Analyses of Bark Proteins in Blister Rust-Resistant and Susceptible Western White Pine (*Pinus monticola*).

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ABSTRACT

This study was undertaken to determine whether significant differences exist in the bark protein profiles of phenotypically blister rust-resistant and susceptible *Pinus monticola*. White pine blister rust is a devastating disease of 5-needle pines caused by the recently introduced (early 20th century) fungal pathogen, *Cronartium ribicola*. Several resistance mechanisms have been identified in both foliage and stem tissue and although most of these resistance mechanisms have been shown to be under oligogenic or polygenic control, little else is known of their physiological and/or biochemical nature. Because no biochemical 'resistance' markers are available to use in early progeny screenings, the selection and testing processes involved in the breeding of rust resistant pine are costly in terms of time and labour. Predicting which trees will survive rust infection 'in the field' remains one of the challenges of white pine breeders. Identification of significant quantitative or qualitative protein differences between blister rust resistant and susceptible groups is an important first step in the isolation and functional characterization of potential resistance markers. The bark reaction known as 'slow canker growth' (SCG) was selected for study because of its delayed onset and because seedlings displaying this reaction tend to survive well in the field. In addition 'mature tree resistance' in well characterized clones was also studied. Individually extracted bark proteins from a total of 16 trees were separated by SDS-PAGE and two-dimensional (2-D) gel electrophoresis. After silver staining, gels were scanned and analyzed with the aid of a laser scanner interfaced with one and two-dimensional software. SDS-PAGE separated and electroblotted proteins were further characterized by immunodetection with barley and petunia anti-chitinase polyclonal antibodies and antibodies raised against a white pine photosystem II protein (PSII) and cold hardiness-related protein (Pin m III). Two-dimensional gel electrophoresis demonstrated *multiple* qualitative and quantitative

protein differences between resistant and susceptible groups. Generally, susceptible groups had more *unique* and quantitatively increased proteins than resistant groups. The most dramatic and consistent differences were seen between resistant ('SCG') versus susceptible seedlings, while trees displaying 'mature tree resistance' had fewer significant changes. Immunodetection with anti-chitinase polyclonal antibodies revealed several bands between 28 kDa and 35 kDa. Qualitative band differences were noted between resistant and susceptible half-siblings (seedlings) but were not consistent for all families tested. Chitinase activity was also detected in mature, lyophilized bark extracts using a 2-step colourimetric assay which employed N-acetyl-glucosaminidase as the second enzyme. Immunodetection with anti-PinmIII demonstrated that *susceptible* mature trees were selectively *enriched* in a **19.2 kDa** protein. Three significant bands were isolated and characterized by N-terminal amino acid sequencing; a **10.6 kDa** band shown to be selectively enriched in resistant (SCG) seedlings from 4 families tested, the **19.2 kDa** band selectively enriched in mature susceptible trees, and a **26.2 kDa** band found to be enriched near the canker tissue of a susceptible seedling. The latter band may correspond to a unique 26.0 kDa immunoreactive (anti-chitinase) band seen in the same individual tree. The significance of these findings is discussed.

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Dedication

To my husband Greg, and my children Matthew and Geoffrey.

Soli Deo gloria.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

General Introduction

The White Pine Blister Rust Problem

Western white pine (*Pinus monticola* D. Don) is a commercially important, fast growing pioneer species that is widely distributed throughout the Pacific Northwest, Idaho, Montana, and as far south as California. Economically, the species yields more merchantable timber and has a higher dollar value per hectare than many other forest trees, and has long been valued as an ornamental tree [Detwiler, 1928]. Because of its sparse representation (5-30%) in mixed conifer stands throughout southern B.C. [Krajina, 1970], the historically high susceptibility of *P. monticola* to devastating blister rust disease [Lachmund, 1934] tempts one to regard the conifer as little more than a weed. However, its concomitant resistance to a variety of root rot diseases has sparked renewed interest for its utilization in reforestation projects. Furthermore, selection for blister rust-resistant western white pine has met with a measure of success in Idaho while a lower incidence of cankers per tree in B.C. has held out the promise of even more success for similar selection programs here [Hunt, 1990]. A program designed to incorporate resistant *P. monticola* into seed orchards has been operating in B.C. for approximately 10 years [Meagher and Hunt, 1985]. Currently, white pine improvement programs require a sizable time committment, due to complicating factors such as genetic variation in the pathogen, limited seasonal availability of basiodiospores for artificial inoculation, and long-term evaluation of host resistance [Diner and Mott, 1985].

Although specific mechanisms of host resistance in the *P. monticola: C. ribicola* pathosystem have been previously identified and partially characterized for needle, short

shoot, and bark [Hoff and McDonald, 1980, 1971; Hoff, 1986; McDonald 1979; Struckmeyer and Riker, 1951], the *physiology and molecular biology* of such resistance mechanisms remains poorly understood. Consequently there is no quick biochemical test available that will enable researchers to identify blister rust-resistant individuals at an early development stage. The current time and labour-intensive selection and progeny testing process remains the workhorse of the forest pathologist and tree breeder.

A wealth of information has accumulated over the last 10-15 years in relation to pathogenesis related (PR) proteins and their role in plant defense. Many of these proteins (including a variety of chitinases) have been characterized as will be discussed later, and the potential of using such proteins as molecular markers in early progeny tests is an attractive prospect. Unfortunately most, if not all, of the studies have been carried out using annual or other short rotation crops. Nonetheless, recent studies of poplar (*Populus*) have shown expression of wound-inducible chitinase genes [Davis *et al.*, 1991], which suggests that the likelihood of finding similar genes in other woody plants is high. Another study of Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) has shown that distinct 'stress proteins', stilbene phytoalexins, and enzymes (i.e. chalcone synthase, stilbene synthase, and cinnamyl alcohol dehydrogenase) are specifically induced in response to ozone exposure- a finding which parallels the well characterized defense responses found in agricultural plants [Sandermann *et al.*, 1989].

To date, no general comparative studies of bark *protein* have been made in disease resistant versus susceptible western white pine. Perhaps this is partly due to the difficulties inherent in working with conifer tissue, i.e. phenolics, resins, and other interfering substances which make it difficult to extract any but the most hardy of enzymes. 'Total protein' extraction methods employ harsh, denaturing conditions rendering extracts useless for functional assays, and protein yields tend to be in the order of 1-2% or less of lyophilized, ground tissue. Despite the above challenges, evidence

from the literature supports the effort to search for and characterize significant proteins in conifers, specifically blister rust-resistant and susceptible white pine, if only to further our understanding of their possible functions in a generalized defense response.

Objectives of Study

The major objectives of this study were:

(i) To compare the bark protein profiles of phenotypically blister rust-resistant (mature tree resistance and 'slow canker growth' bark reaction) and diseased (susceptible) *P. monticola* using one and two-dimensional protein electrophoresis. The working hypothesis, based on studies done in agricultural host:pathogen systems, is that there are unique and/or quantitatively significant proteins that can distinguish blister rust-resistant from susceptible groups.

(ii) To qualitatively screen SDS-PAGE separated and Western blot immobilized bark proteins for immunoreactivity to chitinase polyclonal antibodies and to functionally assay bark proteins for chitinase activity. To also characterize resistant and susceptible trees by their immunoreactivity to selected available polyclonal antibodies to a few proteins. The questions posed are two-fold, 1) is chitinase present in white pine bark? and 2) can other existing antibody probes be used as potential 'resistance markers'?

(iii) To isolate and sequence significant proteins identified by one and two-dimensional electrophoresis and/or immunodetection. Sequence analysis would facilitate development of antibody and nucleotide probes which could be used to screen larger populations and further elucidate the molecular biology of the host response.

Literature Review

Introduction

White pine blister rust (WPBR), caused by the fungal pathogen *Cronartium ribicola* J.C. Fisch. ex Rabenh., is the only stem rust of 5-needle pines in North America. It is native to Asia, from whence it spread throughout Europe. The rust came to the Pacific Northwest in 1910 on a French container shipment of *P. strobus*, and has since spread throughout coastal B.C., the interior, and into the southwestern United States, infecting not only *P. monticola*, but other commercially important white pines.

Natural resistance to WPBR varies among pine species [Hoff *et al.*, 1980]. For example, the Armand pine (*Pinus armandii* Franch.), native to parts of central and southeast Asia, has been listed as either immune or highly resistant by a number of researchers [Spaulding, 1925; Childs and Bedwell, 1948; Bakshi, 1972; Bingham, 1972]. Eurasian species of pine generally display high levels of resistance, with much of it stemming from *one trait*- failure to develop needle spots [Hoff *et al.*, 1980]. Of North American species, *P. aristata* (for some unknown reason) appears to display remarkably high resistance to blister rust infection, ranking '2' on a scale of 1 to 6 ('1'= highest resistance) for trees displaying primary needle spots post-inoculation [Hoff *et al.*, 1980]. The same study gave *P. monticola* a rank of '3' for the same category (primary needle spots) and an overall rank of 5.7 (1-11) for all resistance mechanisms (needle and bark reactions) observed [Hoff *et al.*, 1980]. Studies have also shown coastal selections of resistant *P. monticola* to be more resistant than their interior counterparts [Hunt and Meagher, 1989]. Although Eastern white pine (*P. strobus*) is generally more resistant to blister rust than its western cousin, the former lacks the desirable growth, branching, and

bole characteristics of the latter [Hunt and Meagher, 1989], thus its commercial substitution is not something to be considered.

In order to gain an appreciation for the *complexity* of *rust-resistance* in white pine, it is necessary to look at some of the basic host-pathogen interactions involved during early and established infection. Because there are multiple points along the infection path that host defense responses may potentially be triggered, there are as many ways of modulating resistance. As will be seen, the importance of species heterogeneity, environmental-host genome interactions, pathogen race-host interactions, age-related effects (host) and other confounding variables cannot be underestimated in the search for biochemical and molecular resistance 'tags'.

The purpose of this literature review is to discuss host-pathogen relationships of rusts in general, with special emphasis given to the white pine blister rust pathosystem. After a brief description of the life cycle of *C. ribicola* and its concommitant host pathology, a discussion of physiological and biochemical host-pathogen interactions at the pre-infection and initial infection stages will follow. The role of cell wall degrading enzymes in the *establishment* of pathogenesis is followed by a discussion of the *effects* of rust infection on selected metabolic functions of photosynthesis, respiration, and hormone (i.e. auxin) balance. With this as a background, a discussion of host *resistance* mechanisms (*mechanical* and chemical, *pre-formed* and induced), including the genetics of host-pathogen relationships is given. Attention is given to such examples as the 'hypersensitivity response', and the concepts of monogenic, polygenic, horizontal, and vertical resistance mechanisms are explored. Included in this is a description of the resistance phenotypes of *P. monticola*, along with their proposed mode of inheritance. The importance of phenolic compounds (and thus the phenylpropanoid pathways), pathogenesis-related proteins, and cell wall proteins is also discussed and the review

concludes with a brief discussion of the rationale for looking at *bark protein* profiles in resistant versus susceptible white pine.

Rust Life Cycle

Like many rust fungi, *C. ribicola* has a complex lifecycle with five separate spore stages that colonize two unrelated hosts [Day, 1972]: pines in the subgenus Strobus (white pines), and gooseberries and currants in the genus *Ribes*.

The initial infection court of the fungus is the needle tissue. Fragile basidiospores require high relative humidities (95-99%) in order to germinate [Patton, 1972]. This climatic requirement is usually met within the first 2 weeks of September on the West Coast, when cool, low pressure systems predominate. Although germinal hyphae normally advance to penetrate the needle by way of the stomata [Patton and Johnson, 1970], direct penetration through the epidermal cell had been reported in axenic culture [Diner and Mott, 1982a;1982b].

Initial (needle) symptoms of WPBR infection are apparent within 4-10 weeks post-inoculation, while stem and bark symptoms take months to years to develop. On average, it takes 12-18 months for mycelia to extend from needle to short shoot, and then into the cambial tissue of branch or stem.

Invasion of host tissues from an established infection centre and sequential canker development is a three-stage process [Welch and Martin, 1974a;1974b]. During formation of the canker margin, invading hyphae grow passively through the extracellular spaces of the host for a couple of centimetres beyond the discolored bark regions [Ehrlich and Opie, 1940; Krebill, 1968], whilst obtaining nutrients either from host cell exudates, or directly from the host cell by altering membrane permeability. Formation of the midcanker area is accomplished when the rust ramifies from the initial invading hyphae and spreads throughout the bark [Welch and Martin, 1974a;1974b].

Stem or branch symptoms appear initially as small, discolored, spindle-shaped swellings (= cankers) bordered by a narrow advancement of orange-yellow bark [Ziller, 1974; Agrios, 1988]. Pycnial fluid containing mononucleate pycniospores exudes from receptacles (pycnia) that have formed within the canker tissue. Within a short time, these pycniospores fuse with flexous hyphae of the opposite mating type [R. Hunt, personal communication] and go on to form the sac-like aecial blisters which eventually burst during the spring, releasing characteristically orange, binucleate aeciospores. The physiology of specific morphogenesis in the pine host is considered by some to coincide with aecial development i.e. it has been observed that aecia develop after bud break, but not during dormancy in white pine [Wicker and Harvey, 1969] making *C. ribicola* appear to be physiologically 'out of phase' with its perennial white pine host [Martin, 1987]. However, the legitimacy of the conclusion (i.e. that *C. ribicola* is 'out of phase' with *P. monticola*) based upon the above observation is questionable when it is considered that the fungus is in fact 'in phase' with its alternate *Ribes* host [R. Hunt, personal communication].

After the aecia rupture, aeciospores are windborne, sometimes for several hundred kilometres, until some land upon and successfully infect their alternate *Ribes* host (i.e. *Ribes nigrum* L., the cultivated English black currant, is the most highly susceptible.) The overwintering uredial (repeating) stage gives rise to the telial stage which eventually produces the haploid basidiospores that re-infect the pine.

Mortality rates from WPBR in B.C. white pine stands have approached 100% in the past [Lachmund, 1934; Hunt and Meagher, 1989] with the earliest and greatest degree of kill occuring in the smallest trees (i.e. 1-2 year old seedlings).

In order to understand the nature of host *defense* responses, one must also understand the general physiology and biochemistry of host-pathogen relationships. Changes in host physiology and biochemistry following infection may point to the

metabolic 'actors' engaged in the host defense response. Because specific rust-resistant host phenotypes may vary according to host genetics, age, and species, and be affected also by climate and other environmental factors, it is important to know where to start looking for molecular clues. If, for example, it is known (hypothetically) that the level of phenolic compounds is increased in the bark of WPBR-resistant white pine displaying the 'slow canker growth' (SCG) bark reaction, then one of the logical places to look for molecular tags would be those enzymes which control phenylpropanoid metabolism in bark tissue. It could be that constitutive phenolic levels are higher, that enzymes involved in their metabolism are more rapidly activated, or that highly active isoforms of the same enzymes are present in resistant hosts. Knowledge of the roles of various chemical compounds (i.e. phytoalexins), enzymes, and other proteins in general defense responses will help to identify potential avenues for comparative research.

Early Host-Pathogen Interactions

Studies of pine tissue cultured cells growing adjacent to an invading colony of *C. ribicola* have demonstrated ultrastructural changes similar to those seen in rapidly senescing tissue [Robb *et al.*, 1975a, 1975b]. It would appear that, at least *in vitro*, diffusable compounds produced either by the rust, or by previously colonized host cells, effect acceleration of degenerative metabolic changes in uninfected host cells [Kosuge, 1969; Offord, 1940; Rohringer and Samborski, 1967].

Postgerminational hyphal growth is likely guided by needle surface topography. Oriented hyphal growth along grooves or cell wall junction depressions may facilitate non-random location of stomata, with a minimum energy expenditure [Staples and Macko, 1980], although location by random chance is also possible. One study using subcultures of *C. ribicola* as inocula for white pine propagules demonstrated that *bent* hyphae were not prone to encounter and enter stomata [Diner and Mott, 1985].

How do the growing hyphae perceive physical and/or chemical signals? Microtubules may play a role in signal transduction, demonstrated by the observation that treatments inhibiting microtubule cytoskeletal changes also inhibit appressoria formation [Hoch *et al.*, 1986]. Other candidates include cell surface allosteric receptors that either activate signal transduction pathways, or regulate calcium channel gates. Increases in cytosolic free calcium ions are known to locally alter cytoskeletal elements and secretions [Gunning and Hardham, 1982; Hepler and Wayne, 1985]. Fungal tip growth is also sensitive to changes in extracellular calcium [Jackson and Heath, 1989].

Other signal receptors may be located *within* the cell wall or be associated with the plasma membrane. Lectin-like molecules in the cell wall of some fungal hyphal tips are able to specifically bind fucosyl or galactosyl residues [Hardham, 1992]. Similarly, extracellular proteins have been shown to be a requirement for substratum germling adhesion, germ tube orientation and thigmodifferentiation in the bean rust fungus *Uromyces phaseoli* (Pers.) Wint. [Epstein *et al.*, 1987].

The Role of Cell Wall-degrading Enzymes

The role of cell wall degrading enzymes in pathogenesis has been extensively investigated [Albersheim and Valent, 1978; Kirk, 1971]. The author chooses to briefly examine these enzymes with one question in mind, "*How* might such enzymes (i.e. proteins) differ in blister rust-*resistant* versus susceptible individuals?".

To understand how these enzymes affect the establishment of infection, it is necessary to have a fundamental knowledge of the structure and chemistry of the cell wall and its components. Extensive (and excellent) reviews on these subjects have been prepared by Cassab and Varner [1988], Albersheim [1976], Albersheim and Anderson-Prouty [1975], Talmadge and co-workers [1973], Northcote [1972], Albersheim and coworkers [1969], and Bateman and Miller [1966].

Briefly, the cell wall is composed of an orderly structure of polysaccharides (cellulose, hemicellulose, and pectic compounds), structural glycoproteins, and (in older tissue) lignin. It is compositionally and architecturally tripartite, with a middle lamella, primary cell wall and secondary cell wall. No clear demarcation separates these three regions [Misaghi, 1982]. Although primary cell wall structure is likely conserved from plant to plant, considerable constitutive variation may be found in the secondary wall (both between plants and within the same plant) [Albersheim, 1976; Talmadge *et al.*, 1973].

The distinguishing feature of the secondary cell wall is the presence of lignin, a branched polymer formed by the condensation of three substituted cinnamyl alcohols- sinapyl, coniferyl, and *p*-hydroxy cinnamyl alcohols [Dehority *et al.*, 1962; Freudenberg, 1968; Kirk, 1971; Gross, 1979; Hahlbrock and Grisebach, 1979]. Lignin deposition increases structural integrity and may make other cell wall constituents less susceptible to enzymatic degradation [Cowling and Kirk, 1976].

Cell wall degrading enzymes may be grouped into three categories- cellulases, hemicellulose-degrading enzymes, and pectolytic enzymes. Pectolytic enzymes are rather ubiquitous and *not necessarily* pathogenic. Cellulose degrading enzymes are produced by both non-pathogenic decomposing Basidiomycetes and a number of well known pathogenic fungi: *Rhizoctonia solani* [Bateman, 1964], *Colletotrichum orbiculare* [Porter, 1969], and *Fusarium oxysporum* f. sp. *lycopersici* [Cooper and Wood, 1975] to name a few of the pathogens. Hemicellulose-degrading enzymes do not have a well defined role in pathogenesis [Misaghi, 1982].

Cell wall-degrading enzyme production occurs sequentially, with pectolytic enzymes appearing and 'paving the way' for subsequent enzyme digestion [Bateman and Basham, 1976]. Research by Neil Martin [1980] suggests that pectinase activities in the *C. ribicola:P. monticola* pathosystem may be regulated by the physiological conditions

of host cells. By utilizing 'undesirable' pectic compounds, the rust fungus can prolong the life of the host cell. One striking reaction between bark cells of *P. monticola* and hyphal cells of *C. ribicola* is the fixation of the latter to host cell walls. Evidence given by transmission electron microscopy also suggests the possible involvement of *cellulases and pectinases* in the zone of fixation [Welch and Martin, 1975b]. Perhaps one of the strategies involved in the 'slow canker growth' bark reaction (seen in resistant *P. monticola*) is a constitutive reduction in the level of 'undesirable pectic compounds', or an increase in the production -or constitutive level of -enzyme-quenching substances (such as quinones).

Effects of Pathogen on Host Metabolism

i) Photosynthesis

Small bole cankers in white pine do not appear to affect net photosynthesis as demonstrated by foliar sugar levels found in diseased versus healthy trees [Martin, 1987]. Nevertheless, once the tree is girdled and translocation is effectively cut off, foliar deterioration becomes readily apparent. Bark tissues sampled from the advancing margin of blister rust cankers has been found to contain lower levels of glucose, fructose, and sucrose compared to bark samples proximal or distal to the lesion [Martin, 1987]. Rust lesions may also produce an accumulation of inorganic nutrients. Stem cankers in white pine have been shown to act as metabolic sinks for N, K, and P [Martin, 1972].

Other studies, using labeled carbon dioxide, suggest that decline in photosynthetic activity coincides with fungal sporulation. Decreases in photosynthetic activity may be compensated for by increased photosynthesis in uninfected tissue [Livne, 1964]. Sporulating fungi may also be responsible for increases in dark CO_2 fixation [Mirocha, 1972].

Increases in the carbon dioxide compensation point have been observed in rust infected tissue and are thought to be due to an increase in respiration rather than a decrease in photosynthesis [Raggi, 1980; 1978].

ii) Respiration

In healthy plants, the major (aerobic) respiratory pathway is biphasic [Bonner and Varner, 1976]. Glucose is first converted to pyruvate through the plant glycolytic pathway. Pyruvate is then oxidized to carbon dioxide and water through the reactions of the Kreb's cycle. The rate of carbohydrate catabolism is lower under aerobic versus anaerobic conditions. This phenomenon, known as the 'Pasteur effect' reflects the higher carbohydrate economy of aerobic metabolism [Manners, 1982].

In plants with sporulating rust lesions, respiration rates may show an increase of up to 100%. Early in an infection, increases in respiration may be host-related; however later increases associated with sporulating tissue may be pathogen related [Daly, 1967].

Observed increases in respiration following pathogenesis were originally thought to be primarily due to the uncoupling of oxidative phophorylation from electron transport by phytotoxin activity [Allen, 1953]. This hypothesis, based upon evidence of depressed anaerobic/aerobic CO_2 levels, was later dismissed when it was found that (1) dinitrophenol (DNP, an uncoupling agent) did not produce the same responses as those found in rust infected leaves, (2) the respiratory quotient (ratio of evolved CO_2:O_2 uptake), which increases in DNP uncoupled tissue, did not change in infected tissue, (3) other metabolic ratios which were known to be associated with DNP-uncoupled phosphorylation remained unaffected in the case of diseased tissue, and (4) reported increases in growth rate and metabolic activity did not support the uncoupling hypothesis [Daly, 1976, 1967; Wheeler, 1975]. Other research supports a *partial* uncoupling of oxidative phosphorylation [see review by Shaw, 1963; Agrios, 1988].

Increased respiration in rust-infected tissue may result from increased activation of the hexose monophosphate shunt (HMP) pathway. Studies using labeled carbon show that the C_6:C_1 ratio is lower in rust-infected versus healthy cereal leaves [Shaw and Samborski, 1957]. Increases in the activity of peroxidases and other non-cytochrome

oxidases (i.e. polyphenol, ascorbic acid, and glycolic acid oxidases) also contributes to the increased respiratory demand [Misaghi, 1982; Uritani, 1971]. These enzymes catalyze the conversion of phenolics to their more toxic quinone products and are thus lead actors on the stage of general plant defense.

iii) Alterations in Hormone Balance

The effect of pathogenesis on hormone metabolism has been reviewed by Sequeira [1973]. Pathogens can produce their own hormones or modulate host production of the same. Rust related growth hormones include the auxins (indoleacetic acid [IAA] and related compounds), cytokinins, and gibberellins (GA). These hormones promote growth through the stimulation of cell division, cell elongation, and nutrient accumulation. While auxins and gibberellins are more frequently associated with cell elongation and cytokinins tend to be mitogenic, such classifications are not absolute [Manners, 1982].

Rusts are capable of synthesizing (and oxidizing) IAA [Oaks and Shaw, 1960; Srivastava and Shaw, 1962]. This hormone, in conjunction with cytokinins, seems to be important for the establishment of 'nutrient islands' early in infection [Shaw and Hawkins, 1958; Király *et al.*, 1967; Sziraki *et al.*, 1975]. The 'green island' spots formed on blister rust infected white pine needles [McDonald and Hoff, 1975] are likely the result of this type of hormonal interaction.

Applications of IAA and GA to stems of white pine seedlings produce symptoms characteristic of blister rust disease [Boyer, 1967]. This same study demonstrated the formation of wound periderm in blister rust infected seedlings after application of benzimidazole, a mitogenic kinin-like compound also known to delay senescence in excised leaves. This periderm response was enhanced by IAA and GA, but was not observed in healthy (uninfected) seedlings exposed to the same hormonal treatment [Boyer, 1967]. Wound periderm formation in blister rust resistant white pine has been well documented [Struckmeyer and Riker, 1951].

Host Resistance

i) Genetics of Host-Pathogen Relationships

Disease resistance in plants is the rule rather than the exception. This must be the case as one considers that plants have no way of 'running away from' their pathogens and predators. Although the *basic* tenets of disease resistance biology have been formulated through the study of annual and short rotation crop:pathogen interactions, it is assumed that many of these basic tenets apply *equally* to *forest tree*:pathogen systems [Bingham *et al.*, 1971].

Early studies by Flor [1953] of the flax rust *Melampsora lini* led to the generally accepted 'gene for gene' theory, which basically states that host 'resistance genes' have matching and reciprocal genes for pathogenicity in the fungus. In other words, if a disease state is to occur, 'virulence genes' of the fungus must *compatibly* interact with *matching* 'susceptibility genes' in the host.

Monogenic, Oligogenic, and Polygenic Resistance

Monogenic resistance is under the control of a single gene. This type of resistance is relatively easy to study in detail. Resistance may be controlled by dominant or recessive alleles, and frequently the gene can be localized to a chromosome (if the genetics of the host plant are understood) [VanderPlank, 1968].

Polygenic resistance, on the other hand, describes resistance that is under the control of many genes- sometimes too many to count. Plants in segregating populations tend to show continuous (quantitative) variation and do not fall into disjunct groups. No single gene has an effect large enough to be phenotypically 'felt'. What one sees is the combined effect of multiple genes [VanderPlank, 1968]. All things being equal, polygenic resistance is generally considered to be a 'safer' (i.e. more long term) biological strategy than monogenic resistance as the latter places intense selective pressure on the pathogen.

Oligogenic resistance is under the control of few genes (intermediate between the first two types of resistance), and is strongly familial. A general relationship exists between the first two types of resistance , summarized in the following table (Table 1.). It must be remembered that the relationships described are not absolutely correlative. There are always exceptions to every 'rule'.

Table 1.

General relationship between two types of host resistance [adapted from Carson and Carson, 1989; Manners, 1982].

Criterion	Type I	Type II
Gene number	mono/oligogenic	polygenic
Gene expression	qualitative	quantitative
	(major)	(minor)
	(nonadditive)	(additive)
Specificity	specific	general
	(vertical)	(horizontal)
Environmental influence	small (stable resistance)	large
Age effects	slight	great (resistance increases with age)
Durability	low	high

Vertical and Horizontal Resistance

Vertical resistance exists when a host is more resistant to some races of a pathogen than to others. Such resistance tends to be unstable and subject to breakdown. Conversely, horizontal resistance tends to be manifested evenly against many races of a pathogen. This mechanism reduces the frequency and severity of infections while at the same time permitting the pathogen to complete its lifecycle [VanderPlank, 1968].

Other types of resistance that are worth mentioning include 'field resistance', which is generally age and environmentally dependent and expresses itself in a continuous (quantitative) fashion. The genetics and physiology of field resistance are not as well known, likely because of the confounding effects of age-environmental interactions. It may be safe to assume that such resistance is 'Type II' in nature (see Table 1). Similarly, 'environmentally determined resistance', which also operates in the mature plant, may mask any 'gene-for-gene' effects that might be present [Manners, 1982; see review by Namkoong, 1991]. 'Durable resistance' is basically a breeder's concept describing resistance that persists for the commercial life of a cultivar. Such resistance can only be evaluated in the field [Johnson and Law, 1973]. 'Tolerance' is defined by Schafer [1971] as the "capacity of a cultivar resulting in less yield or quality loss relative to disease severity or pathogen development when compared with other cultivars or crops". The plant basically 'puts up with' the pathogen. It is important not to confuse what appears to be a *passive* mechanism with intermediate degrees of active resistance.

In most cases, disease resistance in forest trees is based on a combination of polygenes and major genes. Resistance based on polygenic inheritance tends to maintain a large gene pool while resistance based on major genes may increase the selective pressure on the pathogen [Heimberger, 1962]. The situation in forest pathosystems is, however, far from clear-cut. Resistance mechanisms may operate at different stages of development, independently of each other [Carson and Carson, 1989]. When a species is

moved to a different environment, resistance or virulence can change, therefore *simple* virulence/resistance relationships may not be as common in forest trees, even at the species level, as they are in agricultural cultivars [Namkoong, 1991]. Major gene resistance mechanisms may be as abundant in forests as they are in agricultural systems; however, it is more difficult to identify such mechanisms in the former because of the greater genetic diversity of host and pathogen populations and the length of time required to make the necessary crosses [Carson and Carson, 1989]. Isozyme studies in western white pine demonstrate that 65% of all *tested* loci are *polymorphic* [Steinhoff *et al.*, 1983].

Generally speaking, resistant host species are usually found in or near pathogen gene centres. Both *C. ribicola* and *Pinus* spp originate from central Asia, so it is not surprising that Eurasian species of pine generally display moderate to high levels of blister rust resistance relative to their North American cousins [Garrett, 1986].

***Pinus monticola:* Bark and Needle Reactions**

There are likely to be 20-30 genes that control blister rust resistance in white pine. Some genes are effectively fungicidal while others retard the disease process [Hoff and McDonald, 1980]. A variety of phenotypic needle and bark resistance mechanisms have been documented in *P. monticola* in response to inoculation with and subsequent infection by *C. ribicola* [summarized by Hoff and McDonald, 1980]. These mechanisms include differential foliar resistance to yellow, yellow-green island, or red lesion forming races [McDonald, 1978]; absence of lesions; reduced needle lesion frequency; premature needle shedding; fungicidal short shoot reactions; fungicidal stem reactions; slow fungus growth in needles or stem, and stem tolerance to cankering [Hoff and McDonald, 1980].

Bingham and co-workers [1971] reported gene-for-gene relationships for the *P. monticola:C. ribicola* pathosystem and suggested that 3 independent host loci interact with 2 independent pathogen loci to produce 4 pathogenic races on pine foliage (one of

the pathogenic loci has alternate alleles that interact with two of the independent host loci). The possibility of a third virulent race of *C. ribicola*, thought to modify the action of red and yellow spot producing races, has also been reported [Garrett, 1986; McDonald and Hoff, 1975]. The reaction known as 'needle spots only' is believed to be controlled by two (recessive) independent and sequential resistance factors, one of which controls early shedding of infected needles [McDonald and Hoff, 1971; *ibid,* 1970]. Fungicidal short shoot and stem reactions are believed to be vertical mechanisms under recessive and (?) oligogenic control respectively, while disease tolerance, and the slow growth reactions of needle and stem are believed to be horizontal mechanisms under (?) polygenic control [Hoff and McDonald, 1980]. Because terpene analysis [Hunt *et al.*, 1985] has suggested that blister rust has not significantly depressed the *P. monticola* gene pool, a measure of intensive breeding selection can be safely allowed.

Pre-formed and Induced Resistance Mechanisms

Defense responses can be generally placed into one of two categories, 1) constitutive responses which depend upon pre-formed mechanical or chemical factors and 2) induced resistance reactions which depend upon factors that are present only after the host is challenged. These categories become somewhat ambiguous if we consider that induced resistance is not possible except for the presence of pre-formed interactive compounds in both host and pathogen [Sequeira, 1983]. Furthermore, resistance reactions are not always immediately apparent and must therefore be quantified by measuring and comparing the same responses in susceptible hosts. Given that all plants respond to challenges by potential pathogens, differences in the speed and intensity of a response (especially in a woody plant) may be what distinguishes a resistant from a susceptible reaction.

Constitutively, an agricultural plant's first line of defense against *rust* infection is its outer covering. Such is the same for conifers. Structural defenses such as the amount and

quality of leaf/needle wax and cuticle coating the epidermal cells, the structure and thickness of epidermal cell walls, and the size, location, and shape of stomata/lenticels will affect the efficacy of pathogen invasion [Agrios, 1988].

Patton and Spear [1980] reported that wax on the needles along with inhibitory substances in the stomatal subchamber hinders infection by *C. ribicola*. Intuitively, the structure of stomata (i.e. narrow entrance and broad, elevated guard cells) will also confer resistance in some host-pathogen systems.

Spaulding [1925] in his study of the relative susceptibility of various white pine species to blister rust infection suggested that a positive correlation existed between bark thickness and disease susceptibility. Comparing *P. monticola* and *P. strobus*, Spaulding noted that the inner bark layer of the former was considerably thicker than that of the latter and concluded that the greater abundance of phloem in *P. monticola* favored growth of the fungus. In the same study, Spaulding noted a positive correlation between the number of stomata per needle, the retention time of needles, and blister rust susceptibility.

Inhibitory levels of fungitoxic compounds commonly occur in the heartwood and in other dead tissues of perennial plants. In living tissues, pre-formed resistance factors are either more fungitoxic than phytotoxic, or they are bound in non-toxic form, sequestered into vacuoles that release toxic moieties upon infection or injury.

Induced resistance may be localized or systemic. Localized resistance is detected only in the immediate areas surrounding the initial invasion site and is frequently characterized by a rapid necrotic collapse of host tissue. This type of reaction is referred to as a 'hypersensitive response' (HR) and is frequently accompanied by an accumulation of antibiotic-like phytoalexins [Bol and Linthorst, 1990; Sequeira, 1983] or lignin deposition [Keen, 1992]. HR reactions may occur in the needles and bark tissue of blister

rust infected sugar pine and western white pine respectively [Kinloch and Littlefield, 1977; Kinloch, 1981; Hoff, 1986].

Systemic resistance occurs at host sites distant from the initial point of infection. An inducing inoculation elicits the local necrotic reaction while systemic resistance is detected later by a challenging inoculation. Systemic accumulation of proteins, terpenoids, and non-phytoalexin compounds may also occur [Sequeira, 1983].

Local and systemic resistance can both be characterized by time and temperature dependence, persistence, and relative non-specificity with respect to the organisms that induce the response and the range of pathogens against which the host is protected [Sequeira, 1983].

Hypersensitivity response (HR)

The HR response, sometimes referred to as an 'incompatible response' in *agronomic* contexts, ensues rapidly upon pathogen penetration of a resistant host cell wall. Once hyphal contact with the protoplast is established, the host nucleus moves toward the invading pathogen and disintegrates, triggering formation of brown, resin-like cytoplasmic granules. Necrotic tissue effectively walls off the obligate pathogen from living tissue thus preventing mycelial extension. The faster the host cell dies after invasion, the more resistant the plant [Agrios, 1988].

It would appear that by altruistically 'sacrificing' a few cells, the whole host organism is protected against the obligate rust. However, dead cells observed at the end of the HR are simply an *ipso facto* marker. Furthermore, hypersensitive cell death appears to require a period of host protein synthesis *a priori*. Protein biosynthesis is likely induced by recognition of some sort of pathogen-related elicitor molecule. Thus, early signal recognition and induction of defense response genes are probably more important in the HR response than the terminal cell necrosis [Graham and Graham, 1991; Schmelzer *et al.*, 1989; Collinge and Slusarenko, 1987].

Foliar HR to blister rust in *P. lambertiana* (sugar pine) possessing the major gene for resistance [Kinloch *et al.*, 1970] is expressed in primary and secondary needles, basidiospore-inoculated cotyledons, and in axenic culture systems (embryos and vegetative hyphae) [Kinloch and Comstack, 1980; Diner and Mott, 1982a, 1982b; Diner *et al.*, 1984].

Bark resistance reactions (not all of which are HR) in response to fungal, bacterial, viral, and insect infestation have been well documented [Struckmeyer and Riker, 1951; Boyer, 1967; Mullick, 1977; Bostock and Stermer, 1989]. Structural wound healing responses include callose deposition, periderm formation, wound gum synthesis, lignification, and suberization. In periderm formation, which is hormonally triggered through a balance of auxins and cytokinins, several layers of cork cells are formed beyond the point of infection. These cork layers (i) inhibit spread of infection, (ii) block spread of toxic substances, and (iii) stop nutrient and water flow from healthy to infected areas. Dead tissues are effectively walled off, forming characteristic necrotic spots or scabs that are eventually sloughed [Mullick, 1977; Raffa and Berryman, 1982; Agrios, 1988].

Three types of hypersensitive bark reactions have been described for fusiform rust-inoculated slash pine [Miller *et al.*, 1976]. *Superficial* corticular hypersensitivity (CH) manifested as a circumscribed, necrotic, and darkly stained reaction zone. CH, similar to the superficial reaction, was slower to develop and produced a larger reaction zone with a distinct cell layer, separating affected from non-affected cells. The third type of hypersensitive reaction confined the fungus to a morphologically 'gall-like' region. All three HR responses were seen more frequently in progeny of selected, phenotypically resistant slash pine. Susceptible pine also displayed similar morphologies but were unable to wall off the fungus. Miller and coworkers suggested that resistance may have been due (as previously mentioned) to a more *rapid* response and a *greater* accumulation of toxic

substances. It should be emphasized at this point that 'rapid response' criteria for agricultural/herbaceous plants may not apply to their woody counterparts.

Role of Phenolics and Lignification

Phenolics and quinones are both known to accumulate in host tissue in response to disease and/or mechanical injury [Rohringer and Samborski, 1967; Farkas and Kiraly, 1962]. Activation of the HMP shunt pathway (an alternate pathway of energy production utilized by the plant under conditions of general stress and during senescence) provides the precursors (phosphoenolpyruvate and erythrose-4-phosphate) for shikimate synthesis. Although synthesis of phenolics is accomplished primarily through the shikimic acid pathway, the acetate-malonate and acetate-mevalonate pathways may also be utilized [Neish, 1964; Kosuge, 1969]. Increased utilization of quinate and shikimate has been seen in rust-susceptible wheat leaves [Rohringer and Samborski, 1967] along with increased accumulation of phenylalanine and tyrosine (products of the former metabolites).

Phenyalanine ammonia lyase (PAL), a key enzyme in the shikimic acid pathway, is responsible for the reductive deamination of L-phenylalanine to trans-cinnamic acid and thus regulates (along with a few other enzymes) the biosynthesis of phenolics committed to this pathway [Kosuge, 1969]. Polyphenol oxidase (PPO) is responsible for oxidizing phenols to their more toxic quinone products while peroxidase not only oxidizes phenolics, but also increases the polymerization rate of such compounds into lignin-like substances [Agrios, 1988]. Inhibition of PPO has been reported to decrease disease resistance in certain plants [Hare, 1966].

Phenolic compounds affect the biochemistry of vital processes such as redox reactions and the modulation of auxin activity. Phenols and quinones are powerful enzyme inhibitors, metal chelators, and uncouplers of oxidative phosphorylation. Many phenolic compounds and their derivatives (i.e. lignans, flavonoids and stilbenes,

alkaloids, and terpenoids) exhibit antibiotic (phytoalexin) properties and are therefore thought to play some role in disease resistance.

High levels of lignans have been found to accumulate in the sapwood of perennial woody plants subsequent to fungal attack or injury [Johansson *et al.*, 1976; Chen *et al.*, 1976]. Many of these lignans have been shown to inhibit the activity of fungal enzymes, while others appear to be synthesized de novo in response to injury.

Flavonoid and stilbene synthesis are also induced in response to wounding or pathogen attack in sapwood [Kemp and Burden, 1986; Hart and Shrimpton, 1979]. Because stilbenes do not normally occur in healthy sapwood (although they are constitutive in heartwood), their induced synthesis in stressed sapwood classifies them as phytoalexins.

Rapid accumulation of phenolics associated with the HR response has been reported in wheat [Kiraly and Farkas, 1962; Beardmore *et al.*, 1983] and sugar pine [Kinloch *et al.*, 1970; Kinloch and Littlefield, 1977] suggesting that either lignification occurs in the necrotic cells during the HR response, or the initial phenolic concentration in host cells may, itself, be sufficiently fungitoxic [Beardmore *et al.*, 1983].

Increased production and accumulation of phenolics has also been observed in juvenile tissue of *P. contorta* within 7 days of inoculation with spores of *Endocronartium harknessii* (a pathogen related to *C. ribicola*) [Allen *et al.*, 1990]. However, in this case, the presence of these compounds did not affect growth of the fungus.

Isolated studies of the *C. ribicola-P. monticola* system have been unable to conclusively demonstrate a quantitative or qualitative difference in phenolics in susceptible versus resistant trees; however, preformed inhibitory compounds (presumably phenolic?) are known to be present in ether fractions of pine foliage [Hanover and Hoff, 1966; Hoff, 1970]. Furthermore, the localization of phenols in host cell vacuoles

following rust basidiospore infection are characteristic of early disease development in white pine needles [Boyer, 1964].

Role of Phytoalexins

There is a stark paucity of information addressing the topic of phytoalexins in perennial woody plants. To date, no concrete evidence has been published that would establish a significant role for phytoalexins in the disease resistance response of conifers or woody plants in general.

Pathogenesis related (PR) and other Cellular Proteins

PR-proteins

Viral and/or fungal infection may elicit host defense responses that range from mild to very severe. Mild responses may fail to affect host gene expression but severe host reactions, especially those of the HR type, will often induce *de novo* synthesis of novel proteins followed by a host immunity to subsequent pathogenic challenges. The correlation of induced, novel protein synthesis with acquired non-specific host resistance suggests that these proteins may be important to the defense response.

Literature on PR proteins has virtually exploded in the last 10 years [see reviews by Linthorst, 1991; Bol and Linthorst, 1990; Kolesnik, 1991; Collinge and Slusarenko, 1987]. Most if not all of the research has, again, been restricted to fast growing commercial (i.e. tobacco) or agricultural species. For example, PR proteins have been reported in pea tissue infected with *Fusarium solani* [Mauch *et al.*, 1988a], sugar beet infected with *Cercospora beticola* [Rousseau-Limouzin and Fritig, 1991], groundnuts infected with leaf rust [Bama and Balasubramanian, 1991], roots of *Fusarium oxysporum*-infected tomato [Benhamou *et al.*, 1990], and oats infected with wheat rust [Fink *et al.*, 1990].

Although PR proteins are generally considered to be present in most plant species, they have been studied most extensively in tobacco, where they were initially discovered (by two independent research groups) in cultivars infected by the tobacco mosaic virus (TMV) [Gianinazzi *et al.*, 1970; Van Loon and Van Kammen, 1970].

PR proteins were originally described as small polypeptides of low molecular weight (10-40 kDa) that were extractable at low pHs (i.e. acidic pH=3), stable to protease digestion, and localized in the extracellular fluid (ECF) [Van Loon, 1985]. Since developing that functional definition, basic and intracellular PR proteins have been isolated and characterized in tobacco and in many other plant species [Collinge and Slusarenko, 1987]. Space limits this discussion to a summary of the main groups of PR-type proteins characterized to date.

Five types of serologically related PR proteins have been characterized in tobacco [Linthorst, 1991]. **PR-1** type proteins are characterized by low molecular weight (≈**15 kDa**) and are induced by necrotizing pathogens and salicylate treatment. However, their function remains unknown.

PR-2 type proteins have been shown to possess β-1,3-glucanase activity. These proteins have both acidic and basic isoforms which are located extracellularly and within vacuoles, respectively. Basic isoforms are constitutively expressed in the roots and developing flowers of noninfected plants and also display a greater hydrolytic activity *in vitro*. β-1,3-glucanases may play a signaling role in the HR response by releasing β-1,3-glucan elicitors from fungal cell walls [Linthorst, 1991]. A soybean PR protein with β-1,3-glucanase activity has also been shown by Kyung-Sik and coworkers [1991] to release phytoalexin elicitor-active fragments from fungal cell walls. PR protein synthesis was induced in this case by treatment with mercuric chloride, or inoculation with *Phytophthora megasperma* H20 (pathogenic to Douglas-fir).

PR-3 type proteins include group I, group II and group III chitinases which will be discussed in more detail in a later chapter.

PR-4 type proteins have molecular weights of 13-14.5 kDa and show homology to the *win* proteins of potato; however, the PR-4 proteins lack the wheat germ agglutinin-like domains of the latter. Their function is not known [Linthorst, 1991].

PR-5 type proteins, including osmotin, and PR-S (induced by salicyclic acid), have a molecular weight of 24-25 kDa and show sequence homology to thaumatin, a sweet tasting protein found in the African shrub *Thaumatococcus danielli*. They are also serologically related to the α-amylase/trypsin inhibitor of maize. Recent studies have shown these proteins to have direct and specific antifungal activity *in vitro* [Vigers *et al.*, 1992]. Osmotin is especially effective, causing rapid bursting (possibly via membrane permeabilization) of the hyphal tips of *Neurospora crassa* [Vigers *et al.*, 1992].

Although many PR-proteins have been shown to inhibit fungal growth *in vitro*, their established role *in vivo* awaits the completion of experiments that combine site-directed mutagenesis with transformation of host cells through recombinant DNA technology.

Cell Wall Proteins

Cell wall proteins are generally rich in hydroxyproline, and are post-translationally glycosylated (galactose and arabinose being the main carbohydrate moieties). Because of their abundance in the structural proteins of animals, it was suggested that hydroxyproline-rich glycoproteins (HRGP's), some otherwise known as 'extensins', possessed a structural function in plants [Lamport, 1965 (review); Lamport, 1969; Lamport *et al.*, 1973].

Extensins are constitutively found in the cell wall of higher plants [Cassab and Varner, 1988]. They are extremely difficult to extract from cell walls and are thought to be insolubilized by intermolecular isodityrosine cross-links [Epstein and Lamport, 1984].

Extensin mRNA and protein has **also** been observed to increase in response to **stress** conditions that include wounding, elicitor and ethylene treatment, and pathogen infection [Mazau and Esquerré-Tugayé, 1986; Rumeau *et al.*, 1988; Ecker and Davis, 1987; Lawton and Lamb, 1987]. Extensin gene expression is readily detected in the stems and roots of wounded tomato plants. Thus it is thought that these proteins may inducibly augment barrier functions and promote wound healing in addition to their structural functions [Showalter *et al.*, 1992]. Consequently, extensins may also be legitimately included as PR proteins.

Glycine-rich proteins (GRPs) have recently been shown to exist in the cell wall [see Condit and Keller, 1990, for review]. Structural, cellular, tissue localization, and regulatory data from studies in petunia and bean indicate that many (but not all) GRPs are structural cell wall proteins with putative wound healing, or vascular system-related functions [Gomez *et al.*, 1988; Keller *et al.*, 1988; Keller *et al.*, 1989; Mundy and Chua, 1988]. Although many structural proteins are GRPs, the maxim does not hold that all GRPs are biological scaffolds. Indeed, chitinase has also been shown to be rich in glycine [Broekaert *et al.*, 1988]. GRPs have been shown to be induced in response to chemical stress (0.2% mercuric chloride) in maize [Didierjean *et al.*, 1992] and may also act as nucleation sites for lignification in some plants [Ye and Varner, 1991]. Gene expression of both GRPs and HRGPs has been shown to be developmentally regulated in a tissue specific manner [Ye and Varner, 1991].

Why Bark Proteins?

There are a number of compelling reasons to start looking at bark proteins in blister rust resistant and susceptible white pine, some being mercenary and others of more noble academic interest. First of all, one of the phenotypic resistance reactions of interest ('slow canker growth') is bark-specific, and individuals displaying this reaction *tend to perform better in field trials*. Slow canker growth (SCG) also has a fairly respectable heritability

(h^2) value of 0.21-0.46, which makes this phenotype attractive to the tree breeder [Hoff and McDonald, 1980]. One would like to know what is (or isn't) happening at a biochemical level to achieve the field effect of this resistance phenotype. Significant differences in protein/enzyme profiles could reasonably be expected between rust-susceptible and rust-resistant white pine because (to recapitulate) (1) heritable resistance factors depend on genes which may code for specific proteins or enzymes that ultimately prevent or retard pathogenesis [Shaw, 1963] and (2) a number of enzymes (i.e. peroxidases, dehydrogenases, chitinases, glucanases) have already been associated with resistance mechanisms against many fungal diseases in plants. It is *reasonable* to assume that similar strategies may be employed in woody plants and particularly conifers, albeit complicated somewhat by their enormous genetic heterogeneity (i.e. high percentage of polymorphic loci), and perenniality (which must therefore take into account age and environmental effects).

Because the activity of certain enzymes may increase in response to infection, attempts have been made in times past to use isozymes as **markers** to identify disease resistant cultivars in some agronomic crops [Kedar, 1959] and in certain species of pine [Hubbes *et al.*, 1991]. Novel proteins may represent isozymes of enzymes already present. Such isozymes may in fact be more effective fungistatics or may be more resistant than the original enzyme(s) to degradation. In Southern pine, differences in zymogram patterns (glucose-6-phosphate dehydrogenase, active in the HMP shunt) associated with fusiform rust resistance have been found [Hare, 1972; Farkas and Kiraly, 1962], however such a finding is unfortunately rare in forest species [Hare, 1972].

Unique, constitutive proteins could potentially be used as molecular markers in pine seedlings at the nursery stage *if* strong correlations could be found between their presence in plant tissue and phenotypic resistance. If *PR-like* proteins can be isolated and

characterized in white pine, it would certainly help to shed useful light on the physiological and biochemical mechanisms of blister rust-resistance in conifers.

CHAPTER 2

MATERIALS AND METHODS

Introduction:

This chapter outlines the sample selection, protein extraction and protein determination methods, one and two-dimensional electrophoresis techniques, Western blot immobilization, visualization procedures (staining and immunodetection), and amino acid analyses used in this study. A separate chapter details the methods used and results obtained from studies on chitinase.

Sample Selection

Bark samples (i.e. all tissue outside the xylem, including epidermis, periderm, and phloem) were collected from *Pinus monticola* grown at two different plantation sites, 1) an abandoned field site located at Lens Creek (LC), B.C. (Plantation 4/Plot B) [Hunt *et al.*, 1987] and a progeny test established at the Puckle Rd (PRd) Forest Research Station in Saanichton, B.C. At both locations, bark was collected from 8 individual trees, 4 of which displayed documented phenotypic resistance, while the remaining 4 were susceptible (i.e. they were cankered). At the LC site, selection for resistance was based upon so called 'mature tree resistance' [Hunt, personal communication]. Such trees were difficult to infect in a *Cronartium ribicola* disease garden [Porter, 1960] and have maintained high field resistance relative to other trees [Hunt and Meagher, 1989]. Selection for resistance at the PRd site was based on phenotypic expression of a localized bark reaction known as 'slow canker growth' (SCG).

Four out of 8 of the LC individuals were ramets of previously rust-screened [Porter, 1960] cloned coastal selections ("Porter's clones"), originally obtained from the Boot Lake and Garibaldi regions of B.C. A 5th ramet from an interior clone (originally from

New Denver) was also sampled. All 5 trees had been re-grafted from the Duncan disease garden and Robertson River plantation between 1958 and 1961, and were subsequently planted at the LC site between 1960 and 1962 [Hunt *et al.*, 1987]. The first bark samples were taken in April of 1991, the field age of the trees being roughly 31 to 33 years. Three 'natural' trees- 1 canker-free ('resistant') and 2 cankered ('susceptible')- were sampled along with the clonal stock to make a total sampling size of 8 individuals for this location. It should be emphasized that the selected *resistant* phenotypes, both natural and clonal, were *all* canker free. Resistant trees were identified as follows: G8, G161, B643 (clones) and N0-7 ('natural, no cankers, age 7). Susceptible trees were identified as G27, 6149 (clones), N6-23 ('natural', 6 cankers, age 23) and N2-15 ('natural', 2 cankers, age 15).

At the PRd progeny test site, contrasting seedling pairs from within the same family (i.e. family = open pollinated, from the same seedlot) were selected. There were 4 pairs, each with a SCG phenotype and a highly virulent cankered phenotype. For each individual, bark samples were taken from a canker-free region and within ≈ 1 cm distal to the canker margin/bark reaction zone. These zone-specific samples were analyzed separately. Collections were made from a total of 8 seedlings, with 2 sample sites per seedling. At the time of bark collection (November, 1992), the seedlings were 7 years old. Identification of resistant trees is as follows (seedlot number in bold): **2413**-20-6; **2391**-39-1; **2411**-11-7; **2398**-128-8. Susceptible trees include **2413**-20-3; **2391**-113-2; **2411**-72-5; **2398**-128-6.

LC bark samples were initially stored at -20°C, freeze dried for 72-168 hours, and ground (individually) with a Wiley Mill (Thomas-Wiley, Intermediate Model, VWR Scientific Canada Ltd, London, Ontario) using #20 mesh screening. Once ground, bark tissue was kept frozen at -20°C until needed. PRd bark tissue was treated in similar

fashion, except that grinding was done under liquid nitrogen with a mortar and pestle in order to maximize yield of the limited sample quantities.

Protein Extraction

Proteins were extracted from individual bark samples using a modified procedure described by Ekramoddoullah [1993]. Unless stated otherwise, all procedures were done at room temperature. All chemicals used in the extraction solutions were electrophoresis purity grade. Briefly, 1 ml of extraction solution (ES-1) composed of 5% sucrose (Bio-Rad, Hercules, CA)/ 5% mercaptoethanol (Sigma, St. Louis, Missouri, U.S.A.)/ 4% sodium dodecyl sulfate (SDS; Bio-Rad) at pH ~ 3.8 was added to 50mg of lyophilized, ground bark tissue and either vortexed at high speed for 12-15 minutes or placed on a shaker platform for the same length of time. The mixture was spun at 14,000 rpm for 15 minutes using a microcentrifuge (Biofuge 15, Heraeus Instruments, Baxter, CanLab). The supernatant was collected and if necessary spun for a second time to clarify. Crude extract was heated for 3 minutes over a boiling water bath (100°C) and allowed to cool to room temperature. Proteins were then precipitated by adding 8x ice cold (-20°C) reagent-grade acetone (i.e. 1.2ml of acetone added to 150µl of extract) and allowing the solutions to sit for an hour at -20°C. Proteins were pelleted by centrifugation at 14,000 rpm for 10 minutes. Protein pellets were resuspended in a total volume of 200-250µl of a second extraction solution (ES-2) consisting of 5% sucrose/ 5% mercaptoethanol/ 4% SDS and 1% nonidet (NP-40; Millipore, Toronto, Canada), pH ~4.2. To facilitate solubilization, the extracts were heated for 3 minutes over boiling water, and vortexed. A final 5-10 minute centrifugation at 14,000 rpm was used to bring down any insolubles and the clear, lightly pigmented extracts were subsequently stored at -20°C until required. Protein extracts were concentrated as necessary using a Microcon-3 (molecular weight cutoff =

3000 daltons) microconcentrater (Amicon Canada Ltd., Oakville, Ontario) according to the manufacturer's instructions.

Protein Determination

Because of the interfering reagents used in the protein extraction solutions (i.e. SDS, mercaptoethanol) and the presence of residual phenolic compounds in the extract, a 'dot blot' method of protein determination using polyvinylidene diflouride (PVDF) membranes (Millipore), Coomassie Brilliant Blue (CBB) R-250 stain (Sigma), and a computer assisted, laser-based densitometer was employed [Ekramoddoullah and Davidson, 1994]. A standard curve was generated by making serial dilutions (i.e. 0.1 to 1.0μg/μl) of a stock solution (1mg/ml) of bovine serum albumin (BSA; Boehringer-Mannheim, Laval, Quebec, Canada). The extracts to be tested were diluted 1:10. The BSA stock solution and all dilutions were made with ES-2.

Using a 96 well ELISA plate as a template, 1μl volumes of each dilution were applied to a dry PVDF membrane. Four replicates of each standard dilution, and four replicates of each tested extract were used. The membrane was dried under a fume hood for 30 minutes, stained for 10 minutes in a solution of 0.1% CBB: 50% methanol, and destained in 50% methanol: 10% acetic acid for 10 minutes. After rinsing with distilled water for 10 minutes, the stained membrane was scanned by a laser scanner (Molecular Dynamics, Model 110A, Sunnyvale, CA) interfaced with a workstation (SPARK 1, Sun Microsystems of Canada Inc., Vancouver, B.C.) and Protein + DNA imageWare systems (PDI, Huntington Station, NY) for blot analysis using the ONED™ software package. Scanning, detection, and quantitation were performed according to the manufacturer's instructions (PDI).

Electrophoresis: SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE was performed at room temperature using a Protean™ 16 cm dual vertical slab gel electrophoresis tank according to the manufacturer's instructions. The Laemmli gel and buffer system protocol [Laemmli, 1970] was employed, using a separating gel of 12% acrylamide overlaid with a 4% stacking gel. All buffer reagents were obtained from Bio-Rad Laboratories. The Bis/acrylamide monomer solution was obtained from Millipore. Analytical gels were 12 cm by 15 cm and 0.75 mm thick. Individual samples were loaded on an equal protein basis as previously determined by dot blot (5-10 μg per lane, silver stain; 30-50 μg per lane, CBB triple stain). Low molecular weight protein standards (Bio-rad) ranging from 14.4 kDa to 97.4 kDa were run alongside the bark proteins and were used to calibrate and calculate relative molecular mass. Electrophoresis was carried out at a constant current of 10 mA per 0.75 mm stacking gel and 15 mA per 0.75 mm separating gel using a Model 3000/300 power source (Bio-Rad). The average time taken to complete a run was 3½ hours. Gels were fixed and either silver stained according to the method of Hochstrasser *et al.* [1988] or stained with CBB utilizing a triple staining procedure [Fairbanks *et al.*, 1971].

Hochstrasser Silver Stain

Unless stated otherwise, all steps were carried out in glass trays at room temperature, with gentle agitation on a shaker platform. Upon completion of SDS-PAGE, gels were individually fixed in a 40% ethanol:10% acetic acid solution for 1 hour after which they were placed into 5% ethanol: 5% acetic acid for up to 3 days. Gels were washed in deionized water for 5 minutes and then incubated in a 2.5% glutaraldehyde solution for 30 minutes. After this additional fixing step, gels were washed 3 times (5-10 minutes per wash) in deionized water and then washed 4 times (30 minutes per wash) in the same in order to clear the glutaraldehyde. Gels were then placed into a 0.8% ammoniacol silver stain solution (composition: 3 g silver nitrate, 5 ml concentrated ammonium hydroxide, 0.75 ml of 10N NaOH, 375 ml deionized water) for 7 minutes and

subsequently washed in 3 changes of deionized water (5 minutes per wash). Gels were developed in a sodium citrate: formaldehyde solution (0.05 g sodium citrate, 500 ml deionized water, 0.5 ml 37% formaldehyde) for 1½-2½ minutes and placed into a 5% acetic acid stop solution for 10 minutes. After scanning, gels were placed into a gel drying solution (7% glycerol, 15% isopropanol, 5% acetic acid) until vacuum drying could be carried out.

CBB Triple Stain

After SDS-PAGE, gels were immediately (and individually) placed into stain #1 (0.05% (w/v) CBB, 25% isopropanol, 10% acetic acid) overnight. The following day gels were fixed and stained for 6 hours in stain #2 (0.005% (w/v) CBB, 10% isopropanol, 10% acetic acid) and then placed into a third stain (0.0025% (w/v) CBB, 10% acetic acid) overnight. The staining reaction was stopped by placing the gels into a 10% acetic acid stop/destaining solution until protein bands could be visualized with a minimum of background staining. Gels were then scanned and stored either in 20% glycerol or in gel drying solution.

SDS-PAGE analysis

Scanning, band detection and matching, molecular weight estimation, calibration and quantitation were performed using the instructions provided in the ONED™ (version 2.4) software manual. Calibrated quantity of proteins was expressed in nanograms. Statistical analysis of quantitated bands (Student-t test; P value≤0.05) was performed using the Microsoft® Excel program (version 5.0; Microsoft Corporation, Frontline Systems, Inc., P.O. Box 4288, Incline Village, Nevada) according to the manufacturer's instructions.

Electrophoresis: 2-D electrophoresis

Analytical two dimensional electrophoresis was carried out using the Millipore Investigator™ 2-D system. Except where mentioned, all chemicals and reagents used

were obtained from Millipore. In the first dimension, the isoelectrofocusing (IEF) gel solution was composed of 9.5M urea, 2% NP-40, 4.1% acrylamide, and 20mM cholamidopropyl-dimethylhydroxypropanesulfate (CHAPS; Sigma). To 5.65 ml of this gel solution was added 350 µl of 2-D optimized ampholyte solution (pH range 3-10) and 40 µl of 10% ammonium persulfate (APS) to effect gel polymerization. Gels were cast in 26 cm x 1 mm glass tubes and allowed to polymerize for 1-2 hours. The final dimensions of the IEF gels were 20 cm x 1 mm. Gels were pre-focused at 110 mA per gel for approximately 1 hour, to a maximum voltage of 1500V. Cathode buffer (upper chamber) consisted of 100 mM NaOH while the anode buffer (lower chamber) consisted of 100 mM phosphoric acid. Individual bark protein samples were pooled on an equal protein basis and layered on top of pre-focused IEF gels. Total protein loaded per gel was 20-30 µg in a total volume of 20 µl, using a sample buffer (Solution 'E'; composed of 0.1 g dithiothreitol (DTT), 0.4 g CHAPS, 5.4 g urea, 0.5 ml ampholyte solution (pH range 3-10), and 6.5 ml deionized water) recommended by Hochstrasser *et al.* [1988] as diluent. A typical run consisted of pooled 'susceptible' and pooled 'resistant' samples in duplicate. Low molecular weight/pI standards (Bio-Rad) were run on a separate gel together with 4 sample gels (2 susceptible and 2 resistant). Isoelectric focusing was performed at room temperature for 18,000 volt hours (17.5 hours). Maximum voltage attained during isoelectric focusing was 2000V using a maximum current of 110 mA per gel.

In the second dimension, the gel tubes were removed after completion of IEF and placed on ice for 10-30 minutes. Gels were extruded into gel trays using a 3ml syringe and syringe adapter filled with Milli-Q® water (Millipore). Gels were equilibrated for exactly 2 minutes in a buffer consisting of 0.3M Tris(Hydroxymethyl)aminomethane (Tris) base, 0.075M Tris-HCl, 3% SDS, 50mM DTT and 0.01% bromophenol blue (BPB), after which they were carefully overlaid onto 20 cm x 20 cm, 12.5% Duracryl slab gels (0.375M Tris, pH 8.8, 0.1% SDS). The slab gels were loaded into a tank containing

10L of pre-cooled (12°C) electrode buffer (25mM Tris base, 192mM glycine, 0.1% SDS, pH 8.8). Top loading buffer (cathode) consisted of 50mM Tris base, 384mM glycine and 0.2% SDS. Gels were run at 20W per gel to a maximum total voltage of 500V. The average duration of a second dimension run was 5 hours.

Upon completion of the second dimension, gels were fixed in a 50% methanol: 10% acetic acid solution overnight. Gels were left in this solution until silver stained.

Silver staining of 2-D gels was accomplished using a modified Morrissey [1981] procedure. Unless stated otherwise, all steps were performed at room temperature with washing and incubation performed on a shaker platform. Briefly, gels were individually washed 3 times in deionized water (20-30 minutes per wash) to remove SDS and fixative. Gels were then placed into a DTT solution (0.02 g DTT in 4L deionized water) for 30 minutes. Following this, gels were incubated in 0.2% aqueous silver nitrate for 40 minutes after which they were washed with deionized water for 5 minutes and rinsed in approximately 300 ml of a 3.5% (w/v) sodium carbonate: 0.05% formaldehyde developer for 10-30 seconds with continuous rocking. After the solution developed a homogeneous brown color (usually within 15-20 seconds) it was immediately drained and the gel placed into approximately 500 ml of fresh developer. Gels continued to develop for approximately 5 minutes after which the reaction was stopped with a 3% acetic acid solution. After 10 minutes in the stop solution, gels were placed into 20% glycerol where they remained until scanning and subsequent drying.

Scanning of gels and computer analysis of separated proteins.

Stained gels were scanned by a laser scanner (Molecular Dynamics) interfaced by PDI's PDQuest™ software (version 4.0) for the processing of 2-D gels and ONED™ (version 2.4) software for the processing of blots and SDS-PAGE gels. An original version of the 2-D software has been described [Garrels *et al.*, 1984]. The scanner was calibrated with a 21-step (0.05-3.05 optical density range) photographic strip (Eastman

Kodak Company, Rochester, N.Y.). Image resolution, in terms of pixel size, was set at 176 x 176 for X and Y axes respectively. Each pixel of a gel scan was automatically assigned an optical density (O.D.) value based upon step tablet calibration, and linear interpolations of O.D. were used to express spot quantitation. Out of a total of 50 2-D gels, 36 gels (8 individual bark samples in triplicate; 3 separately run pooled susceptible and resistant samples in duplicate) were scanned and processed, and 4 matchsets were created.

Two dimensional gel analysis

Scanning, spot detection and quantitation, gel matching, estimation of isoelectric point (pI) and relative molecular weight (Mr), and statistical analysis of resistant and susceptible groups was performed according to the PDI instruction manual.

Gel images (*gim*) obtained from gel scans were initially converted into gel spots (*gsp*) by an autodetect function. Careful manual editing of *gim*/*gsp* images (i.e. valid spots added to *gsp* by marking crosshairs on *gim* while artifactual spots removed from *gsp* by erasing crosshairs on *gim*) was done by comparing the *gim* with the original gel and adding or erasing crosshairs when appropriate. All *gim*/*gsp* images were exhaustively edited prior to gel matching.

Quantitation of gel spots was performed automatically, using a 2-D Gaussian model, where spot quantity was determined by Gaussian volume and expressed in protein data units (PDU).

Matchsets were generated by selecting one gel- usually the most highly resolved gel with the greatest number of spots- to use in the construction of a reference *gsp*. A reference *gsp* consisted of all spots detected in all replicate gels from both susceptible and resistant groups. Such an image was constructed by landmarking recognizably common protein spots in both reference and member gels and then automatically matching the

remaining spots. Matchsets were re-edited by re-checking for match offsets, partial matches, and erratic responses, and manually correcting and rematching *pro re nata.* A total of 4 matchsets were generated. The first matchset was composed of a reference *gsp* and 24 *gsp* members, representing data from triplicate samples of bark proteins from 8 individual trees (4 susceptible and 4 'mature tree' resistant). The remaining 3 matchsets represented data from separate pooled sample experiments. Each of the latter 3 matchsets was composed of a reference gsp, 4 gsp members, and 4 gim (resistant and susceptible duplicates).

Statistical analysis of matchset data was performed with the Student t-test provided in the software package, using a confidence level of 95% (P-value≤0.05). Significant spots were further subjected to Boolean analysis using both Student t-test and an unbounded foldchange as qualifiers in order to narrow down the choice of potential marker proteins. Once Boolean analysis established the number and identity of significant spots in the pooled samples of an experiment, individual samples (i.e. original individual gels) were examined for the presence or absence of the same spots. Candidate spots were then selected on the basis of their 1) relative abundance and location in the gel (for subsequent ease of isolation) and, 2) their demonstrated presence in individual sample gels (i.e. present in at least 2 out of 4 samples in a group).

Western Blot

Western blotting was performed by standard (vertical tank, complete immersion) and semi-dry methods. In both cases, a transfer buffer consisting of 10% methanol: 10mM 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS), pH 11, was used. Following one or two-dimensional electrophoresis, gels were equilibrated for 5-10 minutes in transfer buffer and proteins were electroblotted to PVDF membranes. In the standard blotting method, proteins were electrotransferred for 20-22 hours in a vertical tank (Model #EBU-100, C.B.S. Scientific Co., Delmar, Calif., U.S.A.) at constant voltage

(Model PS500x, DC Power supply, Hoefer Scientific Instruments). Using 50V (2 x 0.75mm-1.0mm thick gels) or 80V (2 x 1.5mm thick gels) at a constant temperature of 4°C resulted in the most efficient transfer. Semi-dry blotting was achieved using the Milliblot-Graphite Electroblotter System Type II (Millipore) at a constant current of 1.25 mA/cm^2 (gel surface) for 90 minutes (ambient temperature). Pre-stained protein standards (Rainbow standards; Amersham International plc., Amersham Place, Little Chalfont, Bucks, England) with a molecular weight range of 14.3 kDa to 200 kDa were run together with bark proteins. Following transfer to PVDF membranes, immobilized proteins were either stained with CBB (see 'protein determination') for band sequencing or blocked with 3% gelatin in Tris-buffered saline (TBS: 20mM Tris-HCl, 500mM NaCl, pH 7.5) for subsequent immunodetection with 4 separate polyclonal antibodies.

Immunodetection

The immunodetection protocol according to Tan and Ekramoddoullah [1991] was used. Briefly, following removal from the Western blot apparatus, PVDF membranes containing the immobilized protein spots/bands were blocked for 30 minutes in 3% gelatin in TBS, pH 7.5. Membranes were washed in 3 changes of cold (4°C) TBST (1% Tween-20 in TBS, pH 7.5) for 10-15 minutes per wash, and then placed in one of 4 possible polyclonal antibody solutions; (1) barley anti-chitinase, 1:750-1:500 dilution in incubation buffer (IB: 1% gelatin in TBST, pH 7.5), 2) petunia anti-chitinase, (a kind gift from Y. Zhang, Simon Fraser University, B.C.) 1:2500 dilution in IB, 3) anti-Pin m III (19 kDa cold protein of 5-needle pine [Ekramoddoullah *et al.*, 1994]), 1:500 dilution in IB and, 4) anti-PSII (23 kDa photosystem II protein of western white pine [Ekramoddoullah, 1993]), 1:500 dilution in IB. Membranes were incubated in 50-75 ml of antibody solution, at room temperature with constant shaking, for a period of 16-20 hours. Following 10 minute washes in 3 changes of cold TBS, the blots were incubated for 1 hour in a 1:2000 dilution of goat anti-rabbit serum in IB (GAR: light and heavy

chain immunoglobins conjugated to an alkaline phosphatase enzyme substrate; Bio-Rad). After washing the blots in cold TBS as above, they were developed in an NBT:BCIP solution (Bio-Rad) according to the manufacturer's instructions. In the case of chitinase immunoblot(s), the color reaction was allowed to continue until a faint purple background was achieved (3-4 minutes), whereas the anti-Pin m III and anti-PSII blots were allowed to develop only until visualization of discreet bands (1-2 minutes). Quantitation of immunoreactive bands (anti-Pin m III) was done using the scanning and processing techniques described earlier (ONED™). Quantity was expressed in O.D. x MM. Statistical analysis of bands was performed using the Excel 5.0 program as previously described.

N-terminal Sequence Analysis

CBB stained protein bands were cut from PVDF membranes and placed directly into a gas-phase microsequencer (Model 470A, Applied Biosystems, Foster City, CA) with an on-line PTH-analyzer and 900A system controller and data analyzer (Applied Biosystems). Chemicals and protocols used for N-terminal sequence and composition analysis were from Applied Biosystems.

Amino Acid Composition Analysis

After hydrolysis in 6M HCl, composition analysis of 3 protein bands (22-25 kDa) was performed using an automatic amino acid derivatizer-analyzer (Applied Biosystems, Model 420) according to the manufacturer's instructions.

CHAPTER 3

RESULTS: ONE AND TWO DIMENSIONAL ELECTROPHORESIS, IMMUNODETECTION AND AMINO ACID ANALYSES

SDS-PAGE

Bark protein extracts from individual samples were separated into between 32-49 well resolved and matched bands by SDS-PAGE. A single **PRd** experiment consisted of duplicate gels of either (1) 4 seedling pairs ('families') per gel and 4 samples per seedling pair (2 'susceptible' and 2 'resistant' sampled from healthy and lesioned areas) or (2) one seedling pair ('family') per gel, 4 samples per family with 3 replicates per sample (16 sample lanes and 2 molecular weight standard lanes). The former experiment(s) were run to examine *group* differences (resistant versus susceptible) whereas the latter experiment(s) were run to examine 'within tree' and 'within family' differences. All samples were run on an equal protein basis as previously determined by dot blot analysis. LC and PRd experiments were treated separately. Individual LC bark protein samples (4 resistant and 4 susceptible) were run in duplicate on a single gel.

Lens Creek (LC)

There were ***no*** consistent qualitative or quantitative differences between resistant and susceptible groups from the LC location. Considerable variation was seen in banding patterns between individual trees within a group.

Puckle Rd (PRd)

Figures 1 and 2 show the SDS-PAGE separated and silver stained protein bands of the PRd families. PRd samples visually demonstrated both qualitative and quantitative differences both within and between families. Indeed, multiple rather than singular differences were observed in the protein profiles of resistant versus susceptible groups as

a whole (see Table 2). Few *consistent* protein differences were observed from one seedling pair to the next, however there were obvious visual differences in the intensity of protein bands from healthy versus lesioned tissue in both resistant and susceptible individuals of a given 'family'. The number of ***unique*** proteins identified by SDS-PAGE was roughly equivalent for both resistant and susceptible groups.

A comparison of all 4 PRd families (in duplicate), in which **32** well resolved bands were matched, revealed a number of interesting protein differences. In individuals from families 2391 and 2411, a **28.3 kDa** band was increased in susceptible versus resistant tissue. A **27.3 kDa** band was found to be *absent* in samples taken from *near the canker site* (*susceptible*) in 3 out of 4 families (2413, 2391, and 2411) and present in the remaining samples (both healthy and lesioned). A **25.9 kDa** band was *absent* in *resistant* individuals from families 2413, 2411 and 2398, while the same band in family **2391** was weaker in the resistant versus the susceptible individual. The presence of this band was later confirmed in an individual experiment of family 2391 (≈ 26 kDa band). A **25.2 kDa** band was found *only* in samples taken near canker site (versus healthy tissue) in susceptible individuals from families 2391, 2411, and 2398. Of samples taken near the lesion sites (versus healthy sites), *resistant* individuals in families 2413 and 2391 tended to have significantly higher levels of protein than *susceptible* individuals (for 26 and 28 band matches respectively out of a total of 32 matches). Comparison of all 4 families revealed that extracts from 'resistant' *healthy* bark tissue tended to be more protein-rich than 'susceptible' *healthy* bark tissue. Other notable results from experiments comparing proteins from all 4 PRd families include, (1) a 24 kDa protein which was found to be selectively enhanced near canker/reaction zones versus healthy tissue in all 4 families (susceptible individuals from 2411 and 2391 were especially enriched), (2) a 23.4 kDa protein found to be absent in all susceptible tissue sampled near the canker margin, and present in healthy tissue from both susceptible and resistant groups. This same protein

was also present in samples taken near SCG zones in families 2413 and 2391. Attempts to sequence these proteins were not successful.

A **10.5-10.6 kDa** protein was found in significantly larger amounts in resistant individuals from all 4 families. This same protein, confirmed in triplicate in individual samples, was found in tissue sampled from healthy sites and reaction sites in 3 out of 4 resistant individuals, whereas the fourth resistant individual expressed the protein in healthy tissue only. The N-terminal sequence analysis of this protein is shown in Figure 11. **Table 3** gives the molecular weight details of proteins found to be ***unique*** to resistant or susceptible individuals. It can be seen at a glance that a great deal of variability exists between individuals within a group.

When experiments were run on individual families (in triplicate), significant (Student t-test, P-value≤0.05) specific quantitative differences were frequently seen in bark samples taken from healthy regions of a given individual versus those taken near the canker or bark reaction zone (see Table 2). *Susceptible trees* often had higher amounts of specific proteins in tissue sampled near the cankered versus healthy regions (see families 2411, 2413, and 2398). An exception to this trend was found in family **2391**, where, in the *susceptible* individual, *healthy* tissue tended to show higher levels of protein for most of the bands matched. A **26.2 kDa** band was significantly (P-value = 0.006) increased in tissue sampled near the canker versus that sampled from the healthy area (2391-113-2, susceptible). Although the amount of this particular protein was not *significantly* higher in the *susceptible* versus *resistant* sample, the slightly higher amount in the cankered individual represented a *reversal* of a nearly uninterrupted trend of 'res>sus' and 'sus-H>sus' for this particular family. This band was also found to be comparable with a unique **26.0 kDa immunoreactive band** (barley antichitinase) seen in the *susceptible* but not the *resistant* individual from family 2391 (to be discussed later) and is believed to be

the same band identified as the '25.9 kDa' band in the experiment comparing all 4 PRd families. This particular protein has been partially sequenced (see Figure 12).

Figure 1.

Representative silver stained SDS-PAGE gel of bark protein samples from 4 PRd seedling pairs ('families';experiment run in duplicate). Total protein loaded per lane = 5μg

(A) = Family 2413, canker/reaction zone; (a) = Family 2413, healthy region

(B) = Family 2391, canker/reaction zone; (b) = Family 2391, healthy region

(C) = Family 2411, canker/reaction zone; (c) = Family 2411, healthy region

(D) = Family 2398, canker/reaction zone; (d) = Family 2398, healthy region

(r) = resistant; (s) = susceptible, (k)= kilodaltons

Low molecular weight marker proteins shown in first lane (left of 'A'). Arrows point to significant protein bands that were later sequenced. Open arrow points to **26.1 kDa** protein (2391-113-2, susceptible); upper solid arrow points to **19 kDa** 'cold' protein (immunoreactive to anti-Pin m III); lower solid arrow points to **10.5 kDa** protein found in resistant versus susceptible tissue (2411-11-7).

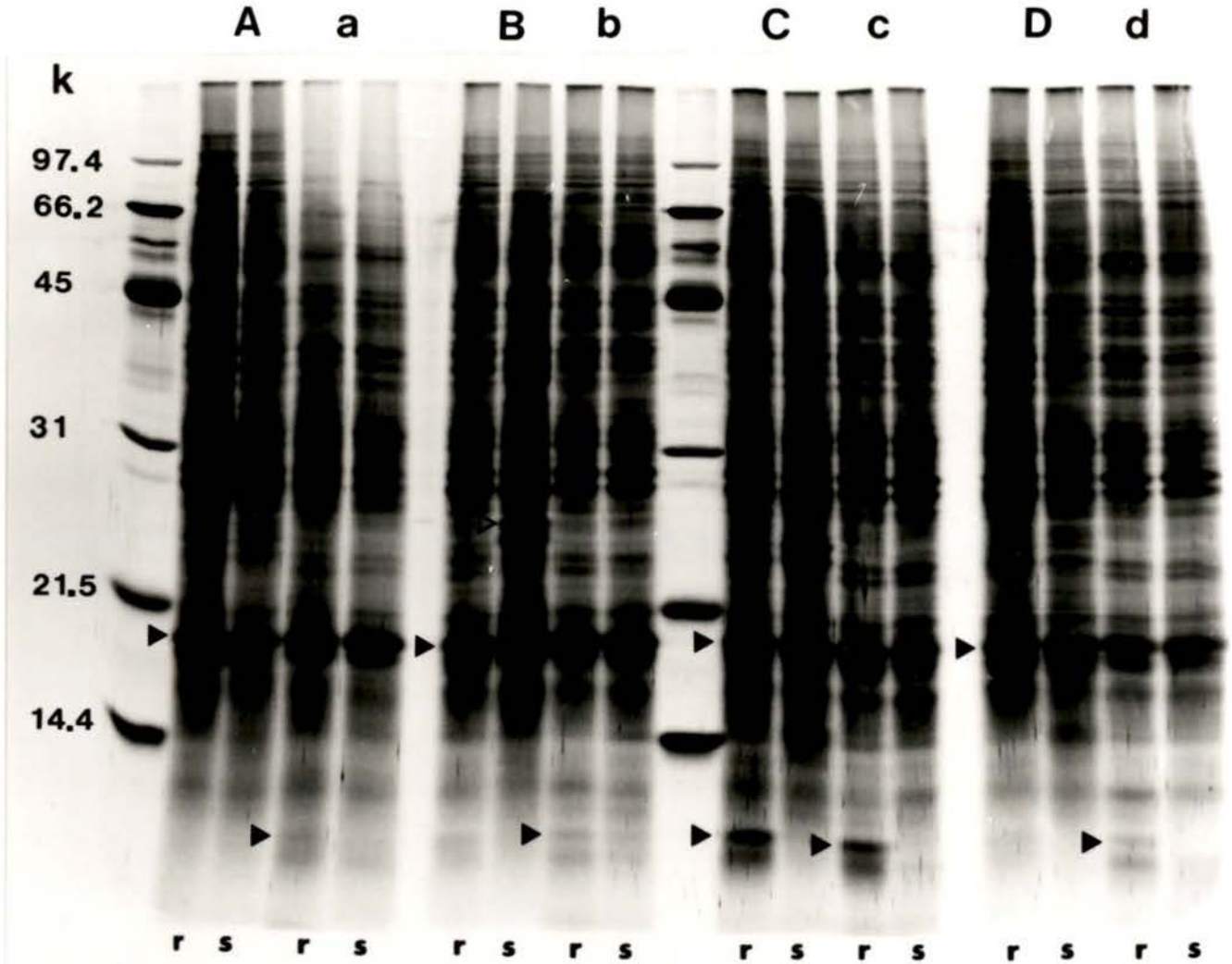

A
a
B
b
C
c
D
d
k
97.4
66.2
45
31
21.5
14.4
r s r s r s r s r s r s r s r s

Figure 2.

SDS-PAGE separation of bark proteins from Family 2411 (A) and Family 2391 (B). Gel was silver stained by the Hochstrasser method [1988]. Total protein loaded per lane = 7.5μg (in triplicate). (a) = resistant; (b) = susceptible; (c) = resistant, healthy tissue; (d) = susceptible, healthy tissue. Arrows highlight *some* of the subsequently sequenced bands. Bio-Rad low molecular weight protein standards in left-most and centre lanes. In gel (A), large arrow points to 10.5 kDa protein band (sequenced) and small arrow points to ≈26 kDa band similar to that sequenced in Family 2391 (gel B). In gel (B), 2 small arrows point to protein bands of ≈26 kDa (upper) and ≈25 kDa (lower). (The ≈26 kDa was also sequenced.) Large arrows point to protein bands of 10.5 kDa (sequenced) and ≈ 10.0 kDa.

(k) = kilodaltons

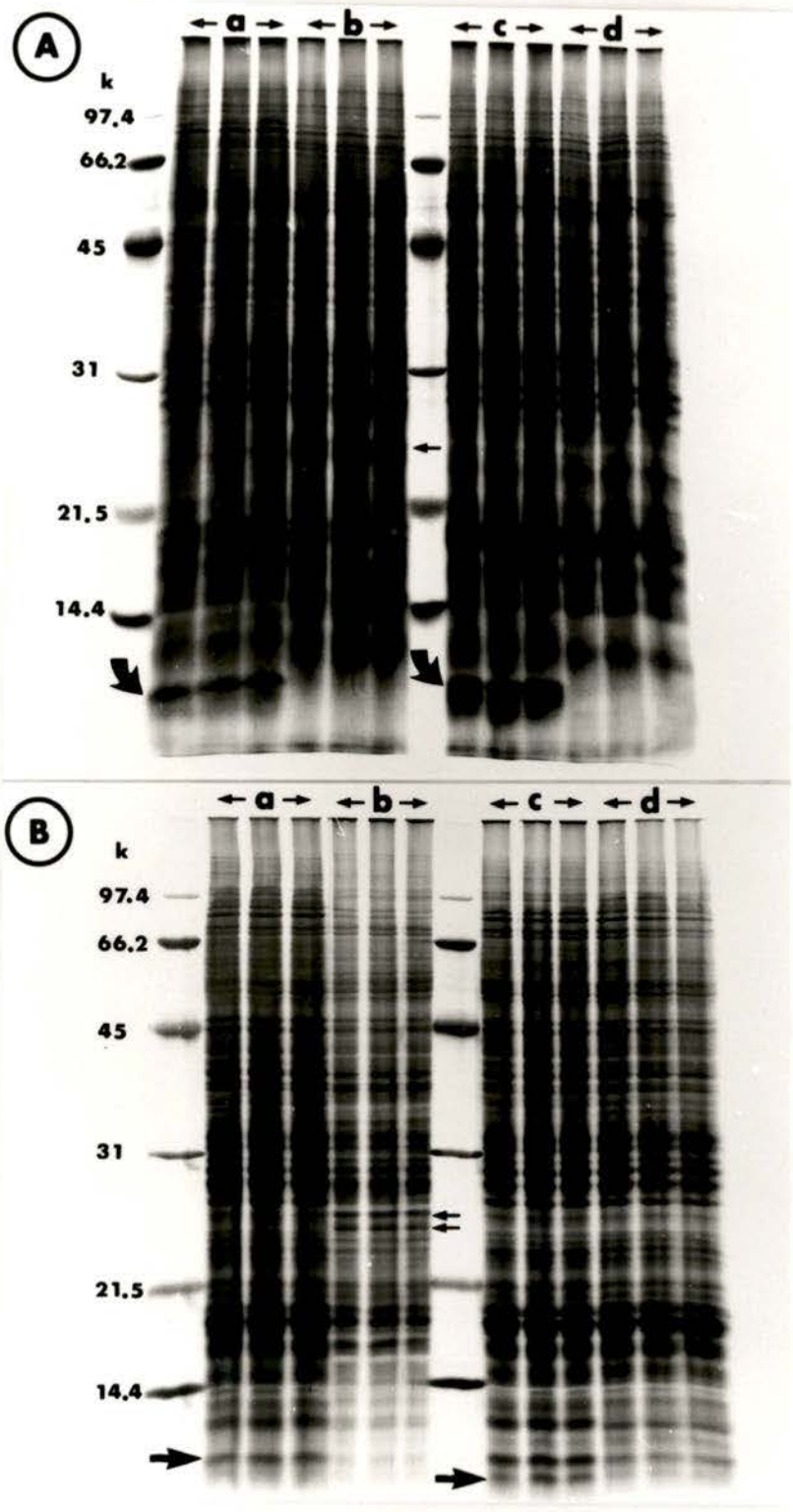

A
a
b
c
d
k
97.4
66.2
45
31
21.5
14.4
B
a
b
c
d
k
97.4
66.2
45
31
21.5
14.4

Table 2.

Summary of the significant (Student t-test, P-value≤0.05) ***quantitative*** differences in *SDS-PAGE* separated proteins found in Puckle Rd (PRd) families (separate experiments; individual families, extracts from resistant and susceptible, lesioned and healthy tissue; in triplicate). Values in the columns represent the *total number* of *significant* proteins found in a specific *category* for a specific family. Family identification is by seedlot (2411, 2413, 2391, 2398). Value in bold represents **26.2 kDa** band found in 2391-113-2 (susceptible). Student t-tests done using triplicate values obtained for each protein band. Res = resistant tree, sampled near bark reaction; Sus = susceptible tree, sampled near canker margin; ResH = resistant tree, healthy tissue; SusH = susceptible tree, healthy tissue.

	Family I.D.			
Category	**2411**	**2413**	**2391**	**2398**
res-unique	3	5	0	4
sus-unique	4	2	4	1
res>sus	1	26	28	0
res<sus	6	0	**1(n.s)**	2
res>resH	1	14	7	4
res<resH	11	2	8	5
sus>susH	5	7	1	24
sus<susH	4	2	19	0
resH<susH	2	0	0	0
resH>susH	6	12	13	13

Table 3.

SDS-PAGE separated proteins found to be **unique** to resistant or susceptible PRd seedlings. Proteins in bold have been sequenced. Data taken from experiments done using individual families (in triplicate).

Family I.D.	Comments
2411-res	54.5 kDa, in healthy tissue
	45.3 kDa, **10.9 kDa**, healthy and SCG zone
2411-sus	33.8 kDa, 33.0 kDa, 25.7 kDa, canker zone
	12.5 kDa, healthy and canker zone
2413-res	44.1 kDa, 22.1 kDa, 20.3 kDa, SCG zone
	24.7 kDa, **10.7 kDa**, healthy and SCG zone
2413-sus	52.0 kDa, canker zone
	19.1 kDa, healthy and canker zone
2391-sus	34.4 kDa, 25.4 kDa, canker zone
	61.9 kDa, 58.2 kDa, healthy and canker zone
2398-res	36.4 kDa, 20.1 kDa, 13.5 kDa, SCG zone
	10.5 kDa, healthy and SCG zone
2398-sus	25.3 kDa, canker zone

Two Dimensional (2-D) Gel Electrophoresis

Figure (3) shows representative silver stained gels of pooled resistant and pooled susceptible bark proteins separated by 2-D electrophoresis (PRd samples). Electrophoretic separation was carried out in duplicate for each site (LC and PRd), and sites were analyzed separately. The number of well resolved spots per gel (pooled samples) was between 300-400.

Lens Creek (LC)

Final analysis of 24 gels revealed 44 proteins to be statistically significant by Student t-test (P-value≤0.05) and a quantitative unbounded foldchange of 2. However, examination of the original gels revealed that most of these protein spots were either located in such a way as to make isolation extremely difficult (i.e. protein located within a cluster of spots), were present in very faint quantities, or were inconsistently represented between or within groups, or in replicates of a given individual. One significant protein (26.5 kDa, pI=6.0) with a foldchange of 3.2 (res>sus) was found to be present in all 24 gels, however with considerable variability both between replicates and between individuals within a group. Other 'qualitative' differences were later discovered to exist only for 1 or 2 individuals within a group rather than for the group as a whole.

Analysis of LC pooled samples revealed both qualitative and quantitative differences between resistant and susceptible groups. From a matchset standard containing 388 spots, 46 spots were found to be significant by Student t-test (P-value≤ 0.05). Of these spots, 5 were unique to the resistant group, 18 were unique to the susceptible group, and 23 were common to both groups. Sixteen of the common proteins were enhanced in susceptible versus resistant groups.

Out of 46 significant spots, 25 spots were found to be significant by *both* Student t-test and a foldchange of less than (-4) and/or greater than 5 (see Table 4). Of these spots, 5 were unique to the resistant group, 18 were unique to the susceptible group, and 2 were common to both groups but significantly increased in the susceptible group. The proteins unique to the resistant group had acidic isoelectric points with molecular weights ranging between 24-38 kDa, while those unique to the susceptible group fell within a wider molecular weight range (14-86 kDa). The 2 'common' proteins had relative molecular masses of 24.1 kDa (pI= 6.0) and 35.3 kDa (pI= 6.6).

While all of these proteins could be considered important, 4 proteins (highlighted in bold, Table 4) were selected on the basis of their qualitative significance, relative abundance, and location on the gel. Gel spot 305 (**25.2 kDa, pI= 5.0**) was present in 2 resistant clones (B643 and G8) while gel spot 8504 (**36.0 kDa, pI= 6.6**) was present in G161 and possibly G8. The native 'resistant' tree displayed neither protein spot. A **39.4 kDa (pI= 5.9**) protein was found in all 4 susceptible individuals (gel spot 6603), while a **32.5 kDa (pI= 5.6**) protein was found in 3 out of 4 of the susceptible individuals (2 'natural' trees, N623 and N2-15, and clone 6149). Attempts to isolate and sequence these proteins were not successful.

Figure 3.

Two dimensional gel electrophoresis separation of pooled bark proteins (representative gel, PRd). Gels were silver stained. Molecular weight markers (17.5 kDa to 76 kDa) are shown to the left (vertical axis). On the horizontal axis, pH increases from left to right (acidic to basic). (A) = pooled resistant; (B) = pooled susceptible. Arrows point to *significant* proteins that were selected on the basis of their relative amount and location in the gel. (k) = kilodaltons

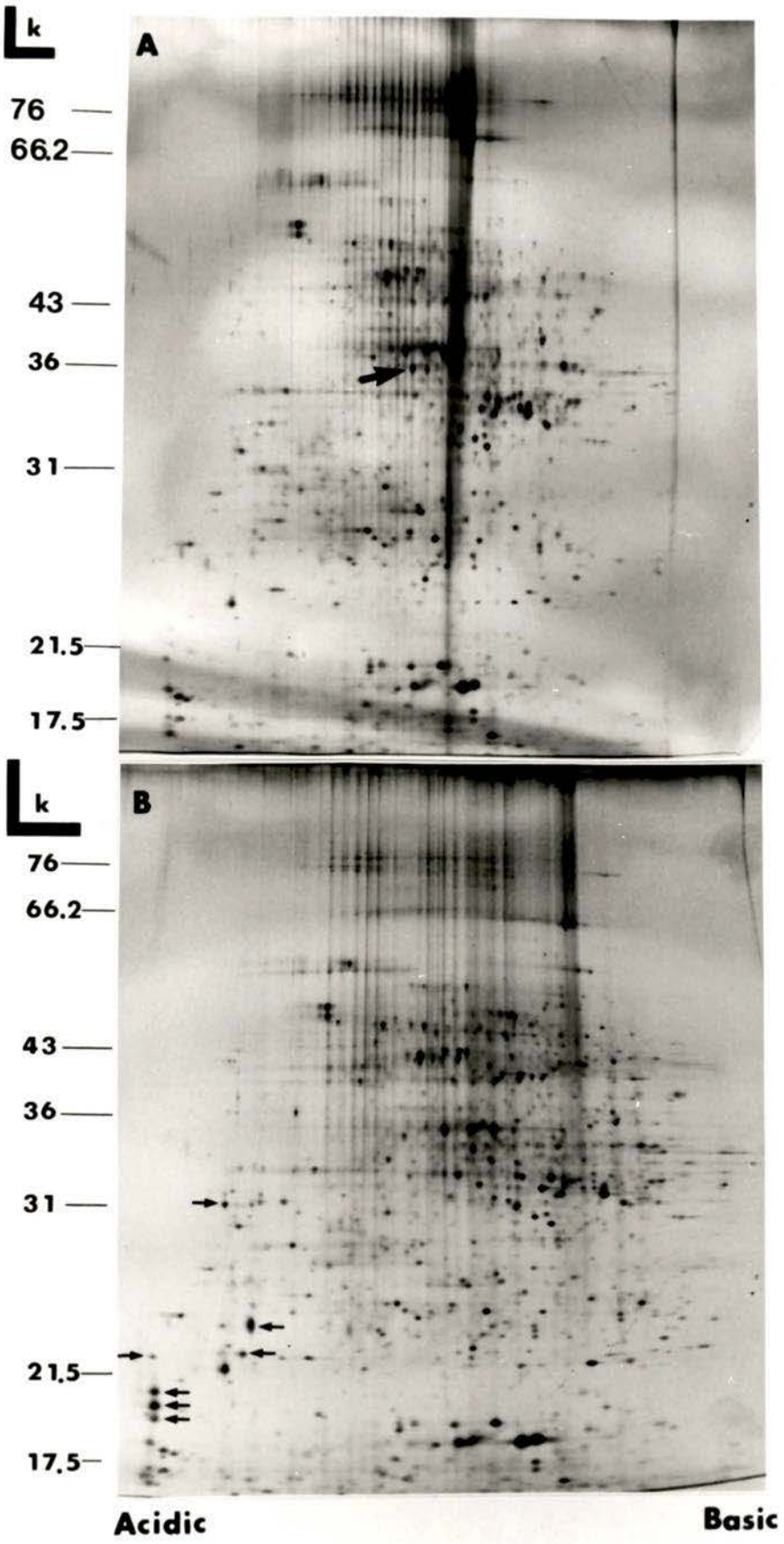

k
A
76
66.2
43
36
31
21.5
17.5
k
B
76
66.2
43
36
31
21.5
17.5
Acidic
Basic

Table 4.

Significant[a] bark proteins in LC pooled susceptible versus pooled resistant *P. monticola* displaying 'mature tree resistance'. Values in bold highlight proteins selected on the basis of relative abundance and position in the gel.

		Protein amount[b] expressed in protein data units (PDU)		
SSp[c]	**pI[d]**	**Mr[e]**	**Sus[f]**	**Res[g]**
305	**5.0**	**25.2**	---	**100.8 ± 6.5**
601	5.2	37.4	---	26.6 ± 3.4
1902	5.4	77.2	30.0 ± 3.3	---
2404	**5.6**	**32.5**	**108.8 ± 11.1**	---
2503	5.5	34.0	---	46.8 ± 5.6
2902	5.6	85.8	80.5 ± 7.7	---
3307	5.7	24.4	62.1 ± 1.2	---
3406	5.7	32.7	36.5 ± 7.7	---
3703	5.7	46.4	39.0 ± 5.6	---
4605	5.7	38.9	35.7 ± 4.4	---
4709	5.7	47.1	52.4 ± 2.7	---
5404	5.9	33.4	61.3 ± 10.7	---
6305	6.0	24.1	270.6 ± 37.2	63.7 ± 13.2
6308	6.0	24.7	---	19.2 ± 0.7
6603	**5.9**	**39.4**	**131.0 ± 3.9**	---
6707	5.9	44.8	53.7 ± 5.8	---
7104	6.4	14.8	104.9 ± 13.2	---
7605	6.3	37.0	91.2 ± 8.1	---
7607	6.5	36.4	262.9 ± 10.1	98.4 ± 24.5
7803	6.2	68.2	34.1 ± 7.1	---
8304	7.0	25.0	43.3 ± 8.1	---
8501	6.6	35.3	155.6 ± 14.0	30.2 ± 23.4
8504	**6.6**	**36.0**	---	**126.7 ± 12.2**
8601	6.5	37.0	74.2 ± 2.2	---
8613	6.6	36.2	24.3 ± 3.7	---

[a] Boolean (Student t-test, P-value≤0.05; foldchange less than -4 and greater than 5.
[b] PDU values are the mean and s.d. of duplicated pooled samples (4 trees per sample, pooled on an equal protein basis).
[c] SSp = sample spot number
[d] pI = isoelectric point
[e] Mr = relative molecular mass (kilodaltons, kDa)
[f] Sus = pooled susceptible bark proteins
[g] Res = pooled resistant bark proteins ('mature tree resistance')

Puckle Rd (PRd)

From a matchset containing 842 spots, significant differences (Student t-test, P-value≤0.05) were found for 146 proteins in the pooled PRd 'resistant versus susceptible' samples. Of these proteins, 14 were unique to the resistant group, 88 were unique to the susceptible group, and 44 were common to both groups. Within the common group, 30 out of 44 proteins were significantly enhanced in the susceptible versus the resistant group.

Table 5 displays 21 proteins selected from the previous 146 on the basis of relative amounts, quantitative foldchange, and position in the gel (for possible future isolation). Sixteen of the 21 spots were significant by a quantitative foldchange of greater than 3. Of those proteins selected, 5 were unique to the susceptible group, 2 were unique to the resistant group, and 14 were common to both groups. Of the 14 common proteins, 7 were enhanced in the resistant group and 7 were enhanced in the susceptible group.

Two-dimensional separation and examination of *individual* bark protein samples revealed that spot 4613 (33.6 kDa, pI= 5.9) was strongly present in 3 out of 4 resistant individuals and weak-moderately present in the fourth (see Figure 4). Spot 1208 (23.3 kDa, pI= 5.1) was strongly present in all four susceptible individuals. However, other spots were not consistently present in all individuals within a group. For example, 1209 (21.7 kDa, pI= 5.1), 402 (31.1 kDa, pI= 5.0), 201 (21.6 kDa, pI= 4.5), 111 (19.0 kDa, pI= 4.5), and 110 (18.0 kDa, pI= 4.5) were strongly present in only 2 out of 4 susceptible individuals. Of 7 selected and flagged (in bold, Table 5; see also Figure 4) spots found in the *pooled* susceptible group, one individual (2413-20-3) was found to have 6 of the protein spots, two other susceptible individuals had 3 of the spots and the remaining susceptible individual had only 1 of the 'significant' flagged proteins. Attempts were made to isolate and sequence spots 4613 (res), 110, 111, 112, and 201 (flagged, see Table

5) without success.

Table 5.

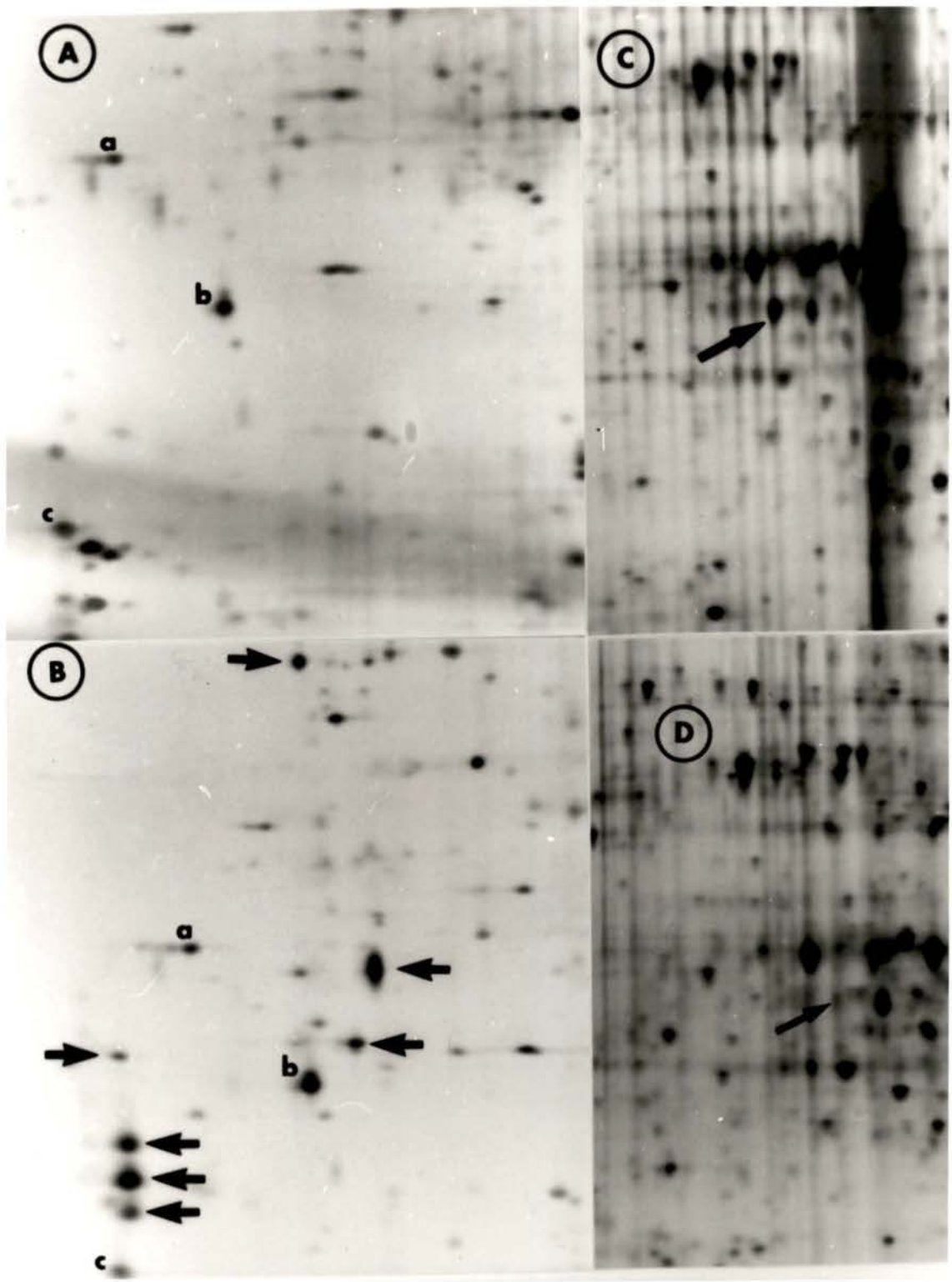
Significant[a] bark proteins in PRd pooled susceptible versus pooled resistant *P. monticola* seedlings displaying the SCG bark reaction. Values in italics are significant by Student t-test only. Values in bold indicate proteins selected for further study on the basis of their relative amounts and their location in the gel.

		Protein amount[b] expressed in protein data units (PDU)		
SSp[c]	pI[d]	Mr[e]	Sus[f]	Res[g]
16	4.5	13.9	76.2 ± 11.9	---
106	---	13.9	314.5 ± 65.2	---
110	**4.5**	**18.0**	**1040 ± 118**	**18.9 ± 5.3**
111	**4.5**	**19.0**	**620.6 ± 104**	**18.5 ± 7.8**
112	**4.5**	**17.2**	**419.0 ± 73.6**	---
201	**4.5**	**21.6**	**125.4 ± 23.9**	**18.6 ± 0.9**
204	5.0	21.8	135.5 ± 22.4	---
205	5.0	23.3	101.8 ± 16.4	23.7
402	**5.0**	**31.1**	**237.0 ± 13.7**	**14.8**
1208	**5.1**	**23.3**	**829.4 ± 92.1**	**46.4 ± 2.0**
1209	**5.1**	**21.7**	**331.8 ± 54.9**	---
3304	*5.8*	*27.1*	*134.0 ± 16.6*	*274.5 ± 3.9*
4610	*5.9*	*34.6*	*333.7 ± 14.3*	*497.3 ± 13.2*
4613	***5.9***	***33.6***	***124.1***	***347.9 ± 16.9***
6412	6.7	31.1	---	101.3 ± 6.2
7108	7.2	16.5	72.7 ± 2.6	17.5 ± 9.3
7511	7.1	31.8	102.6 ± 17.8	479.2 ± 9.6
7518	7.0	32.4	---	209.3 ± 31.4
8008	8.0	13.8	31.9	152.6 ± 5.6
8518	7.7	32.4	20.7	167.9 ± 16.2
8604	7.8	33.6	*192.9 ± 58.1*	*484.5 ± 3.8*

[a] Boolean (Student t-test, P-value≤0.05; foldchange greater than 3)
[b] PDU values are the mean and s.d. of duplicated pooled samples (4 trees per sample, pooled on an equal protein basis)
[c] SSP = sample spot number
[d] pI = isoelectric point
[e] Mr = relative molecular mass (kilodaltons, kDa)
[f] Sus = pooled susceptible (proteins from 4 trees)
[g] Res = pooled resistant (proteins from 4 trees)

Figure 4.

PRd pooled resistant and pooled susceptible bark proteins separated by two-dimensional electrophoresis and silver stained. 'A' and 'B' are left lower corner close-ups of resistant and susceptible samples respectively (see Figure 3.). Small letters 'a,b, & c' identify landmark proteins present in both samples. Arrows in 'B' point to significant proteins in the 'susceptible' group (from top to bottom, ssp 402, 1208, 1209, 201, 111, 110, 112). 'C' and 'D' are centre-gel close-ups of resistant and susceptible PRd pooled samples (*cf* Figure 3.). Single arrow in 'C' and 'D' points to ssp 4613. Ssp 4613 is visually more enhanced in pooled resistant versus susceptible samples.


A
a
b
c
C
B
a
b
c
D

PRd-Healthy tissue samples

From a matchset containing 526 spots, a total of 81 proteins were found to be significant by Student t-test (P-value≤0.05) in pooled bark extracts taken from the healthy tissue of resistant and susceptible seedlings. Out of this total, 18 proteins were unique to pooled resistant, 35 were unique to pooled susceptible and 28 were common to both. Of the common proteins, 24 out of 28 were enhanced in resistant versus susceptible groups. This represents a reversal in the trend observed for tissue sampled near the lesioned areas, where it was seen that susceptible rather than resistant groups tended to be enriched in 'shared proteins'.

Table 6 shows 21 proteins selected from the above 81 significant spots, using previously described criteria. The quantitative foldchange used in this case was greater than 2. Five proteins were unique to the resistant group and 4 were unique to the susceptible group. Of the 13 common proteins, only 3 were significantly enhanced in the susceptible group. Proteins highlighted in bold represent those sufficiently distanced from other spots, making future isolation somewhat more feasible. Isolation of significant proteins was not attempted due to difficulties encountered with earlier attempts at preparative electrophoresis.

Table 7 and Figure 5 briefly summarize results obtained from the 3 independently made matchsets (LC, PRd, PRd-healthy). The PRd susceptible group had by far the greatest number of unique proteins (88). In all three matchsets there were more *unique* proteins in susceptible versus resistant groups. In the LC matchset, common proteins represented 50% of the significant spots whereas in the PRd matchsets, common proteins represented 30% and 35% of the total number of significant spots in pooled samples from near-lesioned, and healthy tissue respectively. In all but the PRd-healthy tissue samples,

common proteins were more frequently significantly increased in susceptible versus resistant groups.

Table 6.

Significant[a] bark proteins in PRd pooled 'healthy' susceptible versus pooled 'healthy' resistant *P. monticola* seedlings displaying the SCG bark reaction. Bark sampled from disease-free regions. Values in italics are significant by Student t-test only.

		Protein amount[b] expressed in protein data units (PDU)		
SSp[c]	**pI[d]**	**Mr[e]**	**Sus[f]**	**Res[g]**
213	---	**17.2**	**181.1 ± 50.0**	**405.6 ± 13.7**
502	---	28.6	86.1 ± 10.1	148.0 ± 4.0
1111	4.8	16.1	---	59.1 ± 7.6
1401	**5.0**	**25.3**	**102.7 ± 26.9**	**232.2 ± 12.1**
1503	4.6	31.5	200.9 ± 29.2	---
1805	4.6	49.8	58.7 ± 0.6	23.7 ± 3.7
2507	5.3	29.4	166.0 ± 3.4	---
2606	5.3	31.8	113.8 ± 10.6	---
3502	5.5	29.3	219.3 ± 45.9	---
3603	**5.5**	**33.7**	**188.2 ± 21.3**	**74.9 ± 2.4**
5704	5.7	34.6	117.8 ± 0.9	---
6205	**5.8**	**16.5**	**213.2 ± 14.1**	**36.7 ± 0.9**
6402	5.7	26.7	122.2 ± 6.3	274.9 ± 34.0
6604	**5.8**	**32.9**	**55.0 ± 4.5**	**207.0 ± 4.4**
6707	5.8	35.5	---	490.2 ± 105.6
7702	5.8	34.4	69.8 ± 4.8	150.3 ± 11.9
7709	*5.9*	*35.4*	*254.9 ± 5.9*	*405.6 ± 23.8*
7715	5.9	35.0	---	399.3 ± 45.7
8606	6.5	33.8	---	141.3 ± 1.3
8707	6.2	35.6	---	149.8 ± 5.6
9209	6.6	16.3	89.7 ± 9.2	41.4 ± 1.9

[a] Boolean (Student t-test, P-value≤0.05 and foldchange of 2

[b] PDU values are the mean and s.d. of duplicate pooled samples (4 trees per sample pooled on an equal protein basis)

[c] SSp= sample spot number

[d] pI= isoelectric point

[e] Mr= relative molecular mass (kilodaltons, kDa)

[f] Sus= pooled susceptible bark proteins

[g] Res= pooled resistant bark proteins

Table 7.

Classification (i.e. unique or common) of statistically significant[a] bark proteins separated by two-dimensional (2-D) electrophoresis. Separation done on pooled protein extracts (4 trees per sample).

Bark source	Unique	Unique	Common	Total
	Sus	***Res***		
LC[b]	18	5	23	46
PRd[c]	88	14	44	146
PRd-H[d]	35	18	28	81

[a] Significant by Student t-test, P-value≤0.05
[b] Lens Creek pooled samples
[c] Puckle Rd pooled samples (near reaction/canker site)
[d] Puckle Rd pooled samples (healthy bark tissue)

Figure 5.

Composition Profile of Significant Proteins from Lens Creek (LC), Puckle Rd (PRd), and Puckle Rd-Healthy (PRd-H) matchsets. Horizontal axis = bark source (i.e. location of sampling); Vertical axis = number of proteins; 'res' = proteins unique to resistant group; 'sus' = proteins unique to susceptible group; 'shared' = proteins common to both groups but enhanced in one or the other.

A comparison of the total number of bark proteins (unique & shared) found in blister rust-resistant versus susceptible white pine.

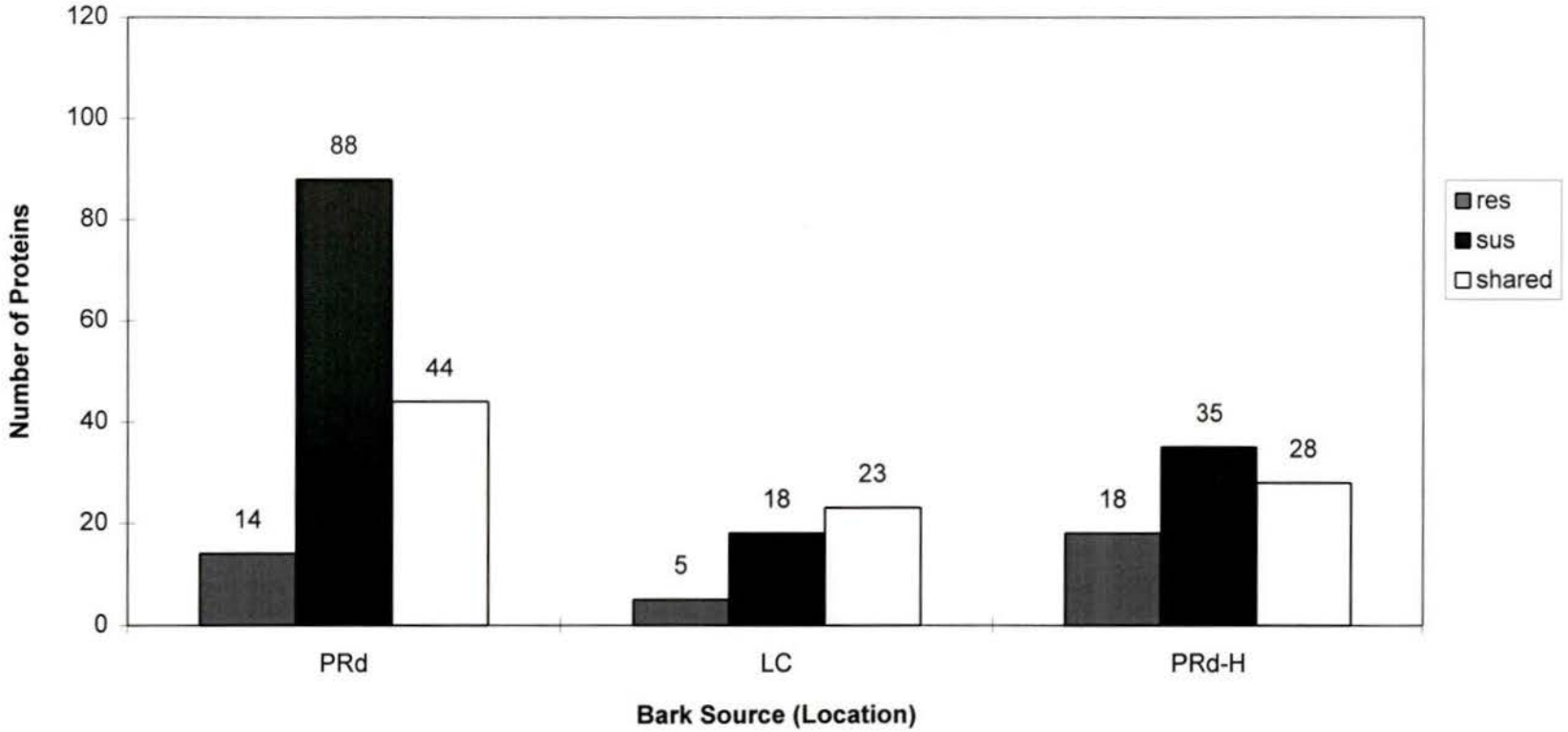


Figure 6.

Anti-Pin m III immunoblot (LC). Total protein loaded per lane = 5μg. Arrow points to ≈19 kDa band. 'sus' = susceptible; 'res' = resistant. Upper blot consists of bark proteins from October (1993) harvest while lower blot consists of bark proteins from April (1991) harvest. Susceptible lanes visually more intense in both immunoblots. (k) = kilodaltons

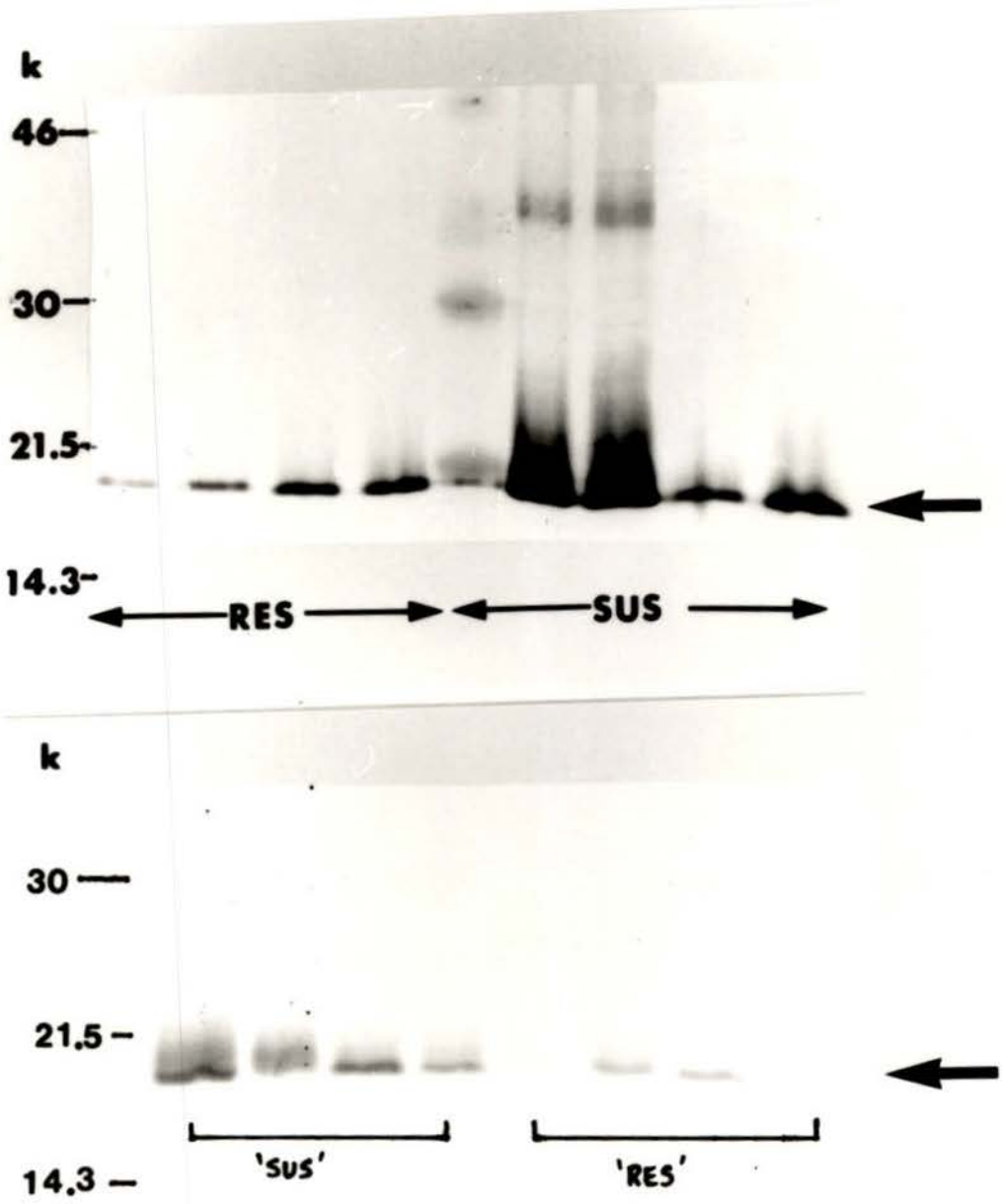

k
46
30
21.5
14.3
RES
SUS
k
30
21.5
14.3
'SUS'
'RES'

Figure 7.

Comparison of the relative amounts of a 19 kDa immunoreactive protein in LC susceptible versus resistant individuals. Quantity expressed in optical density units (O.D.xMM).

Relative abundance of a 19 kDa 'cold hardiness' related protein (PinmIII) in susceptible versus resistant Western white pine from Lens Creek. Susceptible (N2-15, N6-23, G27, 6149). Resistant (G161, G8, B643, N0-7).

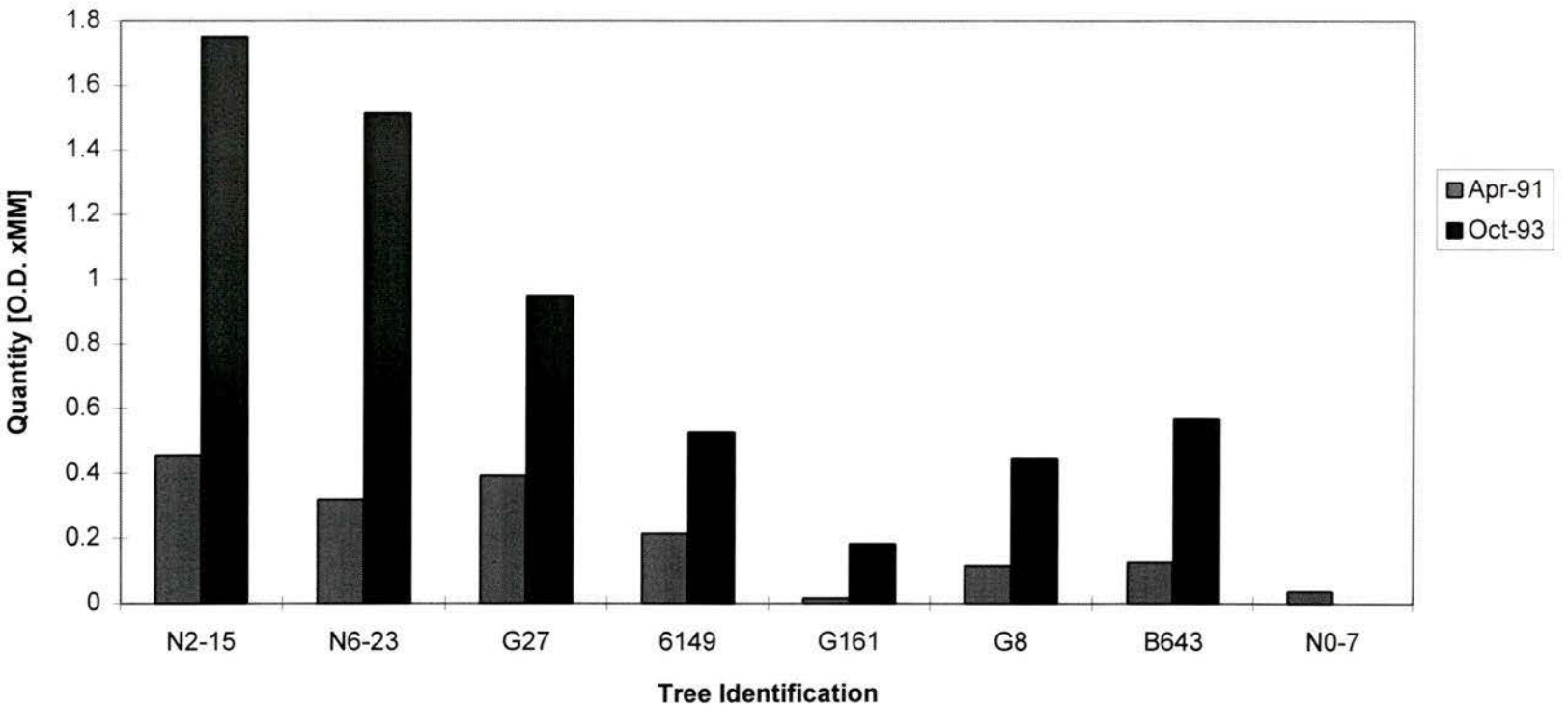


Figure 8.

Isoforms of a 19 kDa immunoreactive (Pin m III) protein. Total protein loaded per sample (2-D gel electrophoresis and subsequent Western blot) was 50 μg. 'A'= PRd seedling (2413-20-6, resistant); small arrows point to 5 acidic isoforms. 'B' = comparison of 'susceptible' (left upper = '6149'; left lower = G27) and 'resistant' (right upper = G161; right lower = G8) individuals. Susceptible individuals (6149 and G27) have 5 isoforms while resistant individuals (G161 and G8) have 1 and 3 isoforms respectively. 'C' = identification of the isoforms of the 19 kDa immunoreactive band in a silver stained 2-D gel. Four open arrows point to 4 identifiable isoforms.

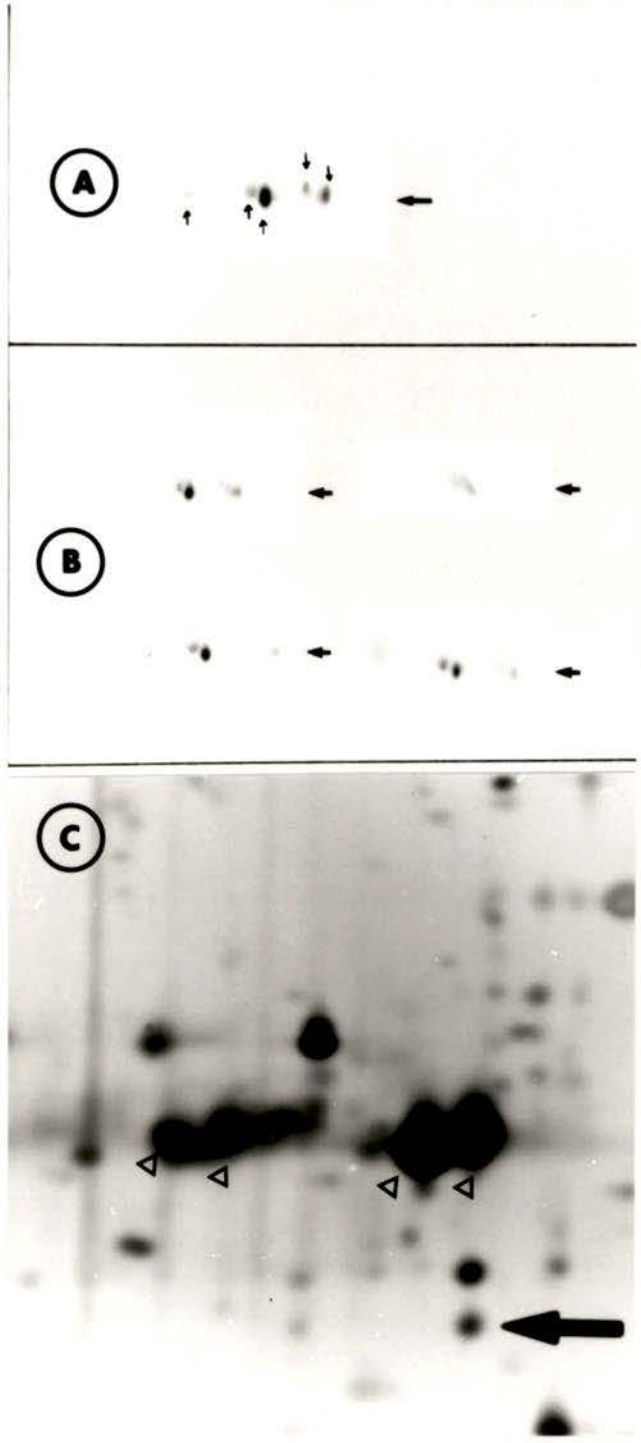

A
B
C

Figure 9.

Anti-PSII immunoblot of LC samples. Total protein loaded per lane = 20μg. Lanes 'a,b,c, & d' represent 'susceptible' individuals. Lanes 'e,f,g, & h' represent 'resistant individuals. Arrow points to single immunoreactive band of approximately 22-23 kDa.

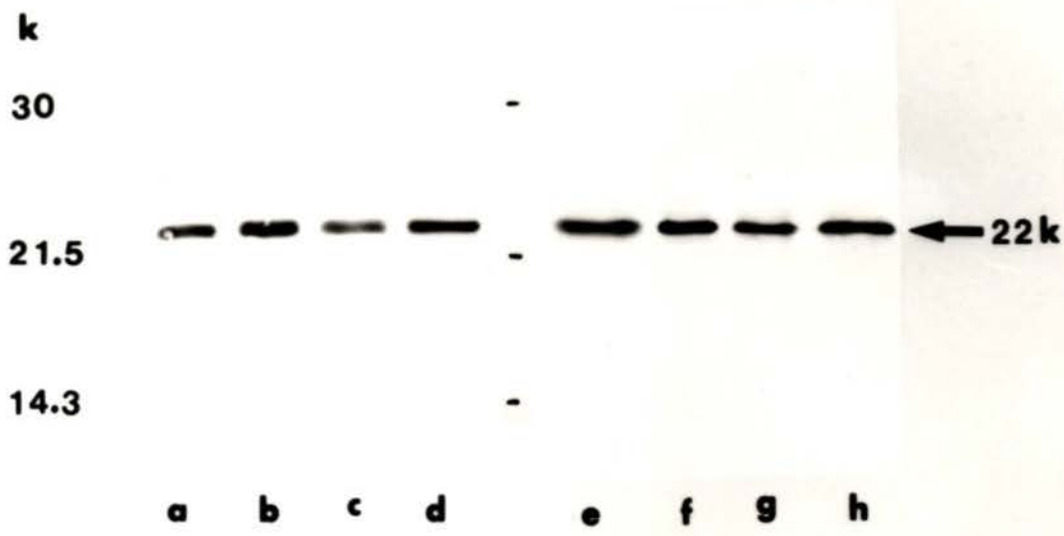
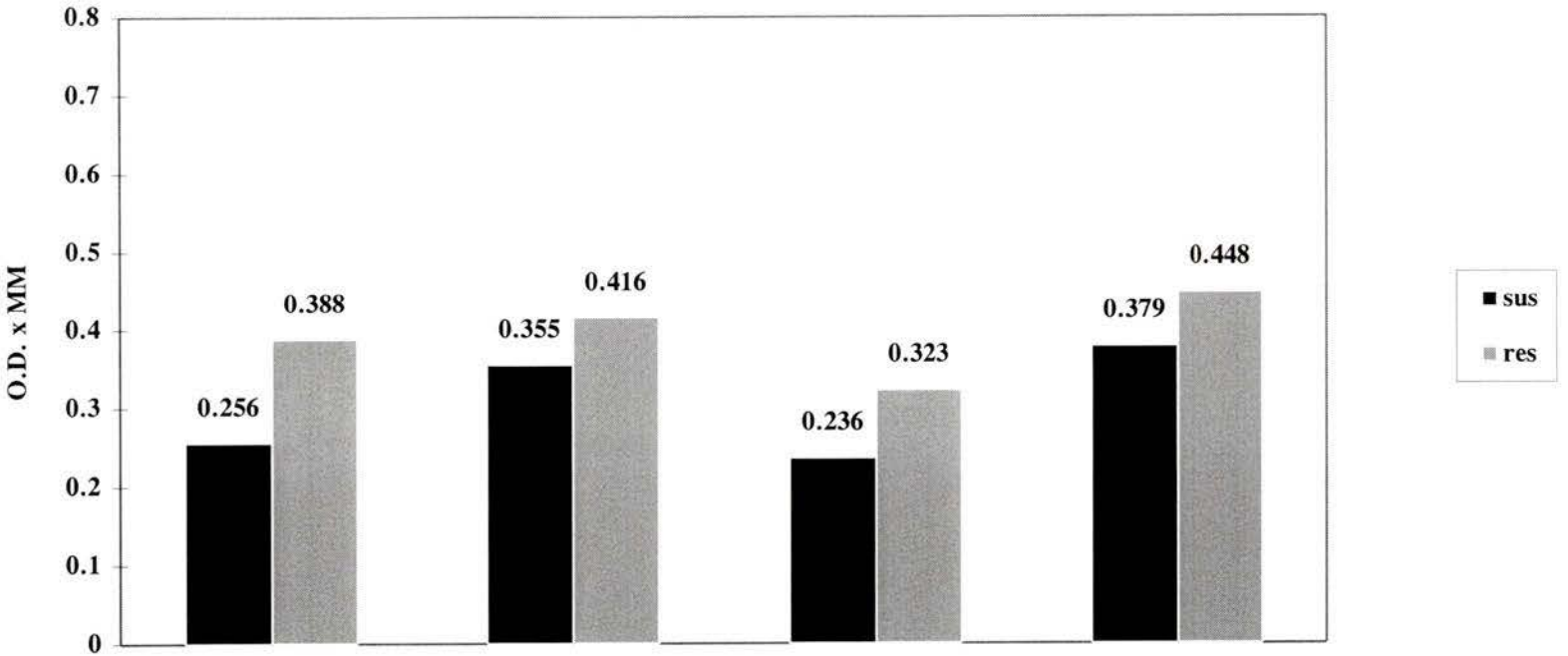

k
30
21.5
14.3
22k
a
b
c
d
e
f
g
h

Figure 10.

Relative quantity (O.D.xMM) of a ≈23 kDa immunoreactive band (anti-PSII) in LC individual bark samples.



PSII immunoblot: Quantitation in Optical Density (O.D.) units

Immunodetection: Anti-PinmIII and Anti-PSII polyclonal antibodies.

Anti-Pin m III

Figure 6 shows an anti-Pin m III immunoblot of LC individuals sampled during October of 1993 and April of 1991 (same individuals sampled on both dates). It can be seen for both dates that immunoreactivity of an approximately 19 kDa band is visually more intense in the susceptible individuals versus individuals displaying 'mature tree resistance'. This observation was quantified (O.D. x MM) using the ONED™ software described earlier. Figure 7 shows the quantitative relationship between phenotype and immunoreactivity to this antibody. It is of note that no crossreactivity was seen in April samples, while minimal crossreactivity was observed in the October samples from 2 susceptible trees ('natural', N6-23 and N2-15).

The difference between PRd 'susceptible versus resistant' individuals was not as visually pronounced as it was for the LC samples (not shown). It is noteworthy that the PRd samples were harvested in November.

Immunodetection with anti-Pin m III after two-dimensional separation and Western blot immobilization revealed 5 acidic immunoreactive isoforms (Figure 8) of the 19 kDa 'cold' protein. Although these isoforms were present in both LC and PRd samples, resistant LC samples appeared to have fewer isoforms than their susceptible counterparts.

Anti-PSII

Immunodetection with anti-PSII was done only with the LC samples (April 1991 bark harvest). Figure 9 shows an immunoblot with a single band of approximately 22-23 kDa. There was no obvious visual difference in color intensity between susceptible (a,b,c, & d) and resistant (e,f,g, & h) individuals. Quantitative analysis of the scanned blot revealed a slight increase in resistant over susceptible (Figure 10), and a paired t-test of

these results was significant (P = 0.012). Investigations were not pursued further because of time constraints.

Amino Acid Analyses

N-terminal sequence analysis

Figure 11 shows the N-terminal sequence analyses of the 10.5 kDa, 26.1 kDa, and 19.2 kDa bands, respectively. No significant sequence homologies were obtained when the sequences of the first two were submitted to the data bank. The 19.2 kDa protein displayed 94% identity with the cold ("dormancy related protein") protein isolated from sugar pine. Out of 20 amino acids, only valine at position 13 differed from that found in sugar pine.

Figure 11.

A.) N-terminal sequence analysis of **10.5 kDa** major band from '2411-11-7' (res) *Pinus monticola* bark protein. Band present in resistant PRd seedlings.

B.) N-terminal sequence analysis of **26.1 kDa** major band from '2391-113-2' (sus) *Pinus monticola* bark protein.

C.) N-terminal sequence analysis of **19.2 kDa** immunoreactive protein (anti-PinmIII) from '2411-72-5' (sus) *Pinus monticola* bark. Amino acid in bold is the only one that differs from that found in the N-terminal sequence of *P. lambertiana*

A. 10.5 kDa

1 5 10 15 20

ser-tyr-phe-ser-ala-trp-ala-gly-pro-gly-X-asn-asn-his-asn-ala-arg-tyr-ser-lys-lys-gly-val

B. 26.1 kDa

1 5 10 15

ala-val-ser-asp-ile-gln-asn-gln-asp-phe-phe-asn-gly-ile-leu-ser-ala-ala-X

C. 19.2 kDa

1 5 10 15 20

val-ser-gly-thr-ser-ser-thr-glu-glu-val-val-gln-**val**-glu-ala-arg-arg-leu-tryp-asn-ala-

25

thr- thr-lys-asp-X-his

Composition analysis of Bands immunoreactive to anti-PSII

Three 'candidate' bands were chosen for composition analysis. All 3 bands were very close together and within the 22-25 kDa molecular weight range. Because of their close proximity to each other, it was difficult to determine which of the 3 bands corresponded to the immunoreactive band, thus the decision to analyze al of them. The results for each band are taken from 6 replicates- a replicate being an individual tree. Tables 8 to 10 show the results obtained for the 3 bands. It is readily apparent that none of the bands demonstrates a high proline content, however the approximately 24 kDa band appears to be relatively glycine-rich (see Table 8).

Table 8.

Amino acid composition (mole%) of ≈ 24 kDa immunoreactive band (anti-PSII) for 6 LC samples.

	Tree I.D.						
Residue	**G8**	**G161**	**N07**	**G27**	**6149**	**N2-15**	**Avg±σxn**
asx	10.9	8.5	9.9	8.1	7.8	9.4	9.1 ± 1.1
glx	13.3	12.9	14.2	13.1	13.1	12.1	13.1 ± 0.6
ser	13.7	9.0	9.1	9.3	8.7	11.1	10.2 ± 1.8
gly	**27.8**	**20.2**	**20.5**	**19.2**	**19.9**	**23.4**	**21.8 ± 3.0**
his	---	1.4	---	---	---	---	n.d.
arg	---	4.3	3.6	3.1	3.1	3.7	3.6 ± 0.4 (n=5)
thre	---	3.9	3.9	4.2	3.8	3.7	3.9 ± 0.2 (n=5)
ala	**10.0**	**6.9**	**9.4**	**8.4**	**7.5**	**7.3**	**8.3 ± 1.1**
pro	---	**3.5**	**3.2**	**4.1**	**4.2**	**3.0**	**3.6 ± 0.5 (n=5)**
tyr	---	3.4	3.3	3.2	3.5	3.4	3.4 ± 0.1 (n=5)
val	6.8	5.1	5.6	7.1	8.0	5.5	6.4 ± 1.0
met	---	1.0	---	---	---	---	n.d.
ileu	---	3.6	3.2	4.1	4.4	3.3	3.7 ± 0.5 (n=5)
leu	6.7	7.6	6.6	8.1	8.3	5.9	7.2 ± 0.9
phe	---	3.4	2.8	4.0	4.2	3.2	3.5 ± 0.5 (n=5)
lys	10.8	5.3	4.8	4.0	3.6	5.0	5.6 ± 2.4

Table 9.

Amino acid composition (mole%) of a ≈ 25 kDa immunoreactive protein band (anti-PSII) for 6 LC bark protein samples.

	Tree I.D.						
Residue	**G8**	**G161**	**N07**	**B643**	**G27**	**N2-15**	**Avg±σxn**
asx	9.8	3.6	10.0	7.8	9.2	4.2	7.4 ± 2.6
glx	10.8	4.9	12.7	12.4	11.7	6.2	9.8 ± 3.1
ser	8.2	7.0	8.2	6.2	7.3	5.8	7.1 ± 0.9
gly	**14.1**	**12.9**	**12.0**	**10.6**	**10.0**	**12.1**	**12.0 ± 1.4**
his	---	---	---	0.8	---	---	n.d.
arg	5.2	3.9	5.3	4.1	5.1	2.9	4.4 ± 0.9
thre	4.2	5.5	4.6	4.1	4.7	5.4	4.8 ± 0.5
ala	**10.7**	**12.6**	**10.7**	**11.7**	**8.9**	**12.2**	**11.1 ± 1.2**
pro	**3.8**	**6.3**	**4.1**	**3.8**	**5.3**	**7.0**	**5.1 ± 1.3**
tyr	2.2	2.2	2.1	1.7	2.4	1.9	2.1 ± 0.2
val	8.2	10.8	9.5	10.5	8.7	12.1	10.0 ± 1.3
met	---	---	---	---	---	---	---
ileu	6.0	8.8	7.2	7.7	6.6	9.2	7.6 ± 1.1
leu	8.6	12.5	10.0	10.7	9.0	12.7	10.6 ± 1.6
phe	2.7	4.5	3.5	3.1	5.4	5.0	4.0 ± 1.0
lys	5.7	4.9	0.3	4.9	5.8	3.1	4.1 ± 1.9

Table 10

Amino acid composition (mole%) of a ≈ 23 kDa immunoreactive (anti-PSII) band found in bark protein extracts from 8 LC individuals.

	Tree I.D.								
Residue	**G8**	**G161**	**N07**	**B643**	**G27**	**N2-15**	**6149**	**N6-23**	**Avg±σxn**
asx	11.2	12.5	6.3	12.0	10.7	12.2	8.3	11.9	10.6±2.1
glx	10.2	11.3	6.0	10.3	9.8	11.9	8.1	11.6	9.9±1.9
ser	6.6	7.0	6.0	7.1	6.5	6.8	6.0	7.1	6.6±0.4
gly	**10.8**	**11.7**	**12.1**	**11.4**	**10.6**	**11.6**	**10.7**	**11.9**	**11.4±0.5**
his	---	---	---	---	---	---	---	---	---
arg	5.8	5.7	---	4.9	5.0	5.4	4.7	4.9	5.2±0.4
thre	4.4	4.6	3.8	4.3	4.1	4.4	4.0	4.4	4.3±0.2
ala	**11.6**	**11.2**	**13.2**	**12.1**	**11.4**	**11.0**	**12.1**	**12.2**	**11.9±0.7**
pro	**3.5**	**3.7**	**6.1**	**3.3**	**3.5**	**3.8**	**4.5**	**4.0**	**4.1±0.8**
tyr	2.8	3.2	3.2	2.9	2.7	3.1	3.0	2.7	3.0±0.2
val	7.1	6.9	12.0	6.9	7.8	7.4	9.2	7.6	8.1±1.6
met	2.3	---	---	1.5	1.7	---	---	---	n.d.
ileu	4.3	3.9	6.7	3.9	4.9	4.0	5.6	3.9	4.7±1.0
leu	7.6	6.8	12.7	7.2	8.4	7.6	9.6	6.9	8.4±1.9
phe	3.8	3.4	6.0	3.6	4.3	3.6	5.1	3.2	4.1±0.9
lys	8.1	8.2	6.0	8.8	8.6	7.4	9.3	7.5	8.0±1.0

CHAPTER 4

DISCUSSION

SDS-PAGE

With the exception of the **19.2 kDa** band detected by anti-Pin m III, there were *no consistent* qualitative or quantitative one-dimensional protein profile differences between resistant and susceptible individuals from Lens Creek. Subtle differences were seen *within* groups; however, for the purposes of this study it was not possible to identify with confidence any 'significant' proteins associated with resistance or susceptibility. However, from the *many* qualitative and quantitative protein differences found within and between PRd families, **3** significant proteins were selected and successfully sequenced. Because considerable variation was seen within groups *and* families, only those proteins found to be present and/or significantly modulated in at least 3 out of 4 families were chosen.

A **26.2 kDa** band found to be enriched near cankered tissue in a susceptible (family 2391) individual did not share amino acid sequence homology with any known protein, but appeared to line up with a unique **26.0 kDa** band detected by barley anti-chitinase in the same susceptible individual. This same protein was also shown to be present in *susceptible* and absent in *resistant* individuals (sampled near lesion) from the remaining 3 families. Even *if* this protein should prove to be fungal in origin rather than a host product, it may still provide a valuable marker for the detection of mycelial extension before bark symptoms become obvious. However, the possibility that the same protein is of host origin, but is being *suppressed* in resistant individuals cannot be entirely ruled out as corresponding bands have been shown to be present in the *healthy* tissue from 2 families (2411 and 2398; both resistant and susceptible individuals). Further investigation is warranted.

An approximately **10.5 kDa** band found to be selectively enriched in resistant versus susceptible PRd families has been characterized by partial N-terminal amino acid sequencing. Although it does not share sequence homology with known proteins, it may potentially serve as a *marker* if further studies with larger sample sizes demonstrate a positive correlation between phenotypic blister-rust resistance and the presence of this protein. Furthermore the presence of this protein in *young seedlings* facilitates its potential utilization as an early screening device.

The third protein selected and characterized by N-terminal sequence analysis was a single**19.2 kDa** band detected by anti-Pin m III polyclonal antibodies and subsequently shown to demonstrate 94% homology with a dormancy-related protein in sugar pine. *Susceptible* individuals from Lens Creek were especially enriched in this band compared to resistant individuals as will be discussed later. Although the abundance of this protein has been shown to be seasonally affected [Ekramoddoullah *et al.*, 1994] in western white pine foliage, bark collections made from the same LC individuals in April (1991), October (1993), and January (1994) appeared to demonstrate *similar* patterns (i.e. susceptible individuals showed a greater relative abundance of the 19.2 kDa protein).

Two-dimensional (2-D) gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis is a powerful analytical (and preparative) technique that has been in use for almost 20 years to resolve complex mixtures of proteins and polypeptides [O'Farrell, 1975], and has contributed -with the assistance of automated gel scanning and computer-based analysis- to the development of a large consortium of protein databases [Komatsu *et al.*, 1993]. Nevertheless, 2-D gel electrophoresis is not without its technical drawbacks, some of which may profoundly affect the interpretation of protein data if not accounted for. There are as many entry points for artifactual variation as there are steps in the process of getting from bark sample to silver-stained, scanned and analyzed two dimensional gel. To illustrate, LC

bark samples were originally run *individually*, in triplicate (24 scanned gels used in a matchset). Unfortunately, limitations imposed by the amount of available computer memory made it impossible to create a matchset that would incorporate both gel images and gel spots, necessitating omission of 24 gel images. Furthermore, unavoidable gel to gel variation (in the same gel run, using the same protein sample) contributed greatly to the amount of confounding artifact (the final standard gel spot image had 1249 spots!). Because *meaningful* comparisons of 2-D gels are possible only when 1) streaking, smearing, and background staining are minimal, 2) gels lack artifacts due to proteolysis or other introduced factors, and 3) protein patterns are reproducible, it was decided to *pool* resistant and susceptible individuals in order to 1) effectively push 'common' proteins into the background while lifting out potentially significant proteins and 2) provide a more *manageable* set of gels for each experiment (4 gels and a standard versus 24 gels) so as to facilitate a more meaningful analysis. Pooling of samples also helped to eliminate some of the artifact due to subtle changes in experimental conditions unavoidable with multiple electrophoresis runs.

Factors affecting 2-D gel quality

Sample Preparation

The most important step in determining the eventual quality of 2-D protein separation is that of sample preparation. Incomplete sample solubilization reduces the amount of protein entering the first dimension gel and adversely affects IEF resolution [Dykstra and Wang, 1992]. *Total* protein extracts from tissue samples include cytoplasmic, extracellular, and *membrane*-bound elements. Although *solubilized* proteins from whole plant tissues (and *in vitro* translation products) can be separated with relative ease, separation of *membrane* proteins tends to be complicated by inconsistencies in the degree to which *disruption* and *solubilization* of membrane complexes occurs. Furthermore, tissues that have very low amounts of protein (such as bark) require

extraction procedures that are especially efficient. Thus the extraction protocol should consistently maximize both the yield and solubilization of the proteins represented in a given tissue. The use of *4% SDS* and 5% mercaptoethanol in the currently used extraction protocol facilitated the solubilization and re-dissolution of extracted and precipitated bark proteins. Attempts to re-dissolve bark protein pellets with either an IEF lysis buffer (Solution 'E'), or a modified ES-2 (with 2% SDS) were *not* successful, hence the necessity of using 4% SDS was absolute. The bark protein extraction technique was consistent in terms of protein yield (1-2μg/μl of protein in extract) and in the reproducibility of protein patterns obtained with SDS-PAGE and 2-D electrophoretic separation. Low protein yield (≈1% of lyophilized and ground tissue) may have been due to the formation of insoluble protein-tannin precipitates in the acidic extraction solution (pH≈ 3.0)- which may have formed despite the addition of PVP. Studies have shown that proanthocyanidin contamination of protein molecules is partially dependent on hydrophobic interactions in addition to the reactions between oxidized phenolics and proteins- which helps to explain the persistence of 'self-tanning' reactions of solubilized extracts despite the addition of PVPP [Nyman, 1985].

Other Sources of Artifact

Apart from the quality of sample preparation, most 2-D gel artifact results from subtle differences in *electrophoresis conditions* or in *silver staining* methods. Despite claims that proteins solubilized in SDS are not suitable for application directly onto IEF gels due to the anionic nature of the detergent [Hurkman and Tanaka, 1986], no major difficulties were encountered when the SDS-extracted and re-solubilized bark proteins were applied to the first dimension. This may be partially due to the presence of NP-40 in both ES-2 and solution 'E' (used to dilute protein samples prior to loading onto IEF gels), which may have helped to 'strip' excess SDS from the protein molecules. A distinct 'SDS-bulge' was noted at the acidic end of the IEF gel upon extrusion. Addition of 20mM

CHAPS to the Millipore IEF gel solution recipe appeared to improve spot resolution when compared to early 2-D gels. CHAPS increases the solubility of protein samples as they migrate through the gel during isoelectric focusing [Hochstrasser *et al.*, 1988].

Background gel staining (silver stain), likely due to inadequate clearance of SDS from the second dimension slab gels, was generally uniform with occasional irregularities. Exposing stained gels to incandescent light (from a light table) had the effect of mildly 'smoothing' some of the background silver stain, however it did not eliminate it entirely. Fading of background stain was also noted upon gel drying. Other sources of artifact noted upon silver staining of slab gels included random vertical 'point streaking' as well as an occasional heavy vertical streaking near gel centres. Vertical point streaking is generally attributed to the presence of particulates trapped within the polyacrylamide matrix that are partially solubilized by the use of thiol-reducing agents in the silver staining procedure [Görg *et al.*, 1987]. Studies have shown that the inclusion of *iodoacetamide* in the buffer used to equilibrate IEF gels prior to second dimension electrophoresis reduces such streaking- presumably by scavenging excess thiol-reducers. Similarly, *excluding* DTT from the gel equilibration buffer has the same effect, but at the expense of protein spot resolution [Görg *et al.*, 1987].

The fact that a *broad* range of molecular weights (from less than 17 kDa to greater than 76 kDa) were *consistently* represented in the stained gels, and that protein patterns were *reproducible* indicates that 1) the *SDS*-extraction procedure used in this study facilitated the solubilization of the majority of bark proteins *without* adversely affecting the quality of silver stained 2-D gels and that 2) the same extraction procedure effectively inhibited proteolysis (as shown by reproducibility of spot patterns) and degradative loss of higher molecular weight proteins in sample extracts.

Two major *objections* to the use of 2-D electrophoresis are that 1) only *relatively* abundant proteins are detected and 2) although many simultaneous quantitative or

qualitative comparisons can be made, such comparisons may not be precise enough for some investigations. Many replicate gels may be required to achieve a statistically significant result, and because many steps are involved in making a gel- each of which may introduce spot variability- there may be an initial tendency to detect differences that are technique-related and confuse them with important biological differences [Strahler *et al.*, 1989]. Other technically related spot variability may be due to sample age (new spots may be due to degradation products accumulated during storage), the degree that samples are solubilized, fluctuations of temperature during electrophoresis, protein loss during equilibration of first dimension gels, and spot anomalies at the extremes of pH [Strahler *et al.*, 1989]. Furthermore, in experiments using samples from individuals, *genetic variability* may manifest in the appearance of polymorphic proteins which cause certain spots to 'change size' or result in the appearance of 'new' spots. Such phenotypic variation can be confusing unless one is aware that polymorphisms are the main cause. The results of this study *do not* rule out polymorphisms as a significant source of spot variability.

Generally, qualitative differences can be assessed reliably as the positions of various proteins in a sample vary little from one gel to the next. However, the measurement of *quantitative* differences in an experimental set is affected by the proximity of a given protein spot to neighbouring spots, the relative amount of protein in the spot, and the resolution of the image analysis system [Dykstra and Wang, 1992]. Furthermore, the degree of staining and the colour of the stained spots may vary considerably for different proteins and with different staining methods [Sammons *et al.*, 1981]. Glycoproteins frequently produce red-brown spots by silver staining [Goldman *et al.*, 1980] and are not always detected by some laser-based scanners.

Strategies used to help compensate for potential problems caused by the above included the use of the 'group comparison' experiments (duplicate 'pooled resistant' and 'pooled susceptible') described earlier, standardization of experimental and staining

conditions, and manual detection and editing of spots not detected by the scanner. Nevertheless, subtle differences in protein spot patterns due to variations in gel/buffer conditions, compounds inherent in crude protein extracts, and/or staining technique were unavoidable, making it difficult at times to discern introduced artifacts from genuine protein profile differences. Anomalous spot patterns due to 'bowing' at the extremes of pH, and protein spot 'doubling' of lower molecular weight spots (i.e. spots less than $\approx$ 30 kDa) occured randomly on at least 1-2 gels out of a possible 5 gel run. The former difficulty was likely due to electrical leakage, while the literature suggests that the latter problem was likely due to an excess of SDS in the gel equilibration buffer used to fix the first dimension gel [Bravo, 1984]. It was also suggested by the manufacturers of the Millipore Investigator 2-D system that electrolyte depletion from the cathode tank to the anode might result in spot doubling as observed, however neither this explanation, nor that of 'excess SDS' explains why spot doubling occured in random fashion, affecting only 1 or 2 gels out of 5. The end result was that pooled experiments were often repeated and stained gels were visually compared to confirm the presence or absence of previously detected candidate protein spots and patterns. It is believed that by focusing on protein 'constellations' within a gel rather than on *single* protein spots, and by running pooled *total protein* samples in duplicate, many of the analytical difficulties associated with experimental error and individual genetic variation, respectively, were overcome or at least partially compensated for. However, it needs to be said that in order to establish meaningful *diagnostic* correlations between the presence/absence of any given protein spot (or group of spots) and a resistance phenotype, a much larger population sampling than the one used in this study is required.

Because of the small sample size (8 trees per location) used in this study, it was impossible to make any broad inferences with respect to the potential diagnostic significance of most of the 'significant' proteins identified. Furthermore, because it was

difficult (due to the degree of 'within group' variation) to narrow down the range of *potentially* correlative marker proteins, the task of choosing potential candidates for further study was at times 'hit and miss'. The lack of a *consistent qualitative* protein marker (as determined by 2-D gel electrophoresis) in either 'mature tree resistant' individuals or in those expressing the SCG bark reaction is illustrative of the polygenic nature of these blister-rust resistance phenotypes [Hoff and McDonald, 1980]. Isozyme studies of 10 stable proteins (encoded by 12 loci) indicated that 65% of the loci within *P. monticola* stands (28 stands, representative of most of the range) were polymorphic [Steinhoff *et al.*, 1983]- not an unusual observation for conifers in general. Much of the variation occurs *within* populations as opposed to between populations- necessitating the study of individuals. This study has demonstrated that *multiple* qualitative and quantitative protein profile differences exist between blister rust resistant and susceptible *groups*. However, polygenic traits are controlled by quantitative trait loci, and unfortunately much of the variation seen in these loci tends to be due to *environmental* influences and interactions [Cheliak and Rogers, 1990] so it is extremely difficult at this point to predict with accuracy which of the proteins identified and partially characterized in this study can qualify as *genetic* markers.

Qualitative Differences

Of the 5 unique proteins identified in the LC resistant group, an acidic (pI= 5.0) 25.2 kDa protein and a 36.0 kDa (pI= 6.6) protein were present in 50% of the canker-free individuals. On the other hand, out of 18 proteins expressed exclusively in the LC susceptible group, a 39.4 kDa (pI = 5.9) protein was found in all 4 individuals, and a 32.5 kDa protein (pI= 5.6) was present in 75% of the individuals. These proteins are worthy of further characterization-especially the latter two. It is noteworthy that with 6 exceptions (ssp's 1902, 2902, 3703, 4709, 6707, and 7803), all of the proteins listed in Table 4 (LC

bark proteins) share similar charge and mass characteristics with many so-called "pathogenesis-related" proteins.

Of the many unique proteins detected in resistant and susceptible PRd groups, very few were consistently represented 'across the board' within their respective groups. A 17.2 kDa (pI= 4.5) protein found in the susceptible group has only been detected in 1 and possibly 2 susceptible individuals, as was also the case for a 21.7 kDa (pI= 5.1) 'susceptible' protein. The same litany holds true for PRd bark proteins extracted from healthy tissue. Without further characterization of the many significant proteins identified, it is not possible to allude to any structural or functional relationship between these and other described proteins. Furthermore, the inconsistent representation of many of these proteins underscores one of the main difficulties of working with conifers in general- namely addressing variation which may be due to the vast genetic heterogeneity inherent within populations. Although each of the highlighted spots (see Tables 4, 5 and 6) identified by two-dimensional analysis as being significant (either by qualitative presence or quantitative enhancement) could eventually receive further attention for the purpose of developing simpler screening probes, larger sample sizes would be required to rule out the possibility that these same spot differences are due to normal within population variation. Future N-terminal sequence analysis and amino acid composition analysis of many of these biosynthetically modulated proteins should provide much information leading to their eventual functional and structural characterization.

Pathogenesis related (PR) proteins

Ever since the discovery of tobacco mosaic virus (TMV) induced proteins in tobacco in the early 1970's, the study of PR proteins has expanded to functionally characterize and isolate similar compounds in a range of plants. However, many PR proteins remain functionally uncharacterized. For example, 7 different proteins ranging in molecular weight from 17 kDa to 42 kDa have been shown to accumulate in azuki bean

leaves in response to treatment by ethylene [Ishige *et al.*, 1991], however only one protein (27 kDa) was functionally identified as an acidic chitinase. A 42 kDa protein identified in the same study was characterized as an intracellular glycoprotein (not hydroxyproline-rich) and its function remains unknown. Of 10 major PR proteins originally detected in tobacco, 4 were functionally characterized as acidic (27.5 kDa and 28.5 kDa) and basic (32.0 kDa and 34.0 kDa) endochitinases [Legrand *et al.*, 1987], and 2 thaumatin-like proteins were later shown to have *in vitro* antifungal activity [Vigers *et al.*, 1992]. Four novel basic polypeptides ranging in molecular weight from 16 kDa to 31 kDa have been observed in peanut cotyledons after inoculation with *Aspergillus* spp [Szerszen and Pettit, 1990]. Electrophoresis of sugar beet intercellular fluid (leaves) under denaturing conditions has shown not only *induced* PR proteins in response to inoculation by tobacco necrosis virus, but reduced amounts of proteins normally found in the intercellular fluid of healthy tissue [Fleming *et al.*, 1991]. Purification and partial N-terminal sequencing of 2 acidic proteins from the aforementioned study revealed that both proteins had an approximate molecular weight of 28 kDa, and shared 80% amino acid sequence homology with induced chitinases from cucumber and *Arabidopsis thaliana.* Zeamatin is a 22 kDa antifungal PR protein found in corn seeds [Vigers *et al.*, 1991]. Its antifungal properties appear to be related to its ability to cause membrane permeabilization. The N-terminal sequence homology of many zeamatin-like proteins with thaumatins and osmotins suggests that they may belong to a larger family of defense-related proteins that also include some PR proteins [Vigers *et al.*, 1991].

Although many of the *molecular weight* and *pI* characteristics of PR proteins are shared by the significant proteins identified in this study, much more research needs to be done to characterize and positively identify these bark proteins before they are grouped as 'pathogenesis-related'. Characterization of potential PR bark proteins by conventional methods (i.e. their resistance to proteolytic degradation (trypsin/chymotrypsin digestion),

their ability to dissolve in acid buffers/solutions, and their inducibility by biotic and abiotic elicitor treatment) may not be necessary given the availability of antibody and nucleotide probes. However, it remains to be seen if any of the significant proteins identified in this study share *functional* homology with some of the well-characterized PR proteins. Elicitor studies and bioassays would eventually be needed to help elucidate the specific or non-specific nature of PR-like proteins in conifers. The largest obstacles at this point in time appear to be 1) successfully *isolating* proteins once they have been selected through analytical 2-D methods and 2) developing a method of protein extraction that maximizes protein yield while minimizing the phenolic contamination that could interfere with a potential bioassay.

Variation due to Age factors

Host age has been shown to affect susceptibility to blister rust infections in *P. monticola* [Lachmund, 1933; Snell, 1936; Patton, 1961; Hunt and Meagher, 1989]. Early studies of *P. strobus* demonstrated that one year old seedlings, despite their comparatively small size, were much more susceptible to infection than their older siblings [Clinton and McCormick, 1919; York and Snell, 1922]. Additional studies [Hunt and Meagher, 1989] with *P. monticola*, using greenhouse inoculation conditions, have shown *stem cankers* to be associated with formation of needle spots on current foliage, confirming earlier hypotheses. There has tended to be a positive correlation between the number of needle spots and the percentage of cankering, however with considerable variability. It is possible that in *P. monticola*, the 'post-inoculation' development of needle spots may be a *juvenile* resistance mechanism [R. Hunt, personal communication]. The final tests seem to be whether or not an individual develops a full blown cankering infection, and the extent to which that cankering process is slowed or halted by host resistance mechanisms. Mature tree resistance, such as that displayed by the LC resistant clones, may be a function of the *inability* of the *rust* to successfully establish a foliar

infection in mature individuals rather than a physiological response of the tree to specific fungal invasion. If such is the case, the role of constitutive needle morphology (e.g. needle waxes, guard cell morphology, needle surface topography) and the biosynthesis of related structural, surface receptor, or other proteins may be more important.

The fact that natural resistance to blister-rust infection *increases* with age may have obscured any *early* biochemical contributions made by other resistance mechanisms. It is of note that only 46 significant (Student t-test) spots were detected between 'resistant' and 'susceptible' groups from the LC location while 146 significant spots were detected between 'resistant' and 'susceptible' PRd groups (sampled near lesioned sites). This 3-fold difference may be *partially* due to age-related effects that influence the efficacy of protein extraction from mature versus seedling/sapling tissue, or they may be the result of age-related differences in metabolism. Evidence to support an age-related hypothesis may come from the observation that samples collected from *healthy* PRd bark *still* had almost twice as many significant protein differences between 'resistant and susceptible' groups compared to LC collections. However, proteins are generally *better* extracted from *young* versus mature tissue due to the lower relative amounts of contaminating resins and phenolics in the former. The greater degree of *spot resolution* seen in the PRd samplings versus the LC collections may be a reflection of this alone. Since bark samples were collected at a time when trees would be entering dormancy (November) it is not as likely that 'normal' and possibly age-related biosynthetic processes would be operating to mask the effects of a host-pathogen metabolic interaction. However, the modulating effect of the former upon the latter cannot be ruled out.

The fact that *novel* proteins were more abundant in *susceptible* groups from *both* LC and PRd sites suggests that 1) some of the proteins may be *pathogen-related* (i.e. of *fungal origin*) or 2) the host defence response in resistant individuals may involve selective gene-inactivation and suppression of protein biosynthesis.

In a manner similar to 'incompatible' agronomic host-pathogen interactions [VanderPlank, 1982], blister rust resistant white pine individuals may constitutively lack some of the protein products necessary to set up a 'successful' infection (i.e. signal proteins, receptor molecules)- and such a deficiency may be reflected by a low relative abundance or diversity of proteins. For example, a membrane protein capable of binding the toxin helminthosporoside (produced by *Helminthosporium sacchari*) has been shown to be present in the leaf tissue of sugarcane clones susceptible to eyespot disease, but absent in the tissue of resistant clones [Strobel and Hess, 1974]. Such *specificity* is not *as* likely to be seen in conifers, however this limited study did demonstrate that *susceptible* white pine from both LC and PRd locations tended to have more *unique* proteins than the resistant western white pine. The possibility of such proteins being fungal in nature was not ruled out.

Another possible explanation for the qualitative protein differential in susceptible groups may relate to the general condition of the diseased host. Heimberger [1962] suggests that the *degree* of attack by such obligate parasites as *C. ribicola* is positively correlated with [pre-inoculation] host vigor. Consequently trees that are to be evaluated for blister rust resistance are grown under environmentally optimum conditions prior to inoculation with basidiospores in order to maximize their natural growth potential. The relative abundance of unique protein spots and the tendency for susceptible groups to be enriched in 'common' significant proteins may be a biochemical indicator of the degree of susceptibility. At the time the PRd samples were taken, susceptible trees had been diseased for 5 years and displayed varying degrees of pathology. It may be that the protein profile picture of this group is a measure of localized disease pathology, although 'healthy' tissue from the susceptible PRd group *still* had almost twice as many *unique* proteins as the resistant group.

Variation due to Resistance Phenotype

It has already been pointed out that LC groups had fewer significant proteins than PRd groups. Although host age may have had some influence, variation due to resistance phenotype (i.e. mature tree resistance versus 'slow canker growth' bark reaction) cannot be ruled out.

Immunodetection studies: anti-Pin m III

Susceptible individuals from the LC location consistently demonstrated a greater degree of immunoreactivity to the anti-Pin m III polyclonal antibody compared to resistant individuals. This effect was especially pronounced in the 'natural' trees (N6-23 and N2-15). Furthermore, 2 of the blister rust-susceptible LC individuals were shown to have 5 serologically related isoforms of the ≈19 kDa protein whereas 1 of 2 resistant LC individuals had but 2 isoforms while the other had 3 (see Figure 8). Despite the limited sample size, these results indicate that some of the quantitative differences observed between susceptible and resistant individuals may be due to the presence of isoforms otherwise masked by SDS-PAGE separation.

The presence of the Pin m III protein in pine bark is interesting because although it appears to behave like a bark storage protein, its expression appears to also be affected by rust-resistance phenotype. Davis and coworkers [1993] have shown that two *wound-inducible* cDNAs (*win4* and BSP) from *Populus* spp show 75% sequence identity to vegetative storage proteins that accumulate seasonally in bark. Although both *win4* and bark storage protein (BSP) genes are systemically wound-induced, transcripts of the former accumulate in leaves and stem tissue while those of the latter accumulate exclusively in the stem and are also regulated in response to short days. It might be possible that the 19 kDa 'cold hardiness related' protein in pine bark *also* accumulates in response to ongoing pathogenic invasion. However, most of the bark storage proteins characterized to date are considerably *larger* than 19 kDa. Poplar BSPs are glycoproteins of between 32-38 kDa [Stepien and Martin, 1992;] that appear to accumulate in ray cells

[van Cleve *et al.*, 1988]. A 32 kDa glycoprotein similar to one of the poplar BSPs has also been shown to accumulate in parenchymatous cells of the inner bark tissues of *Salix microstachya* Turz. [Wetzel and Greenwood, 1991]. A 30 kDa protein has been shown to accumulate in overwintering Douglas fir seedlings while interior spruce seedlings have been shown to demonstrate tissue-specific accumulation of 27 kDa and 30 kDa proteins [Roberts *et al.*, 1991].

The fact that PRd samples did not display as dramatic a difference in the relative intensity of the 19.2 kDa band detected by anti-Pin m III may merely be a reflection of seasonal variation (bark harvested in November) or it may also reflect an age-related difference. Because LC samples harvested in October and January (data not shown) displayed a similar pattern to those harvested in April ('susceptible' relatively more immunoreactive than 'resistant') it seems reasonable to assume that the phenotype-associated differences observed in *this* group were seasonably independent. The relative intensity of this protein increased 'across the board' in autumn and winter collections, yet the tendency for susceptible individuals to have a higher abundance of the protein band remained constant. Further studies need to be done to rule out age effects before deciding that this protein can be used as a *disease* marker. It was not possible to further sample the PRd seedlings to test for seasonal differences due to the time factor and sample attrition (death of susceptible trees). The availability of N-terminal amino acid sequence data will facilitate the future development of nucleotide probes which can be used to test larger sample sizes.

anti-PSII

Because strong and specific immunoreactivity to anti-PSII was demonstrated in bark proteins, it was decided to subject candidate protein bands to composition analysis. This was done because at the time the PSII protein was initially isolated in *P. monticola*, there were actually 2 proteins identified (*Pin m I* and *Pin m II*), both of which had

identical N-terminal sequences but which differed in amino acid composition. The molecular weights of *Pin m I* and *Pin m II* were determined to be 22.6 kDa and 23.4 kDa respectively, with *Pin m I* having a higher proline content than *Pin m II* [Ekramoddoullah,1993]. Proteins with a high proline content have been implicated in enzymatic plant defense (i.e. vacuolar chitinases) and are well known as structural cell wall proteins [Sticher *et al.*, 1992; Keller, 1993; Ridge and Osborne, 1970]. Because of the possibility that the anti-PSII antibody might in fact be picking up a high proline content protein rather than a photosystem II protein, it was decided to subject the immunoreactive band to composition analysis. A single immunoreactive band was detected in bark- indicating that only *one* species with PSII amino acid sequence homology was present. Amino acid composition analysis of the 3 bands located within the immunoreactive area was *negative* for proline rich proteins, however 1 protein band (≈24 kDa relative molecular mass) was shown to be especially glycine rich (see Table 8). The significance of both proline-rich and glycine-rich proteins has already been discussed. A paired t-test showed that the quantitative differences (quantity expressed in O.D. units) between resistant and susceptible individuals for this protein were significant (P= 0.012), however this must be interpreted with caution given the limitations of the sample size.

CHAPTER 5

CHITINASE

Introduction

Chitinase is a hydrolytic enzyme that cleaves chitin, an insoluble polymer of N-acetyl-D-glucosamine found in the exoskeletons of marine invertebrates, insects, and in fungal cell walls [McCormack *et al.*, 1991]. Chitin is not found in the cell walls of plants nor has any endogenous plant substrate been found for purified chitinase [Balasubramanian and Manocha, 1992]. Nevertheless, plant chitinases are induced, increasing in both amount and activity, in response to a number of abiotic and biotic stresses, including salicylic acid and ethylene elicitors, heavy metal ions [Mauch *et al.*, 1984], wounding [Mauch *et al.*, 1988a; 1988b], and fungal, bacterial, and viral infection [Kombrink *et al.*, 1988]. In ectomycorrhizal systems, chitinases may play a role in the partial degradation of fungal cell walls in order to facilitate nutrient exchange between symbionts [Sauter and Hager, 1989]. Active chitinase has been found in extracts of healthy, non-senescent petunia flower tissues (stigma), and has been shown to increase 5-fold following anther dehiscence suggesting that it may also play a role in the sexual reproduction of some higher plants [Leung, 1992].

The chitinase family was originally grouped into three broad classes [Shinshi *et al.*, 1990]; however, chitinases can be grouped into at least six classes based upon structural characteristics (Z. Punja, personal communication). Four classes are officially described in the literature (see review by Graham and Sticklen, 1994). Both exo- and endo-chitinases have been characterized [Boller *et al.*, 1983], with the former cleaving single glucosamine residues from the end of a chitin chain, and the latter cleaving internally to produce oligomers.

Class I chitinases (with *primarily* basic isoforms) contain a signal peptide followed by two structural domains; a 'hevein' domain (rich in cysteine) separated by a variable 'hinge' region from a conserved catalytic domain. Evidence indicates that a C-terminal extension targets these chitinases to the vacuole and they generally have high specific activities relative to their class II counterparts. It is thought that class I chitinases account for most of the chitinolytic activity in plants containing basic and acidic isoforms [Graham and Sticklen, 1994].

Class II chitinases (*primarily* acidic isoforms) are extracellularly secreted and consist solely of a hydrophobic signal peptide followed by a monomeric catalytic domain which lacks both the hevein domain and all, or part of, the variable region found in class I enzymes. The chitinolytic (catalytic) domain of class II chitinases shares *strong* homology with that of class I enzymes. It is hypothesized that these enzymes act in a 'first line of defense' as signaling molecules, releasing elicitors from fungal pathogens. Compared to class I chitinases, which are strongly anti-fungal *in vitro*, class II chitinases have no anti-fungal activity alone, and are only moderately anti-fungal in synergy with β-1,3-glucanases [Graham and Sticklen, 1994].

Class III chitinases are also extracellular hydrolytic enzymes classed primarily on the basis of homology to previously described lysozymes with chitinase activity. The catalytic domain of class III chitinases shares little or no homology with that of class I or class II. Some members of class III chitinases show weak similarity to chitinase D from *Bacillus circulans*. Class I, II, and III chitinases may have basic and acidic isoforms [Lawton *et al.*, 1992; Davis *et al.*, 1991; Collada *et al.*, 1992] and representatives from all three classes may be present in the same plant [Van Damme *et al.*, 1993].

A fourth class of chitinases contain chitin-binding, hinge region, and catalytic domains-*similar to class I chitinases*- however, with the exception of 7 out of 8 conserved cysteine positions, the chitin-binding regions display little homology to those

of class I chitinases, and the *overall homology* of class IV to class I chitinases is low. Chitinases in this class also lack the C-terminal extension which is presumed to target the molecule to the vacuole, thus resulting in an extracellular buildup of the enzyme [Graham and Sticklen, 1994]. Transcription of mRNA for these chitinases occurs in response to fungal infection, and it is proposed that they may fulfill an anti-fungal role similar to that of class I *within the apoplast*.

Generally speaking, acidic chitinases are secreted into the extracellular space while basic isoforms are sequestered into vacuoles; however, a *fifth* class of chitinases are *basic* (similar to class I), but have no chitin-binding domain and are *extracellular* rather than vacuolar. A *sixth* class includes *acidic class I-type* chitinases such as those encoded by *win6* and *win8* genes (from *Populus*) and CHITAS1 and 2 (from garlic).

Other chitinases do not fall neatly into the above categories. For example, mRNA encoding an 8.5 kDa stinging nettle agglutinin also encodes a C-terminal chitinase-like catalytic domain and *two* tandem N-terminal chitin-binding domains [Lerner and Raikhal, 1992; Graham and Sticklen, 1994] that share homology with several class I chitinases. Double chitin-binding domains flag this protein as unique and studies are currently underway to elucidate the function of this domain (which is lost during post-translational processing). Another class I-type chitinase distinguishes itself by dimerizing to form a bifunctional 52.5 kDa enzyme with insect α-amylase inhibitor activity. The specific function of the dimer relative to the monomer is not known, however the 26.5 kDa size of the monomer is considered small for a class I chitinase [Graham and Sticklen, 1994].

Chitinolytic enzymes have been extensively investigated over the past two decades in an attempt to elucidate their roles in plant defense [Van Loon, 1985; Lawton *et al.*, 1992; Mauch *et al.*, 1988a, 1988b; Kombrink *et al.*, 1988]. Chitinase has anti-fungal activity *in vitro* [Verburg and Huynh, 1991] which is enhanced by the presence of β-1,3-glucanase. Both chitinase and glucanase have been shown to be coordinately induced by

pathogen infection and are well established PR-proteins. Chitinolytic enzymes have been isolated and characterized in a variety of biological systems including bacteria [Jones *et al.*, 1986; Watanabe *et al.*, 1992], fungi [Di Pietro *et al.,* 1993; Harman *et al.*, 1993; Kunz *et al.*, 1992], vegetable, flower, cereal and other short rotation crops [Joosten and De Wit, 1989; Vogelsang and Barz, 1993; Jacobsen *et al.*, 1990; Esaka *et al.*, 1990; Fink *et al.*, 1990; Yamagami and Funatsu, 1993; Van Damme *et al.*, 1993; Pan *et al.*, 1992; Vad *et al.*, 1991; Boller *et al.*, 1983; Kurosaki *et al.*, 1987]. Chitinases are found constitutively in chestnut seed storage tissue [Collada *et al.*, 1992]. Relative molecular masses for *plant* chitinases range from 24 kDa to 38 kDa.

Chitinase *activity* has been detected in a number of woody plants, including grape vine (*Vitis vinifera)* [Clarke and Stone, 1962], roots and suspension cultures of *Picea abies* following exposure to cell wall fractions of the mycorrhizal fungus *Amanita muscaria* [Sauter and Hager, 1989], and healthy stem and root tissue from sugar maple (*Acer saccharum*), red oak (*Quercus rubra*), black oak (*Q. velutina*) and white oak (*Q. alba*) [Wargo, 1975]. Partially purified chitinolytic enzymes from the latter 4 forest species have been shown to lyse the hyphal walls of *Armillaria mellea*, suggesting a possible role in host resistance against this and other pathogens. Yields of chitinase from stem tissue were shown to be greater than from root tissue [Wargo, 1975]. Poplar trees (*Populus sp.*) have at least two chitinase genes, *win6* and *win8*, that are *systemically* wound-inducible and that display sequence homology to herbaceous chitinases [Davis *et al.*, 1991].

Thus far, there has not been any report of chitinase activity or immunodetection of chitinase bands in mature Western white pine bark tissue. Because of the reports of the presence of this enzyme in other woody plants, and because of its demonstrated anti-fungal properties *in vitro* and hypothesized role in plant defense, it was decided to test

bark protein extracts of *P. monticola* for both chitinase activity (colorimetric assay) and immunoreactivity to heterologous (barley and petunia) chitinase polyclonal antibodies.

Materials and Methods

Western blot and immunodetection methods have been described earlier. Anti-sera raised against an acidic petunia [Linthorst *et al.*, 1990] chitinase and a barley [Leah *et al.*, 1991] anti-chitinase polyclonal antibody were used. The protein extraction method used to provide samples for the above has already been described. All 16 trees (both LC and PRd locations) were individually screened.

Protein Extraction for Chitinase Assay

Because the protein extraction procedure described earlier employed harsh, denaturing conditions (i.e. 4% SDS, 5% mercaptoethanol, high temperatures), it was not suitable to use as a method for extracting potentially functional enzymes. To compound this problem, most extraction procedures for chitinase (herbaceous or otherwise non-woody plant systems) required little more than simple 0.01-0.05M acetate buffers of low pH. Such buffers were unable to (1) solubilize bark proteins and (2) scavenge interfering phenolic/resinous compounds that might irreversibly complex with and inactivate potential chitinases. To attempt to solve this problem, a modification of the extraction procedure used by Anderson and coworkers [1992] was used to provide a crude enzyme extract from both fresh foliar (secondary needles, randomly collected samples from at least 5- 2 year old greenhouse seedlings) and lyophilized bark tissue (G161, resistant clone, LC). All steps were carried out at 5°C. Unfortunately, there was not sufficient tissue, nor time available to attempt extractions of the PRd samples.

Foliage

Secondary needles were immediately placed into liquid nitrogen to fast-freeze. Needles (2g fresh weight) were ground to a powder in liquid nitrogen with the addition of

0.5-1g of PVPP, transferred to a 50 ml Falcon tube, and homogenized with a Polytron (Brinkmann Instrument Co., Westbury, N.Y.) at half-speed for 30 seconds in 30 ml of freshly made extraction buffer (composition: 50mM piperazine-N,N'-bis-[2-ethane-sulfonic acid] (PIPES) buffer (pH 6.8), 6mM cysteine HCl, 10mM D-isoascorbate, 1mM disodium ethylene diamine tetracetate (EDTA), 0.3-1% Triton X-100, 1-6% PVP-10, 1 drop of antifoam A emulsion, and 0.1% BSA). The homogenate was transferred to Beckman JA-14 tubes for use with a 50.2Ti rotor (model 1926; Beckman, Ontario, Canada) and centrifuged for 20-30 minutes at 20,000x *g*. The brilliant green supernatant was retained and the pellet was then re-homogenized and extracted with an additional 20 ml of extraction buffer, spun as above and the supernatant pooled with that of the first extraction. A rapid dot blot protein determination (qualitative) was done on the crude extract before proceeding with ammonium sulfate (AS) precipitation. Crude extract was divided into 5 ml aliquots and, if not used immediately, was stored at -20°C.

A preliminary 'clean-up' precipitation was performed on the crude extract by adding saturated AS to a final concentration of 30%, with constant stirring. A greenish, gummy precipitate immediately formed which adhered to the sides of the glass beaker. The solution was stirred for 30 minutes and then centrifuged at 20,000x *g* for 30 minutes. The supernatant was retained and then brought to 90% saturation with additional AS. After stirring this solution for at least 1 hour, it was centrifuged for 45 minutes at 20,000-50,000x *g*. The pellets obtained from both 30% and 90% AS precipitations were individually resuspended in 0.5-1.0ml of either 0.01M or 0.05M sodium acetate buffer (pH 5.0). Extracts were stored at 4°C for up to 2 weeks.

Bark

The extraction procedure for bark tissue was essentially the same as that used for foliage, except that the bark had been previously freeze-dried. An acetone powder was prepared by adding 20ml of ice-cold acetone to 0.5g of lyophilized and ground bark

tissue, homogenizing the slurry for 1 minute at full speed using a Virtis-23, and filtering the mixture through Whatman #3 paper. The residue was washed with an additional 25ml of ice-cold acetone and allowed to dry at room temperature. This acetone powder, as well as 'regular' lyophilized and ground bark, was used in the above extraction procedure. The ratio of bark powder to PVPP was approximately 1:1.

Colorimetric Chitinase Assay

A two-step colorimetric enzymatic assay utilizing chitinase and N-acetyl-glucosaminidase (Sigma, unpublished assay currently under review) was employed. All reagents used in the assay were obtained from Sigma. Unless stated otherwise, all steps were carried out at room temperature.

A 200mM potassium phosphate, 2mM calcium chloride buffer (Reagent A, pH 6.0 at 25°C) was used to prepare the following solutions: 1.25% chitin (Reagent B), chitinase enzyme solution (Reagent D, 1 unit/ml), β-N-acetylglucosaminidase (NAGase) at a concentration of 35 units/ml (Reagent E), and 0.1% N-acetyl-D-glucosamine (NAG) stock solution (Reagent F).

Final assay concentrations of a 2.5 ml reaction mix were 160mM potassium phosphate, 1.6mM calcium chloride, 1% colloidal chitin, 1 unit of NAGase and 0.5 units of chitinase (positive control) or 0.5 ml of test extract (2-10mM sodium acetate). A unit was defined as that which would liberate 1.0 mg of NAG from chitin per hour at pH 6.0 at 25°C in a two-step reaction with NAGase.

A color reagent solution (Reagent C) was prepared by mixing a sodium potassium tartrate solution (12.0 g of sodium potassium tartrate tetrahydrate in 8.0 ml of 2N NaOH, heated over boiling water to dissolve) with a 3,5-dinitrosalicylic acid solution (438 mg 3,5-dinitro-salicylic acid in 20ml MilliQ® water, heated over boiling water to dissolve), and bringing the final volume to 40 ml. This solution was placed in an amber jar and stored at room temperature (stable for 6 months).

Procedure:

The following reagents were pipetted (ml) into suitable containers (i.e. 15ml poly-propylene vials, with screw tops).

	Volume (ml)		
	(+)'ve control	(-)'ve control	Test
Reagent B	2.0	2.0	2.0
MilliQ®	---	0.5	---
Reagent D	0.5	---	---
Test extract	---	---	0.5
Total volume(ml)	2.5	2.5	2.5

Additional negative controls were used for crude (pigmented) test extracts to account for any non-specific absorption of the extract at the same wavelength as that of the final product of the enzyme reaction was measured. These controls consisted of 0.5 ml of the test extract and 2.0 ml of Reagent A (no chitin).

Vials were capped securely and placed on their side on a rotary platform at a speed sufficient to keep the chitin in suspension. The reaction mixtures were incubated at room temperature (~ 23°C) for 2 hours. Reactions were stopped by placing the vials into a boiling water bath for 5 minutes. After cooling the reaction mixtures to room temperature, 1 unit of NAGase (29 µl of Reagent E) was added to each vial and vials were incubated for an additional hour with mixing. The suspensions were then centrifuged for 10-15 minutes at 3000 x *g* and the supernatant retained for the colorimetric assay.

Standard Curve:

A standard curve (1mg-5mg) was generated by pipetting (in ml) the following reagents into 15ml heat-resistant tubes.

	Blank	#1	#2	#3	#4	#5
Reagent F	0.0	0.1	0.2	0.3	0.4	0.5
MilliQ®	3.0	2.9	2.8	2.7	2.6	2.5
Reagent C	1.5	1.5	1.5	1.5	1.5	1.5
Total volume	4.5	4.5	4.5	4.5	4.5	4.5

Samples:

The following reagents were pipetted (in ml) into 15ml heat-resistant tubes.

	Test(s)	Blank(s)
Test supernatant	2.0	---
Blank supernatant	---	2.0
MilliQ®	1.0	1.0
Reagent C	1.5	1.5
Total volume	4.5	4.5

All of the tubes (standards, tests, and blanks) were placed into a boiling water bath for 5 minutes. The solutions were allowed to cool to room temperature before reading their absorbance at λ=540 nm. Absorbance readings were done using an ELISA plate reader (Model EL309, Bio-Tek Instruments Inc., Burlington, Vermont) with at least 3-200 µl replicates of each standard and sample.

Calculations:

Standard Curve:

$$\Delta A_{540nm}\ \textbf{Standard} = A_{540nm}\ \textbf{Standard} - A_{540nm}\ \textbf{Standard blank}$$

The slope (**M**) was calculated by plotting the ΔA_{540} against the NAG concentration.

Sample determination:

The concentration of NAG liberated during the reaction was calculated using the following equation.

$$\textbf{mg NAG released} = (A_{540nm}\ \textbf{Test} - A_{540nm}\ \textbf{Blank}) \div \textbf{M}\ (\text{slope})$$

Results:

Table 11 shows the results of the colorimetric chitinase assay of both bark and foliar protein extracts from *P. monticola*. Protein extracts from lyophilized, mature bark (G161, LC) displayed slightly *higher* activity than freshly extracted seedling foliage. Crude extract from foliage also displayed chitinase activity, although to a lesser degree compared to that shown by the 90% AS precipitated fraction. The sample values displayed are corrected values using the negative controls (blanks) as a baseline (negative control values not shown).

Tables 12 and 13 list the immunoreactive bands detected by petunia and barley anti-chitinases, respectively, while Figures 12 and 13 are illustrative of the visual intensity of detected bands. Five bands were detected by petunia anti-chitinase while 11 different immunoreactive bands were detected by barley anti-chitinase. With the latter antibody, no more than 7 immunoreactive bands were detected in a single individual and of those bands, perhaps 3-4 appeared to be strongly immunoreactive.

Petunia anti-chitinase appeared to consistently react with a **28.1 kDa** protein in resistant LC individuals and a **30.4 kDa** protein in susceptible individuals (LC and PRd). A **17.2 kDa** protein was detected in all positively reacting individuals. Two other strongly immunoreactive bands (**34.9 kDa** and **29.2 kDa**) were detected in 2391-113-2 (susceptible). Interestingly, bands of comparable relative molecular weight (**35.0 kDa** and **30.0 kDa**) were detected by *barley* anti-chitinase in this same individual. Similar bands were seen in 2391-39-1 (resistant), however they were somewhat fainter by comparison.

Of note is the relative intensity of a **51.2 kDa** band (*barley* anti-chitinase) in LC samples compared to PRd samples. A **36.9** kDa band was detected strongly in 3 out 4 resistant LC individuals and moderately in both susceptible LC individuals and PRd individuals (both resistant and susceptible). A **28.7 kDa** band was immunoreactive to

barley anti-chitinase in LC samples, but not in PRd samples, similar to the **28.1 kDa** band immunoreactive to petunia anti-chitinase in LC but not PRd individuals. Other low molecular weight bands (**14-17 kDa**) were detected by both anti-chitinases.

For LC and PRd individuals, immunoreactive bands detected by *both* petunia and barley anti-chitinase were within the **17-37 kDa** molecular weight range.

Qualitative differences were noted between susceptible and resistant individuals of PRd families detected with barley anti-chitinase. However, these differences were not *consistently* observed in the 4 families. For example, within family **2391**, a 26 kDa band was detected in *susceptible* versus resistant, while in family **2398**, a 35 kDa band was found in *susceptible* versus resistant. In family **2413**, a 30 kDa immunoreactive band was found in *resistant* versus susceptible while in family **2411**, a 31 kDa band was observed in *resistant* versus susceptible.

Table 11.

Results of colorimetric chitinase assays of *P. monticola* foliage and bark protein extracts. Activity is expressed as milligrams (mg) of NAG released during a two-step reaction with β-NAGase from *Aspergillus niger* (Sigma). AS = ammonium sulphate precipitation.

Sample	Activity (mg NAG)
chitinase control (+)	0.488 ± 0.105[a]
90% AS foliage	0.163 ± 0.035[b]
crude extract (foliage)	0.142[c]
90% AS bark	0.171 ± 0.014[b]
30% AS bark	0.140[c]

[a] average of 7 separate experiments with 3 replicates per experiment
[b] average of 2 separate experiments with 3 replicates per experiment
[c] one observation with 3 replicates

Table 12.

Estimated molecular weight (Mr) in kilodaltons (kDa) of Western white pine bark proteins immunoreactive to ***petunia*** anti-chitinase polyclonal antibodies. Bold text indicates that the protein band was highly immunoreactive relative to other bands.

Tree I.D.	Mr (kDa) of Immunoreactive Proteins
B643 (res)	28.1, 17.2
G8 (res)	28.1, 17.2
N2-15 (sus)	17.2
G27 (sus)	30.4, 17.2
2391 (sus)	**34.9, 30.4, 29.2, 17.2**
2398 (sus)	30.4, 29.2, 17.2

Table 13.

Estimated relative molecular weight (Mr) in kilodaltons (kDa) of Western white pine bark proteins immunoreactive to ***barley*** anti-chitinase polyclonal antibodies. Bold text indicates that the band was highly immunoreactive relative to other bands.

Tree I.D.	Mr (kDa) of Immunoreactive Proteins
2391 (res)	51.2, 36.9, 35.0, 30.0, 17.0, 15.0
2391 (sus)	51.2, 36.9, **35.0, 30.0, 26.0**, 17.0, **15.0**
2398 (res)	51.2, 36.9, 30.0, 17.0, 15.0
2398 (sus)	51.2, 36.9, 35.0, 30.0, 17.0, 15.0
2413 (res)	51.2, 36.9, 35.0, 30.0, 17.0, 15.0
2413 (sus)	51.2, 36.9, 34.9, 17.0, 15.0
2411 (res)	51.2, 36.9, 31.0, 17.0, **15.0**
2411 (sus)	51.2, 36.9, 17.0, 15.0
B643 (res)	**51.2, 36.9**, 28.7, 17.0, 15.0, **14.3**
G8 (res)	**51.2, 36.9**, 28.7, 17.0, 15.0
G161 (res)	**51.2, 36.9**, 28.7, 17.0, 15.0
N-07 (res)	**51.2**, 17.0
G27 (sus)	**51.2**, 36.9, 28.7, 17.0, 15.0
6149 (sus)	**51.2**, 36.9, 28.7, 17.0
N215 (sus)	**51.2**, 36.9, 28.7, 17.0
N623 (sus)	**51.2**, 36.9, 28.7, 17.0

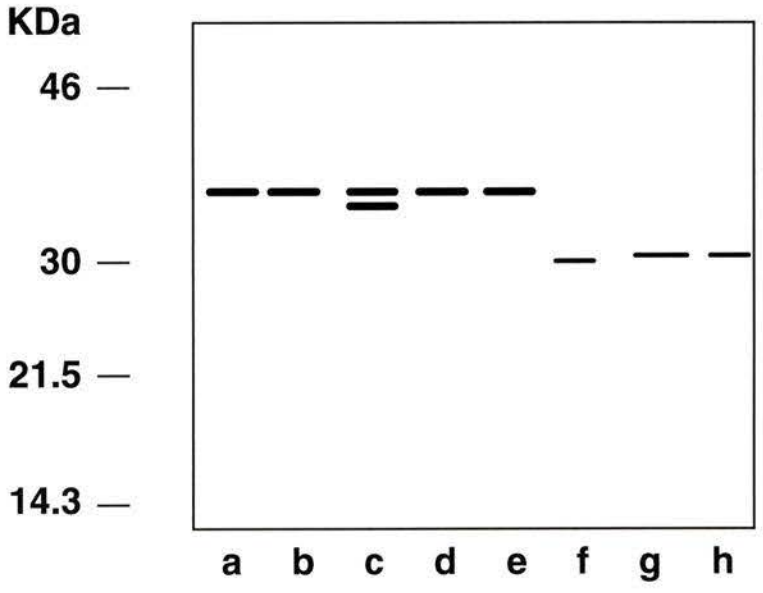
Figure 12.

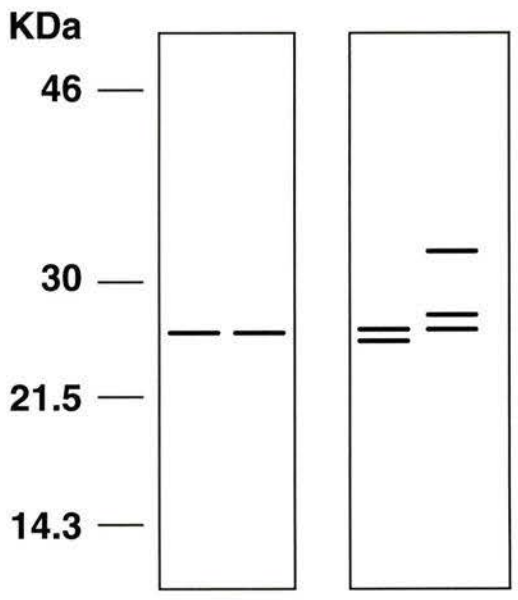
Immunoreactive proteins detected by *barley* anti-chitinase (top; a-h) and *petunia* anti-chitinase (bottom; A and B).

Top half of Figure 12 presents PRd (healthy) individual samples from all 4 families immunodetected by barley anti-chitinase, 1:750 dilution. Family 2391 (a & b; sus & res); family 2398 (c & d; sus & res); family 2413 (e & f; sus & res); family 2411 (g & h; sus & res). Arrows point to immunoreactive bands (30.0 kDa-≈37.0 kDa). Bands in 'f', 'g' and 'h' are less visible in the photograph than in the original blot.

Bottom half of Figure 12 presents 2 resistant LC individuals ('A'; G8 and B643 respectively) and 2 susceptible PRd (healthy) individuals ('B'; 2398-sus and 2391-sus respectively) immunodetected by *petunia* anti-chitinase, 1:5000 dilution. Four arrows point to immunoreactive proteins (**34.9 kDa, 30.4 kDa, 29.2 kDa, 28.1 kDa**). Bands in the right-hand lane of 'B' are visually more intense (***Family 2391, susceptible; healthy tissue***).

(k) = kilodaltons


KDa
46
30
21.5
14.3
a b c d e f g h


KDa
46
30
21.5
14.3

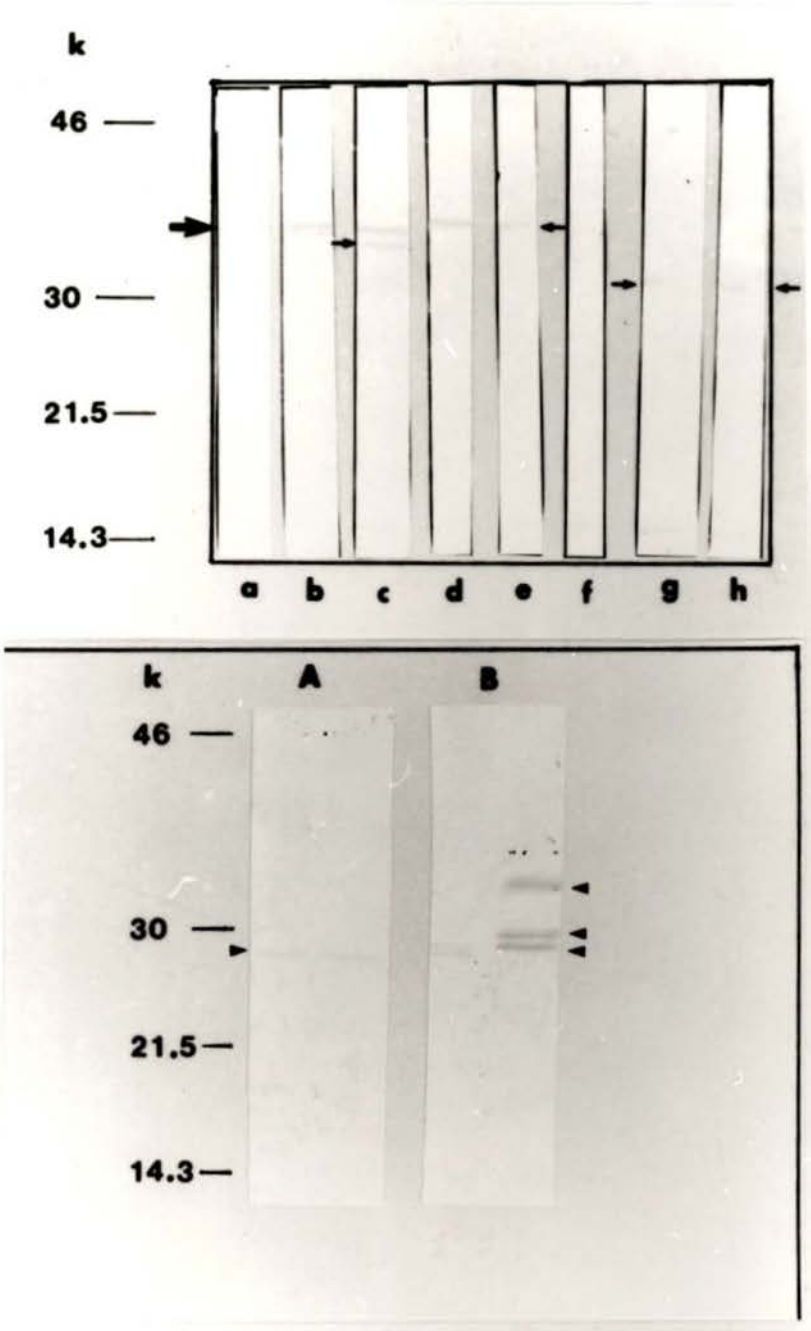

k
46
30
21.5
14.3
a b c d e f g h
k
A
B
46
30
21.5
14.3

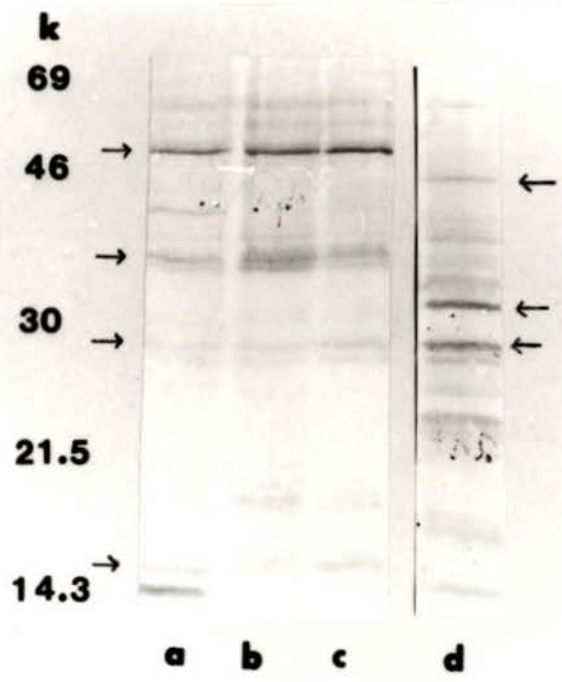
Figure 13.

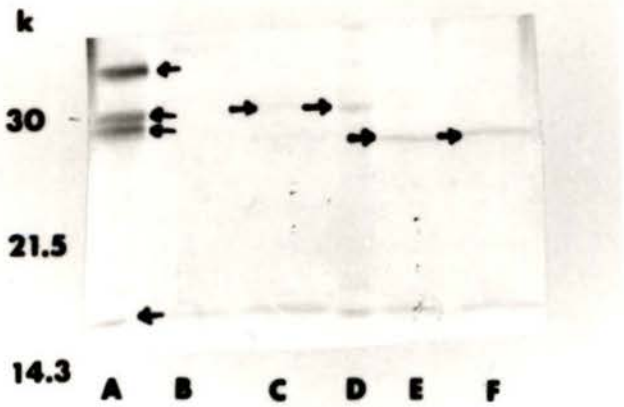
SDS-PAGE separated and Western blot immobilized and immunodetected proteins from LC and PRd samples. Top half of Figure 13 represents protein bands immunodetected by *barley* anti-chitinase and bottom half of the same figure represents protein bands detected by *petunia* anti-chitinase.

Top: *Barley* anti-chitinase immunoblot, using freshly prepared (from lyophilized powder) anti-chitinase antibody 1:750 dilution. Lanes 'a,b, & c' show immunoreactive bands detected in resistant individuals from LC (B643, G8, and G161 respectively) while lane 'd' shows bands detected in 2391-113-2 (PRd-susceptible, healthy tissue). Lane 'd' is also representative of the banding profiles observed for most PRd individuals. Arrows point to immunoreactive bands.

Bottom: *Petunia* anti-chitinase immunoblot (1:2500 dilution). Total protein loaded per sample = 30μg. 'A' = PRd pooled 'susceptible-healthy', 2391/2398; 'B' = N2-15 (LC, 'susceptible'); 'C' = G27 (LC, 'susceptible'); 'D' = pooled PRd 'susceptible-healthy' 2413/PRd 'resistant-healthy' 2411; 'E' = G8 (LC, 'resistant'); 'F' = B643 (LC, 'resistant'). Arrows point to immunoreactive bands. Pooling of resistant and susceptible extracts (lane 'D') was unintentional.

(k)= kilodaltons


k
69
46
30
21.5
14.3
a
b
c
d


k
30
21.5
14.3
A
B
C
D
E
F

Discussion

Much of the time spent in this study involved the development of a useable enzyme extraction procedure that could yield a product whose activity could be measured by colorimetric assay. Several difficulties are encountered in isolating catalytically active proteins from woody tissue. Such tissues are heavily lignified, containing few living cells per unit mass, and also contain high levels of phenolics- many of which are detrimental to enzyme activity and isolation [Haissig and Schipper, 1978]. By using vinylpyrrolidone polymers to bind phenolic substances, monoterpene cyclases along with some of the more robust enzymes (peroxidases and esterases) have been demonstrated in wood (conifer) extracts [Lewinsohn *et al.*, 1991a, 1991b]. Both PVPP and PVP were used in the chitinase-extracting buffer employed to provide a range of possible protection for both soluble and membrane-bound proteins [Loomis, 1974], however because of the nature of the biochemical assay (colorimetric), and in order to provide as potentially optimum a buffer system as possible, ammonium sulfate precipitated proteins were resuspended in simple (0.01M or 0.05M sodium acetate) buffers- without the addition of PVP. This method of using a conservative extraction buffer together with a 'chitinase-friendly' resuspension buffer was moderately successful as shown by the evidence that chitinase activity was colourimetrically detected in the bark tissue. Additionally, demonstration of enzyme activity in extracts derived from freeze-dried and ground bark suggests that the lyophilization and grinding processes did not completely destroy enzyme function in this tissue. Chitinase activity was slightly higher in extracts prepared from freeze-dried bark compared to extracts prepared from fresh foliage, however this may be a *tissue-specific* difference rather than one due to the use of fresh versus lyophilized material. If future studies demonstrate negligible differences in chitinase activity in fresh versus freeze-

dried *bark*, it would enable collection and safe storage of large amounts of assayable material (by freeze drying) without concern for sample deterioration.

Time limitations did not permit replication of chitinase assays on a wider sampling of resistant and susceptible bark protein extracts, neither was it possible to fractionate and further purify or characterize enzymatically active component(s) of the crude extract. Nevertheless, evidence from both *preliminary* chitinase activity assays, *and* immunodetection studies using both petunia and barley anti-chitinase polyclonal antibodies, suggests that several chitinase or chitinase-like proteins are *constitutively present* in white pine bark, and that they share serologic homology with other plant chitinases. This is the first report of chitinase activity in Western white pine bark tissue, and it is the first time that immunoreactivity to heterologous anti-chitinase polyclonal antibodies has been reported for the same species.

Some qualitative immunodetected protein differences that are of note include the presence of a **28.7** kDa band detected by *barley* anti-chitinase in 7 out of 8 LC samples but not in PRd samples. Likewise, several bands between **30.0 kDa** to **35.0 kDa** were observed in PRd individuals, but not LC individuals (using the same polyclonal antibody). Similar differences were noted with petunia anti-chitinase. These qualitative differences may be developmental (age-related), may represent polymorphisms of the same protein, or they may be a result of the more active host-pathogen interactions observed in the PRd collection.

While some of the immunoreactive bands detected in bark protein (especially with barley anti-chitinase) may have been due to non-specific binding, the possibility of the presence of more than one chitinase isoform in pine bark cannot be entirely ruled out. Multiple isoforms of chitinase are known to exist for several plant species. For example, Kombrink and coworkers [1988] were able to isolate 6 different basic isoforms from elicitor-treated potato leaves. The 6 isoforms ranged in molecular weight from **32.6 kDa**

to **38.7 kDa** [Kombrink *et al.*, 1988]. Two basic barley *seed* chitinases of molecular mass **33 kDa** and **28 kDa** have been purified and partially characterized with respect to N-terminal sequence, localization, physical/chemical properties and enzyme activity [Jacobsen *et al.*, 1990], while the predicted acidic *win6* proteins of *Populus* (*win6.2b* and *win6.2c*) both have a molecular mass of **34 kDa** following removal of a 21-22 amino acid signal peptide [Davis *et al.*, 1991]. Tobacco chitinase has been shown by SDS-PAGE to migrate as 2 distinct bands of relative mass **34 kDa** and **32 kDa** [Broekaert *et al.*, 1988]. Autoclaved *Fusarium oxysporum* has been shown to induce *acidic* chitinases of molecular weight **33.5 kDa** in yam callus tissue, while larger *basic* chitinases (**38 kDa**) have been induced by treatment with oligosaccharide and ethylene elicitors in the same tissue [Koga *et al.*, 1992]. Furthermore, the amino acid sequence of the N-terminal domain of *acidic* chitinase from *unstressed* aerial tubers of yam has been determined and shows sequence homology to the N-terminal domain of both wheat germ agglutinin and a tobacco basic (class I) chitinase [Araki *et al.*, 1992]. The significance of the latter finding is that chitinases have been found to be present constitutively as well as being induced. The fact the present study demonstrates immunological detection of bands of similar molecular weights (i.e. from **28 kDa** to **35 kDa**) with both petunia and barley antichitinases, together with the observation that plant chitinases in general tend to have similar biochemical/physicochemical properties [Broekaert *et al.*, 1988], suggests that the currently detected bands in *P. monticola* might be worthy of further investigation and characterization.

A 51.2 kDa band detected by *barley* antichitinase in both LC and PRd individuals- shown to be of weaker intensity in PRd seedlings- shares approximate molecular weight characteristics with a protein found to be *suppressed* in the compatible reactions of wheat rust-infected leaves [Heinz *et al.*, 1990]. Notably, *fungal* chitinases tend to be found within a similar molecular weight range. For example, 3 chitinase isoforms from

Phascolomyces articulosus range between 53 kDa and 69.5 kDa [Balasubramanian and Manocha, 1992]. However, it is unlikely that the 51.2 kDa immunoreactive band represents a fungal chitinase as all 'resistant' LC bark samples were taken from *canker-free* trees. Furthermore, if the protein were fungal in nature, one would expect to see a relative increase in the same from 'susceptible' (cankered) individuals, especially from samples that were collected near lesioned sites (PRd). Such samples would have a higher risk of being 'contaminated' by mycelial elements.

An approximately **37 kDa** band detected by the barley anti-chitinase polyclonal antibody, which gave a visually more intense reaction in 3 out of 4 resistant LC trees, bears molecular weight resemblance to an acidic **36.7 kDa** protein found to be significantly enhanced in *resistant P. lambertiana* seedlings 9 days after inoculation with *C. ribicola* basidiospores [Ekramoddoullah and Hunt, 1993]. Although it is speculative that both bands represent chitinases, the molecular weight similarities between the 2 bands, the immunological evidence given by the barley antichitinase for *P. monticola* bark, and the fact that the 36.7 kDa *P. lambertiana* band is significantly *enhanced* in response to basidiospore inoculation warrant further investigation.

Chitinases with molecular masses between **25 kDa** and **35 kDa** have been stimulated in stressed barley and cucumber, however *chitosanases* with molecular masses between **10 kDa** and **24 kDa** have also been stimulated in stressed barley, cucumber and tomato [Grenier and Asselin, 1990]. Four acidic chitosanases (10 kDa, 12 kDa, and 2- 14 kDa) have been reported in cucumber [Grenier and Asselin, 1990]. It is possible that some of the smaller immunoreactive bands detected by the anti-chitinase polyclonal antibodies could be chitosanases- although thus far, the partial N-terminal amino acid sequence of the **10.6 kDa** 'resistant' band in this study does not appear to demonstrate homology to any *known* plant chitosanases.

Attempts were made to match *strongly* immunoreactive bands with their corresponding bands on silver-stained SDS-PAGE gels. Unfortunately many of the immunodetected bands within the area of interest (28 kDa to 35 kDa) were either *faintly* detectable or poorly separated in the gel, making it difficult to assign positive identifications. *Increasing* the amount of total protein loaded per sample (i.e. to 50-100 μ g per lane) did *not* increase the intensity or gel resolution of the *bands of interest*. The use of CBB (triple stain method) also did not positively affect visualization of bands within this molecular weight range. Future attempts to separate these proteins using a gel with a 20% bis-acrylamide concentration, and thus an increased sieving capacity, may help to resolve this problem. It is possible that these particular proteins are *only* present in weakly detectable quantities, however that does *not* explain why increasing the amount of total protein loaded did *not* increase the relative quantity of the bands of interest.

Phenolic Interference

Apparent depletion of lower molecular weight proteins may have been due to the gradual formation of insoluble protein-phenolic compounds *either* during *concentration* or *storage* of protein extracts. Phenols combine with proteins reversibly by hydrogen bonding and *irreversibly* by oxidation followed by covalent condensations [Loomis and Battaile, 1966]. Because proteins analyzed in the *immunodetection* studies were extracted in the absence of PVP/PVPP (using ES-1 and ES-2), the formation of some protein-phenolic complexes was unavoidable. It should be noted at this point that bark protein extractions *were* attempted using PVPP (ratio of 2:1 w/w of PVPP to lyophilized and ground bark) during initial extraction with ES-1, however the total protein yield (determined by dot blot) was *less* when PVPP was included than when it was omitted (data not shown). Lewinsohn and coworkers [1991a] noted that when PVPP was used at 5% (w/v) to extract *pine bark* proteins, the level of extractable protein did not increase relative to its omission, suggesting that pine bark might contain elements that influence

both protein extraction and enzyme stability. Notably, bark extracts were less pigmented when PVPP was used, however *no* appreciable difference was noted in the one or two-dimensional protein profiles of proteins extracted with or without PVP/PVPP in this or other studies [Ekramoddoullah, 1993]. It is also notable that the gradual changes in the appearance of bark protein extracts (no PVPP) over time (e.g. darkening) and repeated 'freeze-thaw' cycles did *not* qualitatively affect one or two-dimensional protein profiles. The decision to leave PVPP out of the extraction protocol (for analytical one and two-dimensional electrophoresis procedures) was based on maximization of protein yield (as determined by dot blot analysis). Hence, because of the unavoidable presence of phenolic contaminants it is likely that over a period of time, some protein-phenolic complexes within this particular weight range (28 kDa to 35 kDa) may have precipitated out of solution.

Soluble bark protein extracts (extracted *with* and *without* PVPP) were generally observed to darken significantly and precipitate following *concentration* by the use of membrane microconcentrators (Amicon). This suggests that formation of protein-phenolic complexes during extraction procedures may have been both immediate *and* ongoing and that the addition of PVP or PVPP early in the extraction process did *not* completely scavenge phenolics. This observation is in agreement with that of Lewinsohn and coworkers [1991a] who found that PVP was able to sequester most phenolics, but did not remove them from solution. Likewise, PVPP was able to remove most phenolics responsible for browning, but was unable to protect enzyme activity. Subsequent oxidization of solubilized residual phenolics to quinones in the absence of reducing additives (i.e. ES-2 solution) increases the likelihood of forming further protein-phenolic precipitates [Loomis and Battaile, 1966]. Studies of protein-tannin interactions have shown that the formation of tannin-protein precipitates is *time* and temperature dependent [Hagerman and Robbins, 1987] with more precipitates forming after 24 hours at 4°C than

after 15 minutes at room temperature. The ratio of protein to phenolics in solution also affects the degree to which soluble versus insoluble complexes are formed. If *protein* is present in excess, then protein-phenolic complexes are more likely to be soluble [Hagerman and Robbins, 1987]. Concentrating protein extracts did not selectively remove phenolics- rather the concentration of soluble protein-phenolic compounds may have altered the protein to phenol ratio such that the formation of insoluble complexes was facilitated. A method of selectively extracting, *concentrating*, solubilizing and electrophoretically separating lower molecular weight (i.e. < 40 kDa) bark proteins is required to facilitate further study of those protein bands detected by barley and petunia polyclonal antibodies. Ideally as little time as possible should elapse between extraction and electrophoretic separation. A method of detecting chitinase activity *after* polyacrylamide gel electrophoresis is described by Trudel and Asselin [1989]. Using this method, the molecular weights of chitinolytic enzymes have been directly estimated, and it has been shown that *some* microbial chitinases are resistant to denaturation by both SDS and β-mercaptoethanol [Trudel and Asselin, 1989]. The advantage of being able to use SDS and mercaptoethanol without inactivating chitinase is obvious-however, tests still need to be done with plant chitinases to ensure that they share the hardiness of those from microbes.

CHAPTER 6

CONCLUSIONS AND FUTURE RESEARCH

This study has shown that there are ***multiple*** qualitative and quantitative differences between bark protein profiles of blister rust-resistant versus susceptible Western white pine. Although profile differences were more pronounced in 7 yr old seedlings (PRd) versus mature clones (LC), *susceptible* groups from both locations tended to display a higher relative abundance of unique and quantitatively enhanced proteins. Considerable variability was observed *within* resistant and susceptible groups from both locations, making the selection of putative 'marker' proteins difficult. Many of the statistically significant proteins identified through two-dimensional electrophoresis shared similar molecular weight and charge characteristics with the PR proteins described in the literature. Time and expense may preclude the use of two-dimensional electrophoresis as a meaningful screening tool for large populations (i.e. forest species); however, biochemical screening devices such as polyclonal antibodies can be used to qualitatively detect and/or quantify resistance markers in statistical sample sizes. The serendipitous availability of anti-chitinase, anti-PSII, and anti-Pin m III polyclonal antibodies has facilitated both the identification of serologically related (anti-chitinase) proteins in bark extracts, and comparison of the relative intensity of immunoreactive bands (anti-Pin m III) in susceptible and resistant white pine groups. Three 'significant' proteins (SDS-PAGE) have subsequently been isolated and characterized by N-terminal sequence analysis, thus providing the information necessary for the future development of other valuable molecular probes.

Other proteins that warrant further attention have been identified in this study; however, future investigations must first refine bark protein extraction methods to

accomodate preparative two-dimensional electrophoretic separations. Using probes developed from the 10.5 kDa 'resistance' protein and the ~26 kDa 'susceptible' protein, larger white pine populations can be screened and the utility of these markers (by their correlation with phenotype) confirmed. Future chitinase studies should focus on the refinement of assay techniques, and the screening of inoculated and uninoculated seedlings (bark and needle proteins) with heterologous probes from a wider species representation. Heterologous nucleotide probes could be used to screen white pine genomic libraries, facilitating the structural and functional identification and classification of this increasingly ubiquitous enzyme.

The fact that *susceptible* trees (i.e. seedlings) had greater numbers of unique proteins and higher relative amounts of shared proteins deserves further attention. In order to identify which of these proteins were in fact pathogenesis-related proteins (i.e. those induced by rust infection versus those related to host age or genotypic variation), I propose that studies should be done using cohort ramets (identical genotypes, same age; minimum sample size = 4) of rust-screened clones ('resistant' and 'susceptible' tested separately). After artificial inoculation of 50% of the ramets, bark samples would be taken at appropriate intervals (i.e. at the first sign of canker or bark reaction formation) from both control (uninoculated) and inoculated ramets. The extracted bark protein profiles from both groups would be compared to identify *induced* and possibly pathogenesis related proteins. Variations of such protein profile comparisons could be done by using already available antibody/nucleotide probes to screen inoculated and uninoculated ramets. Elicitor studies could also be performed to determine the specificity or non-specificity of induced protein synthesis. For example, seedlings could be exposed to treatment with mercuric chloride, bark wounding, exposure to ethylene, and wounding coupled with IAA/GA application (enhances wound periderm formation). The advantage of using this approach is that would enable the researcher to examine the *manifold*

biosynthetic host defense *response* to *rust infection, or other biotic/abiotic stresses* (in blister rust-susceptible or resistant phenotypes) without introducing the variation due to use of multiple genotypes. Ramets can be treated as 'replicates', enabling the researcher to 'pool' bark proteins with greater confidence. Significant proteins identified, isolated, and characterized through this method could be used as *specific* probes to test larger populations. Developing nucleotide probes to such proteins would permit the study of their differential regulation in resistant versus susceptible phenotypes.

Are there indeed proteins that can distinguish phenotypically resistant from susceptible western white pine? An alternative strategy used to answer this question would be to focus only on identifying a *common* protein for *many* genotypes that is associated with resistance (SCG) or susceptibility. Novel proteins are *preferred* to quantitative proteins, however, such proteins are rare and usually controlled by major genes. Host material of a *specified age* and from a specific *location* would be required to minimize variation due to age and environmentally related factors. The use of *full-sibling* families containing *constrasting* seedling pairs is much preferred to the use of half-siblings (open-pollinated), however, the reality of the situation is that such families are rare. Constrasting seedling pairs from at least 10 families would provide a sound base, with 4 seedling pairs as the absolute mimimum. A control tree (same family, same age, uninoculated) might be included for each seedling pair to provide a 'baseline' protein profile; however, because **only** those proteins common to, or significantly enhanced within, a given *phenotype* would be marked as candidates, such a control would not be necessary. The advantage of this approach is that it narrows down the choice of potential 'molecular markers' by selecting only those proteins that consistently co-segregate with a given phenotype. The disadvantage is that it would be considerably labour intensive.

Many heterologous probes for established 'pathogenesis related' proteins are available, and it might be enlightening to see if these probes can be used to distinguish

resistant from susceptible groups in the same way that *Pin m III* was able to distinguish resistant from susceptible Lens Creek individuals. Many enzymes and isozymes involved in the synthesis of polyphenolics, terpenes, growth hormones, and other phytoalexin-like compounds in woody plants could be differentially regulated- especially in phenotypes displaying resistance bark reactions. For example, current studies of *Pin m III* isoforms (enriched in LC susceptible bark tissue, SDS-PAGE) have shown that 2 isoforms are selectively enhanced in susceptible individuals and that 1 isoform is enhanced in resistant individuals [A.K.M. Ekramoddoullah, *unpublished data*]. These results suggest that differential regulation of isoforms may be used to select one phenotype from another.

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