

## ABSTRACT

WONG, MUI-YUN. Physiological and Molecular Analysis of Nitric Oxide Synthase During Bacterial Infection of Pea (*Pisum sativum* L.). (Under the direction of Jeng-Sheng Huang and Eric L. Davis.)

Nitric oxide (NO) and reactive oxygen species are two key components in the induction of the hypersensitive response (HR) during plant defense against pathogen infection. In animal cells, the production of NO is catalyzed by nitric oxide synthase (NOS). Although NOS activity has been documented in plants, the process of NO synthesis in plants is not well understood. Isolation of the NOS protein and/or cloning of the corresponding gene will greatly facilitate the understanding of NO synthesis and its role in plant defense. The objectives of this research were to analyze the physiological and biochemical properties of a NOS-like protein (peaNOS) of pea (*Pisum sativum* L.), to purify and characterize peaNOS, and to clone the gene(s) encoding peaNOS and relate its expression to NOS activity in pea-bacteria interactions. The application of abiotic agents that induce systemic defense in plants [copper chloride, Actiguard<sup>®</sup>, Triton-X100 and salicylic acid (SA)] to pea leaves did not induce NOS activity and verified reports that NO and NOS function upstream of SA in the signaling pathway of defense responses. Maximum (two-fold) NOS activity was detected three hours before the onset of HR in pea leaves infiltrated with incompatible bacteria (*Ralstonia solanacearum*), which is consistent with the effect of NO in the activation of HR after interaction with H<sub>2</sub>O<sub>2</sub>. The compatible bacteria (*Pseudomonas syringae* pv *pisii*) induced NOS activity significantly, suggesting that NO generation may also be a general response to biotic stress in plants. Antibodies raised against mammalian NOS did not have apparent specificity and utility for isolating peaNOS and should be used with caution in non-mammalian systems. The peaNOS protein was most efficiently extracted under alkaline conditions (pH 8.5 and 9.0) as compared to the neutral conditions (pH 7.4-7.5) in animal systems. Precipitation of the peaNOS protein with various concentrations of ammonium sulfate, sodium citrate and sodium chloride caused

rapid loss of NOS activity. The peaNOS protein did not bind to 2',5'-ADP-Sepharose and calmodulin (CaM)-agarose indicating that the protein lacks binding sites for NADPH and CaM. Cloning of a peaNOS gene based on mammalian NOS was unsuccessful suggesting that the structure of peaNOS gene may be significantly different from mammalian NOS. Analysis of the *Arabidopsis thaliana* genome database identified two gene sequences related to animal NOS, i.e., accessions At4g09680 (similar to NOS of *Rattus norvegicus*) and At3g47450 (similar to NOS of *Helix pomatia*). Gene At4g09680 is probably not expressed since attempts to clone cDNA of this gene using reverse transcription-polymerase chain reaction (RT-PCR) consistently failed, even when RNA of *Arabidopsis* was used as a template. A potential expressed peaNOS gene was successfully cloned using RNA template of pea HR tissues in RT-PCR. The 784-bp peaNOS cDNA sequence had 50% nucleotide identity to the At3g47450 coding sequence and had no other significant match in the database. The correlation of the gene expression of P protein of glycine decarboxylase complex (GDC) of pea (peaP) and NOS activity during HR in pea was not demonstrated here but peaP gene was highly expressed concomitant with NOS activity during disease development. The NOS-like protein involved in NO production during HR in pea appears to be more related to At3g47450 sequence, and is possibly encoded partially by the cloned 784-bp pea cDNA.

**PHYSIOLOGICAL AND MOLECULAR ANALYSIS OF NITRIC OXIDE  
SYNTHASE DURING BACTERIAL INFECTION OF PEA (PISUM SATIVUM L.)**

by  
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**APPROVED BY:**

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**Co-Chair of Advisory Committee**

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## **DEDICATION**

To my Lord and Savior, Jesus Christ, my beloved husband, Boon-Kien, and my precious daughters, Lois and Hannah.

## BIOGRAPHY

Mui-Yun Wong was born in Sabah, Malaysia on December 4, 1970. She received her primary and secondary education in Sabah. In May 1994, she received a bachelor's degree in Agricultural Science from Universiti Putra Malaysia (UPM), Kuala Lumpur. After graduation, she taught general science in a private secondary school in Sabah for six months. She then joined a state-owned timber processing company in Sabah as a Quality Control Officer for a year before she continued a Master's degree in Agricultural Science at UPM under the direction of Sariah Meon. Her Master's thesis was on "Strain Improvement of *Trichoderma harzianum* Rifai through Protoplast Fusion". Upon graduation in August 1999, she was offered a position as an assistant instructor at the Department of Plant Protection, Faculty of Agriculture, UPM. Mui-Yun was offered a scholarship for a doctoral study by UPM in the year 2000. In August 2000, Mui-Yun began her PhD program in the Department of Plant Pathology at North Carolina State University, Raleigh, under the direction of Jeng-Sheng Huang and Eric L Davis. While working on her PhD program, she received a student travel award to the American Phytopathology Society Meeting in July 2002. Mui-Yun had completed her PhD program in May 2004.

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## LIST OF ABBREVIATIONS

ADP	adenine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BH <sub>4</sub>	tetrahydrobiopteridine
cADPR	cyclic adenosine 5'-diphosphate ribose
CaM	calmodulin
cDNA	complementary DNA
cGMP	3',5'-cyclic guanosine monophosphate
DAF-2DA	diaminofluorescein diacetate
DEAE	diethylaminoethyl
DIG	digoxigenin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene bis(oxyethylenenitrilo)tetraacetic acid
FAD	flavin adenine dinucleotide
FITC	fluorescein isothiocyanate
FMN	flavin mononucleotide
GDC	glycine decarboxylase complex
GTP	guanosine 5'-triphosphate
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
HCl	hydrochloric acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HR	hypersensitive response
JA	jasmonic acid
kb	kilo-base pair
kDa	kilo-Dalton
L-NAME	N <sup>G</sup> -nitro-L-arginine methyl ester
mRNA	messenger RNA
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
O <sub>2</sub> <sup>•-</sup>	superoxide
ONOO <sup>-</sup>	peroxynitrite anion
PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine ammonia lyase
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
PR	pathogenesis-related
PVPP	polyvinylpolypyrrolidone
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
RT	reverse transcription
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
TMV	tobacco mosaic virus
Tris	Tris(hydroxymethyl)aminomethane

## **GENERAL INTRODUCTION**

Humans depend upon plants as a primary food and fiber source. About 30 plant species make up 90% of our food supply (Lucas et al., 1992). The domestication of selected plant species from wild plants for agricultural development has emphasized improvement in crop yield and quality. However, plant diseases cause significant economic loss in agriculture in terms of reduced yield and quality. Annual losses to plant diseases in the United States average about 15% of the total agricultural production, or more than \$15 billion (Lucas et al., 1992).

Management options for pathogens depend upon the crop and disease agent. In general, management options include cultural practices, pesticides, biological control, physical control such as heat treatment, the use of resistant cultivars, and environmental manipulation. In natural ecosystems, successful plants have the capabilities to protect themselves from a variety of pathogens including bacteria, viruses, fungi, and nematodes. Disease development is the exception rather than the rule in natural environments.

### **Plant Pathogenesis and Disease Resistance**

Among the 1600 bacterial species known, about 100 species of bacteria cause diseases in plants (Agrios, 1997). At least two types of interactions can occur between phytopathogenic bacteria and plants: compatible and incompatible interactions (for reviews see Lindsay et al., 1993; Long & Staskawicz, 1993; and Staskawicz et al., 1995). The compatible interactions occur between pathogenic

bacteria and susceptible hosts and lead to disease development. Conversely, interactions of pathogenic bacteria and resistant hosts can lead to the induction of hypersensitive response (HR), a localized cell death of the host cells at the site of infection induced by a pathogen. The ability to produce either of these reactions in plants appears to be directed by *hrp* (HR and pathogenicity) and *hrc* (HR and conserved) genes that encode a type III protein secretion pathway that functions to deliver effector proteins encoded by *avr* (avirulence) and *hop* (Hrp-dependent outer protein) genes directly into the plant cells (Collmer et al., 2000). These effector proteins (Avr) may also betray the pathogen to the *R* (resistance) gene surveillance system of plants, thereby triggering the HR (Keen, 1990).

The response of a host plant to invading pathogenic bacteria is determined by specific interactions between pathogen *avr* gene product(s) and the corresponding plant *R* (resistance) gene product(s) in a gene-for-gene manner (Keen, 1990). When corresponding *avr* and *R* genes are present in both pathogen and host, the result is the induction of HR (incompatible interaction), and if either is inactive or absent, disease (compatible interaction) results. The recognition of the Avr signal by *R*-mediated proteins triggers a chain of signal-transduction events that ultimately lead to the activation of defense mechanisms and an arrest of pathogen growth.

Within the last decade, more than 30 *R* genes have been characterized from various plant species that can be divided into five classes (Hulbert et al., 2001). The largest class of *R* genes encodes structurally similar proteins containing a nucleotide-binding site (NBS) and a leucine-rich repeat (LRR) regions (NBS-LRR) while the other four classes are structurally diverse (Dangl and Jones, 2001). In

contrast to R proteins, Avr proteins lack common motifs and share little sequence homology. Avr proteins have been implicated to play a bifunctional role in the plant-pathogen interactions: 1) elicit defense responses in resistant host, and 2) promote virulence in susceptible hosts (White et al., 2000; Luderer and Joosten, 2001).

Resistance mechanisms that plants employ to defend themselves from pathogen attack are generally divided into two categories: 1) preformed defenses such as cuticle and lignin layers and preformed antimicrobial compounds (Osborn, 1996); and 2) inducible defense responses including HR, the production of reactive oxygen species, cell wall fortification, and the production of phytoalexins and pathogenesis-related (PR) proteins (Hammond-Kosack and Jones, 1996). Phytoalexins are low molecular weight, antimicrobial compounds that accumulate rapidly around sites of incompatible pathogen infections and in response to an extensive array of biotic and abiotic elicitors (Smith, 1996). PR proteins are intra- and extracellularly localized proteins that accumulate in intact plant tissue after pathogen attack (Bowles, 1990).

The activation of defense mechanisms can be the result of multiple signaling pathways. Initial defense responses in tissues undergoing HR include oxidative burst, change in membrane ion fluxes ( $K^+$ ,  $H^+$  and  $Ca^{2+}$ ), extracellular alkalization and activation of signaling cascades (Doke et al., 1983; Goodman and Novacky, 1994; Hammond-Kosack and Jones, 1996; Heath, 2000). In addition, the efficient induction of HR requires a balanced production of nitric oxide (NO) and reactive oxygen species (ROS), specifically hydrogen peroxide ( $H_2O_2$ ) (Delledonne et al., 2001). The accumulation of salicylic acid (SA) is also essential in the induction of

local (e.g. HR) and systemic (e.g. systemic acquired resistance, SAR) disease resistance (Gaffney et al., 1993).

### **History of Hypersensitive Response in Plants**

The occurrence of HR in response to plant pathogenic bacteria was first recognized by Klement and coworkers in 1964. They demonstrated that plant pathogenic bacteria could induce HR in resistant host and non-host plants by testing 22 different pseudomonad species or pathovars including the tobacco pathogen (compatible) *Pseudomonas syringae* pv. *tabaci* and five saprophytic species in tobacco leaf tissue. The avirulent (incompatible) pathogens injected at a concentration of more than  $10^6$  cells/mL induced localized tissue necrosis within 12 to 24 hr and subsequent tissue desiccation, symptoms that are indicative of HR. These symptoms were precisely confined to inoculated tissues. The bacterial population declined and the surviving bacteria remained confined to the site of inoculation. Neither inoculation of saprophytic bacteria nor compatible bacteria *P. syringae* pv. *tabaci* resulted in HR symptom development. In contrast, the compatible bacteria spread into the uninoculated tissues causing the slowly developing, progressively enlarging, necrotic lesions of bacterial disease with advancing time.

Once induction has initiated, living bacteria are no longer needed to sustain the HR, as the phenomenon is no longer repressible with antibiotics (Klement and Goodman, 1967). Following HR induction, some physiological and structural changes in the affected cells that occur include cessation of cytoplasmic movement,

cell discoloration, and electrolyte leakage that signifies severe plasma membrane damage (Bushnell, 1981; Tomiyama, 1956; Cook and Stall, 1968).

Although most phytopathogenic pseudomonads, xanthomonads and non soft-rot erwinias were found to be HR elicitors, the critical number of bacteria necessary for induction varies considerably, and some species may not elicit HR at all in incompatible interactions (Klement, 1982). The mechanisms of the HR are nonspecific and may develop whenever any bacterial pathovar is in physical contact with cells of an incompatible host or non-host plant (Klement, 1982). However, bacterial pathogenicity is highly specific where each bacterial pathovar can cause disease in only one or a few closely related hosts (Fahy and Persley, 1983).

Some researchers questioned the use of high bacterial concentrations in the study of HR induction and claimed that the amount was far higher than the number in natural inocula (Ercolani, 1973; Rudolph, 1975). Studies demonstrating that individual host cells developed HR symptoms in tissues inoculated with low numbers of incompatible bacteria dispelled doubts about the validity of bacterial HR as a resistance phenomenon (Turner and Novacky, 1974; Essenberg et al., 1979; Holliday et al., 1981). Klement (1982) demonstrated that even compatible interactions (e.g. *P.s. pv. tabaci* in tobacco) at high concentrations of bacteria may exhibit HR-like necrosis, i.e., using inoculum concentrations that are approximately 100x higher than a number sufficient to induce HR in incompatible interactions.

## Nitric Oxide in Biological Systems

In the 1980's, nitric oxide (NO) was discovered as a signaling molecule involved in the cardiovascular system of mammalian cells (Ignarro et al., 1987). Later, NO was also found to exert a series of other functions including its involvement in physiological and immune responses, such as relaxation of smooth muscle, neurotransmission, cell proliferation, cytotoxicity, inflammation, antimicrobial and antitumor (Furchgott and Zawadzki, 1980; Bredt and Snyder, 1989; Hibbs et al., 1987; Bredt and Snyder, 1994).

NO is a simple molecule consisting of a single oxygen bonded to a nitrogen atom. NO is stable in gas phase for at least 40 years without decomposition (Beckman, 1996). Because it has an unpaired electron, NO is very reactive and rapidly reacts with transition metals such as iron, and oxygen species to form other more reactive compounds such as peroxynitrite anion ( $\text{ONOO}^-$ ) which cause toxicity to the cells. In animal cells, the biosynthesis of NO is primarily catalyzed by the enzyme nitric oxide synthase (NOS, EC 1.14.13.39) during the conversion of L-arginine to L-citrulline in a complex reaction requiring molecular oxygen, calcium, calmodulin, FAD, FMN, tetrahydrobiopterin and NADPH (Bredt and Snyder, 1994).

The discovery of the existence of NO and NOS activity in higher plants in the 1990's (Leshem, 1996; Durner et al., 1998; Delledonne et al., 1998; Huang & Knopp, 1998), particularly their involvement in defense responses against pathogens, sparked the interest of many plant pathologists. Plants had been shown not only respond to atmospheric NO, but also produce substantial amounts of *de novo* NO (Wildt et al., 1997). Similarly, NO exerts multiple functions involved in various

developmental and defense processes in plants. NO has been demonstrated to promote plant growth, such as leaf expansion (Leshem and Haramaty, 1996) and root elongation (Gouvea et al., 1997); regulate stress (Leshem and Haramaty, 1996), endogenous maturation, and senescence in plants. For example, NO inhibits the function of ethylene (Leshem et al., 1998). NO also functions as a signal in the induction of hypersensitive cell death, the initial plant defense response to pathogen infections; expression of pathogenesis-related (PR-1) protein and phenylalanine ammonia lyase (PAL); and phytoalexin accumulation (Durner et al., 1998; Delledonne et al., 1998; and Noritake et al., 1996).

Plants use NO as a signaling molecule via pathways remarkably similar to those found in mammals (Wendehenne et al., 2001). Fig. 1 shows a model of NO-mediated signaling pathway in plant defense against pathogens. After pathogen attack, NO induces the expression of early and late defense genes such as phenylalanine ammonia lyase (PAL) and pathogenesis-related protein 1 (PR-1), respectively. Key enzymes in the signaling cascade are guanylate cyclase, nitric oxide synthase and other putative NO-generating enzymes (Wendehenne et al., 2001). Important signaling molecules are NO, cyclic GMP (cGMP), cyclic ADPR (cyclic ADP-ribose),  $Ca^{2+}$  and salicylic acid. NO signaling in animal cells can be classified as either cGMP-dependent or –independent. Activation of guanylate cyclase increases the cGMP level that alters the activity of three main cGMP-dependent proteins (Wendehenne et al., 2001).

## **Purification of Nitric Oxide Synthase (NOS) and Molecular Cloning of NOS**

Nitric oxide synthase has been isolated and purified from tissues of mammals (Bredt and Snyder, 1990; Stuehr et al., 1991; Forstermann et al., 1991), snails (Ogunshola et al., 1995; Huang et al., 1997), insects (Elphick et al., 1995; Regulski and Tully, 1995; Ogunshola et al., 1995), bacteria (Chen and Rosazza, 1995), fungus (Song et al., 2000), and slime mold (Golderer et al., 2001).

Accumulating evidence had demonstrated the presence of NOS-like protein in plants including the detection of NOS activity via the formation of L-citrulline from L-arginine (Cueto et al., 1996; Ninnemann & Maier, 1996; Huang & Knopp, 1998; Durner et al., 1998);  $\text{Ca}^{2+}$ -dependent NOS activity (Cueto et al., 1996; Delledonne et al., 1998; Ribeiro et al., 1999); NO burst within minutes of HR induction (Foissner et al., 2000); sensitivity to mammalian NOS inhibitors (Delledonne et al., 1998; Durner et al., 1998; Foissner et al., 2000; Pedroso et al., 2000); and positive immunoreactivity to antibodies raised against mammalian NOS (Huang & Knopp, 1998; Barroso et al., 1999; Ribeiro et al., 1999). Moreover, real-time imaging of NO production in epidermal tobacco cells treated with the fungal elicitor cryptogein from *Phytophthora cryptogea* using fluorophore diaminofluorescein diacetate (DAF-2DA) in conjunction with confocal laser scanning microscopy was able to detect NO in the cytosol, along the plasma membrane, in chloroplasts, and in organelles probably representing peroxisomes (Foissner et al., 2000).

However, purifying NOS protein or cloning a NOS gene from plants had remained a challenge until very recently. Identification of a gene encoding a protein with NOS activity from pathogen-induced tobacco, *varP* (Chandok et al., 2003) and

constitutively expressed AtNOS1 from *Arabidopsis thaliana* (Guo et al., 2003) overcame the many difficulties encountered in the attempts to isolate this enzyme from plants. Both genes showed little similarity to any mammalian NOS, but encoded proteins with NOS activity. The NO-synthesizing enzyme isolated from pathogen-induced tobacco is a variant form of the P protein (*varP*) of the glycine decarboxylase complex (GDC) (Chandok et al., 2003). The *varP* protein had an estimated molecular mass of 115-120 kDa and lacks most of the binding domains present in mammalian NOS but shares biochemical and kinetic properties to those of its animal counterparts (Chandok et al., 2003). The AtNOS1 protein had a molecular mass of 62 kDa (Guo et al., 2003) and showed sequence similarity to the protein implicated in NO synthesis in the snail *Helix pomatia* (Huang et al., 1997).

The glycine decarboxylase complex is involved in the glycine cleavage system that catalyzes the degradation of glycine. The GDC is composed of four proteins: P (containing pyridoxal phosphate, PP), T (a transferase responsible for producing 5,10-methylenetetrahydropteroyl-L-glutamic acid), H (a carrier protein containing lipoamide), and L (lipoamide dehydrogenase required to complete the cycle). The P protein, which is considered the true glycine decarboxylase (Hiraga and Kikuchi, 1980), binds the alpha-amino of glycine through its pyridoxal phosphate cofactor, CO<sub>2</sub> is released and the remaining methylamine moiety is then transferred to the lipoamide cofactor of the H protein. The PP binding site of the P protein was implicated to participate in NO synthesis as was demonstrated by *varP* (Chandok et al., 2003).

## Hypothesis and Rationale

There may be multiple forms of NOS-like protein in plants that differ from their counterparts in animal cells. The NOS-like protein in pea may be a different form from those in tobacco and *Arabidopsis*. Isolation of a NOS-like protein in pea will provide another source for comparison of NOS-like protein in plants. In addition, isolation of a NOS-like protein in pea may be necessary in order to successfully clone the corresponding genes since the nucleotide sequence of NOS in plants has limited similarity to animal NOS. This was the case with the first NOS-like protein and gene reported from plants, *varP* (Chandok et al., 2003). With the genes cloned, we may investigate the role of NO and NOS in plants at the genetic level. Furthermore, we may apply this knowledge to develop transgenic plants with increased resistance by over-expressing NOS upon pathogen attack.

Previously, in our lab, leaf tissues from pea (*Pisum sativum* L.) demonstrated the highest NOS activity when compared to soybean, tobacco, tomato, pepper, corn and *Arabidopsis thaliana* (unpublished data). Therefore, efforts on the isolation of a NOS-like protein and cloning of the corresponding gene were focused on pea.

Pea is a host plant for *Pseudomonas syringae* pv *pisii*. Thus, it was selected for the study of compatible interactions. *Ps* pv *pisii* causes bacterial blight disease on peas. The pathogen penetrates the host cells through stomates, hydathodes and injuries where penetration is greatly enhanced during heavy rain (Lawyer, 1984). The pathogen multiplies on walls of hosts intercellularly and secrete enzymes and toxins that breakdown and cause the collapse of the parenchyma cells in the infected leaf tissues, thus, causing lesions on all aboveground parts of peas

(Lawyer, 1984). For the study of incompatible interactions, the initial intention was to use the same pathogen but a different pathovar that can cause HR in pea in order to minimize the effect of genetic background. However, due to problems related to quarantine regulations, we were not able to import the avirulent *P. syringae* pv *pisii* strains. Therefore, *Ralstonia solanacearum*, which is a close taxonomic species to *P. syringae*, and has been demonstrated previously in our lab to cause HR symptoms in infiltrated pea leaves was used in the study of incompatible interactions. Furthermore, *R. solanacearum* is an interesting pathogen due to its wide host range and complete information regarding its genome is available (Salanoubat et al., 2002).

When we started this work in 2000, there was no report on the isolation and identification of NOS-like protein or gene in plants. Subsequently, a database search of the completed *Arabidopsis* genome identified two potential plant NOS genes (At4g09680 and At3g47450). As this thesis progressed, the At3g47450 accession was found by an independent lab to encode a protein associated with NOS activity in plant hormonal response (Guo et al., 2003).

Experimental conditions to optimize NOS activity from pea and purify the NOS-like protein for sequencing represented one of the means to identify the gene(s) from pea. In addition, the gene encoding a P protein of GDC in pea similar to *varP* (Chandok et al., 2003), was previously cloned (Turner et al., 1992; GenBank accession #: P26969), and potential At4g09680 and At3g47450 homologues in pea were also likely candidates. Based upon the information on NOS activity in pea, and the gene sequences of the NOS-like proteins in other plants, purification conditions

of a NOS-like protein from pea and a correlation study between the expression of candidate NOS-encoding genes in pea and NOS activity in both incompatible and compatible plant-bacteria interactions were investigated.

### **Specific Research Objectives**

1. Analyze the physiological and biochemical aspects of a NOS-like protein (peaNOS) of pea (*Pisum sativum* L.).
2. Purify and characterize peaNOS.
3. Clone the gene encoding peaNOS and relate its expression to NOS activity in pea-bacteria interactions.

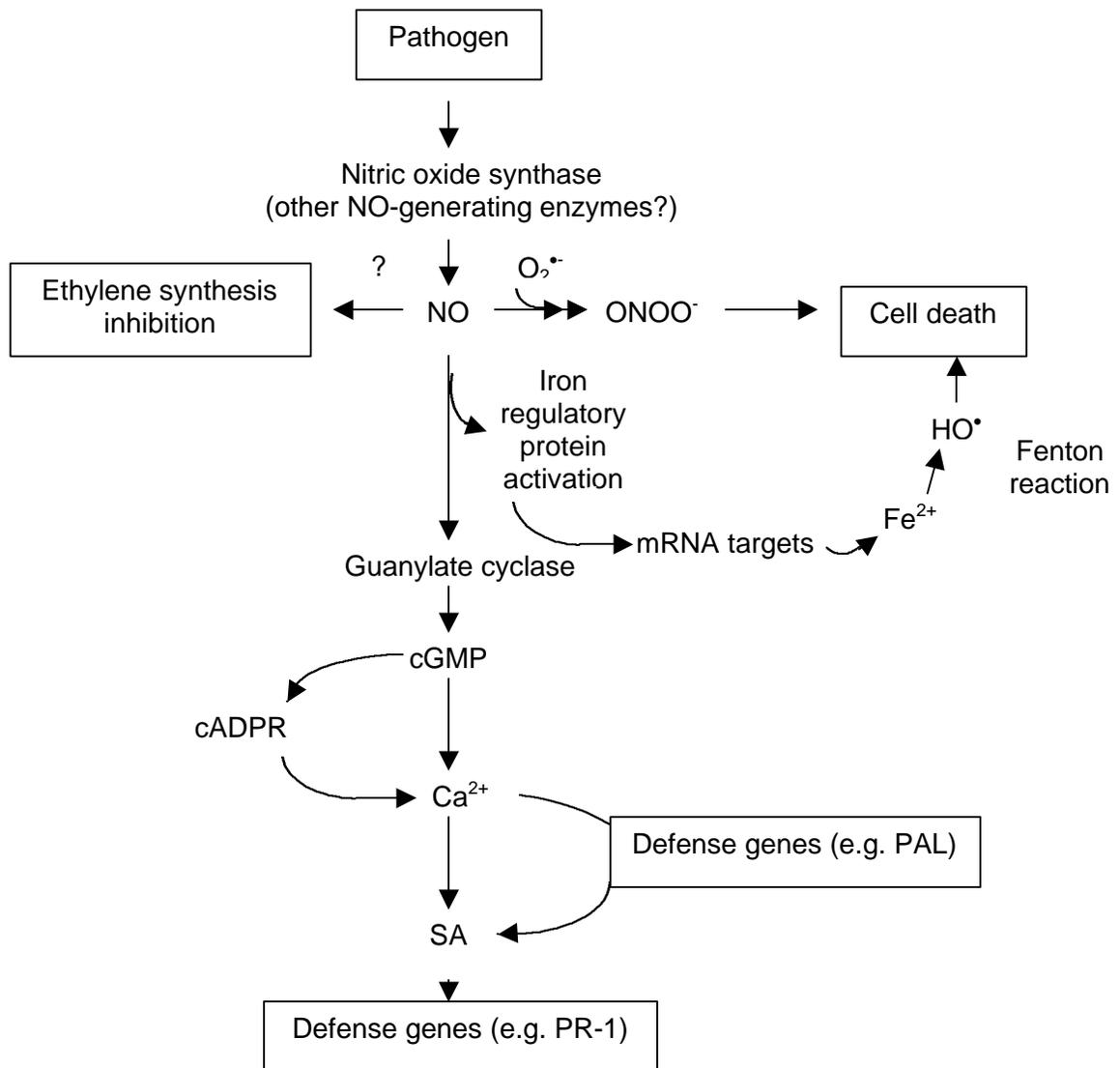


Fig. 1 Nitric oxide (NO)-mediated signaling pathway in plant defense against pathogens. After pathogen attack, NO induces the expression of early and late defense genes such as PAL and PR-1, respectively. Key enzymes in the signaling cascade are guanylate cyclase, nitric oxide synthase (NOS) and other putative NO-generating enzymes. Important signaling molecules are NO, cGMP, cADPR,  $\text{Ca}^{2+}$  and salicylic acid. The model suggests salicylic acid-dependent as well as salicylic acid-independent gene induction. For some genes, full activation might require the simultaneous presence of both cADPR and cGMP. In addition to defense gene activation, NO could mediate cell death by at least two pathways. In the first pathway, NO might convert cytosolic aconitase into an iron regulatory protein (IRP), which modulates the translation and stability of mRNAs encoding proteins involved in the intracellular iron homeostasis. The resulting increase in free iron concentration promotes the Fenton reaction, leading to formation of the hydroxyl radical  $\text{HO}^\bullet$ .  $\text{HO}^\bullet$  creates a killing environment for both host and pathogen. In the second pathway,  $\text{NO}^\bullet$  reacts with  $\text{O}_2^{\bullet-}$  to yield peroxynitrite (ONOO<sup>-</sup>), which could play an important role in the activation of the cell death program. NO could also affect ethylene production during pathogen attack, although there is currently no evidence for this. Abbreviations: cADPR, cyclic ADP-ribose; cGMP, cyclic GMP; PAL, phenylalanine ammonia lyase; PR-1, pathogenesis-related protein 1. Modified from Wendehenne et al., 2001.

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## **CHAPTER 1**

**PHYSIOLOGICAL AND BIOCHEMICAL ANALYSIS, AND PARTIAL  
PURIFICATION OF NOS-LIKE PROTEIN OF PEA (PISUM SATIVUM L.)**

## INTRODUCTION

A hypersensitive response (HR) in plants is defined as a localized death of host cells at the site of infection induced by a pathogen and thought to be responsible for limiting the growth of the pathogen in incompatible plant-microbe interactions (Agrios, 1997). The HR in plant-bacterial interactions consist of initial water-soaked infected tissue followed by rapid necrotic host cell collapse within 8 to 12 hours after inoculation (Agrios, 1997). The phenomenon is 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 and Jones, 1996).

Nitric oxide (NO) and reactive oxygen species (ROS) are two key early components in the induction of HR in plants (Delledonne et al., 2001; del Rio et al., 2004). Specifically, the HR is activated after the interaction of NO with  $H_2O_2$  instead of  $O_2^-$ .  $O_2^-$ , which reacts with NO to produce peroxynitrite ( $ONOO^-$ ), is not a plant cell death signal (Delledonne et al., 2001) in contrast to what happens in animal cells where  $ONOO^-$  induces apoptosis in tumor cells (Lin et al., 1995) and is involved in direct killing of pathogens (Ischiropoulos and al-Mehdi, 1995). Although NO has been detected in plants, the process of NO synthesis in plants is not well understood.

In mammalian cells, NO is produced during the conversion of L-arginine to L-citrulline catalyzed by nitric oxide synthase (NOS, EC 1.14.13.39) (Bredt and

Snyder, 1994). Three isoforms of mammalian NOS have been identified and named on the basis of the tissue source from which they were originally extracted: neuronal NOS (nNOS), also known as Type I; inducible NOS in macrophages (iNOS), Type II; and endothelial NOS (eNOS), Type III (Nathan and Xie, 1994). A fourth isoform of NOS was subsequently found in mitochondria (mtNOS) isolated from rat liver (Tatoyan and Guilivi, 1998), and is similar to iNOS based on kinetic parameters, molecular weight, requirement of cofactors, and cross-reactivity to monoclonal antibodies against macrophage NOS. All NOS isoforms show 50-60% identity in their amino acid sequences (Wendehenne et al., 2001).

Each NOS is a bi-domain enzyme consisting of an N-terminal oxygenase and a C-terminal reductase (Alderton et al., 2001). The oxygenase domain contains a cytochrome P-450 type heme center and a binding site for the cofactor BH<sub>4</sub>. The reductase domain contains NADPH, FAD and FMN binding sites. Both oxygenase and reductase domains are connected by a calmodulin (CaM) binding site in the middle of the enzyme. Each NOS has a different N-terminal extension determining the intracellular localization of the enzyme. In the active form, all NOS enzymes are homodimers.

The expression of nNOS and eNOS is constitutive whereas iNOS is inducible. The activity of constitutive NOS is strictly dependent upon the elevation of intracellular free Ca<sup>2+</sup> and requires the binding of CaM. Thus, both nNOS and eNOS show fast and transient activation (in minutes) (Nathan and Xie, 1994; Mayer and Hemmens, 1997). The activity of iNOS is independent of the intracellular free Ca<sup>2+</sup>

concentration and is sustained for a longer period of time ranging from hours to days (Nathan and Xie, 1994; Mayer and Hemmens, 1997).

A key step in purification of the three isoforms of NOS includes 2',5'-ADP affinity chromatography eluted with NADPH (Bredt and Snyder, 1990; Stuehr et al., 1991; Forstermann et al., 1991). Neuronal NOS, eNOS, and iNOS have a molecular mass of approximately 160, 135, and 130 kDa respectively. Both nNOS and eNOS require  $\text{Ca}^{2+}/\text{CaM}$ , NADPH and  $\text{BH}_4$  for their activity. On the contrary, iNOS requires NADPH,  $\text{BH}_4$ , FAD, FMN but not  $\text{Ca}^{2+}/\text{CaM}$  for its activity. Under physiological conditions, nNOS is both soluble and particulate, eNOS is particulate, and iNOS is soluble.

Nitric oxide synthase genes have also been described in other eukaryotic species: snails (Huang et al., 1997), insects (Regulski and Tully, 1995), fungus (Song et al., 2000), slime mold (Golderer et al., 2001), and very recently in plants (Chandok et al., 2003; Guo et al., 2003). Bacteria (*Nocardia* spp.) also synthesize a NOS-like protein which is smaller than those produced by eukaryotic species (Chen and Rosazza, 1995).

NOS from the bacteria, *Nocardia* spp., has a molecular mass of approximately 52 kDa which exists as a homodimer (Chen and Rosazza, 1995). The enzyme was purified using a combination of 2',5'-ADP agarose affinity chromatography and hydroxylapatite chromatography. The enzyme was tightly bound to 2',5'-ADP agarose and was best eluted with 10 mM NADPH. Its activity was dependent on the presence of NADPH,  $\text{Ca}^{2+}$ , FAD, FMN, and  $\text{BH}_4$  as cofactors. Physical and catalytic properties of this enzyme differ from the mammalian NOS.

The 15-amino acid sequence of the N-terminal was demonstrated to be different from known mammalian NOS.

NOS from the snail *Helix pomatia* has a molecular mass of 60 kDa, and was purified using DEAE anion-exchange chromatography, 2',5'-ADP agarose affinity chromatography and S-300 Sephacryl gel filtration (Huang et al., 1997). Though the activity of the *Helix* NOS was dependent on NADPH and  $\text{Ca}^{2+}$ , the cDNA sequence did not show homology with any NOS isoform and did not show consensus binding sites for NADPH and CaM (Huang et al., 1997). The findings indicated that the protein is associated with NOS and affects its activity, but it may not be the enzyme itself. However, the protein has an ATP/GTP binding site and putative myristoylation sites (Huang et al., 1997).

NOS from the fungus *Flammulina velutipes* has a molecular mass of 100 kDa, exists as a dimer, and its activity is related to the formation of fruiting bodies (Song et al., 2000). The enzyme was purified by three chromatographic steps including an ion exchanger (DEAE-Sephadex A-50), 2',5'-ADP agarose affinity chromatography, and mono P chromatofocusing. The enzyme requires NADPH, FAD, FMN,  $\text{O}_2$ , and  $\text{BH}_4$  as cofactors in the generation of nitric oxide, but not  $\text{Ca}^{2+}$  and CaM (Song et al., 2000).

A NOS protein and its corresponding genes were also isolated from the slime mold *Physarum polycephalum* (Golderer et al., 2001). Expression of *Physarum* NOS is induced during sporulation. The protein has a molecular mass of approximately 120 kDa and was purified using precipitation by 50% (w/v) ammonium sulfate, 2',5'-ADP-Sepharose 4B affinity chromatography, Sephacryl S-300 HR gel filtration, and

Q-Sepharose ion exchange chromatography. Though the *Physarum* protein contains conserved binding motifs for all mammalian NOS cofactors, it shares only approximately 39% amino acid homology to mammalian NOS. However, it lacks the sequence conferring calcium dependence of both mammalian nNOS and eNOS, and thus, shows similarity to mammalian iNOS. Two cDNAs, designated phynosa (3571 bp) and phynosb (3316 bp), were isolated which encoded a 1055 amino acid protein of 118.2 kDa and a 1046 amino acid protein of 117.6 kDa respectively (Golderer et al., 2001). Purified *Physarum* NOS did not react with antibody raised against the three isoforms of mammalian NOS (Golderer et al., 2001).

Chandok et al. (2003) first reported the isolation of a protein with NOS-like activity and its corresponding gene from plants. The NO-synthesizing enzyme is a variant form of the P protein (*varP*) of the glycine decarboxylase complex (GDC) and was isolated from pathogen-challenged tobacco, and thus, designated iNOS. The protein was isolated using G-25 Sephadex chromatography, ammonium sulfate precipitation (0-35%), and four other chromatography steps including DEAE-Sepharose, ARG-Sepharose, ADP-Sepharose and CaM-Sepharose (Chandok et al., 2003). The tobacco iNOS has an estimated molecular mass of 115-120 kDa and its activity required H<sub>4</sub>B, FAD, NADPH, O<sub>2</sub>, Ca<sup>2+</sup> and CaM. The cDNA (3114 bp) sequence, designated *varP*, contained binding sites for CaM, flavin, pyridoxal phosphate/putative NADPH-Rossmann fold, and a leucine zipper. Despite the lack of sequence homology to mammalian NOS, the biochemical and kinetic properties of the *varP* enzyme share similarity to those of its animal counterparts (Chandok et al., 2003). The pyridoxal phosphate domain of the *varP* protein implicated in NO

production during pathogen challenge (Chandok et al., 2003) shares significant amino acid similarity to the corresponding domain predicted from the cDNA sequence of the P protein in pea (Turner et al., 1992).

The *Arabidopsis thaliana* NOS-like protein predicted from the AtNOS1 gene (Guo et al., 2003) had significantly different amino acid sequence differing from its mammalian counterparts, but showed sequence similarity to the protein implicated in NO synthesis in the snail *Helix pomatia* (Huang et al., 1997). The 561 amino acid AtNOS1 sequence had a molecular mass of 62 kDa (Guo et al., 2003). The AtNOS1 protein was expressed in bacteria as a fusion protein with glutathione-S-transferase and purified (80-fold) by glutathione-affinity chromatography. The activity of AtNOS1 was dependent upon NADPH, CaM and Ca<sup>2+</sup>, and inhibited by L-NAME, properties similar to mammalian eNOS and nNOS (Guo et al., 2003). However, AtNOS1 activity was not stimulated by BH<sub>4</sub>, FAD, FMN or heme, which are all cofactors of mammalian NOS.

The goal of this study was to optimize NOS activity and isolate a NOS-like protein in pea. This work was initiated prior to the identification of the NOS-like *varP* protein from tobacco (Chandok et al. 2003) and AtNOS1 from *Arabidopsis* (Guo et al., 2003). Pea had been previously demonstrated in our lab to have the highest NOS activity as compared to other plant species. Previous results (Huang, unpublished data) also showed that the incubation of crude extracts of pea with known NOS inhibitors [aminoguanidine, N<sup>G</sup>-monomethyl-L-arginine (L-NMMA), N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME), and 7-nitroindazole] resulted in the inhibition of NOS activity in a concentration-dependent manner between 1 to 10 mM. At the

concentration of 10 mM, NOS activity was inhibited 35% by aminoguanidine and L-NMMA, 65% by L-NAME, and 25% by 7-nitroindazole. In addition, NOS activity was reduced in the absence of NADPH, FAD, FMN, BH<sub>4</sub>, and Ca<sup>2+</sup> and calmodulin by 62%, 33%, 23%, 15%, and 41%, respectively. Furthermore, adding the small molecular weight (SMW) filtrate (<8 kDa) prepared from supernatants of crude pea leaf extracts significantly enhanced NOS activity at various degree of significance (Huang, unpublished).

Since NOS activity in healthy pea tissues was detected at low levels, tissues with increased concentrations of NOS-like protein were needed for protein isolation. The experiments on the effects of abiotic and biotic interactions on NOS activity in pea were conducted here with two primary goals: 1) to prepare tissue samples with significantly higher NOS activity level for maximal NOS-like protein and RNA (for expressed gene cloning) isolation, and 2) to study the relationships of abiotic and biotic interactions of pea with chemicals and pathogens respectively on NOS activity. To normalize the effect of the presence of the bacteria in the infiltrated tissues, the same concentration of both the incompatible (*Ralstonia solanacearum*) and compatible bacteria (*Pseudomonas syringae* pv. *pisii*) was used, i.e., 10<sup>8</sup> cfu/mL.

In addition, optimum conditions for protein extraction and sample preparation were determined to facilitate the isolation process. Western blot analysis was performed to characterize the molecular size of pea NOS-like protein and to determine the reactivity of the protein with antibodies raised against mammalian NOS.

## MATERIALS AND METHODS

### Effects of Abiotic and Biotic Interactions on NOS Activity

For abiotic treatments, pea (*Pisum sativum*, cv Alaska) plants were grown in a greenhouse at a temperature cycle of 30/26±2°C, and 12 hr light cycle. Leaves of 12±2 day-old pea plants were sprayed with four types of chemicals that induced systemic resistance in plants (Okuno et al., 1991; Hammerschmidt and Kuc, 1995; Rakwal et al., 1996): Actiguard (25 mg a.i./L), copper chloride (10 mM), salicylic acid (2.5 mM), and Triton-X100 (0.1%). Tween 20 (0.05%) was used as surfactant in the preparation of the four chemicals, and applied alone as a control. Treated plants were kept in greenhouse and leaves were harvested at 0, 3, 5, and 7 days after spray.

For biotic interactions, leaves of 10±2 day-old pea plants were vacuum infiltrated with aqueous suspensions of incompatible bacteria (Hayward, 1994), *Ralstonia solanacearum* (7.0x10<sup>8</sup> cfu/mL), compatible bacteria (Lawyer, 1984), *Pseudomonas syringae* pv *pisii* (7.0x10<sup>8</sup> cfu/mL), and distilled water as a control. Pea leaves were harvested at intervals of 0, 3, 6, 9, 12, and 24 hr after infiltration with bacterial suspension. Pea leaf tissues harvested from both abiotic and biotic treatments was immediately frozen in liquid nitrogen, and stored at – 80°C before use. Both abiotic and biotic experiments were repeated four times. NOS activity for each time point was determined as described below.

## **NOS Activity Assay**

Treated pea leaf tissues (0.5-1.0 g) 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  $\mu$ M leupeptin, 1.0 mM PMSF, and 1% polyvinylpolypyrrolidone (PVPP). The homogenate was filtered through one layer of Miracloth and centrifuged at 4°C for 30 min at 20,000g. Protein concentration of crude extracts was determined using protein assay reagent (Bio-Rad, Hercules, CA) and bovine serum albumin (BSA) as standard. NOS activity in extracts was determined by the conversion of L-arginine to L-citrulline (Bredt and Snyder, 1990).

Each reaction mixture (165  $\mu$ L) contained 85  $\mu$ L protein extract, 1  $\mu$ L FAD (2  $\mu$ M), 1  $\mu$ L FMN (2  $\mu$ M), 1  $\mu$ L tetrahydrobiopterin ( $\text{BH}_4$ ) (5  $\mu$ M), 1  $\mu$ L  $\beta$ -NADPH (1 mM), 1  $\mu$ L Calmodulin (40 units), 10  $\mu$ L  $\text{CaCl}_2$  (2 mM), 10  $\mu$ L nonradio-labeled arginine (0.2 mM), 10  $\mu$ L Hepes (30 mM, pH 7.4), 10  $\mu$ L DTT (3 mM), 10  $\mu$ L EDTA (0.6 mM), and 25  $\mu$ L [ $^{14}\text{C}$ ]L-arginine (1.25  $\mu$ Ci/mL) (Amersham Biosciences, Piscataway, NJ), and was incubated at room temperature (23-25°C) for 60 min. Each reaction was stopped by adding 2 mL stop buffer (20 mM Hepes and 2 mM EDTA, pH 5.5), and the mixture was passed through a 1-mL Dowex AG50W X-8 ( $\text{Na}^+$  form) ion exchange column. Positively charged L-arginine was exchanged with  $\text{Na}^+$  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  $\mu$ L (10% of the total mixture) of the flow-through was added to 5 mL of scintillation liquid and radioactivity was counted using a liquid

scintillation counter (LS 7500 Beckman). Specific activity was expressed as picomole of [<sup>14</sup>C]L-citrulline per mg protein per min after subtracting the background value, i.e., value of similarly prepared reaction mixture terminated immediately after adding protein sample.

### **Statistical Analysis**

All statistical data were analyzed using SAS software and General Linear Models (GLM) procedure assuming a completely randomized experimental design. Analysis of variance (ANOVA) was determined using the Least Squared Means and Waller-Duncan K-ratio t Test procedures.

### **Western Blot Analysis**

Protein from crude extracts of leaf tissues from untreated, compatible and incompatible interactions, were separated by 10% (w/v) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-Rad Mini-PROTEAN II slab cell (Bio-Rad, Hercules, CA). Samples (100 µg) were prepared in 0.5 M Tris-HCl (pH 6.8), containing 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-Mercaptoethanol, and 0.125% (w/v) Bromophenol Blue, and were heated at 95°C for 4 min. Gels were run at 180V for 45 min and stained with 0.1% Coomassie brilliant blue R-250 to monitor for uniform sample loading. Polypeptides were transferred to a nitrocellulose membrane (Hybond<sup>TM</sup> ECL<sup>TM</sup>, Amersham Biosciences, Piscataway, NJ) using Bio-Rad Trans-Blot cell (Bio-Rad, Richmond, CA) following the

instructions of the manufacturer, with buffer containing 25 mM Tris and 192 mM Glycine at 70V for three hours.

Immunodetection was conducted using rabbit polyclonal antibodies against brain NOS (bNOS), endothelial NOS (eNOS), and inducible macrophage NOS (iNOS) (Calbiochem, San Deigo, CA) of human, mouse and rat at 1:1000, 1:1000, and 1:2000 dilutions respectively, and Amplified Alkaline Phosphatase Goat Anti-Rabbit Immun-Blot Assay Kit (Bio-Rad, Hercules, CA) according to the manufacturers' instructions. A similar Western blot was conducted using non-denaturing PAGE (5%) and all steps were performed at 4-8°C. Samples (100 µg) were prepared in 1 M Tris-HCl (pH 6.8), containing 50% glycerol (v/v) and 0.05% Bromophenol Blue. Non-denaturing gels were run at 100V for 90 min.

### **Effects of pH, Reducing Agents, Metal Chelators, Protease Inhibitors and Phenolic Chelator on NOS Activity During Protein Extraction**

One gram of ground tissues of untreated pea leaves was homogenized with 50 mM Tris-HCl separately at pH 7.0, 7.5, 8.0, 8.5, and 9.0, and extracted as described above. The pH which provided the highest relative NOS activity was used for the subsequent experiment. Similarly, one gram of ground tissues was homogenized with 50 mM Tris-HCl at the selected pH. The following chemicals were added as separate NOS activity treatments during homogenization: dithiothreitol (DTT, 1 mM), and 2-mercaptomethanol (10 µL/10mL) as reducing agents; EDTA (1 mM), and EGTA (1 mM) as metallo-proteases inhibitors; *p*-Aminobenzamidine and  $\epsilon$ -Amino-n-caproic acid (1 mM and 5 mM respectively),

leupeptin (1  $\mu$ M), and PMSF (1 mM) as protease inhibitors, and polyvinylpolypyrrolidone (PVPP, 1%) as a phenolic chelator. NOS activity was determined and the data were statistically analyzed as described above.

### **Precipitation of Crude Extracts**

Proteins in crude extracts of non-treated pea leaves were precipitated using both salts and organic solvents. Concentrations of 0.5, 1.0, 1.5 and 2.0 M of ammonium sulfate, sodium citrate and sodium chloride; 10, 20, 30, 40 and 50% acetone; and 10, 20 and 30% polyethylene glycol (PEG) were added separately in each sample and centrifuged at 4°C for 30 min at 20,000g. Pellets were resuspended in 200  $\mu$ L of extraction buffer. NOS activity for supernatants, pellets, and pellets added with small molecular weight (SMW) filtrate (<8 kDa) was determined as described above. SMW filtrate was prepared from supernatants of crude pea leaf extracts filtered through Centricon Plus-20 cartridge (Amicon, Millipore Corp., Bedford, MA) and included as a treatment because it significantly enhanced NOS activity at various degree of significance (Huang, unpublished).

### **Partial Purification of NOS-like Protein**

To prepare samples for purification, protein extraction from treated pea leaves was scaled up accordingly. Seventy grams of leaf tissues in 140 mL of extraction buffer was homogenized in a blender and centrifuged at 4°C for 30 min at 20,000g. Supernatant, after chemical precipitation as described above, was passed through ion exchange chromatography with DEAE-Sepharose Fast Flow (Amersham

Pharmacia Biotech, Piscataway, NJ) packed into a Pharmacia column (2.6x10 cm) according to the manufacturer's protocol at a flow rate of 66 mL/hr and equilibrated with equilibration buffer (50 mM Tris-HCl (pH 8.5), 10 mM EGTA, 1  $\mu$ M leupeptin, and 1 mM PMSF). The protein was eluted with a linear gradient of 0 to 0.3 M NaCl in equilibration buffer. Fractions of 3.4 mL were collected and each fraction was assayed for NOS activity and total protein content as described above. Fractions with NOS activity were pooled and concentrated in Centricon<sup>®</sup> Plus-20 cartridges (Amicon, Millipore Corp., Bedford, MA) to half volume.

The pooled, concentrated sample containing NOS-like protein was subjected to affinity chromatography with 2',5'- ADP-Sepharose 4B (Amersham Biosciences, Piscataway, NJ) packed according to the manufacturer's protocol at a flow rate of 1.8 mL/hr, equilibrated in binding buffer containing 50 mM Tris-HCl (pH 8.5), 150 mM NaCl, 10 mM EGTA and 1  $\mu$ M leupeptin, and recirculated through the column five to seven times. The protein was eluted with 10 mM NADPH in binding buffer. Fractions of 3.4 mL were collected and each fraction was assayed for NOS activity and total protein content. Alternative to 2',5'- ADP-Sepharose 4B, Arginine-Sepharose 4B (Amersham Biosciences, Piscataway, NJ) column was used according to the manufacturer's recommendations and Phosphodiesterase 3',5'- Cyclic Nucleotide Activator Agarose (Sigma, Saint Louis, MO) column was used according to Glenney and Weber (1983). All fractions (3.4 mL) collected were assayed for NOS activity and total protein content as described above. All purification steps were carried out in a chromatography chamber maintained at 4 to 8°C.

## RESULTS

### Effects of Abiotic and Biotic Interactions on NOS Activity

For the abiotic interaction, NOS activity of tissues treated with copper chloride, Actiguard<sup>®</sup>, Triton-X100, salicylic acid, and control were 7.9, 7.4, 7.0, 6.7 and 6.3 pmol/min/mg respectively (Fig. 1). There was no significant difference ( $P < 0.05$ ) in NOS activity among chemical treatments, experiments, or interactions of time and treatment (Appendix A). The lack of significant difference in NOS activity between the Tween-20 control and any chemical treatment at any time point indicated that none of the abiotic treatments selected could significantly influence plant NOS activity.

For the biotic interactions, NOS activity of the water control remained relatively constant over 24 hr at levels of 2.1, 2.9, 2.8, 2.4, 2.8 and 2.4 pmol/min/mg respectively (Fig. 2). In the incompatible interaction, NOS activity of the treated tissues of 0, 3, 6, 9, 12 and 24 hr post infiltration (hpi) were 2.5, 4.1, 4.5, 4.1, 3.2 and 2.8 pmol/min/mg respectively (Fig. 2). At six hpi, NOS activity in the incompatible interaction reached maximum level, gradually declined at nine hpi, and reached near control level at 12 and 24 hpi. An initial water-soaked symptom was observed between 9-12 hpi followed by the localized necrosis, typical of HR response, between 12-16 hpi. At 24 hpi, necrosis was severe but infected areas were by the HR.

In the compatible interaction, NOS activity of the treated tissues of 0, 3, 6, 9, 12 and 24 hpi were 2.7, 4.5, 4.3, 5.6, 5.4 and 6.1 pmol/min/mg respectively (Fig. 2).

NOS activity increased throughout the 24 hr period. Maximum NOS synthesis was observed at 24 hpi as compared to that in the incompatible interaction, which occurred at six hpi. Disease symptoms were first observed at nine hpi, beyond that time point, necrosis became severe and spread to adjacent cells.

### **Western Blot Analysis**

Polyclonal antibodies raised against bNOS, eNOS and iNOS of either human, mouse or rat bound non-specifically to many pea proteins separated by both SDS and non-denaturing PAGE (Fig. 3A and B). One differential protein band appeared only in tissues challenged with incompatible bacteria using antibody raised against iNOS after separation by SDS PAGE (Fig. 3A). The protein had a molecular weight of approximately 30 kDa. Immunoblot analysis of non-denaturing PAGE showed no specific binding to high molecular weight proteins that were relatively the same size (130-160 kDa) as mammalian NOS dimers (Wendehenne et al., 2001) with the three types of antibodies (Fig. 3B).

### **Effects of pH, Reducing Agents, Metal Chelators, Protease Inhibitors and Phenolic Chelator on NOS Activity During Protein Extraction**

NOS activity in crude extracts from leaves and stems of normal tissues homogenized with 50 mM Tris-HCl was highest at pH 8.5 and 9.0 (8.5 and 8.6 pmol/min/mg respectively) (Fig. 4A), and significantly different ( $P < 0.05$ ) from NOS activity at pH 8.0, 7.5 and 7.0 (7.0, 5.9 and 5.9 pmol/min/mg respectively) (Appendix

B). pH 8.5 was selected to be used in subsequent experiment due to its near physiological pH range.

At pH 8.5, NOS activity was increased significantly ( $P < 0.05$ ) (Appendix C) by adding EGTA or leupeptin to the homogenization buffer (10.5 and 10.8 pmol/min/mg respectively) as compared to all other treatments, including the control where no chemical was added (6.0 pmol/mg/min) (Fig. 4B). Significant increase in NOS activity compared to control was also observed by the addition of PVPP, PMSF, or EDTA (8.0, 7.8, and 7.3 pmol/min/mg respectively), while the addition of 2-mercaptoethanol or  $\rho$ -Aminobenzamidine and  $\epsilon$ -Amino-n-caproic acid decreased NOS activity as compared to the control (4.2 and 5.0 pmol/min/mg respectively).

### **Precipitation of Crude Extracts**

After extraction, crude protein extracts were precipitated to increase the relative concentration of NOS-like protein. It is common to use salts such as ammonium sulfate, sodium citrate, and sodium chloride to precipitate a particular protein of interest for further isolation step (Bollag et al., 1996). However, in this work, precipitation with ammonium sulfate, sodium citrate or sodium chloride at concentrations of 0.5, 1.0, 1.5 and 2.0 M caused substantial loss of NOS activity as compared to that in crude extract (Fig. 5A).

NOS activity in the supernatant was 2.5-29%, 14-23%, 4-18%, and in the pellets, 0-7%, 7-25.5%, 20-33% of that in crude extract after precipitation with ammonium sulfate, sodium citrate or sodium chloride respectively. Though various concentrations of sodium chloride were able to precipitate more NOS protein than

ammonium sulfate and sodium citrate, maximum NOS activity detected was only 18% of that in crude extract. Adding SMW pea extract to the pellets increased NOS activity by 37-77%, 30-31%, and 16-63% after precipitation with various concentrations of ammonium sulfate, sodium citrate and sodium chloride respectively.

Contrary to precipitation with salts, NOS activity was detected mostly in the supernatant and minimal in the pellet after precipitation with various concentrations of acetone and PEG (Fig. 5B & C). NOS activity in the supernatant was increased by 24-79% after precipitation with 10, 20, 30, 40 and 50% of acetone and 27-71% after precipitation with 10, 20 and 30% of PEG as compared to that in crude extract. The precipitation of non-NOS proteins in the pellet resulted in the increase of NOS activity in the supernatant which indicated that NOS protein of pea was soluble in organic solvent such as acetone and PEG. Addition of SMW pea extract to the pellets after precipitation with both acetone and PEG did not increase NOS activity as it did in precipitation with salts, and probably was due to the presence of NOS protein in the supernatant rather than in the pellets. Precipitation with 40% acetone resulted in the highest increase in NOS activity (79% or 4.7-fold) in the supernatant as compared to control (63.2 and 13.5 pmol/min/mg respectively), therefore, this step was included in the purification procedure.

### **Partial Purification of NOS-like Protein**

Table 1 describes the purification process of pea NOS-like protein, including treatment of crude extracts with 40% ethanol and DEAE-Sepharose ion-exchange

chromatography. NOS activity increased from 16 pmol/min/mg in the crude extracts to 255 pmol/min/mg in the DEAE eluted fractions that represented purification of 16-fold. Treatment with acetone was substituted with ethanol due to the acetone-sensitivity of Centricon Plus-20 cartridges. Treatment with 40% ethanol gave similar result in NOS activity as with 40% acetone.

Two protein peaks were observed in the DEAE-Sepharose elution profile, but NOS activity was detected only in the first peak as shown in Fig. 6. Fractions with NOS activity were eluted at between 0.135 to 0.20 M NaCl. These fractions were pooled, concentrated with Centricon Plus-20 cartridge and applied to affinity columns including 2',5'-ADP-Sepharose, arginine-Sepharose and calmodulin-agarose. Pea NOS-like protein did not bind to any of these columns as all NOS activity was detected in the flow-through. In addition, both protein concentration and NOS activity were minimal in the fraction eluted with 10 mM NADPH in the 2',5'-ADP-Sepharose chromatography (Table 2). The working condition of 2',5'-ADP-Sepharose column was verified previously by applying homogenate of sheep cerebellum to the column and substantially high NOS activity was detected in the fraction eluted with 10 mM NADPH.

NOS-like protein in the eluted fractions is stable when stored at -80°C in either 20% or 40% sucrose overnight and for three days as compared to the control in which sucrose was not added (Table 3). The profile of proteins isolated from each purification step was shown in Fig. 7. SDS-PAGE revealed the presence of more than 20 proteins, including NOS-like protein, in the DEAE eluted fractions.

## DISCUSSION

Systemic acquired resistance (SAR) is characterized by induction of a long-lasting, broad-spectrum, systemic resistance to subsequent infection (Gaffney et al., 1993). A signal is generated that is transported throughout the plant and that predisposes the plant to respond more effectively to subsequent infection. SAR is often associated with the development of a HR, followed by the accumulation of SA and pathogenesis-related (PR)-1 proteins in tissues distant from the infection site (Gaffney et al., 1993). SA accumulation is required but unlikely to be the initial or translocated signal for systemic induction (Rasmussen et al., 1991).

Copper chloride, Actiguard<sup>®</sup>, Triton-X100 and SA had been used to induce resistance in various species of plants in a systemic manner (Okuno et al., 1991; Hammerschmidt and Kuc, 1995; Rakwal et al., 1996). Copper chloride induced phytoalexin production in rice via the jasmonic acid (JA) metabolic pathway (Rakwal et al., 1996). Actiguard<sup>®</sup> contains active ingredient (i.a.) acibenzolar-s-methyl which functions through the same metabolic pathway as SA. Triton-X100 and Tween 20 (used in the control treatment) are surfactants used as wetting agents. JA and SA pathways are two different signaling pathways activated after the induction of defense responses to insects and microbes, respectively. However, the pathways converge at a point controlled by NPR1 (a regulatory protein) and confer similar enhanced defensive capacity against a broad spectrum of pathogens (van Loon et al., 1998).

NO also functions as a signal in plant disease resistance (Delledonne et al., 1998; Durner and Klessig, 1999). However, its effects are located upstream of SA and (PR)-1 proteins (Wendehenne et al., 2001). Fig. 1 in the General Introduction section illustrates a model for NO-mediated signaling pathway in plant defense against pathogens that includes pathways for defense gene activation (PAL and PR-1) and cell-death mediation. Applications of copper chloride, Actiguard<sup>®</sup>, Triton-X100 and SA to pea plants did not increase NOS activity significantly as compared to the control (Fig. 1). This verified the hypothesis that NO functions upstream of SA in the signaling pathway.

In the incompatible pea-bacteria interactions, NOS activity reached maximum level (~2-fold) at six hpi (Fig. 2). This event occurred approximately three hours before visible HR development. The result suggested that maximum synthesis of NOS, and correspondingly NO, occurred immediately before the onset of HR. This is consistent with the observation that HR is activated after the interaction of NO with reactive oxygen species (ROS), specifically with H<sub>2</sub>O<sub>2</sub> (Delledonne et al., 2001). However, the maximum level of NOS activity (~2-fold) was relatively low as compared to three-fold and several-fold increase in NOS activity in the incompatible interactions of *R. solanacearum*-tobacco (Huang & Knopp, 1998) and TMV-tobacco (Chandok et al., 2003), respectively. Although the occurrence of maximum NOS activity and HR symptoms development was consistent, the observation of relatively low level of NOS activity suggested that NO may also be generated during HR by enzymes other than the pea NOS-like enzyme.

The pattern of NOS activity in pea observed over time in the incompatible pea-bacteria interactions suggested an inducible form of NOS (Fig. 2). A similar temporal pattern of NOS activity during the incompatible TMV-tobacco interactions was also reported (Chandok et al., 2003). Though the pattern of NOS activity throughout the infection process was consistent among experiments in this study, the level of NOS activity was highly variable, similar to that reported by Chandok et al. (2003).

In the compatible pea-bacteria interactions (disease development), NOS activity continued to increase to a maximum level (~2.5-fold) at 24 hpi (Fig. 2). The result was in contrast to other reports where no increase in NOS activity was observed in the compatible interactions (Chandok et al., 2003; Durner et al., 1998). The maximum level of NOS activity observed in the incompatible pea-bacteria interactions was similar to the level of NOS activity in the compatible interactions at six hpi. The higher levels of NOS activity observed in compatible interactions suggested that the pea NOS-like enzyme was involved in the general response of pea to biotic stress. This may be due to the use of a high concentration of bacteria cells ( $10^8$  cfu/mL), similar to that of the incompatible bacteria, which may cause overwhelming physiological responses from the host cells. Under field conditions, disease occurred when *Pseudomonas syringae* populations exceeded approximately  $10^4$  cfu per gram on symptomless leaflets (Lindemann et al., 1984).

Pea leaf tissues at six hpi with *R. solanacearum* were used to extract NOS-like protein for purification at maximal NOS activity levels in HR tissues. Typically, NOS protein extractions from tissues of animals, fungi, bacteria, or plants were

performed at pH 7.4-7.5 which reflects the physiological environment of the cells (Huang et al., 1997; Pollock et al., 1991; Bredt and Snyder, 1990; Mayer et al., 1990; Chen and Rosazza, 1995; Ninnemann and Maier, 1996; Cueto et al., 1996; Ribeiro et al., 1999). In this report, NOS activity was optimal when protein extractions were performed at pH 8.5 and 9.0 at 23-25°C. These conditions differed from those of *Flammulina velutipes* (pH 8.0 at 50°C), *Norcadia* sp. (pH 7.0-7.5 at 30°C), and mammals (pH 7.5-7.8 at 37°C) (Song et al., 2000; Chen and Rosazza, 1995; Hevel et al., 1991; Stuehr et al., 1991). The finding indicated that different species of NOS protein performed optimally at different conditions. NOS-like protein of pea was efficiently extracted under alkaline conditions.

In addition, NOS protein extractions from tissues of the above mentioned organisms typically include a cocktail of protease inhibitors, metal ion chelators, reducing agents, and phenolic chelators. These chemicals are important components in the isolation process and they protect the protein of interest from proteolysis and oxidation. This is especially important in the isolation of NOS-like protein of pea since it loses its activity rapidly in each step of the isolation process. In this work, adding 10 mM EGTA and 1 mM EDTA (metal ion chelators and metalloprotease inhibitors), 1  $\mu$ M leupeptin and 1 mM PMSF (serine and thiol protease inhibitors), and 1% PVPP (phenolic chelator) in the extraction buffer retained NOS activity significantly (Fig. 4B). Using high doses of EGTA (10 mM) also help to disrupt and extract membrane-associated proteins (van Renswoude and Kempf, 1984).

The precipitation of NOS-like protein of pea with various concentrations of salts (ammonium sulfate, sodium citrate, and sodium chloride) was either minimal or the salts caused the precipitated NOS-like protein to lose activity rapidly (Fig. 5A). The protein did not seem to be present in the supernatant because NOS activity was only 2.5-29% of that in crude extract. On the contrary, NOS proteins of *Physarum polycephalum*, *Flammulina velutipes*, tobacco, and animals were successfully precipitated with 30-75% of ammonium sulfate and with minimal loss in NOS activity (Golderer et al., 2001; Song et al., 2000; Chandok et al., 2003; Mayer et al., 1990; Fossetta et al., 1996).

Substantial increase in NOS activity in the supernatant and minimal activity in the pellets after precipitation with organic solvents (acetone and PEG) indicated that NOS-like protein of pea was aqueous-soluble (Fig. 5B and C). Proteins that are not precipitated by organic solvents are categorized as hydrophobic proteins, particularly those that are located in the cellular membranes (Bollag et al., 1996). The result indicated that the NOS-like protein of pea might be located within cellular membranes. However, further verifications need to be made regarding this aspect. The detection of NOS activity in the soluble fraction of the crude extracts after centrifugation may be due to the release of NOS-like protein bound to the membranes when a high dose of EGTA (10 mM) was used during protein extraction.

In mammals, bNOS is typically localized in neurons in both soluble and particulate forms, iNOS in macrophages in soluble form, while eNOS in endothelial cells in particulate form (Bredt and Snyder, 1990; Stuehr et al., 1991; Pollock et al., 1991). An isoform of NOS localized in the inner membrane of rat liver mitochondria

was also reported (Tatoyan and Giulivi, 1998). A NOS-like protein was detected either in the cytosol of cells in the division zone or nucleus of cells in the elongation zone of maize root tips (depending on the growth phase of cells) using antibody against mouse macrophage NOS labeled with fluorescein isothiocyanate (FITC) (Ribeiro et al., 1999). NOS-like protein of pea plants was localized in the matrix of peroxisomes (perNOS) and chloroplasts using electron microscopy of immunogold-labeling with antibody against murine iNOS (Barroso et al., 1999). Moreover, real-time imaging of NO production in epidermal tobacco cells treated with the fungal elicitor cryptogein from *Phytophthora cryptogea* using fluorophore diaminofluorescein diacetate (DAF-2DA) in conjunction with confocal laser scanning microscopy was able to detect NO in the cytosol, along the plasma membrane, in chloroplasts, and in organelles probably representing peroxisomes (Foissner et al., 2000).

The purification of mammalian NOS has been conducted with relative ease using 2',5'-ADP-Sepharose affinity chromatography after crude sample preparation with ammonium sulfate precipitation or ion-exchange chromatography (Bredt and Snyder, 1990; Stuehr et al., 1991; Pollock et al., 1991; Tatoyan and Giulivi, 1998). NOS proteins from other species (*Nocardia* sp., *Flammulina velutipes*, *Physarum polycephalum*, and tobacco) have been purified using a combination of chromatography techniques including affinity, hydroxylapatite, ion exchange, mono P chromatofocusing, and gel filtration chromatographies (Chen and Rosazza, 1995; Song et al., 2000; Golderer et al., 2001; Chandok et al., 2003). Song et al. (2000) reported that NOS of *Flammulina velutipes* did not bind to an anion exchanger, and

purification of *Nocardia* NOS was more successful using hydroxylapatite chromatography than anion-exchange or gel filtration chromatographies as a final step in addition to 2',5'-ADP-agarose chromatography (Chen and Rosazza, 1995).

In this work, the NOS-like protein of pea bound to DEAE-Sepharose ion-exchange column and was eluted successfully with a linear gradient of 0.135 to 0.20 M NaCl. However, the protein did not bind to any of the affinity columns, i.e., 2',5'-ADP-Sepharose, arginine-Sepharose or calmodulin-agarose columns. Similarly, Lo et al. (2000b) found that homogenates of 3 day-old mung bean roots did not bind to 2',5'-ADP-Sepharose or arginine-Sepharose. The successful isolation of a tobacco NOS-like protein which co-purified with three other proteins using affinity columns successful for mammalian NOS (Chandok et al, 2003) contrasts to the results above with a pea NOS-like protein.

The findings suggest that pea NOS-like protein may be significantly different in structure from animal NOS and a couple of reports support this hypothesis. Chandok et al. (2003) reported that the pathogen-induced, NO-synthesizing enzyme in tobacco and *Arabidopsis* (*varP*), designated iNOS, is a variant form of the P protein of glycine decarboxylase (GDC). Plant iNOS activity required H<sub>4</sub>B, FAD, NADPH, O<sub>2</sub>, Ca<sup>2+</sup> and CaM which may account for the success in affinity substrate purification. The cDNA sequence of *varP* of *Arabidopsis* contained binding sites for CaM, flavin, pyridoxal phosphate/putative NADPH-Rossmann fold, and a leucine zipper. Another NOS protein identified in *Arabidopsis* (AtNOS1) was constitutively expressed and its activity depended upon NADPH, Ca<sup>2+</sup> and CaM but not H<sub>4</sub>B, FAD, FMN or heme (Guo et al., 2003). The cDNA sequence has homology to a sequence

in the snail *Helix pomatia* that encodes a protein that has been implicated in NO synthesis and contained ATP/GTP binding domains (Huang et al., 1997). Thus, both NOS-like enzymes are distinct from those in mammals and this may explain the difficulties encountered in attempts to isolate NOS-like protein from plants.

Although perNOS could be detected in the peroxisomes and chloroplasts of pea (Barroso et al., 1999), to date neither its purification nor the identification of the gene encoding the protein has been reported. In addition, although NOS gene has been identified in *Arabidopsis* through screening of mutants (Guo et al., 2003), the protein itself was not purified from crude extracts.

The fact that NOS-like protein of pea did not bind to 2',5'-ADP-Sepharose and CaM-agarose indicated that the protein lacks binding sites for NADPH and CaM. This suggests that the NOS-like protein of pea is more similar to AtNOS1 rather than *varP*. The cDNA of AtNOS1 does not contain binding sites for any known mammalian cofactors while cDNA of *varP* contains binding sites for both NADPH and CaM. In addition, the cofactors requirement for NOS activity of the pea NOS-like protein was similar to that of AtNOS1 in that both proteins dependent upon NADPH, Ca<sup>2+</sup> and CaM as cofactors while *varP* protein requires H<sub>4</sub>B, FAD, NADPH, Ca<sup>2+</sup> and CaM as cofactors.

Another aspect of NOS activity besides the involvement of cofactors is the interactions of the enzyme and other proteins in the cells. In mammalian cells, NOS activity (i.e. NO formation) is affected by the interactions of the enzyme and more than 20 interacting proteins (Nedvetsky et al., 2002). For example, calmodulin binding is required for electron transfer (Bredt and Snyder, 1990), NOSTRIN (NOS3

traffic inducer) binding is important for intracellular trafficking of NOS3 (Zimmermann et al., 2002), and Dynamin-2, Porin and protein kinase B/Akt are required for activation (Cao et al., 2001; Sun and Liao, 2002; Dimmeler et al., 1999). The stability of NOS homodimers is also affected by protein-protein interactions. For example, the binding of PIN (a protein inhibitor of NOS1) to NOS1 or NAP110 (a protein inhibitor of NOS2) to NOS2 prevent dimerization of these enzymes, a configuration required for activation (Jaffrey and Snyder, 1996; Ratovitski et al., 1999).

The rapid loss of activity of pea NOS-like protein observed in the purification process may be due to the disruption of protein configuration, or the dissociation of some interacting proteins or unidentified cofactors, e.g., the SMW(<8 kDa) filtrate of pea leaf extract. In this report, adding the SMW filtrate to the proteins pelleted after precipitation by various concentrations of salts restored NOS activity at various degree of significance. These phenomena had also been reported by Huang et al. (1997) during the purification of NOS protein from *Helix pomatia*. Although the 60-kDa protein was eluted from 2',5'-ADP-agarose column with NADPH, and its activity was NADPH dependent, the cDNA sequence revealed no consensus binding site for NADPH. Furthermore, a calmodulin antagonist blocked enzyme activity although the predicted peptide did not show a consensus binding site for calmodulin. The authors mentioned that the 60 kDa protein is an unusually low molecular mass for NOS and suggested that the protein is associated with NOS and affects its activity but may not be the enzyme itself. The protein had an ATP/GTP binding site and putative myristoylation sites that suggested that it may be associated with

membrane binding of NOS. The authors also explained the unusual properties of the 60 kDa protein by assuming that the protein binds to NOS and under denaturing conditions liberate the protein from NOS. Similarly, during the purification of pea NOS-like protein, denaturing conditions may cause some interacting proteins or cofactors be dissociated from the NOS-like protein.

The monomer molecular mass of mammalian NOS are in the range of 130 to 160 kDa (Wendehenne et al., 2001). Huang and Knopp (1998) reported the detection of a single immunoreactive band in both extracts of the control and hypersensitive tobacco leaves after incubation with antibody raised against mammalian NOS. The relative intensity of the immunoreactive band in HR tissues appeared to be much higher than that in control tissues. The molecular mass of the protein band was estimated to be 55 kDa. Pea NOS-like protein may have different structure than its animal counterparts, and thus, the size of NOS-like protein and the ability to bind mammalian anti-NOS antibodies are unpredictable. The detection of multiple immunoreactive bands in this report implied that using antibodies raised against mammalian NOS to detect plant NOS must be interpreted cautiously and verified by functional assays of NOS activity in immunoreactive protein bands. Huang et al. (1997) and Lo et al. (2000a) also suggested caution in using antibodies raised against mammalian NOS for immunodetection of NOS.

Barroso et al. (1999), however, detected a single band of approximately 130 kDa when peroxisomal fractions of pea were probed with polyclonal antibody against murine iNOS. Using samples containing only peroxisomal fractions for NOS detection would enrich for proteins that could potentially bind to the iNOS antibody

as compared to the crude extracts that we used in this work. This resulted in a much greater degree of specificity for the binding of iNOS antibody. Based on the result of Western blot analysis, we were not able to use antibodies raised against mammalian NOS to specifically detect the localization of NOS-like protein of pea. No protein band of ~130 kDa in the crude pea leaf extracts separated here by PAGE bound any mammalian NOS antibodies used, but in contrast, a single band of 30 kDa bound the mammalian iNOS antibody in SDS-PAGE separations.

A 784-bp partial cDNA from pea with 50% nucleotide similarity to AtNOS1 was successfully cloned from pea (Chapter 2). Since the cDNA encoding a 105 kDa P protein of pea similar to *varP* (Chandok et al., 2003) has been cloned (Turner et al., 1992), a direct comparison of pea P protein gene expression compared to an AtNOS1 homologue in pea could be considered in compatible and incompatible pathogen interactions (Chapter 2).

In summary, the application of systemic resistance-inducing chemicals did not induce NOS activity. NO generation was involved in both incompatible and compatible interactions of pea and bacteria although the source of NO generation may involve multiple NO-synthesizing enzymes including a NOS-like protein of pea, thus, suggesting that NO generation may be a general response to biotic stress in plants. A NOS-like protein exists in pea. Polyclonal antibodies raised against mammalian NOS had no specificity to isolate pea NOS-like protein. The pea NOS-like protein was efficiently extracted from leaf tissues at pH 8.5-9.0 in homogenization buffer added with 10 mM EGTA, 1 mM EDTA, 1  $\mu$ M leupeptin, 1 mM PMSF, and 1% PVPP. Partial purification steps for pea NOS-like protein

purification included treatment of crude extracts with 40% ethanol, supernatant of the treated sample passed through an ion exchange DEAE-Sepharose column and eluting bound proteins (including pea NOS-like protein) with a linear gradient of 0.135-0.2 M NaCl. Pea NOS-like protein did not bind to 2',5'-ADP-Sepharose, Arg-Sepharose and CaM-agarose suggesting that the protein lacks binding sites for NADPH and calmodulin as cofactors. The pea NOS-like protein appears to be more similar to AtNOS1 rather than *varP* based on information related to affinity substrates, and cofactor requirements for NOS activity. The observed similarity to AtNOS1 combined with conditions above for enrichment of maximal NOS activity from pea extracts will be useful for purification of NOS-like protein from pea.

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Table 1 Partial purification of pea NOS-like protein

Step	Total protein (mg)	Total activity (pmol/min)	Specific activity (pmol/mg/min)	Recovery (%)	Purification (-fold)
Crude extract	689	11024	16	100	1
Treatment with 40% ethanol	246	7626	31	69	2
DEAE-Sepharose	136	6231	255	56	16

Table 2 NOS activity during 2',5'-ADP-Sepharose chromatography

Fraction	Total Protein ( $\mu\text{g/mL}$ )	NOS Activity (cpm/hr)
Crude extract	0.526	15140
Flow-through	0.468	14040
Eluate (with 10 mM NADPH)	0.008	180

Table 3 Storage of partially purified NOS protein at  $-80^{\circ}\text{C}$

Sucrose (%)	Specific Activity (pmol/mg/min)	
	Overnight	3 days
0	18.813	17.361
20	17.724	17.724
40	18.329	15.909

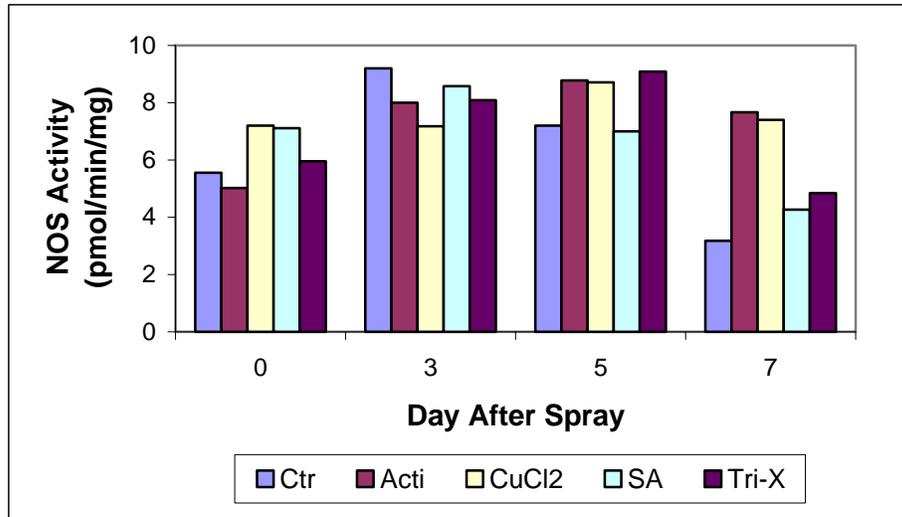


Fig. 1 Effects of abiotic agents on NOS activity of pea. Leaves of  $12 \pm 2$  days old plants were sprayed with four types of chemicals that induced systemic resistance in plants: Actiguard (25 mg a.i./L), Copper Chloride (10 mM), Salicylic Acid (2.5 mM) and Triton-X100 (0.1%). Tween 20 was used as an adhesive agent and also as a control. Treated plants were kept in greenhouse at temperature cycle of  $30/26 \pm 2^\circ\text{C}$  and leaves were harvested at 0, 3, 5 and 7 days after spray. NOS activity for each time point was determined using citrulline formation assay (Bredt and Snyder, 1990). Standard error = 1.22 (n=3).

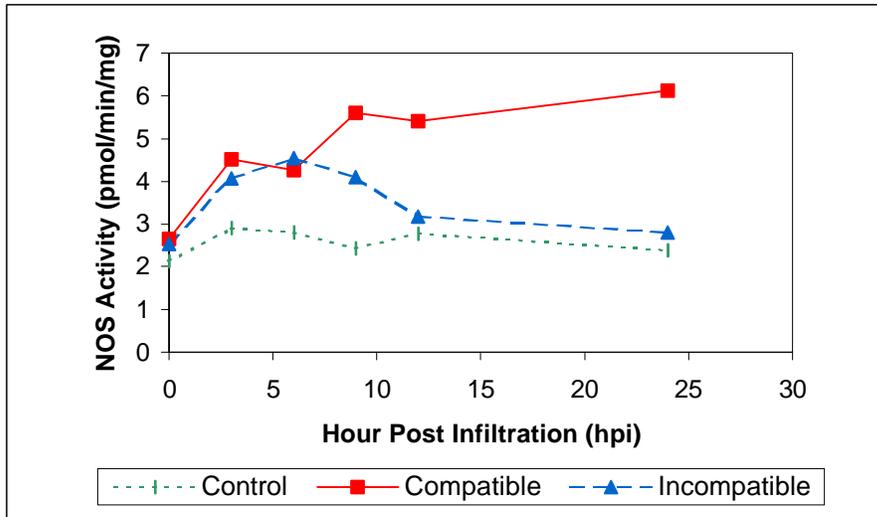


Fig. 2 Effects of biotic agents on NOS activity of pea. Leaves of  $10 \pm 2$  days old plants were vacuum infiltrated with incompatible bacteria *Ralstonia solanacearum* ( $7.0 \times 10^8$  cfu/mL), compatible bacteria *Pseudomonas syringae* pv *pusi* ( $7.0 \times 10^8$  cfu/mL), and distilled water as a control. Treated plants were kept in greenhouse at temperature cycle of  $30/26 \pm 2^\circ\text{C}$  and infiltrated leaves were harvested at intervals of 0, 3, 6, 9, 12, and 24 hpi. NOS activity for each time point was determined using citrulline formation assay (Bredt and Snyder, 1990). Standard errors for control, compatible and incompatible interactions were 0.68, 0.59 and 0.60, respectively ( $n=3$ ).

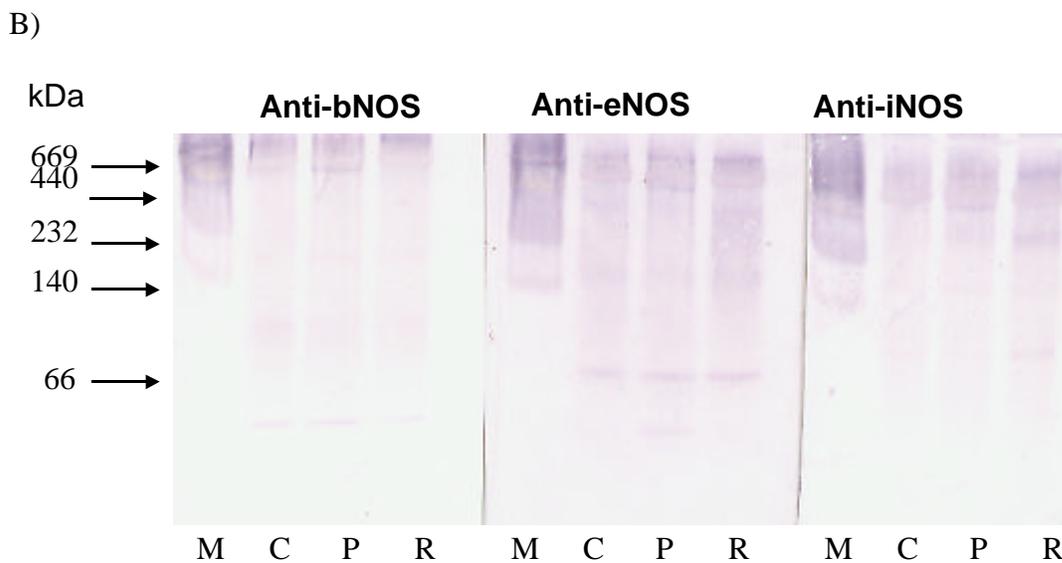
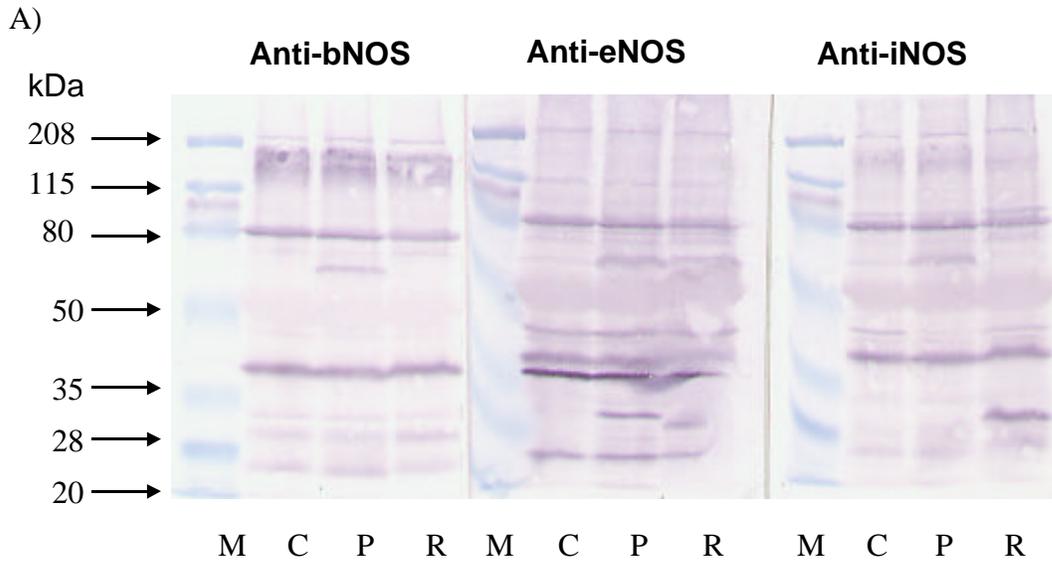
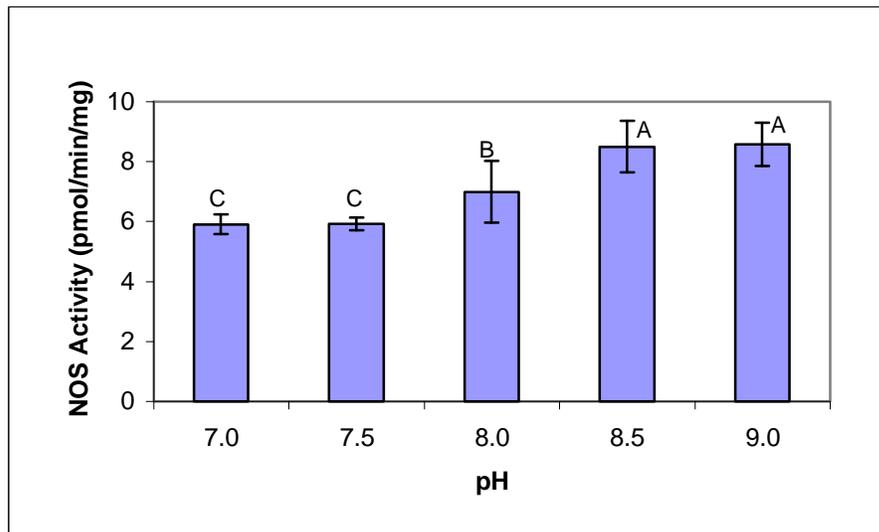


Fig. 3 Immunoblot analysis of pea proteins with antibodies raised against mammalian NOS. Samples loaded in 2% SDS PAGE (A), and 5% non-denaturing PAGE (B) were blotted onto nitrocellulose membranes and probed with three polyclonal antibodies against rabbit mammalian bNOS, eNOS and iNOS. Lane 1, Molecular mass markers (M); 2, Control tissues (C); 3, Tissues from compatible interactions (P); 4, Tissues from incompatible interactions (R). Duplicate experiments were performed.

A)



B)

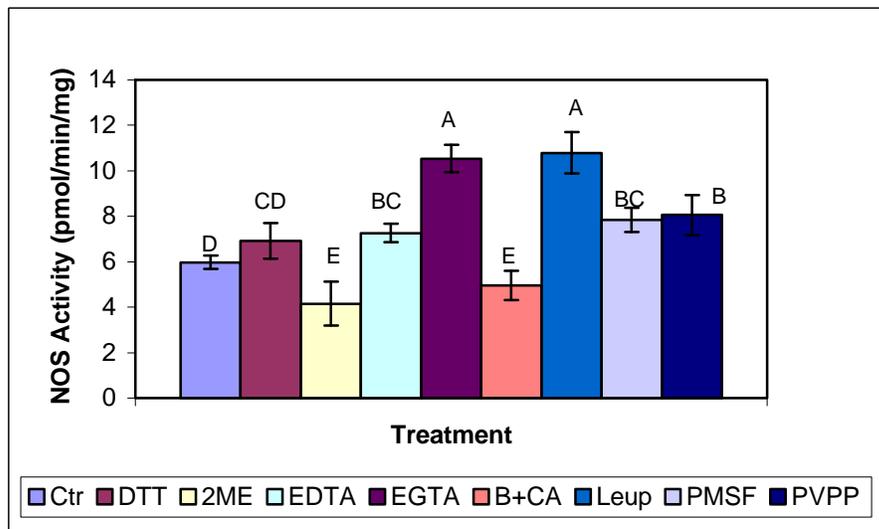
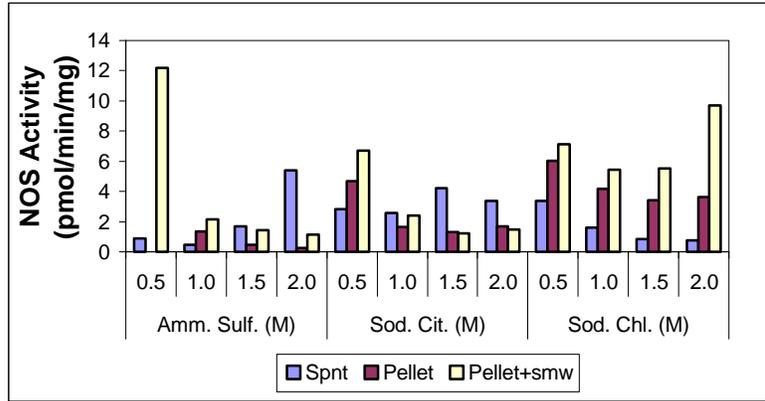
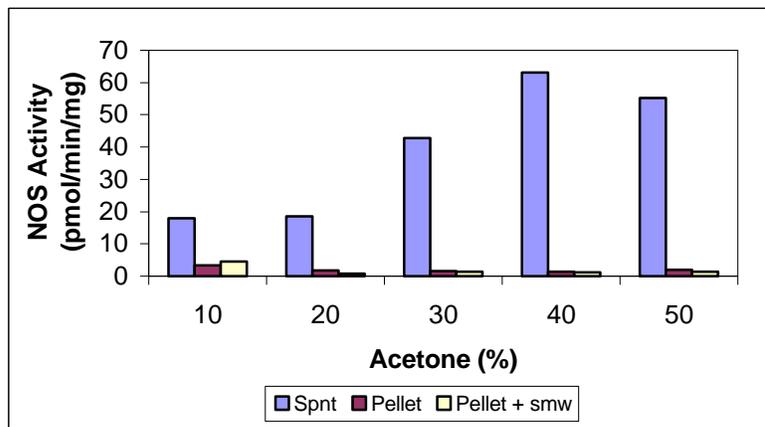


Fig. 4 Effects of pH, reducing agents, metal chelators, protease inhibitors and phenolic chelator on NOS activity during protein extraction. (A) One gram of ground tissue was homogenized with 50 mM Tris-HCl at pH 7.0, 7.5, 8.0, 8.5, and 9.0 independently. pH 8.5 was selected to be used in the subsequent experiment. (B) The following chemicals were added separately during homogenization: dithiothreitol (DTT, 1 mM), and 2-mercaptomethanol (10  $\mu$ l/10ml) as reducing agents; EDTA (1 mM), and EGTA (10 mM) as metallo-proteases inhibitors; *p*-Aminobenzamidine and  $\epsilon$ -Amino-n-caproic acid (1 mM and 5 mM respectively), leupeptin (1  $\mu$ M), and PMSF (1 mM) as protease inhibitors, and polyvinylpolypyrrolidone (PVPP, 1%) as phenolic chelator. NOS activity was determined using citrulline formation assay (Bredt and Snyder, 1990). Error bars indicate standard deviation (n=3). Means with the same letter are not significantly different.

A)



B)



C)

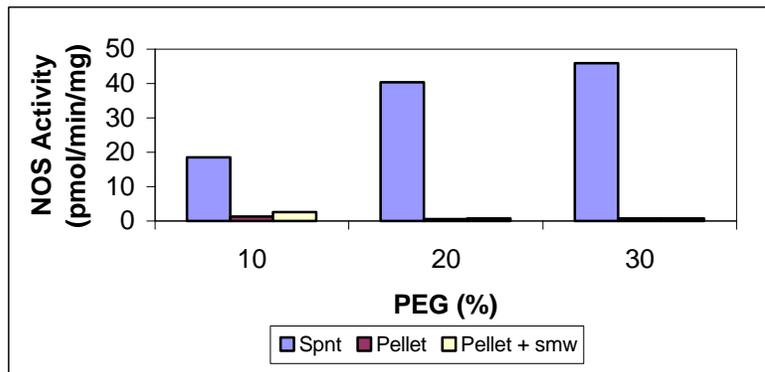


Fig. 5 Precipitation of crude extracts of pea for maximal NOS-like protein isolation. Concentrations of 0.5, 1.0, 1.5 and 2.0 M of ammonium sulfate, sodium citrate and sodium chloride (A); 10, 20, 30, 40 and 50% acetone (B); and 10, 20 and 30% polyethylene glycol (PEG) (C) were added separately in each crude extract and centrifuged at 4°C for 30 min at 20,000g. NOS activity for supernatants, pellets, and pellets added with small molecular weight (SMW) filtrate was determined using citrulline formation assay (Bredt and Snyder, 1990). SMW filtrate was prepared from supernatant of crude extracts filtered through Centricon Plus-20 cartridges. NOS activity for crude extract in experiment A was 18.461 pmol/min/mg, and for experiments B and C was 13.490 pmol/min/mg. Data shown are the means of two experiments.

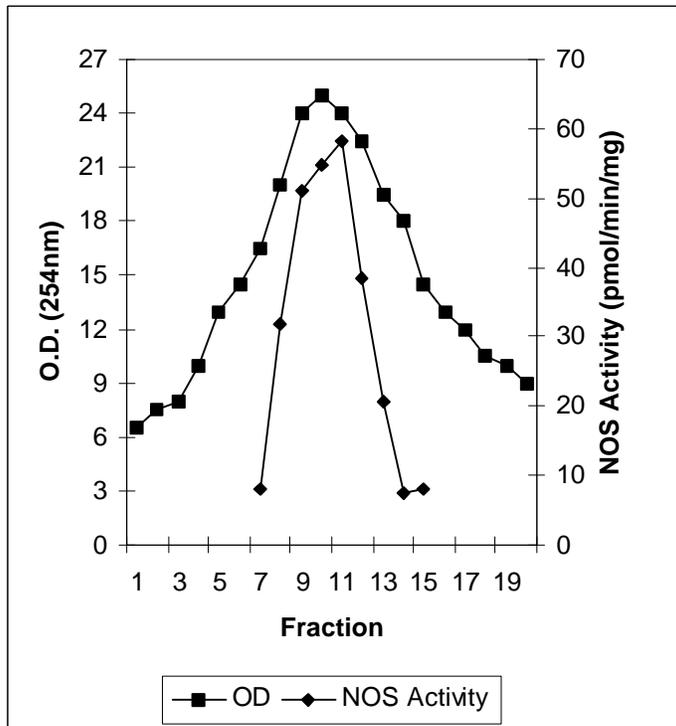


Fig. 6 Partial purification of pea NOS-like protein. Seventy grams of treated tissues was used for protein extraction. Supernatant of crude extract after treatment with 40% ethanol was passed through DEAE-Sepharose fast flow at a flow rate of 66 mL/hr and equilibrated with equilibration buffer containing 50 mM Tris-HCl, pH 8.5, 10 mM EGTA, 1  $\mu$ M leupeptin, and 1 mM PMSF. The protein was eluted with a linear gradient of 0 to 0.3 M NaCl in equilibration buffer. Fractions of 3.4 mL were collected and each fraction was assayed for NOS activity and total protein content. Fractions with NOS activity were pooled and concentrated with Centricon Plus-20 cartridges to half the volume for affinity chromatographies.

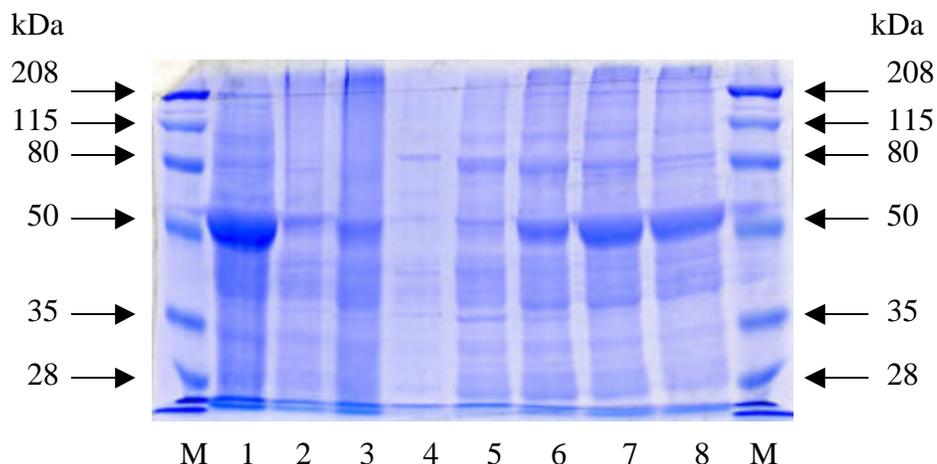


Fig. 7 SDS-PAGE of proteins isolated from each purification step. Proteins from crude extracts of treated tissues, were separated by 10% (w/v) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples (50 $\mu$ g) were prepared in 0.5 M Tris-HCl (pH 6.8), containing 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-Mercaptoethanol, and 0.125% (w/v) Bromophenol Blue, and were heated at 95°C for 4 min. Gels were run at 180V for 45 min and stained with 0.1% Coomassie brilliant blue R-250. Lane M, molecular weight standards; lane 1, crude extract; lane 2, supernatant treated with 40% ethanol; lane 3, concentrated supernatant treated with 40% ethanol; lanes 4 to 8, fractions with NOS activity eluted from DEAE-Sepharose chromatography, lane 7 showed fraction with the highest NOS activity.

## **CHAPTER 2**

### **EXPRESSION AND CLONING OF A GENE ENCODING A NOS-LIKE PROTEIN OF PEA (PISUM SATIVUM L.)**

## INTRODUCTION

In mammalian systems, cloning of cDNA encoding nitric oxide synthase (NOS) of any isoform was performed by constructing a cDNA library from NOS-induced cells and identifying positive clones by plaque hybridization. Probe for plaque hybridization was prepared using PCR and primers designed based on either conserved binding sites for cofactors (Lyons et al., 1992; Nakane et al., 1993; Gnanapandithen et al., 1996), or amino acid sequence from isolated protein (Michel and Lamas, 1992).

In *Helix pomatia*, the cDNA encoding the 60-kDa NOS-like protein was isolated by probing a *Helix* expression cDNA library with an antibody to human neuronal NOS (Huang et al., 1997). The cDNA sequence contained an open reading frame of 1,377-bp (EMBL; accession no. X96994), and had a poly(A) tail but no stop codon. The cDNA sequence also contained an ATP/GTP binding site, and putative myristoylation sites, but did not contain consensus sequences for NADPH, FAD, arginine, and calmodulin (CaM) binding sites.

In *Physarum polycephalum*, two similar cDNA sequences were isolated by screening a *Physarum* cDNA library with a 600-bp probe generated by PCR using primers designed to recognize either nucleotide sequences deduced from isolated peptides or consensus regions of other available NOS sequences from mammals, birds, insects, and a mollusc (Golderer et al., 2001). The two cDNAs were designated physnosa (3571-bp, GenBank accession no. AF145041) and physnosb (3316-bp, GenBank accession no. AF145040) and contained complete reading

frames for NOS. Both sequences also contained binding motifs of mammalian NOS for FMN, FAD, NADPH, CaM, H<sub>4</sub>-B, zinc and caveolin but lack the binding site for calcium.

The cloning of NOS genes from plants has not been straightforward. The direct application of mammalian NOS sequence or binding motifs has not been fruitful to isolate and identify NOS homologues in plants. The first isolation of a plant protein with NOS activity, and its corresponding gene, was only recently reported (Chandok et al., 2003). The NO-synthesizing enzyme is a variant form of the P protein (*varP*) of the glycine decarboxylase complex (GDC) and was isolated from pathogen-challenged tobacco, and thus, designated iNOS. The glycine decarboxylase complex is involved in the glycine cleavage system that catalyzes the oxidation of glycine. The GDC is composed of four proteins: P (containing pyridoxal phosphate), T (a transferase responsible for producing 5,10-methylenetetrahydropteroyl-L-glutamic acid), H (a carrier protein containing lipoamide), and L (lipoamide dehydrogenase required to complete the cycle). The P protein, which is considered the true glycine decarboxylase (Hiraga and Kikuchi, 1980), binds the alpha-amino of glycine through its pyridoxal phosphate cofactor, CO<sub>2</sub> is released and the remaining methylamine moiety is then transferred to the lipoamide cofactor of the H protein. The tobacco iNOS has an estimated molecular mass of 115-120 kDa and its activity required H<sub>4</sub>B, FAD, NADPH, O<sub>2</sub>, Ca<sup>2+</sup> and CaM. The cDNA (*varP*) sequence contained binding sites for CaM, flavin, pyridoxal phosphate/putative NADPH-Rossmann fold, and a leucine zipper. Despite the lack

of sequence homology, the biochemical and kinetic properties of the *varP* enzyme share similarity to those of its animal counterparts (Chandok et al., 2003).

When this project was initiated in the year 2000, there was no report on the isolation and identification of NOS-like protein or gene in plants. Subsequently, a database search of the completed *Arabidopsis* genome identified two potential plant NOS genes (Table 1): At4g09680 homologous to NOS in *Rattus norvegicus* (rat) and At3g47450 homologous to the br-1 protein associated with NOS in *Helix pomatia* (snail). As this thesis progressed, the At3g47450 accession was found by an independent lab to encode a protein associated with NOS activity in plant hormonal response (Guo et al., 2003). The 561 amino acid AtNOS1 protein has a molecular mass of 62 kDa, is constitutively expressed in the cells, and is involved in hormonal signaling. The NOS activity of AtNOS1 was dependent upon NADPH, CaM and Ca<sup>2+</sup>, but not H<sub>4</sub>B, FAD, FMN or heme as cofactors. However, the NOS activity of AtNOS1 was inhibited by L-NAME, properties similar to mammalian eNOS and nNOS (Guo et al., 2003). The cDNA sequence had homology to a sequence in the snail *Helix pomatia* that encodes a protein implicated in NO synthesis and contained ATP/GTP binding domains (Huang et al., 1997).

A cDNA encoding the P protein of GDC in pea had previously been cloned (Turner et al., 1992). The P protein of pea had a predicted molecular mass of 105 kDa and a binding site for cofactor pyridoxal phosphate (PP) at amino acid (aa) 792 of the PLP peptide segment (aa 758-811) (Turner et al., 1992). The PP binding site was implicated to participate in NO synthesis as was demonstrated by *varP* (Chandok et al., 2003). Sequence comparison of the PLP peptide segment of *varP*

and P protein of pea revealed 98% (53/54) amino acid similarity (Chandok et al., 2003). The P protein of GDC of pea was designated peaP and any potential AtNOS1 homologue in pea was designated peaNOS.

The available cDNA of a P protein of GDC in pea (Turner et al., 1992) similar to *varP* (Chandok et al., 2003) and the identification of AtNOS1 (Guo et al., 2003), provided a basis for the cloning of potential NOS gene(s) from pea and to associate their expressions with the NOS activity observed in pea-bacteria interactions in Chapter 1. This study described attempts to clone homologues of At4g09680 and At3g47450 expressed in pea and to monitor the expression of NOS gene candidates (homologues of *varP* and AtNOS1) in pea using real-time RT-PCR (Morrison et al., 1998) as related to NOS activity during pea-bacteria interactions.

## MATERIALS AND METHODS

### Cloning of A Gene Encoding A NOS-like Protein (putative pea-NOS) in Pea

RNA from leaf tissues of pea (*Pisum sativum*, cv Alaska) undergoing hypersensitive response (HR) was used for RT-PCR cloning of a gene encoding a NOS-like protein (putative pea-NOS) in pea. Pea seedlings were grown in a growth chamber at temperature cycle of 30/26°C, and 12 hr light cycle. Leaves of 8±1 day-old plants were vacuum infiltrated with incompatible bacteria, *Ralstonia solanacearum* ( $2.0 \times 10^9$  cfu/mL). Infiltrated leaves were harvested at four hr post infiltration (hpi) just prior to the maximal NOS activity level detected in Chapter 1, frozen in liquid nitrogen, and stored at – 80°C before use.

Treated leaf tissues (100 mg) were ground in liquid nitrogen, and total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Valencia, CA) per the manufacturer's protocol. Residual genomic DNA was removed by on-column digestion using the RNase-Free DNase Set (Qiagen, Valencia, CA). Total RNA was eluted from the matrix with 2x50 µL of RNase-free water. Two micrograms of total RNA was reverse-transcribed to produce single-stranded cDNA in a 20 µL reaction containing SuperScript Reverse Transcriptase (200U/µL; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cDNA was precipitated and resuspended in 20 µL nuclease-free water. Reactions "minus reverse transcriptase" were included to check for DNA contamination.

A number of PCR primer sets based upon conserved binding domains of mammalian NOS were used with pea cDNA in initial (failed) attempts to clone an

expressed NOS homologue from pea (Appendix A). Since cDNA of the P protein of GDC in pea had been cloned (Turner et al., 1992) that was similar to *varP* (Chandok et al., 2003), the attempts to clone a gene encoding a peaNOS protein in pea were based on DNA sequence of GenBank accessions At4g09680 and At3g47450 (encoding potential NOS protein) of *Arabidopsis thaliana*, and using cDNA of pea HR tissues as a template in reverse transcription-polymerase chain reaction (RT-PCR).

PCR was run at a final volume of 25  $\mu$ L using reagents and *Taq* polymerase from Invitrogen (Carlsbad, CA) according to the manufacturer's protocol. PCR conditions were an initial denaturation step at 94°C for 2 min, followed by 40 cycles of 94°C for 1 min, 60°C for 40 sec, 72°C for 1.5 min, and a final elongation step at 72°C for 10 min. cDNA fragments generated from RT-PCR were sequenced at the Interdisciplinary Center for Biotechnology Research DNA Sequencing Core Laboratory at the University of Florida (Gainesville, Florida, FL). Primers were designed using Primer3 program ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) and ordered from Sigma-Genosys (The Woodlands, TX).

### **DNA Sequence Analysis**

DNA sequence search for homology was performed using NCBI GenBank and TAIR databases with BLAST program. Multiple sequence alignment was performed using CLUSTAL-W program of EMBL-EBI and protein motif search using PROSITE program.

## **Preparation of Materials for Correlation Study of Relative Gene Expression of P Protein of Pea (peaP) and NOS Activity**

Pea (*Pisum sativum*, cv Alaska) seedlings were grown in a growth chamber at temperature cycle of 30/26°C, and 12 hr light cycle. Leaves of 8±1 day-old plants were vacuum infiltrated with incompatible bacteria, *R. solanacearum* (2.0x10<sup>9</sup> cfu/mL), compatible bacteria, *Pseudomonas syringae* pv *pisi* (1.0x10<sup>6</sup> cfu/mL), and distilled water as a control. The concentration of *Ps* pv *pisi* used here was lower as compared to that in Chapter 1 (7.0x10<sup>8</sup> cfu/mL) to simulate bacteria populations in the natural environments. Infiltrated leaves were harvested at intervals of 0, 3, 6, 9, 12, and 24 hpi, frozen in liquid nitrogen, and stored at -80°C before use. The experiment was repeated four times. NOS activity for each sample was determined by citrulline formation assay (Bredt and Snyder, 1990) as described in Chapter 1.

## **Real-time RT-PCR and Gene Expression of peaP**

Since the confirmation of an At3g47450 homologue was not achieved in pea, only the relative gene expression of peaP was compared to NOS activity in pea-bacteria interactions. A two-step real-time RT-PCR was performed using Applied Biosystems GeneAmp 7000 Sequence Detection System, ABI Prism (AB SDS) and the SBYR green PCR kit as recommended by the manufacturer (Applied Biosystems, Foster City, CA). Leaf tissues at time zero was used as a calibrator and pea  $\beta$ -actin as an endogenous control. RNA and cDNA samples used were prepared as described in the section above.

Primers for real-time PCR were designed using Primer Express software (Applied Biosystems, Foster City, CA). The following primer sets were used: 1) *peaP* (based on P protein of pea; Turner et al., 1992), forward primer: 5'-GTGACCACCACCGCAATGT-3', reverse primer: 5'-CCATAGCAGCACTAGCAGGATTT-3'; and 2) *pea-actin*, forward primer: 5'-CAATGGCAGAATCCGAAGATATT-3', reverse primer: 5'-CTCCTGCAAATCCAGCCTTAA-3'.

Reaction mixtures contained the following: 12.5  $\mu$ L SBYR Green PCR Master Mix and 0.25  $\mu$ L AmpErase Uracil N-Glycosylate (Applied Biosystems, Foster City, CA), 0.2  $\mu$ M of each primer, 5  $\mu$ L 10x diluted cDNA and nuclease-free water to make a total reaction volume of 25  $\mu$ L. The reactions were performed in MicroAmp 96-well plates capped with MicroAmp optical caps (Applied Biosystems, Foster City, CA) and under the following conditions: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min. Two or three replicates of each sample were performed. Four samples for one treatment were obtained from four independent experiments. The following control reactions were also included: no template control (NTC) to monitor for any contaminants in the reagents, and no-RT control to monitor for DNA contamination in the RNA samples.

Data obtained from real-time PCR was imported into Microsoft Excel software and raw cycle threshold (Ct) values of each sample were converted to mean fold change in gene expression using the  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen, 2001). Relative gene expression of each sample is presented as the fold change in gene expression normalized to  $\beta$ -actin and relative to the untreated control.

## RESULTS

### **Cloning of A Gene Encoding A NOS-like Protein (putative pea-NOS) in Pea**

Cloning of a homologue of At4g09680 in pea failed to identify any potential NOS sequence. No cDNA fragment based on At4g09680 was generated even when cDNA of *Arabidopsis* was used as a template. An attempt to clone peaNOS gene using cDNA library of pea (purchased from ClonTech) as a template, and primer sets targeted to the adaptor sequence of the clones and gene specific primers based on At3g47450 was also not successful.

Cloning based on At3g47450 coding sequence, however, did successfully amplify a 784-bp cDNA fragment from pea (Fig. 1) via RT-PCR using RNA from pea HR tissues as a template. Sequence comparison revealed a 50% nucleotide identity of the 784-bp cDNA to the At3g47450 sequence (Fig. 2), but found no other significant match in NCBI database. PROSITE scan revealed that the 784-bp fragment contains myristoylation sites (225), epidermal growth factor (EGF)-1-like domain, and alanine-rich, cysteine-rich, glycine-rich, and threonine-rich regions.

### **Correlation Study of Relative Gene Expression of P Protein of Pea (peaP) and NOS Activity**

For the water-infiltrated leaves, there was minimal change in the transcript levels of peaP throughout the duration of the experiment (Fig.3A). In the incompatible interactions, the transcript levels of peaP increased to 1.8-fold at three hpi, remained at that level at six hpi and declined to 1.5-fold at nine hpi (Fig. 3A). At

12 to 24 hpi, the transcript levels declined to below base-line level of one-fold (0.8- and 0.3-fold respectively). There was minimal difference overall in the incompatible transcript levels as compared to those in the untreated control.

Conversely, the transcript levels of *peaP* increased to 4.1-fold at three hpi in the compatible interactions and reached a maximum level of 4.7-fold at six hpi (Fig.3A). At 9, 12 and 24 hpi, the transcript levels were 1.5-, 2.2-, and 1.3-fold respectively. There was significant difference in transcript levels at three and six hpi as compared to those in the control and incompatible interactions. No signals of the amplicons (*actin* and *peaP*) were observed in the no-template and no-RT controls. Furthermore, sample from wells randomly picked were visualized with electrophoresis in 2% agarose gel and the result showed a single band with the right size of the amplicon.

The same tissue samples as used in the relative gene expression experiment above were used to measure NOS activity. NOS activity for the control remained between 10.6 to 14.2 pmol/min/mg throughout the 24 hr period (Fig. 3B). In the incompatible interactions, NOS activity increased to 18.4 pmol/min/mg at six hpi, declined gradually to initial level at 12 hpi (14.1 pmol/min/mg) and finally increased to a maximum level (20.3 pmol/min/mg) at 24 hpi (Fig. 3B). In the compatible interactions, NOS activity increased substantially to a maximum level of 24.5 pmol/min/mg at three hpi from 17.2 pmol/min/mg at 0 hpi, returned to near initial level at six, nine and 12 hpi (16.0, 15.9, and 15.3 pmol/min/mg respectively), and finally increased back to maximum level (24.6 pmol/min/mg) at 24 hpi (Fig. 3B). A hypersensitive response was observed between nine to 12 hpi in incompatible

leaves, and compatible disease symptoms were observed between 24 to 36 hpi (Fig.4).

## DISCUSSION

A lack of sufficient homology to mammalian NOS domains was indicated by the failure to amplify any such domains from pea cDNA via RT-PCR (Appendix A). The presence of these domains, however, may not be necessary for NOS activity. The 1.377-kb cDNA of *Helix pomatia* (snail) NOS protein (60 kDa) did not contain consensus sequences for NADPH, FAD, arginine, and calmodulin binding sites (Huang et al., 1997). Although the protein was eluted from 2',5'-ADP-agarose column with NADPH, and its activity was NADPH dependent, the cDNA sequence revealed no consensus binding site for NADPH. Furthermore, a calmodulin antagonist blocked enzyme activity although the cDNA did not show a consensus binding site for calmodulin. The authors suggested that the protein is associated with NOS and affects its activity but may not be the enzyme itself.

The failure to amplify any fragment at all using primers targeted to various region of gene At4g09680 of *Arabidopsis* (homologue of rat NOS), even when cDNA of *Arabidopsis* was used as a template, indicated that gene At4g09680 may just be a hypothetical gene or not expressed in the tissues analyzed.

Guo et al. (2003) were able to verify that the *Arabidopsis* homologue (At3g47450) of the *Helix* NOS gene was expressed *in planta* and did confer NOS activity in association with hormonal response. In the attempt to clone a homologue of At3g47450 in pea, only one primer set out of a total of 20 (Appendix B) was able to amplify a 784-bp cDNA fragment with similarity to At3g47450 (Fig. 1). The successful amplification required 38 to 40 PCR cycles using cDNA of hypersensitive tissues suggesting that the pea homologue of At3g47450 presents at very low

copies in the cells. The high nucleotide similarity of the 784-bp cDNA fragment with the At3g47450 coding sequence suggested that this may be a potential peaNOS gene. Further work needs to be done to clone the full-length of the 784-bp cDNA and to characterize its structure and functional expression.

The inability to timely obtain an At3g47450 homologue in pea left the homologue of *varP* (Chandok et al., 2003) in pea (P protein of GDC, *peaP*) as the only viable candidate to compare expression and NOS activity during pea-bacteria interactions. The levels of NOS activity over time did not correlate with the levels of *peaP* mRNA in both the incompatible and compatible interactions (Fig. 3A and B). If *peaP* gene was the gene responsible for encoding a NOS-like protein, its abundant expression should directly precede maximum NOS activity. This pattern was not reflected in the correlation study. For example, in the incompatible interactions, the expression levels of *peaP* gene did not increase significantly with time but decrease at 24 hpi, while NOS activity increased to a maximum level at six hpi and declined gradually to initial level except at 24 hpi (Fig. 3A and B). In addition, in the compatible interactions, *peaP* gene was highly expressed at three and six hpi while generally NOS activity did not increase with time except at three and 24 hpi (Fig. 3A and B).

In addition, the results showed that the expression of *peaP* gene increased significantly (~4 to 5-fold) during the compatible interactions, instead of the incompatible interactions. This suggested that either the *peaP* was not related to the synthesis of NO during plant defense to pathogen attack, or the expression of *peaP* gene did not coincide with NOS activity as in the case of AtNOS1 mRNA levels did

not increase with the increase of NO generation after induction with abscisic acid (Guo et al., 2003). If *peaP* was not related to the synthesis of NO during plant defense to pathogen attack, the NOS-like protein involved in NO production in pea is probably encoded by a NOS gene different from *varP* as was implicated in NO synthesis in the incompatible interaction of TMV and tobacco (Chandok et al, 2003). The pea NOS-like protein is perhaps more related to AtNOS1 (Guo et al., 2003), and is possibly encoded partially by the 784-bp cDNA cloned using RT-PCR (Figs. 1 & 2). This hypothesis is also supported by the result of the previous experiment (Chapter 1) on the partial purification of NOS-like protein of pea which indicated that the protein is more similar to AtNOS1 rather than *varP* based on information related to affinity substrates, and cofactor requirements for NOS activity. If the expression of *peaP* gene did not coincide with NOS activity, confirmation that *peaP* demonstrates NOS activity by functional expression is needed.

What was interesting is that the expression of *peaP* gene was associated with increased NOS activity in the compatible interaction. *peaP* gene expression was simultaneous with the first peak of NOS activity, and directly preceded the second peak in the disease development phenomenon (Fig. 3A and B). If *peaP* is involved in NO generation, its involvement in the disease development suggested that NO generation may be a general response to biotic stress in plants.

NO had been postulated to function in different physiological, biochemical and molecular processes in plants (del Rio et al., 2004). Furthermore, the accumulation of NO had been implicated in both the abiotic stress such as heat, osmotic, salinity, and wounding (Gould et al., 2003; Garces et al., 2001), and biotic stress such as

hypersensitive response (HR) and systemic-acquired resistance (SAR) (Delledonne et al., 1998; Durner et al., 1998) responses in plants. However, the role of NO in disease development in plants is not fully understood since the literature on this aspect is limited.

To date, only two proteins demonstrating NOS activity have been identified in plants (Chandok et al., 2003; Guo et al., 2003). Both proteins do not have sequence homology to any mammalian NOS isoform. The difficulties in isolating a NOS-like protein in plants prompted the speculation that NO may be produced by many enzymes that are relevant to NO production such as xanthine oxidoreductase, peroxidase, cytochrome P450, and some heme proteins (Millar et al., 1998; Huang et al., 2002; Boucher et al., 1992b; Boucher et al., 1992a), similar to another important free radical, the superoxide anion ( $O_2^{\bullet-}$ ) (Bolwell, 1999). Furthermore, it has been proposed that NO emission in plants can be a generalized stress response similar to reactive oxygen species (ROS) (Gould et al., 2003).

In summary, the findings in this work verified the fact that a NOS-like protein and its corresponding gene in pea are significantly different from mammalian NOS. Homologues of *varP* and AtNOS1 exist in pea. The correlation of the gene expression of P protein of glycine decarboxylase complex (GDC) of pea (peaP) and NOS activity during hypersensitive response (HR) in pea was not demonstrated here but peaP gene was highly expressed concomitant with NOS activity during disease development. The NOS-like protein of pea involved in NO generation during HR is more similar to At3g47450 (AtNOS1), and possibly encoded partially by the cloned 784-bp pea cDNA.

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Table 1 Accessions At4g09680 and At3g47450 of *Arabidopsis thaliana* ecotype Columbia obtained from TAIR and NCBI databases

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Name/Locus	At4g09680	At3g47450
GenBank Accession (cds)	NM_117036	NM_114613
GO Cellular Component	Chloroplast	Mitochondrion
Protein Data		
Length (aa)	1075	569
Molecular weight (kDa)	118.945	62.806
Isoelectric point	6.3917	9.5636
Domains	-	ATP GTP A GTP-binding MMR HSR1
GenBank Accession	CAB39635	CAB51217
Homology	NOS of <i>Rattus norvegicus</i> (rat)	NOS (br-1 protein) of <i>Helix pomatia</i> (snail)

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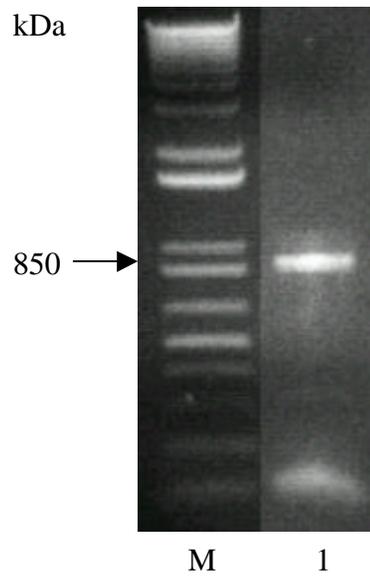
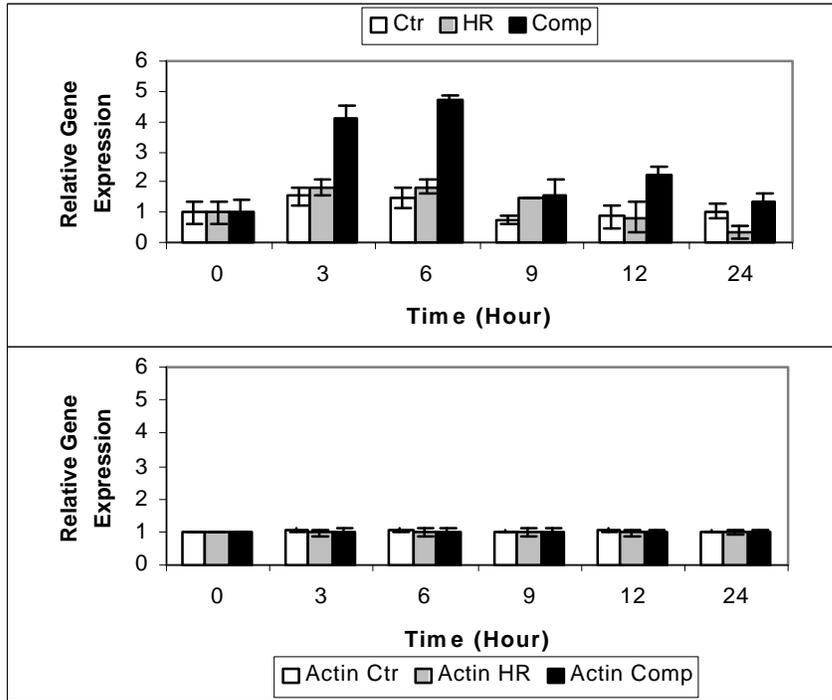


Fig. 1 Cloning of a gene encoding a NOS-like protein in pea using Reverse Transcription-Polymerase Chain Reaction (RT-PCR). cDNA synthesized from total RNA of leaf tissues undergoing hypersensitive response, and primers based on At3g47450 of wild-type *Arabidopsis thaliana* were used. Lane M represents 1-kb plus DNA molecular weight standard, and lane 1, 784-bp fragment.



A)



B)

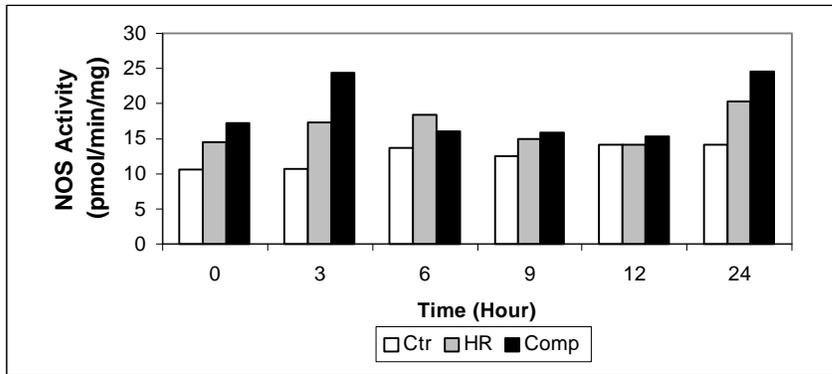


Fig. 3 Correlation study of relative gene expression of P protein of pea (*peaP*) and NOS activity. Leaves of  $8 \pm 1$  days old plants were vacuum infiltrated with incompatible bacteria, *Ralstonia solanacearum* ( $2.0 \times 10^9$  cfu), compatible bacteria, *Pseudomonas syringae* pv *pisii* ( $1.0 \times 10^6$  cfu), and distilled water as a control. Infiltrated leaves were harvested at intervals of 0, 3, 6, 9, 12, and 24 hr post infiltration. A) Real-time RT-PCR was used to determine the level of *peaP* transcript at each time point for each treatment. Relative gene expression of each sample is presented as the fold change in gene expression normalized to  $\beta$ -actin and relative to the untreated control using the  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen, 2001). Error bars indicate standard deviation (n=3). B) NOS activity for each time point was determined using citrulline formation assay (Bredt and Snyder, 1990). Standard errors for control, HR and compatible interactions are 1.41, 1.22 and 2.02 respectively (n=3).



Fig. 4 Hypersensitive response (HR) and disease symptoms in pea leaves. Pea leaves were vacuum infiltrated with incompatible bacteria, *Ralstonia solanacearum* ( $2.0 \times 10^9$  cfu/mL), compatible bacteria, *Pseudomonas syringae* pv *pisi*, ( $1.0 \times 10^6$  cfu), and distilled water as a control. A, Control; B, HR at 9 hpi; C, HR at 12 hpi; D and E, HR at 12 hpi close-up; F, HR at 24 hpi; G, HR at 24 hpi close-up; H, Disease symptom at 24-52 hpi; I, Disease symptom at >52 hpi close-up. hpi denotes hours post infiltration.

Appendices for Chapter 1

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	2	8.2779	4.1389	0.50	0.6112 ns
Day	3	75.8585	25.2861	3.05	0.0403 *
Trt	4	19.2924	4.8231	0.58	0.6781 ns
Day*Trt	12	76.7119	6.3926	0.77	0.6761 ns

Appendix A ANOVA analysis on the effects of abiotic agents-pea interactions on NOS activity. Abiotic agents used were copper chloride, Actiguard®, Triton-X100 and salicylic acid (SA). Denote: ns, no significant difference; asterisk, significant difference at P<0.05.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
pH	4	20.8660	5.2165	18.29	0.0004 ns
Rep	2	2.6449	1.3224	4.64	0.0460 *

Appendix B ANOVA analysis on the effects of pH on NOS activity during sample extraction. pH tested were 7.0, 7.5, 8.0, 8.5, and 9.0. Denote: ns, no significant difference; asterisk, significant difference at P<0.05.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	8	122.3120	15.2890	41.50	<.0001 ns
Rep	2	3.1523	1.5761	4.28	0.0325 *

Appendix C ANOVA analysis on the effects of reducing agents, metal chelators, protease inhibitors and phenolic chelator on NOS activity during sample extraction. Denote: ns, no significant difference; asterisk, significant difference at P<0.05.

## Appendices for Chapter 2

```
1 macpwkflfr vksyqgdike ekdinnvek tpgaipsptt qddpkshkhq ngfpqfltgt
61 aqnvpesldk lhvtpstrpq hvriknwgng eifhdthhkh atsdiscsk lcmsimnsk
121 sltrgprdkp tpveellpqa iefinqyygs fkeakieehl arleavtkei ettgtyqltl
181 delifatkma wrnprciqr iqwsnlqvfd arscstasem fqhicrhily atnsgnirsa
241 itvfpqrsdg khdfriwnsq liryagyqmp dgtirgdpd leftqlcidl gwkptrygrfd
301 vlplvlqahg qdpevfeipp dlvlvtmeh pkyewfqelg lkwyalpaaa nmllevgggle
361 fpacpfngwy mgteigvrdf cdtqrynile evgrrmglet htlaclkdr avteinaavl
421 hsfqkqvnti mdhhtasesf mkhmqneyra rggcpadwiw lvppvsgsit pvfhqemlly
481 vlspfyqqi epwkthiwqd eklrprrei rftvlvkavf fasvlmrkvm asrvratvlf
Domain III
541 atetgkseal ardlaalfsy afntkvvcme qykantleee qlllvvtstf vngdcpnsgq
601 tlkkslfmmk elghtfryav fglgssmypyq fcfaahdidq klshlgasql aptgegdels
661 gqedafrswa vqtfracet fdvrskhiciq ipkrytsnat wepegykltq spesldlnka
721 prsihaknfv tmrlkslqnp qsekssrttl lvqltfegsr gpsylpgehl gifpqntal
781 vqgilervvd csspdqtvc1 evldesgsyw vkdkrlppcs lrqaltyfld ittpptqlql
841 hklarfatee thrqrlealc qpseyndwkf snnptflevl eefpslrvpa aflslqlpil
FAD-isoalloxazine
901 kpryysisss qdhtpsevhl tvavvtyrtr dgqgplhhgv cstwinnlkp edpvpcfvrs
961 vsqfqlpedp sqpciligpg tgiapfrsfw qdrlhdsqhr glkggrmtlv fgcrhpeedh
1021 lyqeemqemv rkgvlfqvht gysrlpgkpk vyvqdilqke ladevsvlh geqghlyvcg
adenine
1081 dvriardvat tlkklvaakl nlseeqvedy ffqlksqkry hedifgavfs ygakkngtle
1141 epkgtrl
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Appendix A Schematic of functional domains and consensus regions of rat astrocyte iNOS. Functional domains (heme, FMN, FAD and NADPH) based on rat astrocyte iNOS (1147 bp) and consensus regions obtained from multiple sequence alignment of animal NOS. Degenerate primers for cloning of pea NOS-like gene were designed based on these domains. Modified from Galea et al., 1994.

4F 10F 7F 12F  
 1 ATGGCGCTACGAACACTCTCAACGTTTCCTTCTCTTCTCTGTGCCACACAACGACGAGA

7F 12F 6F  
 61 CGTGAACCAATCTCAACCGTCATTTACCGTAATCCGACGACATCAATCGTCTGTAAATCA

9F  
 121 ATAGCTAATTCAGAACACCAGTTTCACTCTCGGAACGAGATGGATTTCGGCGGCTGCT

181 CCAACCCTGGAGAAAGGTTCTGGAGAACCAACGAGCTCATGAAGCTCAGAAAGTAGTG

241 AAGAAAGAGATCAAAAAGGAGAAGAAGAAAAAGAAAGAGAGATTATGCACGGAAAGTT

8F 12R  
 301 GTTGATACCTCAGTCTCATGTTGTTACGGCTCGGAGCTCCGTTACAACTTCCGACGTC

12R  
 361 GATTCTCCGGGATTTGTCGATTTGGTTACTTATGAATTGAAGAAGAAGCATCACCAGTTA

421 AGAACTATGATATGTGGAAGATGTCAGCTATGTCCATGGACATATGATTACAGCAGTT

481 GGTGGTAATGGAGTTATCCAGTGGGAAACAATTTGTATCAGCTGATGAACTTCGTGAG

541 AAACCTTCTCATTTACCCATGAGAAAGCTTTGATTGTTAAATTGGTTGATATAGTGGAT

601 TTTAATGGAAGCTTTTAGCTCGTGTTCGTGATTTAGTTGGAGCTAATCCGATTATACTT

2F (GSP1B)  
 661 GTTATACTAAGATTGATCTTCTTCCAAAAGGAACGGATATGAATTGTAACGGGGATTGG

2F (GSP1B)  
 721 GTTGTGGAAGTGAACCATGAGGAAAAAGCTTAATGTCTTGAGTGTCCATCTCACAAGTTCA

1F (GSP1)/8R  
 781 AAGTCCCTGGATGGAGTTAGCGGAGTTGCATCAGAGATCCAGAAGGAGAAAAAGGGACGA

3F  
 841 GATGTCTACATCTGGGTGCAGCTAACGTAGGGAAGTCAGCATTCAATGCTTTGCTG

(GSP2) 10R  
 901 AAAACGATGGCCGAAAGGGATCCTGTTGCAGCAGCGGCACAAAAGTACAAA CCAATTCAA

961 TCTGCTGTCCCTGGAACACCTTGGGTCCAATTCAGATCAACGCTTTCGTCCGAGGAGAG

11F 3R (GSP2)  
 1021 AAGTTGTATGACACACCGGCTGTGCACCTACACCACAGGCAAGCAGCTGTCGTTCAATTCA

2R (GSP1B)  
 1081 GATGATTTACCCGCCCTTGCTCCTCAAATCTCTCAGAGGCAATCTTTTCGATGTAAGT

1R (GSP1)  
 1141 CTGCTTTATTACGGCCAGATTTCAACTTTGCCAACTCAAACGTCAGTAGTCCCAAGGGT

1201 GAGAGCTTAAACGGTTATACATTTTTCTGGGGAGGTCTCGTTAGGATTGACATCTTGAAG

1261 GCTCTACCGGAAACATGTTTACATTTCTATGGACAAAAGCTTTGAGATTCATGCAGTA

1321 CCAACCAAAACAGCGACTGCCTTTTACGAGGCAAACTGGGTGTGCTTCTAACACCTCCA

1381 TCAGGGAAAAATCAGATGCAGGAGTGAAAGGGTTACAATCTCACCGGTTACTTCAAATC

1441 GAAATCAACGATGCAAAAAGACCGGCTAGTGATGTGGCAATATCAGGGTTAGGATGGATT

1501 TCAATTGAACCAATCCGCAAAACACGAGGAACTGAACCGAGAGATCTCAATGAAGCAGAG

1561 CATGAGATACATATTTGTGT<sup>5R</sup>CAGTGTGCCAAAACCAGTTGAAGTTTTTCTTC<sup>11R</sup>GACCAACA

1621 <sup>11R</sup>TTGCCAATTGGTACTTCAGGTACTGAATGGTATCAGTATCGTGAGTTAACCGATAAGGAA

1681 GAAGAAGTAAGACCCAAATGGTACTTTTGA

Appendix B Schematic map of primers used in cloning of At3g47450 homologue in pea. The length of At3g47450 sequence in *Arabidopsis thaliana* is 1.7 kb. A total of 20 primer sets were tested.

Note: Combination of primers used in RT-PCR cloning of At3g47450 homologue in pea

- Set 1 Primer 1F/primer 1R, 401 bp
- 2 Primer 2F/primer 2R, 403 bp
- 3 Primer 3F/primer 3R, 178 bp
- 4 Primer 4F/AUAP, 1710 bp
- 5 Primer 1F/AUAP, 912 bp
- 6 Primer 4F/primer 1R, 1199 bp
- 7 Primer 1F/primer 5R, 802 bp
- 8 Primer 6F/primer 1R, 1123 bp
- 9 Primer 6F/primer 5R, 1524 bp
- 10 Primer 6F/AUAP, 1694 bp
- 11 Primer 7F/primer1R, 1159 bp
- 12 Primer 8F/primer8R, 505 bp
- 13 Primer 9F/primer 1R, 1065 bp
- 14 Primer 10F/primer 12R, 947 bp
- 15 Primer 11F/primer 11R, 612 bp
- 16 Primer 12F/primer 12R, 327 bp
- 17 Exon11F/Exon11R, 103 bp
- 18 Exon11BF/Exon11BR, 174 bp
- 19 Exon12F/Exon12R, 150 bp
- 20 Exon12BF/Exon12BR, 329 bp

Primer sets 17-20 were designed based on exon 11 and exon 12 of the *Arabidopsis thaliana* genomic sequence for gene T21L8.200 (similar to br-1 protein (NOS) in *Helix pomatia*) in chromosome 3 BAC clone T21L8 (GenBank accession no. AL096860). Primer sets tested and worked for *Arabidopsis* were sets 1, 2, 3, and 13. Primer set 12 did not work for either *Arabidopsis* or pea. Primer sets that worked inconsistently for pea were sets 1, 2, and 3. AUAP was a special primer for 3' Rapid Amplification of cDNA Ends (RACE) (Invitrogen, Carlsbad, CA) cloning that consisted of oligo (dT)s and an adapter sequence.