Association of a Novel *Pinus monticola* Chitinase Gene (*PmCh4B*) with Quantitative Resistance to *Cronartium ribicola*

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ABSTRACT

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Multiple families of pathogenesis-related (PR) proteins are believed to contribute to plant quantitative resistance to various pathogens. Along with other host PR proteins, PR3 chitinase is one protein component participating in genetic resistance of western white pine (*Pinus monticola*) to the white pine blister rust (WPBR) pathogen (*Cronartium ribicola*). In the present study, we characterized a novel *P. monticola* class IV chitinase gene (*PmCh4B*) and further analyzed its nucleotide variations in the open-pollinated seed families of diverse geographical distribution and variable levels of quantitative resistance to *C. ribicola*

infection. *PmCh4B* showed high haplotype diversity ($Hd = 0.94$) and nucleotide diversity ($\pi = 0.00965$), similar to those of other conifer genes related to environmental stresses. A low level of intragenic linkage disequilibrium (LD) (but most of the levels with statistical significance) was found within a distance of ≈ 800 bp. Based on *PmCh4B* haplotype frequency, moderate to high levels of population structure were observed among *P. monticola* seed families currently used in breeding programs for WPBR resistance (average $F_{ST} = 0.163$, $P < 0.001$). Association analysis revealed that allelic variants and multiple single-nucleotide polymorphisms of *PmCh4B* were significantly associated with quantitative levels of *P. monticola* resistance against *C. ribicola*. This work represents the first association study for quantitative resistance in western white pine pathosystem and provides a potential for marker-assisted selection in white pine breeding.

Forest conifer trees generally contain at most intermediate levels of both genetic and phenotypic variation due to long life spans with large natural out-crossing populations (35). Most economically and ecologically important characteristics of forest trees are quantitative traits with continuous variations resulting from interactions of genetic and environmental factors. Linkage disequilibrium (LD)-based association mapping does not require specially designed crosses between inbred parents and can be applied to collections of genotypes with unknown genetic relationships. Therefore, it is very useful as an alternative to traditional linkage-based methods for the identification of quantitative trait loci (QTL) by associated DNA markers in plants. By resequencing positional or functional candidate genes and performing sequence analysis of single-nucleotide polymorphism (SNP) data, the candidate gene-based association approach is an effective strategy to determine genetic effects of alleles (haplotypes) on complex phenotypes (8,29). To reveal the genetic variation of potential candidate genes is a key step for marker-trait associations, which would lead to marker-assisted selection applicable in genomics-based breeding and plant health diagnostics (29). This is particularly useful in forest trees because pedigree-based mapping populations cannot be easily generated (38).

The ultimate objective of candidate gene-based association mapping is to identify functional genetic variants that are responsible for phenotypic variation. How to obtain large numbers

of genotypes of functional genes from individuals of many families or large populations is still a challenge in forest conifers. SNPs provide the richest gene variation, with great potential for the detection of genotype-phenotype associations (33). Thus far, genetic variations at the nucleotide level have only been investigated for a few conifer genes (12,13,16,42), with special interest in association study of wood property traits (14).

Western white pine (*Pinus monticola*, Dougl. Ex D. Don) is a valuable, fast-growing tree of interest in the western United States and British Columbia (BC), Canada for forest plantations and sustainable forest ecosystems. This conifer also exhibits excellent genetic resistance to root rot caused by *Onnia tomentosus*, *Armillaria* spp., or *Phellinus* spp., with wide distribution in western North America. However, since it was introduced in the early 1900s, the invasive pathogen white pine blister rust (WPBR, *Cronartium ribicola*) has caused severe reductions in populations of *P. monticola* and other native five-needle pines in the United States and Canada. Breeding programs in the past few decades have found that *P. monticola* possesses both qualitative and quantitative resistance mechanisms against *C. ribicola* (19). Knowledge about molecular mechanisms underlying genetic resistance to WPBR is very limited in western white pine and related conifers (16,26). SNP characterization has provided potential functional markers to tag candidate genes related to disease resistance in angiosperms. Candidate gene markers based on resistance- or defense-related genes provide useful tools for molecular characterization of host resistance in crops and model plants (24,34). Therefore, investigation of genetic variations of host defense-related genes would provide necessary information for identifying QTL involved in complex traits of partial resistance in conifer pathosystems.

In the present study, we selected chitinase genes as candidate genes contributing to partial resistance to *C. ribicola* in western

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*The e-Xtra logo stands for "electronic extra" and indicates that Figure 3 appears in color online.

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white pine because such plant enzymes are likely involved in the host defense response by upregulation under attack by various pathogens and pests (25). Plant chitinases are divided into several classes (I to VII) because they show a wide diversity in protein structures, enzymatic properties, and subcellular localizations (31). Because their gene expression is induced following infection, plant chitinases are also considered to be pathogenesis-related (PR) proteins and classified into four PR families (PR3, PR4, PR8, and PR11) (31). The important role that chitinases play in the plant-defense system has been evidenced by in vitro antifungal activity of purified plant chitinases as well as enhanced resistance against fungal infections in transgenic plants over-expressing related genes (31). One 26-kDa isozyme of the *P. monticola* class IV chitinase (*PmCh4*) family was discovered to exclusively accumulate in partially resistant (slow canker growth [SCG]) seedlings but not in susceptible seedlings following fungal challenge (25), suggesting that *PmCh4* is one of the defense components against *C. ribicola* infection.

The objective of this work was to characterize nucleotide variations of the *PmCh4* gene in western white pine. Nucleotide polymorphisms of one novel *P. monticola* class IV chitinase gene (*PmCh4B*) and its SNP association with quantitative resistance are described here for the first time for this conifer species. Experimental exploration of marker-trait association will shed new light on fundamental research questions important for planning future studies on genetic variation at the genome scale in larger numbers of seed families of this species from throughout western North America.

MATERIALS AND METHODS

Plant materials. Twenty open-pollinated seed families of *P. monticola* collected in breeding programs with a wide geographical distribution were used in the present study. Each seed family represented a wild collection because the pollen cloud was from local geographic areas to the parent (Fig. 1). Among four BC-originated seed families (numbers 1 to 4), BL3110 was tested with quantitative stem resistance phenotypes described as SCG (15) while Wh3115 was shown to be a susceptible family (25). The other two BC seed families (WK3671 and WK3674) were selected in breeding program but the resistance levels of their progenies were not available. Sixteen U.S.-originated seed families were collected from the Colville (number 5), Mt. Baker-Snoqualmie (numbers 6 to 10), Gifford Pinchot (numbers 11 to 17) (Washington), and the Mt. Hood National Forests (Oregon) (numbers 18 to 20). Fifteen of them showed variable phenotypes of quantitative stem resistance described as bark reaction (BR) (36,37). The family BS81-003 (number 7) displayed phenotypes termed as resistance mechanism “X” (36) and its progeny showed resistance levels ranging from complete susceptibility (rust dead) to complete stem resistance (stem symptom-free), including BR, delayed stem infection, fewer stem infections, and longer time to mortality compared with susceptible controls. These 20 seed families represent a sample of genetic resources with partial resistance against white pine blister rust in North America. In total, 290 trees from the 20 seed families were sampled and analyzed in this study.

Fig. 1. Sites in western North America where 20 western white pine seed families were collected. Seed families corresponding to the numbers (1 to 7) are shown in Table 2.

White pine seedling inoculation and phenotypic measurement of quantitative resistance. Seed of 16 U.S.-originated families (numbers 5 to 20) were sowed in 2003 and the seedlings were inoculated artificially by basidiospores of *C. ribicola* in fall 2004 at the United States Department of Agriculture Forest Service, Dorena Genetic Resource Center (Cottage Grove, OR). Disease symptoms were assessed on a yearly basis for the next 5 years using a series of parameters, including presence or absence of aecia, canker damage and severity, and number of stem symptoms by type, such as normal canker, partial BR (with incomplete inhibition of fungal growth), complete BR (without fungal activity in bark), or “clean” or stem-symptom free (36,37). Partial resistance levels (Table 1) were quantitatively ranked for resistance phenotypes from level 0 (rust dead) to level 10 (stem symptom-free) for each seedling of 18 seed families (numbers 1 to 2 and numbers 5 to 20).

DNA extraction, polymerase chain reaction cloning, and sequencing. Needle samples were collected individually from each seedling. Genomic DNA was extracted using a Qiagen DNeasy kit (Qiagen Inc., Mississauga, ON, Canada). Of 20 seed families, 7 of them (numbers 1 to 7) with a total of 115 trees (Table 2) were used for SNP discovery in western white pine chitinase genes. Genomic DNA fragments were amplified by polymerase chain reaction (PCR) using a pair of primers CH5 (5'-CAG AGT TTC TTC AAT GG-3') and CH3 (5'-ATT CTT CAT CCA GAA CCA-3'), which targeted two conserved motifs (QSFFNG and WFWMKN) of the plant class IV chitinase family. PCR was performed using a PCR Master Mix Kit (Qiagen Inc.) in a final volume of 25 µl, containing 5 to 10 ng of genomic DNA on a Perkin-Elmer Thermocycler (Perkin-Elmer Applied Biosystems, Foster City, CA). Thermal cycling conditions were set up as an initial template denaturation step at 94°C for 3 min; followed by 35 cycles of denaturation at 94°C for 40 s, primer annealing at 45°C for 1 min, and primer extension at 72°C for 1.5 min; with a final 10-min extension at 72°C.

The PCR fragments were resolved by 1% agarose gel electrophoresis. The DNA bands with expected sizes (0.7 to 0.9 kb) were

excised and purified using a MinElute gel extraction kit (Qiagen Inc.) and cloned into the pGEM-T easy vector (Promega Corp., Madison, WI). Considering potential PCR error from Taq DNA polymerase, five recombinant clones were selected averagely from each PCR fragment for DNA sequencing. Genomic DNA sequence was used to search for the cDNA sequence of the gene in an expressed sequence tag (EST) data set randomly sequenced from western white pine cDNA library (*unpublished data*).

Nucleotide sequences of genomic DNA inserts were determined on both strands on an ABI310 DNA sequencer (Applied Biosystems, Foster City, CA) using a Thermo-cycle sequence kit (Amersham, Baie d'Urfe, QC, Canada). Nucleotide sequence data were compiled and analyzed using Sequencher software (Gene Codes Co., Ann Arbor, MI). Gene identities were determined by ExPASy Proteomics tools (Swiss Institute of Bioinformatics) and BLAST programs (the National Center for Biotechnology Information) (1).

Genotyping of *PmCh4B* alleles by genomic PCR. To verify marker-trait associations resulting from SNP data analysis, 265 trees from 18 seed families (numbers 1 to 2 and numbers 5 to 20) were used for direct PCR genotyping of the *PmCh4B* alleles. Phenotypic data was available for each of the 265 seedlings for levels of quantitative resistance to *C. ribicola* using the above inoculation assessment criteria (Table 1). Based on nucleotide variations of the *PmCh4B* genomic sequences, two *PmCh4B*-specific primers were designed for genotyping alleles with an indel of 74 bp in the second intron: forward primer Ch4Bf (5'-GTC AAA CTA CTG TCA GAG TTC GA-3') and reverse primer Ch4Br (5'-CCA CCT TCT CTG GAT TAT TCA AC-3'). PCR was performed as described above with some exceptions: an initial template denaturation step at 94°C for 3 min; followed by 35 cycles of denaturation at 94°C for 40 s, primer annealing at 62°C for 1 min, and primer extension at 72°C for 1 min; with a final 10-min extension at 72°C. The PCR fragments were resolved by 1.5% agarose gel electrophoresis. According to PCR fragment sizes, the *PmCh4B* genotype of each seedling was determined with one of three expected genotypes: L/L (single band, 318 bp), L/S (double bands, 318 and 244 bp), and S/S (single band, 244 bp).

Genetic data analysis. The program DNASP5.0 (23) was used for genetic diversity analysis. To exclude possible PCR errors, SNPs and indels at a frequency of >1 or 5% were considered for further analysis. Conserved *PmCh4B* haplotypes with a frequency of >5% were identified in populations and used for further analysis. The number of polymorphic sites (*S*) and haplotype diversity (*H_d*) were calculated for the gene locus. Nucleotide diversity was estimated as Watterson's estimator of θ (θ_w) from *S* (43) and as π , the average number of nucleotide differences per site among sequences in a sample (30). A statistical test of the standard neutral evolution model was performed using both Tajima's *D* statistic (40) based on differences between π and θ_w and *F_s* statistic (11) based on the haplotype frequency distribution.

LD values (*r*²) between SNP sites of the same gene were calculated using the trait analysis by association, evolution and linkage (TASSEL) software package, version 2.01 (3), and the DNASP 5.0 program. For multiple SNPs, a weighted average of *r*²

TABLE 1. Phenotypic ranking of stem partial resistance

Res/Sus ^a	Ranking ^b	Phenotype ^c	Tree (<i>n</i>) ^d
Sus	0	Rust dead	33
Sus	1	NC + aecia	47
Sus	3	NC only; no aecia	10
Sus	4	NC and PBR; no aecia	17
Sus	5	NC, BR, and PBR; no aecia	5
Res	6	PBR only; no aecia	71
Res	7	PBR and BR; no aecia	14
Res	8	BR only; no aecia	20
Res	10	SS-free (clean)	48

^a Res = resistant and Sus = susceptible.

^b Resistance levels were based on the assessment in the third year postinoculation.

^c BR, bark reaction; NC, normal canker growth; PBR, partial bark reaction; SS, stem symptom.

^d In total, 265 trees were collected from 18 seed families (numbers 1 to 2 and numbers 5 to 20).

TABLE 2. *Pinus monticola* open-pollinated seed families and trees for single-nucleotide polymorphism discovery

Seed family ID	Trees (<i>n</i>)	Chitinase clones (<i>n</i>)	Phenotype ^a	Geographical origin	Latitude, longitude	Altitude (feet)
1. BL3110	6	31	SCG	Buttle Lake, BC	49.71, -125.59	750
2. Wh3115	9	46	Sus	Whistler, BC	50.13, -122.94	1,220
3. WK3671	13	56	nil	West Kootenay, BC	50.06, -118.01	1,190
4. WK3674	12	48	nil	West Kootenay, BC	50.02, -117.05	4,500
5. Cv05-052	16	77	BR	Colville, WA	48.64, -117.63	4,000
6. BS82-413	29	113	BR	Mt. Baker-Snoqualmie, WA	48.68, -121.75	1,700
7. BS81-003	30	118	“X”	Mt. Baker-Snoqualmie, WA	48.73, -121.66	800
Total	115	483	...	...	...	...

^a SCG = slow canker growth, Sus = susceptible, nil = none, and BR = bark reaction.

was calculated between each SNP pair (10). P values were determined by permutations based on calculations for the proportion of permuted gamete distribution that were less probable than the observed gamete distribution under the null hypothesis of independence, and the Bonferroni correction was performed to avoid spurious rejections of the null hypothesis in multiple tests (44). Genetic structures of open-pollinated seed families were assessed using an analysis of molecular variance (AMOVA) by calculating F_{ST} (45) with haplotype frequency and their significance was tested to determine the probability of a random F_{ST} with a value the same as or different from the observed one using a permutation-based method using the ARLEQUIN 3.11 program (9). The tests of associations between molecular polymorphism (individual SNP and indel sites, and gene haplotypes) and quantitative resistance phenotype were computed using a mixed linear model (MLM) ($y = \text{marker} + Q + K + e$), with accounting the percentages of admixture of each accession (Q matrix) as cofactors of seed family structure in the software TASSEL (3). With 19 pairs of simple-sequence repeat (SSR) primers developed in eastern white pine (7), a set of SSR markers was developed and each allele of the SSR markers was scored as present or absent (score 1 or 0) in the individual tree. SSR marker data were used for the calculation of both K and Q matrixes as population structure following the user manual of TASSEL (3). Principal components were used to capture major population subdivisions as Q matrix. Kinship was used to capture more subtle relationships as K matrix.

RESULTS

***PmCh4B* characterization.** *PmCh4* genomic DNA fragments were amplified and cloned by PCR using a pair of primers targeting conserved motifs of class IV chitinases. One DNA fragment with the expected size of ≈ 0.8 kb was revealed in each seedling. We obtained sequence data from >500 recombinant clones, and 483 of them were identified as class IV chitinase gene sequences by BLAST search. The majority of chitinase clones (349 of 483 clones) showed almost identical nucleotide sequences and represented a novel gene (designated as *PmCh4B*) for the western white pine class IV chitinase family. Based on the genomic DNA sequence, the full-length cDNA sequence of *PmCh4B* was found in an EST data set randomly sequenced from a *P. monticola* cDNA library. *PmCh4B* displayed only 68% nucleotide identity and 80% amino acid identity to *PmCh4A* reported previously in western white pine (25). A BLASTP search in GenBank found that the putative *PmCh4B* protein also shared high identity with those class IV chitinases from other conifers; 90% with PsCh4 (ABK22417) in *Picea sitchensis*, 88% with Ch4-2 (ACP43363) from *Pseudotsuga menziesii*, and 86% with Chia4 (AAQ17048) from *Picea abies*. Compared with cDNA sequences, both *PmCh4A* and *PmCh4B* genes have the same genomic structure based on gene intron number, position, and phase. These data indicate that *PmCh4B* is a class IV chitinase gene from western white pine.

Nucleotide variations of the *PmCh4B* gene. Because three *PmCh4B* clones were averagely sequenced from each of 115 trees of seven open-pollinated families following PCR amplification

(Table 2), this gene was subjected to a further analysis of genomic variation. In total, ≈ 280 -kb *PmCh4B* genomic DNA fragments were sequenced in 115 trees. The total *PmCh4B* aligned region, including indels, covered partial exon1, intron1, exon2, intron2, and partial exon3 with a length of 756 to 839 bp, averaging 815 bp. The exons of *PmCh4B* had identical lengths with a total coding region of 420 bp but sizes of introns varied greatly, from 336 to 419 bp, because of the presence of indels in the intron regions (Table 3).

In total, 53 SNPs and 3 indels were identified in the *PmCh4B* gene with at least 1% minor frequency. *PmCh4B* showed high haplotype diversity ($H_d = 0.94$) (Table 3). For nucleotide diversities in all sequences, estimates were calculated for *PmCh4B* ($\pi = 0.00965$, $\theta_w = 0.01121$). There is a threefold greater variation in polymorphisms at silent sites than at nonsynonymous sites (Table 3).

Tests of both Tajima's D and Fu's F_s test statistics were performed to determine significant departures from the null hypotheses of neutral evolution. The negative values for Fu's F_s and significant deviations from zero (at least $P < 0.001$) indicate that *PmCh4B* exhibited a larger number of rare frequency haplotypes than expected (Table 3).

LD. In total, 2,213 pairwise LDs were estimated among pairs of SNP sites within 483 genomic sequences of the *Pinus monticola* chitinase (*PmCh4*) genes. A Fisher's exact test revealed that $\approx 90\%$ of them were not significant because some *PmCh4* sequences were available only from a limited number of seedlings. LD estimates (r^2) with significance were selected and plotted versus distance (bp) within the *PmCh4* genomic regions. LD decayed $\approx 40\%$ over a short distance across all sequences by an r^2 decrease of ≈ 0.5 to 0.3 within ≈ 700 bp (Fig. 2).

Fig. 2. Scatter plot of the squared correlation of allele frequency (r^2) versus distance in nucleotide base pairs between polymorphic sites of chitinase genes. In total, 483 genomic DNA sequences were analyzed in 115 western white pine trees of seven seed families. Decline in linkage disequilibrium is shown by a second-order polynomial best-fit curve ($y = 4E-07x^2 - 0.0005x + 0.5075$).

TABLE 3. Nucleotide polymorphisms and neutrality test of the *PmCh4B* gene

	SNP ^a	Indel ^b	π	θ_w ^c	H_d (Std)	Fu's F_s ^d	Tajima's D
Total region	53	3	9.65	11.21	0.94 (0.01)	-30.979***	-0.401
Nonsynonymous	21	0	4.05	10.23	...	...	...
Synonymous	32	3	13.96	11.96	...	...	...

^a Single-nucleotide polymorphism.

^b All indels were present in the introns.

^c Nucleotide diversity estimates (π and θ_w) are $\times 10^3$.

^d Significant neutral test at *** $P < 0.001$.

PmCh4B was revealed to contain 19 common SNP sites and two indel sites with at least 5% minor allele frequency. The *PmCh4B* intragenic average r^2 over all 21 pairwise estimates was 0.13. Out of 231 tests, 74.9% of them were significant ($P < 0.01$) based on a weighted average of r^2 value (Fig. 3). The average r^2 over 19 SNP pairwise estimates was 0.18, and 56.6% of the 171 tests were highly significant with the Bonferroni correction ($P < 0.001$).

Differentiation of *PmCh4B* haplotype among *P. monticola* seed families. The seed families showed considerable variation in phenotypic resistance to WPBR in breeding programs. F_{ST} index is a common pairwise measurement of population genetic differentiation and we used it to reveal genetic structure based on *PmCh4B* haplotype frequency. Six *PmCh4B* (*a* to *f*) haplotypes were identified with at least 5% frequency in the populations. An AMOVA demonstrated that there was a low but significant seed family subdivision within 115 trees of seven seed families (numbers 1 to 7) ($F_{ST} = 0.163$, $P < 0.001$), indicating that most of the genetic variation (83.7%) occurred within the seed families whereas only 16.3% of the total variance was attributable to diversity among seed families. There was little and a nonsignificant amount of genetic diversity (0.27%, $P = 0.348$) between coastal (numbers 1, 2, 6, and 7) and interior (numbers 3 to 5) subgroups. According to the F_{ST} estimates of a pairwise comparison of seven seed families (Table 4), genetic differentiation varied from 0.79% (between BL3110 and BS82-413) to 39.49% (between WK3671 and WK3674). These observed genetic differentiations were significant ($0.0912 < F_{ST} < 0.3949$, $P < 0.001$) among most seed

families, except pairwise comparisons of family BL3110 with three other families: Wh3115, WK3674, and BS82-413, respectively ($0.0079 < F_{ST} < 0.0767$, $0.07 < P < 0.37$).

Association of *PmCh4B* variants to quantitative resistance to *C. ribicola*. Quantitative resistance levels for each tree from 18 seed families were variable from rust dead to stem symptom-free (Table 1). *PmCh4B* SNP and haplotype data were collected from 115 trees of seven seed families (numbers 1 to 7) for association analysis. Of the 21 total SNPs and indels, 10 of them (including 8 SNPs and 2 indels) were significantly associated with quantitative resistance levels ($P < 0.05$) as analyzed using five seed families with available phenotypic data (Table 2). *PmCh4B* genetic variants could explain 5.58% (SNP A695G, $P < 0.05$) to 9.13% (Indel200 and four SNPs: T218C, C282A, C397T, and G412T; $P < 0.01$) of total phenotypic variation as related to partial resistance to *C. ribicola* in the white pine families (Table 5). Among 21 SNPs, only 1 SNP (G787C) resulted in amino acid change (Gly to Ala) but it made no significant contribution to phenotypic variation ($P = 0.15$).

Quantitative resistance levels for tree groups with each of six *PmCh4B* haplotypes (*a* to *f*) are shown in Figure 4A. Haplotypes *d* and *f* (with population frequencies of 8 and 7%, respectively) had high partial resistance levels, whereas haplotypes *a*, *b*, *c*, and *e* (with population frequencies of 18, 20, 11, and 7%, respectively) showed resistance levels in the low to medium ranges. On average, partial resistance levels of trees with *PmCh4B-d* and *PmCh4B-f* were significantly higher than those with *PmCh4B-a* and *PmCh4B-b* (Tukey's highly significant difference [HSD] test, $P < 0.05$). Trees with *PmCh4B-c* and *PmCh4B-e* exhibited medium levels of partial resistance, with no significant difference from trees of the four haplotypes (*a*, *b*, *d*, and *f*) (Fig. 4A).

To verify the significant contribution of *PmCh4B* variants to quantitative resistance, the indel613 was transferred into a gene-specific PCR marker because it spans an insertion/deletion of 74 bp. PCR genotyping revealed three expected genotypes: L/L (single band, 318 bp), L/S (double bands, 318 and 244 bp), and S/S (single band, 244 bp), with population frequencies of 76, 18, and 6%, respectively. Association of this *PmCh4B* PCR marker with quantitative resistance levels in a total of 265 trees from 18 seed families (numbers 1 to 2 and numbers 5 to 20) was highly significant ($R^2 = 0.051$, $P < 0.0001$) (Table 5). The mean partial resistance level of the tree group with genotype L/S was significantly higher than that with genotype L/L (Tukey's HSD test, $P < 0.05$). However, the S/S group showed no significant difference from either the L/L or L/S group (Fig. 4B).

DISCUSSION

The diversity pattern of nucleotide sequences varies widely among genes encoding defense- or resistance-related proteins for plants to protect themselves against attacks from pathogens and pests. Relatively high levels of nucleotide polymorphism have been observed in a few genes in *Pinus* spp. (4,12,35). Despite the fact that plant chitinase genes play an important role in host defense (31), thus far, genetic diversity of chitinase genes has only been investigated in two angiosperms (17,41). In this study,

Fig. 3. Plot of intralocus linkage disequilibrium (LD) between 21 polymorphic DNA sites in the *PmCh4B* region estimated using software package TASSEL. Graphic LD estimates (r^2) were presented in the upper right and P values were indicated in the lower left.

TABLE 4. F_{ST} statistic for *Pinus monticola* open-pollinated seed families based on *PmCh4B* sequences^a

	BL3110	Wh3115	WK3171	WK3174	Cv05-052	BS82-413	BS81-003
BL3110	...	...	...	...	...	...	...
Wh3115	0.06261	...	...	...	...	...	...
WK3671	0.33494***	0.14010*	...	...	...	...	...
WK3674	0.07667	0.13377**	0.39487***	...	...	...	...
Cv05-052	0.15558***	0.13435***	0.17890***	0.26217***	...	...	...
BS82-413	0.00793	0.12710***	0.26355***	0.15285***	0.14547***	...	...
BS81-003	0.09124***	0.15472***	0.23877***	0.22546***	0.13384***	0.09632***	...

^a Significant F_{ST} values: *, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively.

we found that *PmCh4B* had nucleotide diversity ($\pi = 0.00965$) (Table 3) in a range similar to that of *Arabidopsis* chitinase genes (*AtChiA* and *AtChiB*) (17) and other environmental stress-related genes from loblolly pine (12), Scots pine (42), and Douglas-fir (21). The nucleotide diversity estimates showed variability in conifer species, but not higher than reported in angiosperms (35).

High levels of haploid diversity ($Hd = 0.94$) of the *PmCh4B* gene in white pine seed families were comparable with most chitinase genes in *Zea* spp. (41). High haplotype diversity was also revealed at the *pall* locus in *P. sylvestris* (6) and other conifer loci (12,42). Similar to *PmCh4B*, other chitinase genes from *Arabidopsis* and *Zea* spp. also showed neutral selection as estimated by Tajima's *D* test (17,41). However, *PmCh4B* was found to have significantly negative Fu's F_S test values, indicating a greater number of rare haplotypes than expected. A potential selection sweep was detected at the drought-related *erd3* gene in *P. taeda* (12); genes for dehydrin1 and peroxidase in *P. densata* (27), cold-related *dhn4*, *ef*, *abaR*, and *gst1* genes in *P. sylvestris* (42); as well as a water-deficit induced *F3H1* gene in Douglas-fir (21); however, further evidence is required for a conclusion on the evolutionary selection of these conifer candidate genes related to environmental stresses (12). *P. monticola* seed families show a wide range of phenotypic variation in genetic resistance to WPBR (20). Our analysis of molecular data confirmed this diversity among seed families used in breeding programs by SNP characterization of the *PmCh4B* gene.

Pinus is considered to be one of the most genetically variable plant genera. Highly outcrossed species are expected to house heterozygosity levels relatively higher than self-pollinated species and demonstrate little among-population differentiation. *P. monticola* shows low levels of interpopulation variation but high intra-population variation based on analyses of morphological traits, chemicals and isozymes, and DNA markers of various types (amplified fragment length polymorphism [AFLP], random amplified polymorphic DNA [RAPD], and SSR) (2,18,28,39). Our study, based on *PmCh4B* haplotype frequency, also revealed a similar pattern for white pine population structure. *P. monticola* genetic structure has been affected by glaciation during the late Pleistocene (39) and the introduction of the exotic pathogen *C. ribicola* since early 1900s (18). Significant negative F_S in the *PmCh4B* gene is a potential indicator of selective pressures or historic expansion in *P. monticola* populations. As glaciers

retreated northward, *P. monticola* invaded and adapted in new environments in mountain regions of western North America from a few southern refugia. During this process, any adaptive selection would have led to western white pine populations in different geographical regions with differential genetic components. Heavy mortality in many natural populations by *C. ribicola* as well as the geographical topology of the sampling regions may have led to limited gene flow between the remaining white pine stands in the past 100 years. All of these factors contribute to genetic differentiation among those seed families grown in isolated geographical sites.

Fig. 4. Association of *PmCh4B* variants with partial resistance levels. **A and B.** Mean level of partial resistance was shown for major *PmCh4B* haplotypes (-a to -f), or genotypes (L/L, L/S, and S/S), respectively. Standard deviation (SD) was calculated based on the entire population of each tree group. Statistical difference is significant (Tukey's highly significant difference test, $P < 0.05$) between groups labeled with different letters (a and b).

TABLE 5. Association tests of variants of *PmCh4B* with quantitative levels of genetic resistance to *Cronartium ribicola*

Variation name ^a	Location	<i>P</i> marker ^b	df error	<i>R</i> ² marker
A84G	Exon1	0.0510	85	0.0462
A112G	Exon1	0.1134	86	0.0197
T180C	Exon1	0.9620	85	0.0001
T218C	Intron1	0.0023**	85	0.0913
A227G	Intron1	0.9003	85	0.0017
T247C	Intron1	0.0580	86	0.0281
G252T	Intron1	0.2233	85	0.0237
C282A	Intron1	0.0023**	85	0.0913
C397T	Intron1	0.0023**	85	0.0913
G412T	Intron1	0.0023**	85	0.0913
A428C	Intron1	0.0048**	85	0.0805
A467T	Exon2	0.0775	85	0.0399
A468C	Exon2	0.0775	85	0.0399
T573C	Intron2	0.0803	85	0.0391
C610T	Intron2	0.8730	85	0.0022
G691C	Intron2	0.0222*	85	0.0585
A695G	Intron2	0.0266*	85	0.0558
G787C	Exon3	0.1545	85	0.0294
C806G	Exon3	0.0072**	85	0.0748
Indel200	Intron1	0.0023**	85	0.0913
Indel613	Intron2	0.0163*	85	0.0631
L/S	Intron2	0.00008***	255	0.0513

^a Number in the variation name indicates the position. *PmCh4B* variations (19 single-nucleotide polymorphisms and two indels) were detected by DNA sequencing in seven families, and the alleles (L/S) were detected by polymerase chain reaction directly in 18 seed families.

^b Significant differences in bold numbers: *, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively.

The seed families used in the current study exhibited highly variable levels of quantitative stem resistance to *C. ribicola* based on their survival rates 4.5 years after inoculation; for example, 88.2% for BS81-003, 38.5% for Cv05-052, and 27.1% for BS82-413. At the molecular level, *P. monticola* showed high F_{ST} among seed families (average 0.163) as measured by *PmCh4B* haplotypes. This result is higher than those measured in western white pine Idaho populations by AFLP ($G_{ST} = 0.094$) (18) and BC populations by ISSR ($F_{ST} = 0.057$) (28). The level of structural differentiation revealed among conifer populations is affected greatly by the types of molecular markers (such as allozymes, RAPD, restricted fragment length polymorphism, or SNP) used in the investigation (22) and even by the candidate genes selected in SNP genotyping (32). A genetic structure differentiation was found at 14 isozyme loci in Mexican pine (*P. rzedowskii*) populations with an average F_{ST} of 0.175 (5) and at seven functional gene loci in *P. densata* populations with an average F_{ST} of 0.105 (27). Diversity values detected in this study by molecular data for *PmCh4B* indicated a large amount of genetic variation (83%) within each seed family. No *PmCh4B* haplotype was common among all tested seed families. Each seed family surveyed from *PmCh4B* SNPs had a specific combination of haplotypes. Inducible expression in response to *C. ribicola* infection, as well as the exclusive presence of a 26-kDa chitinase isozyme in surviving SCG seedlings of the BL3110 family as opposed to its absence in the completely susceptible family Wh3115 (25), suggests a defense role of the class IV chitinases in quantitative resistance against *C. ribicola*.

In recent years, association genetics has been extensively applied to the dissection of complex adaptive traits and to the discovery of the underlying candidate genes in humans and crops. Our association test revealed that about 50% of *PmCh4B* variants (SNPs and indels) significantly contributed to stem quantitative resistance in the seed families currently applied in the white pine breeding programs (Table 5). A similar result was reported in rice, where five candidate defense response genes (including chitinase and PR1 genes) accounted for 5.5 to 30% of the diseased leaf area variation and co-localized with resistance QTL identified by interval mapping (24).

LD structure as well as genetic correlation among polymorphic loci determines the dissecting resolution of the association study in sampling populations. Recombination and the time period required for recombination events to occur are the major elements affecting the LD unit pattern. Of 483 genomic sequences, LD decayed $\approx 40\%$ within a 700-bp distance of the western white pine chitinase genes (Fig. 2). LD also decayed rapidly in a few other conifers measured thus far; LD usually declines within ≈ 200 bp in Scots pine (42) and $\approx 2,000$ bp in loblolly pine (12), suggesting that candidate gene-based association mapping in these conifers would be especially powerful. In western white pine seed families used in *C. ribicola*-resistance breeding, a low average level of LD ($r^2 = 0.136$) was observed over an ≈ 700 -bp *PmCh4B* sequence. The LDs and LD decay of the *PmCh4* family revealed in western white pine populations may provide valuable information for candidate gene-based association mapping in a future work.

Candidate gene-based association analysis could reveal what alleles, haplotypes, or SNPs affect quantitative traits directly, without relying on knowing the LDs if genes are chosen as candidates based on their putative functions or other selection criteria. Because chitinase is well known for its function in degradation of fungal cell walls (31), in this study, members of the *PmCh4* family were selected as candidates to investigate their contribution to quantitative resistance to WPBR. SNPs and molecular markers derived from the *PmCh4B* gene will be useful for exploring their biological roles as defense-related genes in quantitative resistance to WPBR. Significant association of *PmCh4B* SNPs with quantitative resistance levels provides a potential to develop a diagnostic tool from candidate-based poly-

morphisms for making future selection of trees with polygenic resistance to *C. ribicola* to include in white pine seed orchards. Application of other and more genetic loci related to disease resistance could be more powerful and precise in the investigation of intraspecific genetic variation to reveal a solid association with resistance phenotypic variation. With the increasing availability of inexpensive DNA sequencing, a modern genomics approach for high-throughput SNP discovery through deep resequencing can identify the most important candidate genes that control complex trait adaptation in tree species (8,29). Such an approach in western white pine could theoretically greatly increase the efficiency of breeding programs for genetic resistance to WPBR.

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