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REVIEW

Current and future molecular approaches to investigate the white pine blister rust pathosystem

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Summary

Molecular genetics is proving to be especially useful for addressing a wide variety of research and management questions on the white pine blister rust pathosystem. White pine blister rust, caused by *Cronartium ribicola*, is an ideal model for studying biogeography, genetics, and evolution because: (1) it involves an introduced pathogen; (2) it includes multiple primary and alternate hosts occurring in large, relatively undisturbed ecosystems; (3) some hosts exhibit endemic resistance; and (4) the disease interaction is long enduring. Molecular techniques are used to investigate population genetics, phylogenetics, hybrids, and proteomics in white pine (*Pinus*, subgenus *Strobus*) and blister rust (*Cronartium*) and the genetics of resistance and virulence in the blister rust pathosystem. These techniques include genetic markers, mapping, microarrays, sequencing, association genetics, genomics, and genecology. Molecular genetics contributes to gene conservation, breeding for resistance, and ecosystem management.

1 Introduction

Molecular genetic tools are proving to be especially useful for a wide variety of research questions in forest pathology (Kim et al. 2005). They have a great potential to quickly provide insights for pathosystems with long-lived individuals, where other experimental approaches would require decades. Various disciplines within genetics (phylogenetics, population genetics, ecological genetics, and functional genomics) use shared molecular tools derived from rapidly developing technologies. Over the last few decades, these technologies have greatly increased the capacity to generate large numbers of high-resolution genetic markers (Neale and Ingvarsson 2008). Previous studies were often limited to only a few loci (positions on a chromosome), whereas current genetic analyses routinely assess hundreds to thousands of polymorphic loci. This analytical power continues to increase rapidly, owing to advances in DNA sequencing technologies, high-throughput sequencing, and increased computing power—catalysts for improved capabilities in diverse aspects of molecular genetic research. Numerous techniques can be tailored to meet the research needs for a particular organism and/or hypothesis.

In this review, we focus on technology-driven approaches that have developed within molecular genetics and discuss their ongoing and potential contributions for examining the white pine blister rust pathosystem (*Cronartium ribicola* J.C. Fisch. in Rabh., pathogen). We review the common molecular techniques presently used in white pine blister rust research and discuss their general utility. We then discuss the rapidly changing disciplines of genomics and proteomics and describe how these can be integrated with other

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disciplines and technologies for application to blister rust research and ecosystem management.

2 Population genetics

Population genetics is the study of the organization of genetic variation among individuals within a species (Hartl and Clark 1997). The evolutionary forces of gene flow, genetic drift, mutation, recombination, and natural selection influence the genetic structure of populations. Each of these evolutionary forces can drive changes in gene frequencies, that is, contribute to differentiation or homogenization of allele (alternative gene forms at the same locus) frequencies among populations (Richardson et al. 2005). Traditional population genetics was confined to investigating neutral, genome-wide effects of gene flow and genetic drift. In contrast, functional genomics now examines locus-specific effects caused by natural selection (Vasemägi and Primmer 2005) given either prior knowledge of the gene(s) involved in selection or a high number of variable loci to detect selection.

Presently, the most commonly used techniques in population genetics are based on a PCR. These include simple sequence repeats (SSRs or microsatellites), amplified fragment length polymorphisms (AFLPs), and single-nucleotide polymorphisms (SNPs). Each of these techniques has unique qualities and limitations (Glaubitz and Moran 2000). The choice of a marker system depends on the organism and hypothesis. Below, we describe the utility of these genetic markers using examples with white pine (*Pinus* subgenus *Strobus*) or *C. ribicola* (adapted from Kim et al. 2005).

2.1 Population genetics of white pine species

Knowledge of genetic diversity and structure of forest tree species or populations is fundamental for genetic resource management (King et al. 2010). This information is basic for the conservation of genetic diversity critical for long-term adaptability and sustainability of forest tree species (Ledig 1988; Yanchuk 2001). Data on the organization of genetic diversity fit within a spatial hierarchy from a species-wide distribution to local populations. Different molecular tools are employed to address specific issues and species. For example, Rajora et al. (2000) used 13 SSR markers to assess the impacts of timber harvest on genetic diversity of eastern white pine (*Pinus strobus* L.). They showed that harvesting caused stand level losses in unique and rare alleles and reduction in overall allelic richness. But, they left unresolved whether regeneration would perpetuate these losses or whether gene flow from nearby stands would restore lost allelic richness. Kim et al. (2003) used AFLPs to assess genetic diversity in western white pine (*Pinus monticola* Dougl. ex D. Don) populations within environments of contrasting hazard for *C. ribicola.* A population grown on a low hazard site had higher heterozygosity and twice as many unique alleles compared with a population grown on a high-hazard site.

Genetic studies of forest trees have provided insights into aspects of gene flow by seed and pollen. Uniparentally inherited, organellar DNA markers have been particularly useful in the Pinaceae. As pines predominately inherit mitochondrial (mt)DNA maternally and chloroplast (cp)DNA paternally (Wagner 1992; Bruns and Owens 2000), the independent assessments of maternal and paternal gene flows are possible by examining seed or pollen. Latta and Mitton (1997) found that mtDNA revealed the strongest differentiation among populations of limber pine (*Pinus flexilis* James) whereas cpDNA and nuclear markers (RAPDs and allozymes) revealed considerably less differentiation between populations. The differences in allelic frequencies between mtDNA and the other markers were attributed to limited gene flow by seed relative to pollen. This kind of information is useful for identifying interbreeding populations defined by either maternal or paternal gene flow.

Other studies have used mtDNA markers to investigate seed dispersal distances at various geographical scales for the coevolved whitebark pine (*Pinus albicaulis* Engelm.) and Clark's nutcracker (*Nucifraga columbiana*). Whitebark pine regeneration depends on seed caching by Clark's nutcracker (Tomback 2001). Distributions of mtDNA haplotypes suggest that nutcracker caching mixes seeds from different populations, but large gaps (>20 km) in subalpine habitat pose a significant barrier to seed dispersal by Clark's nutcracker (Richardson et al. 2002b). This information helps restoration programmes by assessing whether natural regeneration would be suitable for specific areas where whitebark pine has been lost (Tomback and Achuff 2010).

Population genetic structure and diversity, when cross-referenced with paleoecological data, have contributed valuable insights into white pine biogeography. For example, Mitton et al. (2000) proposed eight different glacial refugia for limber pine from RFLP mtDNA data. Richardson et al. (2002a) used mtDNA and cpDNA patterns in whitebark pine to infer Pleistocene refugia and post-glacial expansions. Eckert et al. (2008), using a molecular clock on organellar and nuclear DNA sequences, proposed that early- to mid-glaciation events in the California Sierra Nevada created the disjunct distributions of foxtail pine (*Pinus balfouriana* Grev. and Balf.). Such studies provide data on past demographic and emigrational changes which aid in understanding how white pines have responded to historical climatic changes and large-scale disturbances and provide relevant information for developing seed-transfer guidelines.

2.2 Population genetics of *Cronartium ribicola*

Population genetic studies of *C. ribicola* have found varying degrees of genetic structure in North America. The result of a genetic structure study is likely affected by geographic area, detection method, and hypothesis.

Using isozymes, RAPDs, and RFLP markers, Kinloch et al. (1998) found that genetic diversity and differentiation among populations of *C. ribicola* in North America were generally low and that genetic distances were not correlated to geographic distance. The largest genetic distance was found between Happy Camp, California, and other populations sampled in western North America. The *C. ribicola* population at Happy Camp was the only one sampled that possessed the *vcr1* gene (Kinloch and Comstock 1981) which defeats the *Cr1* R gene in sugar pine (*P. lambertiana* Dougl.). Of interest to resistance screening programmes, these techniques suggest that blister rust inoculum sources are broadly similar genetically except for those genes which are strongly selected, such as a virulence gene.

Recent studies have employed AFLPs to investigate the dynamics of population genetic changes in *C. ribicola*. Richardson et al. (2008) found significant differentiation among rust populations associated with rust-resistant plantations and natural stands of either western white pine or sugar pine. The population with the least genetic diversity was sampled from the Happy Camp plantation of sugar pine carrying the *Cr1* gene; the low genetic diversity of this population suggested a genetic bottleneck caused by selection for a virulent rust carrying the *vcr1* gene. The sample population with the highest genetic diversity was an Idaho plantation of western white pine selected for multigenic (partial) resistance to blister rust (see King et al. 2010). This more sensitive technique implies that artificial selection for resistance in white pine is met with rapid natural selection by *C. ribicola*.

Hamelin et al. (1995) and Et-touil et al. (1999) used RAPD analyses to examine the population genetic structure of *C. ribicola* in eastern Canada. They observed that the majority of genetic diversity was found within populations; low-level genetic differentiation occurred among populations within a region, but regional differentiation was not evident among populations of the eastern provinces. The authors proposed this pattern of

genetic structure in *C. ribicola* was influenced by a founder effect from the pathogen's recent introduction. Hamelin et al. (2000) subsequently demonstrated that *C. ribicola* populations in eastern and western North America are distinct. Movement of *C. ribicola* that bridges eastern and western North America could have greater genetic significance than has been observed within the two epidemics.

McDonald et al. (2006) reported discovery of *C. ribicola* on *Pedicularis racemosa* Dougl. ex Benth. and *Castilleja miniata* Dougl. ex Hook. in Idaho. Ribosomal DNA sequencing [internal transcribed spacer (ITS) and 5.8S] and artificial inoculations of *R. nigrum* L. and western white pine seedlings were used to confirm the determination. AFLP profiles of rust isolates from three telial hosts (*Ribes hudsonianum* Richards., *R. lacustre* (Pers.) Poir., and *P. racemosa*) and two aecial hosts (western white pine and whitebark pine) from this Idaho site detected no genetic differentiation among rust isolates derived from different hosts. Additional rust isolates from elsewhere in the western or the eastern United States were demonstrated by artificial inoculation capable of infecting *P. racemosa*, a western endemic. Genetic analyses together with artificial inoculations suggest that North American *C. ribicola* has long possessed a capacity to infect these hosts (Richardson et al. 2007), but the epidemiological consequences of this are unknown.

3 Hybridization

Molecular genetic tools have been very effective for the detection of intraspecific and interspecific hybrids in the Pinaceae (Latta and Mitton 1999; Rajora and Dancik 2000). Host hybrid zones are known to affect disease resistance (Wu et al. 1996) and the population genetic structure of a pathogen (Jerome and Ford 2002). Both plant and pathogen hybrids have been discovered in poplar plantations. For example, one poplar hybrid (*Populus trichocarpa* × *P. deltoides*) became infected by a leaf rust, *Melampsora* ×columbiana that arose following hybridization between *M. occidentalis* and *M. medusae* (Newcombe et al. 2000).

Joly et al. (2006) demonstrated that *C. ribicola* in stands of limber pine can hybridize with comandra blister rust (*C. comandrae* Peck) to form aeciospores. Because these spores have not been observed to infect *Ribes* or other hosts, the pathogenicity and biological implications of this interspecific hybridization, such as the contribution to gene flow, are unknown. Hybridization could be very important for blister rust management if it increased host range or environmental tolerance of the pathogen.

4 Phylogenetics

In comparison with population genetics which uses allelic frequencies to quantify relationships among species populations, phylogenetics typically investigates evolutionary relationships among taxa (groups of organisms related at any rank) by reconstructing the history of nucleotide substitutions within a gene family (Graur and Li 2000). Genetic loci, like the ITS of the nuclear ribosomal (r)DNA, are often used in phylogenetic studies among sister taxa (Vogler and Bruns 1998; Senters and Soltis 2003). For research on more distant (older) phylogenetic relationships, highly conserved genes, such as the ribosomal large subunit, are examined (Maier et al. 2003). New DNA sequencing technologies, increased computing power, and mathematical modelling methods could fundamentally change phylogenetics and other disciplines in genetics.

Phylogenetic analyses using mtDNA, cpDNA, and nuclear (n)DNA sequences are proving especially valuable tools for resolving the complex evolutionary histories of the pine taxa infected by *C. ribicola*. Price et al. (1998) presented a classification of the genus *Pinus*, including the subgenus *Strobus* with its constituent sections and subsections (see

Tomback and Achuff 2010; Table 1). According to Price et al. (1998), section *Strobus* includes subsection *Strobi* (white pines like *P. strobus*) and subsection *Cembrae* (stone pines like whitebark pine); section *Parrya* includes subsection *Balfourianae* (foxtail pines), subsection *Cembroides* (pinyon pines, North America), and subsection *Gerardianae* (Asian pines of uncertain affinity). Although phylogenic analysis supported *Strobi* as a monophyletic group (exclusively share a single common ancestor), the placement of *Balfourianae* as a sister taxon to section *Strobus* was unresolved (Liston et al. 1999; Wang et al. 1999 ; Wang et al. 2000; Gernandt et al. 2001). More recent studies concluded that *Balfourianae* is more closely related to *Cembroides* than *Strobus* (Gernandt et al. 2005; Syring et al. 2005).

Detailed phylogenetic studies with more sampling within taxa have shown that alleles among taxa of subgenus *Strobus* are not always monophyletic (Syring et al. 2007). This non-monophyly can be a result of one or more processes such as introgression, recombination, or incomplete lineage sorting. Taxonomic distinctions such as between limber pine and southwestern white pine may have to yield to a more complex but realistic understanding of biological populations.

Although phylogeny within subgenus *Strobus* is complex, knowledge of genetic relationships and host ranges of *C. ribicola* can increase our understanding of the evolution of the white pine blister rust pathosystem. Blister rust infects many species within several distinct taxa, including *Strobi*, *Cembrae*, and *Balfourianae*. Such a broad host range suggests that all species within these taxa would be susceptible but leaves unclear if species within sister taxa such as *Gerardianae* and *Cembroides* would also be susceptible.

Phylogenetic approaches are used to assess evolutionary relationships within the *Cronartium*. Vogler and Bruns (1998) used ITS rDNA sequences to study the phylogenetic relationships of 24 Eurasian and North American pine stem rusts. They found that closely related rust species (as determined by molecular phylogenetics) generally shared a common plant family for their telial hosts, implying that switches to different telial host families are important for speciation for pine stem rusts. The exceptional case involves *C. ribicola* and *C. occidentale*, species that share a common telial genus—*Ribes* (although *C. ribicola* alternates to *Strobi*, *Cembrae*, and *Balfourianae* and *C. occidentale* alternates to *Cembroides*). These two stem rusts are more phylogenetically distant from each other than are several other species with different telial host species. Although *C. occidentale* might be taken as a native stem rust model for a naturalized *C. ribicola* as they are both pathogens of *Ribes* and *Strobus*, other *Cronartium* pathosystems could also be informative.

Ongoing work seeks to elucidate the phylogeographic relationships between *C. ribicola* in North America and Eurasia (Richardson et al. 2009) and to gain insight to the origin(s) of the rust that spread throughout Europe and subsequently to North America (see Geils et al. 2010). Richardson et al. (2009) found at least two distinct phylogenetic groups in Asia—one in Japan and the other in South Korea–northeastern China (see Kim et al. 2010; Zhang et al. 2010). These Asian groups were also distinct from rust collected in western Europe or North America (Fig. 1). The North American white pine blister rust introduced from Europe represents only a fraction of the genetic diversity of *C. ribicola*; its greater potential might be unexpressed but able to emerge as a result of climatic changes or introduction of an Asian blister rust.

Studies have also addressed the phylogenetics of telial hosts—*Ribes* species (Senters and Soltis 2003; Schultheis and Donoghue 2004) and *Pedicularis* species (Ree 2005). Characterization of white pine blister rust resistance in *Ribes* and *Pedicularis* in light of this phylogenetic data should help our understanding of the evolution of resistance (see Zambino 2010).

A molecular clock is a means to estimate species divergence dates. The premise of a molecular clock is that the number of nucleotide substitutions (mutations) between gene

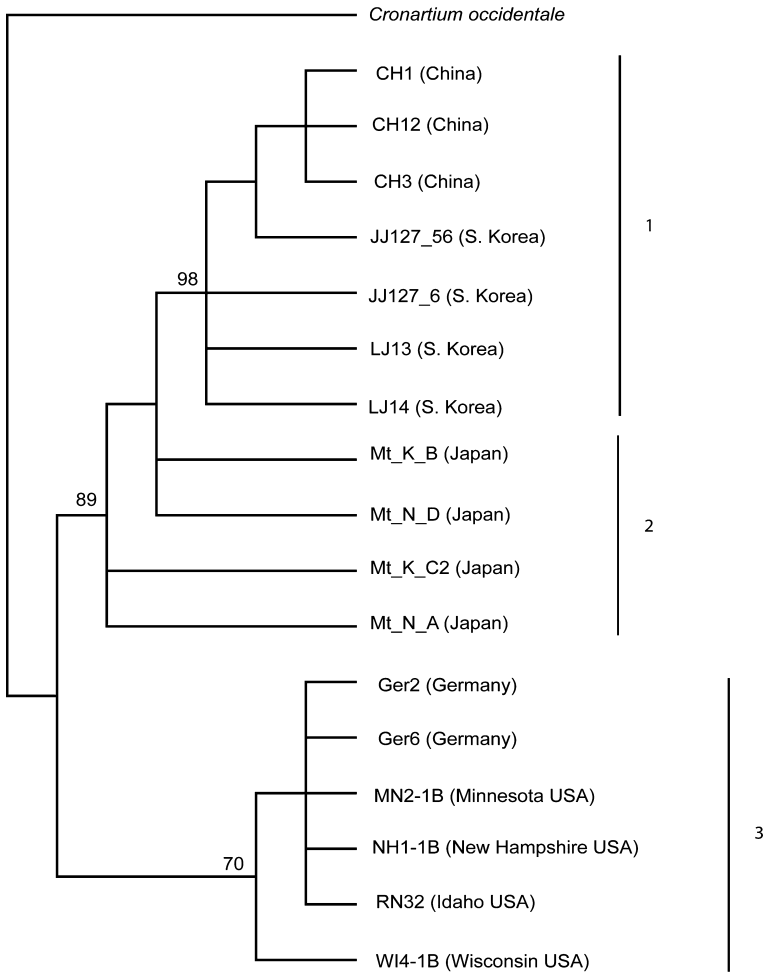

Fig. 1. The phylogenetic relationships of *Cronartium ribicola* isolates developed from maximum likelihood analysis. Three distinct clades are represented and supported by high-bootstrap support. These clades represent three distinct geographic regions: (1) eastern Asia (Korea and northeastern China), (2) Japan, and (3) North America and western Europe (adapted from Richardson et al. 2009).

sequences of species increases with time since divergence. This assumes that mutations occur at a relatively constant rate over time. A molecular clock is calibrated by fossil evidence or the timing of biogeographical events such as the separation of continents or climatic change. As mutation models for particular gene sequences improve, so should the accuracy of divergence estimates (reviewed in Arbogast et al. 2002). However, the inaccurate estimations of divergence times and potential fluctuation in mutation rates challenge an apparent precision of molecular clocks; thus, molecular clock data warrant cautious interpretations (Graur and Martin 2004). Presently, molecular clocks have been used to date the divergence of genera in the Pinaceae (Wang et al. 2000; Willyard et al. 2007) but not for divergence times in the *Cronartium*.

5 Proteomics

Proteomics is the study of proteins expressed by a genome interacting inside an organism, tissue, or cell at a specific developmental stage or in response to an external or internal stimulus. Profiles including several thousands of proteins are typically generated using two-dimensional gel electrophoresis for separation of proteins by charge and size. Separated protein spots are then cut from a gel and digested with trypsin; and the resulting peptides are characterized by mass spectrophotometric techniques such as matrix-assisted laser densitometry ionization tandem-mass spectrophotometer (MALDI-MS). Cataloguing is accomplished by database searching for probable matches using either peptide mass or more expensive peptide sequences. Identifying and resolving the mode of action for the proteins coded by resistance or virulence genes provides us with a better understanding of the genetics and physiology of white pine blister rust that could be useful for developing new screening and breeding approaches.

Davidson and Ekramoddoullah (1997) detected >800 proteins in white pine seedlings and quantified 146 as expressed differentially between white pine seedlings either susceptible or resistant to *C. ribicola*. Further investigations have revealed there are many defence-responsive proteins involved in blister rust resistance (Liu et al. 2004). Proteome profiling of >1200 proteins from spruce attacked or not by weevils found that 104 were differentially expressed; 72% were identified by LC–MS/MS analysis and database searching (Lippert et al. 2007).

Ekramoddoullah (2004) reviewed an additional strategy for detecting and characterizing proteins related to host defence. This technique used either a synthetic peptide identified from a proteomic study or a known DNA coding sequence to make a corresponding antibody that attaches to a protein with the peptide of interest. Such proteomic studies of western white pine infected with *C. ribicola* have led to discoveries of defence-response proteins belonging to the pathogenesis-related (PR) protein groups PR3, PR5, and PR10 and to an anti-fungal peptide (Ekramoddoullah 2004).

White pine PR10 was identified as one of 19 plant PR families. Differential response to biotic and abiotic stimuli among protein members of this gene family indicated that different members of this gene family have evolved to assume diverse functions (Liu and Ekramoddoullah 2003b). Gene promoter analysis of two PR10 genes revealed characteristics of *cis*-regulatory elements (nearby DNA sequences that promote or silence expression) including a fungal elicitor and cold-responsive elements (Liu and Ekramoddoullah 2003a; Liu et al. 2005b). Hunt (2005) showed that western white pine stock resistant to blister rust at cooler, high-elevation sites was susceptible at warmer low-elevation sites; the resistance response could have been induced by PR10 proteins stimulated by exposure to low temperature.

Proteomics is useful for identifying DNA markers associated with genes involved in specific resistance mechanisms. A large-scale screening programme at the Canadian Forest Service found two partial-resistance mechanisms in western white pine—a slow-canker-growth trait and a difficult-to-infect trait (Liu et al. 2004; King et al. 2010). Two proteins, an anti-microbial peptide (PmAMP1) and one isoform of class IV chitinase, were associated with the slow-canker-growth trait (Ekramoddoullah 2004). The peptide PmAMP1 is degraded by *C. ribicola* as infection advances in cankered, susceptible western white pine; whereas the levels of this protein are unaffected in trees with the slow-canker-growth trait and a receding canker margin (Ekramoddoullah et al. 2006). The regulation of PmAMP1 is presently the subject of intensive research by the Canadian Forest Service as it might provide a key for development of a DNA-based marker to screen western white pine for the slow-canker-growth trait.

After infection by *C. ribicola*, the isoform of 27-kDa class IV chitinase from western white pine PR3 family accumulated in both susceptible seedlings and those with the

slow-canker-growth trait, whereas a different 26-kDa PR3 isozyme was expressed specifically in seedlings with the slow-canker-growth trait (Liu et al. 2005a). The difference between seedlings with the slow-canker-growth trait and susceptible seedlings was not only measurable at the chitinase protein level but also at the genomic DNA level. Re-sequencing of chitinase genes revealed a genetic difference between susceptible families and those with the slow-canker-growth trait. The introns of class IV chitinase genes showed a SNP and multiple intron-length polymorphisms. Future work on class IV chitinases in western white pine could provide another tool for marker-assisted selection in white pine breeding and determine whether class IV chitinases are involved as a general defence factor in the slow-canker-growth mechanism.

6 Genetics of resistance and virulence

Molecular characterization of the genes and proteins involved in resistance and virulence is critical for understanding the function, evolution, and stability of the white pine blister rust pathosystem and therefore for designing effective management strategies. One of the most well-studied and understood resistance-virulence systems is the hypersensitive reaction (HR) that conforms with the gene-for-gene concept of Flor (1971). The genes involved in the HR response and the encoded proteins from both host and pathogen have been characterized in several model pathosystems. The HR response is triggered by interaction of a plant receptor (presumably coded by an R gene) with a pathogen elicitor. The result of this resistance reaction is an activation of signal cascades that lead to programmed cell death of infected and adjacent cells. The process is typically initiated by a single, dominant, R gene that encodes a cytoplasmic or membrane-bound receptor (reviewed in Hammond-Kosack and Jones 1997; Gilchrist 1998; Ekramoddoullah and Hunt 2002). In the plant systems studied thus far, the R genes controlling an HR response have a sequence homology across diverse plant taxa (Meyers et al. 1999; Meyers et al. 2002). Because these resistance genes are conserved among plant taxa, readily available PCR primers can be used to characterize these genes in white pine families.

Several white pine families appear to express an HR phenotype coded by an R gene (King et al. 2010). Determining the molecular characteristics of such R genes is a daunting task owing to the large size of a conifer genome (Wakamiya et al. 1993). One method uses PCR-based amplification of candidate R genes (Kim and Brunsfeld 2000; Liu and Ekramoddoullah 2003a). Liu and Ekramoddoullah (2003a) used this approach to selectively amplify PCR products and create a clonal library from western white pine families carrying *Cr2*, the R gene associated with HR in western white pine. Jermstad et al. (2006) completed similar work for sugar pine. R genes are potentially under evolutionary pressure as novel virulence gene products are produced.

A resistance gene analogue (RGA) is a genetic loci that putatively translates a resistance-protein motif. The cloned nucleotide-binding (NB) sequences of western white pine RGAs correspond to two subfamilies of R genes—toll and interleukin-1 (TIR)-NB-leucine-rich repeat (LRR) (Liu and Ekramoddoullah 2003a) and coiled-coil (CC)-NB-LRR (Liu and Ekramoddoullah 2007). The observed high degree of variation in these western white pine NB domains suggests that it contributes to genetic fitness in conifers. By precise domain swapping, various studies have shown that the numbers of LRRs are critical determinants of interactions with avirulence elicitors (Van der Vossen et al. 2000; Xiao et al. 2001). One RGA found in western white pine has almost identical TIR and NB domains but variable LRR lengths (Ekramoddoullah and Liu 2008). These LRR regions are rich in DNA polymorphisms and could be used to develop DNA markers for the saturated genetic mapping of the *Cr2* gene (Liu and Ekramoddoullah 2008).

The generation of novel resistance mechanisms by somatic mutation or recombination would be particularly advantageous for conifer species which are composed of individual

trees that live for hundreds to thousands of years (Michelmore and Meyers 1998). Ontogenetic resistance increases with plant development or age and has been observed in several white pine species (Patton 1961; Kinloch and Byler 1981; Hunt 2004a). Comparison of R-protein structures that determine pathogen specificity could provide useful information on the mechanisms by which ontogenetic resistance operates.

Elicitors usually are proteins excreted by pathogens, such as the protein Cro rI excreted by *C. ribicola* (Yu et al. 2002). This protein has only been detected in the haploid stage during mycelial growth either in the pine host or *in vitro*. As the Cro rI is only present in symptomatic infections, Yu et al. (2002) postulated the protein is involved with pathogenicity. The Cro rI protein might function as a virulence factor and elicitor of HR in the *Cr2* resistance. When extracts containing Cro rI are introduced by injection into western white pine carrying the *Cr2* gene, an HR response is induced that mimics resistance to the intact pathogen (Ekramoddoullah, unpublished data). The Cro rI protein would have to be further purified to determine its mode of action as either an elicitor or virulence factor. A population genetic study of SNPs of the *cro r*I gene might be able to explain the role of the Cro rI protein in the white pine blister rust pathosystem. A single amino acid sequence difference could allow the virulence protein to avoid recognition by the host. Schürch et al. (2004) suggested that deletion or alteration of an avirulence gene might allow a fungal pathogen to avoid recognition by the corresponding resistance gene of the host.

7 Future and ongoing approaches

The white pine blister rust pathosystem has several characteristics useful for clarifying evolution of the invasive pathogen, *C. ribicola*. First, the epidemiological interactions of blister rust often occur in natural ecosystems, providing an *in vivo* experiment from local to continental spatial scales across diverse landscapes and in different communities. Second, resistance to an exotic pathogen is seldom found in native forest pathosystems (e.g., chestnut blight, Dutch elm disease); but such inherited resistance occurs in most white pine species. Third, plant–pathogen interactions of decades or longer have rarely been investigated. The history of blister rust investigations provides researchers with long-term data on ecology and evolutionary biology.

In this section, we discuss new approaches and tools that could aid in the dissection of the blister rust plant–pathogen interactions and evolution. This knowledge is critical for making science-based management decisions that might favour stabilization of disease epidemics and encourage pathosystem naturalization.

7.1 Molecular plant–pathogen interactions

Genomic and proteomic approaches using cDNA libraries are emerging that link the molecular and phenotypic bases of plant defences and pathogen infection. A cDNA library is a collection of individual DNA sequences derived by reverse transcription from messenger (m)RNA of expressed genes in pathogen-challenged and unchallenged hosts. The expressed genes or expressed sequence tags (ESTs) are sequenced, and hybridization experiments reveal the upregulation or downregulation of genes (increase or decrease in gene expression owing to change in sensitivity or number of receptors). Our understanding of the molecular basis of plant–pathogen interactions in white pine blister rust should improve as more genes are discovered in the various resistance and virulence pathways and associated with proteins of known function. In a practical sense, this means being able to describe at a molecular level how plant genes code for proteins that provide a defence and how rust genes defeat it.

A microarray consists of thousands of single-stranded cDNAs fixed onto a glass slide, called a DNA chip. Gene expression is quantified by hybridizing control and experimental samples and assessing relative fluorescence of target gene sequences (reviewed in Gibson 2002; Bryant et al. 2004). A microarray approach allows tens of thousands of genes to be examined in a single pass for changes in regulation. The approach has diverse applications at individual, population, and species levels (Whitehead and Crawford 2006). For example, Ralph et al. (2006) studied genetic interactions resulting from herbivory of hybrid poplars by forest tent caterpillars. Sequence technology, however, could soon replace microarrays as sequencing can now be conducted simultaneously on a whole cDNA library and provide information unavailable from microarrays.

Gene expression has been studied in several pathosystems. In the blister rust pathosystem, a large number of proteins were differentially regulated following *C. ribicola* challenge of western white pine (Ekramoddoullah and Hunt 1993; Ekramoddoullah and Tan 1998). Differential gene expression was examined for fusiform rust [*Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. (*fusiforme*) on slash pine (*P. elliottii*) Engelm.] by comparing RNA extracted from host gall tissue to RNA from healthy tissue and axenic rust cultures (Warren and Covert 2004). In another study, gene expression was assessed from the interaction of slash pine with *Gibberella circinata* Nirenberg and O'Donnell, causal agent of pitch canker. Pine genes upregulated or downregulated in the fusiform rust pathosystem differed considerably from those in the pitch canker pathosystem. These differences were expected, as there are fundamental phytopathological differences between biotrophic *Cronartium* fungi and necrotrophic *Gibberella* fungi (Morse et al. 2004).

New strategies of molecular characterization of white pine R genes (*Cr1, Cr2,* and *Cr3*) could be developed. A biochemical model for the gene-for-gene interaction predicts that an avirulence-gene product (presumably an HR elicitor) would directly or indirectly interact with an R-gene product to trigger the signal transduction pathway leading to activation of defence-related genes. Acting in concert, these gene products give rise to the HR response.

Bendahmane et al. (2000) described a method to establish the functionality of RGAs. Test plants are transformed with an avirulence gene and then infiltrated with transiently expressed RGA cDNA using *Agrobacterium*-based methods. A plant exhibiting HR is taken as evidence the RGA is an R gene interacting with an avirulence gene. If avirulence gene(s) of *C. ribicola* could be identified and cloned, then this approach could identify R genes among white pine RGAs.

A yeast two-hybrid analysis screens for interacting proteins using a 'bait and fish' strategy (Lodish et al. 2004). For example, a 'bait' is a protein of interest such as the Cro rI protein produced by *C. ribicola* and the 'fish' are proteins from a white pine cDNA library. The method requires fusing proteins to binding and activation domains, constructing plasmids with selectable markers, transforming the plasmids into yeast cells, growing and plating the yeast on selective media, and sequencing the DNA of plasmids isolated from the selected cells. The process screens for those white pine proteins that bind to the Cro rI protein, thereby identifying which host proteins interact with a pathogen protein of known or putative function. Alternatively, a protein produced by a host could be used to screen for interacting pathogen proteins.

7.2 Genetic mapping and association genetics

Traditional genetic mapping employs genetic markers such as AFLPs, SNPs, and microsatellite markers to associate qualitative and quantitative traits through segregation of alleles and traits. Traits tightly linked to markers can be used for cloning a region containing a gene of interest or for marker-assisted selection (reviewed in Mackay 2001). Cloning from linked markers works well for organisms with relatively small genomes, mutant lines, or transformation approaches.

Several studies have mapped traits of interest among the Pinaceae. Maps have been developed for qualitative traits such as R-gene resistance to *C. ribicola* in sugar pine, *Cr1* (Devey et al. 1995; Harkins et al. 1998) and in western white pine, *Cr2* (Liu and Ekramoddoullah 2006). Maps are also developed for quantitative traits such as wood properties (Brown et al. 2003) and timing of bud break or bud set (Jermstad et al. 2003). The utility of comparative maps has also been demonstrated with loblolly pine (*P. taeda* L.), Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco], and other Pinaceae (Krutovsky et al. 2004). Mapping of resistance traits in white pines and virulence in *C. ribicola* should be simplified as additional molecular markers and newer, high-throughput sequencing technologies become available (Echt et al. 1999; Kim et al. 2003; Richardson et al. 2008).

An alternative to traditional mapping is association genetics which was developed for human disease research (Goldstein and Weale 2001) but is especially practical for the conifers which characteristically have large genomes and long generations times. Association genetics relies upon life-history characteristics (for conifers, random mating with high-gene flow) and the use of numerous candidate genes to determine associations with phenotypes (Neale and Savolainen 2004; González-Martínez et al. 2006; Neale and Ingvarsson 2008). Given that resistant phenotypes and quantitative traits are well characterized in several white pine species, association genetics should be readily applicable to the white pine blister rust pathosystem.

7.3 Population genomics and genecology

Population genomics is the study of genome-wide patterns of genotypes for addressing natural selection and demographic processes. Approaches in population genomics vary from analyses of specific adaptive traits (such as association genetics) to elucidating genetic patterns to order to make inferences of demographic history and identify putative targets of selection. This later approach is known as a genome scan (Vasemägi and Primmer 2005; Biswas and Akey 2006). Genome scans have been performed over a wide range of organisms such as humans, fish, and oak (Akey et al. 2002; Campbell and Bernatchez 2004; Scotti-Saintagne et al. 2004). Implementing hundreds of molecular markers to saturate the genome, researchers have the capability to detect putative loci under positive selection based on the overall level of genetic differentiation among loci. Statistics test whether loci have genetic differentiation that is greater than expected under neutrality and therefore suggests operation of genetic drift, mating, or gene flow.

Genecology, the study of associating genetic responses to environment, has been traditionally addressed with the use of quantitative traits. Such studies have provided important information regarding climatic adaptation of white pines (Rehfeldt et al. 1984; Bower and Aitken 2008) and other conifer species. More recently, the use of climate models (Hijmans et al. 2005; Rehfeldt 2006) has enabled researchers to discern associations between quantitative traits and climatic variables mapped at high-spatial resolution, providing climate-based genecological models (Rehfeldt et al. 2001; St Clair et al. 2005). Given the volume of molecular data that can be generated, such genecolgical approaches could be used to identify putative selective signatures; and comparisons could be drawn between adaptive traits and molecular markers.

As the knowledge of genes and their functions increase in white pine, blister rust, and related pathosystems, the abilities to link genes to processes of disease, evolution, and ecological adaptation should improve. Currently, efforts are underway to create a knowledge base in the white pine blister rust pathosystem to address these questions. For example, the American Phytopathological Society (2003) has listed *C. ribicola* as a high-priority organism for DNA sequencing, and plans are ongoing to develop an EST library (R. Hamelin, personal communication). Genomic sequencing of white pines is a daunting task owing to the large genome size. However, comparative genomics could help

elucidate a molecular basis of resistance mechanisms and thereby suggest how to improve resistance for natural or artificial regeneration of white pine.

8 Conclusion

The white pine blister rust pathosystem presents unique opportunities to research fundamental questions in biology and to support management for healthy ecosystems. Phylogeny and population genetics reveal clues to the biogeography and evolution of *Strobus* and *Cronartium*—ideas useful for genetic conservation and management. Genetics and proteomics generate information on the inheritance and expression of resistance and virulence—practical data for regenerating white pines. Studies of invasive ecology and co-evolution enhance our understanding of host and pathogen interactions—knowledge valuable to sustain resilient populations. Molecular techniques provide the keys to addressing these questions.

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