Emerging technologies / Technologies naissantes

Challenges and opportunities in studies of host–pathogen interactions in forest tree species

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Abstract: Root pathogens and rust diseases can cause extensive damage to Canadian forest tree species. Loss of growth, although difficult to visualize, is substantial over the life of a tree. Understanding host–pathogen interactions is important in managing yield loss and can aid in the identification of disease-resistant trees. However, studying the interactions involving forest pathogens offers both challenges and opportunities. Some of these issues are described from our perspective through working on white pines – blister rust pathosystem and pathogens that infect the roots. Several resistance mechanisms to the white pine blister rust fungus, *Cronartium ribicola*, have been identified in pine. At the molecular level, several defence responsive proteins and their genes have been characterized. Some of these are identified to be potential candidates for markers associated with resistance or susceptibility. Current research activities and future directions and application of technologies to isolate and characterize resistance genes in white pine are discussed.

Key words : white pine blister rust, *Cronartium ribicola*, root pathogens, pathosystem, marker proteins, resistance genes.

Résumé : Les agents pathogènes racinaires et les rouilles des arbres peuvent causer d'importants dommages aux espèces forestières canadiennes. Une diminution de croissance, bien que difficile à observer, peut s'avérer considérable sur la durée de vie d'un arbre. La compréhension des interactions hôte–parasite est importante pour la gestion des pertes de rendement et peut contribuer à l'identification d'arbres résistants aux maladies. Cependant, l'étude des interactions impliquant des agents pathogènes forestiers offre à la fois des défis et des occasions. Certains de ces aspects sont décrits de notre point de vue, à la suite de nos travaux sur le pathosystème rouille vésiculeuse – pins blancs et les agents pathogènes qui infectent les racines. Plusieurs mécanismes de résistance au champignon de la rouille vésiculeuse du pin blanc, le *Cronartium ribicola*, ont été identifiés chez le pin. Au niveau moléculaire, plusieurs protéines de réponse défensives et leurs gènes ont été caractérisés. Certains d'entre eux sont identifiés comme des candidats possibles pour devenir des marqueurs associés à la résistance ou la sensibilité. Nous abordons aussi les activités de recherche actuelles et les orientations et applications futures des technologies d'isolement et de caractérisation des gènes de résistance du pin blanc.

Mots clés : rouille vésiculeuse du pin blanc, *Cronartium ribicola*, pathogènes racinaires, pathosystèmes, protéines marqueurs, gènes de résistance.

Introduction

Studies on host–pathogen interactions in forest tree species have focused on the most economically and environmentally damaging diseases in Canadian forests, which are more likely to attract stable funding, rather than focusing on a minor pathogen, even though a minor pathogen may be easier to manipulate. There is no shortage of important forest diseases in British Columbia (BC), as stem-rust and root pathogens can cause extensive damage (Neville and Winston 1994). These pathogens are probably of a high priority across Canada, with other pathogens such as dwarf mistletoe (*Arceuthobium* spp.) and casual agents of hypoxylon canker [*Entoleuca mammata* (Wahlenb.) J.R. Rogers & Y.-M. Ju], red band needle blight [*Mycosphaerella pini* Rostr.], and some needle casts being important in specific geographic regions. Many of the diseases caused by these pathogens can be insidious and not well recognized even by experienced forestry workers. Loss of growth is difficult to visualize and mortality may be minor on an annual basis. However, over the life of a tree crop, minor growth losses and sporadic mortality can add up to be substantial, and when these losses are put into forest yield models, their impact is better appreciated. Exotic diseases, such as chestnut blight [*Cryphonectria parasitica* (Murrill) Barr] and scleroderris canker [*Gremmeniella*

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abietina (Lagerb.) M. Morelet], that are now present, and others that are not yet established, such as pitch canker [*Fusarium circinatum* Nirenberg & O'Donnell] and sudden oak death [*Phytophthora ramorum* Werres, Marwitz & Man], have the potential to be devastating and will require research funding.

The challenges

For root pathogens, inoculation protocols need to ensure infection and death of all, or nearly all, primary hosts through natural infection courts, preferably in a short time. For some root diseases, such as tomentosus root rot, the infection court is unconfirmed. For others, good inoculation methods are wanting. For instance, laminated root rot [*Phellinus weirii* (Murr.) Gilb.] research has occurred for over 60 years in BC and the Pacific Northwest, but only recently has an acceptable protocol for inoculation been developed (Sturrock and Reynolds 1998). Such protocols would permit the ranking of species into primary and lesser hosts. Similar protocols need to be developed for most forest pathogens. Bypassing the natural infection court and using inconsistent inoculation methods may yield false interpretations of host–pathogen interactions. Consistent inoculation protocols are also necessary when assessing virulence of strains and evaluating resistance of host genotypes.

Some diseases have restricted geographic distributions, such as stalactiform rust [*Cronartium coleosporioides* Arth.], delphinella blight [*Delphinella abietis* (Rostr.) E. Muell.], elytroderma broom [*Elytroderma deformans* (Weir) Darker], hypoxylon canker, and tomentosus root rot [*Inonotus tomentosus* Teng], which are absent from the BC coast, even though the hosts are present. Undetermined epidemiological factors are probably responsible for the host–pathogen interaction being geographically limited. Keen field observations under various climatic conditions are needed to reach a starting point on which to base experimental epidemiological studies. A clear understanding of epidemiology is a necessity to obtain good repeatable inoculations when studying host–pathogen interactions.

Availability of inoculum is frequently limiting for epidemiological studies in forest pathology. Production of inoculum can be difficult, particularly with rusts and needle cast fungi, as they are obligate pathogens. Natural inoculum may be only available for a short period each year. Quantifying the inoculum dosage for repeat inoculations can be frustrating to researchers. Host–pathogen studies must consider host age, phenology, and cell and tissue type to obtain a clear picture of the interaction at the whole host level. Ontogenetic resistance is common in conifers (older conifers are immune to some rusts), older larch is tolerant of armillaria root rot [*Armillaria ostoyae* (Romag.) Herink], and spraying to control red band needle blight may cease in older pines. Phenology may assure needle cast infection or it may help to avoid disease by the host flushing new growth after the pathogen has dispersed its spores. Volatiles in the substomatic chamber of unique host genotypes may halt some pathogens from invading further. Resistant responses can be unique in different tissues as the pathogen invades deeper into the host. Within certain host genotypes, necrophylactic periderms may form in response to stem rusts and dwarf mistletoes.

White pine blister rust — a challenging pathosystem

Inoculation methods

In screening for resistance to white pine blister rust (WPBR) [*Cronartium ribicola* J.C. Fisch.] in white pines, many of the above problems have been encountered, and after considerable research, they have been solved, some more satisfactorily than others. The infection court has been confirmed as the stomates on needles. Basidiospores are the inoculum, and these are produced on telia attached to alternate host leaves (*Ribes* spp.); they cannot be produced on culture medium. Telia germinate best if they have been initiated at less than 20°C (Van Arsdel et al. 1956). This largely limits inoculum production to once each year, although small quantities of inoculum can be produced in growth rooms and glasshouses. Basidiospores can germinate near 0°C, making cold storage difficult, and they are delicate to the point of splattering when sprayed from most spray equipment (Matthews and Miller 1986). Inoculum density is crudely controlled when spores are cast from natural sources; however, an inoculation protocol that assures >95% infection of susceptible genotypes has been described (Hunt 1988). Techniques should be developed to allow production of basidiospores as needed, storage for future use, and uniform application.

Production of large numbers of uniformly grown seedlings is a requirement for a large screening trial. This work is not suitable for graduate thesis research. Seed cones are not produced on candidate field trees until they are about 15 years old; the cones need two growing seasons to ripen and they are not produced annually. To collect the seeds, trees need to be climbed and cones bagged prior to seed fall. Subsequently, trees need to be reclimbed to collect the seeds, which, in the meantime, may have been destroyed by insects or squirrels. Seeds need to be stratified for months before they germinate (Hunt 1988). First-year pines have solitary primary needles, which are radically different from the common secondary needles, the latter being produced in clusters of five. Primary needles are many times more susceptible to infection (Hunt 1991). Often, nursery-produced seedlings have a second flush in a season and this usually consists of primary or atypical needles, which are highly susceptible to infection. To maintain uniform seedlings, these and first-year needles must be removed by hand before inoculating 2-year-old seedlings. Inoculation screenings occur on current secondary needles, but if older plants are inoculated, infection diminishes on current needles. There is some data to indicate that 1- and 2-year-old needles are more susceptible than current year needles; in young plants, the reverse seems to be true (Hunt and Jensen 2000). Sorting out this host–pathogen interaction requires good control over inoculum density, uniform host genotypes of various ages, various stages of phenological development, and, perhaps, various epidemiological conditions. This has yet to be done.

Some host genotypes may produce surface waxes of unique form, which hinder basidiospore germ tubes from

locating stomata. Water droplet size is critical for good germination (Hansen and Patton 1977). Spores occurring in large droplets germinate abnormally, but they may germinate well at the periphery of large droplets and in small droplets. We abandoned an automatic watering system for wetting pine foliage because at the beginning of the water line, the droplets amalgamated into unacceptably large droplets, while at the end of the water line, there were too few droplets. Water application with hand-held misters delivered fine droplets, as judged visually, and were more satisfactory. Water droplet size and number are still poorly controlled factors affecting basidiospore germination, which may be critical in studying effects of surface waxes or host–pathogen interactions during penetration.

Sometimes, germ tubes penetrate between the guard cells, form substomal vesicles, but fail to ramify through the needle parenchyma. At other times, ramification through the parenchyma is extensive, causing a conspicuous yellow infection spot before penetrating the endodermis and into the vascular bundle. Yet, at other times, the pathogen readily penetrates the endodermis and only a small infection spot is formed. Hypersensitive reactions caused by single genes are also known to occur in needle tissues (Kinloch and Dupper 2002). Variability seems to be the rule in the first phase of this host–pathogen interaction.

Identifying disease resistance

Slow-canker-growth resistance (Hunt 1997) is a "partial resistance" phenomenon that is believed to confer durable resistance. It occurs in the main stem or branches, but not in the needles. In screening for this, a composite inoculum of aeciospores is collected in the spring to inoculate the blister rust alternate host (*Ribes* spp.), which in turn produces heterogeneous inoculum for screening white pine seedlings in the fall. Additionally, single-gene resistances are sought. If the pathogen has genes that overcome the single-gene resistance, they may be easily picked up in a composite inoculum and nullify the single-gene resistance. It is a challenge to have diverse inoculum to adequately screen for a particular trait, yet not discriminate against other traits, some of which are yet to be identified. For instance, it appears that in screening a particular white pine family from the U.S.A., we have identified a single dominant gene for resistance, while this family is classified as susceptible using composite inoculum in Oregon. Additionally, identifying and maintaining an inoculum that overcomes single genes is important as it can be used to unmask single genes to confirm that resistance genes have been stacked in a breeding program designed to pyramid several resistance genes. These sets of contrasting resistant and susceptible host genotypes versus virulent and avirulent pathogen genotypes are desirable for studying host–pathogen interactions. These are only just now being recognized for the WPBR host–pathogen system.

The durable slow-canker-growth resistance is usually confirmed in a blister rust screening program 5 to 6 years postinoculation, or sometimes longer. It is a challenge to reduce the time to identify this resistance. An understanding of the host–pathogen interaction conferring a slow-canker-growth response is important in developing markers. There are different phenotypes of slow-canker-growth resistance, which may be attributable to parts of the same set of genes or to entirely different genes. A suite of marker probes may be needed to recognize the full range of slow-canker-growth responses. A quick test based on molecular probes for slow-canker-growth resistance would be useful for breeding programs trying to pyramid resistance genes. Because single-gene resistances occur in the needles before slow-canker-growth occurs in the stem, they may mask the slow-canker-growth trait. Probes for slow-canker-growth resistance would permit recognition of slow-canker-growth resistance in seedlings with single-gene resistance traits. Once there is a good quick test to confirm slow-canker-growth resistance, there would be an opportunity to develop it for production use in culling seedlings that lack slow-canker-growth resistance so that these are not used for reforestation.

White pine blister rust — molecular understanding of resistance mechanisms

Protein analysis

Classical breeding for tree improvement has been very useful and will continue to be an approach in the future. However, this process can be aided immensely if a molecular understanding of different resistance mechanisms is obtained. This knowledge would allow breeders to sustain tree improvement on a long-term basis. Molecular analysis of this host–pathogen interaction can lead to the discovery of defense response genes and their protein products. These products could provide probes for screening trees for resistance. In one approach, pathogenesis-related (PR) proteins that are differentially regulated between susceptible and resistant pine seedlings following inoculation were studied. Pathogenesis-related proteins are among the most widely studied components of plant defense responses. Eleven groups of PR proteins have now been described (van Loon et al. 1994). We discovered that conifer proteins were extremely difficult to extract (Ekramoddoullah 1991). Therefore, extraction procedures from white pine needles had to be developed (Ekramoddoullah 1993). This method allowed the extraction and electrophoresis of proteins without interference from phenolic substances. However, the protein extract contained a high amount of detergent and a reducing agent, which interfered with commonly used protein determination methods. Subsequently, a method for the determination of conifer proteins extracted with detergent and reducing agents was developed (Ekramoddoullah and Davidson 1995).

Another challenge was to look for proteins, either preformed or induced, that were uniquely associated with resistant or susceptible phenotypes. Having successfully developed a methodology for extraction and determination of proteins, we decided to use a well established procedure of O'Farrell's 2-dimensional (2-D) gel electrophoresis. Although this procedure can resolve thousands of proteins, its use has been limited because it is a painstaking task to manually score the 2-D gel data and the data is only qualitative. However, qualitative and quantitative analyses were made possible by coupling the laser gel scanner with specialized software for processing 2-D gel data. This allowed us to build a 2-D gel data base consisting of thousands of conifer proteins from resistant, susceptible, pathogen-induced and

noninduced seedlings (Ekramoddoullah and Hunt 1993). We developed a 2-D gel protocol (Ekramoddoullah and Tan 1998) that greatly reduced gel streaking and background staining of protein samples by phenolic compounds. This increased the number of protein spots per gel and helped resolve spots with computer-assisted image software. With the recent advances in matrix-assisted laser-desorption ionization time of flight mass spectrometry for direct sequencing of proteins from stained 2-D gels, this has become an important tool in proteomics soon to be used in conifer genomics and proteomics.

Detection of unique proteins

Electrophoretic analysis of bark revealed two proteins (26.0 and 10.6 kDa) associated with slow-canker-growth resistant and susceptible western white pine (*Pinus monticola* Dougl. ex D. Don) trees, respectively (Davidson and Ekramoddoullah 1997). Partial protein sequencing revealed that the 26.0-kDa protein had a strong homology to chitinase, and the 10.6-kDa protein, a strong homology to an antifungal protein isolated from *Macadamia integrifolia* Maiden & Betche (Marcus et al. 1997).

During a 2-D gel electrophoretic analysis of sugar pine (*Pinus lambertiana* Douglas) proteins (Ekramoddoullah and Hunt 1993), we observed two intensely stained protein spots of low molecular mass in the acidic region of the gel. We obtained a partial N-terminal sequence of one protein (GenBank accession No. A40451) from both 1-D and 2-D gels that showed no homology with known proteins and named this *Pin l* I. Recently, the deduced amino acid sequences from *Pin l* I cDNA revealed that this protein belonged to the PR-10 family (Ekramoddoullah et al. 2002). We developed a specific anti-*Pin l* I antibody (Ekramoddoullah et al. 1995) and this antibody was used to detect a homologous protein of similar size in western white pine. The homologous protein was named *Pin m* III. These proteins are also induced by cold stress (J.J. Liu, A.K.M. Ekramoddoullah, and X. Yu, unpublished data).

We have analyzed bark proteins of mature resistant (i.e., free of cankers) and susceptible (with several cankers) western white pine trees (Davidson and Ekramoddoullah 1997) to detect the levels of *Pin m* III protein. The level of this protein was significantly lower in resistant trees than in susceptible ones, and higher in winter than in summer (Ekramoddoullah et al. 1998). In diseased trees, cankered tissues had high levels of *Pin m* III, while samples collected from the outside edge of the canker had low levels of *Pin m* III. The high level of *Pin m* III in infected tissues was postulated to be a consequence of fungal infection. However, the level of *Pin l* I was significantly higher in the foliage of inoculated sugar pine seedling carrying a single dominant gene for resistance (*Cr1*) than in the foliage of inoculated susceptible sugar pine seedlings (Ekramoddoullah and Tan 1998). Stem samples of both resistant and susceptible sugar pine seedlings had similar levels of *Pin l* I.

We have examined the localization of *Pin m* III in an infected needle spot of susceptible western white pine immunocytochemically with anti-*Pin l* I antibody using both light and electron microscopy (A.K.M. Ekramoddoullah, D.W. Taylor, G. Jensen, and L. Manning, unpublished data). These results illustrated that the *Pin m* III protein was bound to the blister rust fungus regardless of whether the fungus was inside or outside the host cell. Similar results were found under light microscopy utilizing a silver enhancement technique. This is the first time that a PR-10 protein has been shown to bind to the pathogen.

To gain more insight into the up-regulation of PR-10 proteins in other conifers, we have extended our research to the pathosystem Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) – *Phellinus weirii*. We devised a unique strategy to develop probes to identify and characterize PR-10 proteins in Douglas-fir. This strategy involved polymerase chain reaction (PCR) cloning of Douglas-fir genomic DNA utilizing primers based on a white pine *Pin m* III cDNA sequence. The deduced amino acid sequence of Douglas-fir PR-10 was compared with that of the white pine PR-10 protein and showed about 80% homology. Since the primer sequence of white pine PR-10 protein was reflected in N- and C-terminal sequences, the homology was overestimated. We synthesized a peptide based on the sequence information distal to the N-terminal region but well conserved between white pine and Douglas-fir PR-10 protein. An antibody made to this synthetic peptide detected PR-10 protein in Douglas-fir tissues and showed seasonal variation; it was up-regulated following infection with *Phellinus weirii* (Ekramoddoullah et al. 2000). When compared with five-needle pines, Douglas-fir and white spruce (*Picea glauca* (Moench) Voss) had extremely low amount of protein expressed, suggesting differences in the regulation of PR-10 gene expression in different conifer species.

Gene analysis in conifers

Stress induction

Since our work clearly showed that the expression of PR-10 genes is induced by both biotic and abiotic stress, we are investigating whether *cis*-acting regulatory elements in the gene promoter region are responsive to different stresses. In particular, we are interested in delineating cold and fungal responsive regulatory elements in the promoter, which may shed light on the cross-communication between these two different stimuli. Few PR-10 gene promoters have been isolated and characterized. Some regulatory elements responsive to pathogens have been tentatively characterized (Eulgem et al. 1999). These studies revealed that the process of transcriptional activation by elicitation differed among various PR-10 genes within the same species. DNA-binding proteins (WRKY) have been identified in parsley, which bind to an elicitor-response element of its PR-10 gene promoter (Rushton et al. 1996). An elicitor-response element in the potato PR-10 promoter has also been identified (Matton et al. 1993). An accumulation of the PR-10 mRNA was correlated with the binding of two transcription factors to these elicitor-response elements, indicating elicitor-dependent activation of the PR-10 gene (Despres et al. 1995). Hajela et al. (1990) presented evidence that a cold-regulated gene, *corI* 5a of *Arabidopsis*, had a cold-inducible promoter, and subsequently a *cis*-acting regulatory element that can impart cold-regulated gene expression (Baker et al. 1994).

Since no other PR-10 genes, other than those in conifers, were shown to be induced by cold stress, studying conifer PR-10 gene promoters would be of value in understanding

the mechanisms of cross-communication between different external stimuli, such as cold stress and pathogen infection. Thus, we started a systematic analysis of PR-10 gene promoters in white pine. Recently, we have isolated, cloned, and sequenced three PR-10 gene promoters, each having between 85 and 100% homology with *Pin m* III cDNA in an open-reading frame (J.-J. Liu and A.K.M. Ekramoddoullah, unpublished data). The sequence analysis of the promoters revealed that they have characteristic regulatory elements responsive to various stresses. Deletion analysis of one of the western white pine PR-10 gene promoters, *PmPR10-1.14*, utilizing a transient expression system, showed that the promoter activity could be increased three-fold. The study also revealed the presence of enhancer and silencer elements in the promotor region. Fluorometric β-glucuronidase (GUS) assays of transgenic tobacco plants demonstrated that the longest promoter of 1675 base pairs directed GUS expression constitutively at high levels in the roots of mature plants, but in other tissues such as leaves, stems, and flower organs, expression levels were too low to be detectable in histochemical assays. Histochemical localization analysis showed that *PmPR10-1.14* promoter directed a tissue-specific expression exclusively during the initiation and development of the lateral roots. The distal 5′-deletion of promoter to –311 did not decrease the expression level significantly in the roots, suggesting that the *cis*-acting regulatory elements necessary for a high level of gene expression reside in the proximal fragment from –311 to +69. As one striking feature, *PmPR10-1.14* promoter contains two copies of direct repeat sequence of 281 base pairs at its distal 5′ region. Deletion of one copy (–1326 to –1045) or both copies (–1675 to –1045) of the repeat sequence increased gene expression significantly in leaves and stems, which was regulated developmentally. Further deletion to –820 erased the increased gene expression in leaves and stems. These experiments have revealed that the root-specific expression of *PmPR10-1.14* gene in transgenic tobacco plants is mediated by different promoter regions with both negative and positive regulatory mechanisms. This type of promoter offers a wide range of applications in expressing endogenous or foreign proteins for the enhancement of plant resistance to pathogens and pests or of stress tolerance to heat, salt, and drought and in improving the nutritive value of edible root plants and the production of recombinant proteins aimed at molecular farming and phytoremediation.

In the wound-induced expression of PR-10 genes in western white pine, we isolated 13 different PR-10 cDNAs. Northern blot analysis showed that PR-10 gene expression was activated by both local and systemic wounding, and the accumulation of PR-10 transcript was much more abundant and rapid in locally wounded needles than in systemically damaged tissues. Western immunoblot analysis following isoelectric focusing and 2-D electrophoresis revealed that nine PR-10 proteins accumulated to different extents after wounding. Wound-inducible PR-10 proteins were differentially expressed in response to cold-hardening and pathogen infection. The wound-induced PR-10 protein accumulation was enhanced by the wound-signal compound methyl jasmonate and by okadaic acid, a specific inhibitor of type 1 or type 2A serine/threonine protein phosphatases. However, it was partially suppressed by salicylic acid and abscisic acid. These data provide an opportunity to elucidate further the signal transduction pathway involved in the activation of PR-10 protein synthesis in the defense response of white pine to mechanical injury. Characterization of key genetic elements in disease-inducible and wound-inducible promoters is essential for developing genetically engineered disease- and insect-resistant conifers.

Pathogen induction

We are currently conducting a molecular study of the Douglas-fir – armillaria root rot pathosystem. Protein was extracted from root bark of 11- and 25-year-old interior Douglas-fir (*Pseudostuga menziesii* var. *glauca* (Beissn.) Franco) trees that were naturally infected with *Armillaria ostoyae* (Robinson et al. 2000). The SDS–PAGE profiles of healthy and infected bark, or with adjacent-to-root infection, revealed significant differences in the concentration of a 29.3-kDa protein. The N-terminal sequence of this protein displayed significant homology to a white pine 26.0-kDa protein (Davidson and Ekramoddoullah 1997) and to an internal sequence of a basic endochitinase isolated from *Brassica napus* L. Use of a polyclonal antibody produced against N-terminal synthetic peptide of this putative endochitinase-like protein (ECP) indicated differences in the quantities of ECP in healthy roots compared with roots infected by *A. ostoyae*. The amount of ECP was elevated in root bark of coastal Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco var. *menziesii*) in response to *Phellinus weirii* infection, although in lower quantities relative to those found in the *A. ostayae* – Douglas-fir pathosystem. Western immunoblot analysis using anti-ECP resulted in the detection of 27- and 29-kDa polypeptides in both control and inoculated samples. Two-dimensional gel electrophoresis followed by Western immunoblot analysis revealed that the apoplastic fluid contained multiple ECP isoforms with pIs ranging from 5.3 to 5.8 and molecular masses of 27–30 kDa. The activity of native ECP was determined using a colorimetric assay and a gel overlay technique. The gel overlay assay confirmed that the antibody specific to the ECP recognized an enzymatically active protein. Because cold-induced chitinases in winter cereals are also known to have antifreeze activity (Hon et al. 1995), it was of interest to examine the active chitinolytic apoplastic fluid obtained from Douglas-fir during the winter for antifreeze activity. Antifreeze activity was confirmed and seasonal sampling of needle tissue showed some accumulation of ECP in winter months. While microsequencing these proteins of apoplastic fluid, we have also detected a thaumatin-like protein, a PR-5. We have produced antibody to this PR-5 protein, which allowed the detection of a similar protein in white pine – blister rust pathosystem. Like PR-3 protein (i.e., endochitinase), PR-5 is also induced in both pathosystems in response to infection. We have recently sequenced cDNAs encoding PR-3 and PR-5 proteins (J.J. Liu, N. Piggott, and A.K.M. Ekramoddoullah, unpublished data).

Monoclonal antibodies and their use in the study of host–pathogen interaction

We have produced monoclonal antibodies to the white blister rust fungus (Ekramoddoullah and Taylor 1996). The

reason for producing monoclonal as opposed to polyclonal antibodies was to have antibodies with specificity unique to the pathogen and not the host. Specific antibodies are useful for distinguishing the origin of PR proteins. They may be useful in evaluating host resistance or susceptibility by quantifying immunochemically the fungal mass in the infected host. However, the production of monoclonal antibodies is an expensive proposition and is time consuming. Another problem was the source of fungal tissues for producing monoclonal antibodies. Mycelium would have been ideal, but culturing the obligate pathogen is difficult. We used basidiospores to produce a large number of monoclonal antibodies. A majority of the antibodies cross-reacted with white pine proteins or other pine fungi, with a few that exclusively recognized *C. ribicola* antigens. Two fungal-specific monoclonal antibodies were reactive to antigens extracted from mycelium and infected needles. More importantly, following inoculation of seedlings, about 90% of the susceptible and 5% of the resistant seedlings had fungal antigen detected by these monoclonal antibodies (Ekramoddoullah and Tan 1998), suggesting their potential use in screening for resistance. We were interested in genetically engineering one of the monoclonal antibodies into white pine to provide another method for rust resistance. Recently, the genes encoding one monoclonal antibody (Mab 7) were cloned and sequenced and an scFv cDNA suitable for introduction into western white pines to generate potential resistance to WPBR through plantibody immunomodulation approach was constructed (Wilde 1998). Since the antigen reactive to Mab 7 was found to be a homologue of HSP70 (Yu et al. 2001), several points would need to be considered if this Mab 7 was to be engineered into white pine. It may be envisioned that the Mab 7 produced against the fungal HSP70 could cross-react with the host's HSP70 and thus be detrimental to the host by interfering with the function of its HSP70. Since Mab 7 did not cross-react with white pine proteins (Ekramoddoullah and Taylor 1996), it possibly recognizes an epitope unique to the fungal HSP70. One strategy would be to design an appropriate vector so that the plantibody is secreted out of the host's cell as is done for molecular farming of plantibodies (Haitt et al. 1989). Whether our single chain antibody to fungal HSP70 would likewise be able or not to confer resistance to WPBR is the subject of our ongoing research. Because there are few other mechanisms (except major gene resistance) available to enhance resistance to the blister rust, plantibodies may provide another layer of resistance.

Opportunities for future research

Over the last 5 years, we have repeatedly inoculated western white pines containing the hypersensitive *Cr2* gene (Kinloch et al. 1999) without obtaining infection, and it has not failed in over 15 years of field testing in BC, demonstrating that it provides resistance against BC isolates of the pathogen. There is now a major effort by the Forest Genetics Council of British Columbia to identify individuals homozygous for *Cr2* to use them as pollen sources in BC seed orchards. In general, a hypersensitive response is the hallmark of a single-gene interaction with the avirulence gene of the pathogen, whereby the host cells immediately surrounding the initial pathogen invasion undergo sudden cell death. According to the gene-for-gene theory (Flor 1971), for each resistance gene identified in the plant, there exists a corresponding avirulence gene in the pathogen. The most common biochemical model for this gene-for-gene relationship presumes a recognition process between a pathogen-derived elicitor of the plant defense and a plant-derived receptor for the elicitor. A wide variety of such genes have been isolated from agronomic crops. Deduced protein sequences revealed several common motifs. Recently, these common motifs leucine-rich repeat (LRR), nucleotide binding site (NBS), and leucine zipper (LZ) have been exploited as PCR primers to amplify resistance gene analogues (RGAs) from a number of plants. Resistance gene analogues have been cloned, sequenced, and linked to known disease resistance gene loci by mapping on various genetic maps. More recently, a technological breakthrough has been made to functionally analyze RGAs (Bendahmane et al. 2000). This method requires that the elicitor from the pathogen and the corresponding gene of the host be well characterized. Utilizing the RGA approach, we propose to isolate these *Cr* genes from both western white pine and sugar pine. Characterization of these genes would pave the way for creating trees with multiple resistance specificity, including slow-canker-growth resistance, spots-only resistance, and newly identified *Cr* genes.

Based on the conserved sequences of resistant gene signature motifs (i.e., NBS, LZ, or LRR), a large number of primers will be designed and used in PCR reactions to isolate RGAs from genomic DNA and cDNA libraries of western white pine and sugar pine. We have recently constructed 12 subgenomic DNA libraries for western white pine and used them as templates for genomic DNA walking to clone PR-10 genes and their promoters. Once RGA fragments are identified using PCR with above-mentioned degenerate primers, we can use a similar strategy (i.e., subgenomic library and DNA walking) to clone full-length genes and RGAs. cDNA libraries for inoculated resistant western white pine and sugar pine foliage will be constructed as we have done previously for white pine and fungal cDNA libraries. Although RGAs have been isolated from genomic DNA, the purpose of utilizing cDNA libraries in this study is two-fold. First, there is some evidence that the expression of resistance genes is enhanced following exposure to the pathogen, and second, these libraries will also be used for cloning genes encoding PR proteins identified in the proteomic analysis. Resistance gene analogues will be fully sequenced and characterized. In addition to their functional analyses (see below), RGAs will be included in the sugar pine gene linkage map. Resistance gene analogues will be functionally analyzed using *Agrobacterium* transient expression whereby the elicitor-like *Cro r* I (Ekramoddoullah et al. 1999; Yu et al. 2002) and the candidate R gene (i.e., RGA) will be expressed in transgenic tobacco, resulting in a hypersensitive response. In this procedure, transgenic tobacco will be generated by *Agrobacterium*-mediated stable transformation with cDNA encoding *Cro r* I, and then, cloned RGA will be agroinfiltrated into these transgenic tobacco plants to elicit hypersensitive response. This approach was chosen because it is easier than conventional map-based cloning or transposon tagging, which is ex-

tremely difficult for *Pinus* spp. because their physical genome size is estimated to be >31 pg. This size would correspond to a genetic distance of 30 Gb.

We also plan to identify the disease, wound-responsive and cold-inducible *cis*-acting regulatory sequences in cloned PR-10 promoters that are involved in the signal transduction pathway of white pine defense response to blister rust. Two strategies will be used to identify the regulatory sequences. We will analyze promoter activity using a transgenic *Arabidopsis* or tobacco system that is well established in our laboratory. A series of 5′-deletions of promoters will be constructed and fused to the reporter gene GUS in transgenic plants. β-Glucuronidase activity will be measured upon infection (disease or wounding) to determine which promoter region is responsible for gene expression. Furthermore, we will use electrophoretic mobility-shift assays to determine crucial *cis*-acting regulatory sequences. We will extract nuclear proteins from infected or wounded needles and incubate them with nonradioactive labeled promoter. We will use the identified *cis*-acting regulatory sequences of PR-10 gene on the affinity chromatogragh column to purify the transcription factor(s) (TFs) specifically expressed upon fungal infection or wounding. These purified TFs will be characterized at the molecular level and microsequenced to assess their biological function in the disease-activated signal transduction pathway. In the meantime, we will clone their cDNA sequence via RT–PCR or southwestern screening of white pine cDNA library using the identified *cis*-acting regulatory sequences as probes. Finally, an expression study of TFs will provide information on their interaction with other proteins or regulators that occupy the intermediate positions in the signal transduction pathway between western white pine *Cr2* gene and PR gene expression

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