AN ABSTRACT OF THE THESIS

Kamesh R. Vyakaranam for the Master of Science

in Chemistry presented on December 8, 1998

Title : Binding studies of the *avrXa10* gene product from *Xanthomonas orzyae pv. orzyae* to the promoter of a rice defense-response peroxidase gene

Abstract approved : Michael V. Keck

Xanthomonas orzyae pv. orzyae , the causal agent of the bacterial blight in rice, interacts in a race-specific manner with rice cultivars containing different resistance genes. *Xanthomonas orzyae pv. orzyae* containing the AVRXa10.F1 protein is known to elicit a hypersensitive response (HR) in rice cultivars containing the resistance gene *Xa-10*, but not on cultivars lacking *Xa-10* gene. Recent research has shown that there is possible interaction of the AVRXa10 protein and the peroxidase promoter of the *avrXa10* gene. In the present work, the binding of the AVRXa10 gene product from *Xanthomonas orzyae pv. orzyae* to the promoter of a rice defense-response peroxidase gene was studied. The peroxidase encoded by this gene is known to be a component of the defense response. The AVRXa10.F1 protein was isolated from *Escherichia coli* containing pET-avrXa10.F1 and identified by western blot. The binding of this protein to the PCR-amplified and 5' end- labeled 117.10/3.5 promoter region of a defense-response peroxidase gene was tested by mobility shift assay. The results showed possible binding of the AVRXa10.F1 protein to the promoter region of the peroxidase gene. This finding provides insight into the mechanism of disease resistance and plant-bacteria interactions.

Binding Studies of the *avrXa10* Gene Product from *Xanthomonas oryzae pv. oryzae* to the Promoter of a Defense-Response Peroxidase Gene.

A Thesis

Submitted to

The Division of Physical Sciences

Emporia State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Kamesh R. Vyakaranam

December, 1998

Michael V. Keck

Approved by Major Advisor

Michael V. Keck

Approved by Committee Member

Billie L. Johnson

Approved by Committee Member

Arthur M. Lee Sr.

Approved by Committee Member

Dwayne Baeckman

Approved by Division Chair

Timothy W. Sawyer

Approved by the Dean of Graduate Studies and Research

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank the faculty and graduate students of the Division of Physical Sciences for their overwhelming support throughout my study at Emporia State University. Also I would like to extend word of thanks to Dr. Jan Leach and her group for their help in this project.

A special word of thanks to Dr. Keck, my major advisor, for his pateince, support and advice throughout my study. Also I would like to thank my graduate committee members, Dr. Landis, Dr. Johnson and Dr. Leach for their criticism and encouragement. I would also like to thank my fiancee, Geeta for her help in writing the thesis.

Lastly, I would like to thank the Emporia State University Research and NASA-EPSCoR for their financial assistance for this project. Furthermore, I would like to dedicate this degree to my family for their encouragement and support they gave me throughout my study.

PREFACE

This thesis was written in the style required by the ACS Style Guide: A Manual for Authors and Editors (2nd edition).

TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

Like humans and other animals, plants are susceptible to pathogenic infection. Also like humans, plants have the capability of producing a defense response. There are many different molecular mechanisms for this response, depending on the plant type, pathogen type, or other factors. Rice (*oryza sativa*), for example, can and does produce different responses against the pathogens, (e.g. *Xanthomonas oryzae pv oryzae* the bacterial blight pathogen) to minimize losses (Leach and White, 1996). It is important to agricultural industry to understand the plant resistance mechanisms, as this knowledge can aid in developing disease resistance in crop, and in developing insecticides.

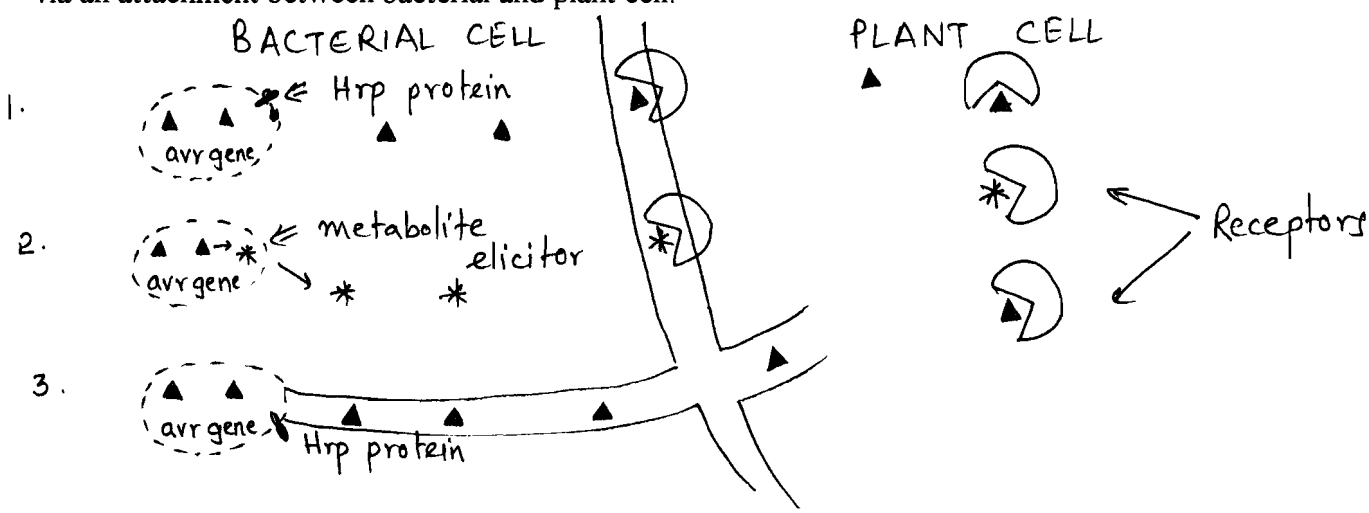
Because rice is a major food staple for much of the world population, studies of its interaction with pathogens such as *Xanthomonas oryzae pv oryzae* has been a major focus of study in many laboratories. General strategies for improving disease resistance in rice, which have been developed based on an understanding of these mechanisms, include introducing cloned disease resistance genes in rice cultivars, and improving the expression of plant derived genes (Young *et.al.*, 1994).

The National Aeronautics and Space Administration (NASA) is also quite interested in the plant-pathogen interaction. Long term space occupation is a major goal of NASA. In order to do this, methods must be developed to generate renewable food supplies in space, as it is impractical to carry multi-year food supplies to space stations. Experiments conducted on space shuttle flights have shown that plants grown in space exhibit an increased susceptibility to pathogenic infection over plants grown in the unit gravity conditions on the earth's surface (Jan Leach, unpublished results). The reasons for this

difference are unknown, and are in fact very difficult to ascertain, because as indicated above, the resistance mechanisms are not well understood. It is thus important to NASA's mission that plant-pathogen interactions and resistance responses be well-defined mechanistically.

The current model for the genetic basis of pathogenic elicitation of resistance in plants was first described by Flor, who proposed the " gene for gene" hypothesis (Flor, 1971). According to Flor's hypothesis , plant resistance depends on the presence of specific host genes for resistance (R genes) and corresponding pathogen genes for avirulence (avr). A resistance response occurs only when both the specific R genes and corresponding avr genes are present. It is important to emphasize that, although several different R genes and avr genes have been identified, for a resistance response to occur, specific R and avr genes (the corresponding pair) must be present. Three models have been proposed to explain the genetically specific recognition between avr and R genes (or gene products) (Leach and white, 1996). These models are shown schematically in Scheme1. In the first model, the dimer model, the avr gene product (a protein whose true function is unknown) is the elicitor. It is exported from the bacterial cell via Hrp proteins (Hypersensitive reaction/pathogenic) to the plant, where it enters the plant cell and binds directly to the protein product of the R gene, or to the R gene itself. In the second, or elicitor/receptor model, the avr gene product has an enzymatic function; it synthesizes low molecular weight signal molecules or metabolites (elicitors) which are identified by a receptor in the plant cell encoded by corresponding R genes. In the third model, the transfer model, the

avr gene product is the elicitor, but is directly delivered to the cytoplasm of the plant cell via an attachment between bacterial and plant cell.



Scheme1 The three models for interaction between R and avr genes (based on Fig.3 in Leach and white, 1996).

Determining which of these models operates in what situation is a main goal of the research in this area. In general, all of these models are consistent with what has been learned concerning specific defense response mechanisms of the plants. These include, but are not limited to: 1. lignin deposition; 2. cell wall reinforcement; 3. production of active oxygen species; 4. hypersensitive response (HR), defined as rapid plant cell death (Leach and White, 1996). Each of these mechanisms will be discussed briefly below.

1. Lignin deposition Lignin or other phenolic polymers serve as physical barriers to prevent fungal penetration of host cells, although such lignin deposition is not likely to provide much protection against *Xanthomonas oryzae pv oryzae*, as it doesn't typically

penetrate the cell. However, the chemistry of lignin deposition may still be a useful defense response against this organism, as biosynthetic intermediates may be toxic to pathogen. Some of these reactive intermediates are active oxygen species generated by a class of enzymes known as peroxidases. Peroxidase activity thus seems to be intimately linked to the defense response. One example of this type of response is the incompatible interaction between rice cv Cas 209 and race 2 of *Xanthomonas oryzae pv oryzae*. The accumulation of lignin polymers and phenolic polymer deposition upon pathogenic infection was correlated with a decrease in bacterial multiplication. This response is believed to be controlled by the effect of the plant gene Xa-10, which offers resistance to some races of pathogen (Reimers *et.al.*, 1992).

2. Cell wall reinforcement Another defense mechanism is cell wall reinforcement of host cells. The bacterial plant pathogens, such as *Xanthomonas oryzae pv oryzae*, are found in vascular tissues or extracellular spaces. Host cells can produce an effective defense against these pathogens if lignified materials block extracellular spaces thus preventing bacterial spread.

3. Production of active oxygen species The production of active oxygen species and H_2O_2 , collectively called oxidative burst, is known to occur during a defense response (Bolwell et.al., 1995). Several rules for these oxidative burst products have been proposed, including crosslinking the cell wall structural proteins and generating signal molecules. Recent studies have shown that H_2O_2 generated by oxidative burst can also

cause hypersensitive death of challenged cells and induce cellular protectant genes in surrounding cells (Levine et.al., 1994).

4. Hypersensitive response A hypersensitive response is a rapid but localized necrotic response of a plant to microbial invasion. Current work toward understanding the sequence of events underlying the induction of hypersensitive cell death and defense gene transcription after pathogen attack is intensely being pursued (Lamb, 1994). Very little is known about the mechanism of hypersensitive cell death, but possible early events during hypersensitive cell death are ion fluxes (K^+/H^+) and loss of cellular compartmentalization (Atkinson *et. al.*, 1990). K^+/H^+ response was initially described as K^+ efflux/ H^+ influx exchange, but H^+ influx is unproven. ATPase inhibitors and increases in extracellular pH inhibits hypersensitive response and causes cell death (Murphy *et.al.*, 1989). Concomitant with the onset of hypersensitive response is transcriptional activation of defense genes subsequently encoding enzymes of phytoalexin and lignin synthesis (Lamb *et.al.*, 1994). Transcriptional activation of defense genes is initiated rapidly after the pathogen elicitor signal, but transcriptional dependent responses takes several hours for effective cumulative transcription and translation (Lamb *et.al.*, 1994).

The potentially important role of peroxidases in the defense response has been mentioned above. Peroxidases are known to be involved in the last enzymatic step of lignin biosynthesis, oxidation of cinnamyl alcohol into free radical intermediates which then crosslink. While a direct role of these isoenzymes in defense response has not yet been proven, an increase in peroxidase activity is correlated with infection in many plant

species. Reimers et.al.(1992) monitored peroxidase activity in extracellular extracts of rice leaves during compatible and incompatible interactions. It was observed that in incompatible interactions, cultivars containing the Xa-10 gene for bacterial blight resistance show an increase in extracellular activity of a cationic peroxidase (PO-Cl) and two anionic peroxidases, accompanied by a decline in the number of bacteria recovered from inoculated tissue, and by accumulation of lignin at the inoculation site. Thus, increases in three specific peroxidase isoenzyme activities is correlated with a resistance response of rice to *Xanthomonas oryzae. pv. oryzae*. No increase in PO activity was observed in compatible interactions. Current efforts toward the molecular characterization of PO-Cl will provide further insight into the significance of this isoperoxidase in the defense response in rice (Reimers *et.al.*,1992).

Another interesting factor concerning defense responses of rice plants to *Xanthomonas oryzae. pv. oryzae* is that light seems to be important (Reimers *et.al.*, 1993). When plants were exposed to more than 8 hours of light within the first 24 hours after infiltration with avirulent strains of *Xanthomonas oryzae. pv. oryzae*, leaf tissues showed resistance to pathogens as bacterial multiplication declined, hypersensitive response occured, and accumulation of lignin was observed. However, if plants infiltrated with avirulent bacteria were incubated in less than 8 hours of light within first 24 hours after infiltration, little cationic peroxidase was detected, lignin did not accumulate, and final bacteria numbers were higher than in tissues exposed to more than 8 hours of light. These

results suggest that light is essential for rice plant defense responses. The reason for the light effect is unknown, but may be related to increased energy needs.

As described above, these defense responses are elicited through a genetically specific interaction between the avirulence and resistance gene products. The mechanism of elicitation of the defense response is currently under much scrunity, and is the subject of intense investigation in many laboratories. Much progress has been made towards cloning and characterization both avr and R genes and gene products. However, this often does not provide much information about the function of gene or the gene product. The models previously discussed suggest that either the *avr* gene product must function as an elicitor, or it must modify some other protein or small molecule so as to produce an elicitor (Leach and White, 1996). In the enzymatic mode of action (the elicitor/receptor model), activity may occur within the bacterium, within the plant cell, or in extracellular spaces. The dimer model, proposed by Leach and White (1996) to explain the *X. o. pv. oryzae*/ rice interactions, defines the avr gene product (protein) as the elicitor. It predicts that avr gene products are located outside the bacterial cells, where they can interact with the plant cell.

Several experiments have suggested that this is not the case. Immunoelectron microscopy experiments suggest that the product of the avrXa10 gene is located in the cytoplasm of the bacterial cells (Reimers *et al.*, 1994). Young et.al. (1994) introduced an immunological tag (FLAG) into the C-terminal coding domain of the avrXa10 gene product in order to be able to specifically detect this protein. They found that the AVRXa10 protein was detected in the cytoplasm of *Xanthomonas oryzae pv. oryzae* but

not outside the bacterial cells. Also, no hypersensitive response could be elicited by treating rice cells with AVRXa10 protein isolated from a clone and expressed in *E.* Coli (Murphy *et.al.*, 1989). These results strongly support a hypothesis that AVRXa10 protein does not directly elicit the defense response, thus implying that it must be responsible for generating some mediator.

Later studies (Leach and White,1996), however, suggested that avrXa10 protein could be transported outside of the bacteria cell, but might not have been detected by Reimers *et.al.* or Young *et.al.* Reasons for this lack of detection, as suggested by Leach and White, could include poor antibody recognition, degradation of the avrXa10 protein by host proteases, or direct transfer of the protein from the bacterium into the plant cell. Thus, the models utilizing avr proteins as elicitors are still possible.

If the avr protein is in fact the actual elicitor, the mechanism by which it activates the various defense responses remains an important problem to be solved. The peroxidases discussed above are one possible target of activation. White et.al.(1996) have suggested that peroxidase genes of the host plants are activated during a defense response in rice elicited by avrXa10. One possible mechanism for induction of the peroxidase response is that the avrxa10 protein actually binds to the promoter and functions as a transcription factor, thus stimulating transcription of the peroxidase gene. Consistent with this hypothesis is the presence of nuclear localization signals within the avrXa10 protein (Leach *et.al.*, 1992). This hypothesis is readly tested. Plasmid DNA's containing peroxidase promoters have been previously constructed by Leach and colleagues (Leach *et.al.*, 1992).

With the peroxidase promoter DNA in hand, its binding to the avrXa10 protein can be readily assessed using a gel mobility shift asssay. The basis for this assay is the slower migration through a gel of a DNA fragment which has a protein bound to it. The mobility shift DNA-binding assay provides a simple, rapid and extremely sensitive method for detecting sequence-specific DNA-binding proteins. In the present investigation, an effort has been made to analyze the DNA binding properties of the *avrXa10* gene product from *Xanthomonas oryzae pv. oryzae.*

MATERIALS:

Protocols used were standard procedures used in Dr. Jan Leach's lab or found in molecular biology protocol manuals, except as noted. The plasmid DNA isolation kit was purchased from Qiagen Inc. Restriction enzymes (EcoR1 and Xho1) were obtained from Promega. Sephadex PD-10 columns were procured from Pharmacia. Bacterial cells (strain DH5□') containing the plasmids (pBS 6.1/7.5-Xho1, pBS 117.10/3.5-Xho1 and pBS G 6.1/x5.5), [^{32}P] labeled nucleotide (ICI), *Escherichia coli* (strain118-1 BL21(DE3)) bacteria containing the plasmid pET-avrXa10 and *Escherichia coli* (BL21(DE3)) bacteria containing the plasmid pET-22b+ were graciously donated by Dr. Jan Leach, Department of Plant Pathology, Kansas State University. The Anti-FLAG IgG M2 monoclonal antibody, which detects the FLAG peptide of the *avrXa10* gene, was purchased from Kodak. The secondary antibody (Alkaline Phosphatase conjugated antimouse-IgG) was obtained from Sigma Chemicals. The substrate kit for detection of the secondary antibody and the high range molecular weight markers were purchased from Biorad. The Coomassie Plus protein assay reagent was purchased from Pierce Biochemicals. The *Hin*dIII digested □DNA molecular weight ladder and 1 kb molecular weight ladder was obtained from BRL Life Tech. The optical absorption measurements were made using a McPherson EU-707D spectrophotometer. The recipes for media and buffers used are provided in the appendix. Additional background articles are included in the reference section.

Plasmid DNA isolation:

The plasmids containing the rice genomic clones pBS 6.1/7.5-Xho1, pBS

117.10/3.5-Xho1 and pBS G 6.1/x5.5 were isolated using the QIAGEN plasmid purification kit according to the protocol provided. Plasmid concentrations were then calculated from the absorbance at 260 nm using Beer's law (A = □ lc) and letting $□_{260}$ = 6600 L/cm· mol per nucleotide. Yields were then calculated based on an average molecular weight (MW) of 330 g/mol per nucleotide.

Agarose gel electrophoresis of plasmid samples:

A 0.05 μg sample of each plasmid prepared above was analyzed by 1% agarose gel electrophoresis in TAE buffer containing ethidium bromide at concentration 2 μg/mL. The bands were visualized by UV transillumination.

Digestion of DNA with Restriction Endonuclease Xho1:

Xho1 restriction analysis was carried out to further verify the identity of the isolated DNA. A 0.05 μg quantity of each plasmid was incubated with 5 units of Xho1 restriction enzyme for one hour at 37 ^{0}C in buffer provided by the enzyme supplier. The products were analyzed by agarose gel electrophoresis as above.

Purification of the protein AVRXa10.F1:

The *Escherichia coli* bacteria (strain BL21(DE3)) containing pET-avrXa10 plasmid (contains the gene coding for the AVRXa10.F1 protein) were inoculated in 15 mL of LB media containing carbenicilin at a concentration of 100 μg/mL and incubated for 16 hours at 37 ^{0}C on a rotary shaker. Then 1 mL of bacteria was subcultured in 250 mL of LB media and incubated for 3 hours under the same conditions. To this culture IPTG (isopropyl-□-thiogalactopyanoside) was added to a final concentration of 1 mM, and incubation was continued for an additional 2 hours. The cells were harvested by

centrifugation at 10,000 rpm for 10 minutes at 4 ^{0}C. The pellet was washed with 10 mM phosphate buffer and centrifuged as before. It was then resuspended in 5 mL of 10 mM phosphate buffer and the cells were disrupted using a French press. The solution containing the disrupted cells was centrifuged for 30 minutes at 12,000 rpm at 4 ^{0}C. The supernatant containing the soluble proteins was fractionated with ammonium sulfate. A 1-mL portion of a chilled saturated ammonium sulfate solution was added dropwise. At 20 % ammonium sulfate by weight, the precipitated proteins were isolated by centrifugation at 12,000 rpm for 10 minutes at 4 ^{0}C. The supernatant was further combined with additional ammonium sulfate in a similar fashion until 40 % ammonium sulfate concentration was reached. Precipitated proteins were isolated by centrifugating in the same way. The final pellet from each fraction was dissolved in 1 mL of 10 mM phosphate buffer. The protein fractions were then desalted on a Sephadex PD-10 column, eluting very slowly with the 10mM phosphate buffer, and analyzed by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Biorad mini-Protean).

Western Blot of the AVRXa10.F1 protein:

Proteins from the gel were transferred to a nitrocellulose membrane in order to probe and identify the AVRXa10.F1 protein. The blotting apparatus was assembled as shown in Figure 2. The transfer was carried out for 1.5 hours at 100 mA using Towbin buffer. The membrane was blocked with 1% (w/v) dry milk solution overnight in order to prevent non-specific protein binding. The blot was overlayed with 6 μL of primary monoclonal antibody (M2) (which detects the flag peptide) in TBS buffer and 8 μL of secondary antibody (alkaline phosphatase conjugated antimouse-IgG) which detects the

primary antibody. The blot was then washed with TBS buffer. A substrate kit containing BCIP (5-bromo-4-chloro-3-indolyl phosphate)/ NBP (nitroblue tetrazolium in DMS) substate and alkaline phosphatase color was used for the detection of the secondary antibody.

DNA Probe Radiolabeling Reaction:

In a 0.5 mL microcentrifuge tube were combined 10 pmol (2 μL) of the POX 22.3 PCR amplified region of the peroxidase promoter gene, 10 pmol (3 μL) of □- ^{32}P labeled ATP, 1 μL of T4 polynucleotide kinase 10X buffer, 5 units (1 μL) of T4 Polynucleotide Kinase (5-10 u/μL), and 3μL of sterile water. The contents were incubated at 37 ^{0}C for 30 minutes. The kinase was deactivated at 90 ^{0}C for 2 minutes. The end-labeled DNA was stored at -20 ^{0}C.

Mobility shift assay using pET-avrXa10.F1 protein:

The mobility shift assay was carried out on a 4 % nondenaturing polyacrylamide gel. For the binding reaction, 4 fmol (~ 4 ng) DNA probe, 2 μg non specific carrier DNA, 10% glycerol, 25 mM HEPES, pH 7.5, 40 mM KCl, 0.1 mM EDTA, and 15 μg pET-avrXa10.F1 extract (20-40 % fraction) were combined in a 0.5 mL microcentrifuge tube. The binding mixture was incubated for 20 minutes at room temperature. The gel was loaded and electrophoresed at 30-35 mA for 15 minutes in tris-glycine buffer at room temperature. After electrophoresis, the gel was dried and autoradiographed.

Mobility shift assay using pET-22b+ proteins:

A mobility shift assay was also carried out using protein extracts from *E-coli* cells containing the pET vector without the insert as a control. The analysis was carried out on

a 4% nondenaturing polyacrylamide gel. For the binding reaction, 4f mol (~ 4 ng) DNA probe, 2 μg non specific carrier DNA, 10 % glycerol, 25 mM HEPES, pH 7.5, 40 mM KCl, 0.1 mM EDTA, and 15 μg crude extract of pET-22b+ were combined in a 0.5 mL microcentrifuge tube. The binding mixture was incubated for 20 minutes at room temperature. The gel was loaded and electophoresed at 30-35 mA for 15 minutes in tris-glycine buffer at room temperature. After electrophoresis, the gel was dried and autoradiographed.

Estimation of plasmid DNA pBS 6.1/7.5-Xho1, pBS 117.10/3.5-Xho1 and pBS G 6.1/x5.5-Xho1:

The plasmids pBS 6.1/7.5-Xho1, pBS 117.10/3.5-Xho1 and pBS G 6.1/x5.5-Xho1 in *E. coli* bacteria, all donated by Dr. Jan Leach, were isolated using the QIAGEN plasmid purification kit. The plasmid DNA pBS 6.1/7.5-Xho1, isolated in this way, was quantified spectrophotometrically. A 10 L aliquot of the original solution was diluted to 1.0 mL with water. The resultant dilution yielded an absorbance of 0.078 AU at 260 nm. Using Beer's law and an extinction coefficient of 6,600 L/mol·cm, the concentration was determined to be 1.18×10^{-5} moles of nucleotide/liter. After accounting for the initial dilution, this yielded an original amount of nucleotide of 4.44×10^{-7} moles of nucleotide. Using an average molecular weight of 330 g/mol per nucleotide, a total yield was 146g was calculated.

Using a procedure identical to that described above, final yields of plasmids pBS 117.10/3.5-Xho1 and pBS G 6.1/x5.5-Xho1 were determined to be 558g and 369g, respectively.

Restriction digest of the plasmids pBS 6.1/7.5-Xho1 and pBS G 6.1/x5.5-Xho1:

The restriction digest of the plasmids was performed to verify the identity of the clones. The lane designations of the restriction digest are shown in Table I. The *Hin*dIII digested DNA molecular weight ladder and 1 kb molecular weight ladder are the standard markers used to estimate the molecular weight of the DNA fragments. A 0.5 g sample of the plasmids isolated above were loaded. The electrophoresis results are shown in Figure 3. The approximate MW of the fragments of the plasmid pBS 6.1/7.5-Xho1 was

12.2, 8.1 and 4.0 kb respectively and that of plasmid pBS 6.1/x5.5-Xho1 was 10.2, 5.0 and 4.0 kb respectively. In the absence of the *avrXa10* insert, only two bands, the linear and the supercoiled, are expected. The digestion gave three bands for each of the plasmid DNA samples, showing that the insert was cloned into the *Xho*1 site of the respective plasmids. Also, the comparison of the molecular weights of the fragments of plasmid pBS 6.1/7.5-Xho1 and pBS 6.1/x5.5-Xho1 with lab models showed these were the correct clones.

Electrophoretic properties of AVRXa10.F1:

The AVRXa10.F1 protein was isolated from *Escherichia coli* (strain118-1 BL21(DE3) transformed with pET-avrXa10.F1). The soluble proteins were partitioned with ammonium sulfate into 2 fractions, those that precipitated in the presence of 20 % ammonium sulfate and those that precipitated in the presence of 40 % ammonium sulfate. The pellet obtained after the 0-20 % fractionation was small compared to the other. Upon redissolving in phosphate buffer, the proteins were desalted on a Sephadex column to remove ammonium sulfate. Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to analyze the protein content of the two fractions defined above. The high molecular weight markers (Biorad) were used to estimate the molecular weight of the putative AVRXa10.F1 protein. The SDS-PAGE gel is shown in Figure 4. The putative AVRXa10.F1 protein is indicated. The approximate molecular weight is 119 kd.

Protein estimation using the Coomassie plus protein assay reagent:

The protein extracts were assayed for total protein content by the Bradford method

(Bradford M.M., 1976). The concentrations and their respective absorbances of the standards (Bovine Serum Albumin (BSA)) and the unknown (AVRXa10.F1 protein extract) are given in Table II. A standard curve of absorbance vs. protein concentration was generated using the Corel Quattro Pro program and is shown in Figure 6. Linear regression analysis was carried out in order to determine the equation of the line that best fit the experimental data points. Concentration of the unknown was then estimated by comparing its absorbance to the standard curve. The estimated concentration of the total proteins in the 20-40 % fraction was 1.44mg/mL.

Western blot analysis of AVRXa10.F1 protein:

To confirm the presence and location of AVRXa10.F1 protein, the SDS-PAGE gel was electroblotted to nitrocellulose and immunostained with primary monoclonal (M2) antibody (which is raised to the FLAG tag) and secondary antibody (alkaline phosphatase conjugated antimouse-IgG). This substrate yielded a purple color upon hydrolysis by the alkaline phosphatase . The antibodies cross-reacted specifically with the AVRXa10.F1 protein in the western blot from SDS gel, consistent with the putative assignment above, as shown in Figure 5.

Mobility shift assay using the end-labeled primer:

The PCR product 22.3 peroxidase promoter of the plasmid pBS 117.10/3.5-Xho1 was donated by Dr. Jan Leach. The DNA was subsequently end-labeled using -[^{32}P]-ATP and polynucleotide kinase. A 4% nondenaturing PAGE gel was used for the mobility shift assay. The autoradiograph of the gel showing DNA migration in the presence and absence of the AVRXa10.F1 protein is shown in Figure 7. The comparison of the band of

the DNA protein complex (lane 1, top of the gel) and DNA alone (lane 4) indicate possible binding of the AVRXa10.F1 protein and the DNA.

Mobility shift assay using plasmid pET-22b+ as control:

The experiment was performed to incorporate the plasmid pET-22b+ (lacking the avrXa10.F1 insert) as a control. The DNA used for this experiment was PCR product 22.3 region of the peroxidase promoter which was 5' end labeled with -[^{32}P]-ATP, as described above. The mobility shift assay was carried out on a 4 % native PAGE gel. Binding reactions were carried out using 4 ng of the above DNA with 15 g of the 20-40 % protein fraction containing the AVRXa10.F1 protein, and also with 15 g of a 20-40 % protein fraction isolated from bacteria containing plasmid pET-22b+ (same cloning vector but lacking the avrXa10.F1 insert), which was used as the control. The results are shown in Figure 8. The quantitative differences in the band patterns in the lanes containing DNA and protein fraction with AVRXa10.F1 (lane 3) and that of the DNA and protein containing no *avrXa10* insert used as a control (lane 4) show the possible binding of the DNA and AVRXa10.F1 protein.

The *avrXa10* gene is one of approximately 12 related bacterial avirulence genes in the *Xanthomonas oryzae pv. oryzae* genome (Young *et al.* 1994) involved in elicitation of resistance. *Xanthomonas oryzae pv. oryzae*, the pathogen causing bacterial blight of rice, interacts with rice cultivars containing different resistance genes. The mechanisms of host-pathogen interaction leading to elicitation of the resistance response are yet to be identified, although studies suggest an extrabacterial role for at least some of the Avr proteins (Leach and White, 1996). One postulated mechanism for this interaction is the direct activation of resistance genes by the AVRXa10 protein. The function of proteins as transcription factors is well known (Garrett and Grisham, Biochemistry). Given the sequence of the AVRXa10 protein, particularly the identification of possible nuclear localization sequences within the protein, the intriguing possibility that the AVRXa10 protein fortuitously acts as a general transcription factor inside the plant cell was examined. If such an activation occurs to elicit a defense response, a likely target is the set of peroxidase genes, as their participation in the response is well established (Leach and White, 1996). In particular, a possible target in rice is the POX 22.3 peroxidase gene promoter, the cationic peroxidase for which this gene code is known to show increased concentrations upon infection. A study of a possible interaction between AVRXa10.F1 protein and POX 22.3 peroxidase promoter interaction was thus undertaken in the hope that it would provide insight into a mechanism of elicitation of resistance in rice.

Because of the importance of this bacterial gene product with respect to pathogenicity of the organism, it has previously been cloned into a pET vector by Leach and co-workers. The clone is approximately 2.96 kb in size. An abbreviated restriction

map of the *avrXa10* gene is given in the Figure 9. The clones of the three peroxidase promoters, pBS 6.1/7.5-Xho1, pBS 117.10/3.5-Xho1 and pBS G 6.1/x5.5-Xho1, are all Xho1 fragments cloned into the Xho1 site of the vector pBluescript. The restriction map of pBluescript (pBS) vector is given in Figure 10. The pBS 6.1/7.5-Xho1 plasmid is a rice genomic clone 7.5, kb in length, containing N-terminal coding sequence of peroxidase gene POX 8.1 and its promoter region. pBS 117.10/3.5-Xho1 plasmid is also a rice genomic clone, 3.5 kb in length. It contains the promoter, coding regions, and 3' untranscribed region (3' UTR) of the POX 22.3 peroxidase gene. pBS G 6.1/x5.5-Xho1 is a clone containing the above 3.5 kb fragment, as well as C-terminal half of POX 8.1 peroxidase gene.

Each of these pBS constructs containing peroxidase promoters were obtained as gifts from Dr. Jan Leach. Each clone was amplified in *E.Coli* and isolated by standard procedures. A restriction digest of each of the clones, pBS 6.1/7.5-Xho1 and pBS G 6.1/x5.5-Xho1, was carried out with the Xho1 restriction endonuclease in order to verify the identity and integrity of the isolated clones. The digestion gave three bands of varying lengths for each of the clones, as indicated in Figure 3. The restriction digest of pBS 6.1/7.5-Xho1 gave bands corresponding to molecular weights of approximately 12.2, 8.1 and 4.0 kb, respectively and that of pBS G 6.1/x5.5-Xho1 gave bands corresponding to molecular weights of approximately 10.2, 5.0 and 4.0 kb. The largest fragment in each case corresponds to some undigested plasmid DNA. The 4.0 kb fragment corresponds to the cloning vector itself, while the 8 and 5 kb fragments correspond to the expected sizes of the cloned inserts. The restriction enzyme Xho1 thus is shown to cleave the plasmids

pBS 6.1/7.5-Xho1 and pBS G 6.1/x5.5-Xho1 at two positions, yielding the expected fragments and sizes for the clones. The analysis verified that the correct clones were generated and isolated for study.

The AVRXa10.F1 protein was expressed from the pET vector in *E-Coli* and isolated using a protein purification protocol developed in the lab of Dr. Jan Leach. Use of French press instead of sonication for cell lysis has been reported to yield better results (Young *et. al.* 1994). The ammonium salts of the proteins obtained after fractionation were desalted using Sephadex PD-10 column rather than by the cumbersome dialysis procedure. The estimation of the quantity of the isolated proteins was carried out using the Coomassie Plus Protein Assay Reagent. The estimated total protein concentration was 1.44 mg/mL, for a total yield of 2.88 mg.

Unfortunately, a good method for isolating pure functional AVRXa10.F1 protein has not yet been developed. The protein is rather unstable, and the extra time required for additional purification steps results in a dramatically decreased protein activity. Thus, DNA binding experiments were carried out using the partially purified mixture obtained at this point.

SDS-PAGE analysis was used to assess the purity of this protein mixture and to verify the presence of the AVRXa10.F1 protein within it. The protein mixture is shown in lane 1 of Figure 4. Tentative assignment of the band corresponding to the target protein is indicated by the arrow. The experiment clearly shows that most of the protein is contained in the 20-40% ammonium sulfate fraction. In order to verify the assignment of the

AVRXa10.F1 band, advantage was taken of the fact that an immunological tag (FLAG) has been previously introduced (Young *et. al.* 1994) into the C-terminal region of the *avrXa10* gene to detect the gene product specifically. Western blot analysis could thus be performed to identify the AVRXa10.F1 protein. Anti-FLAG IgG M2 monoclonal antibody, used for detection of FLAG peptide, was shown to specifically bind to a protein of molecular weight of approximately 119kDa (Figure 4, lane 3), consistent with the previous tentative assignment.

Once the AVRXa10.F1 protein and the peroxidase promoters were obtained, electrophoretic mobility shift assays were performed in order to identify possible interactions between the protein and the DNA. Specifically, nondenaturing PAGE was used to detect the formation of a specific complex between the POX 22.3 peroxidase promoter and the AVRXa10.F1 protein. The assays detect proteins binding specifically to the ^{32}P-end labeled DNA fragment under study, as the protein binding will retard the mobility of the fragment during electrophoresis. The PCR amplified 22.3 region of the peroxidase promoter was used as the DNA probe. This DNA was used because it contains the promoter, coding regions and 3' UTR region of the peroxidase gene POX 22.3. The autoradiograph (Figure 7), indicates possible binding of the AVRXa10.F1 protein to the 22.3 promoter region of the peroxidase gene. This can be concluded by the comparison of the band of the DNA protein complex (lane 1) and the DNA band alone (lane 4). Figure 8 also indicates a possible interaction between the DNA and the AVRXa10.F1 protein, which can be concluded from the quantitative differences between the band in the lane containing the DNA alone (lane1) and the band pattern in the sample containing DNA and

the protein (lane 3). There is also a quantitative difference between the band patterns in the lanes containing DNA and protein fraction with AVRXa10.F1 (lane 3) and that of the DNA and protein containing no *avrXa10* insert used as a control (lane 4). The differences in the mobilites of the DNA substrates in the presence and absence of protein is consistent with what would be expected for protein binding.

An understanding of the molecular basis for the ability of a plant to recognize when it has been infected by a pathogen and subsequently generate a defense response is a difficult but important goal. It is difficult because a plant undoubtedly utilizes many different mechanisms for doing so, and the molecular interactions are quite complex. It is important because the ability of humans to protect important food crops from pathogenic destruction is increasingly important as the world's population grows. An understanding of the molecular interactions that are involved in this complex process is necessary in order to rationally develop interventions to fight pathogenic infection by augmenting and broadening the defense response. Rice is a particularly important plant to study in this regard, as it represents a major food source for much of the world's current population. The binding of the *Xanthomonas* AVRXa10.F1 protein to the cloned peroxidase promoter DNA, as indicated by this study, would represent substantial support for a unique pathogen recognition and defense response mechanism utilized by at least some races of rice. This is the first experimental evidence supporting the hypothesis that the AVRXa10 gene product ellicits a defense response in rice by acting as a general transcription factor, thereby actually inducing the expression of plant proteins which participate in pathogen destruction. If future experiments can verify this interaction, it would be an exciting and

significant contribution to the field of plant pathology because: 1) it is the first evidence for such a signalling mechanism; 2) it would motivate a search for similar mechanisms in other plant systems; 3) the mechanistic information may lead to new strategies for pesticide development via chemical or genetic means.

The molecular interactions observed in this study are in some ways surprising. By producing proteins that act as rice peroxidase transcription factors, *Xanthomonas* is actually elliciting its own destruction, a strategy that does not seem evolutionarily sensible. Why, then, might this interaction occur?

One possibility is that the binding of this protein to peroxidase promoters is fortuitous, and that the true (but as yet unknown) function of the AVRXa10 gene product is critical to some other event in the pathogen infection process. Such a scenario would at least provide a logic for evolutionary preservation of an otherwise apparently disadvantageous protein. Notably, the ellicitation of the defense response by the avrXa10 gene product only occurs in strains of rice that carry the corresponding resistance genes. Thus, pathogenicity is not compromised in other strains or races, and the survival of the pathogen as a species is not substantially deleteriously affected. The maintainance of this unidentified but critical function would be more impotant to *Xanthomonas* than a compromised pathogenicity in a subset of rice strains.

Another possibility is that the enhancement of the transcription of certain genes may actually be in some way beneficial to *Xanthomonas*. Given our considerable lack of understanding of the molecular interactions involved in the infection process and defense response, one cannot rule such a possibility out. In this case, the peroxidase gene may or

may not be the primary target for such enhancement, but may fortuitously contain similar enhancer or promoter elements as does the targeted gene. This explanation is perhaps more consistent with the apparent nuclear localization signals present on the avrXa10 gene product.

Irrespective of the reasons for the possible activation of the peroxidase gene by the Xanthomonas avirulence protein, its existence presents a potential target for intervention. The details on how this might be accomplished are not yet clear, but will emerge upon further examination of this system. Agricultural biotechnology has already siezed the opportunity to generate genetically engineered crops which are resistant to herbicides as well as to various types of diseases. Two things are necessary for successful implementation of such strategies, namely the identification of an appropriate molecular target, and the ability to genetically manipulate this target in a useful way. These studies have identified one such molecular target.

Although the results of this study are strongly suggestive, they are not conclusive that the AVRXa10.F1 protein actually binds to the peroxidase promoter. A number of follow-up experiments need to be carried out to verify this interaction and to exploit the results. The mobility shift assays need to be modified and refined to give clearer results. Cleaner, more precisely defined DNA samples which are uniquely end-labeled should be used rather than the PCR amplified and labeled DNA's utilized in these experiments. If possible, pure protein should be used rather than the protein mixture, and a much more extensive set of controls needs to be done, including competition experiments with non-specific proteins. Conditions need to be varied to define any dependence of the protein-

DNA interaction on temperature, pH, metal ions, or co-inducers. Also, other rice genes which yield enhanced expression under these conditions need to be identified. Once the binding parameters have been identified and verified, this information can be used to systematically search for similar interactions in other systems, and to develop methodologies for practical applications with respect to crop enhancements.

In conclusion, a putative binding interaction between the avrXa10 avirulence gene product from Xanthomonas oryzae pv. oryzae and the promoter region of the peroxidase gene in a resistance strain of rice has been identified. It is suggested that the avirulence protein may function as a transcription factor. The binding of the protein to the DNA thus represents the molecular mechanism by which the rice defense response is ellicited in this system. This mechanism, when verified, would be unique among all plant-pathogen interactions studied to date, and would also represent the first defined role for an avirulence gene in elliciting plant defense responses. Results such as these should eventually lead to more efficient and sustainable crop production.

Abbott, M.K., Bennett, R.L., & Denell, R.E. (1994). Insect gravitational biology: Ground-Based and shuttle flight experiments using the beetle *Tribolium castaneum*. *J. Exp. Zool.* 269, 242-252.

Akiyama, H., Kanai, S., Hirano., Kumei, Y., Mukai, C., & Nagaoka, S. (1996). An improved quantitative RT-PCR flourescent method for analysis of gene transcripts in the STS-65 space shuttle experiment. *J. Biotechnol.* 47, 325-333.

Armbrust, T.S., Odom, W.R., & Guikema, J.A. (1994). Structural analysis of photosystem I polypeptides using chemical crosslinking. *J. Exp. Zool.* 269, 205-211.

Armstrong, J.W., Chapes, S.K. (1994). Effects of extracellular matrix proteins on macrophage differentiation, growth, and function: Comparison of liquid and agar culture systems. *J. Exp. Zool.* 269, 178-187.

Atkinson, M.M., Keppler, L.D., Orlandi, E.W., & Mischke, C.F. (1990). Involvement of plasma membrane influx in bacterial induction of the K^+/H^+ exchange and hypersensitive responses in tobacco. *Plant Physiol.* 92, 215-221.

Bolwell, G.P., Butt,V.S., Davies, D. R., & Zimmerlin A. (1995). The origin of oxidative burst in plants. *Free Rad. Res* 23, 517-532.

Bradford, M..M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254

Bradley, D.J., Kjellbom, P., & Lamb, C.J. (1992). Elicitor and wound-induced oxidative cross-linking of a protein-rich plant cell wall protein: a novel, rapid defense response. *Cell* 70, 21-30.

Chang, D., Paulsen, A., Johnson, T.C., & Consigli, R.A. (1993). Virus protein assembly in microgravity. *Adv. Space Res.* 13, 251-257.

Clejan, S., O'Connor, K.C., Haque, S., & Primavera, A.C. (1995). Effects of simulated microgravity on DU 145 human prostate carcinoma cells. *Biotechnol. Bioeng.* 50, 587-597.

Cogoli, A. (1996). Biology under microgravity conditions in Spacelab International Microgravity Laboratory 2 (IML-2). *J. Biotechnol.* 47, 67-70.

Cogoli, A. (1993). The effect of hypogravity and hypergravity on cells of the immune systems. *J. Leukoc. Biol.* 54, 259-268.

Flor, H.H. (1971). Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* 9, 275-296.

Fuchs, B.B., & Medvedev, A.E. (1993). Countermeasures for ameliorating in-flight immune dysfunction. *J. Leukoc. Biol.* 54, 245-252.

Gubler F., Kalla R., Roberts, J.K., & Jacobsen, J.V. (1995). Gibberellin-regulated expression of a myb gene in barley aleurone cells:evidence for myb transactivation of a high-pl □-amylase gene promoter. *The plant cell* 7, 1879-1891.

Hilaire, E., Paulsen, A.Q., Brown, C.S., & Guikema, J.A. (1995). Microgravity and clinorotation cause redistribution of free calcium in sweet clover columella cells. *Plant cell physiol.* 36, 831-837.

Hilaire, E., Paulsen, A.Q., Brown, C.S., & Guikema, J.A. (1995). Effects of clinorotation and microgravity on sweet clover columella cells treated with cytochalasin D. *Physiol. Plant.* 95, 267-273.

Horneck, G., Rettberg, P., Rink, H., Kozubek, S., & Schmitz, C. (1996). DNA repair in microgravity: studies on bacteria and mammalian cells in the experiments REPAIR and KINETICS. *J. Biotechnol.* 47, 99-112.

Konstantinova, I.V., Rykova, M.P., Lesnyak, A.T., & Antropova, E.A. (1993). Immune changes during long-duration missions. *J. Leukoc. Biol.* 54, 189-201.

Kumei, Y., Shimokawa, H., Katano, H., Whitson, P.A., & Sams, C.F. (1996). Microgravity induces prostaglandin E2 and interleukin-6 production in normal rat osteoblasts: role in bone demineralization. *J. Biotechnol.* 47, 313-324.

Lamb, C.J. (1994). Plant disease resistance genes in signal perception and transduction. *Cell* 76, 419-422.

Levine, A., Tenhaken R., Dixon, R., & Lamb, C. (1994). H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79, 583-593.

Leach, J.E., & White, F.F. (1996). Bacterial avirulence genes. *Annu. Rev. of Phytopathol* 34, 153-179.

Marthy, H.J., Gasset, G., Tixador, R., Schatt, P., Eche, B., & Gorand, D. (1996). The sea urchin larva, a suitable model for biomineralisation studies in space. *J. Biotechnol.* 47, 167-177.

Meehan, R., Whitson, P., & Sams, C. (1993). The role of psychoneuroendocrine factors on spaceflight-induced immunological alterations. *J. Leukoc. Biol.* 54, 236-244.

Murphy, T., & Huerta, A.J. (1989). Effects of extracellular pH on UV-induced K+ efflux from cultured rose cells. *Plant Physiol.* 90, 749-753.

Ohme-Takagi, M., & Shinshi, H. (1995). Ethylene-inducible DNA binding proteins that

interact with an ethylene-responsive element. *The Plant Cell* 7, 173-182.

Pippia, P., Sciola, L., Cogoli-Greuter, M., Meloni, M.A., & Cogoli, A. (1996). Activation signals of T lymphocytes in microgravity. *J. Biotechnol.* 47, 215-222.

Reimers, P..J., Guo A., & Leach, J.E. (1992). Increased activity of a cationic peroxidase associated with an incompatible interaction between *Xanthomonas oryzae pv. oryzae* and Rice (*Oryza sativa*). *Plant Physiol* 99, 1044-1050.

Schmitt, D.A., & Schaffar, L. (1993). Isolation and confinement as a model for spaceflight immune changes. *J. Leukoc. Biol.* 54, 209-213.

Seaman, G.V.F., & Snyder, R.S. (1996). Electrophoresis in space. *J. Biotechnol.* 47, 335-339.

Sonnenfeld, G., & Miller, E.S. (1993). The role of cytokines in immune changes induced by spaceflight. *J. Leukoc. Biol.* 54, 253-258.

Spooner, B.S., Metcalf, J., DeBell, L., Paulsen, A., & Guikema, J.A. (1994). Development of the brine shrimp *Artemia* is accelerated during spaceflight. *J. Exp. Zool.* 269, 253-262.

Spooner, B.S., Hardman, P., & Paulsen, A. (1994). Gravity in mammalian organ development: Differentiation of cultured lung and pancreas rudiments during spaceflight. *J. Exp. Zool.* 269, 212-222.

Spooner, B.S. (1994). Introduction: Gravitational cellular and developmental biology. *J. Exp. Zool.* 269, 177.

Tamagnone, L., Merida A., Parr A., Mackay S., Culianez-Macia, F.A., Roberts K., & Martin C. (1998). The AmmYB308 and AmMYB330 transcription factors from antirrhinum

regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. *The plant cell.* 10, 135-154.

Taylor, G.R. (1993). Overview of spaceflight immunology studies. *J. Leukoc. Biol.* 54, 179-188.

Taylor, G.R. (1993). Immune changes during short-duration missions. *J. Leukoc. Biol.* 54, 202-208.

Thevenet, D., D'Ari, R., & Bouloc, P. (1996). The signal experiment in BIORACK: *Escherichia coli* in microgravity. *J. Biotechnol.* 47, 89-97.

Young, S. A., White, F.F., & Leach, J.E. (1994). AVRXa10 Protein is in the cytoplasm of *Xanthomonas oryzae pv. oryzae*. *MPMI* 7, 6, 799-804

LB (Luria-Bertani) medium

Per Liter:

10g Bacto-tryptone
5g Bact.-yeast extract
10g NaCl

10% SDS

10g SDS plus 90 mL water. The solution was autoclaved to sterilize.

TAE (Tris-Acetate EDTA) Buffer pH 7.4 (Working Solution: 50x)

Tris-base 242 g
Glacial Acetic Acid 57.1 ml
EDTA (0.5 M pH 8) 100 ml

TE (Tris-EDTA) buffer pH 7.4

10 mM Tris, 1 mM EDTA

Agarose Gel (1%)

1g agarose per 100 ml TAE Buffer.

Phosphate Buffer A pH 7.4

10 mM Pi (8.1 mM K_2HPO_4 and 1.9 mM KH_2PO_4)
1 mM EDTA
1 mM DTT (Dithiothreitol)
0.5 mM PMSF

Tris/Glycine/SDS electrode buffer (Towbin buffer) pH 8.3

Tris base 3g
Glycine 14.4g
10% SDS soln. 10 mL
Deionized water to 1000 mL

TBS (Tris-Borate) buffer

50 mM Tris (pH 7.4)
150 mM NaCl

Electrode buffer for mobility shift assay (pH 8.3)

Tris base 3g
Glycine 14.4g
EDTA 0.372g
Deionized water to 1000 mL

TABLES

Table I. Restriction digest of plasmids pBS 6.1/7.5-Xho1 and pBS G 6.1/x5.5-Xho1 with the restriction enzyme Xho1.

Table I corresponds to figure 3 (page27).

Lane number	Contents
1	0.1μg of 1 kb ladder
2	0.05μg of plasmid pBS 6.1/7.6-Xho1
3	0.15μg of plasmid pBS 6.1/7.6-Xho1
4	0.5μg of mass ladder
5	0.05μg of plasmid pBS G 6.1/x5.5-Xho1
6	0.15μg of plasmid pBS G 6.1/x5.5-Xho1
7	0.1μg of 1 kb ladder

Table II. Protein estimation of the AVRXa10.F1 over expressed from pET-avrXa10.F1 in *E-coli*.

Absorbances are measured at 595 nm.

Table II corresponds to figure 6 (page 30)

well number	concentration (mg/ml)	Net absorbance
1	2.0	1.625
2	1.5	1.406
3	1.0	1.467
4	0.75	1.326
5	0.50	1.098
6	0.25	0.780
7	0.125	0.718
8	unknown sample(AVRXa10.F1)	1.459

FIGURES

Figure 1. The pET-avrXa10.F1 clone containing pET-22b+ *T7lac* promoter (P3). The restriction sites are: B, *Bam*H1; E,*Eco*R1; K,*Kpn*1; P,*Pst*1; S,*Sma*1; X,*Xba*1. The FLAG peptide is shown as DYKDDDDK.

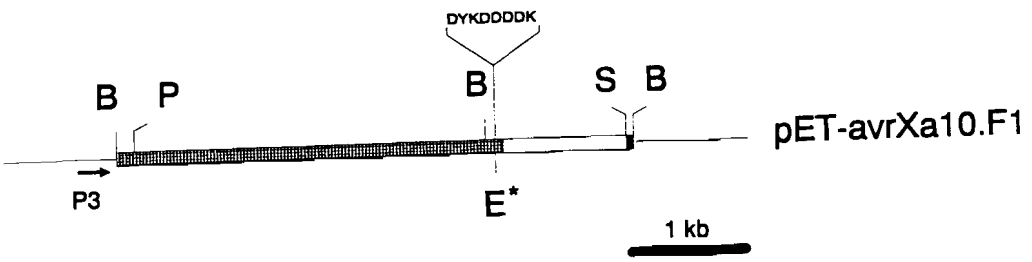

DYKDDDDK
B
P
B
S
B
pET-avrXa10.F1
P3
E*
1 kb

Figure 2. Assembly of the blotting apparatus. The filter paper was cut to the same size as the sponge and the SDS-PAGE gel was placed between them on the black side. The nitrocellulose membrane was placed on top of the gel and the cassette was closed.

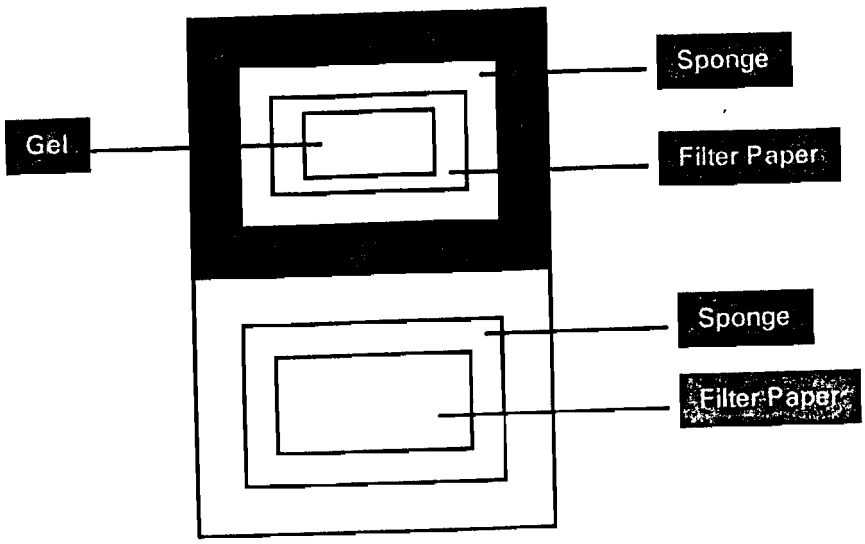

Sponge
Gel
Filter Paper
Sponge
Filter Paper

Figure 3. Agarose Gel showing the restriction digest of the plasmids pBS 6.1/7.6-Xho1 and pBS G 6.1/x5.5-Xho1 with the restriction enzyme Xho1. Lane designations are given in Table I.

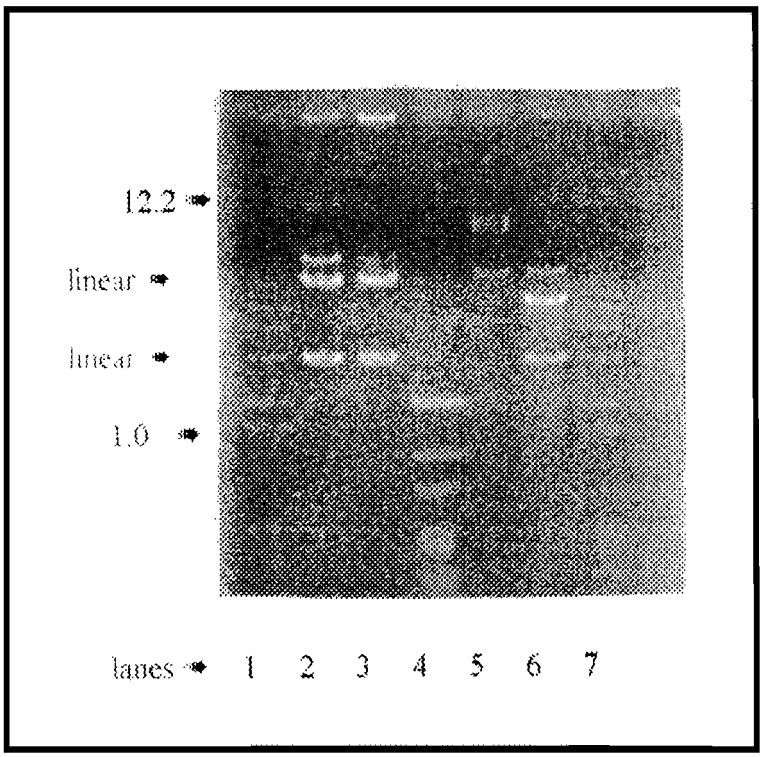

12.2
linear
linear
1.0
lanes 1 2 3 4 5 6 7

Figure 4. The SDS-PAGE gel showing protein fractions precipitated by ammonium sulfate during the AVRXa10.F1 protein isolation procedure. Lane1: 20-40% fraction. Lane 2: 0-20% fraction. Lane 3: High molecular weight marker (MW range 40-250 kd). The MW of the five bands are 205, 118, 95, 80 and 49 kd respectively.

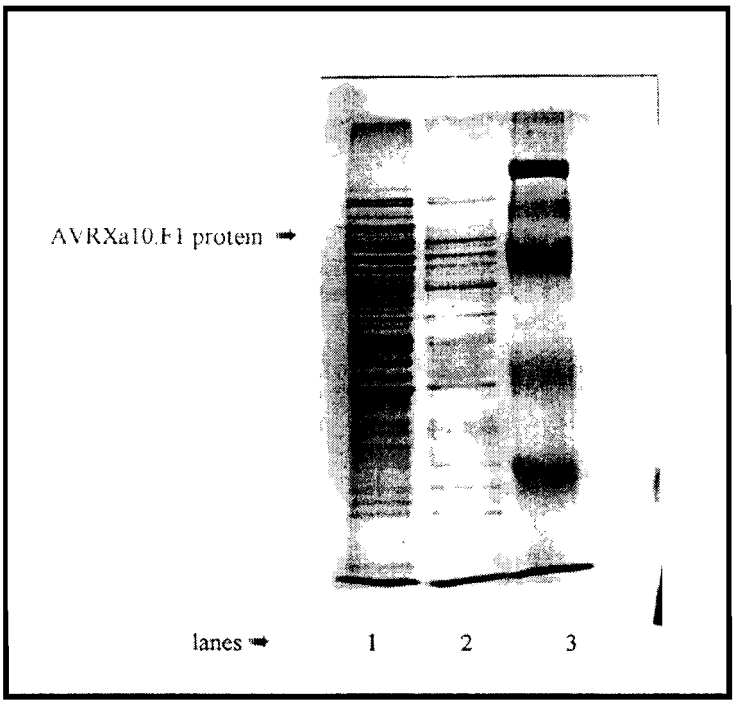

AVRXa10.F1 protein
lanes
1
2
3

Figure 5. Western blot confirming the presence of AVRXa10.F1 protein. Lane 1: High molecular weight marker. Lane 2: Band corresponding to the AVRXa10.F1 protein.

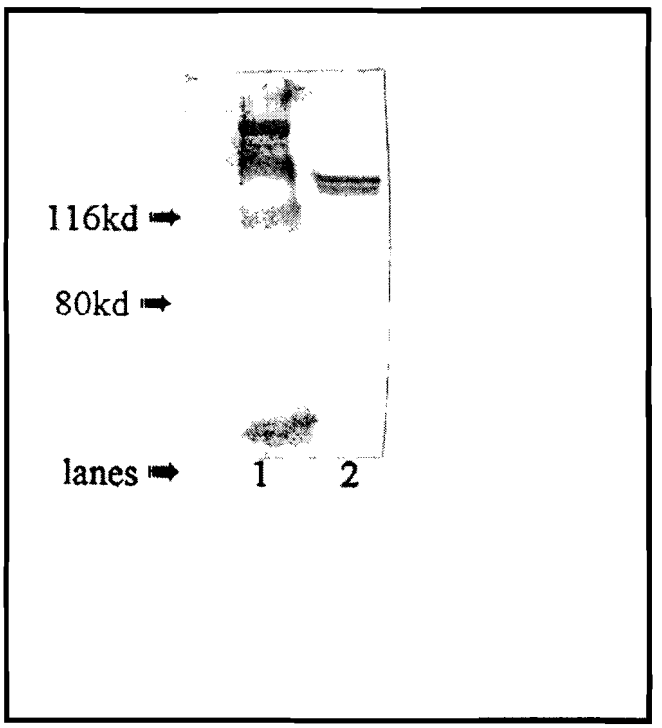

116kd
80kd
lanes
1
2

Figure 6. Graph showing the curve for the net absorbance (corrected 595nm) and best fit. X-axis is concentration in mg/mL and Y-axis is absorbance.

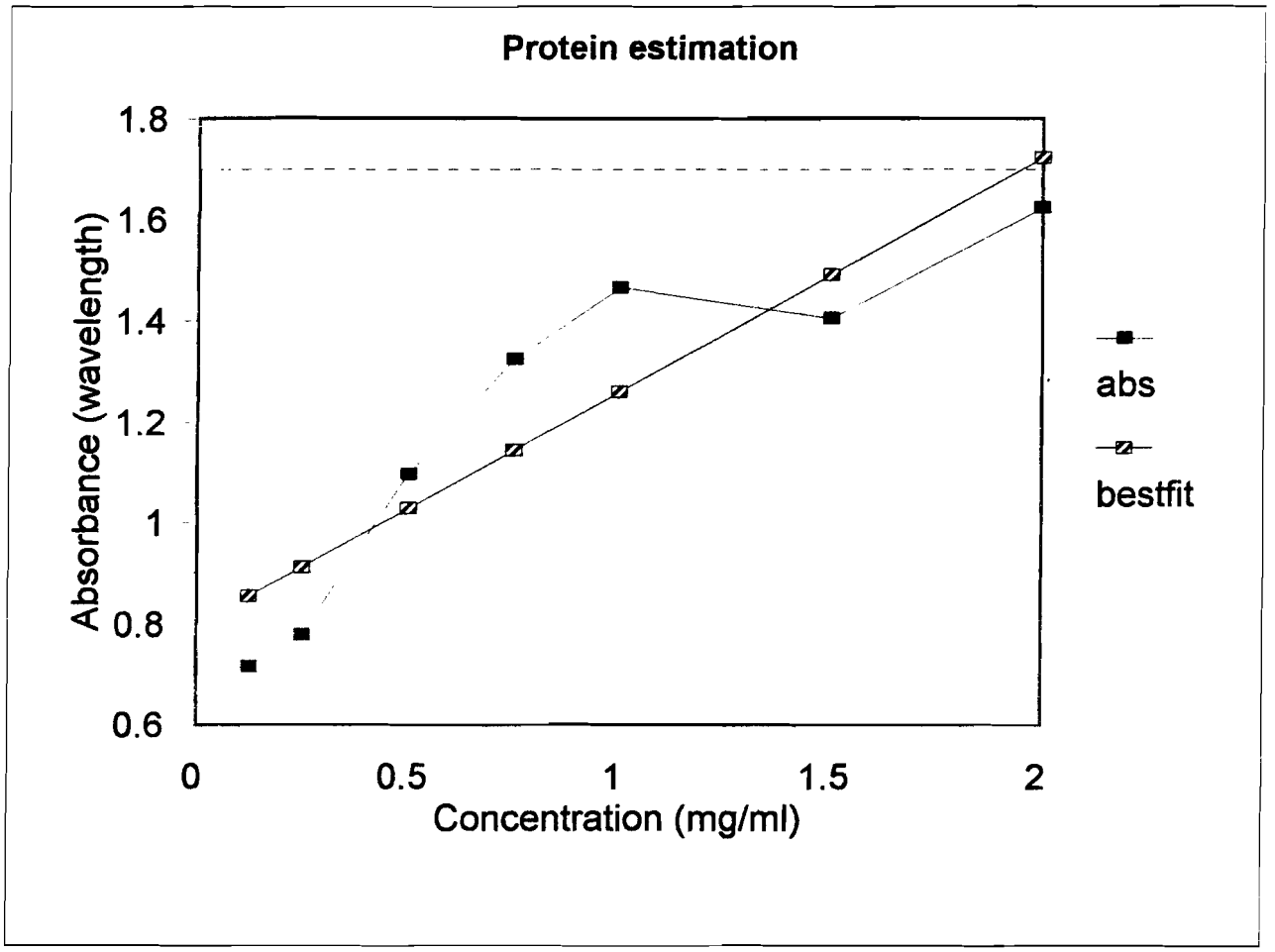

Protein estimation
Absorbance (wavelength)
1.8
1.6
1.4
1.2
1
0.8
0.6
0
0.5
1
1.5
2
Concentration (mg/ml)
abs
bestfit

Figure 7. Autoradiograph of the mobility shift assay. Lane 1: 4fmol DNA probe + 15μg 20-40% fraction of pET-avrXa10. Lane 2: 1/10 fraction of the above DNA probe + 15μg 20-40% fraction of pET-avrXa10. Lane 4: 4fmol DNA probe. Lane 6: 1/10 fraction of the above DNA probe. Lanes 3, 5 and 7 are empty.

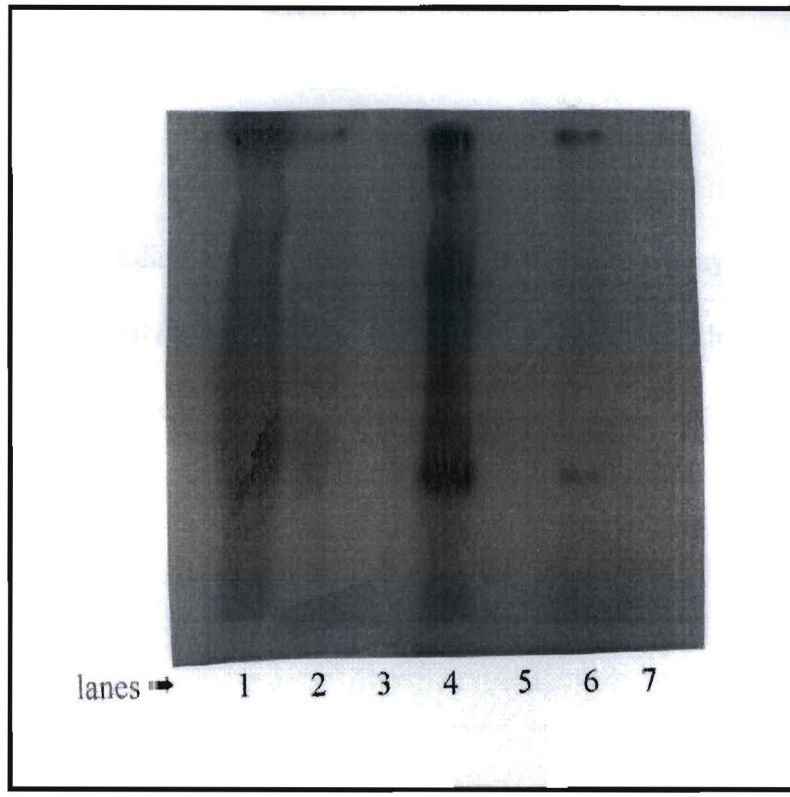

lanes
1
2
3
4
5
6
7

Figure 8. The autoradiograph of the controlled mobility shift assay. Lane 1: 4fmol DNA. Lane 3: 4fmol DNA + 15μg of 20-40% fraction containing the AVRXa10.F1 protein. Lane 4: 4fmol DNA + 15μg of the 20-40% protein fraction isolated from bacteria containing plasmid pET-22b+ (not having the avrXa10.F1 insert).

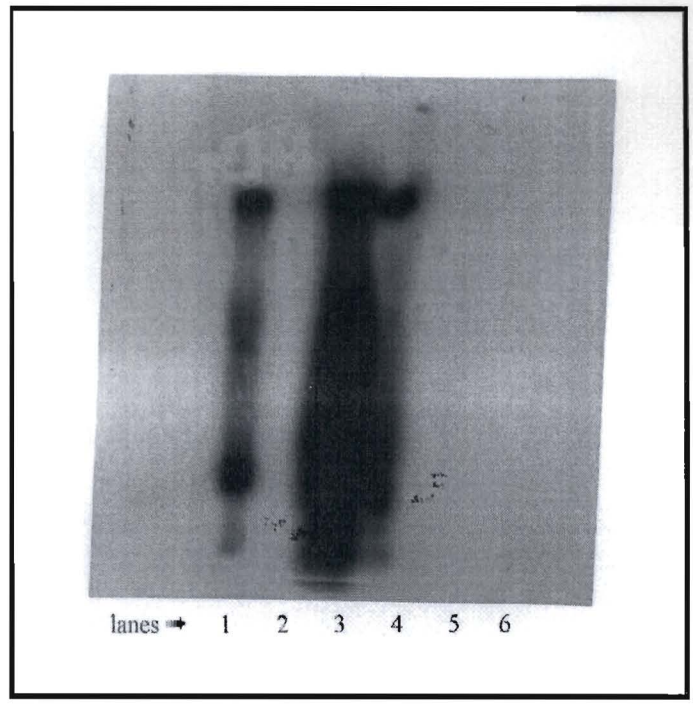
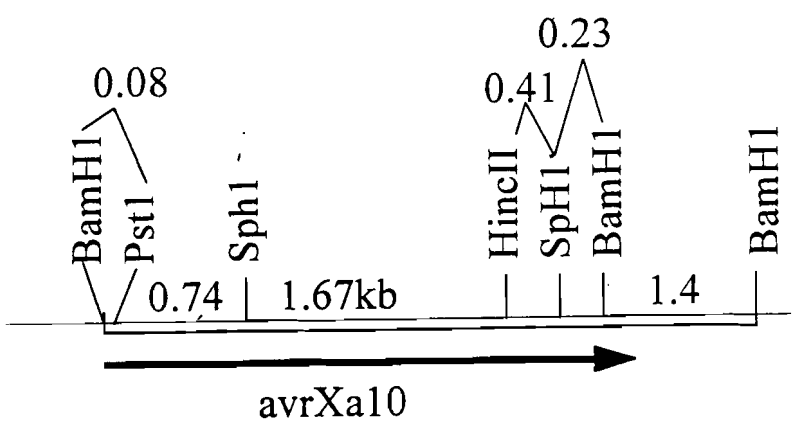

lanes ➡ 1 2 3 4 5 6

Figure 9. The restriction sites of the *avrXa10* gene.

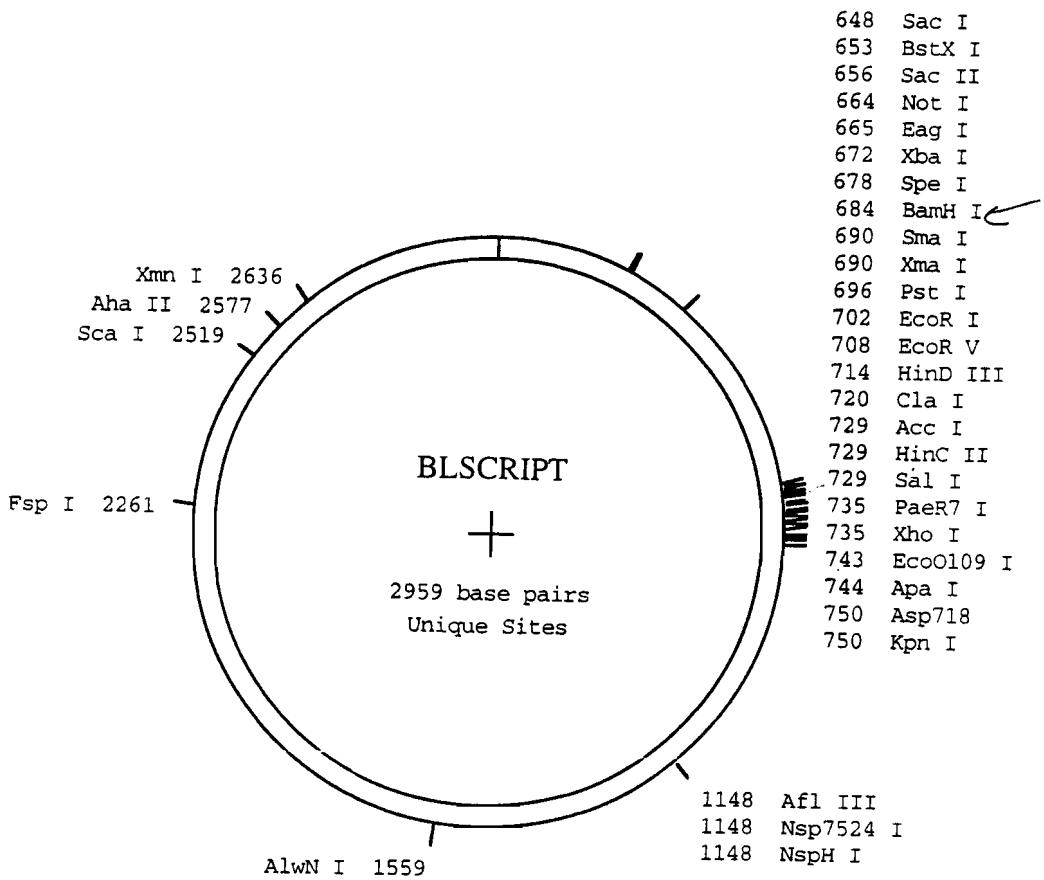


Insert of *avrXa10* subclone

pBluescript vector

Gene region of *avrXa10*

Figure 10. The restriction map of the vector pBluescript.


BLSCRIPT
2959 base pairs
Unique Sites
Xmn I 2636
Aha II 2577
Sca I 2519
Fsp I 2261
AlwN I 1559
1148 Afl III
1148 Nsp7524 I
1148 NspH I
648 Sac I
653 BstX I
656 Sac II
664 Not I
665 Eag I
672 Xba I
678 Spe I
684 BamH I
690 Sma I
690 Xma I
696 Pst I
702 EcoR I
708 EcoR V
714 HinD III
720 Cla I
729 Acc I
729 HinC II
729 Sal I
735 PaeR7 I
735 Xho I
743 EcoO109 I
744 Apa I
750 Asp718
750 Kpn I

Signature of Graduate Student

Signature of Major Advisor

I, Kamesh R. Vyakaranam, hereby submit this thesis to Emporia State University as partial fulfillment of the requirements of an advanced degree. I agree that the Library of the University may make it available for use in accordance with its regulations governing materials of this type. I further agree that quoting, photocopying, or other reproduction of this document is allowed for private study, scholarship (including teaching), and research purposes of a non profit nature. No copying which involves potential financial gain will be allowed without written permission of the author.

Signature of Author

12/16/98
Date

Binding studies of the *avrXa10* gene product from *Xanthomonas oryzae pv. oryzae* to the promoter of a defense-response peroxidase gene.

Title of the Thesis Report

Signature of Graduate Office Staff Member

May 4, 1999
Date Received