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# PHYSIOLOGICAL AND IMMUNOBLOT ANALYSES OF A NITRIC OXIDE SYNTHASE (NOS)-LIKE PROTEIN OF PEA *(PISUM SATIVUM L.)*

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**Abstrak:** Nitrik oksida (NO) berfungsi sebagai molekul signal dalam pelbagai proses biologi tumbuhan, termasuk rintangan terhadap penyakit. Pengeluaran NO adalah berkaitan dengan nitrik oksida sintase (NOS). Penyemburan bahan kimia yang merangsang rintangan sistemik dalam tumbuhan tetapi tidak merangsangkan aktiviti NOS pada kacang manis *(pea)* mencadangkan bahawa NO berfungsi pada sebelah atas molekul asid salisilik (SA) dalam rangkaian signal respons pertahanan tumbuhan. Aktiviti NOS telah dirangsangkan dalam kedua-dua interaksi tidak serasi dan serasi kacang manis masing-masing dengan *Ralstonia solanacearum* dan *Pseudomonas syringae* pv *pisi*, antara 3 jam dan 6 jam pos-inokulasi. Ini menunjukkan NOS terlibat dalam kedua-dua respons rintangan dan perkembangan penyakit dalam kacang manis. Antibodi terhadap NOS mamalia didapati tidak cukup spesifik untuk mengesan suatu protein seperti NOS kacang manis dan ini mencadangkan bahawa protein seperti NOS kacang manis mungkin berbeza struktur daripada NOS mamalia, serta pengesanan protein seperti NOS pada tumbuhan menggunakan kaedah antibodi patut dilakukan dengan cermat dan disahkan dengan aktiviti pencerakinan NOS.

**Abstract:** Nitric oxide (NO) functions as a signal molecule in different biological processes in plants, including disease resistance. Its production is related to nitric oxide synthase (NOS). The application of chemicals that induce systemic resistance in plants did not induce NOS activity in pea, suggesting that NO functions upstream of salicylic acid (SA) in the signaling pathway of defense responses in plants. NOS activity was induced in both the incompatible and compatible interactions of pea with *Ralstonia solanacearum* and *Pseudomonas syringae* pv *pisi*, respectively, between 3 h to 6 h post-infiltration, indicating that NOS was involved in both resistance and disease development responses in pea. Antibodies raised against mammalian NOS did not have specificity in detecting a NOS-like protein in pea, suggesting that the pea NOS-like protein could be structurally different from mammalian NOS, and immunodetection of a plant NOS-like protein must be conducted with caution and verified with functional assays.

**Keywords:** Pea, Nitric Oxide Synthase (NOS), *Ralstonia solanacearum*, *Pseudomonas syringae* pv *pisi*

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## INTRODUCTION

Nitric oxide (NO) is involved in different biological processes in plants, including biotic interactions resulting in defense responses such as hypersensitive response (HR) and systemic-acquired resistance (SAR) (Delledonne *et al.* 1998). Plant defense mechanisms are associated with a rapid burst of oxidative reactions, change in membrane ion fluxes, extracellular alkalinization, activation of signaling cascades, cell wall fortification, and phytoalexin and pathogenesis-related (PR) proteins production (Hammond-Kosack & Jones 1996). The accumulation of NO and salicylic acid (SA) are essential in the induction of HR and SAR (Delledonne *et al.* 2001; Gaffney *et al.* 1993). Induced resistance such as SAR, can be triggered by certain chemicals, non-pathogens, avirulent forms of pathogens, incompatible races of pathogens, or by virulent pathogens under circumstances where infection is stalled due to environmental conditions (van Loon *et al.* 1998).

In animal systems, most of the NO produced is due to the enzyme nitric oxide synthase (NOS) (Moncada *et al.* 1991; Ignarro 2000; del Rio *et al.* 2004) which occurs in three isoforms (Nathan & Xie 1994). In plants, accumulating evidence had demonstrated the presence of a NOS-like protein including NOS activity via the formation of L-citrulline from L-arginine method used in mammalian system (Huang & Knopp 1998; Durner *et al.* 1998; Barroso *et al.* 1999); and positive immunoreactivity to antibodies raised against mammalian NOS in tobacco (Huang & Knopp 1998), maize (Ribeiro *et al.* 1999) and pea (Barroso *et al.* 1999). Although NOS activity has been documented in plants, the process of NO synthesis is not well understood. Thus, isolation of a NOS protein and/or cloning of the corresponding gene will greatly facilitate the understanding of NO synthesis and its role in plant defense against pathogen infection.

Pea, which previously demonstrated the highest NOS activity as compared to other plant species such as soy bean, tobacco, tomato, pepper and corn (Huang, Pers. Comm.), was used in this system. *Pseudomonas syringae* pv *pisi*, which causes bacterial blight disease on pea was selected for the study of compatible interactions. *Ralstonia solanacearum*, which is a close taxonomic species to *P. syringae* and previously demonstrated to cause HR symptoms in infiltrated pea leaves (Huang, Pers. Comm.), was used in the study of incompatible interactions. Due to limited information on the involvement of NOS (correspondingly NO) in plant interactions, this study was conducted to analyze the effects of abiotic and biotic interactions in pea on NOS activity. Detection of a NOS-like protein in pea using antibodies raised against mammalian NOS was also attempted.

## MATERIALS AND METHODS

### Materials

Pea (*Pisum sativum* L., cultivar Alaska) seeds were obtained from Floridata Marketplace (Tallahassee, USA). Actiguard$^{TM}$ was obtained from Syngenta Corporation (Greensboro, USA). Copper chloride ($CuCl_2$), SA, Triton-X100,

Tween 20, Tris-HCl, ethylene diamine tetraacetic acid (EDTA), ethylene bis(oxyethylenenitrilo) tetraacetic acid (EGTA), leupeptin, phenylmethylsulfonyl fluoride (PMSF), polyvinyl polypyrrolidone (PVPP), glycerol, bromophenol blue, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin ($BH_4$), β- nicotinamide adenine dinucleotide phosphate (NADPH), calmodulin (CaM), calcium dichloride ($CaCl_2$), nonradio-labeled arginine, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St. Louis, USA). Protein assay reagent and Amplified Alkaline Phosphatase Goat Anti-Rabbit Immun-Blot Assay Kit were obtained from Bio-Rad Laboratories (Hercules, USA). Bovine serum albumin (BSA) was obtained from Merck & Co. (Whitehouse Station, USA). Dowex AG50W X-8 ($Na^+$ form) and liquid scintillation cocktail were obtained from Fluka (Seelze, Germany). Radio-labeled L-[U-$^{14}$C]arginine and nitrocellulose membrane (Hybond$^{TM}$ ECL$^{TM}$) were obtained from Amersham Biosciences (Piscataway, USA). *Ralstonia solanacearum* and *Pseudomonas syringae* pv *pisi* were kind gifts from Dr Peter Lindgren, Department of Plant Pathology, North Carolina State University. Brain NOS (bNOS), endothelial NOS (eNOS) and inducible macrophage NOS (iNOS) were obtained from Calbiochem (San Deigo, USA).

**Protein Extraction**

Leaf tissues (0.5–1.0 g) in each experiment were ground in liquid nitrogen and homogenized in two volumes (1–2 mL) of extraction buffer which contained 50 mM Tris-HCl (pH 8.5), 1.0 mM EDTA, 10.0 mM EGTA, 1.0 μM leupeptin, 1.0 mM PMSF, and 1% PVPP. The homogenate was filtered through one layer of Miracloth and centrifuged at 4°C for 30 min at 20,000 × *g*. Protein concentration of supernatant from the crude extracts was determined using ready-to-use protein assay reagent and BSA as standard.

**Nitric Oxide Synthase (NOS) Activity Assay**

NOS activity in all preparations was determined by the conversion of L-[U-$^{14}$C]arginine to L-[U-$^{14}$C]citrulline using the method of Bredt and Snyder (1990) with modifications. Each reaction mixture (165 μL) contained 85 μL protein extract, 1 μL FAD (2 μM), 1 μL FMN (2 μM), 1 μL $BH_4$ (5 μM), 1 μL β-NADPH (1 mM), 1 μL CaM (40 units), 10 μL $CaCl_2$ (2 mM), 10 μL nonradio-labeled arginine (0.2 mM), 10 μL Hepes (30 mM, pH 7.4), 10 μl DTT (3 mM), 10 μL EDTA (0.6 mM) and 25 μL L-[U-$^{14}$C]arginine (1.25 μCi/mL), and was incubated at room temperature (23–25°C) for 60 minutes. Each reaction was stopped by adding 2 mL stop buffer (20 mM Hepes, 2 mM EDTA, pH 5.5) and the mixture was passed through a 1-mL Dowex AG50W X-8 ($Na^+$ form) ion exchange column. Positively charged L-arginine was exchanged with $Na^+$ and bound to the Dowex column, thus, allowing non-charged radio-labeled L-citrulline to pass through the column. The column was washed with 2 mL distilled water. A volume of 416.5 μL (10% of the total mixture) of the flow-through was added to 5 mL of liquid scintillation cocktail and radioactivity was counted using a liquid scintillation counter (LS 7500 Beckman). Specific activity was expressed as picomole of L-[U-$^{14}$C]citrulline $min^{-1}$ $mg^{-1}$ protein after subtracting the background value i.e., value of similarly

prepared reaction mixture terminated immediately after adding protein sample. Detection of each sample was replicated three times.

**Preparation of Materials for Abiotic Interactions**

Pea seedlings were grown in a greenhouse at a temperature cycle of 30/26 ± 2°C and 12 h light cycle. Leaves of 12 ± 2 day-old plants were sprayed with one of four compounds used to induce systemic resistance in plants: Actiguard™ 25 mg a.i./L, $CuCl_2$ (10 mM), SA (2.5 mM) and Triton-X100 (0.1%). Tween 20 (0.05%) was used as a surfactant in the preparation of the four chemicals and applied alone as a control. Treated plants were kept in a greenhouse according to completely randomized design (CRD), and leaves were harvested at 0 day, 3 days, 5 days, and 7 days after spray. Harvested leaves were immediately frozen in liquid nitrogen and stored at –80°C before use. Each treatment consisted of three replicates. Protein extraction and NOS activity assay for each treatment and time point were conducted as described above.

**Preparation of Materials for Biotic Interactions**

Pea seedlings were grown in a growth chamber at temperature cycle of 30/26°C, and 12 h light cycle. Leaves of 8 ± 1 day-old plants were vacuum-infiltrated with incompatible bacteria, *R. solanacearum* ($2.0 \times 10^9$ cfu/mL); compatible bacteria, *P. syringae* pv *pisi* ($1.0 \times 10^6$ cfu/mL); and distilled water as a control. Treated plants were kept according to design CRD; and infiltrated leaves were harvested at intervals of 0 h, 3 h, 6 h, 9 h, 12 h and 24 h post-infiltration, immediately frozen in liquid nitrogen and stored at –80°C before use. Protein extraction and NOS activity assay for each treatment and time point were conducted as described above.

**Immunoblot Analysis**

Total proteins extracted as described above from healthy leaf tissues, and incompatibly- and compatibly-challenged leaf tissues were separated by 10% (w/v) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-Rad Mini-PROTEAN II slab cell according to the manufacturer's instructions. A total of 100 μg protein of each sample was used. Polypeptides were transferred to a nitrocellulose membrane using Bio-Rad Trans-Blot cell following the instructions of the manufacturer. The detection of NOS-like protein in pea was conducted using the Amplified Alkaline Phosphatase Goat Anti-Rabbit Immun-Blot Assay Kit. Briefly, membranes were incubated in blocking solution (see manufacturer's protocol) for 1 h at room temperature, washed for 5–10 min before incubated with primary antibodies, i.e., rabbit polyclonal antibodies against three isoforms of mammalian NOS: bNOS, eNOS and iNOS of human, mouse and rat at 1:1000, 1:1000 and 1:2000 dilutions, respectively, according to the manufacturer's recommendations for 2 h. Membranes were washed again before incubated with secondary antibody, i.e., biotinylated goat anti-rabbit antibody solution for 2 h. After the final wash, the target antigens (bNOS, eNOS and iNOS) were visualized using colorimetric method utilizing 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrates according to the manufacturer's protocol. All incubation and washing steps were conducted with

agitation at room temperature. A similar immunoblot was conducted using non-denaturing PAGE (5%) and all steps were performed at 4–8°C. One hundred μg of each sample was prepared in 1 M Tris-HCl (pH 6.8), containing 50% glycerol (v/v) and 0.05% (w/v) bromophenol blue. Non-denaturing gels were run at 100 V for 90 min prior to transfer to nitrocellulose for immunoassay. Both the immunoblot analyses using SDS and non-denaturing PAGE were repeated three to four times.

**Statistical Analysis**

All statistical data were analyzed using the SAS program version 9.1 (SAS Institute, Cary, NC) and General Linear Models (GLM) procedure. Analysis of variance (ANOVA) was determined using the Least Squared Means and Waller-Duncan K-ratio for the t-Test procedures at P = 0.05 level.

## RESULTS

In the interactions of pea and abiotic agents, Figure 1 shows the mean NOS activity detected in the treated leaves as compared to the controls. Statistical analysis showed that overall, there was no significant difference in NOS activity among the treatments. NOS activity in leaves of control, SA, Triton-X100, Actiguard$^{TM}$ and $CuCl_2$ were 6.3 pmol, 6.7 pmol, 7.0 pmol, 7.4 pmol and 7.9 pmol L-[U-$^{14}$C]citrulline min$^{-1}$ mg$^{-1}$ protein, respectively, at any time point. However, there was a significant difference in time. Regardless of treatments, a general increase in NOS activity in all treatments occurred at day 3 (8.2 pmol L-[U-$^{14}$C]citrulline min$^{-1}$ mg$^{-1}$ protein) and day 5 (8.1 pmol L-[U-$^{14}$C]citrulline min$^{-1}$ mg$^{-1}$ protein) after treatment, and decreased by day 7 (5.7 pmol L-[U-$^{14}$C]citrulline min$^{-1}$ mg$^{-1}$ protein;) as compared to day 0 (6.2 pmol L-[U-$^{14}$C]citrulline min$^{-1}$ mg$^{-1}$ protein). NOS activity at 0 day, 3 days and 5 days after treatment showed no significant difference from one another, but NOS activity at 3 days and 5 days after treatment were significantly higher than that at 7 days after treatment. There was no significant difference in the interactions between time and treatment.

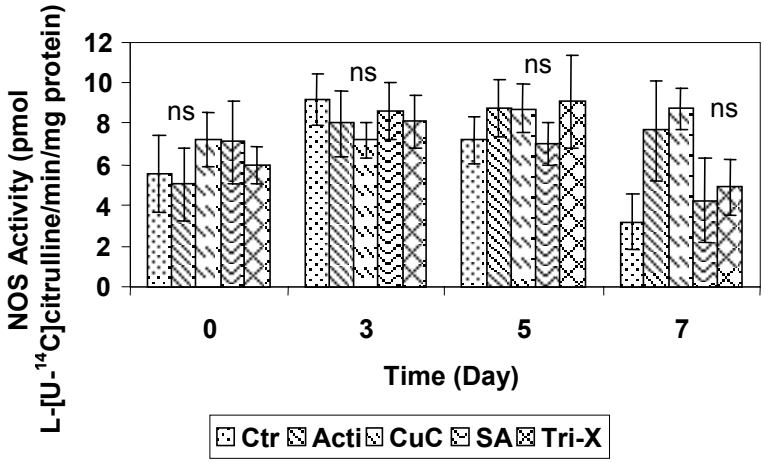


**Figure 1:** Effects of abiotic agents on NOS activity in pea leaf tissues. Ctr, control; Acti, Actiguard™; CuC, copper chloride; SA, salicylic acid; Tri-X, Triton-X100; and ns, no significant difference at $P < 0.05$ between treatments.

In the interactions of pea and pathogens, Figure 2 shows the mean NOS activity detected in leaves of incompatible and compatible interactions as compared to the control. Overall, there was a highly significant ($P < 0.005$) difference in NOS activity among the treatments. NOS activity in the water-infiltrated leaf control was between 10.6 pmol to 14.2 pmol L-[U-$^{14}$C]citrulline min$^{-1}$ mg$^{-1}$ protein throughout all time points monitored within the 24 h period (Fig. 2). In the pea incompatible interaction with *R. solanacearum*, NOS activity peaked at 6 h and 24 h post-infiltration (18.4 pmol and 20.3 pmol L-[U-$^{14}$C]citrulline min$^{-1}$ mg$^{-1}$ protein, respectively, Fig. 2), and was significantly greater ($P < 0.05$) than that in the control. In the pea compatible interaction with *P. syringae*, NOS activity was significantly greater ($P < 0.05$) than that in tissues of the control and incompatible interactions, and peaked at 3 h and 24 h post-infiltration (24.5 pmol and 24.6 pmol L-[U-$^{14}$C]citrulline min$^{-1}$ mg$^{-1}$ protein, respectively, Fig. 2). NOS activity was consistently highest in leaf tissues during compatible interaction at all time points except at 6 h post-infiltration, as compared to that of the control and incompatible interactions. There was also a significant ($P < 0.05$) difference in time. Regardless of treatments, NOS activity was highest at 24 h post-infiltration and showed no significant ($P < 0.05$) difference from that at 3 h post-infiltration, but showed significant ($P < 0.05$) difference from that at 0, 6, 9 and 12 h post-infiltration. However, there was no significant ($P < 0.05$) difference in the interactions of time and treatment. A HR was observed (data not shown here) between 9 to 12 h post-infiltration in leaves infiltrated with *R. solanacearum*, and necrotic disease symptoms were observed (data not shown here) between 24 to 36 h post-infiltration in leaves infiltrated with *P. syringae,* demonstrating the occurrence of resistance response and disease development, respectively, in the host plant.

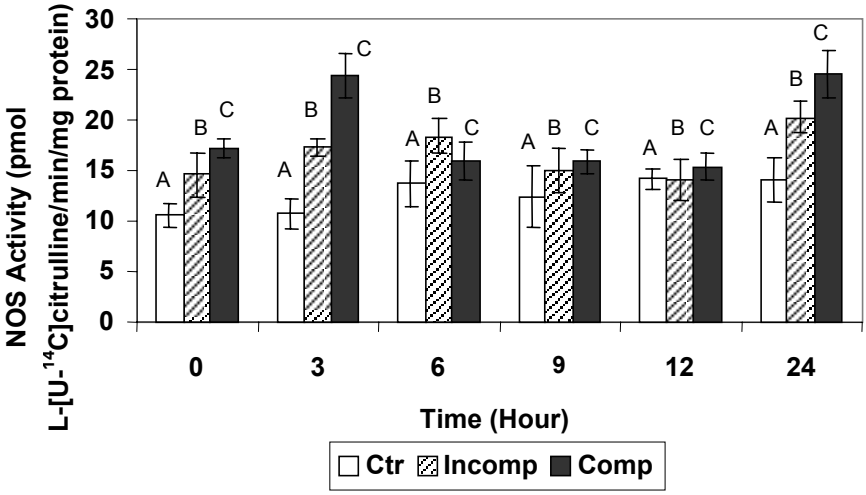


**Figure 2**: NOS activity during incompatible and compatible pea-bacterial interactions. Ctr, control; incomp, incompatible interaction; Comp, Compatible interaction. Means with the same letter shows no significant difference at $P < 0.05$.

Polyclonal antibodies raised against bNOS, eNOS and iNOS of either human, mouse or rat bound non-specifically to more than ten pea proteins separated by SDS-PAGE (data not shown here). The experiment was repeated three to four times and showed identical protein band profiles. However, one differential protein band appeared only in tissues challenged with incompatible bacteria (*R. solanacearum*) using antibody raised against iNOS (data not shown here). The protein had a molecular weight of approximately 30 kDa. Immunoblot analysis of proteins separated by non-denaturing PAGE using the same antibodies as used in SDS-PAGE analysis revealed non-specific binding of low molecular weight proteins (data not shown here), instead of the expected high molecular weight proteins that had similar size to mammalian NOS dimers (260–320 kDa).

## DISCUSSION

Copper chloride, Actiguard®, Triton-X100 and SA had been used to induce resistance in various species of plants in a systemic manner. $CuCl_2$ induced phytoalexin production in rice via the jasmonic acid (JA) metabolic pathway (Rakwal *et al.* 1996). Actiguard® contains the active ingredient acibenzolar-s-methyl that functions through the same metabolic pathway as SA (Okuno *et al.* 1991). Triton-X100 and Tween 20 are surfactants used as wetting agents. The JA and SA pathways are two different signaling pathways activated after the induction of defense responses to insects and microbes, respectively (van Loon *et al.* 1998). NO also functions as a signal in plant disease resistance

(Delledonne *et al.* 1998). The applications of $CuCl_2$, Actiguard®, Triton-X100 and SA to pea leaves did not increase NOS activity significantly as compared to the control, suggesting that NO functions upstream of SA in the signaling pathway of plant defense responses. The result supports previous similar observations (Wendehenne *et al.* 2001). The differential pattern of NOS activity between time points in all treatments suggests that NOS activity may be affected by the age of pea plants as NO was demonstrated to play a vital role in plant growth, fertility, stomatal movements and hormone signaling in *Arabidopsis* (Guo *et al.* 2003).

Information is limited to the involvement of NO in disease development as compared to its involvement in disease resistance. NO functions as a signal in the induction of hypersensitive cell death, expression of early and late defense genes such as phenylalanine ammonia lyase (PAL) and pathogenesis-related (PR-1) protein, respectively; and phytoalexin accumulation (Delledonne *et al.* 1998; Durner *et al.* 1998; Noritake *et al.* 1996). HR is activated after the interaction of NO with reactive oxygen species (ROS), specifically with $H_2O_2$ (Delledonne *et al.* 2001). The temporal pattern of NOS activity in pea, i.e., the occurrence of two peaks representing two NO bursts observed over time in both the incompatible and compatible pea-bacteria interactions suggests an inducible form of NOS, in contrary to a constitutive form in *Arabidopsis* (Guo *et al.* 2003). The two peaks of NOS activity in both the incompatible and compatible pea-bacteria interactions occurred at similar time points suggesting that NOS was related to not only hypersensitive cell death in resistance response, but also necrotic cell death in disease development of the host. The occurrence of two NO bursts and the production of NO during a compatible interaction were also demonstrated by Conrath *et al.* (2004). The results indicated that NO generation may be a general response to biotic stress in plants, a phenomenon similar to that of ROS (Bolwell 1999).

The monomer molecular mass of mammalian NOS is in the range of 130 kDa to 160 kDa (Wendehenne *et al.* 2001). Huang and Knopp (1998) reported the detection of a single immunoreactive band (~55 kDa) in both extracts of the control and leaves undergoing HR in tobacco after incubation with antibody raised against mammalian NOS. The intensity of the immunoreactive band in HR tissues was much higher than that in control tissues. Ribeiro *et al.* (1999) reported the detection of an approximately 166 kDa protein band after incubation with two different isoforms of mammalian anti-NOS antibodies in soluble fractions from young maize leaves and root tips. However, two bands of lower molecular weight were also detected in which they claimed to be degradation products of the 166 kDa protein.

Barroso *et al.* (1999) also reported the detection of an immunoreactive polypeptide of about 130 kDa in pea peroxisomal fractions using antibody raised against mammalian iNOS. The result is in contrast to that reported in this paper where multiple immunoreactive protein bands were detected using three different isoforms of mammalian anti-NOS antibodies. No protein band of approximately 130 kDa in the crude protein extracts bound any antibodies raised against mammalian NOS used; but in contrast, a differential protein of ~30 kDa bound the mammalian anti-iNOS antibody in SDS-PAGE separations using protein extracts of HR tissues. Further analysis need to be carried out to characterize

this protein and determine its role in plant defense. The difference in the two reports could be due to the source of extracted protein and antibodies used. In the work of Barroso *et al.* (1999), using samples containing only peroxisomal fractions for NOS detection would resulted in a much greater degree of specificity for the binding of iNOS antibody as compared to the crude extracts that we used in this work. However, they did not verify that the 130 kDa immunoreactive protein possessed NOS activity. Although both works used polyclonal antibodies for the immuno-detection, they were from different sources, and thus, the degree of specificity of the antibodies was not comparable.

The detection of multiple immunoreactive bands in this report conferred two implications. First, the pea NOS-like protein may have different structure than its animal counterparts; and thus, the size of the NOS-like protein and the ability to bind mammalian anti-NOS antibodies are unpredictable. Similar conclusion was made by Barroso *et al.* (1999) that reported none of the NOS activities detected so far in plants (Cueto *et al.* 1996; Delledonne *et al.* 1998; Ribeiro *et al.* 1999) have been found identical to the NOS isoforms present in mammals. To date, the only NOS protein of plant identified was isolated from *Arabidopsis* (Guo *et al.* 2003), which has sequence similarity to the protein implicated in NO synthesis in the snail *Helix pomatia* (Huang *et al.* 1997) and was related to NO production in hormonal responses in plants. Second, using antibodies raised against mammalian NOS to detect a plant NOS-like protein must be interpreted cautiously and verified by functional assays of NOS activity in immunoreactive protein bands to counter false immunoreactivity. Butt *et al.* (2003) used proteomic approach to demonstrate the mammalian NOS antibodies that recognize many NOS-unrelated plant proteins; and of the opinion, it is inappropriate to infer the presence of plant NOS using immunological technique.

In summary, the results in this report suggested that NO functions upstream of SA in the signaling pathway of plant defense responses. NO production may be a general response to biotic stress in plants that leads to both resistance and disease development responses of the host plant. Antibodies raised against mammalian NOS did not have specificity in detecting a NOS-like protein in pea, suggesting that the pea NOS-like protein could be structurally different from mammalian NOS, and immunodetection of a plant NOS-like protein must be conducted with caution and verified with functional assays.

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