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The Characterization of Peroxidase Associated with induced Resistance and the isolation of TN5 mutants of Pseudomonas syringae pv. Syringae unable to induce resistance

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THE CHARACTERIZATION OF PEROXIDASE ASSOCIATED WITH INDUCED SYSTEMIC RESISTANCE IN CUCUMBER AND THE ISOLATION OF TN5 MUTANTS OF PSEUDOMONAS SYRINGAE PV. SYRINGAE NO LONGER ABLE TO INDUCE RESISTANCE

By

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ABSTRACT

THE CHARACTERIZATION OF PEROXIDASE ASSOCIATED WITH INDUCED SYSTEMIC RESISTANCE IN CUCUMBER AND THE ISOLATION OF TN5 MUTANTS OF PSEUDOMONAS SYRINGAE PV. SYRINGAE NO LONGER ABLE TO INDUCE RESISTANCE

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Induced systemic resistance in cucumber, muskmelon and watermelon was accompanied by a systemic increase in peroxidase activity. The increased activity was due to an increase in an acidic isozyme localized to the extracellar spaces of leaves. Peroxidase levels reached approximately 6% of total soluble protein in induced cucumber leaves as determined by radial immunodiffusion.

The anodic peroxidase isozymes of cucumber, muskmelon and watermelon appeared as a cluster of three bands (30-33 kd) on native and denaturing gels. The three forms of the cucumber isozyme (33,31 and 30 kd) were purified and found to be differentially glycosylated as determined by their differential affinity for Con A-Sepharose and susceptibility to α mannosidase. The amino acid sequence of three fragments from the 33 kd peroxidase shared homology with sequences in tobacco, turnip and horseradish peroxidase.

Peroxidase levels began to increase systemically

in cucumber seedlings 24-30 hr after inoculation of the first leaf with the HR inducing pathogen Pseudomonas syrinae pv syringae. The rapid increase in peroxidase activity was accompanied by a rapid increase in systemic resistance. Induced resistance was expressed within 2 days of the HR inducing inoculation as measured by a 70% decrease in Colletotrichum lagenarium lesion diameter. Tn5 mutants of P.s. syringae selected for their inability to induce resistance in cucumber had also lost their ability to cause disease on the host plant wheat. In two mutants isolated, Tn5 had inserted into a region of the genomic DNA which shares homology to a functionally similar region cloned from Pseudomonas syringae pv phaseolicola. A genomic clone was isolated from a pLAFR3 library of P.s. syringae which restored the ability of both mutants to induce HR, peroxidase activity and resistance.

In memory of my father, Howard

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

In contrast to the circulatory immune system of animals, plants frequently sequester invading bacterial and fungal pathogens at the sites of attemped infection. Compartmentalization is achieved by the rapid localized activation of defense related metabolism in response to pathogen invasion. Several examples of defense related responses which have been reviewed recently include hypersensitive cell death (11), phytoalexin production (1,2), and lignin biosynthesis (19). Rapid activation of one or more of these processes resulting in compartmentalization of pathogens is determined by the presence or absense of disease resistance genes in the host plant. The mechanism by which disease resistance genes mediate the expression of defensive responses is unknown.

In contrast to genetic resistance, induced resistance involves the activation of defense mechanisms in the absence of specific genes for disease resistance. Resistance may be induced at the site of the resistance inducing treatment (locally) or in tissues distant to the site of treatment (systemically) (12,13,16,18). Resistance can be locally induced by prior inoculation of tissue with non-host pathogens or by treatment with elicitors of defense responses. Elicitors are constituents of pathogens or hosts which appear to act as signal molecules for the elicitation of resistance related metabolism. They have been

characterized as oligosaccharides (17,20,21), glycoproteins (3,21) and lipids (6). With the exception of the proteinase inhibitor inducing factor isolated from tomato, a pectic fragment of MW 5000 (4,17), no elicitor has been identified in induced systemic responses.

Systemic induced resistance has generated considerable interest because of its phenomenological similarity to the process of immunization in animals. The majority of research on induced systemic resistance employs cucumber or tobacco as the host plant. In cucumber, inoculation of the first leaf (inducing inoculation) of seedling plants with necrosis inducing fungal, bacterial, or viral pathogens induces systemic resistance to an array of pathogens (13). Resistance is maximal several days after the inducing inoculation and may be enhanced by additional inoculations. Induced resistance in cucumber persists until flowering. Jenns and Kuc (10) found that resistance to Colletotrichum lagenarium could be induced in susceptible watermelon and muskmelon by grafting stems to the stem of an induced cucumber plant. Further evidence for the vascular transport of a 'signal' for induced resistance was provided by Guedes et al (7). They found that steam girdling of petioles prevented the expression of induced resistance in leaves distal to the induced leaf. Although the induction of systemic resistance in plants appears to involve the transport of signal through the vascular tissue, the mechanism of resistance is the same

as for other plant disease resistance reactions; enhanced ability to compartmentalize invading pathogens. Thus induced resistance is expressed as a decrease in the size and number of fungal lesions or as a decrease in the number of lesions induced by bacteria and viruses (12). This decrease in successful infection by pathogens has been correlated with an enhanced ability of the epidermal cells to lignify at infection sites (8).

Prior to the expression of enhanced lignification, biochemical changes resulting from the inducing inoculation include systemic increases in extracellular chitinase and peroxidase. Both enzymes are similar with respect to their anionic charge, molecular weight (approx. 30,000) and their regulation in response to pathogens (5,8,14,15). Hammerschmidt and Kuc determined that increases in peroxidase activity occur systemically in cucumber tissue as soon as pathogen induced necrotic lesions appear on the inducing leaf (9). They associated the increased peroxidase activity with an anodic triplet localized to the extracellular spaces of leaf tissue. Chapters 1 and 2 are devoted to the further characterization of this peroxidase triplet.

Considerably more is known about the biochemical events that follow resistance inducing inoculations than about the sensing mechanism that the triggers the response. The involvement of elicitors in local induced resistance responses suggests that they may play a role in induced

systemic resistance. A systematic search for elicitors of induced resistance should take into consideration the criteria which have been established for the response by Kuc et al. (7-10,12,13):

1. Resistance can only be induced by living pathogens; abiotic damage to tissue has no resistance inducing effect.

2. The resistance inducing organism must cause a necrotic lesion.

3. The level of resistance induced is directly correlated to the amount of necrosis produced by the inducing organism.

The requirement for a living organism to induce systemic resistance suggests that the triggering mechanism is some process unique to pathogen/host interactions rather than a specific component of either organism alone. Resistance can be induced by viruses, bacteria, and fungi; yet it is unlikely that these diverse organisms share preformed constituents which activate induced resistance. Likewise, the inability to induce resistance through wounding or other abiotic damage indicates that the active factor is not solely of plant origin. Chapter 3 proposes a system in which to study the components of the pathogen/host interaction and their importance to induced systemic resistance.

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CHAPTER I.

COMPARATIVE STUDY OF ACIDIC PEROXIDASES ASSOCIATED WITH INDUCED SYSTEMIC RESISTANCE IN CUCUMBER, MUSKMELON AND WATERMELON

Physiological and Molecular Plant Pathology (1988) **33**, 000–000

Comparative study of acidic peroxidases associated with induced resistance in cucumber, muskmelon and watermelon

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Inoculation of one leaf of cucumber, muskmelon or watermelon with *Colletotrichum lagenarium* induces a systemic increase in soluble peroxidase activity. Increased total activity is reflected in an increase in activity in intercellular wash fluids of leaf tissue. The specific activity of peroxidase extracted from intercellular wash fluids from systemically induced leaves was at least two-fold higher than specific activity extracted from control leaves. Intercellular peroxidase migrates as a cluster of three bands on high pH native polyacrylamide gels, and the patterns are similar for cucumber, muskmelon and watermelon. The peroxidases are *c.* 30 kD as determined by SDS polyacrylamide gel electrophoresis. Antibodies raised against cucumber intercellular peroxidase reacted with muskmelon and watermelon peroxidases in patterns of partial identity in the Ouchterlonoy double diffusion assay.

INTRODUCTION

Inoculation of one leaf of cucumber with any of several different pathogens results in the systemic induction of resistance against subsequent attack by several pathogens [*13*]. Similarly, inoculation of the first leaf of muskmelon and watermelon with *C. lagenarium* also results in the induction of systemic resistance to that fungus [*3, 13, 18*]. Induced resistance in cucumber and muskmelon is associated with an enhanced ability of the host plants to lignify in response to infection by pathogenic fungi [*5, 8, 10, 18*]. Rapid lignin deposition may provide a physical and/or chemical barrier to the invading pathogen [*19*]. Induced systemic resistance in cucumber is also associated with enhanced levels of a group of acidic, extracellular peroxidases which accumulate systemically in plant tissues as induced resistance develops [*11*]. Peroxidases catalyse the final polymerization step of lignin synthesis, and may therefore be directly associated with the increased ability of systemically protected tissue to lignify [*9*]. The purpose of this study was to further characterize the peroxidases induced in cucumber and to determine whether similar peroxidases are induced in watermelon and muskmelon seedlings as a result of resistance inducing inoculations.

EXPERIMENTAL AND RESULTS

Pathogen culture and hosts

Colletotrichum lagenarium (Pass.) Ell and Halst. race 1 was maintained on V-8 juice agar at 18 °C in the dark. Conidial suspensions for use as inoculum were prepared from 7- to 10-day-old cultures as described previously [*14*].

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Muskmelon (*Cucumis melo* L. cv. Iroquois), watermelon (*Citrullis vulgaris* L. cv. sugar baby), and cucumber (*Cucumis sativus* L. cv. SMR 58) plants were grown from seed in the greenhouse.

Inoculation of hosts for peroxidase induction

Peroxidase was induced in 14-day-old plants by infiltrating approximately 500 µl of a *C. lagenarium* spore suspension (1×10^5 spores ml^{-1}) into 10 sites on the first true leaf using a 3-ml disposable syringe appressed to the underside of the leaf. Control plants received no treatment, as it has been determined previously that water injections have no resistance or peroxidase inducing effect [*11*]. Seven days after inoculation, the second true leaves of induced (inoculated) and control plants were collected for peroxidase assays.

Extraction of peroxidase from leaf tissue

Twenty-five to fifty grams of leaves were rinsed with distilled water and placed into a large desiccator one-half filled with ice cold distilled water. Leaves were held under the surface of the water with a ceramic plate and infiltrated under reduced pressure for 15 min. Infiltrated leaves were blotted on cheesecloth, and four to five leaves were rolled together and placed tips down into a 30-ml plastic centrifuge tube one-fifth filled with 5-mm diameter glass beads. Intercellular fluids were collected by centrifugation at 2000 r min^{-1} for 15 min in a Sorvall SS-34 rotor. Extracts were pooled, centrifuged at 10 000 min^{-1} for 20 min and the supernatant lyophilized prior to storage at −20 °C.

Leaf tissue previously extracted by vacuum infiltration was frozen in liquid nitrogen and ground to a powder with mortar and pestel. Powdered leaf tissue was suspended in 0·01 M $NaPO_4$ buffer pH 6·0 (4 ml g^{-1} original fresh weight). The homogenate was then vacuum filtered through Whatman No. 4 filter paper. Homogenates were repeatedly rinsed with the same volume of buffer until no peroxidase activity was detected in the filtrates. The same procedure was used to extract ionically bound peroxidase from the buffer extracted homogenate by using 0·21 M $CaCl_2$ in 0·01 M $NaPO_4$ buffer pH 6·0. Samples of all washes were reserved for determination of total soluble peroxidase extracted.

Peroxidase activity and protein determination

Peroxidase activity of leaf extracts was determined using guaiacol as a substrate as described previously [*11*]. Protein concentrations were determined according to Bradford [*2*] using bovine serum albumin (BSA) as a standard. The fractionation of soluble peroxidase from leaf 2 of induced or control tissue of cucumber, muskmelon and watermelon is summarized in Table 1. Total soluble peroxidase activity from induced tissue was higher than activity from control tissue for all three plant species. Induced cucumber, muskmelon and watermelon leaves had a six-fold, three-fold and 35% increase in total soluble peroxidase activity, respectively, over control tissue activity. The amount of peroxidase activity extracted by vacuum infiltration varied from 8 to 37% of the total soluble peroxidase activity. The specific activity of peroxidase extracted by vacuum infiltration of induced leaves from cucumber, muskmelon and watermelon was 7-, 2- and 4-fold higher, respectively, than the specific activity of peroxidase extracted by vacuum infiltration of control leaves.

TABLE 1

Fractionation of soluble peroxidase activity from control and induced cucumber, muskmelon and watermelon leaves

Species	Treatment[a]	Total soluble activity extracted[b]	% Total soluble activity present in intercellular extract	Specific activity[c]	
				Intercellular extract	Buffer + salt extract
Cucumber	C	8·8	20	71	1·3
	I	49·2	37	478	6·4
Muskmelon	C	23·1	24	150	1·7
	I	31·1	24	298	2·2
Watermelon	C	27·5	8	48	1·7
	I	64·4	25	174	3·1

[a] C, control; I, Induced.

[b] Activity from intercellular extract + activity from buffer and salt washings. Activity expressed as A_{470} min g fresh wt^{-1}.

[c] A_{470} min mg protein.

Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide slab gels, 1·5 mm thick, were prepared for native or denaturing gel systems. For native gels, an anionic (pH 9·3) discontinuous system was used [*12*]. An acrylamide concentration of 7·5% provided optimal separation of peroxidase isozymes. Gels were stained for peroxidase activity or protein upon completion of electrophoresis. For denaturing gels, the SDS system of Laemmli was used with a 15% acrylamide gel [*15*]. Gels were stained for protein following electrophoresis.

Polyacrylamide gel stains

a *Peroxidase activity.* After electrophoresis, gels were placed in a solution containing 50 mg of 3-amino,9-ethylcarbazole, 10 ml of *N*,*N*,dimethylformamide, 200 µl of 30% H_2O_2 and 190 ml of sodium acetate buffer pH 5·0 [*7*]. Bands were visualized within 10 min. Gels were rinsed and stored in a solution of 7% acetic acid in 50% methanol.

b *Protein.* The silver nitrate staining procedure of Morrissey [*16*] was modified as follows. After electrophoresis, gels were placed in 50% methanol, 10% acetic acid for 1–12 h. Gels were then rinsed in many changes of distilled water for one hr followed by 30 min in 200 ml of 5 µg ml^{-1} dithiothreitol. Gels were incubated in 0·1% aqueous silver nitrate for 1 h and protein bands visualized by placing the gel in 200 ml of 3·0% Na_2CO_3 containing 50 µl of 37% formaldehyde. Colour development was stopped by the addition of 12 g of citric acid.

Peroxidase purification

Hammerschmidt *et al.* have shown previously that increased total peroxidase activity in induced cucumber leaves is reflected in the increased activity of three bands of acidic peroxidase readily extracted from intercellular spaces [*11*]. For each cucurbit species

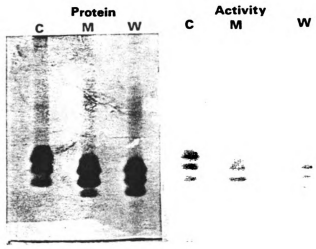

FIG. 1. Native polyacrylamide gel electrophoresis of purified acidic peroxidases from cucumber (C), muskmelon (M) and watermelon (W). Gels were stained for protein using silver nitrate or for peroxidase activity using 3-amino,9-ethylcarbazole as described in the text.

in this study, lyophilized intercellular extract was resuspended in 2–3 ml of 0·05 M Tris–HCl pH 8·0 and applied to a Sephadex G-75 column (1·5 × 25·0 cm) equilibrated in the same buffer. The column was eluted with 0·05 M Tris–HCl pH 8·0 and 2-ml fractions containing peroxidase activity were pooled and applied to a DEAE-Sephadex column (2 × 10 cm) equilibrated with 0·05 M Tris–HCl pH 8·0. Peroxidase was eluted from the column with a linear salt gradient of 0·0–0·2 M NaCl in a total volume of 150 ml. Fractions (1·3 ml each) containing peroxidase activity were pooled, dialysed extensively in distilled water, and lyophilized. Lyophilized samples were re-chromatographed on the DEAE column, dialysed and lyophilized. Peroxidase activity eluted from DEAE as single peaks at 0·13, 0·14 and 0·16 M NaCl for cucumber, muskmelon and watermelon peroxidase, respectively.

Peroxidase eluted two times from DEAE was essentially pure as indicated by gel electrophoresis followed by the silver stain for protein (Fig. 1). Peroxidases purified from cucumber, muskmelon and watermelon were similar with respect to their migration on native and denaturing acrylamide gels (Figs. 1, 2). The peroxidases from each species migrated as three bands of decreasing relative protein and peroxidase staining intensity from the slowest to fastest migrating. The peroxidases have molecular weights ranging from 30 to 33 kD as determined by SDS gel electrophoresis with standards of known molecular weight (Fig. 2).

Purification of cucumber peroxidase for antibody production

Cucumber peroxidase purified by DEAE chromatography was further purified by preparative gel electrophoresis followed by electroelution of peroxidase bands. Lyophilized samples were resuspended to a concentration of 1·0 mg ml^{-1} protein in electrophoresis sample buffer containing 0·5 M Tris–HCl pH 6·8, 10% glycerol and

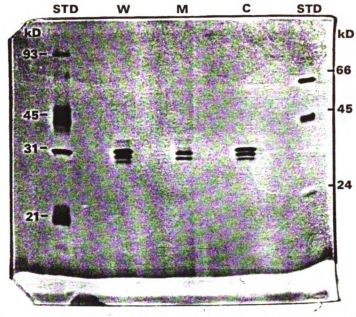

FIG. 2. SDS polyacrylamide gel electrophoresis and molecular weight determination of purified peroxidases from watermelon (W), muskmelon (M) and cucumber (C).

0·05% bromphenol blue. One millilitre of sample containing 1·0 mg of protein was applied to a 3 mm thick native slab gel. Peroxidase bands were detected in the gel directly by their brown colour or by staining a 5 mm strip of gel for peroxidase activity. Bands corresponding to peroxidase were excised with a razor blade and electroeluted using an ISCO electroeluter with 0·05 M Tris–glycine pH 8·9 as the elution buffer [*1*].

Production of antibodies to cucumber peroxidase

For the initial injection, 200 μg of PAGE purified native peroxidase was emulsified in 1 ml of Freund's complete adjuvent (Difco). Two New Zealand white female rabbits were injected intramuscularly with equal volumes of the emulsified peroxidase (100 μg each). Subsequent injections were given every 3–4 weeks using 100 μg of native peroxidase per rabbit emulsified in Freund's incomplete adjuvent. Sera was collected 7–10 days after each injection.

Cross reactivity of cucumber, muskmelon and watermelon peroxidase with anti-cucumber peroxidase sera

Ouchterloney double diffusion was carried out on glass microscope slides coated with a 3 mm layer of 1% agarose in phosphate buffered saline (PBS). The centre well was filled with 10 μl of undiluted anti-cucumber peroxidase sera and outer wells were filled with 10 μg each of cucumber, muskmelon or watermelon peroxidase. Antigen and antibody were allowed to diffuse towards each other at room temperature in a moist Petri dish until precipitate formation was maximal. Unreacted proteins were washed from agarose by placing slides in 3 l of 0·3 M NaCl at 4 °C for 24 h. Slides were allowed to dry at room

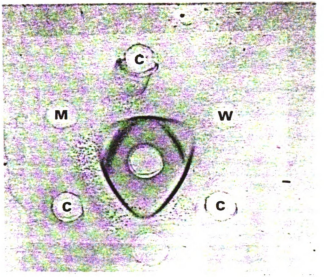

FIG. 3. Cross-reactivity of anti-cucumber peroxidase antisera with muskmelon and watermelon peroxidase. Centre well contains 10 µl of anti-cucumber peroxidase whole sera. Outer wells were loaded with 10 µg in 10 µl each of cucumber (C), watermelon (W) or muskmelon (M) peroxidase.

temperature and precipitation bands visualized by staining with Coomassie blue [6]. Muskmelon and watermelon peroxidase reacted with anti-cucumber peroxidase serum in patterns of partial identity with cucumber peroxidase (Fig. 3). The anti-cucumber peroxidase serum showed a slightly higher degree of cross-reactivity with muskmelon than with watermelon as evidenced by the shorter and less distinct "spur" produced.

CONCLUSIONS

Induced resistance in cucumber, muskmelon and watermelon is accompanied by a systemic increase in peroxidase activity. Although the acidic peroxidases induced in each of the three plant species migrate as three bands on polyacrylamide gels, it is unclear whether they are the products of different genes or result from post-translational modifications of a single gene product. Genetic studies of this peroxidase triplet in cucumber have shown that the three bands are inherited as a single locus [4]. Factors such as differential glycosylation and interactions with phenolics may alter the mobility of a single protein on polyacrylamide gels [17-20].

Peroxidases induced in the three plant species are similar with respect to charge and molecular weight and show a high degree of immunological cross-reactivity. The structural and regulatory similarity of peroxidases induced in cucumber, muskmelon and

watermelon may reflect a similar role for the enzymes in the systemic induced resistance response.

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CHAPTER II.

FURTHER CHARACTERIZATION OF ANODIC PEROXIDASE TRIPLET ASSOCIATED WITH INDUCED RESISTANCE IN CUCUMBER

CHAPTER II. INTRODUCTION

Peroxidase is ubiquitous in higher plants and has been the subject of thousands of journal articles during the past century. Theorell crystallized horseradish peroxidase in 1942 (cited in 24), and biochemists and biophysisists have subsequently documented virtually every nuance of its spectroscopic, catalytic, sequence and structural properties (24,29). In spite of the quantity of data available on the reactions catalyzed by peroxidase *in vitro*, its function in plants has remained obscure due, in part, to its broad substrate specificity and the presence of numerous isozymes. Researchers have, nevertheless, assigned various functions to plant peroxidases based on their tissue localization and activities on plant derived substrates. Several functions suggested for peroxidase include hydrogen peroxide formation (22), ethylene biosynthesis (20,26), IAA oxidation (10,17,28) and lignin biosynthesis (11,13,21).

Although a number of model systems have been proposed for the study of peroxidase regulation and function, two which hold considerable promise are the genetic analysis of peroxidase isozymes in petunia and the molecular analysis of isozymes in tobacco. Berg et al. have greatly simplified the interpretation of complex peroxidase isozyme profiles by providing genetic evidence for post-translational modifications of peroxidase isozymes (1-5,16). They have focused on three structural genes designated prxA, prxB, and

prxC which code for the 10 major peroxidase isozymes found in petunia. The prxA locus is of particular interest, as it codes for an acidic cell wall associated cluster of 3-4 bands similar to those that have also been observed in tomato (25), tobacco (23) and cucumber (9,14). Five alleles of the prxA locus have been identified based on shifts in the electrophoretic mobility of the peroxidase cluster. The prxA alleles are co-dominant, indicating that variations in electrophoretic mobility of the enzymes are due to mutations in the structural gene rather than a modifying enzyme.

Several lines of evidence support the hypothesis that a single gene product of the prxA locus is post-translationally modified to produce the 3-4 bands observed on starch gels. First, only the slowest migrating band of the PRXa cluster is seen in young leaf tissue. The two faster migrating bands appear gradually as the leaf matures and are suggested to be processed forms of the primary band. These secondary bands have been termed 'mozymes' for 'modified enzymes'. Second, in crosses of plants with different alleles of prxA, only the original triplets and combined hexaplets were observed in the progeny. The authors concede that it is possible, though unlikely, that the observed segregation is due to three tightly linked genes. Finally, internal site mutations at prxA that affect temporal expression of the gene simultaneously affect the expression of the entire PRXa cluster. The PRXa clusters were purified from plants homozygous for each of the 5 alleles and tested for cross-

reactivity to antibodies prepared to one cluster. Peroxidases encoded by the 5 alleles were immunologically identical as determined by Ouchterlony double diffusion assays. Antibodies raised to the PRXa cluster did not cross-react with PRXb or PRXc peroxidases. Future work in this system will focus on the differential expression of the PRXa cluster using the antibodies specific for that isozyme.

Lagrimini et al. (18,19) have adopted a molecular rather than genetic approach in their study of the regulation and function of peroxidase isozymes in tobacco. In their initial report, they characterized tissue specific, wound inducible, and virus inducible expression of 12 isozymes by isoelectric focusing. They subsequently isolated a cDNA clone for the acidic (pI 3.5, pI 3.75) isozymes and used Northern blot analysis to determine tissue specific message levels for the isozymes. Message levels for the anionic isozymes were greatest in stem tissue, where they are thought to function in the lignification of xylem. Southern blots of genomic DNA from three species of tobacco probed with the peroxidase cDNA indicate that the acidic isozymes are the products of allelic genes. Similar experiments are planned for the study of cationic and moderately anionic isozyme expression. Lagrimini has continued the study of isozyme function by transforming tobacco with anionic peroxidase clones under the control of a promoter which allows constitutively high levels of expression (personal communication). He has also introduced an 'antisense' sequence, a sequence encoding an

mRNA complementary to the the peroxidase message, which effectively reduced levels of peroxidase activity in the tissue. The physiological effects of such transformations promise to reveal valuable information on the regulation and function of peroxidase isozymes.

The model systems described above are useful because they have simplified the complex problem of assigning function to numerous bands on a gel. The first system simplifies the study of peroxidase isozymes conceptually, by providing conclusive evidence that groups of 'isozymes' are actually the products of a single structural gene. The second study provides another tool with which to study the regulation of peroxidase isozymes without relying on their electrophoretic mobility. The use of specific probes for each isozyme message will allow greater sensitivity in determining the factors that affect their regulation.

The purpose of this chapter is to propose pathogen mediated expression of peroxidase in cucumber as a model system for the study of peroxidase regulation in Cucurbitaceae. Preliminary evidence indicates that peroxidase is regulated similarly in response to pathogens among species of Cucumis, Citrullus (Chapter 1) and Cucurbita (unpublished). The study of peroxidase regulation in cucumber is inherently simplified by the presence of only one anodic and two cathodic isozymes in leaf tissue. The anodic isozyme, Px2, appears as a triplet in starch gels and is similar to PRXa in petunia in that the three bands may be

attributed to a single structural gene (9) . In addition, a specific function for this isozyme in lignification associated with disease resistance has been proposed (15). The systemic increase in peroxidase activity associated with induced systemic resistance in cucumber is due to increases in the anodic isozyme (14). An understanding of factors important in the regulation of this isozyme in response to disease will necessarily contribute to our understanding of the regulation of induced systemic resistance.

CHAPTER II. MATERIALS AND METHODS

Peroxidase purification

Acidic intercellular peroxidase was purified from 200g of the second and third leaves of cucumber seedlings 7 days after the first leaf had been inoculated at 10 sites with a 1x10 spore/ml suspension of Colletotrichum lagenarium as described in Chapter 1. The three forms of the cucumber peroxidase triplet were separated by excising them individually from a preparative native polyacrylamide gel prior to electro-elution. Prior to extraction, one cm leaf discs were removed from leaves for use in radial immunoassays of peroxidase. Native and denaturing gels were prepared and stained as described in Chapter 1.

Radial immunodiffusion

The quantity of native peroxidase protein in leaf extracts was estimated by radial immunodiffusion. Immunodiffusion plates were prepared by diluting crude anti-peroxidase sera 1:80 in 10 ml 1% melted agarose (50C),50mM Barbital buffer pH 8.6. The agarose-antibody dilution was poured out onto an 8X8 cm glass slide and allowed to solidify. Two mm diameter wells were formed with a brass borer and agarose plugs removed by aspiration. Wells were loaded with 5 µl supernatant (2 mg protein/ml) of leaf homogenates from systemically protected or control leaves, or with dilutions of purified peroxidase. Proteins were allowed to diffuse

from wells in a moist petri dish for 5 days at room temperature. Unprecipitated proteins were washed from the agarose and precipitin rings were stained as described in Chapter 1. A standard curve of peroxidase concentration vs. the square of precipitin ring diameter was prepared using the known dilutions of purified peroxidase, and peroxidase content of leaf samples was calculated using this curve.

Isoelectric focusing

Pre-poured ultra-thin (0.15 mm) isoelectric focusing gels (pH 3-10) were obtained from Serva. Electrophoresis was carried out on a horizontal electrophoresis unit (Bio-Rad) to a final voltage of 1700V with a maximum power of 4 watts. Starting current was adjusted to obtain an intial voltage of 200V. Electrode buffers were obtained from Serva. Ten µl of leaf homogenate supernatants containing 10 µg protein were loaded into 20 µl capacity wells placed on the center of the isoelectric focussing gel. A total of 1 µg of intercelluar extract protein was used for IEF gels. Following electrophoresis, gels were stained for peroxidase activity as described in Chapter 1.

Concanavalin A column chromatography

Peroxidase was chromatographed on ConA sepharose according to manufacturers instructions (Pharmacia). Five micrograms of pure peroxidase were applied to a 0.5X 1 cm column

prepared in a 3ml syringe stoppered with glass wool. The column was washed with 0.02 M Tris-HCl,0.5M NaCl pH 7.4 until no peroxidase activity eluted. Bound peroxidase was eluted with 0.5 M α-D-methylmannoside. Peroxidase in column fractions was characterized by native gel electrophoresis.

Treatment of isozymes with α mannosidase

The purified bands of the cucumber peroxidase triplet were treated individually with -mannosidase as described by Gaudreault and Tyson (12). Twenty ug of each peroxidase was incubated in 50mM Na Acetate (pH 4.5), 5mM ZnSO4 containing α mannosidase (Sigma). The mixtures were placed at 37C for 72 hr prior to analysis by native PAGE.

Affinity purification of anti-cucumber peroxidase antibody

Five mg of DEAE purified peroxidase was coupled to 1 g CNBr- activated sepharose according to manufacturers instructions (Pharmacia). The freeze-dried sepharose was washed in 1 mM HCl prior to the addition of peroxidase dissolved in 5 ml coupling buffer (0.1M NaHCO3 pH 8.3, O.5M NaCl). The mixture was incubated overnight at 4C with occasional stirring. Remaining active groups on the CNBr-Sepharose were blocked with 0.2M glycine, pH 8.0 for 2 h at room temperature. Excess peroxidase was removed with sequential washes in coupling buffer followed by acetate buffer (0.1M, pH 4) containing 500 mM NaCl, followed again

by coupling buffer. The gel was washed until no peroxidase activity was detected in the wash fluid. For affinity purification, 2 ml crude sera were diluted 1:1 in 2X PBS-Tween (0.2% Tween 20,20mM NaH_2PO_4, 300 mM NaCl, adjusted to pH 7.2 with NaOH) and added to the peroxidase-sepharose gel material. The mixture was incubated overnight at room temperature with occasional mixing. Unreacted antibody was washed from the gel with PBS-Tween. Bound antibody was eluted from the gel using a modified procedure for antibody elution from nitrocellulose (27). The sepharose mixture was poured into a 3ml syringe stoppered with glass wool. The syringe was suspended into a 15 ml disposable centrifuge tube containing 100 µl of 0.5M Na_2PO_4. One ml of antibody elution buffer (5 mM glycine-HCl, pH 2.3, 500 mM NaCl, 0.1% Tween 20, 100 µg BSA/ml) was added at the top of the syringe prior to centrifugation at 1600g for 2 min. Rapid neutralization of eluted antibody was necessary for the preservation of activity.

Western blots

The individual bands of the peroxidase triplet were tested for their affinity to anti-cucumber peroxidase in a Western blot. Equal quantities (500 ng) of each peroxidase were electrophoresed in SDS polyacrylamide gels as described in Chapter 1. After electrophoresis, the peroxidases were blotted to nitrocellulose and probed with affinity purified anti-cucumber peroxidase (1:100), and stained with alkaline

phosphatase according to the method of Blake et al. (6). Attempts to blot native gels of peroxidase were unsuccessful.

Peroxidase amino acid sequence analysis

The electroeluted peroxidase band with the highest apparent molecular weight was selected for sequence analysis because it was available in the greatest quantity. Peroxidase sequence analysis of endoproteinase Lys-C (Boehringer-Mannheim) fragments was conducted by William Burkhart (Ciba- Geigy Agricultural Biotechnology Unit, Research Triangle Park, NC). Digestion fragments were separated by HPLC and sequenced by automated Edman degradations on the Applied Biosystems 470A gas-phase sequencer. Phenylthiohydantoin amino acids were analyzed using the Applied Biosystems 120A PTH Analyzer.

CHAPTER II. RESULTS

Quantitation of leaf peroxidase by Radial immunodiffusion

Radial immunodiffusion results are summarized in Figs. 4 and 5 and Table 2. Peroxidase accounts for approximately 6% of total soluble protein in homogenates of induced leaves according to this assay. No precipitin rings developed around extracts from control leaves, indicating that constitutive levels of peroxidase are less than 1% of total soluble protein

Purification of acidic peroxidase triplet

The purification scheme for peroxidase is shown in Tables 3 and 4. The high (H), medium (M), and low (L) molecular weight bands of the peroxidase triplet were successfully separated from one another as determined by SDS polyacrylamide gel electrophoresis (Fig. 6). The three bands were eluted in quantities that reflect the intensity of their staining on native and denaturing polyacrylamide gels. The H, M, and L bands represented 65%, 25%, and 10% of the total peroxidase eluted, respectively. The three bands migrate to a single isoelectric point of approximately 3.0 on isoelectric focusing gels (Fig. 7), indicating that differences in mobility on native polyacrylamide gels are due to variations in molecular weight rather than charge.

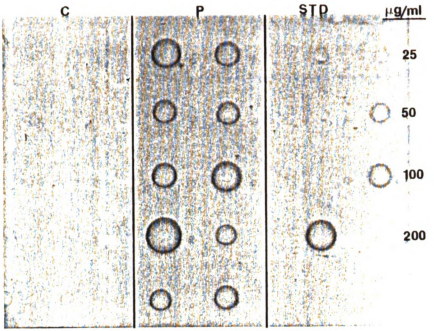

Fig. 4. Radial immunodiffusion of peroxidase from systemically protected (P) and control (C) leaf homogenates. Standards are 5 µl of 200, 100, 50, and 25 µg/ml peroxidase.

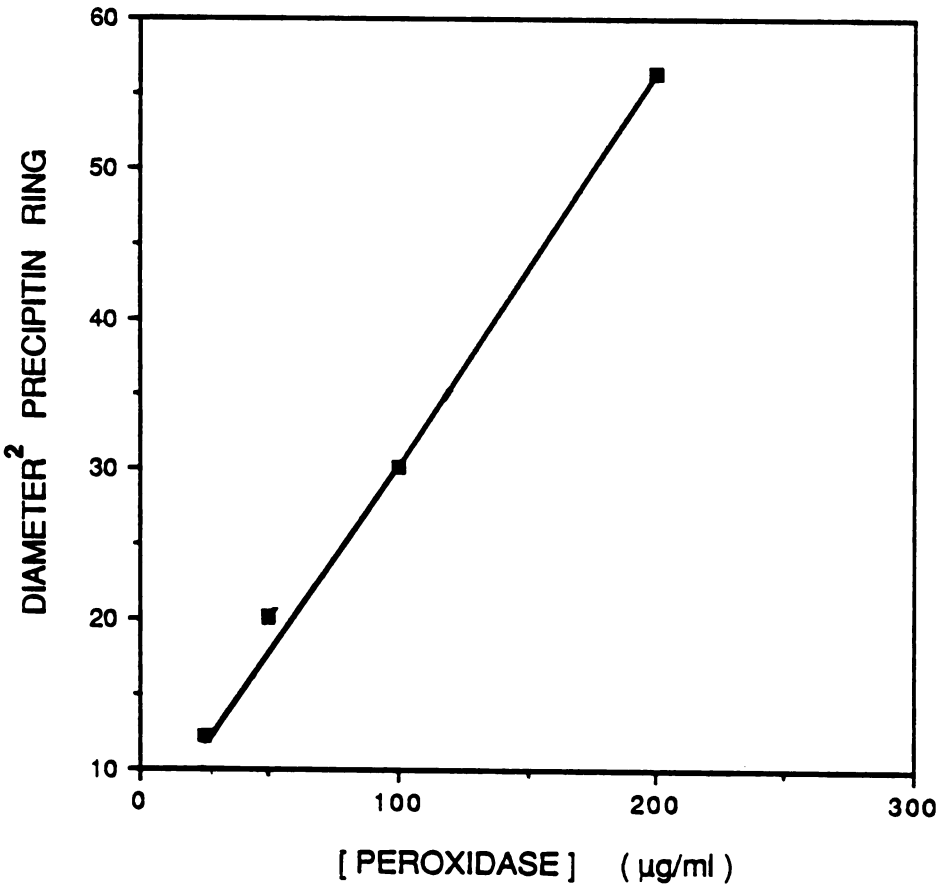

Fig. 5. Standard curve for radial immunoassay of peroxidase.

Table 2. Results of peroxidase immunodiffusion assay of protected leaves.

Sample	Precipitin ring diameter (mm)	(diameter)^2	Peroxidase concentration (ug/ml)	%Total Protein
Standards (ug/ml)				
200	7.5	56.3		
100	5.5	30.3		
50	4.5	20.3		
25	3.5	12.3		
Protected Leaves				
1	5.0	25	72	3.6
2	8.5	72	266	13.3
3	6.0	36	116	5.3
4	5.5	30.3	92	4.6
5	7.5	56.3	202	10.1
6	6.0	36	116	5.3
7	4.5	20.3	52	2.6
8	7.5	56.3	202	10.1
9	6.0	36	116	5.3
10	6.0	36	116	5.3
			AVERAGE	6.6 + 1.0

Table 3. Purification of peroxidase from systemically protected leaves.

FRACTION	SPECIFIC ACTIVITY (Δ A470/min/mg)	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (Δ A470/ml)	%YIELD
Intercellular extract	1480	16.5	25,160	100
Sephadex	1660	13.0	21,580	86
DEAE	4600	2.5	11,500	46
Electroeluted	5700	0.84	4528	18

Table 4. Quantification and specific activity of individual peroxidases isolated by electroelution from a polyacrylamide gel.

PEROXIDASE (Kd)	SPECIFIC ACTIVITY (ΔA_{470} /MIN/MG)	AMOUNT ELUTED (µg)	% TOTAL ELUTED
33	5600	550	65
31	6400	210	25
30	5100	80	10

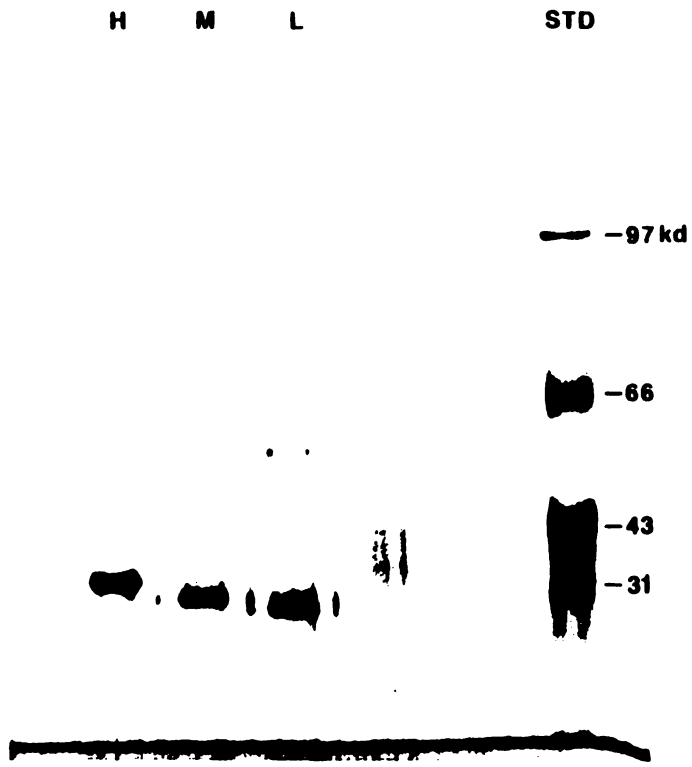

Fig. 6. SDS polyacrylamide gel electrophoresis of isolated high (H), medium (M) and low (L) molecular weight forms of the anodic peroxidase triplet.

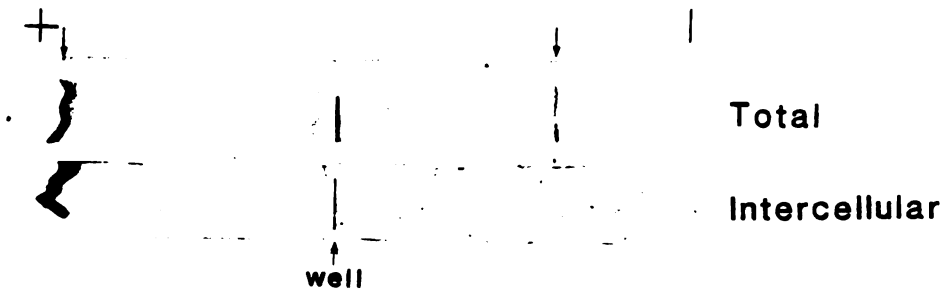

Fig. 7. Isoelectric focusing of peroxidase from intercellular extracts and total leaf homogenate. The gel was stained for peroxidase activity and major bands of activity are indicated by arrows (↓). The anodic end of the gel is indicated by (+) and the cathodic end by (-).

Concanavalin A-sepharose chromatography

Figure 8 shows the results of Con A-sepharose chromatography of the acidic peroxidase triplet. The lowest molecular weight peroxidase had no affinity for Con A while the band with intermediate mobility bound somewhat and the slowest migrating band bound tightly.

α Mannosidase treatment

The effect of α mannosidase treatment on mobility of the three peroxidases is illustrated in Fig. 9 . Treatment of the highest and intermediate molecular weight peroxidases with α mannosidase increased their mobility slightly on native gels while mobility of the fastest migrating band was unaffected.

Western blot

The three peroxidases reacted differentially with antibodies prepared to all three (Fig. 10). The antibody reacted strongly to the highest molecular weight peroxidase, slightly to the intermediate peroxidase, and weakly to the lowest molecular weight band. Smearing of the peroxidase bands is possibly due to the incomplete reduction of disulfide bonds in the presence of β-mercaptoethanol. This problem was eliminated by using dithiothreitol in the sample buffer as the reducing agent (Fig. 6)

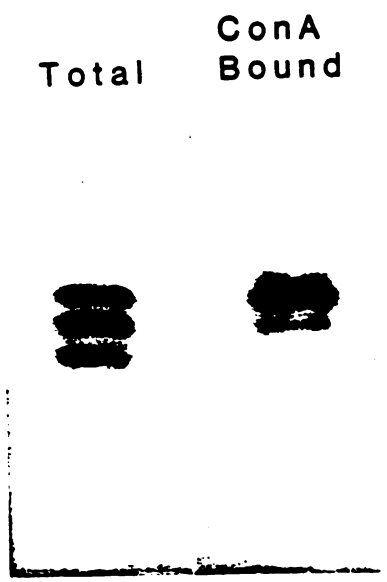

Fig. 8. Affinity of anodic peroxidases for Con A-sepharose.

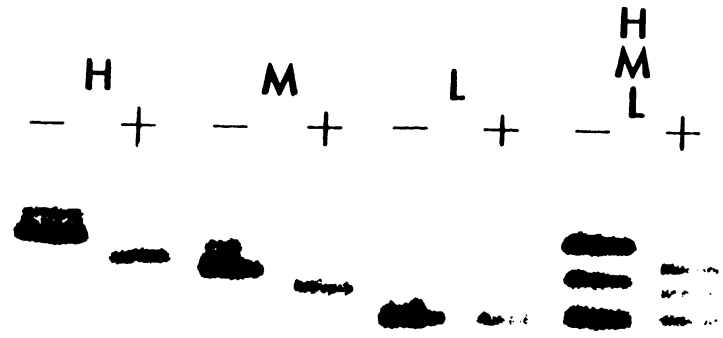

Fig. 9. Susceptibility of high (H), medium (M), and low (L) molecular weight forms of peroxidase to α mannosidase. (+) α mannosidase treated, (-) no treatment.

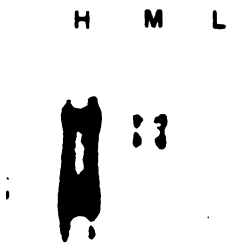

Fig. 10. Affinity of anti-cucumber peroxidase for high (H), medium (M), and low (L) molecular weight forms of anodic cucumber isozyme. Equal quantities (500 ng) of each form are present.

Amino acid sequence of peroxidase

The sequence of three fragments of cucumber peroxidase is compared to the corresponding sequences from horseradish, turnip, and tobacco in Fig. 11. The cucumber peroxidase fragments showed a slightly higher degree of homology to tobacco peroxidase than to horseradish and turnip.

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            36                              62

Tobacco      IIRLHFHDCFVNGCDGSILLD-TDGTQT
Horseradish   L              A     N TSFR
Turnip        L    F               D SSFTG
Cucumber                 D     V  EDQ  IT


            83                                      122

Tobacco      TALENVCPGVVSCADILALASEIGVVLAKGPSWQVLFGRK
Horseradish  A V SA  RT     L TI AQQS T  G    R PL  R
Turnip       S V KA            I ARDS  QLG  N N KV  R
Cucumber       V                E RDA T  S QG T QL


           288          302

Tobacco      TGTNGQIRTDCKRVN*
Horseradish     Q    LN RV  SNS*
Turnip        SS E   KV GKT *
Cucumber       T E    N R L *
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Fig. 11. Comparison of the amino acid sequence of three fragments of cucumber peroxidase with horseradish, turnip, tobacco peroxidase. Amino acids are designated by the single letter code and numbered from the amino terminal of tobacco peroxidase. Blank spaces indicate homology to tobacco peroxidase.

CHAPTER II. CONCLUSIONS

Peroxidase accumulates intercellularly in leaves during induced systemic resistance and may comprise approximately 6% of the total soluble protein in the leaf. Constitutive levels of the enzyme are much lower than this, indicating that peroxidase is probably synthesized de novo in response to stress signals associated with pathogen aggression. In order to determine the involvement of de novo transcription and translation for peroxidase, it will be useful to have antibodies which are specific for the peptide moiety of the peroxidase glycoprotein.

Antibodies raised against the native acidic peroxidase isozyme, which appears as three bands differing in size by 1-2 kd on SDS gels, react preferentially with the largest of the three forms of the isozyme. The basis for this differential reactivity may lie in the observed differential glycosylation of the three bands. Clarke and Shannon (8) have determined that the predominant carbohydrate residues on horseradish peroxidase isozyme C are mannose and glucosamine. Interestingly, Boller and Metraux (7) have recently reported that significant levels of α-mannosidase and β-N-acetyl-glucosaminidase are present in intercellular extracts of cucumber leaves. The two faster migrating forms of peroxidase presumably result from the post-translational modification of the larger

form, as has been suggested for the PRXa isozyme in Petunia (1). Alternatively, the peroxidases differ in the extent to which they are glycosylated initially. It is possible that some peroxidase is exported from the cell before the completion of glycosylation processes. A complete amino acid sequence of the peroxidase isozyme will reveal the potential sites for glycosylation.

The sequencing and subsequent cloning of the gene for this peroxidase isozyme will facilitate definitive experiments designed to answer questions regarding gene copy number, time-course of mRNA synthesis in response to pathogen stress, and the nature of a signal sequence which may initially target the protein for export. Manipulation of peroxidase expression through transformation experiments similar to those being conducted with tobacco may ultimately reveal the function of peroxidase in induced systemic resistance.

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CHAPTER III.

ISOLATION AND CHARACTERIZATION OF TN5 MUTANTS OF *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* NO LONGER ABLE TO INDUCE RESISTANCE

CHAPTER III. INTRODUCTION

Induced systemic resistance in cucurbits was first described by Kuc et al (1975). They reported that inoculation of one leaf of cucumber with Colletotrichum lagenarium induced systemic resistance against subsequent challenge by Colletotrichum lagenarium. Since that initial report, more than 12 pathogens, including viruses, bacteria and fungi have been shown to induce resistance (Kuc 1983). Resistance is nonspecific with respect to the inducing pathogen and the challenge pathogen. Histochemical and ultrastructural studies of infection sites on protected leaves indicate that fungal penetration is arrested in the epidermal cell layer (Richmond et al. 1979, Xuei et al 1987). The inability of pathogens to penetrate the epidermis is associated with an enhanced ability of the outer epidermal walls to lignify at infection sites (Hammerschmidt and Kuc 1982, Basham and Cohen 1983, Dean and Kuc 1987).

Biochemical changes which occur prior to challenge inoculations include systemic increases in extracellular chitinase and peroxidase (Metraux and Boller 1986, Metraux et al. 1988, Hammerschmidt et al. 1982, Smith and Hammerschmidt 1988). Peroxidase has provided a convenient marker for induced resistance, as increases in peroxidase activity precede the expression of induced resistance (Hammerschmidt and Kuc 1982, Stermer and Hammerschmidt

1984). The suggested role for peroxidase in lignin biosynthesis and the potential role of chitinases in the release of elicitors from fungal cell walls (Boller, 1985) indicates that these enzymes may be important components of the induced resistance response.

A fundamental question remaining in the study of induced systemic resistance is the nature of the 'signal' that has been hypothesized to move from the leaf on which the inducing inoculum is applied to other tissues (Jenns and Kuc 1979, Guedes et al. 1980, Dean and Kuc 1986). We have chosen a genetic approach to look for specific signals generated in the pathogen-host interaction which are involved in the induced resistance response. Specifically, we hoped to identify gene products of a pathogen which would potentially have in vitro resistance inducing activity. A bacterial system was chosen for this study because of the relative ease of genetic manipulation. In the initial experiments, we found that the hypersensitive response (HR) elicited by a wheat isolate of Pseudomonas syringae pv syringae was more effective than the disease reaction caused by Pseudomonas syringae pv lachrymans at inducing systemic resistance in cucumber. A Tn5 mediated mutational analysis of P.s. syringae was conducted in order to identify bacterial genes responsible for the efficient induction of resistance.

CHAPTER III. MATERIALS AND METHODS

Media and bacterial strains

Luria-Bertani media was used for culturing E. coli and Pseudomonas spp. overnight cultures. Davis minimal agar plates (Lederberg 1950) were used for growing strains of Pseudomonas spp. Antibiotics were used in the following concentrations: naladixic acid (Nal) 200 ug/ml; kanamycin (Kan) 50 ug/ml; tetracycline (Tc) 15 ug/ml and rifampicin (Rif) 100 ug/ml. Table 1 lists bacteria, plasmids and their relevant characteristics.

Culture of plants

Cucumber seedlings var SMR-58 were grown from seed in 6 inch clay pots in the greenhouse as described previously (Hammerschmidt et al. 1982).

Challenge inoculations

The fungal pathogen Colletotrichum lagenarium, causal agent of anthracnose on cucurbits, was used for all challenge inoculations. Culture of the pathogen was as described previously (Kuc and Richmond 1977). For challenge inoculations, ten 10 µl drops of a 1 x 10^5 spore/ml suspension were placed on leaf 2 of induced or control plants at various times after the inducing inoculation. High relative humidity necessary for spore germination was maintained by placing the plants individually into plastic

Table 5. Bacterial Strains and plasmids

Strain or plasmid	Characterisitics	Reference or Source
Pseudomonas syringae pv syringae (PSSD20)	Nalr; wheat pathogen	Vincent and Fulbright (1983)
PSSD21, PSSD22	Nalr, Kmr; PSSD20::Tn5	This study
Pseudomonas syringae pv lachrymans	Rifr; cucumber pathogen	C.A. John, J.H. Heinz Co. Bowling Green, Ohio
pRK7	Kmr	
pPL6	Tcr; pLAFR3 cosmid carrying the Hrp region of P. syringae pv. phaseolicola (NPS3121)	Lindgren et al. (1986)
pSUP1011	Tn5; Kmr, Cmr	Simon et al. (1983)
pLAFR3	Tcr IncP cos+ repRK2+	Staskawicz et al. (1987)
pRK2013	Kmr, IncP, TraRK2+, repE1+	Ditta et al. (1980)
pPSSD21	Tcr, Kmr; pLAFR3 containing Tn5 EcoRI fragment from PSSD21	This study
pPSSD22	Tcr, Kmr; pLAFR3 containing Tn5 EcoRI fragment from PSSD22	This study
pPS1	Tcr; pLAFR3 containing PSSD20 genomic DNA	This study

bags for 24 hr. Lesion diameter was measured to the nearest millimeter 7 days after the challenge inoculation.

Transposon mutagenesis

The suicide plasmid vector of Tn5, pSUP1011, was used to generate mutants of PSSD20 as described by Anderson and Mills (1985). Colonies of PSSD20 containing Tn5 insertions were selected on minimal media supplemented with 50 ug/ml kanamycin. Prototrophic transconjugates from two separate matings were screened for HR and peroxidase inducing ability. Attempts to select for Tn5 insertions in P. syringae pv lachrymans were unsuccessful.

Screening of Tn5 mutants for loss of ability to induce peroxidase activity

Single colonies of prototrophic kanamycin resistant bacteria were aseptically transfered into sterile test tubes containing 2 ml LB media. The tubes were placed in a test tube rack on a rotory shaker at room temperature. Overnight cultures were diluted to 10 ml with dH20 and infiltrated individually at ten sites into the first leaves of cucumber seedlings. Three days after infiltration, 1 cm leaf discs were removed from each plant and homogenized separately in 1.9 ml microfuge tubes using a modified drill and drill bit. Leaf homogenates were resuspended in 1 ml of 0.01 M phosphate buffer (pH 6), centrifuged in a microfuge for 5 min and the supernatants assayed for peroxidase

activity.

Peroxidase assay

Peroxidase activity in leaf disc homogenates was assayed by adding 2.5 ul of each supernatant to 25 ul dH2O in microtiter plate wells (Nunc) followed by the addition of 100 ul substrate solution containing 220 ug ABTS/ml (2-2'-Azino-di(3-ethyl benzthiazoline sulfonic acid), 50 mM citrate buffer pH 4.0 and 0.02% H2O2. Color development was allowed to proceed for 15 min followed by the addition of 100 µl stopping reagent containing 0.17% HF, 6 mM NaOH, and 5 mM EDTA. An ELISA plate reader (Dynatech) was used to quantitate color development (Δ A405) in wells. Ten control samples and 10 samples from induced plants were included in each plate. Mutants from inoculated plants which gave absorbance values below the average of the positive controls were retested.

In vivo growth of bacteria

Overnight cultures of bacteria were washed in saline and diluted to an OD600 of 0.05 for inoculation (approximately 10^4 cfu/ml). Approximately 200 µl of each bacterial strain was infiltrated into 5 separate plants for each time point. One cm discs containing the infiltration site were removed at various times after infiltration, surface sterilized in 20% bleach, and homogenized in saline. Homogenates were serially diluted in saline and total cfu/infiltration site

determined for each disc.

Cloning of Tn5 insertion sites from PSSD21 and PSSD22

Southern blots revealed that both mutants contained Tn5 in a 17 kb EcoRI fragment. Genomic DNA from mutants was digested to completion with EcoRI and size fractionated on a linear 5-40% sucrose gradient for 5 hr at 25,000K in a Sorvall TH641 rotor. Fractions containing fragments in the 10-20 kb range were pooled, ligated to pLAFR3 and packaged into phage using a commercially available packaging extract (Promega). Phage from the packaging mixture were used to transduce E. coli HB101 according to manufacturers recommendations. Colonies containing Tn5 were selected on LB containing 50 µg/ml kanamycin. Twenty km colonies were selected from each packaging reaction and screened in mini-preps for cosmids containing only the 17 kb EcoRI genomic fragments from PSSD21 and PSSD22. The resulting cosmids, pPSSD21 and pPSSD22, were used for mapping of Tn5 insertion sites.

Construction of PSSD20 genomic library in pLAFR3

A genomic library of PSSD20 was constructed as described by Lindgren and Panopoulos (1986). Total genomic DNA was partially digested with Sau3A and size fractionated on a linear 5-40% sucrose gradient as described above. Fragments of MW 15-30 kb were ligated to the BamH1 site of pLAFR3. The cosmid ligation mixture was packaged in vitro

and the resulting phage were transduced into E. coli HB101. Transconjugants were selected on LB containing tetracyline and streptomycin.

Tri-parental matings

Ten ml overnight cultures of recipient P.s. syringae strains, donor E. coli containing pLAFR3 clones and E. coli HB101 containing the helper plasmid pRK2013 were pelleted at 4000g for 10 min in a Sorval SS-34 rotor. Cells were washed twice in 10 ml 0.85% sterile saline prior to mating. Twenty ul of each bacteria was pipetted onto a sterile 45 µm millipore filter on complete agar plates. After incubation in a 27C incubator for 8 hr, filters were washed in 10 ml sterile saline and serial dilutions were plated on selective media.

Basic molecular techniques

Nick translations were performed using a commercially available kit (BRL). DNA fragments for use as probes were electrophoretically eluted from gels onto DEAE membranes (Schleicher & Schull) according to manufacturers recommendations. Standard procedures, including isolation of genomic and plasmid DNA, agarose gel electrophoresis, ligation, restriction endonuclease digestions and Southern blotting were performed as described by Maniatis et al.(1982).

CHAPTER III. RESULTS

HR vs. disease induced peroxidase activity and resistance

The hypersensitive response elicited by Pseudomonas syringae pv. syringae was more effective than disease caused by Pseudomonas syringae pv. lachrymans at inducing systemic increases in peroxidase activity. Peroxidase induction was maximal after only 2 days for plants induced with P. syringae pv. syringae while those induced with the pathogen P. syringae pv lachrymans required 6 days for maximal peroxidase induction (Fig. 12, A, B). Further time course experiments indicate that peroxidase activity begins to increase at 24-36 hr after inoculation with P.s.syringae, precisely the time HR symptoms begin to appear (Fig. 13). Consistent with the rapid increase in peroxidase activity, near maximal resistance in HR induced plants is expressed 2 days after the inducing inoculation as demonstrated by a decrease in C. lagenarium lesion diameter on challenged leaves (Fig 14) Plants induced with the pathogen P.s.lachrymans required 5-6 days after the inducing inoculation to acquire the same level of resistance.

Mutant isolation

Approximately 2000 prototrophic kanamycin resistant mutants were initially screened, and two were isolated which did not induce peroxidase activity. These mutants were designated PSSD21 and PSSD22. Both mutants had lost the ability to

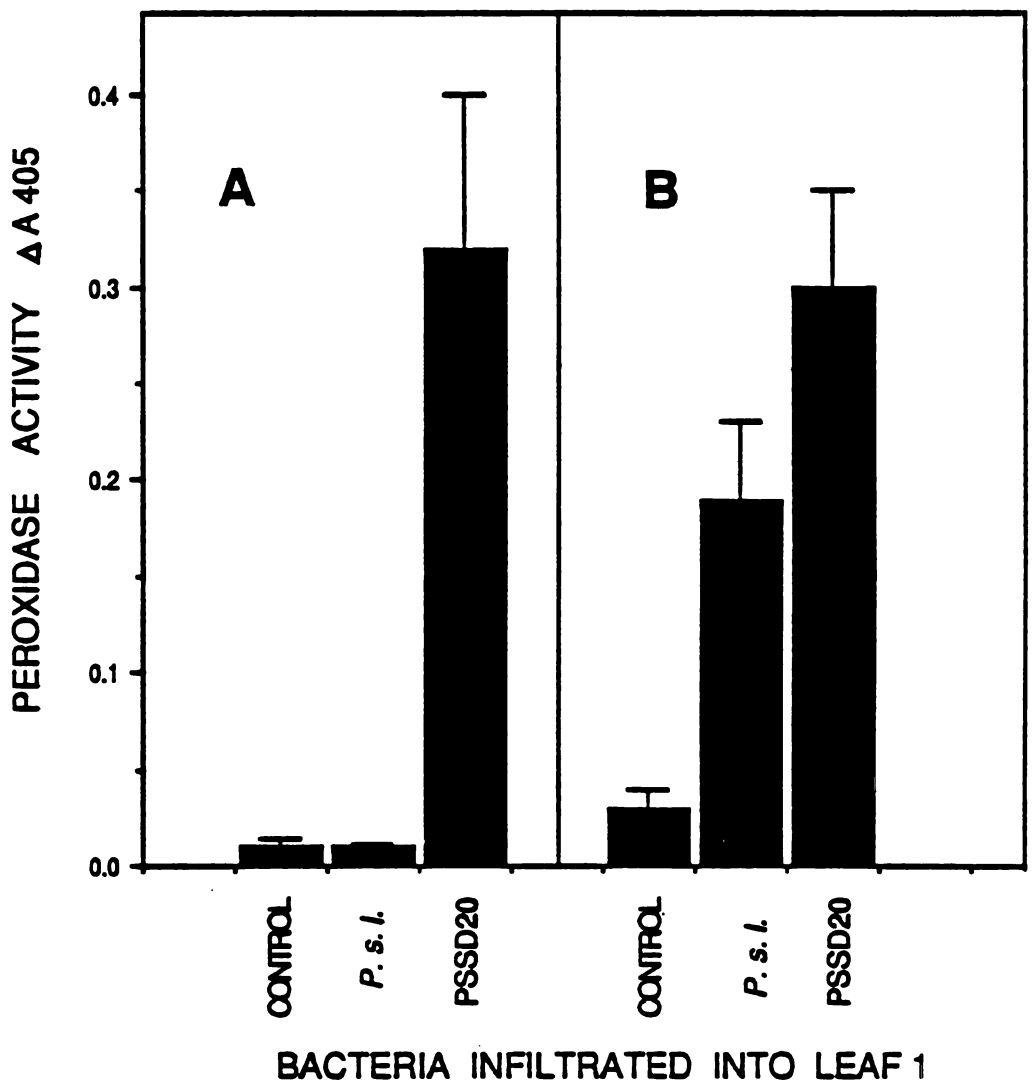


Fig. 12. Systemic increases in peroxidase activity in response to inoculation of leaf 1 with Pseudomonas syringae pv. syringae (PSSD20) or Pseudomonas syringae pv. lachrymans (P.s.l.). Peroxidase activity in leaf 2 of plants was determined at 2 days (A), or 6 days (B) after inoculation of leaf 1. Control plants received no treatment.

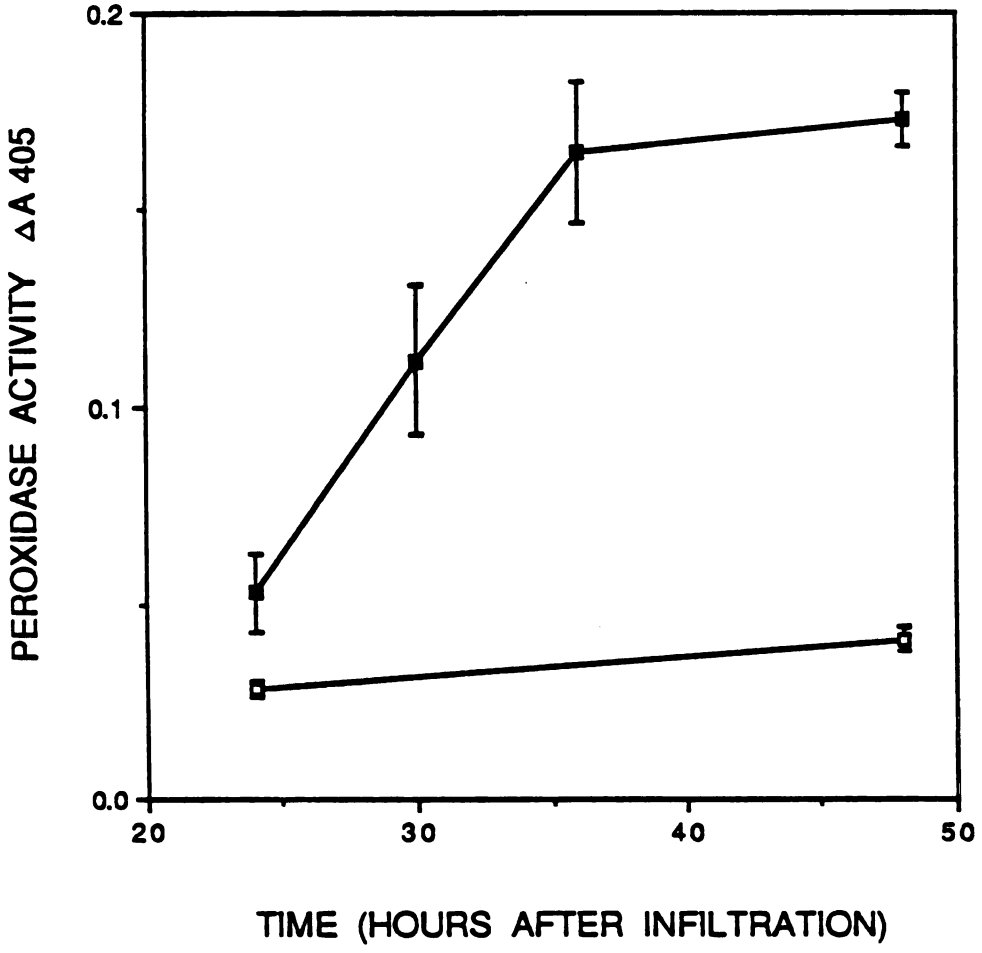


Fig. 13. Time course for the induction of peroxidase activity in the second leaf of plants inoculated on the first leaf with Pseudomonas syringae pv syringae (▬) or control plants (▭).

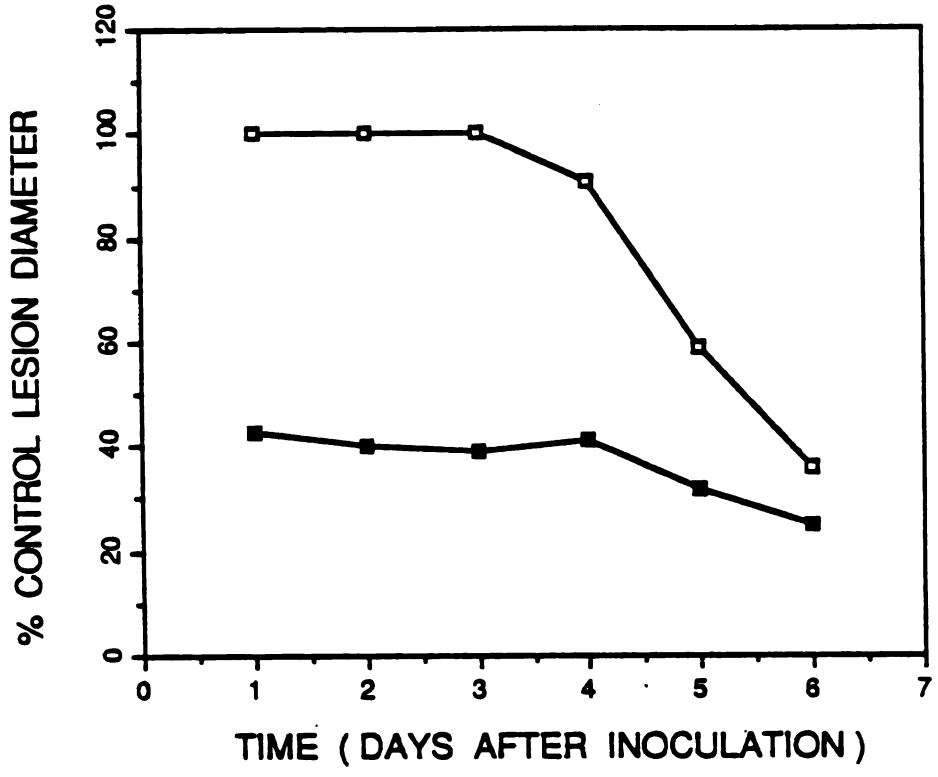


Fig. 14. Time course for the expression of induced systemic resistance in plants inoculated on leaf 1 with *Pseudomonas syringae* pv *syringae* (■) or *Pseudomonas syringae* pv. *lachrymans* (□). Leaf 2 of inoculated or control plants was challenged with 10 drops of a 10^5 spore/ml suspension of *Colletotrichum lagenarium* at various times after inoculation. Lesion diameter was measured 7 days after challenge inoculations and reported as % of control lesion diameter for each time point.

produce a hypersensitive reaction (HR^-) and had simultaneously lost the ability to cause disease on the host plant wheat. Mutant PSSD21 produced chlorosis at the site of infiltration when greater than 10 cfu/ml were infiltrated while PSSD22 produced no reaction at any concentration infiltrated. Neither mutant was able to induce resistance in cucumber (Fig. 15).

In vivo growth of bacteria

In vivo growth of PSSD21 was compared to that of the wild type P.s. syringae and the pathogen P.s. lachrymans. After an initial 10-fold increase the population of mutant remained relatively stable. (Fig. 16 A,B). Wild type P.s. syringae multiplied 10 - 10 during the first 2 days after infiltration while the pathogen P.s. lachrymans multiplied 10 during the same time period (Fig. 16A). Mutant PSSD22 did not multiply in leaf tissue as well as the wild type as determined by subsequent in vivo growth studies (Fig. 16B). The genomic clone pPS1 which restored the ability of the mutants to produce an HR slightly enhanced the ability of the mutants to multiply in leaf tissue (Fig. 16B) This enhancement was only observed during the first 2 days after infiltration, however, possibly due to the instability of the large cosmid in the absence of antibiotic selection. The loss of ability to induce an HR was not solely due to the inability of mutants to grow in leaf tissue, as infiltration of up to 10 cfu/ml still did not produce a hypersensitive response.

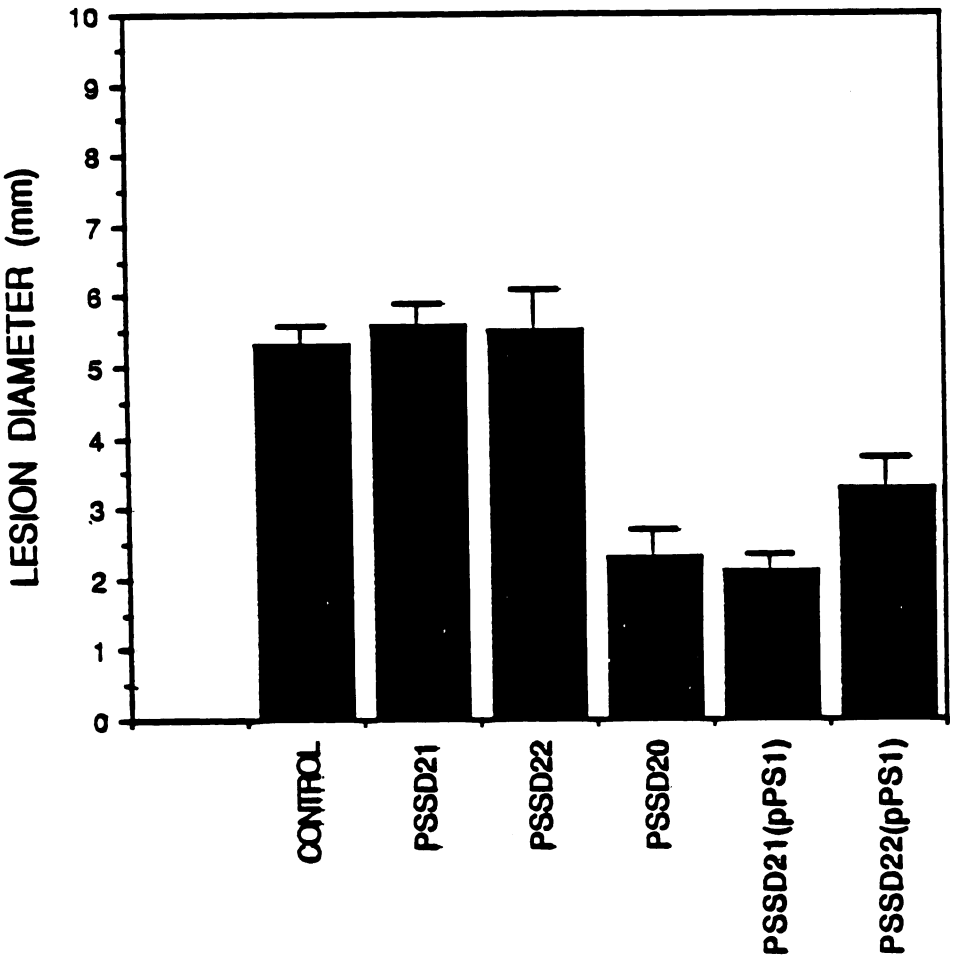


Fig. 15. Comparative ability of wild type, Tn5 mutants, and restored mutants of P.s. syringae to induce systemic resistance. Bacteria were infiltrated at 10 sites into leaf 1 at a concentration of 10 CFU/ml and leaf 2 was challenged 4 days later with 10 drops of a 10^5 spore/ml suspension of Colletotrichum lagenarium. C. lagenarium lesion diameter was determined 7 days after the challenge inoculation.

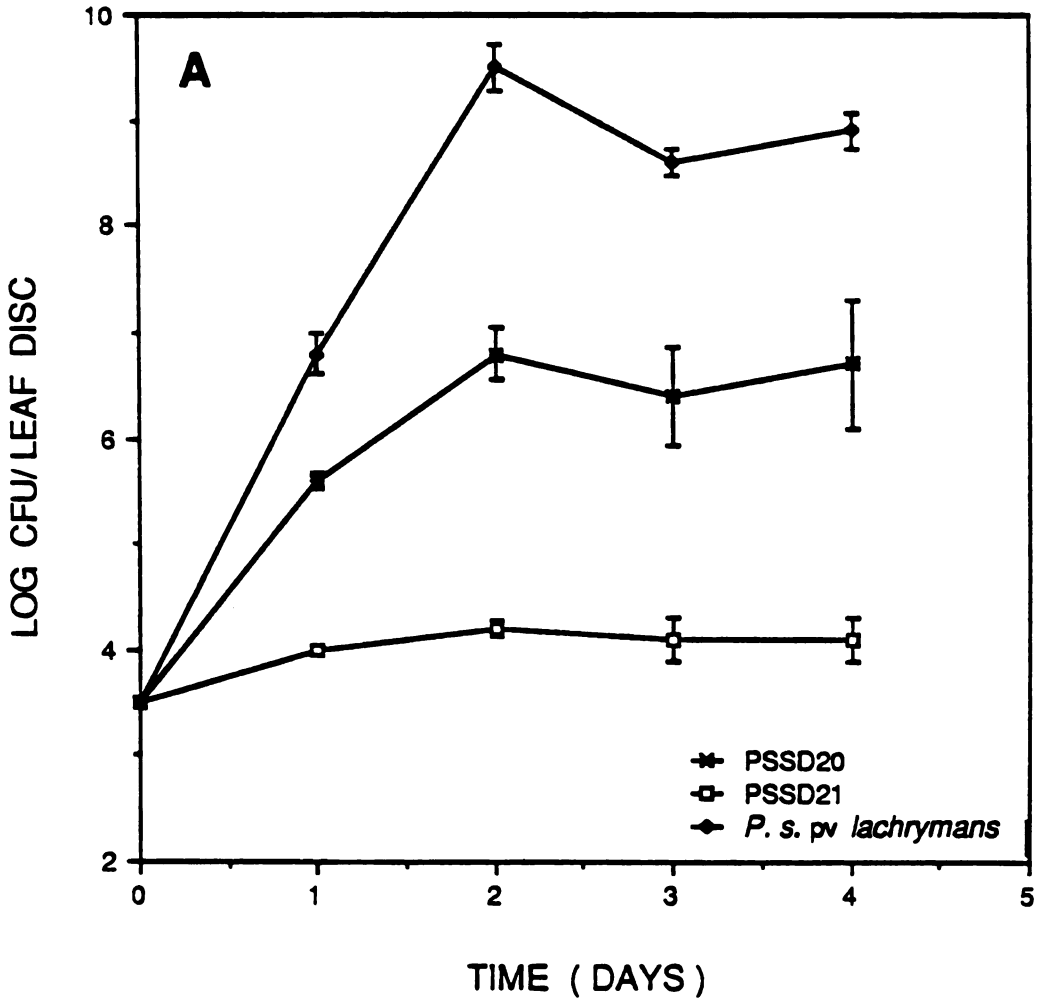
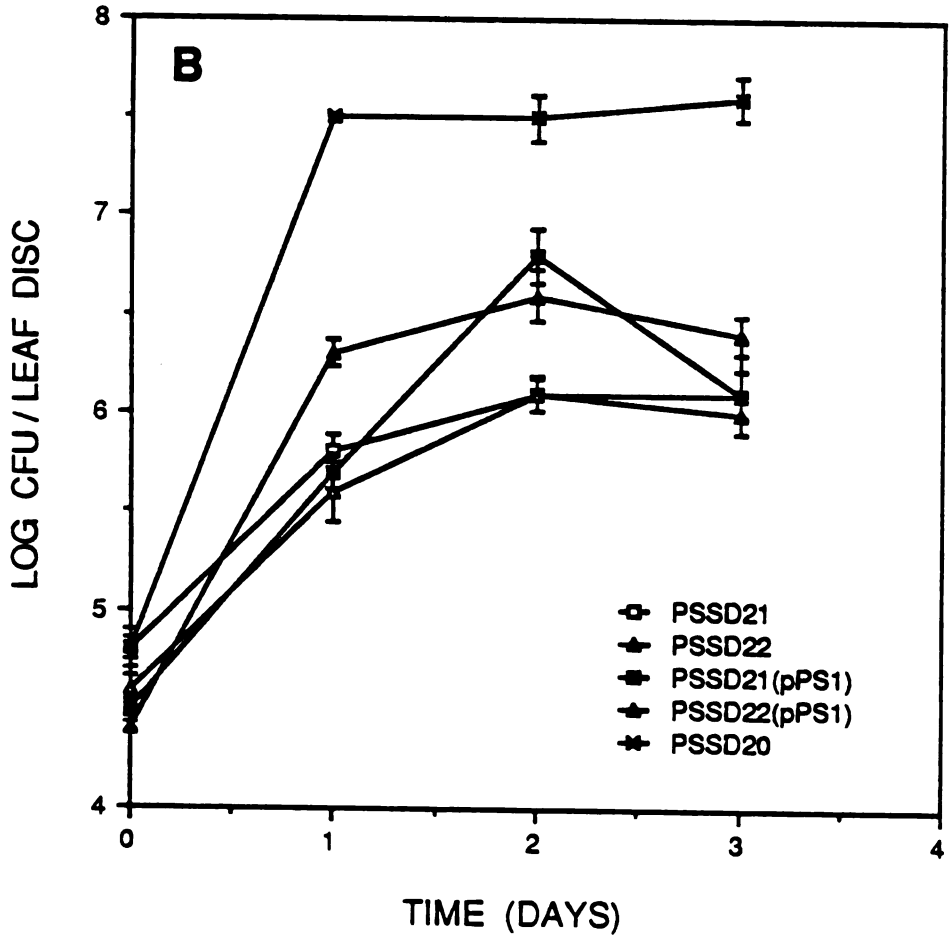


Fig. 16. A,B. In planta growth of bacteria. Each data point represents the mean of the log of total cfu/disc from 5 plants. Standard error bars are shown.


B
LOG CFU / LEAF DISC
TIME (DAYS)
PSSD21
PSSD22
PSSD21(pPS1)
PSSD22(pPS1)
PSSD20
8
7
6
5
4
0
1
2
3
4

Physical characterization of Tn5 insertion sites

Southern blots of Eco R1 digested genomic DNA from the two mutants probed with pRKC7, which contains the Km gene present within Tn5, indicated that Tn5 was present in a 17 kb fragment in each of the two mutants (blot not shown). Tn5 insertion sites were deduced from a restriction map of the cloned fragments using BamHI, and HindIII which cut within Tn5 but did not cut within flanking regions of genomic DNA (Fig. 17). A 10 kb EcoRI HindIII fragment from pPSSD21 indicated in Fig 17, was used as a probe in a Southern blot of EcoR1 digested DNA from PSSD20, PSSD21, and PSSD22 (Fig. 18). The probe hybridized to one 11 kb fragment of PSSD20 and to one 17 kb fragment in each of the two mutants, indicating that Tn5 had inserted into different sites of the same Eco R1 fragment in the mutants.

Isolation of clone from library and complementation of mutants

The 10 kb HindIII fragment from PSSD21 (Fig 17) was used to probe a genomic pLAFR3 library of the wild type P.s. syringae. Approximately 1000 colonies were screened and two were isolated which hybridized strongly to the probe; pPS1 and pPS2. The cosmids were mobilized separately into PSSD21 and PSSD22 and the resulting transconjugates were tested for their ability to induce an HR on cucumber. One cosmid, pPS1

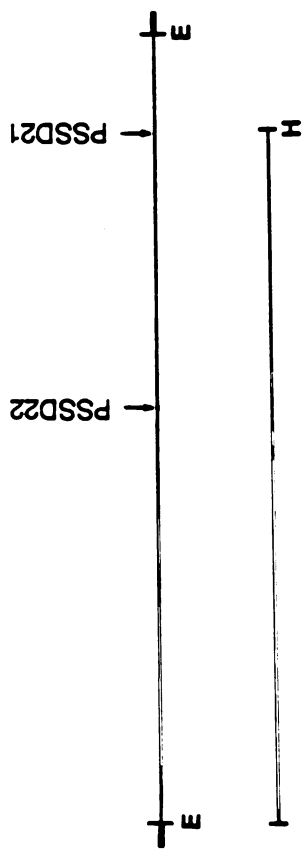


Fig. 17. Location of Tn5 insertion sites (↓) in pPSSD21 and pPSSD22. The 10 kb HindIII probe from PSSD21 is shown.

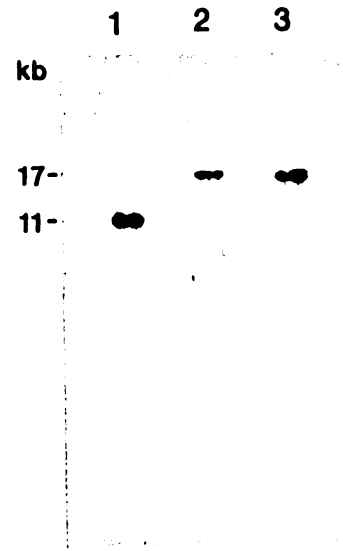


Fig. 18. Hybridization of 10 kb Hind III fragment from pPSSD21 to EcoRI digested genomic DNA from PSSD20, PSSD21 and PSSD22(lanes 1, 2 and 3 respectively).

restored the ability to induce HR to both mutants while the second cosmid, pPS2 restored function to PSSD22 but not PSSD21. As Tn5 was inserted into different sites in the same EcoR1 fragment in the two mutants, we tested the ability of pPSSD21 and pPSSD22 to restore function to PSSD22 and PSSD21, respectively. Introduction of pPSSD21 into PSSD22 restored the ability of PSSD22 to cause HR on cucumber and disease on wheat. However introduction of pPSSD22 into PSSD21 did not restore function to that mutant.

Restoration of Resistance Inducing Activity to Mutants

The genomic clone pPS1 restored the ability of PSSD21 and PSSD22 to induce resistance and peroxidase activity, although the restored mutants were somewhat less effective than PSSD20 (Figs. 15,19).

Homology to HRP cluster from P. syringae pv. phaseolicola

The simultaneous loss of ability to cause disease on wheat and HR on cucumber indicates that mutants PSSD21 and PSSD22 belong to a class termed 'HRP' mutants by Lindgren et al.(1986). The P. syringae pv. phaseolicola hrp gene cluster necessary for disease and HR has been cloned and characterized (Lindgren et. al. 1986; Lindgren et. al. 1988). We obtained plasmid pPL6 containing the entire hrp cluster from P.s. phaseolicola to study functional and sequence homology to the P.s. syringae region. Plasmid pPL6 restored the ability of both PSSD21 and PSSD22 to cause HR on

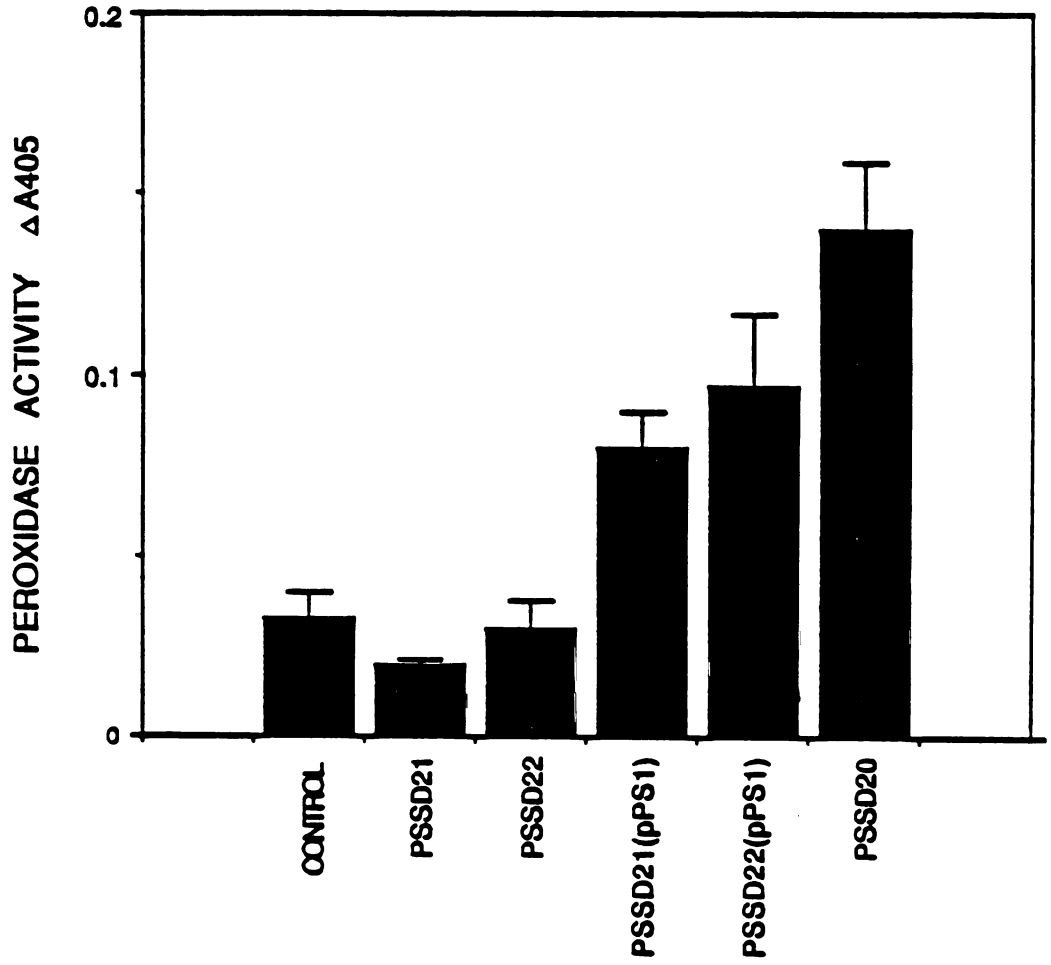


Fig. 19. Comparative ability of wild type, Tn5 mutants, and restored mutants of P.s. syringae to induce systemic increases in peroxidase activity. Plants were inoculated with bacteria as described in Fig. 15. One cm leaf discs were removed for peroxidase determination prior to challenge with Colletotrichum lagenarium.

cucumber. A southern blot of BglII/EcoR1 digested pPL6 was probed with the 10 kb EcoRI HindIII fragment from pPSSD21 and the probe hybridized strongly to the 7.4 kb fragment of pPL6, and weakly to the 4.3 and 4.0 kb bands (Fig. 20).

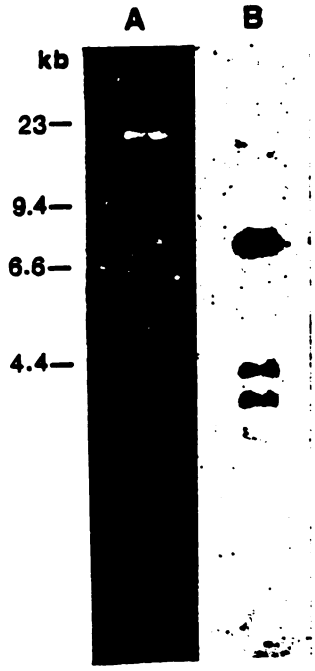


Fig. 20. Homology between Pseudomonas syringae pv. phaseolicola HRP region and a portion of the functionally similar region from PSSD20. A, pPL6 digested with BglII and EcoRI. B, hybridization of 10 kb HindIII fragment from PSSD21 to BglII/EcoRI digested pPL6. The blot was hybridized and washed in the presence of 1.0 M NaCl and 1% SDS at 68C.

CHAPTER III. DISCUSSION

In all cases of induced sytemic resistance in cucumber reported, the level of resistance induced systemically has been quantitatively correlated to the amount of disease necrosis on the inducing leaf (Hammerschmidt et al. 1982, Kuc 1983). Similarly, the expression of induced systemic resistance is related temporally to the appearance of necrosis on the inducer leaf. These observations are supported in our study, in which the HR induced by P. syringae pv. syringae induces resistance more rapidly than the more slowly developing necrotic lesion induced by P. syringae pv. lachrymans. The requirement for necrotic lesion formation has been supported genetically through the generation of Tn5 mutants of P. syringae pv. syringae that no longer induced the HR or systemic resistance. Complementation of the mutations demonstrated that restoration of HR also resulted in the restoration of resistance inducing activity.

The mutants isolated in this study are characteristic of a class of mutants described in other pathovars of Pseudomonas syringae that exhibit a simultaneous loss in the ability to cause an HR on nonhosts and the ablility to cause disease on host plants. The region of the genome necessary for these functions, known as the 'HRP' region, has been shown by Lindgren and Panopoulos (1987) to be conserved among Pseudomonas syringae pathovars. The genomic region of P.

syringae pv syringae that we have identified as important in the induced resistance response shares homology with the cloned HRP region of P. syringae pv. phaseolicola (pPL6) based on complementation tests and DNA hybridization. A functionally similar hrp cluster in Pseudomonas solanacearum has been shown by Boucher et al (1987) to share homology with other strains of P. solanacearum and eight pathovars of Xanthomonas campestris.

The observation that common bacterial genes are involved in pathogenesis and the hypersensitive response suggests that the two reactions may produce common 'signal' molecules in bacteria-plant interactions. Thus with respect to induced systemic resistance, the difference in reponse to disease and HR causing pathogens is quantitative rather that qualitative. Lyon and Wood (1976) have observed similar quantitative differences in the ability of HR and disease causing races of P. phaseolicola to cause electrolyte leakage in bean leaves. Both races induced significant electrolyte leakage, but the HR reaction induced high levels of leakage after only 24 hr while the pathogen required three to four days to induce a similar response. The saprophyte P. fluorescens did not induce any electrolyte leakage. Klement (1982) has also suggested that hypersensitive and disease reactions differ primarily in the speed and magnitude of the response.

A better understanding of the physiology and biochemistry of the HR may provide insight into the nature of the signal

generated in response to pathogens. Physiological changes which have been associated with the HR include lipid peroxidation (Keppler and Novacky 1986,1987) and loss in membrane potential accompanied by a rapid efflux of K+ ions (Pavlovkin et al 1986, Atkinson et al. 1985). Baker et al. (1987) demonstrated that Tn5 mutants of P. syringae pv. pisi that have lost the ability to cause a hypersensitive response on tobacco, also fail to induce the K+ ion efflux. Atkinson and Baker (1987) have also shown that the enhanced K+ efflux, H+ influx associated with the HR helps to promote the growth of bacteria in intercellular spaces of leaves. How these physiological changes associated with the HR may relate to the induction of systemic resistance is unknown and area for future research.

In conclusion, we have demonstrated that rapid coordinated cell death associated with the HR induced by P. syringae pv. syringae induces systemic resistance and peroxidase activity more efficiently than does the necrotic lesion caused by P. syringae pv. lachrymans. In addition, Tn5 mutagenesis and complementation studies have provided the first genetic evidence for the requirement of pathogen induced necrosis for induced resistance expression. We have also demonstrated that the region of the P. syringae pv. syringae genome necessary for inducing resistance is functionally homologous to the hrp region of P. syringae pv. phaseolicola and shares at least some sequence homology. The mutants generated in this study will provide a new tool to

study the physiological and biochemical events underlying induced systemic resistance in cucumber.

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FUTURE DIRECTIONS

FUTURE DIRECTIONS

The plant pathologist's search for a resistance inducing signal has proved as elusive as the plant physiologist's search for florigen. In retrospect, the inability to isolate a 'signal' is of minor significance considering the valuable information induced systemic resistance may contribute to our understanding of disease resistance, peroxidase function, and pathogen mediated plant gene regulation. The following two paragraphs suggest 'the next experiment' to follow those presented in Chapters 1-3. The concluding paragraphs propose several ways in which induced systemic resistance in cucumber may be used to explore more fundamental questions pertaining to plant cell biology.

The use of HR causing bacteria to induce resistance has several advantages over the use of fungal pathogens: bacteria are easier to maintain, they induce resistance more quickly, and the genome is readily accessible for study. Although Pseudomonas syringae pv syringae may be considered 'typical' of HR inducing bacteria, other strains should be tested for their ability to induce systemic resistance in order to ensure that the response is non-specific. Many P. syringae pathovars produce a toxin, syringomycin, and the role such a toxin might play in induced systemic resistance should be assessed. Finally, Grimm and Panopoulos (1988) have suggested that the hrp region regulates the expression of genes involved specifically in disease processes. The premise for

this hypothesis lies in the observation that several genes in the hrp region share homology to regulatory proteins in Rhizobium spp and Klebsiella pneumoniae which mediate plant-bacteria interactions. Sule and Klement (1971), and Lyon and Wood (1976) have reported that viable cells from old cultures of P. phaseolicola have a decreased capacity to elicit an HR. Plant pathogens frequently lose their virulence when they are subcultured for long periods of time in the absence of a plant host, though no molecular explanation for this phenomenon has been presented. One easily tested possibility, at least for bacterial, pathogens is that the functional 'hrp' locus is lost from cultures in the absence of selective pressure. If this is the case, it should be possible to complement bacteria which have lost virulence in culture with the cloned hrp region.

A systemic increase in peroxidase activity is the first detectable evidence that a cucumber plant has responded to a resistance inducing inoculation. This response can by quite rapid, as demonstrated in Chapter 3. The next question to address in this respect is: how do the levels of peroxidase mRNA vary temporally and spatially during induced resistance? Specific probes for the anodic peroxidase may be used in a Northern blot analysis of mRNA prepared from tissue taken at various times in order to determine when and where peroxidase transcription is activated. The gene for cucumber chitinase has also been cloned (Metraux et al., in preparation) and a comparison of the message levels for these two proteins in

response to pathogen stress should indicate whether their regulation is influenced by the same factors.

A recent review has emphasized the need for a more thorough investigation into the processes of protein sorting in plant cells (Della-Cioppa et al. 1987). Both chitinase and peroxidase accumulate in the extracellular spaces of leaves, making them suitable proteins for the study of sorting mechanisms which target proteins for export. Both proteins are specifically regulated by biological stress and are similar with respect to size and charge. Characterization of the respective genomic clones for these proteins should reveal whether they share common regulatory and signal sequences. A comparison of the glycosylation profiles of chitinase and peroxidase will indicate whether this modification is conserved. The size and structure of extracellular proteins must presumably be limited by their need to pass from the cytoplasm through the cell wall. Recent studies on the conformation of cell-cell channels may help to delimit the structural constraints on extracellular proteins (Meiners et al. 1988).

A thorough understanding of the regulation and processing of peroxidase and chitinase may, in the future, provide a variety of useful applications. Both chitinase and peroxidase are putative defense related proteins. The possibility exists that novel, more effective defense proteins may be introduced into plants under the specific regulation of pathogen stress. These compounds would

preferably be designed to meet the criteria which allow their selective regulation and cellular localization. Finally, the use of plant cells in biotechnology for the production and export of commercially valuable products may be facilitated by an understanding of the characteristics of a protein which result in its export from the plant cell.

The 'signal' released by pathogen induced stress governs fundamental processes in plant cell biology. Investigation into the nature of this signal is perhaps best approached through the study of these processes and the elements of their regulation.

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