Phytopathology® • 2025 • 115:44-53 • https://doi.org/10.1094/PHYTO-04-24-0129-R

Fungal Biology and Genetics

A New Threat to Limber Pine (*Pinus flexilis*) Restoration in Alberta and Beyond: First Documentation of a *Cronartium ribicola* Race (*vcr4*) Virulent to *Cr4*-Controlled Major Gene Resistance

Jun-Jun Liu,[1,†] Richard A. Sniezko,[2] Sydney Houston,[1] Genoa Alger,[3] Jodie Krakowski,[4] Anna W. Schoettle,[5] Robert Sissons,[3] Arezoo Zamany,[1] Holly Williams,[1] Benjamin Rancourt,[1] and Angelia Kegley[2]

[1] Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre, 506 West Burnside Road, Victoria, BC, V8Z 1M5, Canada
[2] U.S. Department of Agriculture Forest Service, Dorena Genetic Resource Center, 34963 Shoreview Drive, Cottage Grove, OR 97424, U.S.A.
[3] Parks Canada, Waterton Lakes National Park, Waterton Park, Alberta, T0K 2M0, Canada
[4] Independent Consultant, Box 774, Coleman, AB, T0K 0M0, Canada
[5] U.S. Department of Agriculture Forest Service, Rocky Mountain Research Station, 240 West Prospect Road, Fort Collins, CO 80526, U.S.A.
Accepted for publication 21 September 2024.

Abstract

The coevolution of virulence reduces the effectiveness of host resistance to pathogens, posing a direct threat to forest species and their key ecosystem functions. This is a threat to limber pine (*Pinus flexilis*), an endangered species in Canada due to rapid decline mainly driven by white pine blister rust caused by *Cronartium ribicola*. We present the first report of a new, virulent race of *C. ribicola* (designated *vcr4*) that overcomes limber pine major gene (*Cr4*) resistance (MGR). Field surveys found that three parental trees (pf-503, pf-508, and pf-2015-0070) were cankered with white pine blister rust in Alberta, but their progenies showed MGR-related phenotypic segregation postinoculation with an avirulent race of *C. ribicola* (*Avcr4*). Genotyping of their progenies using *Cr4*-linked DNA markers and a genome-wide association study provided additional support that these cankered parental trees had *Cr4*-controlled MGR. To confirm the presence of *vcr4*, aeciospores were collected from the cankered pf-503 tree to inoculate resistant seedlings that had survived prior inoculation using the *Avcr4* race, as well as seedlings of two U.S. seed parents, one previously confirmed with MGR (*Cr4*) and one without MGR, respectively. All inoculated seedlings showed clear stem symptoms, confirming that the virulent race is *vcr4*. These results provide insights into the evolution of *C. ribicola* virulence and reinforce caution on deployment of *Cr4*-controlled MGR. The information will be useful for designing a breeding program for durable resistance by layering both R genes with quantitative trait loci for resistance to white pine blister rust in North America.

Keywords: co-evolution, major gene resistance (MGR), pathogen surveillance, single-nucleotide polymorphism (SNP), virulence

Virulence of plant pathogens is usually defined as the degree of damage and the corresponding fitness reduction of their hosts following infection by the pathogens (Barrett et al. 2009; Sacristán and García-Arenal 2008; Sacristán et al. 2021). Evolution of novel virulence is defined as any event or process that promotes the (qualitative or quantitative) fitness of a pathogen, causing disease; it includes pathogen adaptation to host immunity and physiology, host species, genotypes, tissues, or the environment (Sacristán et al. 2021). Both genetic diversity within pathogen populations and the processes that maintain variation in pathogenicity provide essential information for predicting pathogen evolution, disease outcomes, and prevalence (Burdon and Thrall 2008). Coevolution between hosts and pathogens is an arms race of constant reciprocal adaptation. Infection outcomes and their economic and ecological impacts in many pathosystems are determined by dynamic interactions among hosts, pathogens, and the environment (Lannou 2012; Tack et al. 2012). Host genetic diversity is a key factor affecting variation of pathogenicity in the pathogen populations. Plant immune response, nutrient availability, and quantitative disease resistance (QDR) may hamper pathogen reproduction after an initial successful infection (Karisto et al. 2018). Thus, novel virulence activity is essential for pathogens to gain new adaptations to host resistance. Despite the central importance of virulence-related traits to pathogenicity, the processes underlying their evolution and the maintenance of intraspecies variation within populations of pathogens are not well understood, especially in forest pathosystems, such as the white pine blister rust (WPBR) pathosystem.

WPBR, caused by *Cronartium ribicola* (J.C. Fisch.) is the most destructive disease of five-needle pines in the subgenus *Strobus*. Originating in Eurasia, it has spread worldwide via human activities and caused significant losses of timber and forest ecosystem integrity, particularly in North America. Limber pine (*Pinus flexilis* James) is a foundation species for high-elevation ecosystems, distributed in the Rocky Mountains and Intermountain Ranges from southern British Columbia and Alberta in Canada to New Mexico, Arizona, and California in the United States. Limber pine is highly susceptible to WPBR, a pathogen that poses a risk for the collapse of associated high-elevation ecosystems (Schoettle et al. 2022). Due to high rates of rust infection and rust-caused mortality, limber pine has been recommended for designation as an endangered species by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC 2014).

To counteract the primary threat to the species, the core of limber pine restoration involves selecting and propagating WPBR-resistant germplasm across its geographical range (Alberta Environment and Parks 2022; Schoettle et al. 2019a, 2022). Major gene resistance

†Corresponding author: J.-J. Liu; jun-jun.liu@NRCan-RNCan.gc.ca

Funding: Support was provided, in part, by the CFS Pest Risk Management and GRDI programs awarded to J.-J. Liu, Waterton Lakes National Park of Canada, and U.S. Forest Service Rocky Mountain Research Station and Forest Health Protection (Research Agreements 20-IJ-11221633-173 and 14-IJ-11221633-126, respectively, from RMRS to CSF-PFC).

e-Xtra: Supplementary material is available online.

The author(s) declare no conflict of interest.

(MGR) and QDR were detected at low frequencies in several five-needle pine species native to North America (Liu et al. 2022a; Sniezko and Liu 2022). Research has identified MGR loci (*Cr1*, *Cr2*, *Cr3*, and *Cr4*) against WPBR in sugar pine (*P. lambertiana* Douglas), western white pine (*P. monticola*, Douglas ex D. Don), southwestern white pine (*P. strobiformis*), and limber pine, respectively (Kinloch and Dupper 2002; Schoettle et al. 2014). Limber pine MGR was first genetically characterized in stands in Colorado and Wyoming and recently also confirmed in stands in Alberta, Canada (Schoettle et al. 2014; Sniezko et al. 2016). Comparative association mapping further confirmed that MGR was controlled by the same genetic locus *Cr4* in limber pine populations from the Southern and Northern Rocky Mountains (Liu et al. 2020). An association study revealed that *Cr3* and *Cr4* were likely the same R locus, orthologous between southwestern white pine and limber pine (Liu et al. 2022b).

Breeding and ecosystem restoration programs of five-needle pines in Canada and the United States are using both MGR and QDR to improve WPBR disease resistance (Alberta Environment and Parks 2022; Schoettle et al. 2019a, 2022; Sniezko and Liu 2022; Sniezko et al. 2011, 2023, 2024). However, the durability of host genetic resistance is affected by the genetic diversity of pathogenic populations (Sniezko and Liu 2023). MGR generally confers a race-specific resistance and can lose its effectiveness when new virulent races evolve. Plant *R* genes typically encode receptors that recognize avirulent (Avr) effectors, leading to activation of effector-triggered immunity for complete disease resistance (Jones and Dangl 2006). A gene-for-gene (*Avr–R*) relationship has not yet been confirmed at the molecular level in the WPBR pathosystem, but R proteins encoded by *Cr1* to *Cr4* are presumed to detect corresponding *C. ribicola* Avr effectors (*Avcr1* to *Avcr4*) in a classic gene-for-gene relationship, resulting in development of MGR-related phenotypes (Kinloch and Dupper 2002; Liu et al. 2022a).

Resistance in host populations, especially MGR, favors evolution of novel virulent effectors in pathogen populations that allow pathogens to overcome the host immune system or mutation of the original *Avr* effector that then allows the pathogen to evade host surveillance mediated by R proteins. Either scenario leads to a disease outbreak in previously resistant host populations. *C. ribicola* virulent races (*vcr1* and *vcr2*) have been documented on infected MGR trees where the fungus had overcome sugar pine *Cr1* (Kinloch and Comstock 1981) and western white pine *Cr2* (Kinloch et al. 2004), respectively. Both *vcr1* and *vcr2* cause fatal stem cankers, killing host trees despite their *Cr1* or *Cr2* genotypes in native forests, field plantations, or seedling inoculation trials (Kinloch et al. 1999, 2004; Sniezko et al. 2020). In contrast to MGR, QDR is usually controlled by quantitative trait loci with phenotypic contribution from multiple genes (Gou et al. 2023). QDR was well maintained when sugar pine and western white pine hosts were challenged with *vcr1* and *vcr2* (Sniezko et al. 2020).

Documentation of MGR in limber pine and southwestern white pine is relatively recent (Kinloch and Dupper 2002; Schoettle et al. 2014; Sniezko and Liu 2022; Sniezko et al. 2016). Although neither *vcr3* nor *vcr4* has previously been reported in Canada or the United States, detection and characterization of either *vcr* race would be useful for developing race-specific DNA markers to detect virulence genes. Effective monitoring of *C. ribicola* evolution and migration of virulent races in North America would help define the corresponding *R* gene (*Cr3* or *Cr4*) in pine breeding and restoration programs (Liu et al. 2021). Knowing which virulence genes are present and where they occur would be beneficial for the design of efficient breeding programs for durable resistance to WPBR in North America.

Knowledge of the evolution of virulence in *C. ribicola* is essential to estimating the long-term outcomes of deploying resistant five-needle pines and the potential role of MGR. In this study, seeds were collected from healthy and cankered limber pine parent trees. Half-sib seedling progeny of their open-pollinated seed families were inoculated using the Avr *C. ribicola* race (*Avcr4*) collected in Oregon. Several seed families had close to 50% canker-free progeny, suggesting the presence of MGR. However, some putative MGR families originating from recently cankered wild parent trees in Alberta suggested the existence of a *C. ribicola* virulent race that could overcome MGR in limber pine. The objective of this study was to determine whether the Alberta *C. ribicola* virulent race is *vcr4* that overcomes MGR controlled by *Cr4*. A more comprehensive understanding of *Cr4*−*vcr4* interactions will promote future selection of novel *R* genes against a broad spectrum of rust races and will also likely highlight the need for development of limber pine populations with QDR to provide seedlings that can have a long-term impact on the successful restoration of limber pine ecosystems.

Materials and Methods

Plant materials

Open-pollinated seeds of half-sib families were collected from four seed parents from Alberta and two from the Southern Rocky Mountains in Colorado and Wyoming for use in this study (Supplementary Table S1). The four Alberta parental trees used were pf-2, pf-503, pf-508, and pf-2015-0070; the latter three parental trees had developed stem cankers. Of these seed families, progeny from pf-2 previously showed Mendelian phenotypic segregation with the stem canker trait (Sniezko et al. 2016), and progeny from pf-508 were previously characterized as *Cr4*-controlled MGR against the *C. ribicola* Avr race collected in Oregon in the MGR-2016 trial (Liu et al. 2020). The other two Albertan parental trees were characterized for the first time in this study. The two Southern Rocky Mountain parental trees were NR-3647, which is located on Niwot Mountain in northern Colorado, and PHA-112, which was located at Pilot Hill, southern Wyoming, but is now dead. In MGR progeny tests using the *C. ribicola* Avr race collected in Oregon, NR-3647 was documented as a *Cr4*-controlled MGR family with 71% stem-symptom-free seedlings (Liu et al. 2016; Schoettle et al. 2014), and PHA-112 was documented as a susceptible (*cr4/cr4*) family with 0% stem-symptom-free seedlings and 0% survivors (Liu et al. 2016; Schoettle et al. 2014). Seed tree PHA-112 had WPBR infection at the time of seed collection in 2003. Seed tree NR-3647 and other seed trees previously inferred to be *Cr4* in and around the Niwot Mountain and Pilot Hill regions have remained canker-free in the field despite rust incidence exceeding 40% at both sites when inspected and assessed in 2023 (A. W. Schoettle, *personal communication*).

Fungal inoculation and assessment of disease symptoms

Seedlings were inoculated at two locations: (i) at the Dorena Genetic Resource Center (DGRC) in Dorena, Oregon, using *C. ribicola* races (*Avcr4*) collected from Oregon, and (ii) at the Canadian Forest Service, Pacific Forestry Centre (CFS-PFC) using the putatively virulent race collected from Alberta cankered tree pf-503 (Supplementary Table S2). *C. ribicola* infects *Ribes* to complete its lifecycle (Geils et al. 2010). The Oregon inoculum source was a heterogeneous mixture of *Avcr4* genotypes from infected *Ribes* spp. leaves collected from multiple field locations in Oregon (Schoettle et al. 2014). Resistant and susceptible phenotypes were determined based on stem canker development as reported previously (Schoettle et al. 2014; Sniezko et al. 2016). Three inoculation trials of Alberta seed families using *Avcr4* rust sources occurred at the DGRC: MGR2016, SY2016, and SY2017, in which 6-month-old seedlings (MGR-2016) and 18-month-old seedlings (SY2016 and SY2017) were inoculated using basidiospore densities of approximately 6,000 or 3,000 spores/cm^2, respectively (Liu et al. 2020). The MGR2016 trial was used to classify a parent or a seed family as MGR or non-MGR, whereas SY2016 and SY2017 trials were used to examine the parental offspring for QDR but also to confirm the MGR nature of a family tested in the MGR trial.

To further clarify if the Alberta virulent race is *vcr4* that overcomes *Cr4*-controlled MGR, aeciospores were collected from one of the MGR parent trees, pf-503. The tree had inactive cankers as early as 2011, yet aeciospores were observed and sampled (race pf-503) on 24 May 2019, and propagated by inoculating black currant (*Ribes nigrum* 'Ben Nevis') leaves of container-grown plants in a greenhouse inoculation room at the CFS-PFC on 6 June 2019. The *Ribes* plants were sprayed with water to keep the abaxial and adaxial sides of the leaves well moistened immediately before inoculation. Spores from pf-503 were applied to the moistened underside of the *Ribes* leaves using a fine paint brush or by hand. The underside of the leaves was subsequently gently sprayed with water, so the spores were suspended in water droplets without washing them off. The inoculated *Ribes* plants were incubated for 24 h under ambient laboratory conditions, inside a moistened, plastic inoculation box with a plastic cover. A fog mist machine worked constantly to keep the inoculation room as damp as possible. After 24 h, the plants were removed from the inoculation boxes and left on the bench in the inoculation room for 4 days until orange blisters were visible, indicating urediniospore development. *Ribes* plants were then moved to a growth chamber maintained at 21/15°C day/night with a 16-h photoperiod where they were watered regularly and monitored for disease development. Urediniospores began shedding after 10 to 12 days and were collected by gently suctioning the spores from the underside of the infected *Ribes* leaves into a small side arm-flask under low vacuum. The spores were transferred from the flask into microcentrifuge tubes and stored at either 4°C for re-inoculation or at −80°C for long-term storage.

Ribes inoculations were repeated to bulk up our spore collection, and urediniospores were used on 16 September 2019, to inoculate *Ribes* with the goal of infecting limber pines. By 9 October, telia were well developed on the *Ribes* leaves, and growth chamber conditions were adjusted to 15°C with a 12-h photoperiod to promote basidiospore development and subsequent shed. The inoculation trial at the PFC in 2019 was initiated on 23 October 2019, to test the virulence of race pf-503 (Supplementary Table S2). Limber pine resistant seedlings of seed families from pf-2 (two seedlings) and pf-503 (six seedlings) that survived the 2016 MGR trial at the DGRC were shipped to Canada in the winter of 2018 and re-inoculated with the race pf-503 (putative *vcr4*) at the CFS-PFC. This inoculation trial also included 10 2-year-old seedlings from each of the seed families PHA-112 and NR-3647, for a total of 28 seedlings. A plastic inoculation box containing a metal grid on top was placed inside a growth chamber set to 15°C with a 12-h photoperiod. Diseased *Ribes* leaves were placed on the metal grid with the spore shedding side facing down. Leaves were sprayed with water, and water-saturated sponges were placed on top of the leaves to keep them wet. The 28 limber pine seedlings were placed underneath the leaves with about a 15-cm distance between them. The inner plastic wall of the inoculation box was sprayed with water to maintain humidity. Vaseline-coated slides were placed in different locations under the leaves to capture spore shed, and the plastic box was kept closed. After 24 h, the slides were removed and inspected under a compound microscope at 200 to 400× magnification. Urediniospores and germinating basidiospores were observed. The seedlings were left a second night under the diseased *Ribes* leaves to facilitate infection of the pine seedling needles.

After inoculation, seedlings were monitored regularly, and disease symptoms were visually assessed a total of five times, once each in March 2020, October 2020, June 2021, October 2021, and January 2022 when most seedlings began dying and were sampled for DNA extraction. The inoculation trial at the CFS-PFC generally followed the same standard measurements for rust inspection at the USDA-DGRC (Johnson and Sniezko 2021; Sniezko et al. 2011), with both the presence of needle disease spots and stem disease symptoms being recorded. Stem disease symptoms included swellings on the main stem or branches of the seedlings; these swellings were often fusiform-shaped with an orange-colored margin on the perimeter of the swollen area. Assessment criteria included seven disease-related morphological traits: (i) types of needle disease spots (a yellow-red center, yellow, or red); (ii) swelling at the base of the needle fascicle (yes or no); (iii) number of branches with swelling per total number of branches; (iv) number of stem swellings on the main stem per number with associated pycnia; (v) rust-caused damage coded in categories (0 = none, 1 = animal, 2 = mechanical, 3 = disease [non-blister rust], 4 = disease [blister rust] and alive, 5 = insect, 6 = general climatic [e.g., snow, frost], 7 = water/drought, 8 = unknown/other, 9 = dead from rust; these categorical codes were not quantitative values, so a missing value (such as 6, 7, or 8), especially under greenhouse inoculation conditions, should not affect the interpretation of the results); (vi) rust-caused disease severity coded based on an ordinal scale (0 = no stem infection, 1 = just beginning to see orange at the base of a needle or infection on limb instead of bole [main stem], 2 = less than 20% of the bole encircled by stem infection, 3 = 20 to 40% of the bole encircled by stem infection, 4 = 40 to 99% of the bole encircled by stem infection, 5 = infection completely encircles the bole with relatively little vertical expansion at less than 10% of bole length, 6 = infection completely encircles the bole and affects 10 to 40% of the length of bole, 7 = infection completely encircles the bole and affects 40 to 90% of the length of the bole, 8 = infection completely encircles the bole and is observed over 90 to 100% of the bole, 9 = dead from rust); and (vii) seedling vigor, where 1 = tree alive and green, 2 = alive with yellow needle tips, 3 = tree chlorotic, and 4 = tree dead. For seed families pf-2 and pf-503, disease symptoms focused on the new stem growth beginning in 2019 to distinguish it from previous infections due to the MGR16 inoculation at the DGRC. A Kruskal-Wallis test was used to detect differences between disease assessment scores among seed families.

TaqMan-based single-nucleotide polymorphism (SNP) genotyping using *Cr4*-linked SNP markers

Following inoculations at the DGRC using Oregon *Avcr4* races (trials MGR2016, SY2016, and SY2017; see Supplementary Table S2), a total of 27, 28, and 53 seedlings from pf-503, pf-508, and pf-2015-0070 families, respectively, were sampled for association studies. Genomic DNA was extracted from needles using a DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol and used to detect SNPs through qPCR-based SNP genotyping and targeted amplicon-seq as outlined below.

Cr4-linked SNP markers characterized in previous genetic mapping and association studies, including snp296815Y, snp204077R1, snp438219K, and snp-M304083-485Y (Liu et al. 2016, 2021), were used to test and confirm if the MGR phenotype was controlled by *Cr4* using TaqMan-based SNP genotyping. TaqMan arrays were designed (Supplementary Table S3) and genotyping was performed as described previously (Liu et al. 2021) using needle genomic DNA from the progeny of the seed families. To determine if SNP loci detected by TaqMan arrays were significantly associated with MGR phenotypes, G × P association was evaluated by χ^2 tests using genotypic frequencies between two seeding groups with susceptible and resistant phenotypes (characterized by presence versus absence of stem symptoms).

Amplicon-seq and SNP detection

For a genome-wide association study (GWAS), SNPs were detected by the same amplicon-seq arrays as reported previously (Liu et al. 2024). In brief, the amplicon-seq arrays targeted a set of 480 limber pine genes with genome-wide distribution that were selected from a previously constructed limber pine genetic linkage group (LG) map (Liu et al. 2019). One exon sequence per gene was used to design Fluidigm custom primers with one amplicon per gene, and multiplex PCR amplification was performed with 10 pooled sets of primers per PCR reaction using the Fluidigm Access Array System (Fluidigm Corporation). Targeted amplicon-seq was performed to

generate 250-bp paired-end sequences using the Illumina MiSeq sequencer as described previously (Liu et al. 2021).

After filtering raw MiSeq data using the Trimmomatic program with default settings (Bolger et al. 2014), clean 250-bp paired-end reads were used to detect DNA variants using FreeBayes as described previously (Garrison and Marth 2012; Liu et al. 2019, 2024). In-house R scripts were developed to merge SNP data from individual samples, as well as to perform statistics for distributions of SNP depth, missing data, and minor allele frequencies prior to association analysis (Liu et al. 2019, 2024).

GWAS of genetic resistance to *C. ribicola*

The seedling family from pf-2015-0070 was selected for GWAS to determine if the Alberta virulent race is indeed *vcr4* that overcomes *Cr4*-controlled MGR. Families from pf-503 and pf-508 were not included because of their smaller family sizes (only 27 and 28, respectively), which was due to poor seed germination rates (Supplementary Table S2). GWAS was performed using a general linear model and a mixed linear model in TASSEL (Bradbury et al. 2007). Because MGR phenotypic data were binary (presence versus absence of stem symptoms), a logistic regression model was also used for GWAS in PLINK (Purcell et al. 2007). A population structure (Q) and a kinship matrix of polygene (K) were considered in the mixed linear model ($y = \text{marker} + Q + K$) to avoid spurious marker-trait associations (Yu et al. 2006). Because the adjacent SNPs within the same gene had high linkage disequilibrium, the number of polymorphic genes rather than the number of total SNPs was used for Bonferroni correction, setting a threshold of $P < 1.58 \times 10^{-04}$ for a significant association of the SNP loci in GWAS. Manhattan and quantile-quantile (Q-Q) plots were used to graphically present results of association tests, and the marker-Rsq values (R^2) were used to estimate the proportion of total phenotypic variation that could be explained by each SNP locus.

Results

Seedling families of cankered Alberta parental trees showed MGR against *C. ribicola* Oregon *Avcr4* races

During seed collection and field surveys, three Alberta parent trees (pf-503, pf-508, and pf-2015-0070) were noted as having one or more cankers. Both pf-503 and pf-508 are localized in the same stand of trees, which had a stand rust incidence (cankered trees) of 83% as surveyed in 2019. By contrast, pf-2015-0070, 34 km northeast, originated in a stand with a rust incidence of 42% in the same survey. Tree pf-503 was recorded with inactive cankers as early as 2011, although aeciospores were only observed and collected in May 2019. Aeciospores were observed on tree pf-508, and samples were collected in June 2020 (Fig. 1). In contrast to heavy infection on pf-503 and pf-508, the tree pf-2015-0070 had one inactive canker in 2017 and 2021, as well as a few possible WPBR needle spots in 2017, but no aeciospores were available to confirm *C. ribicola* infection or WPBR.

Following inoculation at the DGRC using Oregon *Avcr4* races, families pf-503, pf-508, and pf-2015-0070 showed MGR-related phenotypic segregation based on assessments of presence or absence of stem symptoms. Family pf-508 was inoculated independently in the MGR16 trial as reported previously (Liu et al. 2020) and as in the SY2016 trial of this study. The χ^2 test revealed

Fig. 1. Stem cankers and aeciospores of *Cronartium ribicola* (cause of white pine blister rust) on Alberta limber pine parental trees. **A to C,** Cankered branches of tree pf-503. **D to F,** Cankered branches of tree pf-508.

that phenotypic segregation fit the Mendelian segregation ratio as expected with a 1:1 ratio and *P* values ranging from 0.361 to 0.862 for the open-pollinated seed families of trees pf-503, pf-508, and pf-2015-0070, respectively (Supplementary Table S2). The ratios indicate that all three parental trees might have a dominant *R* locus for MGR against the Oregon *C. ribicola* races and suggest that the Alberta *C. ribicola* race that infected and caused stem cankers on the parental trees was virulent, in contrast to the Oregon Avr (*Avcr4*) race.

In contrast to results from Alberta, the field phenotypes of the Southern Rockies parental *Cr4*-resistant tree, NR-3647 (stem-symptom-free), and susceptible tree PHA-112 (cankered and now dead) are consistent with their *Cr4* resistance, or lack thereof, inferred from progeny inoculation trials using the Oregon *C. ribicola Avcr4* race (Schoettle et al. 2014; A. W. Schoettle, *personal communication*). This suggests that *C. ribicola* populations in the Southern Rockies are currently composed of an Avr (*Avcr4*) race (Supplementary Table S2).

TaqMan-based SNP genotyping and GWAS confirmed that the Alberta virulent race was *vcr4* overcoming *Cr4*-controlled MGR

Of the MGR families used in this study, previous resistance screening and association studies revealed that MGR in seed families NR-3647 (Schoettle et al. 2014) and pf-2 (Liu et al. 2020; Sniezko et al. 2016) was controlled by *Cr4*. NR-3647 with *Cr4*-controlled MGR was confirmed here by TaqMan genotyping using *Cr4*-linked SNPs. The snp-M304083Y locus was well segregated in the NR-3647 family, with genotypic frequencies of T/T, T/C, and C/C at 10, 50, and 40% before inoculation with either *vcr4* or *Avcr4*. In contrast, survivors from the *Avcr4* infection shifted genotypic frequencies, with T/T, T/C, and C/C at 40, 50, and 10%, respectively (Fig. 2A). The genotypic frequencies detected by the TaqMan array were fully consistent with the previous resistance screening trial that revealed a stem-symptom-free rate at 71% post *Avcr4* inoculation (Schoettle et al. 2014).

To determine if parental MGR of pf-503, pf-508, and pf-2015-0070 was mediated by *Cr4*, three *Cr4*-SNP genotyping arrays (snp-M304083Y, snp296815Y, and snp204077R1; Supplementary Table S3) were used to genotype their progenies following phenotyping by *Avcr4* inoculation. At least one SNP locus was informative with genotypic segregation in a genotyped seed family (Fig. 2B to F; Supplementary Table S4). Association of *Cr4*-linked SNP genotypes with MGR phenotypes showed the highest significant level in the pf-508 family (snp-M304083Y with χ^2 tested P = 3.7E-44). Family pf-2015-0070 had association levels (snp296815Y with P = 2.2E-23 and snp-M304083Y with P = 3.0E-25) very similar to family NR-3647 with known *Cr4*-controlled MGR (snp-M304083-485Y with P = 2.9E-20). In contrast, pf-503 showed G × P associations at the lowest levels, but still significant (snp296815Y with P = 3.1E-06 and snp204077R1 with P = 1.2E-12, respectively).

For family pf-2015-0070, GWAS was further performed through amplicon-seq-based SNP genotyping. The targeted 480 genes were previously mapped across 12 *Pinus* consensus LGs. After filtering SNPs at a minor allele frequency of 5% or greater and missing data less than 20%, 3,348 SNPs were detected and used for GWAS. Analyses using a general linear model, mixed linear model, and logistic regression model consistently revealed that SNPs within three genes (*m286986*, *m265032*, and *m410333*) were significantly associated with MGR-related phenotypic traits (Fig. 3). The associated

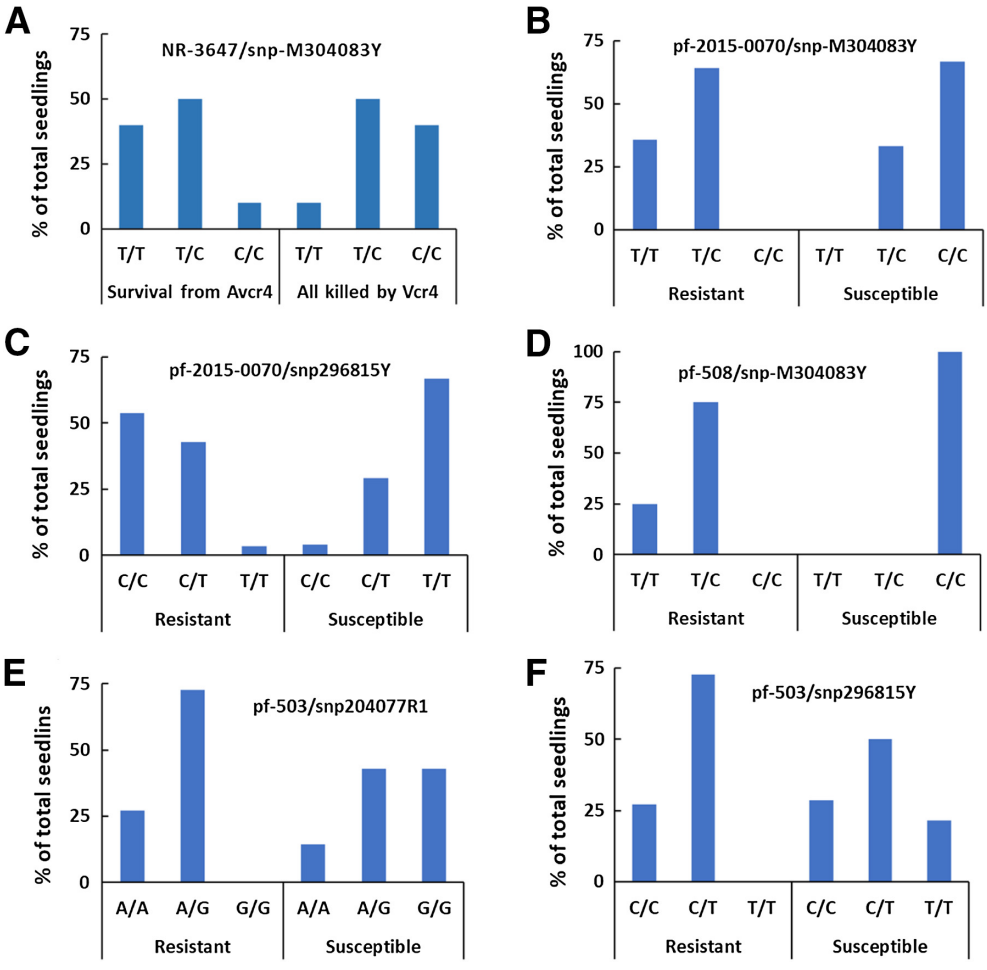

Fig. 2. Genotypic frequency of the polymorphic *Cr4*-linked single-nucleotide polymorphism (SNP) loci as detected by TaqMan SNP genotyping arrays. Phenotypes of major gene (*Cr4*) resistance in limber pine were determined by controlled inoculation using *Cronartium ribicola Avcr4* races collected in Oregon. **A,** The NR-3647 family genotyped at the snp-M304083Y locus. **B,** The pf-2015-0070 family genotyped at the snp-M304083Y locus. **C,** The pf-2015-0070 family genotyped at the snp296815Y locus. **D,** The pf-508 family genotyped at the snp-M304083Y locus. **E,** The pf-503 family genotyped at the snp204077R1 locus. **F,** The pf-503 family genotyped at the snp296815Y locus. All monomorphic SNP loci are not presented here for any of the four seed families.

SNPs included four in gene *m286986* (at nucleotide positions −37R, −144K, −249K, and −314R), one in gene *m265032* (at nucleotide position −171R), and two in gene *m410333* (at nucleotide positions −79Y and −110K). These associated SNP loci explained total phenotypic variations with R^2 values up to 0.65. The three associated genes encoded a putative cytidine/deoxycytidylate deaminase family protein (m286986), beta-amylase (m265032), and a pyridoxal-dependent decarboxylase family protein (m410333). They were previously mapped on the *Pinus* consensus LG-08, and gene *m286986* was close to *Cr4* with a genetic distance of ~3 cM (Liu et al. 2019).

TaqMan-based SNP genotyping and GWAS demonstrated that MGR of pf-503, pf-508, and pf-2015-0070 families was likely controlled by *Cr4*, and the *C. ribicola* race causing cankers on parental stems overcame *Cr4*-controlled MGR. Thus, the virulent Alberta race was designated the *vcr4* race.

Inoculation by Alberta virulent race *vcr4* confirms it can overcome limber pine MGR

Following inoculation using the pf-503 rust race, stem symptoms developed on all seedlings of the four families, with no difference between MGR (pf-2, pf-503, and NR-3647) and susceptible

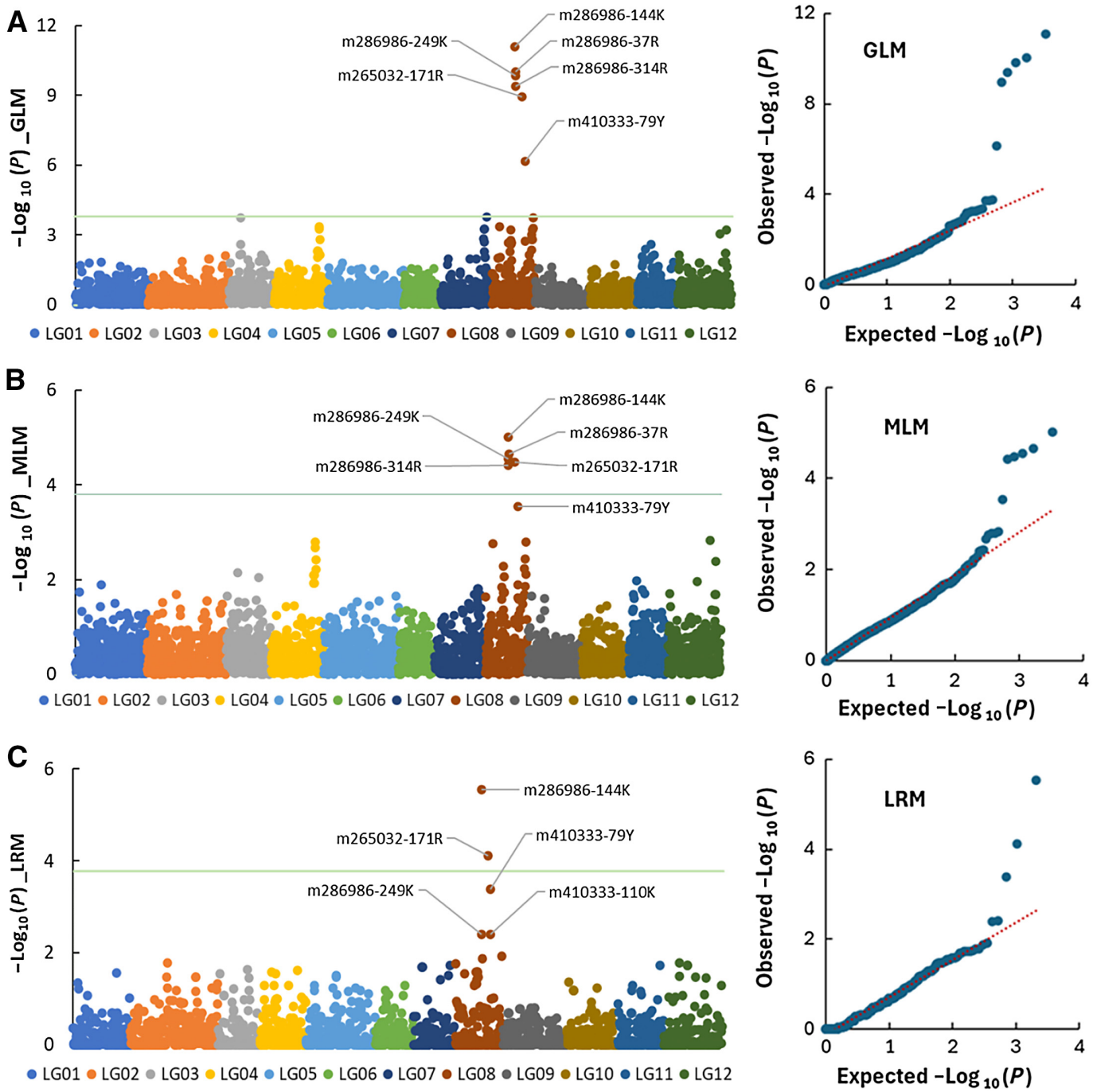

Fig. 3. Manhattan and quantile-quantile (Q-Q) plots depicting single-nucleotide polymorphism (SNP) loci strongly associated with major gene resistance phenotypes of limber pine to white pine blister rust. **A,** Genome-wide association study (GWAS) using a general linear model (GLM). **B,** GWAS using a mixed linear model (MLM). **C,** GWAS using a logistic regression model (LRM). The *x* axis shows relative orders and positions of SNPs on the limber pine genetic linkage groups (LGs), and the *y* axis of $-\log_{10}(P$ values) show association levels. The horizontal green lines in the Manhattan plots (the left panels) indicate the threshold of significant association shown by $-\log_{10}(P$ values) adjusted by Bonferroni correction. The red dotted lines in the Q-Q plots (the right panels) represent the diagonals for the trends of expected association *P* values.

(PHA-112) families. Swelling was observed on all seedlings at needle fascicles or on branches and stems. Pycnia were observed on all inoculated seedlings except two: one seedling in family pf-503 with one large stem symptom that died at the early infection stage and another seedling in family pf-2 with five branches with stem symptoms. These stem disease symptoms and signs indicated that all seedlings were susceptible to the pf-503 race (Supplementary Fig. S1; Supplementary Table S2), with stem infection and high scores for development of rust-caused damage and disease severity (Fig. 4A).

Comparison of disease assessment scores found no significant difference among *Cr4*-controlled MGR families pf-503/pf-2 and NR-3647 and susceptible (*cr4/cr4*) family PHA-112 (*P* values for the Kruskal-Wallis test for rust-caused damage, disease severity, and growth vigor were 0.199, 0.239, and 0.632, respectively), indicating that MGR families with *Cr4* were 100% susceptible when inoculated with the putative *vcr4* race (Fig. 4B). Both the inoculation trials and the association studies confirmed that the Alberta pf-503 *C. ribicola* race infecting progeny from parental MGR trees was *vcr4*, which overcame *Cr4*-controlled MGR, and that it is different from the Oregon Avr (*Avcr4*) race. *C. ribicola* pf-503 race overcame *Cr4*-controlled MGR in three limber pine seed families, two originating from Canada and one originating from the Southern Rockies in the United States.

Discussion

This study is the first to document a *C. ribicola vcr4* race overcoming *Cr4*-controlled MGR in Alberta limber pine field stands. Results of the inoculation trials at the DGRC using an *Avcr4* race confirmed that three Alberta parents used in this study were MGR. The seed families pf-503 and pf-2015-0070 were first reported as being MGR in the present study, but field observations found that their parents were cankered. An association study using TaqMan genotyping arrays and GWAS revealed that MGR was controlled by *Cr4* in three Alberta cankered parent trees. We isolated and propagated the *vcr4* race from one infected Alberta MGR parent tree (pf-503) and used it to successfully infect *Cr4* seedlings under controlled growth chamber conditions. The inoculation with the pf-503 rust race resulted in 100% of seedlings developing stem disease symptoms, with *Cr4* seedlings from both Alberta and Colorado MGR families being moribund or dead at the final sample date at the end of the inoculation trial. Following infection with the pf-503 race, stem disease development showed no difference on seedlings between MGR and susceptible (*cr4/cr4*) families, confirming that the pf-503 race is a *vcr4* race. Documenting the presence of *vcr4* provides additional insight into the durability of MGR against *C. ribicola* and the evolution of fungal virulence in the WPBR pathosystem.

The confirmed Alberta MGR parent trees with cankers were localized at two sites (Cloudy Ridge and Beaver Mines) of southwest Alberta, 35 km apart, where stand disease incidences were 85 and 42% in the 2019 survey, respectively. High infection rates would kill most of the susceptible parent trees and increase frequencies of resistance genotypes in the surviving stands through natural selection. However, higher incidence of MGR parents within a stand increases the selection for emergence of virulent races. Inactive cankers on pf-503 were recorded as early as 2011, indicating that *vcr4* emergence might have occurred earlier than 2011 in this stand.

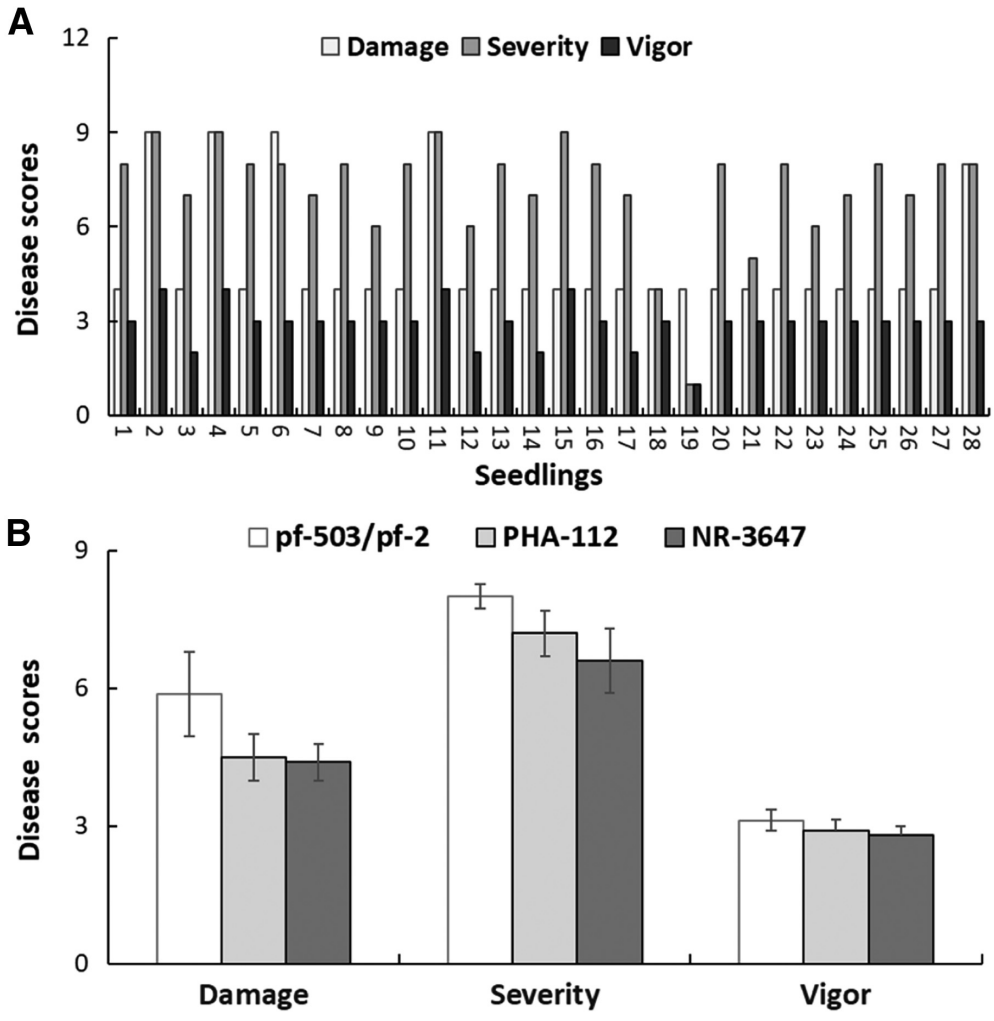

Fig. 4. Assessment of white pine blister rust disease at 1 year postinoculation with *Cronartium ribicola vcr4* race. **A,** Scores of damages, severity, and growth vigor on each seedling of the four seed families, including pf-503 (numbers 1 to 6), pf-2 (numbers 7 to 8), PHA-112 (numbers 9 to 18), and NR-3647 (numbers 19 to 28). **B,** Comparison of diseased traits among families. Families pf-2 and pf-503 were combined as one set and compared with PHA-112 and NR-3647. Rust-caused damage was scored as 0 = none, 4 = rust, and 9 = dead seedlings. Disease severity was scored as 0 = no infection; 1 to 3 = rust, little impact; 4 to 6 = intermediate; 7 to 8 = severe impact; and 9 = dead from rust. Growth vigor of seedlings was scored as 1 = tree alive, green; 2 = alive with yellow needle tips; 3 = tree chlorotic; and 4 = tree dead. Each bar shows the mean of trait scores and the standard deviation of the mean for each family. No significant difference was found among seed families as tested by a Kruskal-Wallis test ($P > 0.05$) for four assessed traits.

The stem canker of pf-2015-0070 was noticed later in 2017 in the stand (Beaver Mines). A collection of aeciospores in nearby trees at this site in coming years will help confirm the continued presence of the *C. ribicola vcr4* race in this stand.

In contrast to *vcr4*, the *vcr2* race that overcomes *Cr2*-mediated MGR in western white pine was originally detected in the 1960s in the Champion Mine region near Cottage Grove, Oregon, where *Cr2* was first documented (Kinloch et al. 1999). It was later found with wide distribution throughout the Oregon Central Cascades (Kinloch et al. 2004; Sniezko et al. 2020). Because western white pine now occurs in relatively low abundance in natural stands or plantings, the *vcr2* distribution pattern has likely been caused by migration of *vcr2* to neighboring areas. However, *vcr2* has not yet been documented in Washington or British Columbia, where some MGR families have been planted over the last few decades (R. A. Sniezko, *personal communication*). MGR has generally maintained its effectiveness in six western white pine progeny trials in western Washington, at least through the first 15 years after planting, even though most of the trials now have more than 85% disease incidence (R. A. Sniezko, *personal communication*). The first documented occurrence of *vcr1* that overcame the sugar pine (*P. lambertiana* Dougl.) *Cr1*-mediated MGR was in 1976 to 1978 near Happy Camp, California, where sugar pine MGR seedlings that had survived WPBR testing had been extensively planted (Kinloch and Comstock 1981). About 20 years later, *vcr1* appeared ~700 km south of Happy Camp (Kinloch et al. 2004). To date, *vcr1* has not been documented in Oregon field trials (R. A. Sniezko, *personal communication*), and *Cr1* is still an important component of the California sugar pine rust resistance program.

Monitoring for *vcr4* has been underway since 2008 in the Southern Rocky Mountains (Cleaver et al. 2017; Schoettle et al. 2014, 2019a). WPBR incidence is 65% in the stand of PHA-112, 7.3% immediately around NR-3647, and 29% in the larger site of the stand. However, no evidence of a virulent race that can overcome *Cr4* resistance has been found as of 2023 in the Southern Rocky Mountain regions (A. W. Schoettle, *personal communication*), but this may be due to the relatively few *Cr4* parents identified and the relatively low prevalence of WPBR in the past. It is important to monitor the incidence, distribution, and migration of *vcr4* and other *vcr* races to determine the future utility of MGR for reforestation with the associated pine species. For this purpose, more MGR parent trees or plantings using MGR seed lots from these parent trees need to be identified and monitored in different host tree regions.

Mutation rates, costs of genetic fitness, the frequency of resistant alleles, and genotype−environment interactions may affect emergence, migration, and distribution patterns of pathogenic virulence. The spontaneous rate for a favorable mutation from *Avr* to *Vir* was estimated at 1×10^{-5} to 1×10^{-7} among spores per generation (Drake et al. 1998; Stam and McDonald 2018). In several crop pathosystems, preexistence of virulence to new *R* genes was detected with low frequencies (ranging from 6×10^{-5} to 1.3×10^{-2}) before the pathogens were exposed to the *R* genes (Balesdent et al. 2013; Degrave et al. 2021). Given the natural life cycle of rust fungi with the development of five distinct spore stages (urediniospores, teliospores, basidiospores, spermatia, and aeciospores), *C. ribicola* may have evolved various *vcr* races in a short time after its introduction into North America. During the lifespan of long-lived perennial plants, genetic variation of associated pathogens can arise and shift in frequency multiple times, increasing the chance of virulence emergence and outbreak (Plissonneau et al. 2017). WPBR can infect and kill five-needle pines of any age. The same tree may be attacked by various WPBR genotypes repeatedly for many years during its long life, until a virulent race is able to infect, resulting in cankers and mortality. Like other rust fungi (Figueroa et al. 2020), both sexual out-crossing of the pathogen on five-needle pines and asexual reproduction on *Ribes* enable generation and proliferation of new combinations of beneficial alleles and purging of deleterious alleles (Gitzendanner et al. 1996). Previous studies found that densities and frequencies of MGR trees in natural stands were low in all tested species of American native five-needle pines, with the average frequency of *Cr1* at 2.2% (range: 0 to 8.9%), *Cr2* at 0.11% (range: 0 to 0.8%), and *Cr4* at 5% in the Southern Rocky Mountains (range: 0 to 13.9%) (Kinloch 1992; Kinloch et al. 2003; Schoettle et al. 2014). However, some field plantings can contain much higher incidence of MGR because this resistance was predominant in both field trial and reforestation plantings of both sugar pine and western white pine in Oregon and California. Changes in genetic diversity of rust populations may result in rapid adaptation of *C. ribicola* to stands with high frequencies of *R* genes or resistant quantitative trait loci in WPBR pathosystems (Sniezko et al. 2020).

To help minimize migration of virulent races and the damage they cause, reforestation plantings in the last several decades in Oregon and California for sugar pine and western white pine included genetically diverse mixtures from seed orchards that incorporate MGR, QDR, and susceptible seedlings (R. A. Sniezko, *personal communication*). In addition, for most of the plantings in Oregon, the western white pine or sugar pine has been planted in relatively low frequency in combination with other tree species. Potential durability of MGR in these species is discussed by Sniezko and Liu (2023). In some western white pine plantations, pruning of the lower branches of high-value trees is done to reduce the area suitable for infection by the pathogen (Singleton and Oblinger 2017). Additionally, it helps further extend the utility of *Cr2* because in many areas, the majority of cankers occur at less than 3 m height (Koester et al. 2018). Deployment strategies to maximize the utility of MGR to *C. ribicola* in limber pine and other white pine species are also being considered (Schoettle et al. 2019a, b).

The evolutionary dynamics of rust virulence are heavily influenced by molecular interactions of host R proteins with pathogen *Avr* effectors. Because of the emergence and migration of virulent races, resistant host genotypes have failed in some forest pathosystems over a short time span (Louet et al. 2023). The frequent outbreak of forest diseases, as driven mainly by adaptation of novel virulence-related traits in populations of pathogens, presents one major limitation for the deployment of resistant germplasm for long-term control of nonnative and native forest pathogens (Ghelardini et al. 2016; Kinloch 2003). Thus, the emergence of the virulent races of *C. ribicola* such as *vcr1*, *vcr2*, and *vcr4* will make restoration of five-needle pines more complex and challenging. WPBR control strongly depends on the durability of resistance in host trees (Sniezko and Liu 2023). There is a serious concern regarding resistance durability in the face of evolving new genes or alleles of rust effectors. A shift from Avr to virulence in the WPBR populations will render MGR deployment ineffective (Kinloch 2003). However, the prevalence of the virulent *C. ribicola* races is still largely unknown in North American populations.

Knowledge of the emergence and frequency of virulence genes across the landscape will support the design of an appropriate breeding program by incorporating both *R* genes and QDR genotypes for durable resistance to WPBR in North America. Considering that there are few known *Cr* genes and the low frequencies of germplasm with QDR to WPBR in some five-needle pine species, there is still a need to explore how to promote resistance while maintaining high genetic diversity in populations of five-needle pines (Jenkins et al. 2022; Schoettle et al. 2022). In some western white pine plantings with MGR seedling stock, disease incidence is 100%, with very high mortality. In contrast, the disease incidence of QDR families has been lower and more durable (Sniezko et al. 2020). In natural outbreaks of forest diseases, the frequency of resistance will increase, as they will be the only survivors. However, natural populations will crash or potentially be extirpated if they contain few/no resistant genotypes (Schoettle et al. 2022). In most five-needle pine species, supplementing stands with rust-resistant genotypes will be needed to raise the level and frequency of QDR for population sustainability. Breeding programs to develop resistant planting stock are already underway for the commercial species of western

white pine and sugar pine, as well as for whitebark pine, a keystone high elevation species (Sniezko et al. 2024). Using seeds from known resistant field trees to enrich seedling stock for the immediate ecological restoration needs of the other five-needle pine species will have to suffice until breeding programs can be developed (Sniezko et al. 2024). To face the threat posed by new, more virulent isolates, future resistance selection of limber pine and other five-needle pines may need to increase their focus on QDR, which is already a component in applied resistance programs (Schoettle et al. 2022; Sniezko and Liu 2022, 2023; Sniezko et al. 2020, 2024). Although *vcr1* and *vcr2* have no obvious impacts on QDR in sugar pine, western white pine, and whitebark pine (Sniezko et al. 2020, 2024), we still need to determine if any newly emerged virulent race (such as *vcr4*) has an effect on the durability of QDR in other five-needle pines by inoculation of their QDR genotypes with virulent races of *C. ribicola*. This is important especially for species (such as whitebark pine) that to date have had no MGR documented. The presence of MGR and QDR and level of QDR vary widely by species (Sniezko and Liu 2022). Before novel *R* genes and quantitative trait loci for durable resistance to WPBR are identified and made available, a more practical and operationally feasible interim solution is to maintain high genetic diversity of host populations by mixing MGR with other genotypes in plantation or restoration areas where high infection and mortality rates are predicted to minimize the selection on rust genotypes and decrease the risk of an outbreak of virulent races. This strategy has been routinely used in Oregon and Washington for western white pine and sugar pine for several decades and recommended in the Southern Rockies for limber pine (Schoettle et al. 2014, 2019a, b). Therefore, the genetic diversity of both plants and pathogens needs to be carefully assessed to benefit sustainable management of plant−microbe interactions. The molecular mechanisms underlying the virulence of pathogens and resistance of their hosts are complex, and related knowledge is still limited in the WPBR pathosystems. Current restoration is mainly based on resistance phenotypes we can identify and propagate, with incorporation of MGR in some western white pine and sugar pine programs. Confirmed emergence of *vcr4* highlights the need to focus on QDR more intensely for limber pine (Liu et al. 2024). Fortunately, resistance breeding programs for western white pine, sugar pine, and whitebark pine, some underway for over 50 years, have emphasized the use of QDR (Sniezko and Liu 2022; Sniezko et al. 2020, 2024).

Acknowledgments

We thank colleagues from the CFS-PFC technical support team for plant growth and rust inoculation, colleagues at Waterton Lakes National Park for collection of tissue samples of trees and rust spores, and colleagues at the DGRC for sample collection and phenotype assessments.

Literature Cited

Alberta Environment and Parks. 2022. Alberta Whitebark Pine and Limber Pine Recovery Plan. Alberta Species at Risk Recovery Plan No. 44, Edmonton, Alberta. https://open.alberta.ca/publications/alberta-whitebark-pine-and-limber-pine-recovery-plan

Balesdent, M.-H., Fudal, I., Ollivier, B., Bally, P., Grandaubert, J., Eber, F., Chèvre, A.-M., Leflon, M., and Rouxel, T. 2013. The dispensable chromosome of *Leptosphaeria maculans* shelters an effector gene conferring avirulence towards *Brassica rapa*. New Phytol. 198:887-898.

Barrett, L. G., Kniskern, J. M., Bodenhausen, N., Zhang, W., and Bergelson, J. 2009. Continua of specificity and virulence in plant host–pathogen interactions: Causes and consequences. New Phytol. 183:513-529.

Bolger, A. M., Lohse, M., and Usadel, B. 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30:2114-2120.

Bradbury, P. J., Zhang, Z., Kroon, D. E., Casstevens, T. M., Ramdoss, Y., and Buckler, E. S. 2007. TASSEL: Software for association mapping of complex traits in diverse samples. Bioinformatics 23:2633-2635.

Burdon, J. J., and Thrall, P. H. 2008. Pathogen evolution across the agroecological interface: Implications for disease management. Evol. Appl. 1: 57-65.

Cleaver, C. M., Burns, K. S., and Schoettle, A. W. 2017. Limber Pine and White Pine Blister Rust Monitoring and Assessment Guide for Rocky Mountain National Park. Final Report prepared by the Rocky Mountain Research Station for Rocky Mountain National Park. Inter-Agency Agreement 15-IA-11221633-157. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fort Collins, CO. https://www.fs.usda.gov/treesearch/pubs/56244

COSEWIC. 2014. COSEWIC Assessment and Status Report on the Limber Pine *Pinus flexilis* in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa, Canada.

Degrave, A., Wagner, M., George, P., Coudard, L., Pinochet, X., Ermel, M., Gay, E. J., Fudal, I., Moreno-Rico, O., Rouxel, T., and Balesdent, M.-H. 2021. A new avirulence gene of *Leptosphaeria maculans*, *AvrLm14*, identifies a resistance source in American broccoli (*Brassica oleracea*) genotypes. Mol. Plant Pathol. 22:1599-1612.

Drake, J. W., Charlesworth, B., Charlesworth, D., and Crow, J. K. 1998. Rates of spontaneous mutation. Genetics 148:1667-1686.

Figueroa, M., Dodds, P. D., and Henningsen, E. C. 2020. Evolution of virulence in rust fungi—Multiple solutions to one problem. Curr. Opin. Plant Biol. 56:20-27.

Garrison, E., and Marth, G. 2012. Haplotype-based variant detection from short-read sequencing. arXiv preprint arXiv:1207.3907.

Geils, B. W., Hummer, K. E., and Hunt, R. S. 2010. White pines, *Ribes*, and blister rust: A review and synthesis. For. Pathol. 40:147-185.

Ghelardini, L., Pepori, A. L., Luchi, N., Capretti, P., and Santini, A. 2016. Drivers of emerging fungal diseases of forest trees. For. Ecol. Manag. 381: 235-246.

Gitzendanner, M. A., Dupper, G. E., White, E. E., Foord, B. M., Hodgskiss, P. D., and Kinloch, B. B., Jr. 1996. Genetics of *Cronartium ribicola*. III. Mating system. Can. J. Bot. 74:1852-1859.

Gou, M., Balint-Kurti, P., Xu, M., and Yang, Q. 2023. Quantitative disease resistance: Multifaceted players in plant defense. J. Integr. Plant Biol. 65: 594-610.

Jenkins, M. B., Schoettle, A. W., Wright, J. W., Anderson, K. A., Fortier, J., Hoang, L., Incashola, T., Jr., Keane, R. E., Krakowski, J., LaFleur, D. M., Mellmann-Brown, S., Meyer, E. D., Pete, S., Renwick, K., and Sissons, R. A. 2022. Restoring a forest keystone species: A plan for the restoration of whitebark pine (*Pinus albicaulis* Engelm.) in the crown of the continent ecosystem. For. Ecol. Manag. 522:120282.

Johnson, J. S., and Sniezko, R. A. 2021. Quantitative disease resistance to white pine blister rust at southwestern white pine's (*Pinus strobiformis*) northern range. Front. For. Glob. Change 4:594-610.

Jones, J. D. G., and Dangl, J. L. 2006. The plant immune system. Nature 444: 323-329.

Karisto, P., Hund, A., Yu, K., Anderegg, J., Walter, A., Mascher, F., McDonald, B. A., and Mikaberidze, A. 2018. Ranking quantitative resistance to Septoria tritici blotch in elite wheat cultivars using automated image analysis. Phytopathology 108:568-581.

Kinloch, B. B., Jr. 1992. Distribution and frequency of a gene for resistance to white pine blister rust in natural populations of sugar pine. Can. J. Bot. 70:1319-1323.

Kinloch, B. B., Jr. 2003. White pine blister rust in North America: Past and prognosis. Phytopathology 93:1044-1047.

Kinloch, B. B., and Comstock, M. 1981. Race of *Cronartium ribicola* virulent to major gene resistance in sugar pine. Plant Dis. 65:604-605.

Kinloch, B. B., Jr., and Dupper, G. E. 2002. Genetic specificity in the white pine−blister rust pathosystem. Phytopathology 92:278-280.

Kinloch, B. B., Jr., Sniezko, R. A., Barnes, G. D., and Greathouse, T. E. 1999. A major gene for resistance to white pine blister rust in western white pine from the western Cascade Range. Phytopathology 89:861-867.

Kinloch, B. B., Jr., Sniezko, R. A., and Dupper, G. E. 2003. Origin and distribution of Cr2, a gene for resistance to white pine blister rust in natural populations of western white pine. Phytopathology 93:691-694.

Kinloch, B. B., Jr., Sniezko, R. A., and Dupper, G. E. 2004. Virulence gene distribution and dynamics of the white pine blister rust pathogen in western North America. Phytopathology 94:751-758.

Koester, H., Savin, D. P., Buss, M., and Sniezko, R. A. 2018. White pine blister rust hazard rating for 265 sites in southern Oregon, USA. Pages 173-180 in: Proceedings of the IUFRO joint conference: Genetics of five-needle pines, rusts of forest trees, and strobusphere; Proc. RMRS-P-76. A. W. Schoettle, R. A. Sniezko, and J. T. Kliejunas, eds. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fort Collins, CO. https://www.fs.usda.gov/treesearch/pubs/56733

Lannou, C. 2012. Variation and selection of quantitative traits in plant pathogens. Annu. Rev. Phytopathol. 50:319-338.

Liu, J.-J., Johnson, J. S., and Sniezko, R. A. 2022a. Genomic advances in research on genetic resistance to white pine blister rust in North America white pines. Pages 163-191 in: The Pine Genomes. Compendium of Plant Genomes. A. R. De La Torre, ed. Springer, Cham, Switzerland.

Liu, J.-J., Schoettle, A. W., Sniezko, R. A., Sturrock, R. N., Zamany, A., Williams, H., Ha, A., Chan, D., Danchok, B., Savin, D. P., and Kegley, A. 2016. Genetic mapping of *Pinus flexilis* major gene (*Cr4*) for resistance to white pine blister rust using transcriptome-based SNP genotyping. BMC Genomics 17:753.

Liu, J.-J., Schoettle, A. W., Sniezko, R. A., Waring, K. M., Williams, H., Zamany, A., Johnson, J. S., and Kegley, A. 2022b. Comparative association mapping reveals conservation of major gene resistance to white pine blister rust in southwestern white pine (*Pinus strobiformis*) and limber pine (*P. flexilis*). Phytopathology 112:1093-1102.

Liu, J.-J., Schoettle, A. W., Sniezko, R. A., Yao, F., Zamany, A., Williams, H., and Rancourt, B. 2019. Limber pine (*Pinus flexilis* James) genetic map constructed by exome-seq provides insight into evolution of disease resistance and a genomic resource for genomics-based breeding. Plant J. 98: 745-758.

Liu, J.-J., Sniezko, R. A., Houston, S., Krakowski, J., Alger, G., Benowicz, A., Sissons, R., Zamany, A., Williams, H., Kegley, A., and Rancourt, B. 2024. Genome-wide association study (GWAS) reveals polygenic architecture for limber pine quantitative disease resistance to white pine blister rust. Phytopathology 114:1626-1636.

Liu, J.-J., Sniezko, R. A., Sissons, R., Krakowski, J., Alger, G., Schoettle, A. W., Williams, H., Zamany, A., Zitomer, R. A., and Kegley, A. 2020. Association mapping and development of marker-assisted selection tools for the resistance to white pine blister rust in the Alberta limber pine populations. Front. Plant Sci. 11:557672.

Liu, J.-J., Sniezko, R. A., Zamany, A., Williams, H., Omendja, K., Kegley, A., and Savin, D. P. 2021. Comparative transcriptomics and RNA-seq-based bulked segregant analysis (BSR-Seq) reveals genomic basis underlying *Cronartium ribicola vcr2* virulence. Front. Microbiol. 12: 602812.

Louet, C., Saubin, M., Andrieux, A., Persoons, A., Gorse, M., Pétrowski, J., Fabre, B., De Mita, S., Duplessis, S., Frey, P., and Halkett, F. 2023. A point mutation and large deletion at the candidate avirulence locus *AvrMlp7* in the poplar rust fungus correlate with poplar *RMlp7* resistance breakdown. Mol. Ecol. 32:2472-2483.

Plissonneau, C., Benevenuto, J., Mohd-Assaad, N., Fouche, S., Hartmann, F. E., and Croll, D. 2017. Using population and comparative genomics to understand the genetic basis of effector-driven fungal pathogen evolution. Front. Plant Sci. 8:119.

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A. R., Bender, D., Maller, J., Sklar, P., de Bakker, P. I. W., Daly, M. J., and Sham, P. C. 2007. PLINK: A tool set for whole-genome association and population-based linkage analyses. Am. J. Hum. Genet. 81:559-575.

Sacristán, S., and García-Arenal, F. 2008. The evolution of virulence and pathogenicity in plant pathogen populations. Mol. Plant Pathol. 9:369-384.

Sacristán, S., Goss, E. M., and Eves-van den Akker, S. 2021. How do pathogens evolve novel virulence activities? Mol. Plant-Microbe Interact. 34: 576-586.

Schoettle, A. W., Burns, K. S., Cleaver, C. M., and Connor, J. J. 2019a. Proactive Limber Pine Conservation Strategy for the Greater Rocky Mountain National Park Area. General Technical Report RMRS-GTR-379. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fort Collins, CO. https://www.fs.usda.gov/treesearch/pubs/57621

Schoettle, A. W., Burns, K. S., McKinney, S. T., Krakowski, J., Waring, K. M., Tomback, D. F., and Davenport, M. 2022. Integrating forest health conditions and species adaptive capacities to infer future trajectories of the high elevation five-needle white pines. For. Ecol. Manag. 521:120389.

Schoettle, A. W., Jacobi, W. R., Waring, K. M., and Burns, K. S. 2019b. Regeneration for resilience framework to support regeneration decisions for species with populations at risk of extirpation by white pine blister rust. New For. 50:89-114.

Schoettle, A. W., Sniezko, R. A., Kegley, A., and Burns, K. S. 2014. White pine blister rust resistance in limber pine: Evidence for a major gene. Phytopathology 104:163-173.

Singleton, R., and Oblinger, B. W. 2017. Prioritizing young western white pine stands for blister rust pruning on the Warm Springs Reservation. J. For. 115:385-392.

Sniezko, R. A., Danchok, R., Savin, D. P., Liu, J.-J., and Kegley, A. 2016. Genetic resistance to white pine blister rust in limber pine (*Pinus flexilis*): Major gene resistance in a northern population. Can. J. For. Res. 46:1173-1178.

Sniezko, R. A., Johnson, J. S., Kegley, A., and Danchok, R. 2024. Disease resistance in whitebark pine and potential for restoration of a threatened species. Plants People Planet 6:341-361.

Sniezko, R. A., Johnson, J. S., and Savin, D. P. 2020. Assessing the durability, stability, and usability of genetic resistance to a non-native fungal pathogen in two pine species. Plants People Planet 2:57-68.

Sniezko, R. A., Krakowski, J., Sissons, R., Alger, G., Liu, J.-J., Lewien, M., Danchok, R., Zitomer, R., and Kegley, A. 2023. Finding and using genetic resistance to white pine blister rust in Alberta populations of limber pine (*Pinus flexilis*). Page 142 in: Proceedings of the IUFRO 2019 Joint Conference: Genetics of Five-Needle Pines and Rusts of Forest Trees. J. Krakowski, W. Strong, and R. A. Sniezko, compilers. Victoria, B.C. Technical Report 142.

Sniezko, R. A., and Liu, J.-J. 2022. Genetic resistance to white pine blister rust, restoration options, and potential use of biotechnology. For. Ecol. Manag. 520:120168.

Sniezko, R. A., and Liu, J.-J. 2023. Prospects for developing durable resistance in populations of forest trees. New For. 54:751-767.

Sniezko, R. A., Mahalovich, M. F., Schoettle, A. W., and Vogler, D. R. 2011. Past and current investigations of the genetic resistance to *Cronartium ribicola* in high-elevation five-needle pines. Pages 246-264 in: The Future of High-Elevation, Five-Needle White Pines in Western North America: Proceedings of the High Five Symposium. Proceedings RMRS-P-63. R. E. Keane, D. F. Tomback, M. P. Murray, and C. M. Smith, eds. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fort Collins, CO. https://www.fs.usda.gov/rm/pubs/rmrs_p063/rmrs_p063_246_264.pdf

Stam, R., and McDonald, B. A. 2018. When resistance gene pyramids are not durable—the role of pathogen diversity. Mol. Plant Pathol. 19:521-524.

Tack, A. J. M., Thrall, P. H., Barrett, L. G., Burdon, J. J., and Laine, A. L. 2012. Variation in infectivity and aggressiveness in space and time in wild host–pathogen systems: Causes and consequences. J. Evol. Biol. 25: 1918-1936.

Yu, J., Pressoir, G., Briggs, W. H., Bi, I. V., Yamasaki, M., Doebley, J. F., McMullen, M. D., Gaut, B. S., Nielsen, D. M., Holland, J. B., Kresovich, S., and Buckler, E. S. 2006. A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. Nat. Genet. 38: 203-208.