Chapter 7

Transcript analysis of nitric oxide inducible pathogenesis related proteins
7.1. Introduction

It is apparent from the previous sections that NO is involved in number of physiological and developmental responses of plants. Thus, there must inevitably be changes in the spectrum of gene expression in plants following exposure to NO. Induction of gene expression mediated by NO was first shown during plant–pathogen interactions of tobacco and tobacco mosaic virus (TMV). Application of NO donors GSNO or SNAP induced the expression of phenylalanine ammonialyase (PAL) and pathogenesis related (PR-1) proteins genes (Durner et al., 1998). PR-1 gene expression was also induced by TMV infection, an effect suppressed by co-injection with the NOS inhibitor, L-NAME, providing evidence for the endogenous mediation of TMV-induced gene expression by NO (Klessig et al., 2000). Delledonne et al. (1998) showed that SNP induced the expression of PAL and chalcone synthase (CHS) in soybean suspension cultures. Mackerness et al. (2001) showed that NO scavenging with C-PTIO or inhibition of NOS by L-NAME inhibited CHS gene expression in Arabidopsis plants.

On the other hand, NO also activates signaling activity of including salicylic acid (SA) production, cGMP (cyclic guanosine monophosphate) synthesis, calcium fluxes, and reversible protein phosphorylation (Klessig et al., 2000). NO induction of PR-1 gene expression in tobacco required SA synthesis and action, whereas induction of PAL gene expression did not (Durner et al., 1998).

Pathogenesis-related (PR) proteins are a group of diverse proteins whose accumulation is triggered by pathogen attack. In a sense, PR proteins constitute a point where the various response networks intersect by reacting with different signals such as salicylic acid, jasmonic acid, systemin, and ethylene including NO. In theory, the constitutive expression of PR proteins, either singly or combined, might confer decreased susceptibility to a specific group of pathogens (Hammond-Kosack and Jones, 1996).

Plants have developed an arsenal of rapid and efficient defense responses against pathogens. A key difference between resistant and susceptible plants is the timely recognition of the invading pathogen and the rapid and effective activation of host defenses. The “gene-for-gene” paradigm describes the dependence of this resistance on matched specificity between a plant disease resistance (R) gene and a pathogen avirulence gene (Avr). In a process that is reminiscent of innate immune responses, these
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genes determine receptor–ligand interactions that activate a complex host defense response. Once activated, these defense responses manifested in part or fully during establishment of systemic resistance (Cohen et al., 2001).

Bioinformatics analysis of the transcriptional changes induced by NO in *Arabidopsis* identified that some genes responsible for jasmonic acid biosynthesis are induced by NO (Palmieri et al., 2008). Microarray analysis of NO-related transcripts in plants detected 990 genes in *Medicago truncatula* roots treated with NO donors during incompatible interaction with foliar pathogen *Colletotrichum trifolii* (Ferrarini et al., 2008). Salicylic acid and NO synergistically act together in cellular signal transduction involved in activation of expression of PR1 in *Arabidopsis* plants (Zottini et al., 2008).

Studies on the transcriptional changes induced by NO made possible identification of genes involved in different functional processes such as signal transduction, defense and cell death, reactive oxygen species (ROS) production and degradation. The transcriptional expression of these genes can be explained by the cooperation of a set of transcription factors that bind a common region in the promoter of the regulated genes. *Arabidopsis thaliana* plants during incompatible interaction with *Pseudomonas syringae* pv. *tomato*, recorded the elevated level of NO which mediated transcriptional activation of PR1 and PAL genes. Further *A. thaliana* with heterologous expression of bacterial nitric oxide deoxygenase (NOD) which blocks the NO production fails to activate the transcriptional action for same genes (Zeier et al., 2004). *A. thaliana* plants and cell suspensions treated with lipopolysaccharides isolated from *P. syringe* elicit the glutathione-complex-related genes, PR-1, other oxidative stress and defense-related genes via NO signaling (Zeidler et al., 2004). *Nicotiana tabacum* cell suspension cultures treated with cryptogein a fungal hyphal cell wall protein elicitor from *Phytophthora* spp elicits expression of lipoxygenase (LOX), PR-3, glutathione S-transferase (GST), and PAL through NO signaling (Lamotte et al., 2004). *A. thaliana* plants treated with gaseous NO record the transcriptional activation of LOX and PR3 genes (Huang et al., 2004). Soybean cells and *Arabidopsis thaliana*, treated either with a bacterial pathogen *P. syringae* or an elicitor induced NO consequently expression of PAL, chalcone synthase and GST (Grun et al., 2006). INF1 purified from the *P. infestans* regulates NO which cascades mapkinase (MAPK) signaling. But blocking of
NO generation resulted in high susceptibility to *Colletotrichum orbiculare* and *P. infestans* (Asai et al., 2008).

In pearl millet, expression pattern of PR proteins such as PR-2 (β-1,3-glucanase), PR-9 (Peroxidases) and PR-10 (RNase) was demonstrated in response to downy mildew pathogen infection (Kini et al., 2000; Shivakumar et al., 2003). Recently, expression of PR-1, PR-5 (thauamatin like protein) and PR-15 (Oxalate oxidase) was demonstrated during induction of resistance mediated by *Pseudomonas fluorescense* (Niranjan Raj, 2005) and also the expression of PR-13 (Thionin-like proteins) was recorded during induction of resistance by abiotic inducers (Chandrashekara, 2007).

But none of these studies implicated NO role in expression of PR proteins during systemic resistance particularly in pearl millet and oomycete obligate parasitism. On the other hand, most of the studies concentrated on PR-1 while implicating the NO in various systems. Hence in the present study, trascriptomic analysis of PR1, PR2, PR3, PR4 and PR5 has been undertaken to understand plant defense responses at molecular level and to figure out the network of signals NO associated for restricting pathogen.

### 7.2. Materials and Methods

#### 7.2.1. Host, pathogen and inoculation

As described in Chapter 3.

#### 7.2.2. Treatments

Treatments consisted of three different sets. The first set includes seedlings raised from untreated resistant cultivar IP18292. The second set includes, susceptible seedlings raised from the seeds treated with SNP (referred to as induced resistant seedlings). The third set includes resistant and induced resistant seedlings that were treated with 10mM C-PTIO separately for 1h prior to challenge inoculation. For all set of seedlings the challenge inoculation was made for two-day-old seedlings by whorl inoculation method.

#### 7.2.3. Sampling

Pearl millet seedlings were harvested from treatments as described above in the different time intervals at 0, 3, 6, 9, 12, 24, 48 and 72 h after inoculation and immediately wrapped in aluminum foil and stored at -70°C until further use.

#### 7.2.4. cDNA Probes used
Heterologous cDNA probes from barley for PR1 (At2g14610, PR2 (At3g57260), PR3 (At3g12500), PR4 (At3g04720) and PR5 (At1g75040) were kindly gifted by Prof. Durner, Professor, Italy.

7.2.5. Plasmid DNA extraction

A single recombinant colony from the master plate was incubated in 1 ml LB medium containing ampicillin (100 µg ml⁻¹). This was grown to saturation at 37°C overnight at 175 rpm and the culture was pelleted in a 1.5 ml eppendorf tube. The pellet was suspended in 200 µl of solution A (4 mg lysozyme ml⁻¹ of GTE solution), 300 µl of solution B (1.0% SDS and 0.2 N NaOH) and 300 µl of solution C (7.5 M ammonium acetate) were added, the contents were mixed well and kept on ice for 10 minutes. The solution was centrifuged for 10 min at 10000 rpm to pellet the chromosomal DNA and cell debris. The plasmid DNA from the supernatant was precipitated with 2.5 volumes of 95 % Ethanol for 10 min on ice. This was followed by a spin at 10,000 rpm at 40°C for 10 min, to pellet plasmid DNA and RNA. The pellet, washed with 70 % ethanol and dried under vacuum, was dissolved in TE buffer. The plasmid was treated with 1 ul (10mg/ml) of RNase (DNase free) at 37°C for one hour to remove RNA. It was followed by phenol: chloroform treatments to remove RNase.

7.2.6. Using PCR amplification for insert purification

Alternatively, PCR was used for amplification and then inserts were purified using Sephadex® 6-50 or Spin Column Elutips® or similar size exclusion media. In this method, the extracted plasmid DNA was diluted 100 times by mixing 1 µL plasmid DNA in 99 µL of water and 5 µL of this was used in a PCR reaction using M13 forward and M13 reversal universal primers. The following recipe was used to make the PCR reaction:

<table>
<thead>
<tr>
<th>PCR components</th>
<th>PCR conditions</th>
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<tbody>
<tr>
<td>Water</td>
<td>32.5 µl</td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>2.5 mM dNTP</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Universal primer</td>
<td>1.0 µl</td>
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<tr>
<td>Reversal primer</td>
<td>1.0 µl</td>
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<tr>
<td>Taq polymerase</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>Total</td>
<td>50.0 µl</td>
</tr>
</tbody>
</table>
7.2.7. Labeling of probes

The random-primed method of Feinberg and Vogelstein (1983) was used for labeling DNA with $\alpha$-$^32$P. The double stranded DNA was denatured, and complementary labelled fragments were synthesized from octadeoxinucleotides primers of random sequence. By including a nucleotide triphosphate with a proximal ($\alpha$)$^32$P-labelled phosphate in the reaction mixture, the DNA polymerase will produce radioactively labelled DNA. Probe labelling was carried out according to Bangalore Genei kit (Bangalore, India) by initially denaturing the probe at 95°C for 10 min, then placed on ice for 5 min followed by incubating in a reaction mixture consisting of Primer and buffer 5 µl, dNTPs 6 µl, $^32$P dATP 4 µl and Klenow 1.5 µl at 37°C for 1 h. The reaction was stopped by adding 400 µl of 200 mM EDTA and denatured at 95°C for 5 min followed by chilling on ice for 10 min prior to hybridization.

7.2.8. RNA isolation

The success of differential gene expression studies depends on the integrity of the RNA and on it being free of chromosomal DNA contamination. The prime concern in isolating high quality RNA are usually attributed to the presence of ribonucleases (RNAses) and a high content of secondary metabolites such as phenolics and polysaccharides, which bind to RNA upon cell lysis resulting in extensive degradation and low yields. To avoid RNAse contamination during RNA extraction a set of protocol was followed which is described as follows: Glasswares were treated with 0.1% diethylpyrocarbonate (DEPC) water, autoclaved and baked at 160°C overnight to inhibit RNase activity. All the reagents were prepared with DEPC treated water. Frozen plant material were ground to a fine powder in liquid nitrogen, to that 10 ml of extraction buffer containing 150 mM LiCl, 5 mM EDTA pH 8.0, 50 mM Tris-HCl pH 9.0, 5 % SDS, and 10 ml of phenol:chloroform (1:1) was added. The tube was shaken thoroughly for 10 min until an emulsion was obtained. The tubes were then centrifuged at 2000 rpm for 15 min at room temperature. Supernatant was transferred to a new centrifuge tube containing 10 ml phenol:chloroform, shaken vigorously and centrifuged at 2000 rpm for 15 min at room temperature. This step was repeated twice. The supernatant obtained was transferred to a centrifuge tube containing 10 ml of chloroform, shaken well and centrifuged at 2000 rpm, 10 min at room temperature. Upper phase was transferred to a
sterile corex tube, 1/10th volume of 3 M NaOAc was added (pH 5.5) and 2.5 volumes of 96% ethanol were added and stored at –20°C overnight. The tubes were centrifuged at 10,000 rpm at 4°C for 30 min. Pellet was dissolved in 0.15 M NaOAc and to that 6 ml of ice cold 4 M NaOAc was added and kept in –20°C overnight. The tubes were centrifuged at 10,000 rpm at 4°C for 30 min. Supernatant was drained off and the pellet was resuspended in 1.0 ml 3 M NaOAc and transferred immediately onto 1.5 ml eppendorf tubes on ice for 1 h. It was centrifuged at 10,000 rpm, 30 min at 4°C, supernatant drained off, 0.5 ml 70% ethanol was added to the pellet and vortexed briefly. The tubes were then centrifuged at 10,000 rpm, 30 min at 4°C. Pellet was dried for 10 min and dissolved in 50 µl of sterile water and stored in freezer (-20°C). Purity of RNA was checked by taking absorbance ratio of 260 and 280 nm and integrity of the RNA sample was checked by electrophoresing on denaturing 1.2% formaldehyde agarose gel.

7.2.9. Northern blotting

Approximately 30 µg of total RNA sample along with loading buffer was kept in boiling water bath for 2 min, chilled on ice for 5 min and electrophoresed on 1.2% agarose gels containing 7.4% formaldehyde, 1x MOPS buffer, 5 mM sodium acetate and 1mM EDTA. After electrophoresis, RNA gel was then shaken in excess water at room temperature for 2 X 15 minutes in order to remove formaldehyde. RNA was then transferred on to Hybond N membranes (Amersham, UK) by capillary transfer using 20 X SSC overnight. After blotting the membrane was washed in 2x SSC at room temperature for 2 X 10 min and exposed to UV- for crosslinking (UV-Stratalinker™ 1800, USA). The nylon membrane was later baked at 80°C for 1 ½ hr.

7.2.10. Northern hybridization

Northern blots were pre-hybridized at 42°C with 10 ml of pre-hybridization solution (0.8 ml of 100X of Denhardt’s solution, 0.5 ml of 10 % SDS, 2.0 ml of 20X SSC, 20 µl of 0.5 M EDTA, 0.2 ml of 10 mg/ml salmon sperm DNA and sterile distilled water to 6.5 ml) for 4 h in case of new blots and 1 h for stripped blots. Pre-hybridization was performed in a Techne Hybridizer (HB-1D).
7.2.11. Hybridization

Labeled probe was added to the hybridization bottles containing blots and pre-hybridization mixture and incubated at 42°C in hybridization oven for at least 16 h. Care was taken to remove air bubbles present between the blot and the hybridization bottle.

7.2.12. Washing of blots

Following hybridization, the blots were washed using four changes of 50 ml each of ³²P-wash solution. Each wash was carried out for 15 min at 42°C in hybridization bottles using the hybridization oven. The first two washes were done using wash 1 solution (2X SSC and 0.1 % SDS) followed by two washes with wash 2 solution (0.1 X SSC and 0.1 % SDS). Membranes were air dried and enclosed in cling films.

7.2.13. Autoradiography

Autoradiography was carried out at -70°C by exposing the membrane to Phosphor Image analyzer (FLA 5000, Fuji Film, Japan) using red lazer. Analysis of transcript intensity was carried out using the Bioprofile image analysis system (Vilber Lourmat, France).

7.3. Results

7.3.1. PR-1 expression

Transcript accumulation of PR1 expression recorded both in the resistant as well as seedlings raised after the SNP seed treatment. At constitutive level the signals are very weak. In resistant seedlings signals begins from 3h and maintained till 72hpi. On the other hand, in SNP treatment signals also recorded at constitutive level i.e. 0hpi and it was instigated at 3hpi and lasted to 12hpi with enhanced signal pattern thereafter signals pattern become weak. However, prior C-PTIO treatment to resistant and induced resistant seedlings, showed the down regulation of PR1 expression. Very faint signals of PR-1 were noticed in the susceptible seedlings and the accumulation pattern was exactly similar to the resistant seedlings (Fig. 7.1).
7.3.2. PR-2 expression

Effect of NO on expression pattern of PR-2 in pearl millet in response to pathogen infection was documented. Constituent level of signals was not detected in resistant, SNP and susceptible seedlings. In resistant seedlings PR accumulation was detected at 6hpi and continued up to 24 hpi. Higher signals were recorded at 9, 12, 24 hpi. In resistant prior treated with C-PTIO banding pattern was similar to the resistant seedlings indicated that NO did not affect the expression and found to be independent of NO. In SNP treated seedlings accumulation started at 6 hpi and terminated at 9hpi, thereafter, no transcript accumulation was not recorded. In SNP+C-PTIO, signals pattern similar to SNP treatment alone and indicated that once after its initiation scavenger fails to block the effect of signals. Very faint signals of PR-1 were noticed in the susceptible seedlings and the expression pattern was exactly similar to the resistant seedlings (Fig. 7.2).

![Figure 7.1. Transcript accumulation of PR-1 in pearl millet seedlings treated with NO to downy mildew pathogen inoculation.](image)
7.3.3. PR-3 expression

PR-3 expression was noticed both in resistant and SNP treated seedling upon pathogen inoculation. In resistant seedlings whilst, at constitutive levels the signals were weak, and gradually the intensity increased from 3 hpi and maintained till end of 72hpi. PR-3 expression shown to depend on NO as the signals was completely blocked in prior treatment of scavenger. In SNP treatment, expression recorded from the beginning of 3hpi and remained to be till end of 48hpi, but higher signals recorded at 6, 9, 12 hpi thereafter very faint signals were noticed. Co-treatment with C-PTIO blocked the expression. In the susceptible seedlings accumulation pattern was weak and pattern was exactly similar to the resistant seedlings (Fig 7.3).
7.3.4. PR-4 expression

PR-4 expression was noticed only in the resistant seedlings and the expression pattern was in bi-phasic manner. In the beginning maximum signals were recorded at 3 and 6hpi and decreased thereafter, again transcript accumulation recorded at 12 and lasted till 72hpi. Prior treatment of NO scavenger did not affect the accumulation pattern. The signals were weak in SNP treated seedlings as well as susceptible seedlings. Very faint signals of PR-4 were noticed in the susceptible seedlings and the accumulation pattern was exactly similar to the resistant seedlings (Fig 7.4).
7.3.5. PR-5 expression

PR-5 expression was noticed only in the resistant seedlings and the expression of transcript accumulation recorded from 12 and continued till 72hpi. Prior treatment of NO scavenger did not affect the signals in resistant seedlings and signals pattern were similar to resistant alone. The signals were completely absent in SNP treated seedlings and very weak in susceptible seedlings. Very faint signals of PR-5 were noticed in the susceptible seedlings and the accumulation pattern was exactly similar to the resistant seedlings (Fig 7.5).

Fig. 7.4. Transcript accumulation of PR-4 in pearl millet seedlings treated with NO to downy mildew pathogen inoculation.
Fig. 7.5. Transcript accumulation of PR-5 in pearl millet seedlings treated with NO to downy mildew pathogen inoculation.

7.4. Discussion

Before employing any inducer or molecule for disease management it is important to study/characterize the expression/regulation of genes that encode various defense enzymes and signaling compounds at molecular level to elucidate and comprehend the network of signals in which pathogen restricted.

In the present study, the pattern of PR transcripts indicated that, NO is involved in regulation of PR-1 and PR-3 expression but not in PR-2, PR-4 and PR-5 expression which showed that they are independent of NO in pearl millet. Interestingly, PR-2 signals were induced by SNP treatment but was not blocked even after treated with NO scavenger. This is attributed to the effect of SNP, which might triggers the defense responses by activating the components of other signaling pathways like salicylic acid, which become independent at later stages. However, the signals of PR-4 and PR-5 were not induced by the SNP, but expressed in the resistant cultivar. Thus, our data further adds evidence that NO might be involved in
SA signaling and/or production as suggested previously. Several lines of evidence point to an inter-relationship between NO and salicylic acid (SA) in plant defense. Treatment of tobacco and *A. thaliana* leaves with NO induces a substantial increase in endogenous SA. In tobacco, this increase is required for PR-1 expression and probably involves NO-dependent induction of the PAL gene (Durner *et al*., 1998). In addition, NOS inhibitors and a NO scavenger attenuate SA-induced systemic acquired resistance (Song and Goodman, 2001). Although these results suggest that NO is involved in both SA biosynthesis and action, other studies have indicated that NO function requires SA. In transgenic tobacco, the ability of NO donors to reduce the size of TMV-induced lesions was abolished by the expression of the bacterial gene nahG, which encodes the SA-degrading enzyme salicylate hydroxylase (Song and Goodman, 2001). Recent evidence suggests that NO also plays a role in the wounding/jasmonic acid (JA) signaling pathway. In tomato, NO donors inhibited both wounding induced H$_2$O$_2$ synthesis and wounding or JA induced expression of defence genes (Orozco-Cardenas and Ryan 2002). This inhibition was independent of SA, which has been shown to antagonize JA synthesis and/or activity. Thus, NO may interact directly with the wounding/JA pathway at a point downstream of JA synthesis and upstream of H$_2$O$_2$ generation. Consistent with this possibility, NO donors delayed and/or reduced wounding-induced generation of H$_2$O$_2$ and expression of the JA-inducible ipomoein gene in sweet potato (Jih *et al*., 2003). Additional evidence that NO cross-talks with the wounding/JA pathway comes from the demonstration that wounding- and/or JA treatment induces NO production in sweet potato and *A. thaliana* epidermal cells, and that exogenous NO induces all of the genes that are required for JA biosynthesis. The relationship between these pathways remains unclear, however, because NO treatment did not increase JA levels and the wounding-induced expression of JA-forming enzymes was independent of NO. In contrast, NO treatment of SA-deficient NahG plants resulted in the activation of JA-responsive genes and JA production, suggesting that SA negatively regulates NO-mediated JA synthesis in wild type plants (Huang *et al*., 2004). Salicylic acid and NO synergistically act together in cellular signal
transduction involved in activation of expression of PR1 in *Arabidopsis* plants (Zottini et al., 2008).

In the present investigation, it can be concluded that NO is involved in PR-1 and PR-3 gene expression of both resistant and induced resistant seedlings upon pathogen inoculation and shown to be dependent on NO for their expression whereas PR-2 activation recorded both resistant and induced resistant but did not shown to depend on NO. NO induced PR-1 and PR-3 expression during induction of resistance in pearl millet against downy mildew pathogen.