Minireview / Minisynthèse

Molecular tools in the study of the white pine blister rust [*Cronartium ribicola*] pathosystem

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Abstract: Tree diseases cause extensive damage to Canadian forests, resulting in severe economic impact. Understanding host–pathogen interactions is important in managing yield loss and can aid in identification of disease-resistant trees. Molecular characterization of the genes and proteins that make up resistance and virulence phenotypes is critical to understand the function, evolution, and stability of a given conifer pathosystem. In this review, a perspective of some of these issues and challenges faced while working on the white pine blister rust [*Cronartium ribicola*] pathosystem is presented. Several defense-response proteins and their genes have been characterized through the use of proteomic and genomic approaches and may serve as candidates for markers associated with resistance or susceptibility to disease in white pine. Current research as well as 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*, pathosystem, virulence, defense proteins, resistance genes, molecular characterization.

Résumé : Les maladies des arbres causent d'importants dommages aux forêts canadiennes et, conséquemment, d'importantes pertes économiques. La compréhension des interactions hôte–pathogène est nécessaire pour limiter les pertes de rendement et peut être utile à l'identification des arbres résistants aux maladies. La caractérisation moléculaire des gènes et des protéines responsables de la résistance ou des phénotypes de virulence est essentielle à la compréhension de la fonction, de l'évolution et de la stabilité de tout pathosystème coniférien. Dans la présente synthèse, nous présentons un aperçu de certains de ces aspects ainsi que des défis à relever lorsque l'on travaille avec le pathosystème de la rouille vésiculeuse du pin blanc [*Cronartium ribicola*]. Plusieurs protéines de défense induites et leurs gènes, caractérisés par des moyens relevant de la protéomique et de la génomique, sont des candidats pour servir de marqueurs liés à la résistance ou à la sensibilité chez le pin blanc. La recherche actuelle ainsi que les futures orientations et applications de technologies pour isoler et caractériser les gènes de résistance du pin blanc sont examinées.

Mots clés : rouille vésiculeuse du pin blanc, *Cronartium ribicola*, pathosystème, virulence, protéines de défense, gènes de resistance, caractérisation moléculaire.

Introduction

Tree diseases are natural components of forest ecosystems. However, even native tree diseases that have coevolved with particular host species present within an ecosystem can cause extensive damage and result in severe economic impact, particularly in coniferous stands. Introduced pathogens can be even more damaging to forest ecosystems. Management of these diseases is critical to sustain the health and productivity of Canada's forests.

In British Columbia, losses due to tree diseases result in an estimated annual growth loss of 17.5%. (approximately Can$2.6 billion) (Jack Woods, Program Manager, Forest Genetic Council, British Columbia, personal communication). Because of this economic impact, several forest pathogens were identified as research priorities in British Columbia (Neville and Winston 1994). These include the root diseases caused by *Armillaria* spp. and *Inonotus tomentosus* (Fr.:Fr.) S. Teng., laminated root diseases caused by *Phellinus weirii* (Murrill) R.L. Gilberton, and white pine blister rust (WPBR) [*Cronartium ribicola* J.C. Fisch.] and gall rust [*Peridermium harknessii* J.P. Moore]. Knowledge of how coniferous hosts interact with pathogens can aid in the identification and deployment of trees with improved resistance to disease. Molecular tools, such as genomics and proteomics, provide an unprecedented opportunity to eluci-

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date the genetic bases of tree defenses to pathogens. My research program has extensively employed these tools to study the WPBR pathosystem.

White pine

White pine refers to 25 different five-needle pine species belonging to three sections of the subgenus *Strobus*. White pine species native to North America include western white pine (*Pinus monticola* D. Don), eastern white pine (*Pinus strobus* L.), and southwestern white pine (*Pinus strobiformis* Engelmann). In Europe, Balkan or Macedonian pine (*Pinus peuce* Gris), Armand pine (*Pinus armandi* Franch.), and Swiss stone pine (*Pinus cembra* L.) are prevalent, while Himalayan pine or blue pine (*Pinus wallichiana* A.B. Jaks.) and Korean pine (*Pinus koraiensis* Sieb & Zucc.) are of Asian origin.

White pine blister rust infects all white pines. During its life cycle, the fungus produces five different spore states on the two unrelated hosts, i.e., pine and its alternate host, *Ribe*s spp., such as currants and gooseberries. The orange aeciospores of blister rust produced on the pine infect *Ribes*, on which urediniospores, teliospore, and basidiosopores are produced. In the fall season, basidiospores released from the *Ribes* host are transported by wind to needles of nearby pines where they germinate, and the mycelia penetrate through stomata. Visual disease symptoms, such as yellow, red, orange, or purple spots, appear on needles the following spring. Usually, it takes 1 to 2 years for an infected tree to develop cankers on its stem.

White pine blister rust originated in Asia, perhaps in association with Korean pine, and spread to Europe when Tibetan monks brought bonsai blue pine to Ukraine (Hunt 2003). White pine blister rust became a serious problem as early as 1865 (Blada and Popescu 2004), and by the early nineteenth century, it had spread throughout Europe. At about that time, it was also introduced into Vancouver, British Columbia, via a shipment of infected eastern white pine seedlings from France.

Western white pine is a fast-growing and valuable component of the timber industry. Since its introduction, WPBR has devastated western white pine. The rust has severely impacted forest ecosystems and has caused reduced planting of *P. monticola* and other related pine species. Western white pine was the predominant conifer species in the Pacific Northwest before the introduction of the blister rust pathogen. This is an important tree species in Pacific Northwest's forest ecosystems. The ecological and economic importance of this species and its susceptibility to the rust were the main factors that prompted our research. We employed the genetic resources available from the western white pine breeding program in British Columbia, Canada, to study the molecular basis of genetic resistance to WPBR infection.

Genetic resistance

Knowledge of the nature and pattern of adaptive genetic variation is required to maintain species viability for conservation goals and to protect genetic structure. Research on resistance to WPBR has focused mainly on screening and selection of open-pollinated seed lots from canker-free parent trees that had survived blister rust infection. With testing throughout the host-species range, a few different resistance types have been found (Hunt 2004).

A large-scale screening program at the Canadian Forest Service, Victoria, British Columbia, found two western white pine "partial-resistance" mechanisms: slow-canker-growth (SCG) (Hunt 1997) and difficult-to-infect (DI) responses (Hunt 2004). Slow canker growth is a stem-resistance phenomenon that occurs following inoculation with *C. ribicola*. Four phenotypes were observed within SCG resistance: no swelling, fusiform, gall, and constricted cankers. Observed as quantitative phenotypes, SCG resistance may be attributable to parts of a set of different genes (Ekramoddoullah and Hunt 2002).

Apart from the aforementioned complex resistance mechanisms, a typical gene-for-gene system has been identified in the white pine pathosystem (Kinloch et al. 1970; Kinloch and Littlefield 1977) for the major resistance gene *Cr1* that triggers a typical hypersensitive response (HR), which results in rapid, localized cell death at *C. ribicola* infection sites on needles of sugar pine (*Pinus lambertiana* Dougl.). A single dominant gene (*Cr2*) was later identified in western white pine (Kinloch et al. 1999). Recently, another dominant gene (*Cr3*) originating from southwestern white pine (*P. strobiformis*) has also been reported (Kinloch and Dupper 2002). Both *Cr1* and *Cr3* are indistinguishable functionally from *Cr2*. Because their reactions to blister rust inoculum sources differ (Kinloch and Dupper 2002), *Cr1*, *Cr2*, and *Cr3* are evidently not from the same allele and are possibly located at different genetic loci (Kinloch et al. 2003). Gene *Cr1* from sugar pine has been mapped within 1 centimorgan (cM) to a randomly amplified polymorphic DNA (RAPD) marker (Devey et al. 1995; Harkins et al. 1998). Recently, we also mapped a *Cr2* gene from western white pine to four RAPD markers within 3 cM on a genetic linkage (Liu 2006). However, conventional map-based cloning or transposon tagging is extremely difficult in *Pinus* spp. because of the species' physical genome size, which is estimated to be about 42.5 to 54.9 pg, with lengths from 41 700 to 53 800 mb (Bogunic et al. 2003). It is laborious and time consuming to transfer a genetic marker into a physical marker for the positional cloning of a specific resistance gene in a conifer species.

Resistance, conferred by single dominant resistance genes, is specific to a particular pathogen race that expresses the corresponding avirulence (*Avr*) gene. Two virulence genes (*Vcr1* and *Vcr2*) are known to overcome resistance conferred by *Cr1* in sugar pine and *Cr2* in western white pine, but no virulence gene has yet been found to negate *Cr3* in *P. strobiformis* (Kinloch and Dupper 2002). The observation of *Cr2* gene performance in seven plantations in British Columbia over 13 years revealed that no virulent pathotype of *C. ribicola* is present in the province, suggesting that, to date, the *Cr2* gene is stable in this region (Hunt et al. 2004). Pathogen *Avr* genes are easily mutated or eliminated, so protection conferred by single resistance genes is not often durable. The pyramiding of several race-specific resistance genes into a single plant genotype increases the ability of single dominant resistance genes to provide long-term protection to the plant. This is particularly true for white pine

because the rust pathogen must go through meiosis before it can attack the pine; that makes it difficult for the pathogen to pyramid its *Vcr* genes. Once such single dominant resistance genes (*Cr1*, *Cr2*, and *Cr3*) are identified and characterized, and since the technology for transforming white pines has been developed (Levee et al. 1999), these genes can be transferred to closely related five-needle pine species to protect them against a wide spectrum of *C. ribicola* pathotypes.

Plant defense response

Trees, as with all plants, are regularly exposed to many microbes. Fortunately, only a few of these cause diseases in their hosts. Trees defend against these pathogenic microbes by using preformed barriers and active defense mechanisms. Active defense mechanisms include oxidative burst and antimicrobial compounds such as phytoalexins, peptides, and proteins. Race-specific recognition mediated by direct or indirect interaction between the pathogen's avirulence-gene product and plant's resistance-gene product triggers a network of signal-transduction cascades that result in a series of plant-defense processes, including induction of pathogenesis-related (PR) proteins, leading to the phenotype of resistance to disease (Martin et al. 2003). However, expression of several downstream genes in this signal-transduction pathway is common in various resistance types and even in cases where the host is susceptible (Kamoun et al. 1999). To what extent the genetic components that underlie responses mediated by single resistance genes are distinct from partial (i.e., SCG resistance) or nonhost resistance remains unclear.

To study the role of proteins in white pine defense, new analysis methods were required because conifer tissues produce large quantities of phenolic substances that interfere with the extraction and electrophoresis of proteins (Ekramoddoullah 1991). Extraction procedures from white pine needles were developed (Ekramoddoullah 1993) to permit the extraction and electrophoresis of proteins without interference from phenolic substances. However, the high amounts of detergent and the reducing agent in the protein extract 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).

Two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) is a powerful technique for resolving components of a protein mixture. We have employed this technique in our investigation of resistance to blister rust in white pine (Ekramoddoullah and Hunt 1993). Unfortunately, widespread streaking occurs in 2-D gel patterns of conifer proteins, and a large number of protein spots are consequently lost during subsequent computer-assisted gel-image analysis. To overcome this problem, we adapted Hochstrasser's method (Hochstrasser et al. 1998) in the Millipore® 2-D Investigator system (Ekramoddoullah and Tan 1998a).

Having developed a methodology for extraction and quantification of proteins, we decided to use the well-established procedure of O'Farell's 2-D gel electrophoresis. Although this procedure can resolve thousands of proteins, its use has been limited, and the resulting data is qualitative at best. We quickly realized that looking for defense-related proteins in 2-D gel patterns of conifer proteins was futile: the patterns were complex and the interpretation was difficult.

Fortunately, around 1990, qualitative and quantitative analyses of proteins were made possible by coupling laser scanners with specialized software for processing 2-D gel data. Within the last few years, sequencing of a protein directly from 2-D gel by mass spectrophotometry has advanced to a point that a global proteomic approach is feasible and is now an important complement to the genomic approach. Stained protein spots are usually cut out from the gel and digested with trypsin, and the resulting peptides are subjected to a mass spectrophotometer such as MALDI–MS-MS (matrix-assisted laser densitometry ionization – tandem mass spectrophotometer), which generates enormous peptide-sequence data. Additionally, the entire process can be automated. It requires expensive computing time to establish the sequences of individual peptides. However, if the peptide is derived from a known protein or the gene of a known protein, one can search databases by peptide mass alone and establish the probable identity of the protein from which the peptide might have been derived. We have, at times, identified proteins as above, but in most cases we used a different strategy as described below.

Use of antibody probes to a given protein

Peptide sequences are analyzed to assess the probability of the peptide being antigenic, or of making antibodies to the peptide. Commercial softwares (e.g., DNASTAR; DNASTAR Inc., Madison, Wisc., USA) are available to run this kind of analysis. We have been successful (95%) in making antibodies to various peptides (19 to date). In one case, the peptide antibody did not detect the protein, perhaps because the peptide was part of a conformational determinant that was destroyed in the native protein during the denaturation in the electrophoresis. There are limitations in using a small peptide of a protein for antigenic analysis. Ideally, if the entire protein sequence (e.g., deduced sequence from cDNA) is known, one can search for the molecule's best antigenic region.

Sequence information is then used to synthesize a peptide. The synthetic peptide is purified to 95% purity. The purity of the peptide is essential to generate a specific antibody. The synthesis of the peptide and its purification can be carried out through a contract with a commercial supplier. In our case, we use Multiple Peptide System (MPS), a company in San Diego, California (there are several commercial suppliers of this service worldwide). Being small molecules, peptides are not immunogenic, i.e., if injected alone into animals, they do not elicit an antibody response. However, when the peptide is coupled to a carrier protein such as KLH (Keyhole Lymphet Hemocyanin), the conjugate will produce antibodies to the peptide and also to the carrier protein. This is known as "hapten vs. carrier" in immunology, whereby the carrier protein will recruit T helper cells and present the hapten (in this case the peptide) to B cells so antibodies to the peptide are produced. To facilitate conjugation, cysteine is added to either the N or C terminus of the peptide (sometimes if cysteine is present in the sequence, then the internal cysteine will be used). The sul-

fhydral group of the cysteine is used for conjugation. Antibodies produced by hapten–carrier conjugates are very heterogeneous and contain antibodies not only to the epitopes of the peptide but also to many epitopes present in the carrier protein. The antibody to the peptide is then purified with affinity chromatography. In this purification method, the peptide is coupled to a solid matrix to obtain an immunosorbent; the affinity column is made of this immunosorbent. The crude antiserum (usually from rabbit) is passed through this affinity column. The antipeptide antibody is bound to the column, and other antibodies pass through the column. Since antigen–antibody interaction is noncovalent, a change in the pH of the solution dissociates the antipeptide antibody from the column, so the antipeptide antibody is then eluted with buffer of high or low pH. The purification of the antipeptide is monitored by enzyme-linked immunosorbent assay (ELISA) plate coated with the synthetic peptide. The specificity of the antibody is determined by western immunoblot by demonstrating that it detects a single band (corresponding to the expected size of the protein) in a crude protein extract.

Identification of defense-response proteins in white pine

PR-10 protein

While studying the WPBR pathosystem in sugar pine, we identified an 18-kD protein that is seasonally regulated (Ekramoddoullah et al.1995). A partial amino acid sequence of this protein was obtained and allowed us to produce an antibody specific to the protein. Using this antibody, we detected the protein in eastern white pine. The protein was also shown to be associated with frost hardiness of western white pine; this led to an antibody-based method to determine frost hardiness of young seedlings prior to planting (Ekramoddoullah et al.1995). Further study revealed that this same protein was also up regulated in western white pine, following blister rust infection (Ekramoddoullah et al. 1998). To clone the gene encoding this protein, the antibody was used to screen a cDNA library prepared from cold-hardened western white pine needles. This led to the successful cloning of the gene, which was shown to belong to the pathogenesis-related (PR)-10 family (Yu et al. 2000).

PR-10 protein accumulates in healthy tree tissues through the fall, reaching maximum levels in the winter months and minimum levels in the summer months. If, however, a tree has cankers as a result of blister rust infection, the level of PR-10 protein remains high even in the summer months. This suggests that PR protein is induced by the rust fungus. Recently, Liu et al. (2003) have shown that there are at least 19 members of the PR-gene family, each with its own promoter. Indeed, pathogen-inducible and cold-inducible regulatory elements have been identified in one member of the gene family (Liu et al. 2005a). Our protein data indicate that there is cross-communication between biotic- and abiotic-stress-related pathways. Blister rust on resistant trees was observed to be reduced at a high-elevation site compared with that on trees at a low-elevation site (Hunt 2005). That proteins such as PR-10 may be induced by cold may explain in part this increased resistance of white pine at high elevation.

We also investigated the molecular evolution of the western white pine PR-10 gene family. Our data indicates that at least one subfamily (subfamily III of western white pine PR-10 genes) experienced strong and steady selective pressure (Liu and Ekramoddoullah 2004). Mapping analysis of the detected amino acid residue substitutions onto the three-dimensional (3-D) structure of western white pine PR-10 revealed that a number of substitutions have occurred in the ligand-binding sites of PR-10 proteins. These analyses suggest that the putative ligand-binding sites of western white pine PR-10 proteins have undergone rapid adaptive evolution (Liu and Ekramoddoullah 2004). Ligand-binding ability and specificity of most PR-10 proteins remain unknown; however, it is believed that the role of ligand-binding sites and transport in PR-10 proteins may be important to plant-defense response in pathological situations, as well as to growth and development (Markovic-Housley et al. 2003). Rapid selective evolution, evidence of which was observed in the western white pine PR-10 family, would permit different protein members to distinguish various ligands for their physiological roles in defense against *C. ribicola* attack.

A function has yet to be assigned to the conifer PR-10 protein. Some PR-10 proteins exhibited ribonuclease activity (Bantignies et al. 2000; Bufe et al. 1996; Wu et al. 2003) or binding activity towards cytokinin (Fujimoto et al. 1998; Gonneu et al. 2001; Mogensen et al. 2002), sterols (Markovic-Housley et al. 2003), fatty acids, and flavonoids (Mogensen et al. 2002). Crystal structures of members of the PR-10 protein family have been determined for a major birch pollen allergen, Bet v 1 (Gajhede et al. 1996), and its low-allergenic isoform, Bet v 1l (Markovic-Housley et al. 2003), for a major cherry allergen, Pru av 1 (Neudecker et al. 2001), and classic PR-10 proteins from yellow lupine, LlPR10-1A and LlPR10-1B (Biesiadka et al. 2002). 3-D structures of these PR-10 proteins are similar: each consists of a long C-terminal α-helix surrounded by a seven-strand β-sheet and two N-terminal short α-helices. However, the structure of the conserved P-loop motif (GxGGxGxxK) in the PR-10 3-D structure (Biesiadka et al. 2002) does not support the suggestion that the P loop is a binding site of nucleotides involved in RNase activity, and thus it renders the RNase hypothesis of PR-10 protein function controversial. On the other hand, a large, hydrophobic, Y-shaped cavity has been found in 3-D structures of PR-10 proteins. It forms between residues lining the interior face of the β-sheet and the three α-helices and it functions as a ligand-binding site. The PR-10 transport of physiologically active ligands may be an important step in plant-defense response, as well as in plant growth and development. Immunogold labeling of white pine PR-10 protein shows that it binds to the cell wall of the blister rust fungus (Ekramoddoullah 2004).

Assigning a function to white pine PR-10 protein has proven very difficult because biological activities of conifer proteins are destroyed by the extraction procedure. Another approach is to clone the gene and express it in a bacterial system to obtain a recombinant protein, then assay the func-

tion of that protein. We have produced and purified the recombinant protein for one member of white pine PR-10 gene family and tested its ribonuclease activity. Although this recombinant protein did not have ribonuclease activity, it had allergenic activity towards patients with white pine pollen allergy (A.K.M. Ekramoddoullah and T. Midoro-Horiuti, unpublished data). Our recent work suggests that multigene members of the PR-10 family underwent great diversification during conifer evolution to assume diverse functional roles (Liu and Ekramoddoullah 2004). One member of the PR-10 protein family may thus retain ribonuclease activity. This concept of the ribonuclease role of PR-10 protein is attractive, particularly if the activity is directed specifically towards fungal RNA (Park et al. 2004). It could then also explain why PR-10 protein is up regulated quickly in white pine carrying the *Cr1* gene compared with how the protein is regulated in susceptible white pine (Ekramoddoullah and Tan 1998b). PR-10, perhaps along with other proteins, might eliminate the fungus in resistance (R)-protein-mediated hypersensitive cell death. To determine this, all recombinant white pine PR-10 proteins must be obtained and their functions established individually. The diverse functions of these natural variants could be compared to help identify molecular domains relating to specific activities.

Promoter analysis showed that one western white pine PR-10 gene, *PmPR10-1.14*, was expressed in a root-specific manner with developmental regulation (Liu and Ekramoddoullah 2003a), and its expression was not affected by wounding (Liu et al. 2005a). In contrast, another western white pine PR-10 gene, *PmPR10-1.13*, was expressed in aboveground parts of the tree and was induced by pathogen infection and wounding (Liu et al. 2005a). A few cis-regulatory elements responsible for inducible expression in angiosperms were identified in the *PmPR10-1.13* promoter. Among these, a typical box-W1 (TTGACC) is the binding site of WRKY transcription factors for the fungal-elicitor response (Rushton et al. 1996). Other cis-regulatory elements included a sequence of TTCGTCC with similarity to another elicitor-responsive element (ERE) (Shah and Klessig 1996), two CGTCA/TGACG motifs identical to those involved in the methyl jasmonate response (Rouster et al. 1997), and an ERE-like motif (ATTTCAAG) that is very similar to an ethylene-responsive enhancer element (Itzhaki et al. 1994). We have identified multiple members of a complex western white pine gene family encoding WRKY transcriptional factors (J.-J. Liu and A.K.M. Ekramoddoullah, unpublished data). Investigation of their regulatory role in activation of western white pine PR-10 gene expression will further our understanding of the western white pine defense mechanism. Promoter 5′-deletion analysis in transgenic *Arabidopsis* reveals that some of these cis-elements are important to disease- and wound-induced PR-10 gene expression (Liu et al. 2005a). This work on western white pine PR-10 promoters suggests that a special subset of the western white pine PR-10 promoter family that is activated by *C. ribicola* is regulated by a combination of a set of transcriptional factors. The investigation of cis-acting elements that regulate the pathogen-inducible expression of western white pine PR-10 genes will shed light on mechanisms of the defense response governed by the resistance gene.

PR-3 protein

Members of this family of PR proteins are also known as chitinases, enzymes that cleave chitin, a structural component of fungi. An acidic chitinase was identified to be up regulated in susceptible white pine (Davidson and Ekramoddoullah 1997). Liu et al. (2005b) recently cloned a class IV chitinase gene from white pine, and an antibody was made to an immunogenic region of the deduced protein. Using western immunoblot, we found two chitinases in white pine trees that possessed SCG resistance, but only one of the chitinases was found in susceptible white pine trees. The difference between SCG and susceptible western white pine seedlings was reflected not only at the chitinase protein level but also at the genomic DNA level. Polymerase chain reaction with isoform-specific primers or class-specific primers of the western white pine PR-3 gene family revealed that there was a gene difference between SCG and susceptible seedlings. The introns of western white pine class IV chitinase genes showed single-nucleotide polymorphisms (SNP) and intron-length polymorphisms. Bishop et al. (2000) found that the molecular evolution of plant class I chitinases is driven by adaptive selection for advantageous mutations. They showed that plant-defense proteins not related to pathogen recognition also evolve in a manner consistent with coevolutionary interactions between plants and pathogens. Our work on western white pine class IV chitinases provides a potential tool for marker-assisted selection in western white pine breeding and suggests that, as a general defense factor, class IV chitinase might participate in SCG resistance. This also provides an immunochemical procedure to screen trees for SCG resistance, potentially reducing screening time, which otherwise may take up to 7 years.

Pm-AMP1

We have identified a 10.6-kDa protein (Davidson and Ekramoddoullah 1997), now designated as Pm-AMP1 (*Pinus monticola* antimicrobial peptide 1), which was enriched at the canker margins of white pine trees with SCG resistance. The sequence of the first 20 amino acids at the terminal amino acid sequence was determined and shown to have no homology with known proteins in the databank. An antibody probe to the synthetic peptide was barely able to detect the Pm-AMP1 from one sample, although the sample contained enough protein to allow us to successfully sequence the N terminus. Recently, we cloned the gene encoding this Pm-AMP1 and ran an antigenic analysis on the deduced protein sequence (Zamani et al. 2005). As suspected, the N-terminal region was the least antigenic region of the sequence. An antibody prepared to one of three potential antigenic sites successfully bound the Pm-AMP1. Near canker margins, the protein was always higher in SCG trees than in susceptible trees. Surprisingly, healthy tissues distal to the canker in susceptible trees contained more of the protein than canker margins did, suggesting that the fungus was able to degrade Pm-AMP1 in advance of its infection front in susceptible trees. Although the healing cankers were in the tree's stem, the foliage of SCG trees had consistently higher levels of Pm-AMP1 than did foliage of susceptible trees. Tests also showed that the protein was barely detectable in current-year needles and was higher in 2-year-

old needles than in 1-year-old needles. Current-year needles of seedlings are more susceptible to blister infection than older needles (Hunt and Jensen 2000).

In an attempt to establish Pm-AMP1's putative antifungal activity, we produced a bacterial recombinant form of the protein and purified it. The recombinant protein was added to an axenic culture of *C. ribicola* in vitro: fungal mycelia stopped growing near the filter paper containing the recombinant protein, whereas the mycelia grew over filter paper containing an unrelated protein. Microscopic examination revealed that in the absence of the inhibitor, i.e., the recombinant protein, the fungal hyphae had broad, robust tips, whereas fungal hyphae treated with the recombinant protein had elongated and narrow tips, with pronounced forking at the ends. These results clearly indicate that Pm-AMP1 has antifungal activity towards *C. ribicola* and that SCG resistance could be due, in part, to the expression of this protein. The genetic elements underlying the expression of this protein need to be investigated to improve this type of resistance.

Thaumatin-like protein

We also discovered a thaumatin-like protein (TLP) (belonging to PR-5 family) in western white pine (Piggott et al. 2004). PR-5 proteins have been identified in numerous plant species, and some have been shown to have antifungal properties, in that they are able to degrade fungal cell walls (Zareie et al. 2002). Thaumatin-like proteins can be subdivided into large TLPs, with molecular masses of 21 to 26 kDa and 16 conserved cysteine residues (these form eight disulfide bonds to stabilize the tertiary structure of the proteins), or small TLPs, with molecular masses of 15 to 18 kDa and, because of an intramolecular deletion of approximately 58 amino acids, only 10 conserved cysteine residues. While the more common large TLPs have been identified in monocots, dicots, and conifers, small TLPs have primarily been described in monocot crop species.

Using an antibody to Douglas-fir PR-5 (Zamani et al. 2004), we have been able to identify two white pine PR-5 proteins with molecular masses of 25 kDa and 17 kDa and approximate pI of 5.4. Protein sequencing with nanospray MS-MS of tryptic digests of 2-D gel protein spots confirmed that these proteins belong to the PR-5 family. During protein sequencing, a basic form of 17-kDa PR-5 was also identified (A.K.M. Ekramoddoullah and A. Zamani, unpublished data). These PR-5 proteins are also up regulated in the WPBR pathosystem. The gene encoding the 17-kDa PR-5 protein has been cloned: the deduced protein sequence is consistent with the observed protein-sequence data. The function of various PR-5 isoforms found in given species has yet to be determined and may yield interesting results.

Resistance genes and analogs

During the past decade, 40 R genes that confer resistance to various plant pathogens such as viruses, bacteria, oomycetes, fungi, and nematodes have been cloned and characterized from model and crop species of angiosperms (Ellis and Jones 1998; Martin et al. 2003; McDowell and Woffenden 2003). Resistance genes are grouped into six types according to their protein product structures (Dangl and Jones 2001). Most resistance-gene products resemble intracellular receptors, containing a nucleotide-binding (NB) domain, plus a stretch of leucine-rich repeats (LRR) at the C terminus. Leucine-rich repeats are supposed to either mediate protein–protein interactions or to control specific recognition of ligands by receptor molecules (Kobe and Deisenhofer 1995). Nucleotide-binding LRR proteins are grouped into two subfamilies with additional N-terminal structures that include either a coiled-coil domain (CC-NB-LRR) or a domain similar to *Drosophila* Toll and mammalian interleukin-1 receptor (TIR-NB-LRR) (Martin et al. 2003; McDowell and Woffenden 2003).

The loblolly pine (*Pinus taeda* L.) genome project initiated by the Forest Service of the United States Department of Agriculture identified some expressed sequence tags of resistance-gene analogs (RGAs), but using them for probing in sugar pine revealed that none were functionally associated with *Cr1* (Kinloch 2003). Therefore, a genomics strategy is necessary to search for RGAs and to study RGA families in conifer species. Analysis of possible genetic links of RGAs to R genes, and functional investigation of the biological roles of proteins encoded by RGAs will provide clues for resistance-gene identification in conifers.

A polymerase chain reaction (PCR) strategy was used to clone NB sequences from the western white pine genome (Liu and Ekramoddoullah 2003b). Our study found that conifer RGAs encoded NB-LRR proteins with structural features of angiosperm resistance proteins. Dicot plants contain both gene subfamilies that encode TIR- and CC-NB-LRR proteins, but in monocot plants such as rice, only resistance or RGA genes belonging to one subfamily of CC-NB-LRR were found (Zhou et al. 2004). We identified both RGA subfamilies encoding for TIR-NB-LRR proteins (Liu and Ekramoddoullah 2003b) and CC-NB-LRR proteins in the western white pine genome (Liu and Ekramoddoullah[1]), which suggests that conifer genomic organization of the RGA family has more in common with that of dicots than with that of monocots. The identification of a NB-LRR superfamily in both conifers and angiosperms also suggests that common ancestral genes existed prior to the evolutionary split of these seed plants about 275 to 300 million years ago (Cannon et al. 2002; Meyers et al. 2002).

Although all of the cloned RGAs belonged to the NB-LRR superfamily, there was a great diversity among them at both nucleotide and deduced amino acid sequence levels. Many western white pine RGAs are expressed because they are cloned from cDNAs directly or because the mRNAs transcribed from them are detected by reverse transcript (RT)-PCR. Therefore, it is reasonable to speculate that some western white pine RGAs now identified may act as functional resistance genes against pathogens. Almost half of the cloned western white pine RGAs of the TIR-NBS-LRR subfamily are pseudogenes (Liu and Ekramoddoullah

[1] Liu, J.J., and Ekramoddoullah, A.K.M. Characterization of a subfamily of resistance gene analogs encoding CC-NBS-LRR proteins in western white pine (*Pinus monticola* D. Don). XIIth International Congress of Molecular Plant–Microbe Interaction, 14–18 December 2005, Cancun, Mexico. [Abstr.] To be published.

2003b). Sequence comparisons suggest that these pseudogenes originated from point mutations, insertions, or deletions of nucleotides. The pseudogenes have been identified in the angiosperm resistance-gene classes of *Xa21*, *Cf9*, *Pto*, and *Dm3* genes. The pseudogenes may be as abundant as functional genes in the human genome (Harrison et al. 2002). Believed to be reservoirs of potential variation, pseudogenes can become functional resistance genes because of their recombination and of gene conversion between alleles or paralogs (Michelmore and Meyers 1998). They could be considered genes-in-waiting, as recombination and mutation could cause reading-frame shifts. Therefore, genes such as *Cr2* in the WPBR pathosystem can arise in the absence of the pathogen. A recent study showed that a mouse pseudogene was expressed as a regulator to stabilize mRNA of its homologous coding gene (Hirotsune et al. 2003). Further investigation of the biological role of western white pine RGA pseudogenes will help in the elucidation of resistance-gene regulation and evolution.

Resistance-gene analogs are found clustered around known resistance-gene loci in angiosperms. For example, some RGAs are genetically linked with these loci in *Arabidopsis* (Aarts et al. 1998). Within a cluster of RGAs, more than one gene may confer resistance against different isolates of a specific pathogen or against biologically diverse pathogen taxa (Cooley et al. 2000; Van der Vossen et al. 2000). Because of the high levels of polymorphism and diversity and the analogs' functional character of transcription activity, western white pine RGAs will provide a genetic source for development of molecular markers, especially markers for selection of resistance traits to *C. ribicola* in forest breeding. The full-length western white pine RGAs already cloned must be either overexpressed or silenced to investigate their biological function in host signal-transduction pathways initiated by *C. ribicola* infection. New genomics and proteomics technologies will provide novel approaches to identify intermediate components in their signaling.

Nucleotide-binding domains play an important role in signaling processes governed by resistance proteins. Diversity among NB sequences may be critical to the specificity of interactions either with a pathogen elicitor or with proteins downstream in distinct signaling pathways (Aarts et al. 1998). The cloned NB sequences of western white pine RGAs correspond to two subfamilies of CC-NB-LRR (Liu and Ekramoddoullah[1]) and TIR-NB-LRR (Liu and Ekramoddoullah 2003b). High variation in these NB domains has been observed, suggesting that it might contribute in part to the evolutionary fitness of conifers. Other studies using precise domain swapping have shown that a number of LRRs are essential for interaction with avirulence factors (Van der Hoorn et al. 2001; Wulff et al. 2001). Our recent research revealed that members of a class of western white pine TIR-NB-LRR proteins have almost identical TIR and NB domains, but with different LRR lengths (Liu and Ekramoddoullah[1]). The diversification of LRR domains may be significant for perception and recognition of pathogen ligands.

Resistance genes are under evolutionary pressure because novel *Avr*-gene products are produced as the pathogen evolves. Within *C. ribicola* populations, at least two virulence genes (*Vcr1* and *Vcr2*) are found to overcome resistance genes *Cr1* and *Cr2*, respectively (Kinloch et al. 2004). Initial work demonstrated that *C. ribicola* affected gene flow in the western white pine population. In two natural western white pine populations from northern Idaho, the population with low pressure from blister rust had higher polymorphism and higher heterozygosity than did the population that had experienced high mortality due to blister rust (Kim et al. 2003). The major evolutionary mechanism for novel polymorphisms at resistance-gene loci is through point mutations (Michelmore and Meyers 1998). Mispairing and unequal crossing-over following point mutations are responsible for resistance-gene diversification (Noël et al. 1999). This is certainly true for crop and model species with short life cycles, but little is known about perennial plants like conifers. Our research revealed that single-nucleotide mutations (DNA-fragment insertions, deletions, or duplications) occur in western white pine RGAs (Liu and Ekramoddoullah 2003b). For these long-lived species (conifers can live hundreds to thousands of years) generation of new resistance via somatic mutations may be particularly advantageous (Michelmore and Meyers 1998). Ontogenetic resistance is observed in western white pine and other five-needle pines. Comparison of variability in the resistance-protein structures that determine pathogen specificity will provide useful information among long-lived individuals with ontogenetic resistance.

The evolutionary development of pathogens must also be considered. Deletion and point mutations in an *Avr* gene are two mechanisms for a pathogen to escape recognition mediated by the corresponding resistance gene. If we assume that this is comparable to what happens in plants, there are two conflicting aspects in managing pathogen evolution:

(1) Mutation in *Vcr* genes to overcome an R gene in conifers occurs by chance in the pathogen population, not because of resistance-gene pressure. Therefore, an increasing presence of the pathogen increases chances for mutation. For example, pollen boosting a conifer-seed orchard with heterozygous resistant-gene pollen would greatly increase the chance for pathogen mutation compared with pollen boosting with homozygous resistant-gene pollen (Liu et al. 2004). Because of this, the homozygous resistant-pollen option should be chosen to lessen the chances of new *Vcr* genes developing.
(2) Once the mutation occurred, resistance-gene pressure will increase the presence of new *Vcr* genes throughout the pathogen population. Plantations then established with the homozygous pollen will be at risk of early attack. In theory, the heterozygous option would help slow the attack; nonetheless, with a disease like WPBR, little background resistance in susceptible trees exists to slow the epidemic on a practical basis. Unlike in agricultural situations, large populations of native conifer hosts that lack the R gene grow nearby; they naturally maintain a diverse pathogen population. These conifer populations would slow the spread of new *Vcr* genes through the pathogen population.

Pathogen-derived proteins

In proteomic analysis of the conifer response to pathogen infection, no suitable method exists for differentiating pro-

teins derived from hosts from those derived from pathogens. Neither the host proteins that we have characterized to date nor the corresponding antibody probes react to any pathogen-derived proteins. Testing has been possible only after producing an antibody probe specific to a well-characterized protein. Monoclonal antibody technology is useful in studying tree–pathogen interaction (Ekramoddoullah 1990). We have produced a series of monoclonal antibodies to the WPBR fungus (Ekramoddoullah and Taylor 1996). Using one of the monoclonal antibodies specific to the white blister rust fungus, we could quantify the fungal mass in infected white pine tissues. Monoclonal antibodies, along with specific polyclonal antibodies, can also be used to immunocytochemically study the involvement of fungal proteins in host–pathogen interaction. We identified a 70-kDa heat-shock protein in the rust fungus by using one particular monoclonal antibody (Yu et al. 2001). This finding led to genetic engineering of a single chain from a monoclonal antibody that was directed against a heat-shock protein (Wilde 1998). Interestingly, the epitope recognized by the monoclonal antibody is not present in white pine heat-shock protein. This opened up a possible new approach of engineering the single-chain mammalian monoclonal antibody into white pine to inhibit growth of the fungus before the tree can produce its own age-related defense proteins.

While evaluating monoclonal antibodies to quantify fungal mass in susceptible white pine infected with blister rust, we observed, in sodium dodecyl sulfate (SDS)-PAGE, a strong protein band corresponding to the size of white pine PR-10 (Ekramoddoullah et al. 1999). Although it was an intensely stained band, it did not react with anti-PR-10 antibody in the corresponding western blot. N-terminal sequence of the protein band confirmed that the band was not a PR-10 protein. At that time, we were also testing monoclonal antibodies produced against blister rust spores to determine whether these antibodies would bind to fungal mycelia derived from rust isolates obtained from various geographical sources (Ekramoddoullah and Taylor 1996). In one isolate, a strong protein band was observed that corresponded to the size of PR-10 protein and whose N-terminal sequence was identical to the one previously found in the infected host. We concluded that the protein, named Cro r I, was of fungal origin. To characterize it, an antibody was prepared to the synthetic N-terminal peptide. Immunochemical quantification of Cor r I showed that levels of the protein were significantly higher in susceptible than in resistant white pines. It is a secreted protein that can be detected only in the haploid life cycle of the fungus, during mycelial growth in the pine host or in haploid culture. It was found also in uninfected needles of susceptible white pines that have stem cankers, suggesting that the protein is translocated to healthy tissues. When crude extracts containing Cro r I was introduced into western white pine that carry the *Cr2* gene, a hypersensive response that mimics resistance to the intact pathogen was induced (A.K.M. Ekramoddoullah, unpublished data). Cro r I protein must be further purified to determine whether it is active as an elicitor or virulence factor. A population-wide genetic study of SNPs of *Cro r I* gene may also shed light into the role of Cro r I protein in WPBR pathosystem, as a single amino acid sequence difference can occur from a virulent-race protein (e.g., elcitor) to avoid recognition by a host. A recent study suggests that deletion or alteration of an avirulence gene might allow the fungal pathogen to escape recognition by the host's corresponding resistance gene (Schurch et al. 2004).

Future research

Molecular understanding of host–pathogen interactions in conifers lags far behind that of similar interactions in model plant pathosystems. A major problem is that conifers have a long generation time and are thus not easily amenable to genetic manipulation techniques such as gene knock-out experiments or the creation of mutants that are necessary to analyze defense-related protein functions. As proteomic and genomic technologies advance, more genes and proteins will be discovered. One of the areas that could be developed is the study of vector systems for producing recombinant proteins for the purpose of assessing function, at least in vitro. If, for example, a protein is found to have antifungal properties, manipulation to up regulate its expression in conifers could be undertaken. To do this, the transcription factor(s) that bind to the cis-acting element in the promoter region of the gene would need to be fully characterized. On the pathogen side, elicitor and virulence factors need to be characterized. The function of these can be assessed by gene knock-out experiments for at least some pathogens, such as root rots. With regards to obligate pathogens such as WPBR, a prime area of research would be to develop a method whereby axenically grown fungal mycelia could be used to infect white pines. Gene knock-out experiments could then be carried out in these obligate pathogens. The elucidation of the 3-D structures of these proteins by methods such as X-ray crystallography could also yield useful information regarding ligand-binding sites; this information might be used to identify and enhance their function.

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