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SHORT COMMUNICATION / *COMUNICAÇÃO*

Nitrate reductase-dependent nitric oxide synthesis in the defense response of *Arabidopsis thaliana* against *Pseudomonas syringae*

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ABSTRACT

Nitrate reductase (NR) was recently shown to play an important role during phytopathogenic interactions by providing substrates for the synthesis of nitric oxide (NO), a key signal for plant defense responses. In order to give additional support to this hypothesis, we compared NO-mediated defense responses of wild-type and NR double-deficient (*nia1 nia2*) *Arabidopsis thaliana* plants inoculated with the IBSBF-1115 (ibs) strain of *Pseudomonas syringae* pv. *maculicola* (Psm) and with genetically characterized avirulent (avr) or virulent (vir) strains of Psm. Inoculation of wild-type leaves with avr or ibs, but not vir, stimulated NO emission, as measured by the indicator 4,5-diaminofluorescein. NO emission induced by avr was higher than that induced by ibs. Wild-type plants displayed the hypersensitive response (HR) when infiltrated with the strains avr or ibs, although a stronger HR was induced by avr. The vir strain did not induce HR in wild-type plants, and leaves developed severe infection symptoms. *nia1 nia2* plants did not show significantly increased NO emission nor did they develop HR to any of the analyzed strains of Psm, but displayed clorotic lesions and higher bacterial growth in their leaves. Overall, these results highlight the importance of NR-dependent NO synthesis for plant defenses against pathogen attack.
Keywords: *Pseudomonas syringae* pv. *maculicola*, avirulent strain, hypersensitive response, *nia1 nia2* mutant.

RESUMO

Síntese de óxido nítrico dependente da nitrato redutase na resposta de defesa de *Arabidopsis thaliana* contra *Pseudomonas syringae*

A nitrato redutase (NR) possui importante papel na interação fitopatogênica por prover os substratos para a síntese do óxido nítrico (NO), um sinalizador essencial para a defesa vegetal. A fim de fornecer suporte adicional a essa hipótese, neste trabalho foram comparadas as respostas de defesa mediadas por NO de plantas de *Arabidopsis thaliana* selvagens e deficientes para a NR (*nia1 nia2*) quando inoculadas com a linhagem IBSBF-1115 (ibs) de *Pseudomonas syringae* pv. *maculicola* (Psm) e com as linhagens geneticamente caracterizadas como avirulenta (avr) ou virulenta (vir) de Psm. A inoculação de folhas selvagens com avr ou ibs, mas não vir, estimulou a emissão de NO, medida pelo indicador 4,5-diaminofluoresceína. A emissão de NO induzida por avr foi maior que aquela induzida por ibs. Plantas selvagens apresentaram resposta hipersensitiva (RH) quando infiltradas com avr ou ibs, mas uma RH mais forte foi induzida por avr. A linhagem vir não induziu RH nas plantas selvagens, e as folhas desenvolveram sintomas severos da infecção. Plantas *nia1 nia2* não tiveram um aumento significativo da emissão de NO nem desenvolveram RH após inoculação com as linhagens analisadas de Psm, apresentando intenso crescimento bacteriano e clorose foliar. Em suma, esses resultados evidenciam a importância da síntese de NO dependente da NR na defesa vegetal ao ataque de patógenos.
Palavras-chave: *Pseudomonas syringae* pv. *maculicola*, linhagem avirulenta, mutante *nia1 nia2*, resposta hipersensitiva.

Nitric oxide (NO) is a signaling molecule that plays an important role in plant defense against pathogens (reviewed by Hong et al., 2007). Together with reactive oxygen species, NO participates in inducing hypersensitive response (HR), which consists of localized cell death and develops in plant tissue to prevent pathogen proliferation from the site of infection (Delledonne et al., 2001). The HR and other defense mechanisms are established in an incompatible interaction in which a pathogen that expresses an avirulence gene is recognized by a plant that has the corresponding resistance (*R*) gene (Dangl & Jones, 2001). In the absence of the *R* gene, the plant is susceptible to infection, constituting a compatible pathogen-host interaction (Lam et al., 2001).

Despite the importance of NO in plant cells, the molecular mechanisms related to NO synthesis in these organisms are not yet completely understood. Although evidence suggests that plants can produce NO from L-arginine, no homologues of mammalian NO synthases have yet been identified in plants (del Rio et al., 2004).

The reduction of nitrite to NO by nitrate reductase (NR), which is an essential enzyme for nitrogen assimilation, is additionally thought to be an important source of NO in plants (Yamasaki & Sakihama, 2000). However, Modolo et al. (2005) have identified a mitochondrial activity that reduces nitrite to NO independently of NR as the major producer of NO in *Arabidopsis thaliana* in response to infection by *Pseudomonas syringae* pv. *maculicola* (Psm). *A. thaliana* mutant plants lacking the two NR structural genes (*nia1 nia2*) have been shown to possess a reduced capacity for NO synthesis in response to inoculation with the IBSBF-1115 (ibs) strain of Psm, which causes an incompatible interaction in the wild-type plant but has not been genetically characterized with respect to virulence (Modolo et al., 2005). The deficient NO production in *nia1 nia2* plants was associated with lower levels of nitrite and L-arginine, when compared to that of wild-type plants (Modolo et al., 2006). These observations suggested a role for NR in producing the substrates for NO synthesis during phytopathogenic interactions (Salgado et al., 2006).

In order to give further support to the proposition that NR-dependent NO synthesis is important for plant resistance to pathogens, in the present work the NO-mediated defense responses were compared for wild-type (WT) and *nia1 nia2* *A. thaliana* plants to infection by different strains of Psm. To this end, we analyzed NO production, HR induction, bacterial growth and disease development in both genotypes of *A. thaliana* plants induced by the ibs strain of Psm, which was originally isolated in the Instituto Biológico de Campinas, and compared these responses to those induced by strain ES4326, avirulent (avr), which carries the gene *avrRpm1*, and isogenic strain ES4326, virulent (vir), which lacks the gene *avrRpm1*.

Bacteria were grown at 28°C in King's B medium containing antibiotics appropriate to each strain (avr: 50 $\mu g.mL^{-1}$ rifampicin, 50 $\mu g.mL^{-1}$ streptomycin and 50 $\mu g.mL^{-1}$ kanamycin; vir, 50 $\mu g.mL^{-1}$ rifampicin; ibs, no antibiotics). After 48 h, bacteria were diluted to the desired concentration and inoculated into the abaxial surface of *A. thaliana* leaves using a syringe without a needle. Thirty- to forty-day-old WT and *nia1 nia2* plants of *Arabidopsis thaliana* L. ecotype Columbia-0, cultivated as described by Oliveira et al. (2009), were used for the experiments.

NO emission by *A. thaliana* leaves was analyzed using the fluorescent indicator 4,5-diaminofluorescein (DAF-2), following the method of Seligman et al. (2008). Briefly, intact leaves (40 mg) were incubated under dark conditions in 0.1 M phosphate buffer (pH 7.2) containing 50 μM DAF-2. After 1 h, the leaves were discarded and the resultant solution was diluted fivefold in phosphate buffer. Fluorescence emission spectra between 500 and 550 nm upon excitation at 495 nm were recorded in a Hitachi F-4500 spectrofluorometer (Hitachi Ltd., Tokyo, Japan). Standard curves were obtained by measurement of known concentrations of a NO saturated solution.

For cell death visualization and HR analysis, *A. thaliana* leaves were immersed for 1 min in a boiling solution of lactophenol containing 0.25 $mg.mL^{-1}$ Trypan blue. The tissues were incubated for 4 min in ethanol/lactophenol (2:1), followed by storage in 50% ethanol. Treated leaves were mounted on microscope slides and examined using a Nikon Alphaphot-2 YS2-H microscope equipped with a Canon Power Shot A95 camera. Macroscopic symptoms were analyzed with the same camera coupled to a Labomed stereomicroscope. The images presented in the figures are representative of at least three independent experiments, each done in triplicate.

Bacterial growth in *A. thaliana* leaves was estimated by homogenizing leaf discs from infiltrated areas, and then plating the homogenate on King's B medium. Colonies were counted 2-3 days after plating. Statistical analyses were done using Student's *t*-test with $p < 0.05$ indicating significance.

As shown in Figure 1, control non-inoculated *nia1 nia2* leaves displayed lower basal NO emission than WT leaves, which confirms the deficient NO production by this mutant (Modolo et al., 2005, 2006; Oliveira et al., 2009). When plants inoculated with the avr strain of Psm were analyzed, there was a significant increase in NO emission by WT leaves, from 36.4 ± 9.1 to 102.3 ± 7.2 $pmol.min^{-1}.g\ FW^{-1}$. The ibs strain also stimulated NO emission by WT leaves, although to a lesser extent (71.1 ± 14.8 $pmol.min^{-1}.g\ FW^{-1}$). In contrast, the avr and ibs strains did not induce significant increases in NO emission when inoculated into *nia1 nia2* leaves. Additionally, there were no significant increases in NO emission by leaves of either genotype in response to inoculation with the vir strain, suggesting that this strain, in contrast to avr and ibs, was not recognized by the plant. The observation that vir inoculation did not induce an increase in NO emission even in WT leaves also shows that NO production is not a consequence of wounding stress caused by the infiltration procedure. Indeed, when plants were mock-infiltrated, no increase in NO emission was observed (not shown).

Since a relationship between NO levels and the induction of plant defense mechanisms has been proposed (Zeier et al., 2004), we then compared the ability of Psm strains to induce the HR in WT and *nia1 nia2* leaves of *A. thaliana* (Figure 2A). As expected for an incompatible interaction, WT leaves inoculated with the avr strain presented many stained patches corresponding to dead cells, indicating a clear induction of the HR. Inoculating WT plants with the ibs strain of Psm led to a weaker HR than that induced by the avr strain, as indicated by a lower density of dead cells. In *nia1 nia2* leaves infiltrated with the avr or ibs strains, dead cells were almost absent, indicating that this mutant did not develop the HR. The vir strain did not induce the HR even in WT leaves, as expected for a compatible interaction. Similar results were observed in control leaves inoculated with water (not shown).

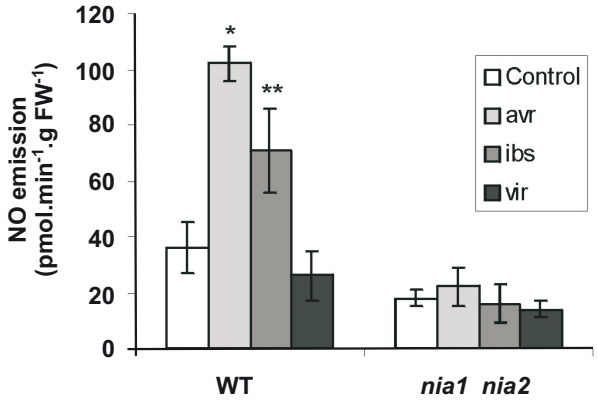

FIGURE 1 - NO emission by *A. thaliana* leaves in response to Psm inoculation. Leaves from WT and *nia1 nia2* plants were inoculated with 5 x 10^7 cfu.mL^{-1} of the avr, ibs and vir strains of Psm. Six hours after inoculation, 40 mg of leaf tissue was incubated for 1 h with 50 µM DAF-2. Basal NO production of non-inoculated leaves (Control) was also analyzed. Bars represent mean ± standard deviation (n = 3). *p < 0.01 and **p < 0.05 compared to controls.

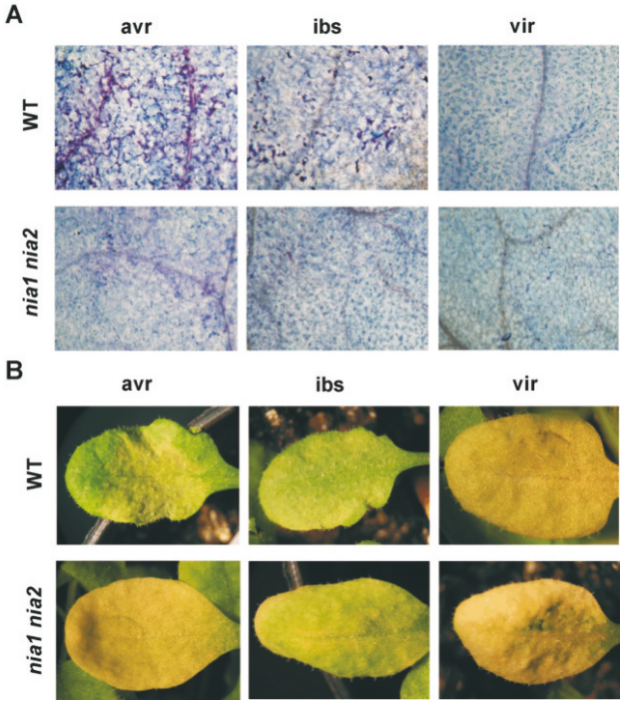

FIGURE 2 - HR induction and macroscopic symptoms in *A. thaliana* leaves inoculated with Psm. WT and *nia1 nia2* plants were infiltrated with 5 x 10^7 cfu.mL^{-1} of the avr, ibs or vir strains of Psm. **A.** Cell death was analyzed by treating leaves with lactophenol and Trypan blue 24 h after inoculation. Slides were observed at 10 X magnification; **B.** Symptoms were recorded 48 h after inoculation.

Macroscopic symptoms were also analyzed in order to follow disease progression in *A. thaliana* plants inoculated with Psm strains. As shown in Figure 2B, 48 h after inoculation with the avr strain, WT showed gray lesions in the inoculated region, which can be considered the macroscopic expression of the HR (Delledonne et al., 1998). Inoculation of the ibs strain induced macroscopic symptoms similar to those induced by the avr strain. However, the HR lesion was lighter, consistent with the microscopic cell death results presented above. In *nia1 nia2* mutant plants, intense leaf chlorosis developed in response to inoculation with avr. Leaf chlorosis was also observed in the *nia1 nia2* mutant inoculated with ibs, although milder symptoms developed than those induced by the avr strain. WT and *nia1 nia2* leaves were completely chlorotic after inoculation with the vir strain, indicating severe disease progression in both plant genotypes.

Leaf chlorosis is often associated with disease progression and bacterial growth in plants (Delledonne et al., 1998; Zeier et al., 2004). We thus determined the number of colony-forming bacteria of the three Psm strains in leaves of both genotypes (Table 1). When this analysis was carried out 6 h after the inoculation, there was no significant difference in colony number among genotypes, indicating that inocula initially contained almost the same amounts of bacteria. However, 48 h after inoculation, the avr strain showed more pronounced growth in *nia1 nia2* leaves (3,214 ± 1,801 cfu.cm^{-2}) than in WT leaves (141 ± 16 cfu.cm^{-2}), in accordance with the more severe symptoms displayed by the *nia1 nia2* mutant (Figure 2B). As expected for a compatible interaction, the vir strain displayed intense growth in leaves, the extent of which did not differ significantly between the WT (5,002 ± 2,878 cfu.cm^{-2}) and *nia1 nia2* (6,772 ± 2,509 cfu.cm^{-2}). For the ibs strain, although a high bacterial growth was observed in WT leaves (5,096 ± 3,690 cfu.cm^{-2}), it was significantly lower than that detected in *nia1 nia2* leaves (23,158 ± 3,983 cfu.cm^{-2}). The higher growth of ibs, when compared to that of avr, even in WT leaves, may result from the growth of epiphytic bacteria, since the determination of ibs growth has been carried out without including antibiotics in the cultivation medium.

Overall, the analysis of NO-mediated defense responses in *A. thaliana* plants showed that the ibs strain of Psm has an avirulent phenotype since inoculation of both the ibs strain and the avr strain induced NO emission (Figure 1) and HR (Figure 2A) in WT leaves. Moreover, neither ibs nor avr inoculation led to severe disease symptoms in WT leaves, in contrast to the chlorosis observed after vir inoculation (Figure 2B). These results clearly indicate that the ibs strain elicits plant defense responses similar to those induced by avr. However, the ibs strain induced a weaker defense response than avr in WT plants.

The comparative analysis of defense responses of *A. thaliana* plants to strains of Psm with different degrees of virulence showed a positive correlation between NO production and plant defense. The milder microscopic and macroscopic HR induced by the ibs strain (Figure 2) was positively

TABLE 1 - Growth of the avr, ibs and vir strains of Psm (expressed in cfu.cm^{-2}) in wild-type (WT) and *nia1 nia2* leaves 6 and 48 h after inoculation with 1 x 10^6 cfu.mL^{-1} of bacteria

Genotype	avr		ibs		vir	
	6h	48h	6h	48h	6h	48h
WT	9 ± 6	141 ± 16	8 ± 2	5,096 ± 3,690	12 ± 6	5,002 ± 2,878
nia1 nia2	20 ± 12	3,214 ± 1,801*	15 ± 9	23,158 ± 3,983**	18 ± 10	6,772 ± 2,509

Data represent mean ± SD (n = 4); * $p < 0.01$ and ** $p < 0.05$ compared to the WT.

correlated with the lower amount of NO produced in WT leaves (Figure 1), when compared to the responses induced by the avr strain of Psm. Moreover, vir inoculation failed to evoke NO production and led to severe disease symptoms in WT leaves (Figure 2B).

Additionally, the present results show the importance of NR for plant defense responses. In contrast to WT leaves, which developed HR, *nia1 nia2* mutant plants were susceptible to the avr and ibs strains of Psm. When inoculated with these strains, *nia1 nia2* plants did not develop HR (Figure 2A), showed chlorosis indicative of disease progression (Figure 2B) and presented more intense bacterial growth in their leaves (Table 1). Since NO has been proposed as an important signaling molecule for plant defenses against pathogens (reviewed by Hong et al., 2007), the impaired response of *nia1 nia2* to Psm might result from deficient NO production. Accordingly, previous work has shown that when NO production is recovered by infiltration of exogenous nitrite, *nia1 nia2* leaves are able to develop HR after inoculation with the ibs strain of Psm (Modolo et al., 2006).

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