a pharmacological study, the channels responsible for NO-induced $[Ca^{2+}]_{cyt}$ elevation include voltage-dependent Ca^{2+} channels of the plasma membrane and intracellular Ca^{2+} channels sensitive to RYR and IP_3R inhibitors. This observation paralleled the situation encountered in animal cells in which almost ail the molecules involved in the. control of Ca^{2+} homeostasis seem to be modulated by NO [3]. By contrast, NO released by the donor did not induce nuclear free Ca^{2+} changes in tobacco cell suspensions (D Lecourieux, personal communication, 2004). Therefore, the modulation by NO of enracellular Ca^{2+}-permeable channels leading to an enrichment of the nuclear free Ca^{2+} concentration seems unlikely. However, caution in the interpretation of these data needs to be exerted. Indeed, a main problem facing experiments based on NO donors is the access of NO to its targets. For example, it is possible that NO entering the cells was metabolised before acting on the nucleus Ca^{2+} homeostasis. Furthermore, NO might not be sufficient to activate the mechanisms involved in the increase of nuclear free Ca^{2+} concentration.

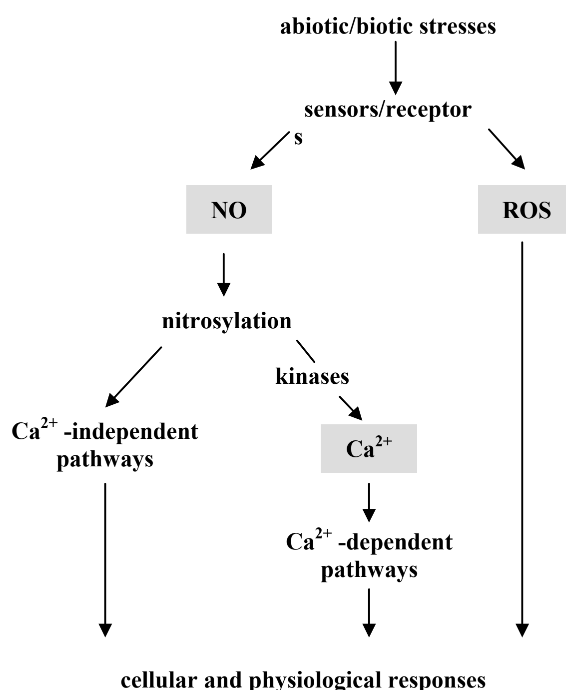

Figure 2. Schematic description of NO involvement in biotic/abiotic transduction pathways

Recent evidence from our laboratory suggest that NO mediates $[Ca^{2+}]_{cyt}$ through multiple mechanisms (O Lamotte, C Courtois, personal communication, 2004) . First, the elevation of $[Ca^{2+}]_{cyt}$ triggered by NO in aequorin-transformed tobacco cells was diminished by dithiothreitol, a reducing agent capable of denitrosylation nitrosothiol groups [34]. This inhibitory effect suggests that NO might partly act by S-nitrosylation or oxidation of cysteine residues. Second, we obtained evidence that NO operates on Ca^{2+} mobilization through phosphorylation-dependent processes, suggesting that the NO signal converges into protein kinases pathways. Accordingly, we demonstrated that NO can induce within minutes the activation of two protein kinases, a serine/threonine protein kinase and a MAPK. Whether both protein kinases are involved in NO signaling leading to Ca^{2+} mobilization is currently unknown. Third, 8-bromo-cADPR, a selective agonist of cADPR-mediated Ca^{2+} release, suppressed the Ca^{2+}-mobilizing action of NO, confirming the existence of a NO/cADPR/ Ca^{2+} signaling cascade in plant cells. Finally, NO treatment depolarised the plasma membrane of tobacco cells, most probably through the inhibition of outward-rectifying K^+ channels as recently reported by Sokolovski and Blatt [35]. This depolarisation may in turn activate voltage-dependent Ca^{2+} channels of the plasma membrane.

3. Conclusion

Plants express adaptive response to allow them to confer tolerance to environmental stresses and ensure survival. NO function is signal transduction pathways during this response (Figure 2). Although the precise signaling functions of NO are poorly understood, its capacity to modulate Ca^{2+} homeostasis provides an extraordinary and remarkably effective way of conveying information. Little is known about the signaling consequence of the NO/Ca^{2+} crosstalk but it is likely that modulation of the expression of stress-related gene may occur. Elucidating the role of NO in plant response to stressons will also require the identification of nitrosylated proteins. A recent novel strategy bas been reported for the identification of such proteins [36] and within the next few years, numbers of them will become available. The challenge will be to understand their functions and to determine the extent to which nitrosylation influences plant cell response to environmental and infection stresses.

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