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Detection and function of nitric oxide during the hypersensitive response in *Arabidopsis thaliana*: Where there's a will there's a way

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ABSTRACT

Nitric oxide (NO) was identified as a key player in plant defence responses approximately 20 years ago and a large body of evidence has accumulated since then supporting its role as a signalling molecule. However, there are many discrepancies in current NO detection assays and the enzymatic pathways responsible for its synthesis have yet to be determined. This has provoked strong debates concerning the function of NO in plants, even questioning its existence *in planta*. Here we gather data obtained using the model pathosystem Arabidopsis/Pseudomonas, which confirms the production of NO during the hypersensitive response and supports is role as a trigger of hypersensitive cell death and a mediator of defence gene expression. Finally, we discuss potential sources of NO synthesis, focusing on the role of nitrite as major substrate for NO production during incompatible interactions.

Contents

1. Introduction

Nitric oxide (NO) is a small gaseous radical, defined as a 'do it all' molecule [1], which is present throughout the plant life cycle. It is involved in physiological processes such as stomatal closure as well as pathophysiological processes such as biotic and abiotic stress responses [2]. In one of its key roles, NO acts as a trigger for the hypersensitive disease resistance response (HR) induced by avirulent pathogens [3], which is characterized by the formation of necrotic lesions at the infection site to inhibit further pathogen entry and spreading [4]. According to the so-called 'balance model', hypersensitive cell death is regulated by the coordinated action of NO and H_2O_2 produced simultaneously in the infected tissues [5]. NO is also thought to regulate a battery of plant defence genes, allowing the establishment of pathogen resistance [6].

An increasing body of evidence indicates that NO signalling can be mediated within the plant cell by its direct interaction with target molecules, e.g., by causing post-translational protein modifications [6,7]. However, as in animal cells, the NO signal may also be relayed by second messengers such as Ca^{2+} and cGMP [8], particularly during the regulation of defence gene expression. Despite

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abundant data concerning the role of NO in plants, its production and reliable detection generally, and during the HR in particular, is a matter of controversy. It is therefore necessary to study the production and function of NO in this specific process using the most suitable and consistent tools.

In this article, we summarize evidence concerning the role of NO as a trigger of hypersensitive cell death and as a regulator of defence gene expression. We discuss the current methods used for NO detection in plants and provide original data showing NO emission from pathogen-challenged Arabidopsis plants using a chemiluminescence-based method. Finally, we discuss potential sources of NO during the HR, focusing on the importance of nitrite for NO biosynthesis. Defence responses are likely be pathosystem-specific [9,10], so we have concentrated on the genetically well-defined host–pathogen interaction between *Arabidopsis thaliana* and *Pseudomonas syringae*.

2. The function of NO during the HR: I do therefore I am!

The first study reporting the importance of NO in pathogen resistance showed a loss of resistance in Arabidopsis plants challenged with an avirulent strain of *P. syringae* (*Pss*) together with inhibitors of animal NO synthase [3]. Since that initial report, the plant scientific community has tried to understand the detailed role of this fascinating reactive molecule in plant–pathogen interactions.

2.1. More NO...

Many studies have reported the physiological effect of exogenously applied NO on plants, and have linked NO to hypersensitive cell death, a key marker of the HR [11]. For example, the exposure of Arabidopsis cell suspension cultures and plants to NO donors induces cell death [12–14], which (like apoptosis in animals) is associated with chromatin condensation [12] and involves caspase-like and cystatin-sensitive protease activities [12,13]. Delledonne and colleagues [5] proposed the so-called 'balance model' based on experiments with soybean cell suspension cultures, in which NO-induced cell death requires the coordinated action of NO and H_2O_2. This model has been confirmed genetically in transgenic Arabidopsis plants in which thylakoidal ascorbate peroxidase (tAPX) activity is perturbed by overexpression or antisense suppression, resulting in the modulation of cell death responses to NO donors by changing the concentration of H_2O_2, and in tobacco cells elicited with the proteinaceous elicitor cryptogein [15–17]. The involvement of NO in hypersensitive cell death has also been demonstrated by applying NO donors to the *dnd1* (*defense no death 1*) mutant, which has an altered HR phenotype. The NO donors restored the cell death response triggered by avirulent *P. syringae* pv. *syringae* (*Pss*) and *P. syringae* pv *tomato* DC3000 carrying the *AvrB* gene (*Pst AvrRpt2*) [18]. Because the *dnd1* mutant lacks a functional cyclic nucleotide-gated channel 2 (CNGC2), this report placed NO generation downstream of Ca^{2+} signalling during the induction of hypersensitive cell death (reviewed in [8]).

The application of exogenous NO donors not only induces cell death, but also modulates the Arabidopsis transcriptome, as determined using different profiling approaches (e.g., cDNA-AFLP, microarrays) and different NO sources (NO donors, NO gas). Thus, several groups have reported the induction of genes related to defence and cell death using such assays [14,19,20]. These large-scale expression studies have revealed that NO upregulates genes encoding enzymes in the phenylpropanoid pathway involved in phytoalexin biosynthesis [14], and also genes related to the synthesis of and response to salicylic acid (SA), a major regulator of systemic defence responses [19]. Recently, NO was described as a key component required to establish SA-dependent systemic acquired resistance (SAR) in Arabidopsis plants challenged with avirulent pathogens [21]. Other NO-regulated genes include receptor-like kinases (RLKs) and plant receptor kinases (PRKs) responsible for pathogen recognition [22], and AtMKK5 [20], a MAP kinase kinase (MAPKK) which induces HR-like cell death [23]. Finally, NO was shown to induce the expression of several WRKY transcription factors, including WRKY46 which is induced by pathogen infection [20]. Together, these data establish a clear molecular link between NO and some of the main components of defence mechanisms governing plant resistance and cell death.

Recent analysis of the promoters of NO-regulated genes identified some common transcription factor-binding sites [24], including ocs-element-like sequences (OCSEs) that are important for the expression of defence genes such as *GST6* and *PR1* [25,26], and TGA/ocs element-binding factors (OBFs) that interact with NPR1 (nonexpressor of pathogenesis-related genes 1), a key regulator of SAR [27]. Furthermore, the W box element is found in the promoters of many plant defence genes [28], such as NPR1 that has been shown to regulate defence responses [29], and this is recognized by WRKY transcription factors. Interestingly, an NO-dependent mechanism is also responsible for the translocation of NPR1 into the nucleus [30], suggesting that NO could regulate NPR1 both at the transcriptional and post-translational levels in order to modulate specific defence genes.

Although these studies showed that defence responses in plants are induced by exogenous NO, it is not clear whether the application of exogenous compounds reflects genuine physiological situations. Exogenously-applied NO may not replicate the function of endogenous NO and may have side effects in plants. Accordingly, it is has been reported that some by-products of exogenous NO donors can strongly interfere with physiological and metabolic processes in plants, e.g., the cyanogenic groups of sodium nitroprusside (SNP), a widely used exogenous NO donor [31,32]. In an attempt to circumvent the use of NO donors, researchers have sought mutants displaying higher endogenous NO levels as compared with wild-type plants. The Arabidopsis *nox1* (NO overproducer 1)/*cue1* mutant accumulates constitutive high levels of NO [33], and experiments involving incompatible plant–pathogen interactions have shown this accelerates HR cell death kinetics and the magnitude of the response to two distinct *R* genes [34]. However, despite the positive correlation between NO content and hypersensitive cell death, the genetic basis of the *nox1*/*cue1* mutant is the loss of function of a gene encoding a chloroplast phosphoenolpyruvate/phosphate translocator, resulting in a pleiotropic phenotype that makes the specific analysis of NO activity challenging. In the same manner, transgenic Arabidopsis plants have been produced expressing the mammalian neuronal NO synthase (nNOS), resulting in the stronger induction of *PR* genes (*PR1*, *PR2*) in response to virulent bacterial pathogens, and correlating with a major resistance phenotype [35]. However, the basis of nNOS activity in plants remains unclear because in animals this enzyme requires BH_4 as a cofactor, which is not present in plant cells. Shi and colleagues [35] speculated that nNOS may use FH_4 as substitute in plant cells but this has not been demonstrated experimentally and therefore it is not yet possible to confirm the reliability of this nNOS-overexpressing plants.

NO bioactivity may not be controlled exclusively at the level of NO biosynthesis during plant defence responses but also by regulating the availability of S-nitrosoglutathione (GSNO), which is thought to function as a mobile reservoir of NO bioactivity [36]. Therefore, the Arabidopsis *gsnor* mutant, which has impaired GSNO reductase activity, has been used by several groups to study the function of NO in plants. In all studies, *gsnor* knockdown/knockout mutants displayed higher levels of S-nitrosothiols (SNOs) as well as SNO proteins [34,37–39]. However, when these mutants were challenged with virulent or avirulent pathogens, there were strong discrepancies among the observed responses. This could reflect dif-

ferences in the genetic material (transgene expression or T-DNA insertion), plant growth conditions or plant age [38], as well as the existence of a compensatory defence pathway [39], but whatever the basis for these observations it was difficult to reach conclusions regarding the role of NO mediated by SNOs during defence. Recent reports demonstrate that GSNOR activity is required for competitive viability [40,41] and that it regulates multiple developmental and metabolic programs, so these phenomena may account for the pleiotropic effects of the *gsnor* mutation [42].

The role of NO in cell death and gene regulation is supported by the analysis of mutants affecting these processes, which produced higher amounts of NO thus linking NO to the compromised phenotypes. For example, ozone (O_3) is known to induce HR-like lesions with the hallmarks of programmed cell death in sensitive Arabidopsis accessions and mutants [43,44] and can therefore be considered as a model to study hypersensitive cell death. In this context, the *rcd1* mutant, which develops severe lesions in the presence of O_3 and shows a constitutive high level of defence gene expression, produces more NO than wild-type plants [45]. This suggests NO may play a role in both the initiation of cell death and the subsequent containment of the lesions. Similarly, the double mutant *nudt6 nudt7* shows a temperature-dependent autoimmune (HR-like) phenotype, *i.e.*, constitutive *PR1* and *PR2* expression, stronger resistance to both virulent and avirulent pathogens, and the development of spontaneous microlesions, and also produces elevated levels of NO [46]. Interestingly the upregulation of *PR1* in this mutant is abolished in the presence of the NO scavenger cPTIO, thus confirming the involvement of NO in this phenotype. Finally, in the context of NO/Ca^{2+} interdependence [8], Arabidopsis mutants overexpressing the pepper (*Capsicum annuum*) calmodulin 1 (CaCaM1) or the pepper receptor-like cytoplasmic protein kinase CaPIK1 (affecting Ca^{2+} sensing and signalling, respectively), produce more NO in response to virulent *P. syringae* pv. *tomato* (*Pst*) and suffer a higher rate of cell death [47,48]. The concomitant accumulation of NO and reactive oxygen species (ROS) in transgenic plants overexpressing CaCaM1 or CaPIK1 [47,48] provides further genetic evidence that NO cooperates with ROS to induce cell death in depending on their concentration ratio [5].

Despite the caution required when interpreting data based on pharmacological compounds or mutants with pleiotropic phenotypes, the results discussed above confirm the observations made with NO donors and collectively demonstrate that NO triggers cell death and regulates defence gene expression during the HR. However, a valid and reliable scientific demonstration requires both gain and loss of function aspects to be considered. Therefore, we must now look at the evidence from plants that have been pharmacologically or genetically depleted for NO.

2.2. NO more...

The first report concerning the production and function of NO during the HR was based on the use of the NO scavenger cPTIO and inhibitors of mammalian NO synthases, such as L-NNA or PBITU [3]. This approach has been applied in diverse pathosystems using cell suspension cultures and elicitors. In contrast, few reports describe the pharmacological inhibition of NO production and activity in Arabidopsis plants challenged with avirulent pathogens. The efficacy of the well-known and valuable NO scavenger cPTIO may be impaired by cellular reactions and is significantly reduced in a time-dependent manner [49]. Therefore, this approach may be unsuitable in whole plants infected with avirulent pathogens because scavenger efficiency declines over the duration of such experiments, which is generally up to 24 h post infection (hpi).

Alternative genetic approaches have been developed to overcome the limitations discussed above and to address the lack of information about NO-producing enzymes. One example is the overexpression of a bacterial NO dioxygenase (NOD), a flavohaemoglobin capable of converting NO to nitrate using NADPH and O_2 [9,50]. Accordingly, Arabidopsis plants overexpressing *Escherichia coli* NOD display a reduced NO burst in response to infection with avirulent pathogens, associated with a much weaker than normal induction of *PAL* expression and a reduction in HR-related cell death [50]. In a similar manner, Boccara and colleagues [9] expressed *Erwinia chrysanthemi HmpX* in avirulent *Pst*, leading to a significant delay in the development of macroscopic symptoms and the appearance of fewer HR patches, thus confirming that the removal of NO by directly targeting NOD at the site of infection compromises HR-related cell death.

Another approach exploited NO turnover mechanisms in plants, such as NO scavenging by non-symbiotic haemoglobins [6]. A primary function of haemoglobins is to protect against nitrosative stress and to modulate NO signalling functions [51]. Indeed, NO can be metabolized to nitrate by non-symbiotic haemoglobins (such as AtHb1), which acts as an NO dioxygenase using NADPH as an electron donor [52]. Remarkably, although AHb1 overexpression reduced NO production induced by transferring light-adapted plants to darkness, it affected neither NO accumulation nor hypersensitive cell death in response to an avirulent pathogen [52]. This suggested that AHb1 effectively protects plants from harmful nitrosative stress but does not interfere with the 'beneficial' massive NO burst required to establish the plant defence response [52]. In contrast, overexpression of the Arabidopsis haemoglobin GLB1 effectively compromised NO accumulation in response to avirulent pathogens, associated with a reduction in hypersensitive cell death [53]. Such discrepancies concerning the effect of haemoglobin may reflect the experimental conditions used in the different studies and remain to be clarified. In line with the NO-dependent initiation of SA biosynthesis [54], supported by gene expression analysis [19], SA concentrations in *PstAvrRpm1*-challenged 35S-GLB1 transgenic plants were significantly reduced compared to wild-type plants. Based on the central role of SA in the potentiation of defence and cell death [55], this clearly establishes a link between NO/SA and the induction of cell death during the HR.

Finally, the Arabidopsis mutant *cml24* is interesting based on the striking Ca^{2+}-dependent NO production recognized in the context of plant defence responses [8]. This mutant lacks a fully functional copy of the CaM-like 24 Ca^{2+} sensor and accumulates less NO than wild-type plants in response to *PstAvrRpt2*, resulting a lower rate of HR-related cell death [56]. This demonstrates that NO production is effectively regulated by CaM/CaM-like proteins during the HR and that the pathway is required to induce cell death. In this context, we speculate that NO levels may be substantially higher in the Arabidopsis mutant overexpressing AtCML43, in which the onset of HR is more rapid than in wild-type plants [57].

All of the NO-related mutants described above have pleiotropic phenotypes so it is difficult to study the effect on NO production in isolation. However, despite the different genetic backgrounds and the diverse pathways that are affected all the mutants have lower levels of NO than usual which correlates with an altered HR, strongly supporting the conclusion that NO triggers HR-related cell death and regulates defence gene expression. In this context, we can apply a simple syllogism: every molecule that has an evident function in a physiological process exists *in planta*; NO has an evident function during the HR; therefore, NO exists *in planta*. And if this is the case, we should be able to detect it and measure it.

3. NO detection in Arabidopsis: catch me if you can!

Several analytical methods, established in animals and adapted for plants, have been developed to detect and quantify NO. Most of them have been already applied for the detection of NO in Arabidopsis during plant–pathogen interactions, including laser-

based infrared spectroscopy, haemoglobin conversion, the detection of diaminofluoresceins, electron paramagnetic resonance and chemiluminescence [58]. These techniques suffer from various limitations in term of specificity, sensitivity and the equipment requires, leading to many discrepancies in the determination of NO levels *in planta* [59].

The oxyhaemoglobin assay, which has an intracellular NO detection with a detection limit of 1–3 nM [60] was used in the initial study reporting the production of NO during plant–pathogen interactions [3]. This has been applied in Arabidopsis cell suspension cultures, revealing a specific increase in NO levels in response to avirulent pathogens in the first hours following infection [12]. However, the ROS produced simultaneously with NO during plant defence responses [3,12] can also oxidize oxyhaemoglobin, and the reaction is also influenced by pH, which is known to vary during the HR [61]. Both these phenomena can interfere with the assay and generate false positive results.

Diaminofluorescein-based dyes (DAF-2DA, DAF-FM-2DA) are the most widely used reagents for NO measurement in the plant research community, as demonstrated by the extensive list of studies reporting their application particularly in the context of the HR. Nevertheless, their use is controversial for several reasons. First, they do not detect NO directly but instead detect its oxidation product N_2O_3 [62]. Furthermore, many different compounds can react with DAF-2 or interfere with DAF-2T fluorescence during NO detection, such as catecholamines, superoxide radicals, dithiothreitol, 2-mercaptoethanol, glutathione, and divalent cations such as Ca^{2+} and Mg^{2+} [63]. Ascorbic acid and dehydroascorbic acid are generated during stress and these can also combine with DAF to form DAF-2-DHAs that generate fluorescence similar to DAF-2T [64]. A new Cu(II) fluorescein-based probe (CuFL) has been developed to detect NO in a more specific manner [65] and has been used successfully to detect NO in plant nodules [66], in Arabidopsis leaves elicited with oligogalacturonides [67] and in tobacco cells elicited with cryptogein [17]. It would be interesting to test the suitability of CuFL for measuring NO in the context of the HR. However, like all fluorescent dyes, CuFL does not allow the production of NO to be monitored in real time during infections, and given the short half-life of NO (3 s) compared to such fluorescent products, the values generally do not represent the actual amount of NO present in cells at any given moment but instead reflect the accumulation of more stable fluorescent products during the infection process.

As an alternative, an electron paramagnetic resonance (EPR) spin trap method with N-methyl-D-glucamine dithiocarbamate (MGD) as a spin-trapping agent was used to monitor NO emission from Arabidopsis leaves in response to infection with the avirulent *P. syringae* pv. *maculicola* [68,69]. However, caution must be exercised when using this method because the use of reducing agents with nitrite overestimates the amount of free NO, and the L-NG-nitroarginine methylester shows the same spectrum as the $Fe^{2+}(DETC)_2NO$ complex under strong reducing conditions [58]. Moreover, alternative nitroxide spin traps, among which the well-known NO scavenger cPTIO, successfully tested *in vitro* and in animal cells, are rapidly reduced in plants into diamagnetic EPR silent products, thus compromising their use for NO detection in plant systems [49]. Finally, this technique also requires expensive equipment and considerable user expertise making it less accessible to the plant research community [59].

Laser-based infrared spectroscopy techniques, namely CO laser-based photoacoustic detection and quantum cascade laser (QCL)-based detection offer the advantage of measuring NO *ex planta* with high sensitivity (pmol range) compared to other methods [59]. Both methods have been successfully used to detect NO emission by Arabidopsis plants elicited with bacterial pathogens [53,69]. Nevertheless, little information is available about these methods, again probably reflecting the limited access to such facilities and the unique expertise required to detect NO.

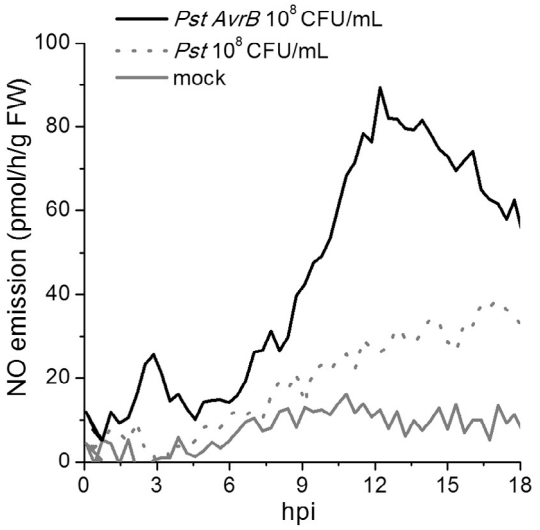

Fig. 1. Gas-phase NO emission pattern of *Arabidopsis thaliana* plants in response to pathogen infection revealed by chemiluminescence. *Arabidopsis thaliana* Col-0 plants were infiltrated with mock (10 mM $MgCl_2$; solid grey line), *Pst AvrB* 10^8 CFU/mL (solid black line), or *Pst* 10^8 CFU/mL (dotted grey line) and placed in the nutrient solution. NO emission was measured by chemiluminescence. Experiments were carried out three times with similar results. FW, fresh weight.

In contrast, chemiluminescence is a simpler method and is the best-established approach for the measurement of gaseous NO [58]. This technique relies on the reaction between NO and O_3, which forms light photons that are measured with a photodetector [70]. The method is specific and sensitive and can be used for the real-time detection of NO gas emissions from intact plants. This allows the precise time-course of NO production to be defined, which is necessary to decipher its function in a given process. Although chemiluminescence was initially used to measure NO in plants during anoxia [52,71], the first attempt to detect NO during plant defence responses (tobacco leaves infected with the necrotizing elicitor cryptogein) was unsuccessful [72]. NO production in this system has been demonstrated using DAF [73] as well as CuFL [63] and NO electrode measurements [74], so it is clear that the problem is technical rather than based on the absence of NO. Accordingly, NO emission from tobacco leaves infected with avirulent pathogens has recently been detected by chemiluminescence [75]. We also describe here the first chemiluminescence detection of NO emitted in the pathosystem Arabidopsis/Pseudomonas (Fig. 1). It is reasonable to assume that measuring emissions from detached leaves during infection may be unsuitable, thus we emphasise that the experiments were carried out using single whole plants under the same conditions used to study molecular basis of the HR. This is important because the reliable and precise measurement of NO allows production to be correlated with downstream events potentially regulated by this molecule. Thus, under these conditions, the emission of NO revealed by chemiluminescence increased significantly during the HR induced by avirulent *Pst AvrB* compared with mock-treated plants, up to 80 pmol NO per hour per gram fresh weight (Fig. 1). A high concentration of virulent *Pst* was also able to induce NO emission in Col-0 plants, but the amount produced was much lower (maximum ~30 pmol/h/g FW) and the onset was delayed as compared with *Pst AvrB*, demonstrating that a strong and anticipated NO burst is specific for the incompatible interaction.

According to these data, it is now clear that the exposure of Arabidopsis leaves to avirulent pathogens triggers an NO burst, which

shows a biphasic trend as previously reported [3,76]. Given the high specificity of chemiluminescence, these data also demonstrate a clear time course of NO production, and the NO emission detected at the onset of the HR in response to the HR-like inducer O_3 [77] confirms the role of NO as an early event in the induction of hypersensitive cell death [3,12,76,78].

Interestingly, the high sensitivity of the method allowed us to detect NO emission from plants infected with virulent pathogens, although delayed and at a much lower level than the HR-related NO burst, as previously reported [76,78]. This confirms the specificity of the strong NO emission observed during the incompatible interaction and is in agreement with previous comparative transcriptomics experiments in Arabidopsis plants exhibiting compatible and incompatible interactions, which revealed that differences in defence-related gene expression between susceptible and resistant plants are temporal and quantitative rather than qualitative [79]. As previously hypothesized, the low-level production of NO during compatible interactions could even be an important requirement for pathogen virulence [78]. This reinforces the idea that the magnitude and timing of NO production are fundamental requirements that favour HR induction over disease. The role of the NO burst as a trigger for cell death was previously questioned based on results obtained with the NO scavenger cPTIO, which prevented cryptogein-induced cell death in tobacco cell suspension cultures when added immediately after elicitation but also after a significant delay [72]. However, according to the tightly regulated and orchestrated NO emission necessary to induce HR as described above, it is reasonable to assume that the compromised cell death observed with cPTIO reflected the premature 'interruption' of the NO burst, which is absolutely consistent with previous hypotheses concerning the function of NO as an inducer of cell death [3,80].

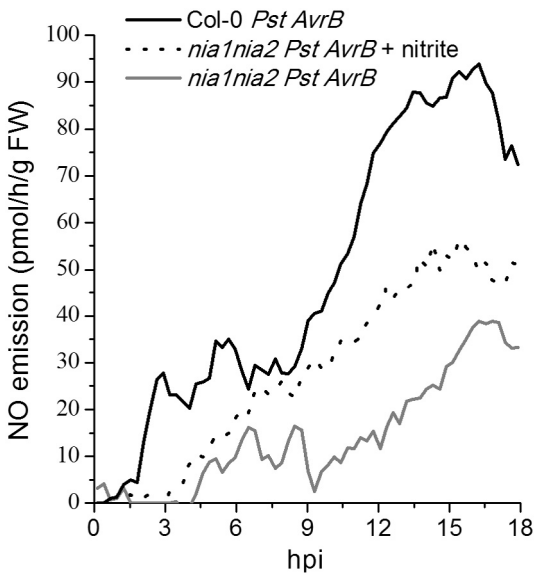

Fig. 2. Gas-phase NO emission pattern of NR-deficient *Arabidopsis thaliana* mutant plants infected with avirulent pathogens in presence or absence of exogenous nitrite. *Arabidopsis thaliana* Col-0 plants (black line) were infiltrated with *Pst AvrB* 10^8 CFU/mL and placed in the nutrient solution without any treatment. The *nia1 nia2* plants were infiltrated with *Pst AvrB* 10^8 CFU/mL and placed in the nutrient solution supplemented (dotted line) or not (grey line) with 100 μM nitrite. Experiments were carried out three times with similar results. FW, fresh weight.

4. NO sources: the origins debate

The experiments described above confirm that NO is involved in multiple physiological and pathophysiological processes in plants, and its emission can be detected directly. Even so, defining the sources of NO in plants, particularly during the HR, has been more difficult than expected based on experience in animal systems [2,81,82]. Two main routes have been identified thus far, namely the oxidative and reductive pathways.

The oxidative pathways involve NO synthase (NOS)-like enzyme activity as well as NO generation mediated by hydroxylamine or polyamine [83,84], with L-Arg acting as the substrate or as an intermediate, respectively. Accordingly, mammalian NOS inhibitors, based on analogues of L-Arg, can block NO production in different plant systems [3,70,85] particularly in response to pathogen infection [3]. However, the putative involvement of such NOS-like activity has been questioned in a recent work reporting the aspecific inhibition of the oligogalacturonide-induced nitrate reductase activity by the widely-used NOS inhibitor L-NAME [67]. Moreover, although a NOS-like activity has been reported in plants [87], a plant NOS has not yet been identified. Thus, the genetic basis of these biochemically-defined pathways has not been elucidated and their precise role in plant defence responses remains to be proven. In this context, after several failures, the recent discovery of a homolog of mammalian NOS in the unicellular green alga *Ostreococcus tauri* [88] will likely rekindle the discussion [89]. This enzyme despite the high similarity with animal NOSs shows some structure variations leading to differences in particular in term of regulation by Ca^{2+} and required co-factors. The authors propose that, according to the heterogeneity in the NOSoxy domain reported for the different NOSs so far identified, the search of new NOS isoforms that differ in such NOSoxy domain could be the key to unravel the presence of this protein in plants [90].

In contrast, the reductive pathway involves the conversion of nitrite into NO, mainly by the best-characterized enzyme nitrate reductase (NR) [71,91], but also by the mitochondrial electron transport chain [92–95], the plasma membrane-bound nitrite:NO reductase (NiNOR) [96] or the peroxisomal xanthine oxidoreductase (XOR) [86]. However, NiNOR has been shown to produce NO in roots but not in leaves [96], whereas NO production in mitochondria or based on XOR activity is restricted to anaerobic conditions or hypoxic stress [86,92]. Thus, among these different candidate enzymes, NR seems the only likely source of NO from nitrite in the context of plant defences. A role for NR during the HR has been suggested based on experiments using the *nia1 nia2* double mutant deficient for NR [97], which has a low NO content in response to bacterial infections [68,69,98,99]. Interestingly, supplementing the *nia1 nia2* mutant with exogenous nitrite restored NO production in response to avirulent *Psm* [68], corresponding to the reduced nitrite level measured in this mutant [69]. Here, by using chemiluminescence, we confirm that NO produced by the *nia1nia2* mutant in response to an infection with *Pst AvrB* is strongly reduced and delayed as compared with Col-0 plants, and that providing the mutant with nitrite partly restored NO emission induced by the avirulent pathogens (Fig. 2) but did not achieve full restoration as previously reported [68].

The missing NO production is unlikely to reflect the depletion of arginine, which would prevent NO synthesis by the oxidative pathway, because full NO production is not restored by providing the mutant with additional arginine [98]. This suggests that NR may also play a minor role as a NO-producing enzyme, and rather it appears clear that the main function of this enzyme is to synthetize nitrite as a substrate for NO production [100]. Together, these published reports [68,69] and our data presented herein confirm the importance of nitrite as a substrate for NO production during the HR, and demonstrate that nitrite can be converted into NO by NR-independent sources that have yet to be identified.

5. Conclusions

It is now clear that NO is produced and has a well-established function in plants undergoing the HR. This could be considered as the prelude for new investigations concentrating on the currently unknown steps in NO synthesis and signalling.

It is reasonable to speculate that the identification of NO sources will continue to intrigue the plant research community, and in this context the results obtained with the NR-deficient mutant suggest that a search for new enzymes that convert nitrite to NO would be fruitful. Moreover, once the function of a molecule is known, it is crucial to understand the mechanism. NO signalling in plant defence responses has been extensively reviewed, from the post-translational modifications induced by NO to the second messengers and partners required for NO signal transduction [6,7]. However, although the list of NO targets is continuously growing, the function of many of these proteins has not been characterized and some of the links from NO to NO-mediated responses are still missing. For example, we can assume that an NO 'transducer' exists playing the same role as calmodulins for Ca^{2+}, *i.e.*, a molecule that senses NO and transmits the information to downstream targets such as transcription factors that regulate defence gene expression. Furthermore, the genetic determinants of the NO-based trigger for hypersensitive cell death have yet to be identified. Finally, the role of the NO derivative peroxynitrite ($ONOO^-$) and $ONOO^-$-mediated tyrosine nitration, both of which occur during the HR, is still a matter of debate. After almost 20 years of research, the story of NO in plant defence responses has only just begun.

6. Materials and methods

6.1. Plant growth

A. thaliana ecotype Columbia-0 (Col-0) was grown in soil, whereas the NR null mutant *nia1 nia2* was grown in soil supplemented with 2.5 mM $(NH_4)_2SO_4$. All plants were placed in a growth chamber with 60% relative humidity, a 9-h photoperiod (light intensity 100 μmol m^{-2} s^{-1}) and a day/night temperature of 24/22 °C.

6.2. Bacterial culture

Virulent *P. syringae* pv *tomato* DC3000 (*Pst*) and avirulent *P. syringae* pv *tomato* DC3000 carrying the *AvrB* gene (*Pst AvrB*) cultures were grown overnight at 28 °C in King's B medium (2% w/v Proteose Peptone, 6.1 mM $MgSO_4$, 8.6 mM K_2HPO_4 and 1% v/v glycerol, pH 7.2) containing the appropriate antibiotics (50 μg/L rifampicin for *Pst*; 50 μg/L rifampicin and 50 μg/L kanamycin for *Pst AvrB*). The bacterial suspensions were infiltrated at $OD_{600} = 0.1$ in 10 mM $MgCl_2$ into the abaxial surface of *A. thaliana* leaves using a hypodermic syringe without a needle. As a control, leaves were infiltrated with 10 mM $MgCl_2$.

6.3. Gas phase NO measurement with chemiluminescence

Whole root-cut plants were infiltrated with pathogens at the indicated concentrations and placed in a Petri dish containing a nutrient solution (pH 5.7) in the presence or absence of nitrite (10 mM) as appropriate. Anhydrous $CaCl_2$ was added to the chamber to reduce humidity during the experiment. A constant flow of measuring gas (air or nitrogen) was pulled through the chamber atg a rate of 0.3 L min^{-1} and subsequently through the chemiluminescence detector (ECO Physics CLD 88et, Switzerland, detection limit 0.5 ppt) using a vacuum pump. The measuring gas (air or nitrogen) was made NO free by passing it through a deNOxer (ECO Physics). Constant light was provided to the plants at an intensity of 100 μmol m^{-2} s^{-1} and the air temperature in the cuvette was maintained at 20–22 °C.

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