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HOW PLANTS RESIST DISEASE - A FOCUS ON "NONHOST RESISTANCE" OF PEA TO A BEAN PATHOGEN

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PREFACE

An appreciation of this earth's botanical beauty exists in most of us. Master gardeners as well as the home based folks realize the magic of what plants do in surviving seasons, adverse soil, insects and pathogens, etc. The simple fact that a plant can develop a seed and this seed can shut down gene activity for a period and then with water magically restart and control the almost 20,000 genes and regulate them in a way that at the appropriate time activates the transcription of the genes for roots, leaves, flowers etc. Anyone that has had a high school biology class knows that the DNA within the nucleus of every cell has this 20 thousand gene information. The structure and gene regulation of plant nuclei and the nuclei of our own cells is not that much different, thus plant scientists and animal scientists both strive to understand this regulatory process. The regulation of this gene activation process is crucial in the plant's resistance to disease. An example of almost complete similarity between plant and human is one defense response gene coding for a product called "defensin" important for many biological entities in defending against pathogens. This small defensin protein (peptide) is part of a multigene product response in pea tissue that can resist plant pathogens that are not pea pathogens e. g. in resisting a bean pathogen. This is called "nonhost" resistance. The pea tries but is not typically successful in resisting true pea pathogens. This ebook encompasses 50 plus years of research which brings to a current conclusion how the plant responds to pathogen and nonpathogen and in some of the ways this defense can be bolstered to make a plant resist a true pathogen.

This is a case-study that looks, mostly at pea, but also at potato and flax host tissue to hypothesize generally "How Plants Resist Diseases".

Brief answer to "How plants resist diseases"

Sometimes the answer to the question of interest to the reader is answered but it requires a lot of input to retrieve so in the next sentences I will assemble a

simple answer for, How Plants Resist disease?

*When pathogen of **bean or potato** contacts a **pea** plant it releases signals that generate a response in plant cells—an activation of "defense genes". These genes code for products such as the peptide (short protein) called defensin. Defensin is strongly antifungal and rapidly slows the growth the fungus. The fungus then accumulates digestive enzymes internally that further dictates its own demise. This resistance is called "nonhost resistance" –i.e. a pea resisting a bean pathogen. When a true **pea** pathogen arrives at the **pea** plant it also releases signals that generate a defense response, however the response to these signals is slower. Although the growth of the fungus is slowed it is not totally stopped and some of its growing points (hyphal tips) continue to grow through the now weaker plant defense response and the fungal digestive enzymes remain mostly in the older tissue where they recycle components utilized for tip growth—through this time when the plant's defense response subsides, leading to a susceptible response or susceptibility.*

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Introduction

The essence of this ebook is to fill in all of the features that are not mentioned in the simple answer given above and relate the molecular mechanisms involved by showing the data that forms the basis of the simple answer.

My lab continued for 50+ years utilizing an assay of pea tissue disease resistance responses initiated by Professor Cruickshank in Australia [1]. A plant such as pea grows in the soil while continually being confronted by all the microbes of the soil. Many of which successfully infect plant species other than pea-- how does the pea survive? I would like to sum up the general conclusions reached by all of us in my lab and some others throughout the decades to date, to answer this and other major scientific questions about disease resistance in plants.

Plants are full of nutrients that can support microbial growth and if the microbe can penetrate the plant defenses and break the cells the nutrients are technically there for the taking. However, the plant defense response makes this difficult. This report focuses on the manner that microbes initiate the response and the factors that make-up the plant's suppressive action [2]. Plant pathogenic fungi and bacteria have evolved enzymes capable of digesting the waxy surface of leaves [3], digesting the carbohydrate within plant walls, lipids within the plant cell membrane, and finally the enzymes of the fungus that can digest proteins, nucleic acids, and other molecules within the plant cell's cytoplasm [4]. More specifically, plants to become resistant must be able to synthesize new RNA and protein which requires it to retain an intact general metabolism. Plants, like us humans, when their immune responses are low, become vulnerable to these pathogens. Humans are indeed subject to infection from almost all microbes when by necessity immune suppressants are used following an organ transplant. Plants subjected to a mutagenesis that incapacitates almost any gene that is directly or indirectly required for intermediate metabolism also incapacitates the plant defense response. Such a mutational event breaking resistance can be interpreted incorrectly by the researcher that he/she has successfully identified a gene for disease resistance. On the pathogen's side, Harold Flor and others found that a "race" of a given pathogen can become virulent by mutating one of its own "avirulence" genes, because the product of that gene had been inadvertently signaling the activation of the plant's defense response [5]. What Flor found by examining races of a pathogen that causes a rust disease on flax plants, has proven true for many other plants and pathogens known to infect them. When plants confront pathogens, not known to normally infect a given plant species, the plant's response is rapid and the pathogen is quickly resisted. This is called "non host resistance". Seldom, if ever, in nature does this resistance break down, apparently because there are many signals besides the "avirulence traits of the fungus" that signal the non host plant's response. There is some corollary to having a kidney transplant from an animal or from a genetically unrelated human. The transferred organ is violently resisted. Even when there is a match up with an organ from a "matched" close relative, immune-suppressors must

be taken by the individual receiving the transplant.

What is Plant Disease Resistance?

There are two general types of plant defense, the non host resistance discussed above (e.g. pea against a bean pathogen) and a race-specific resistance that can directed by a plant species against races of a pathogen that are typically virulent on this species (a true-host, like flax against flax rust races-- rather than the "inappropriate pathogens" that trigger non host resistance). The gene or genes controlling this latter "race-specific resistance" are often found to be single dominant genes and are called R genes. Many such genes have been characterized through classical genetic crosses. If the plant's R gene fails, the plant species can be again given resistance by breeding in new R genes found in wild, genetically-crossable relatives of that species. These new genes can be introduced into the "varieties" of the species that are used in commercial agriculture crops using conventional plant breeding techniques. Once the gene is bred in, to be commercially acceptable, the recipient plant needs to be crossed back to a commercial-like variety---selecting always for the resistance and discarding progeny with wild species genes that would make this new variety less desirable for consumption etc.

R genes-biochemical make-up.

Functional domains of the major classes of plant resistance (R genes) include: Leucine rich repeats; nucleotide binding sites; toll-like receptors; transmembrane; nuclear localization signal; WRKY amino acid sequence domain; toxin degrading enzyme; and receptor for a pathogen molecule (PAMP) (e. g. chitin oligomer from the fungal wall or a flagellin fragment from flagella). The latter reception of the PAMP can be followed by a series of protein kinases that, hypothetically, mediate the activation of transcription factors that are associated with the activation of pathogenesis-related (PR) genes-- whose products are responsible for suppressing fungal growth. The "PAMPs" investigated on the pea system discussed herein activates PR genes by altering the conformation of the plant's chromatin [2]. This DNA-specific, chromatin-altering route of defense gene activation is utilized by many other fungal components in activating the "nonhost" disease resistance response-- and is the central feature of this ebook. PR genes are always a component of the plant disease resistance response even though R genes are manipulative in the intensity of their expression in race-specific resistance.

Gene-for gene hypothesis

Dr. Harold Flor working with flax, and the races of the flax rust fungus (the causal fungal pathogen is *Melampsora lini*) that infected it, sorted out genetic lines that in sum total possessed about 27 R genes known at that time. He eventually identified even more races of the flax rust fungus and through genetics, developed individual race "lines" that each contained a single Avirulence (Avr) gene, enough lines to match up with the 27-plus flax lines with individual R

genes [5]. Thus a flax that had a R gene when matched with a corresponding pathogen *Avr* gene was resistant to that flax rust race. If he mutated away that particular fungal *Avr* gene the plant became susceptible to that flax rust race. Unfortunately, a new flax variety that had acquired the new R gene for resistance often failed to resist after a few growing seasons because of the natural mutation of the corresponding *Avr* gene.

This allowed mutated-flax-rust-types to eventually became prominent in the environment since they were the only ones that could multiply on the flax variety possessing this new R gene (this situation is similar to the development of antibiotic resistance in human pathogens).

The use of Flor's well-characterized material allowed my lab to investigate some of the biochemistry that occurs with a R-gene dictated resistance response. With the availability of double labelling with ^{14}C and ^{3}H labeled amino acids we could follow the synthesis of new protein in both the untreated R gene-containing plant and one that had been inoculated with a race of the rust that would generate the resistance response. Sharon von Broembsen, found that when testing the match up, that gave the disease resistance response, there was a surge in the synthesis of broad-size-ranges of proteins in the plant [6]. This surge was absence in all other combinations that did not conform to the resistance match-up described in Flor's designations. The conclusion was that disease resistance is an induced response involving the enhanced synthesis of many plant genes. This information was developed into an "Induction Hypothesis" [7].

Surprisingly, the prevailing thought on the chemistry of disease resistance at that time included explanations that involved toxin production by the pathogen or nutrients available to the pathogen as the determining factors dictating successful infection by the pathogen. Toxins remain as major determinants of susceptibility. Thus the hypotheses forwarded were labelled the *Nutrition hypothesis* or later the *nutrition-inhibition hypothesis*.

Induction hypothesis

Dr. Martin Schwochau an organic chemist and me a biochemist, formulated Sharon's information into an *Induction hypothesis.* Our thinking was assisted by the work of Jacob and Monod [8], on the activation of genes in bacterial systems. We also attended 6 weeks of lectures by Professor William Q. Loegering (Univ of Minnesota) on plant-rust genetics. It was also known at the time that compounds called phytoalexins (antifungal isoflavonoids) were increased in legumes [1] in response to a pathogen. The phytoalexin, pisatin, in pea was found to be induced by various pathogens and we found it to be generated via the shikimic acid secondary plant pathway and then on from phenylalanine [9] to a continued secondary pathway via chalcone and then on toward pisatin. Dr. Hans Van Etten's lab [10] later resolved more completely the individual steps.

Figure 1: The potato shoot was excised with a razor blade exposing the stem to treatment solutions. The split immature pea pod endocarp surface is exposed to treatment. In both tissues, treatments by-pass the waxy cuticle layer that is a physical barrier, but not one that is decisive in the resistance response.

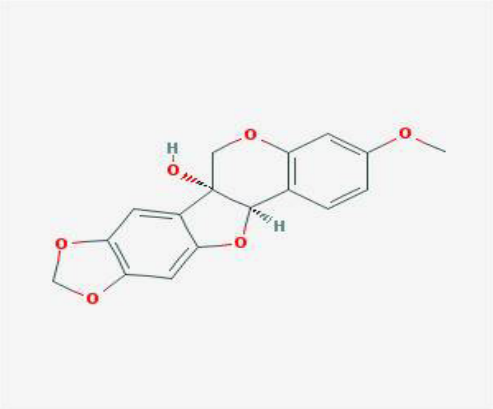

Figure 2: Pisatin structural formula.

Nonhost resistance in pea endocarp tissue

Much of our long-term effort was shifted to the use of pea endocarp tissue, previously described by the Australian, Dr. I. A. M. Cruickshank. For most of the 50 years, we challenged the tissue with a pea pathogen, *Fusarium solani* f. sp. *pisi* (Fspi) to generate the *susceptible response* and the bean pathogen, *Fusarium solani* f. sp. *phaseoli* (Fsph) to generate the ***nonhost*** *resistance response*. This pea endocarp tissue **(Figure 1)** responds rapidly (within 6 h), to generate total resistance to the bean pathogen. Thus every change associated with disease resistance is analyzable within these early hours. Inoculation of an intact pea plant required over 15 days to distinguish resistance or susceptibility. (A similar rapidly responding potato stem tissue has been adapted, namely a 2 cm length of the excised end of a potato shoot shown in **Figure 1**. Other benefits: we can observe the pathogen growth or lack of it clearly in the microscope and see changes in tissue pigmentation when they occur near the pathogen. Other measurements of nuclear diameter; phenylalanine ammonia-lyase enzyme activity; pisatin **(Figure 2)** production; mRNA and protein synthesis; DNA damage; chromatin changes; and defense gene activation could be readily obtained. We studied this system as intensely as labs might study specific cancer cures. We have taken advantage of innovations in molecular biology and advances derived from cancer research.

Detection of functions that affect the non-host defense response:

Both protein and messenger RNA synthesis are required for the generation of the disease resistance response. Cordycepin blocks the synthesis of RNA and cycloheximide that blocks protein synthesis were both able to block resistance if applied at the time of inoculation (Hadwiger, et al. 1974). Short delays in the application of these inhibitors allowed the process of gene transcription and protein synthesis to go to the completion of developing resistance, indicating that gene transcription and protein synthesis are necessary to develop resistance.

Heat shock

Other mechanisms for blocking disease resistance affect other vital functions of the plant cell. A heat shock of 35 degrees centigrade for 1 h will convert the pea tissue from disease resistance to total susceptibility to the pathogen (Fsph) of bean, because the cell prioritizes the production of heat shock proteins and simultaneously abandons defense gene activation [11].

Effect of anti-metabolite compounds

Many chemicals that have anti-metabolite functions block the pea disease resistance response. For example, the drug, novobiocin, can bind to a DNA gyrase molecule that prevents the gyrase-dependent action and results in the blocking the nonhost resistance of pea to the bean pathogen, Fusarium solani f. sp. phaseoli (Fsph) **(Figure 3)**.

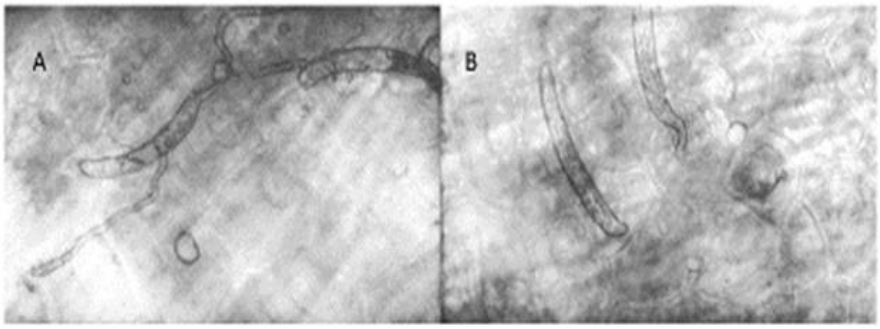

Figure 3: Effect of novobiocin on the pea defense response against the bean pathogen, *Fusarium solani f. sp. phaseoli* (Fsph). A = Fsph growth 20 pi following the application of novobiocin (0.5 mg/ml) that breaks the nonhost resistance. B = The nonhost resistance response of pea observed against Fsph in absence of treatment.

Surprisingly, actinomycin D a compound known to inhibit messenger RNA production, when applied to pea tissue in low concentrations actually enhanced the induction of pisatin production and the activation of the disease resistance response **(Figure 4)**. It turned out that the inhibition of RNA synthesis in peas required much higher concentrations than did other eukaryotic cells. At low concentrations the function of actinomycin was to intercalate into the pea DNA molecule. This loosened the structure of the chromatin within, resulting in activation of defense genes [2]. This observation was the first clue that DNA conformational changes could be responsible for increasing RNA synthesis. Also that these changes could also be the mechanism for how the defense genes are induced in nature. **Figure 5** indicates there

is a clear change in the fibril-like structure in the nuclear structure in pea cells below the surface inoculum compared with the structure of un-inoculated tissue. This information led us to a more complete analysis of the effects of compounds with both known and predictable molecular features with the potential to alter DNA structure.

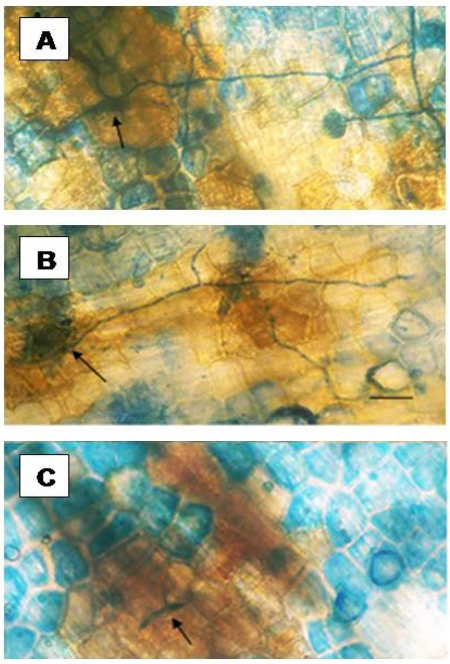

Figure 4: The pea endocarp disease resistance can be manipulated with actinomycin D. A = The pea pathogen, Fspi growth (48 h pi) in the water treated, susceptible pea tissue, B = Fspi growth on pea tissue treated with actinomycin (1 µg/ml) a conc. inadequate to induce resistance; C. Terminated growth of Fspi on pea tissue treated with actinomycin D (3 µg/ml) a concentration adequate to induce resistance. Higher actinomycin concentrations (25 µg/ml plus) progressively inhibit the plant defense response.

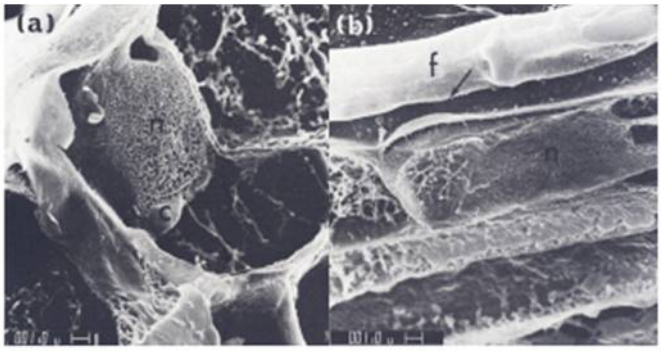

Figure 5: The structure of a pea endocarp cell as seen in a scanning electron microscope 1 h after treatment with (a) water or (b) spores from the bean pathogen, *Fusarium solani f. sp. phaseoli.* Note the nuclear texture change from the fibrous state in the water control toward a dispersed texture under the fungal spore (Hadwiger and Adams, 1978).

Cytological view of nuclear changes in pea cells

A revised freeze-fracture method (developed by Mike Adams, at Washington State University) that retains plant tissue at liquid nitrogen temperatures until there is complete removal of water prior to conventional scanning microscope preparation, enabled the photos of **Figure 5** through **Figure 6**. These photos provide artifact-free views of the fungal spore induced structural changes in plant nuclei within 1-6 h following inoculation.

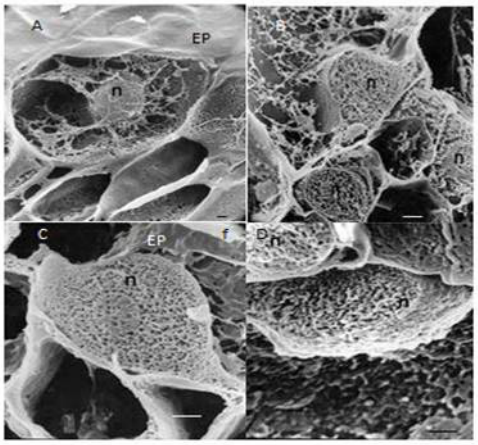

Figure 6: Scanning electron microscope views (Hadwiger and Adams, 1978) of nuclei in cells adjacent to the fungal spores 1 or 6 hour following inoculation or water treatment. A = untreated tissue; B = water; C = Fusarium solani f. sp. phaseoli (Fsph) 1 h post inoculation; D = Fsph treatment 6 h post inoculation. Note the extreme textural change after 6 h. (republished with permission from Physiological Plant Pathology 12:63. 1978).

Cross-sections of surface pea endocarp cells viewed in a transmission microscope further demonstrate nuclear changes. These views are of chromatin structural alterations **(Figure 7)**.

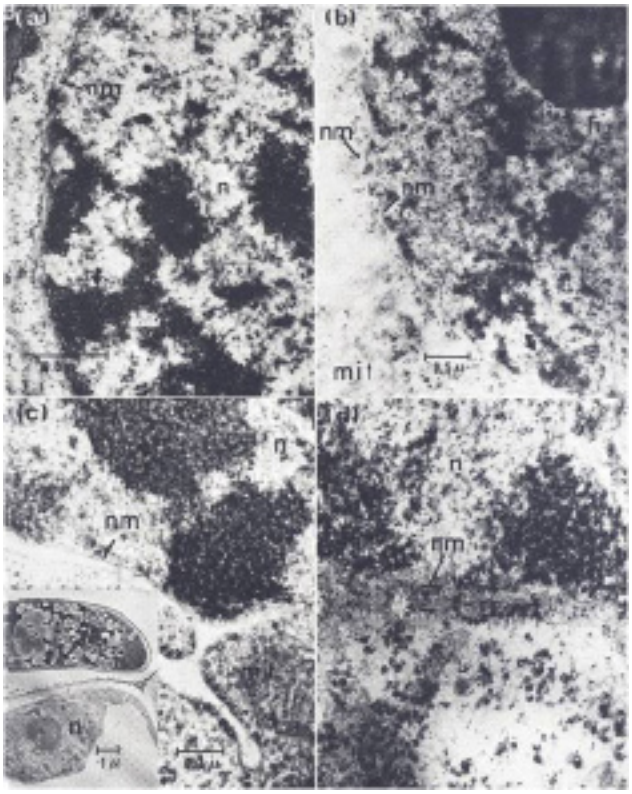

Figure 7: Transmission electron microscope view of intact pea endocarp cells nuclear textures 1 h after treatment with water (a), *F. solani f. sp. phaseoli* (Fsph) spores (b), *F. solani f. sp.pisi* (Fspi)(c) and Fspi 6 h (d)(Hadwiger and Adams, 1978). The sharp organization of the control nucleus is disrupted by Fsph and distorted by Fspi before becoming dispersed at 6 h. Abbreviations: nm = nuclear membrane; n = nucleus; mit = mitochondria.

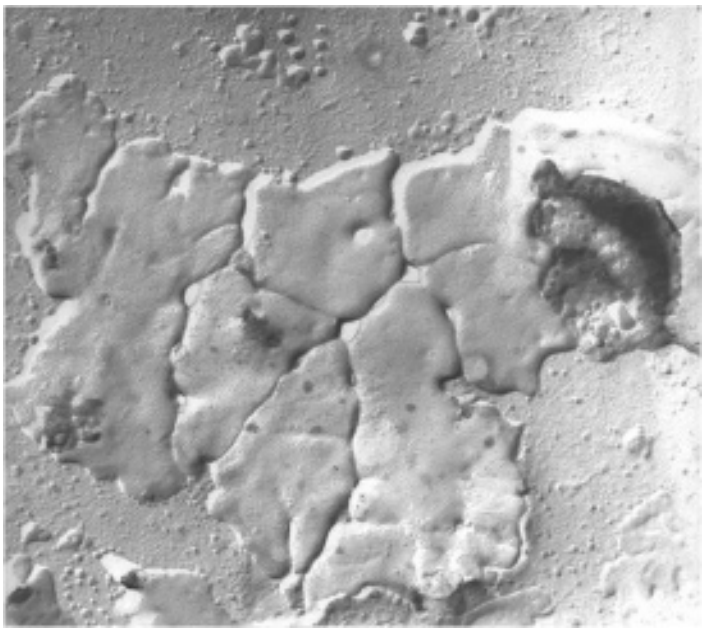

Figure 8: Fragments observed in electron microscope grids prepared as chromatin spreads of isolated pea nuclei.

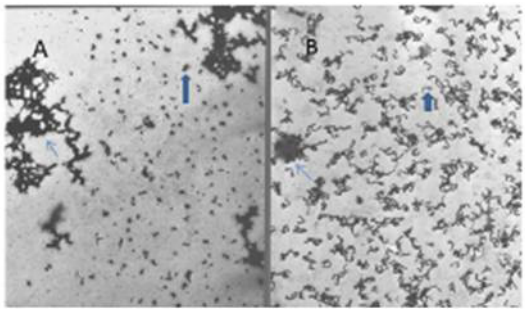

Figure 9: Pea chromatin spread relating the effect of a DNA intercalating molecule. Actinomycin D applied to pea endocarp tissue can enter the cell and affect transcription. Actinomycin can disperse areas of the chromatin within the plant nucleus within 1 hour. Dispersed chromatin is the site of intense RNA synthesis indicating the areas of gene activation influenced by loosening the compaction of the chromatin. Photo A. Flecks are caused by radio-labeled actinomycin and are associated with dispersed chromatin. Photo B. Larger flecks indicate the synthesis of RNA labeled with uridine (Republished from Front. Plant Sci. 6:373) [27] in regions of dispersed chromatin.

Among the dispersed chromatin spreads are occasional fragments that might be interpreted as portions of the pea nuclear membrane that have remained intact **(Figure 8)**. Within the photo of these fragments are regions that match other regions. There are seven pairs of chromosome in pea the nuclear organization required for the DNA to replicate and respond to transcription signals must be retained in an inter phase between cell divisions. These nuclear membrane pieces appear to retain such organization.

A functional view of the effect both of a conformational change caused by the intercalation of the labeled actinomycin D molecule and the development of RNA synthesis regions within the disrupted chromatin **(Figure 9)**. Likewise, the pea nucleus is intimately associated with the cytoplasmic structures **(Figure 10)** that can further influence nuclear structure.

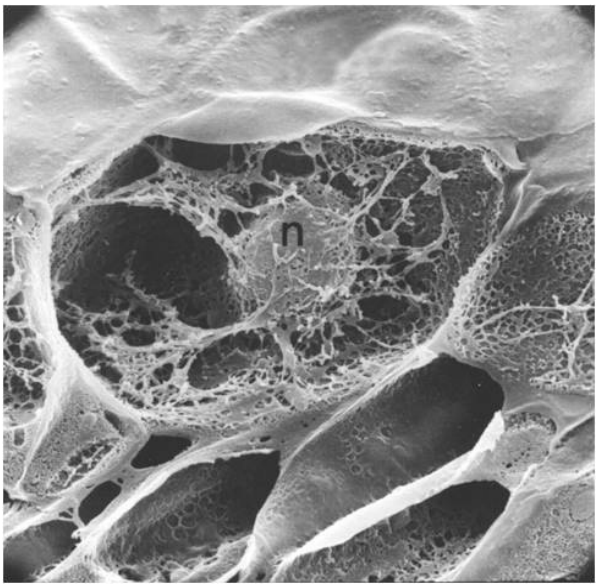

Figure 10: A scanning electron microscope view of the pea nucleus (enlarged from figure 6) and its connection with cytoplasmic components.

Before the water is removed from the cell, cytoplasmic streaming enables signal movement throughout the cell and thus components with nuclear localization signals reach the nucleus **(Figure 10)**. The nuclear preps and chromatin spread protocols are from Hadwiger and Adams, 1978. The overall summary of the cytological views (**Figures. 5 through 9**) of the pea host nuclear structural changes indicate that actions specific for DNA alteration can be confirmed using these multiple techniques.

Since the structural changes are extensive it follows that the effects responsible are do to targeting sites throughout the pea genome. This is born out in analyses of the DNA isolated from pea tissue in the early hours after inoculation [12] that indicate an associated DNA degradation occurs. However, there are specific effects on the regime of genes that are activated. At the biochemical level the production of pisatin is an indication of the activation of multiple genes in the secondary metabolic pathway that formulates this phytoalexin.

Pisatin production

Pisatin is one of many isoflavonoid phytoalexins (anti-fungal compounds) that accumulate in leguminous plants under stress. Following the basic work by the Cruickshank lab on isolation and characterization of pisatin [1], it was straight forward to determine that the compound was newly synthesized from a secondary pathway advancing beyond the shikimic acid pathway through phenylalanine [9].

Shikimic acid→→ phenylalanine →→ chalcones →→ Pisatin

Although pisatin has antifungal properties it is not the silver bullet of disease resistance. Similarly, many other phytoalexins were not sole determinants of disease resistance (Bailey & Mansfield 1982). The induction of pisatin synthesis necessitated that multiple genes in this

secondary pathway be activated. Also, the activation of this pathway is closely associated with the entire nonhost resistance response and thus provides an easy assay [13] for large scale monitoring of the defense response in peas.

Phenylalanine ammonia-lyase (PAL) and chalcone synthetase (CHS)

PAL and CHS enzymes and the genes coding them were useful in monitoring the secondary pathways that culminate in a large number of routes toward a variety of phenolics. Other enzymes, such as the product of gene *DRR 206* that catalyzes a branch pathway toward lignans [14], are also increased in the nonhost resistance response.

Defense gene translation

As indicated above the "nonhost" and "gene-for-gene" responses involve the enhanced synthesis of multiple protein products that may result from individual signaling agents. These products can be directly accessed by recovering the products of all expressed genes at the RNA level and then translate them into protein using *in vitro* and *in vitro* transcription systems. As shown in **Figure 11**, there are increases in product synthesis (followed intercellularly with administered labeled amino acids) representing many genes. These 2-D separations do not identify those gene products that are anti-microbial in function, however there are characteristic arrays reflecting the treatment that differs visually. That is, the array that is a response to heat shock differs from that of heavy metal stress, etc. Surprisingly, there are similarities in these patterns generated by nonhost [11] and gene-for-gene specific pathogen stress [15], chitosan applications [11] and some DNA-specific treatments [9] which suggest that there must be some common stress regions targeted within the chromatin of pea chromosomes. Further, fungal signaling components such as chitosan affect multiple regions within the total mass of chromatin. These regions apparently are also vulnerable to similar conformational changes caused by the DNA intercalator, actinomycin D, that possesses a specific DNA sequence preference [16,17].

The identification of specific DRR (disease resistance response) or PR (pathogenesis-related) genes, that apparently directly affect fungal growth, and increase in synthesis is important. Some of these defense genes from the pea DNA have been shown individually to have disease-resisting potential detected by incorporating them into other plant species [18,19]. It is currently possible to sequence the proteins highly expressed in the above 2-D electrophoresis separations **(Figure 11)** and predict back the probable DNA sequence. However, at the time of the initial research, the selections of the "turned on" genes were based on the hybridization intensity of all genes within a pea library with the labeled mRNA from control tissue compared with mRNA from tissue responding to the pathogen [20].

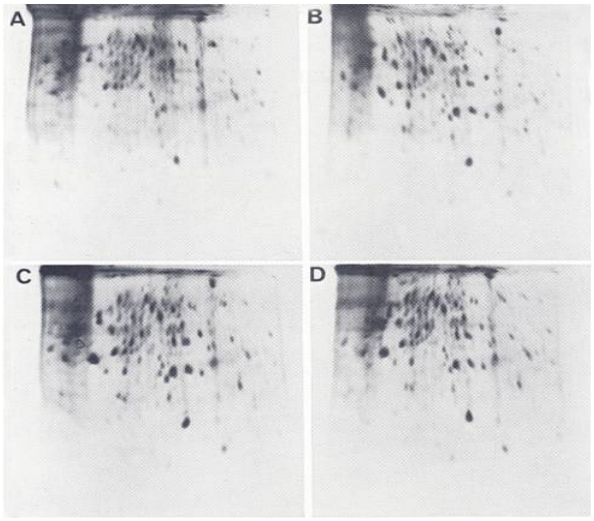

Figure 11: Methionine S^{35}-labeled pea proteins translated in vivo and separated on a 2-dimentional gel representing the mRNAs from pea endocarp tissues treated 8 h as follows: A = water; B = Fspi spores (3x 10^5); C = Fsph spores (3x $10^{6)}$ and D = chitosan (1 mg/ml)(from Wagoner, et al.,1982).

Cloning Activated Defense Genes

A library of pea genes obtained from the Murray lab [21] was used in RNA hybridization assays of genes that are active in plant disease resistance. The defense response was intense in a period 8 hours after challenging pea endocarp tissue with the bean pathogen, *Fusarium solani f. sp. phaseoli* (Fsph). Thus the mRNA at that time point was recovered and radioactively labeled. Similarly, the mRNA was recovered from healthy tissue. When the induced-RNA hybridized with the DNA within large spreads of individual clones of the pea "library" of clones (representing individually, all the genes of pea) the most intensely active genes were identified. These selected clones were further processed in "northern" analyses to verify further which pea genes were most likely to be defense genes [20,22]. My laboratory being the first to clone some of these genes published them as "Disease Resistance Response" (DRR) genes. The labelling of similar defense genes from viral infections by European labs termed these defense genes "PR" or Pathogenesis-related genes, by-passing our initial label. The example "northern analysis"of 3 of these genes in **Figure 12** [12] below uses 3 of the PR pea genes from pea to demonstrate the rapid induction (many fold intensities over water control treatments) within 3-6 hours of defense genes--not only by the pathogens Fsph (bean pathogen) and Fspi (pea pathogen), but by an array of DNA-affecting components such as Actinomycin D, Mitomycin C, Etoposide, Netropsin, Cisplatinum, ultraviolet light, Fluorouracyl, chitosan and fungal DNase.

Such northern analyses provided a screening technique based on nucleic acid sequence and was now available to characterize, genetically, the components of the defense response most likely to relate to disease resistance. Eventually the functions coded by these PR genes became known. Briefly, some known gene products: RNase, defensin, chitinase, β-glucanase, and other enzymes of secondary plant pathways. Of these, the gene coding for RNase, DRR49,

was transferred to potato and found to develop resistance to early blight of potato [18]. A Brazilian lab utilized our clones of two defensin genes DRR230a and DRR39 and translated them to their protein products in "expression vectors" and demonstrated that they were indeed very anti-fungal proteins [23]. Gene DRR230a has recently been transformed into cotton and soybean and contributes to resistance against the plant pathogens in the genera *Fusarium, Colletotrichum* and *Phakopsora* [24]. The pea defensin peptide sequences are highly conserved with those into human blood cells and those present in scorpion toxin.

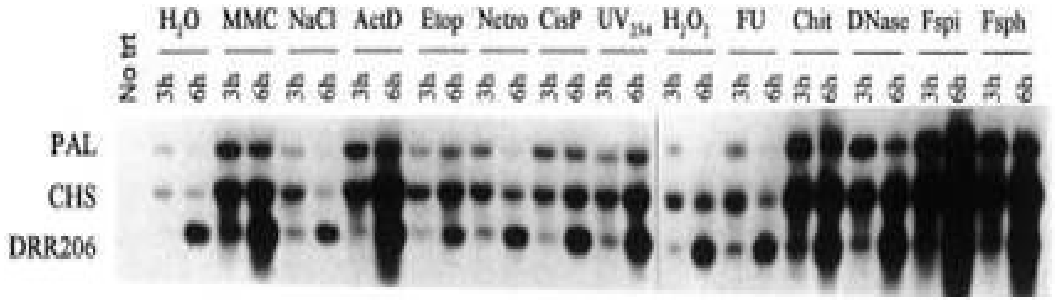

Figure 12: Northern analysis [12] of pea pod RNA following treatments with biotic and abiotic agents at 3 and 6 h. The blot was hybridized with pea DRR206, phenylalanine ammonia lyase (PAL), and chalcone synthase (CHS) probes. The agents: No trt, no treatment; mitomycin C (MMC)(50 µg/mL); NaCl (1.2 mg/mL); actinomycin (ActD)(12.5 µg/mL); etoposide (Etop) (12 µg mL); Netropsin(12 µg mL); Cisplatinum (CisP) (50 µg mL); UV (589 J/m); H_2O_2 (10 mM); Flourouracyl (FU) (200 µg/mL); chitosan (Chit) (1 mg/mL); DNase (20 units/mL);*Fusarium solani f. sp. pisi* (Fspi) and f. sp. *phaseoli* (Fsph) spores(10^7 spores/mL).

TUNEL assays

The DNA damage hypothesized to be associated with the infection of pea endocarp tissue by the *Fusarium solani* pathogens was directly visualized with a TUNNEL assay. The broken ends of the DNA molecules within a nucleus were attacked enzymatically with a derivative that could be detected by microscope **(Figure 13)**.

TUNEL assay

The TUNEL assay demonstrates that there is a rapid cleavage of the DNA within the nuclei of the pea endocarp surface cells (**Figure 13 Plates A & C**). The single DNA strand cleavage capability of fungal DNase [25] is likely responsible for the loosening of chromatin structure seen in the electron microscope transmission photos above. The Fsph fungus DNase is produced with a leader "Signal Peptide" that enables its release and entrance into the plant cell [26]. The fungal DNase that accumulates within 18 h post inoculation within the germinated mycelia of the bean fungus (Fsph) (**Figure 13, Plate C**) has the potential to fragment the nuclei responsible for continued growth. Plate F indicates that some of the nuclei in the pea pathogen (Fspi) remain intact enabling continued growth.

Photos **(Figure 14)** show the effects of the bean pathogen (Fsph) on nuclear states within pea endocarp cells in the vicinity of fungal spores 24 hours after inoculation. The DNA-specific dye, DAPI, indicates that the non-fluorescing nuclei have experienced extensive damage in

both the plant cells and in the resisted bean pathogen.

A more recent analysis of the early nuclear condition of both the pea pathogen (Fspi) and the bean pathogen (Fsph) on pea tissue indicates that it is the inability of these nuclei to remain intact that is the final lethal result of the disease resistance response [27]. Fortunately for the pea pathogen, a few of its advance mycelia do remain intact enabling it to continue to grow after the surge of the resistance response subsides.

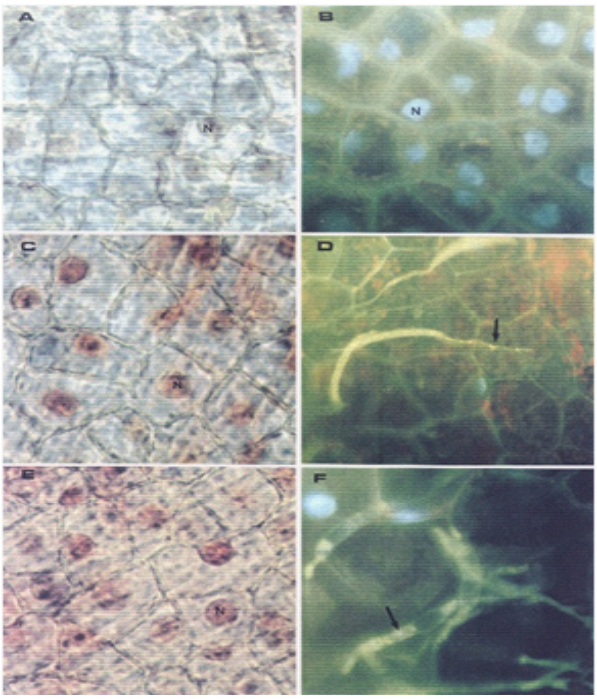

Figure 13: The condition of the DNA in pea nuclei 3 h following treatment with water Plate A; with spores of the bean pathogen (Fsph) Plate C; with spores of the pea pathogen (Fspi)Plate E; (Spores were removed prior to staining). The intensity of DNA strand cleavage is indicated by the red color developed with the accuracy of the TUNEL assay. Fsph spores plate D and Fspi spores Plate F eighteen hours after inoculation on pea endocarp. Plate B = untreated control. At this point these treatments were stained with the DNA-specific stain Hoechst 33258 and viewed in UV Light. Observe that these fungal treatments quickly cause DNA damage (strand breakage) in the plant nuclei (3 hours). After 18 hours post inoculation in the incompatible reaction plant nuclei near the spore stain poorly and the nuclei in the germinated hyphal tip (Fsph) are fragmenting (arrow). Some of the Fspi nuclei are also damaged however some remain intact (photos republished with permission and revised from Klosterman et al., Molec. Plant Pathology 2: 147, 2001).

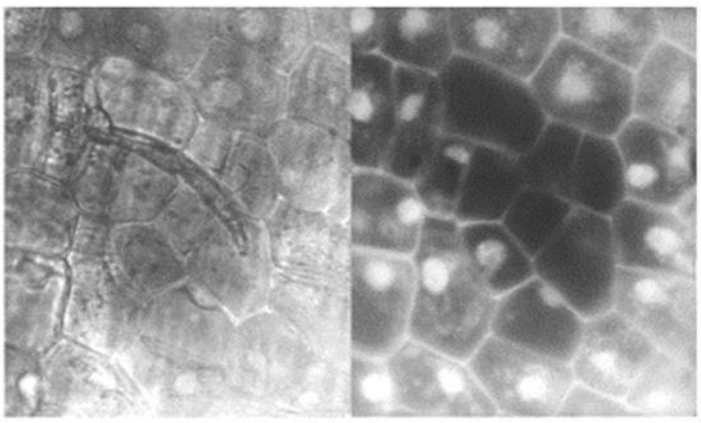

Figure 14: Photos of the incompatible interaction between a spore of the bean pathogen (Fsph) on pea endocarp surface. In the left photo the spore is seen with white light and the same region in right photo under UV light now showing the fluorescence of a DNA-specific DAPI stain. There is no stainable DNA in the fungus and the cells immediately around it 24 h following inoculation, suggesting the action of accumulated and released fungal DNase.

Hydrolytic enzymes and chitosan

Pea β-glucanase and chitinase enzymes can digest the major fungal wall compounds, β-glucan and chitin respectively. The genes coding these two enzymes are expressed in unchallenged pea tissue but become induced to higher levels 10 hours post inoculation [28]. The β-glucanase and chitinase action did not end with fungal wall digestion. David Kendra worked extensively on wall fragments and found that the pea fungus interaction generated chitosan oligomers that could induce the pisatin synthesis and also be directly antifungal [29]. Chitosan, is a derivative of chitin and is released in the interaction **(Figure 15)**. Chitin at physiological levels has little effect on resistance in peas but the removal of its acetyl groups converts chitin to chitosan. Chitosan is both a potent inducer of disease resistance (including multiple resistance components) and is a strong antifungal compound. Chitosan can induce total resistance in pea to pea pathogens (**Figure 16**). Our discovery of these properties of chitosan was as a result of testing other positively charged polymers [30] as inducers of pea defense.

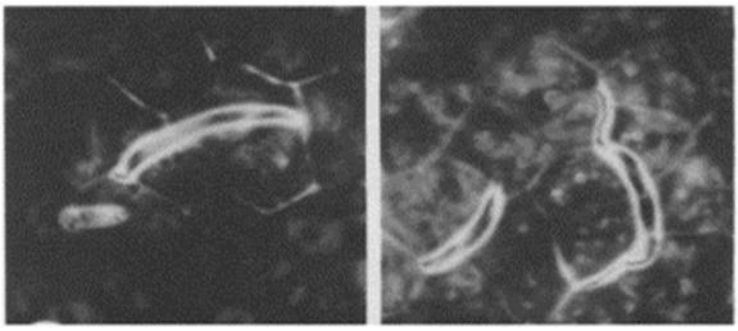

Figure 15: Immuno-fluorescent view of chitosan in the pea/bean pathogen (left) and pea/pea pathogen (right) interactions (5 h pi). The release of chitosan is detected with FITC-conjugated anti-chitosan antisera (all fluorescent material is chitosan).

Immuno-fluorescent techniques based on anti-chitosan anti-sera conjugated with the fluorescent compound FITC were used to detect the chitosan release and plant cell uptake shown in **Figure 15**. These results were augmented with radio-labelled chitosan and cellular fractionation to establish that a portion of the chitosan rapidly appears in the pea nuclei.

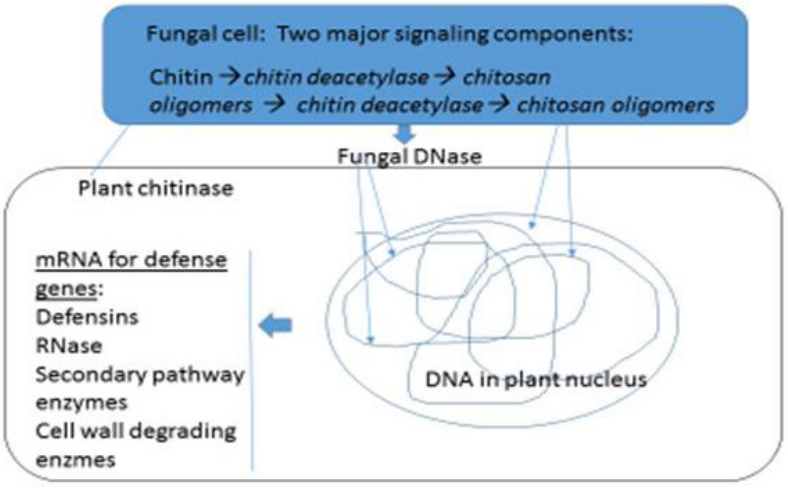

Figure 16: Description of the known actions of two major fungal components, chitosan and DNase, that signal the activation of pea defense genes. Fungal DNase is produced with a leader peptide sequence that enables it to transfer through a membrane. Chitosan is derived from chitin by the enzyme, chitin deacetylase.

Because chitosan is composed of polymerized glucosamine the polymer has alternating amino groups along its length, a positive charged property that affords it a strong affinity for the negative charged phosphates of the DNA molecule. A computer analysis indicated that the chitosan heptamer (seven glucosamines) can reside in the minor groove of the DNA [31]. The chitosan attachment to DNA *in vitro* alters both the melting point and the CD spectra of DNA. The enzymatic action of the fungal DNase is to cleave single strands of the double-stranded DNA. Both chitosan and DNase alter DNA conformation within the chromatin of the plant nucleus apparently removing obstructions of the stalled RNA polymerase complexes enabling their continued transcription of mRNA coded by defense genes.

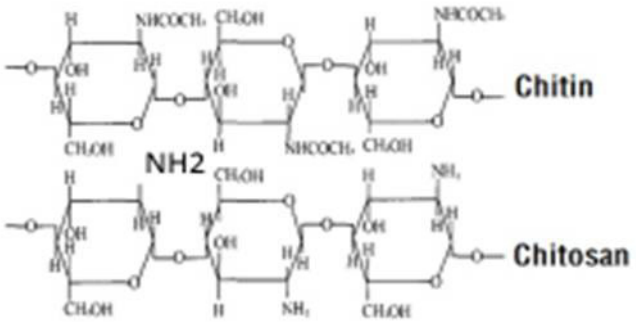

Figure 17: Structures of chitosan and chitin. The optimal size of a chitosan oligomer for induction of defense genes is one with 7 glucosamine sugars [29,55].

Where does chitosan come from?

Many fungi have chitin as a major component of their cell wall. It has been determined that the fungi also produce an enzyme, chitin deacetylase that can convert chitin to chitosan [29]. Chitin is biosynthesized starting from ADP N-acetyl glucosamine. There has not been reported a fungal ADP glucosamine precursor which may mean that chitin is synthesized first and later de-acetylated to form chitosan as depicted in the **Figure 16** cartoon [32].

Thus after plant contact, there is a rich potential source compound for chitosan heptamers that can develop in the interactions between fungal pathogens and the plant host tissue.

Why should fungi have acquired genes for cleaving the DNA molecule?

Indeed all fungi, whether pathogen or not, appear to have a gene for the enzyme DNase [26]. Fungi that exist in a world outside the plant have hydrolytic enzymes such as the DNase that under good growing conditions aids the fungus by digesting large molecules in older mycelia and then transporting these nutrients to the growing fungal tip. However, in general, the plant contact slows even virulent fungi enough to allow DNase to reach the growing tip where it has been shown that in a resistant response the fungus experiences enough DNA degradation that the nucleus in the tip deteriorates. This is a lethal condition for the fungus. This happens to a true pathogen to some extent but there are enough tips with intact nuclei to continue growth in this susceptible response [2] which develops slower than the resistance response.

Chitosan has been available as a by-product, derived commercially from the shells of crab and shrimp. Our research on basic peptides and proteins [20] that activated plant defense responses such as pisatin synthesis led us to discover for the first time that this polymer could activate plant defense responses and further could suppress the growth of a number of plant pathogenic fungi [33]. Our research became devoted to finding agriculture applications for chitosan. In the intervening decades it has become used world-wide in agriculture and horticultural applications. Chitosan was first developed by us as a wheat seed treatment that enhanced yield and increased the lignin content of the wheat cell wall [31]. As a result, it reduced the symptoms of a root rotting disease and increased the yield of wheat by approximately 10 percent.

Chitosan has been shown to provide protection to potatoes from Potato Late blight. In this case it was used as a "sticker" for approved use by organic growers to stick and spread copper containing compounds on leaf surfaces [34]. As a sticker it presented the copper compound on the potato leaf prior to the subsequently arriving "Potato Late Blight**"** organism, *Phytophthora infestans*.

There is a reason to believe that chitosan has an additional future in protecting plants from fungal diseases [35] beyond physically applying it to plants. First the general public has to recognize that engineering genes into plants can not only be safe but provide a means to control diseases without the use of fungicides and other synthetic remedies. We are currently working on the chitin deacetylase enzyme coded by the fungus that converts chitin to chitosan. If the chitin deacetylase gene were transferred to the plant it would produce chitosan naturally in the interaction. This proposed future use of chitosan depends on consumer acceptance or permission to transfer the gene into commercially grown plants. Its production can be engineered using a trigger called a promoter, that we know has the ability to activate any gene when the plant is challenged by a pathogen [19]. The essence of this mechanism is that there is an abundance of chitin in the walls of most fungal pathogens that the chitin deacetylase can attack, releasing chitosan with the result of both suppressing the fungal pathogen's growth and further activating plant defense genes.

Earlier we used the indicated promoter of the plant defense gene DRR206 to an advantage to develop disease resistance against *Pseudomonas syringae* a bacterial pathogen of tobacco [19]. A lab 100 yards from ours investigating lignin and lignin production in plants came across our DRR206 DNA sequence (accession number: U11716) [14] when entering their sequence of an enzyme they identified into **genbank**, a reservoir of DNA and protein sequence information. We then came to know what protein (enzyme) the pea DRR206 actually coded, namely an enzyme in the plant's pathway to lignan and lignin.

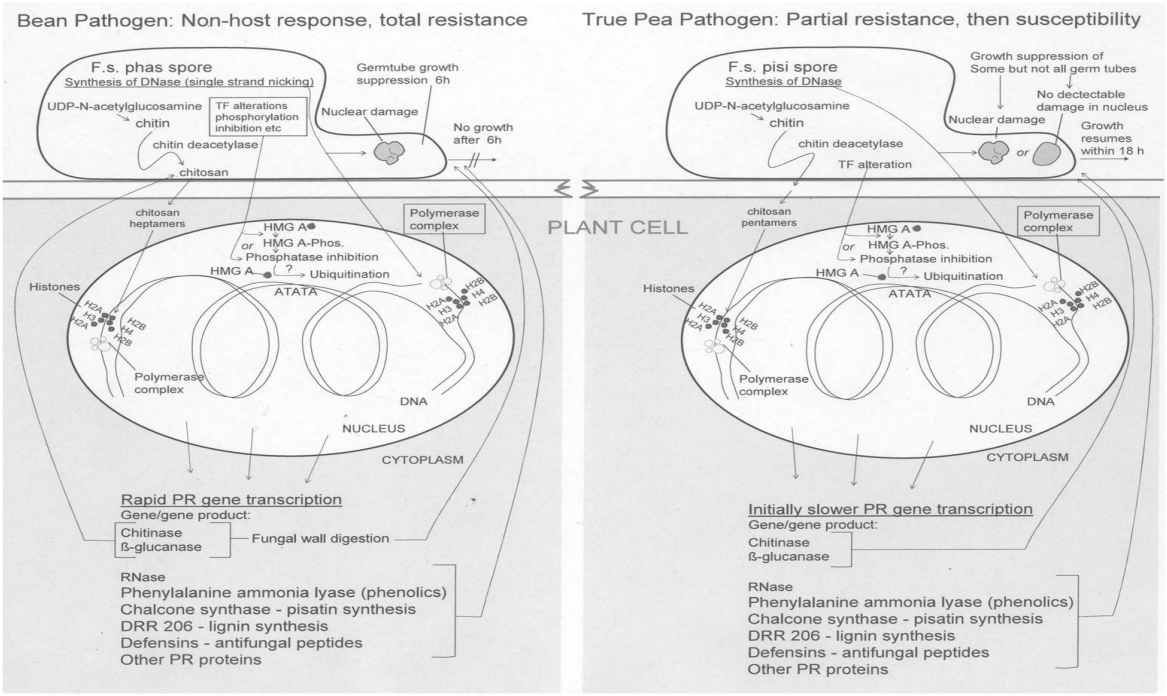

Figure 18: Summary of changes detectable following inoculations of the "cuticle-less" pea endocarp system. Events of the nonhost resistance response following theinoculation with the bean pathogen, *Fusarium solani* f. sp. *phaseoli* (Fsph), areas follows: The elicitors, DNase and chitosan, are produced by the fungi. Thechitosan heptamer from deacetylated chitin is directly antifungal and fungal DNase (Fsph DNase) is associated with nuclear fragmentation in the plant nucleus and in nuclei of the fungal germ tube. Elicitors enter plant cells and plant nuclei, affecting chromatin changes, DNA strand breakage,histone displacement and/or HMG A phosphorylation/dephosphorylation and degradation. These changes enhance the accumulation of pathogenesis-related (PR)-specific RNA. The rapid induction of PR genes/products suppress fungal growth. The presence at zero time & increased synthesis of chitinase andβ-glucanase genes at 10 h contribute to elicitor release. Events associated with the inoculation of the peaendocarp occur with both the pea& bean pathogens but with the pea pathogen (Fspi) appear at a slower rate.The bean pathogen's nuclei are damaged and growth is completely inhibited. A percentage of the pea pathogen's hyphal tip nuclei remain intact and at 12 h it resumes growth.

This model involves three proposed mechanisms forenhancing PR gene transcription. (i) Chitosan heptamers alter chromatin via competition with basic nuclear proteins for DNA attachment sites, displacing H2A/H2B histones. (ii) Fungal infection leads to reduced HMG A phosphorylation and HMG A levels, altering AT-rich regions. (iii) Fungal DNase (single-strand nicking) attacks plant DNA, changing helical stress [56].

Summation of how pea plants resist disease

This summation can't explain all of the plant's schemes to resist disease, but I can surmise many answers derived from the simple pea endocarp/*Fusarium solani* f.sp. interactions **(Figure 18)** The above paragraphs relate some of the components that are antifungal and describe the genes that code for them and make up the steps of the following scenario: The pathogenic fungus arrives to the plant as a spore. The spore requires simple conditions usually moisture and temperature for germination and the commencement of growth. As it contacts a plant root or leaf it confronts a waxy layer call cutin for which it has an enzyme to degrade. Secondly the next layer of the plant cell is a wall which Debra Delmer has shown allows rather large molecules to pass through. The signals for triggering disease resistance once through the wall confront a lipid-containing membrane that can also contain receptor proteins with the capability of transferring signals as a series of cascading events to activate plant genes. Alternately, our research has shown that some major signals locate, through and beyond the

membrane, to the plant nucleus. The nucleus contains the DNA-containing the defense genes that must be activated and my lab has centered on the manner that the chitosan discussed above can alter DNA and an enzyme called DNase that can clip one of the DNA molecules double strands. Both of these actions can result in the subsequent activation of the plant defense genes. The reason this gene activation occurs has also been researched by other biotech labs [36].

Chromatin structure and defense gene activation

At this point it is useful to relate the situation that allows gene activation to come about, starting with a description of how the nucleus is constructed. First all the DNA information for what the plant does is present in every plant cell. When stretched out it is a meter in length but it is assembled in each nucleus that is only 10 microns in diameter. It is assembled tightly with histone proteins into bundles called nucleosomes **(Figure 19)** [37].

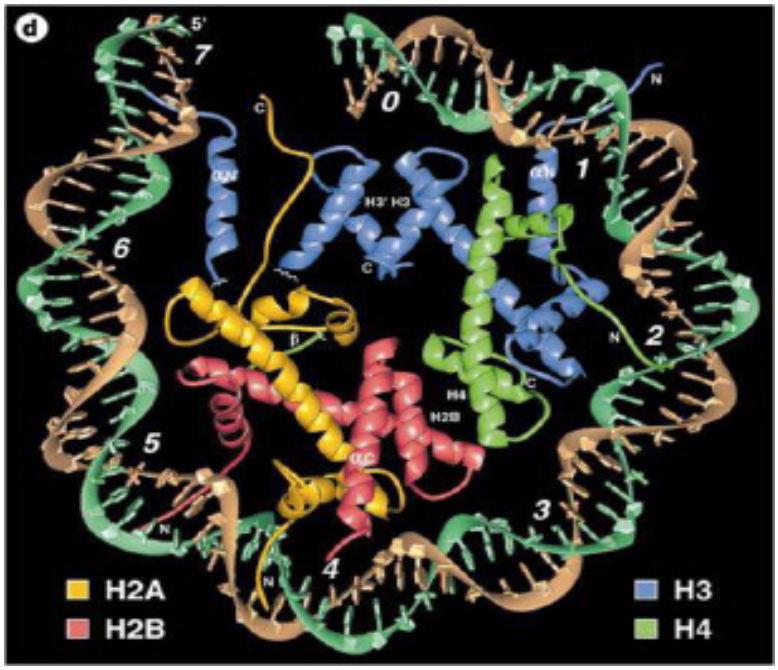

Figure 19: Crystal structure of a nucleosome. The DNA helix (around the outside) encompasses, and is held to its conformation by histones H2A, H2B,H3 and H4. Reproduced with permission from Karolin Luger lab of- Structural Biology:Chromatin Structure & Dynamics, University of Colorado, Boulder,CO [37].

Various other nuclear proteins have roles in gene regulation some called transcription factors. One called HMG A further connects the DNA, hooking up with DNA sequences rich in adenine and thymine bases (Klosterman. et al., 2003) Central to gene activation is a protein enzyme called RNA polymerase that functions in the nucleus. RNA polymerase acts with a complex that transcribes the RNA messages that code for the gene products. The information for these messages is stored in the DNA. This RNA polymerase to start the transcription must thread a single DNA strand through its center and shoot out a messenger RNA **(Figure 20)**. This messenger RNA subsequently leaves the nucleus to be translated into a protein product.

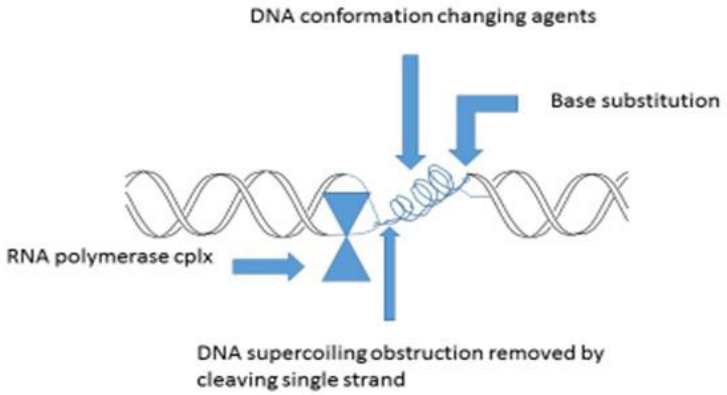

Figure 20: The multiple DNA specific actions that have the potential to release stalled RNA polymerase (RNAP) complexes. DNA within living cells exists as supercoils that can be either positive or negative helixes. Supercoiling must be eliminated enough for a single DNA strand to pass through the RNA polymerase complex. Fungal pathogens can release chitosan fragments that can reside in the minor groove of DNA. These positively charged interface with the negative charges of the DNA molecule and change the DNA conformation. The fungal DNase enzyme can cleave single strands of DNA and release the DNA helical obstructions to the transcription of genes. As the cell replaces damaged DNA, the reconstruction can incorporate errors in base sequence or incorporate alternate bases. All of these actions can enable "stalled" genes to become expressed.

Many of the DNA locations of genes have everything ready for transcription to RNA but are stalled. The stalling can result from the helical tangling of the DNA **(Figure 20)** or by the histones and HMG A that hold the nuclear material together [38]. The two "elicitors" that we indicated activated defense genes, can function to alleviate the stalling. The fungal DNase that enters the nucleus can clip a single DNA strand that relieves the helical tangling. The chitosan molecules composed of seven or more glucosamine possessing positive charges attach firmly to the DNA negative charges and are likely to out-compete the histones for DNA sites again unpacking and loosening the packing of the DNA in the nucleus [38]. The result again is to allow the stalled genes to become activated. Only two such DNA specific elicitors are discussed, but our research has shown that literally hundreds of DNA-specific components can activate defense responses in pea tissue. Many of these components have been investigated by physical chemists who report how each can alter the DNA or its hook up with the adjacent nuclear proteins [36, 39].

Thus, the fungus has components that can enter the plant cell and activate defense genes and the plants defense genes have the potential to inhibit the growth of the fungal pathogen. Some of these components mentioned above can interfere with the functions of the fungal pathogen's metabolism and condition. For example, plant defensins, rich in the amino acid cysteine, are destructive to cell membranes. Plant enzymes can alter crucial fungal compounds; one is the RNase that is produced by the plant gene DRR49. Both of these plant genes when expressed trans-genetically can singly develop disease resistance [18,19]. The plant can also release fungal wall degrading enzymes such as chitinase and β-glucanase that not only weaken the fungal wall but release chitosan that has fungal suppressing and defense gene activating actions [28]. This paragraph contains the essence of how plant resist disease. The following

material is included to explain in more detail the kind of mechanisms that are involved and hypothesize how they affect disease resistance. These hypotheses are from data generated, some on "gene-for-gene reactions", and mostly from the pea endocarp's non-host resistance response.

Plant growth and fungal hyphal development

The development of disease resistance appears not to have the pre-destined organization that the plant has for developing leaves, stems, flowers and roots, or that the fungus has for linear growth of its mycelia. To this point, a fungus growing independently of a plant contact grows mostly from its front hyphal tip and as the mycelial strand gets older it has enzymes to digest aging portions for translocation and use at the growing tip. When on plant tissue, tip growth is suppressed to the point that some of the hydrolytic enzymes migrate close to the growing tip. In the case of the DNase enzyme, it commences clipping its own DNA which can permanently terminate growth of this pathogenic fungus [13].

Scenario of the fungal pathogen.

Out in nature fungal pathogens first contact the plant cuticle containing cutin. As indicated above it releases a cutinase to break cutin down. The gene for this enzyme can be activated when contacting fragments of cutin in *in vitro* culture [3]. The pea endocarp tissue is without a cuticle layer so the first contact is with the pea cell wall, here it confronts hydrolytic enzymes such as β-glucanase, cellulose, polygalacturonase and chitinase. Signals are released via fragments from β-glucan, poly galacturonic acid, chitin and chitosan. Receptors exist for chitin fragments, but have not been reported for pea cells [2].

Plant cell fragments.

Pectate lyase and pectic fragments from pea cell walls elicit low increases in the pea defense genes DRR49, DRR176, DRR206 and pisatin, that might contribute to plant defense, but the mechanism of action is not known [40].

Chitosan and reactive oxygen signaling.

Chitosan polymers in the range of 7 sugar units are very active in signaling defense gene activation and suppressing fungal growth [29]. The gene activation mechanism is due to alterations of nuclear DNA hypothetically by inserting into the minor groove of the DNA molecule. Another series of enzymes generate "reactive oxygen species" called ROS. Individuals of the ROS such as hydrogen peroxide applied externally to the pea endocarp tissue can activate the defense response at a low level, consistently, as well as cause damage to pea DNA [41].

Reactive Oxygen species:

Signaling via reactive oxygen species (ROS) is a part of induced responses in many biological systems and can make substantial

changes in DNA. Likewise, various ROS elicitor concentrations generated within pea endocarp tissue are temporally associated with the activation of pathogenesis-related (PR) genes [41]. Within 10 min, ROS accumulate in the nonhost resistance response to a bean pathogen, *Fusarium solani f*.sp. *phaseoli* (Fsph), to levels 4-fold those elicited by the pea pathogen *Fusarium solani*f.sp. *pisi* (Fspi). Application of hydrogen peroxide H_2O_2 (HP) to pea tissue increases DNA fragmentation and activates

The PR genes (DRR206, defensin, PR10, and PR1b) within 50 min and changes the diameters of the nuclei of pea host cells in 3 h. Moderate increases in levels of the pisatin and the growth suppression of the peapathogen, Fspi, were observed within 24 h after H_2O_2 treatment to the endocarp. Our results indicate that

H_2O_2 contributes to the induction of the defense response in pea endocarp tissue and is mediated by activation of DNA damage responses.

Abiotic DNA-specific elicitors:

Other externally applied DNA-specific components that are not natural signals of the pea-Fusarium interactions can never the less activate many of the same pathogen induced responses. These compounds are available for other purposes from organic chemicals to pharmaceutical purposes but are useful because some of their interactions with DNA are known or can be predicted. The actions include: Intercalation between and spreading base pairs of the DNA, associated binding with the DNA backbone, residing in the major or minor groove of the DNA molecule, cross-linking DNA strands and substituting for base pairs. Some actions have now been verified in cancer therapy research. Surprisingly, most of them at some concentration can activate the plant defense response, indicating that there are some sensitive regions (sensitive to respond providing a level of resistance) of nuclear chromosomes. This diversity of chromatin structure has been observed in the electron microscope. As indicated previously, the chromatin disruption by DNA-specific compounds appear with hot spots of RNA synthesis [2].

Potential sites of sensitivity within the genome/chromosomes:

Observations of QTLs (quantitative traits) in peas have identified regions of chromosomes that add to disease resistance and are often also regions where PR genes for resistance reside [42]. In addition to locating regions of chromosomes with resistance traits, it has also been possible to associate chromatin changes within the region where the PR gene is being activated.

It was first done by showing that the DNA-linking elicitor is located in the area where the DNA sequence of the PR gene is located [43]. A more recent technique,"chromatin-immuno precipitation" (CHIP) enables the monitoring of nuclear proteins at the site of a PR gene [38]. This information enabled the documentation of the depletion of histones H2A and H2B as well as the transcription factor HMG A at the DNA site of the PR genes *DRR230* and *β-glucanase* 5 h following inoculation of the pathogen on pea endocarp tissue. This depletion can mechanically remove obstructions in front of the RNA polymerase complex allowing it to complete the reading off of the gene code.

Characterization of some representative PR genes:

Suppression of fungal growth (*Fusarium solani f. sp. phaseoli* on pea tissue) is detectable and the induction of pea PR genes commences (Hadwiger et al., 1995) within 6 h. Some of the early proteins translated have known functions that may relate to their potential to slow fungal growth and are described as follows:

DRR206 codes for an enzyme associated with a secondary pathway toward (lignan) production [14]. This pea gene when transferred to canola has conferred resistance against a fungal pathogen of canola;

DRR230 and DRR39 code for pea defensins (Chiang and Hadwiger, 1991), with defined antimicrobial activity [23]; and as indicated provides protection in cotton and soybean against Fusarium, Colletotrichum and Phakopsora [24]

DRR49 (PR-10) codes for a product that enters the nucleus (Allaire and Hadwiger, 1994) and is a putative RNase. DRR49 trans-genetically confers resistance in potato to early blight [18].

Genes for secondary enzymes: The genes for some secondary pathway enzymes are induced such as phenylalanine ammonia-lyase (PAL), [9] and chalcone synthetase (CHS), functioning in the production of chalcones. PAL and CHS are also intermediates in the production of lignan [14], lignin, flavonoids and isoflavonoids, e.g. the phytoalexin, pisatin [1].

The pea gene PR-1 is homologous with the PR1b gene in Arabidopsis. In Arabidopsis this gene has a PR-1 function and is a "non-expressor" of NPR1 which reportedly is a master, positive regulator of plant immunity (Yu et al., 2001).

The relationship of heat shock in pea plants to disease resistance:

Our knowledge of the heat shock response has provided a way to implicate the entire group of PR gene products as crucial to the disease resistance function [11]. Heat shock is

known to have an impact on gene expression at different levels, such as inhibition of protein synthesis that blocks translation initiation.At ~40 degrees C the synthesis of normal proteins is greatly decreased [44]. Normal mRNAs are taken into stress granules for storage. Additionally, the heat shock response precludes the induced synthesis of PR genes in peas. Simultaneously the tissue is producing heat shock-related products. Soon the pea endocarp tissue becomes totally susceptible to a bean pathogen. As the decline of heat shock response occurs (~9 h) the tissue regains the ability to synthesis PR genes. Also, all the normal genes are translated in the recovery period [45,46].

Cell death as a resistance factor?

In a study of effects of fungal pathogens, DNA-damaging agents and biotic elicitors of defense genes the time at which cell death occurred was monitored with trypan blue and fluorescein diacetate [12]. Cell death observed in the nonhost resistance reaction against the bean pathogen (Fsph) remained below 5% until 18 h. However with UV light, and actinomycin D cell death exceeded 30% 24 h following treatment. Although cell death can increase within 18-24 h, the cytological evidence of total nonhost resistance occurs before 6 h. Thus cell death in pea endocarp tissue is not an accurate indicator of the resistance response. Additionally, the yellow-green colorationis associated with the resistance response, though this coloration is indicative of the response, it is mostly visible after 10 h and thus after the initial resistance has occurred.

Salicylic acid (SA) affinity to DNA and plant defense responses.

SA-induced reactive oxygen species released in the pea/*Fusariumsolanum* f.sp. *phaseoli* interaction resulted in fragment alterations in pea nuclear DNA and cytologically-detectable nuclear diameter and structural changes in the pea host nuclei. SA-related action increased resistance to the true pea pathogen *F. solani* f.sp. *pisi* and the accumulation of the phytoalexin, pisatin. SA-induced PR gene activation may also be attributed to the host pea genomic DNA damage. Previously published reports [47,48] indicate that SA has an affinity for the DNA molecule it can be a component of the plant immune response [48]. At certain concentrations, SA can be temporally associated with increases in the defense response of pea [13].

Basic cellular components as potential gene activators:

Of the basic components of the plant-pathogen interactionreleased from hydrolytic digestions, short polymers of both protein and carbohydrate that possess a positive charge can influence the nuclear DNA. Further there are polyamines (spermine, spermidine, cadaverine) that are natural components of living cells. Spermine is capable of inserting into the minor groove of DNA, however its action in inducing the defense response is dependent on concentrations that are above those normally occurring in the plant cell [49].

Phosphatase inhibitors

Phosphatase inhibitors found in other biological entities can cause major effects related to increases in PR gene activity, pisatin production and the overall control of both resistance and susceptibility. Calyculin A, a phosphatase inhibitor from sponge when applied in very low concentrations to pea endocarp can stimulate levels of pisatin comparable to those induced by spores of the incompatible pathogen [50]. At levels of 0.3-10 µg/ml it breaks the resistance of pea endocarp tissue to the bean pathogen, Fsph and at the level of 0.03-0.07 µg/ml can activate a strong level of resistance to the pea pathogen, Fspi. The phosphorylation or de-phosphorylation of nuclear proteins may occur prior to the ubiquitinations associated with their removal from DNA and the activation of previously suppressed genes [38].

Toll-like receptor/signaling cascade:

Host/parasite interactions can involve a fungal-elicitor-recognition by a general class of plant receptors [51]. This recognition prompted the development of a more specific terminology especially for interactions between Avr genes of the pathogen and R genes within the plant host [52]. The eliciting component was termed a microbe or pathogen-associated molecular patterns (MAMPs or PAMPs, respectively) and the corresponding plant component, a ‘‘pattern recognition receptor’’ (PRR). This terminology fits some interactions in plant defense. In animal systems, PAMPs in generating immune responses can initiate a proteolytic cascade that generates a protein ligand for Toll called ‘‘Spatzle’’ [53]. Toll and Toll-like receptors (TLRs) interact with serine–threonine kinases, some of which share homology with kinase domains of plant receptors such as FLS2, the receptor for flg22. Subsequently a series of protein kinases mediate the activation of transcription factors, such as the nuclear factor-kB (NF-kB), a family of inducible trans-activators. This mediation occurs through the inactivation of a protein inhibitor (IkB of NR-kB), and results in the expression of immune response genes.

Two components termed PAMPs, the fl22 peptide and the chitin oligomers have been shown to be recognized by plant protein receptors in some plant species. These recognitions have been associated with signaling systems resembling the Toll cascades of animal cells. These PAMPs were administered to the pea endocarp [2]. The chitin preparations were able to generate low level increases in pisatin production and excessive chitin concentrations could affect the resistance response of pea endocarp tissue to the pea pathogen. Flg22 at 62 ug/ml elicited a low level of pisatin and a moderate hypersensitive response. There has been no reported receptor for flg 22 in pea [54]).

Conclusions

Some host/parasite interactions have benefited evolutionarily from a niche that enables a true pathogen to exist and thrive at least temporarily on a given plant. The plant and pathogen

each have regulatory regimes that can be disrupted following contact. This case study indicates the multiplicity of signals that can be either productive or disruptive. The nonhost resistance signaling appears to have the greater potential for disruption and possibly more signals for incompatibility than the susceptibility response. The early discovered realization "that disease resistance is a more rapid response than susceptibility", is in my view the major difference ultimately as many of the eliciting signals have common features. Because of the potent action of chitosan and fungal DNase in activation of PR genes and pisatin synthesis, I consider them major signals. Also because they are present in quantity in most fungi and capable of entering the plant nucleus, the site of gene transcription. The signaling of gene-for-gene controlled responses appear to develop thresholds of resistance activity above this base of interaction. In pea the same defense response genes are usually activated similarly in both gene-for-gene and nonhost resistance. More over common gene products such as defensins have the potential to be directly antifungal and slow fungal growth on the plant. This case study also relates the adverse effects that slow growth has on the accumulation of hydrolytic enzymes within mycelia that can be terminally toxic to the fungus. Finally, the plant's chromatin structural changes are intimately evolved in the activation of defense genes. DNA damage within itself can alleviate the suppression of genes such as those for DNA damage repair and likely those coding for defense products. The removal of DNA helical structure or nuclear protein obstructions to the RNA polymerase complex is a major feature of the transcription of defense genes.

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