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Journal:	<i>Genome</i>
Manuscript ID	gen-2020-0080.R1
Manuscript Type:	Article
Date Submitted by the Author:	17-Dec-2020
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Keyword:	Association analysis, In-vitro anti-fungal array, Quantitative disease resistance (QDR), White pine blister rust (WPBR), Pathogenesis-related protein family 10 (PR10)
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

In-vitro anti-fungal assay and association analysis reveal a role for the *Pinus monticola* PR10 gene (PmPR10-3.1) in quantitative disease resistance to white pine blister rust

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ABSTRACT

Pathogenesis-related (PR) proteins play important roles in plant defense response. However, functional investigation of PR10 genes is still limited and their physiological roles have not been conclusively characterized in biological processes of conifer trees. Here we identified multiple novel members in the western white pine (*Pinus monticola*) PmPR10 family by bioinformatic mining available transcriptomic data. Phylogenetic analysis of protein sequences revealed four PR10 and two PR10-like clusters with a high synteny across different species of five-needle pines. Of ten PmPR10 genes, *PmPR10-3.1* was selected and expressed in *Escherichia coli*. The purified recombinant protein exhibited inhibitory effects on spore hyphal growth of fungal pathogens *C. ribicola*, *Phoma exigua* and *P. argillacea* by in-vitro antifungal analysis. Genetic variation analysis detected a total of 21 single nucleotide polymorphisms (SNPs) within *PmPR10-3.1* in a collection of *P. monticola* seed families. A nonsynonymous SNP (t178g) showed significant association with relative levels of quantitative disease resistance (QDR), explaining about 8.7% of phenotypic variation as the peak value across all SNPs. Our results provide valuable insight into the genetic architecture underlying *P. monticola* QDR, and imply that *PmPR10-3.1* may function as an important component in conifer basal immunity for non-specific resistance to a wide spectrum of pathogens.

Keywords: Association analysis; In-vitro anti-fungal assay; Pathogenesis-related protein family 10 (PR10); Quantitative disease resistance (QDR); White pine blister rust (WPBR)

INTRODUCTION

Pathogenesis-related (PR) proteins are important components involved in host defense responses to protect plants from environmental biotic and abiotic stressors. PR proteins are grouped into different families based on sequence homology and biological activities (van Loon et al., 2006). The PR10 family contains a large number of proteins that share some common features, including low-molecular weight (15-20 kDa), acidic pI, a glycine-rich loop motif (GXGGXGXK), conserved cytosolic location and 3-D structure (van Loon et al. 2006; Fernandes et al., 2013; Agarwal and Agarwal 2014).

PR10 proteins are universal in the plant kingdom as a family with multiple members. They usually show differential expression across plant organs and development stages in response to environmental stressors, suggesting potential roles in various biological processes during plant growth, development, and defense responses against various pathogens and other environmental stress factors (Agarwal and Agarwal 2014). Several angiosperm PR10 genes were expressed in *Escherichia coli* and their recombinant proteins were analyzed by in-vitro assays. These studies revealed that some recombinant PR10 proteins possess RNase, or anti-microbial activity, as well as ligand binding activity (Mogensen et al. 2002; Zhou et al. 2002, Wu et al. 2003; Park et al. 2004; Chadha and Das 2006; Pungartnik et al. 2009; Xie et al. 2010, Gomez-Gomez et al. 2011; Agarwal et al. 2013; Fan et al. 2015). Transgenic plants with over-expression of PR10 genes exhibited enhanced resistance or tolerance to salinity, cold, osmotic stress, and infection by various pathogens (Fan et al. 2015; Jain and Kumar 2015; Agarwal et al. 2016; Castro et al. 2016; Han et al. 2017). Evidence accumulated so far indicates that PR10 proteins have multiple functions (Liu and Ekramoddoullah 2006; Agarwal and Agarwal 2014; Jain and Kumar 2015). Different family members may have adapted different functions although there is high sequence homology and structural similarity among them (Biesiadka et al. 2002). The emerging picture strongly suggests that PR10 proteins bind phytohormones and other plant mediators, including the recently identified melatonin (Sliwiak et al. 2016; 2018). However, functional investigation of PR10 genes is still limited and their physiological roles in plant biological processes have not been conclusively characterized (Jain and Kumar 2015).

The function of the PR10 family in five-needle pines is of great environmental and economic importance because these conifers play key roles in forest industry and ecosystems across North America. However, wild populations of North American native five-needle pines (the subgenus *Strobus*) have been seriously disturbed by the fungal pathogen *Cronartium ribicola* (J.C. Fisch.), which causes white pine blister rust (WPBR). Both major gene resistance (MGR) and quantitative disease

resistance (QDR) were detected in a few species of five-needle pines (Sniezko et al. 2014). Although MGR confers a complete immunity to specific *C. ribicola* races, it can be overcome by virulent strains in the WPBR pathosystems (Kinloch et al. 2004). For example, western white pine (*Pinus Monticola* Douglas ex D. Don) *Cr2*-controlled MGR can be overcome by the *vcr2* pathotype. Compared to MGR, QDR is controlled by polygenic loci, thus generally more durable for disease resistance in plant immune systems (Parlevliet 2002). The genomic complexity for QDR-related traits and non-characterized rust genotypes hinder elucidation of genetic mechanisms underlying five-needle pines' QDR to WPBR. Thus far, quantitative trait loci (QTLs) for genetic resistance to WPBR have not been well characterized in western white pine. Moreover, little is known about functional genes and molecular defense mechanisms underlying host QDR against WPBR (Liu et al. 2016a; Vázquez-Lobo et al. 2017).

Our previous studies identified several PR10 genes and proteins (PmPR10s) in western white pine (Liu and Ekramoddoullah 2004). Expression of the PmPR10 family members was differentially regulated during plant development and in response to biotic/abiotic stressors and treatments of plant hormones (Liu and Ekramoddoullah 2006). However, the molecular functions of PmPR10s in plant response to biotic stressors are still elusive. The aim of this study was to gain a comprehensive understanding of the PmPR10 family expressed during defense response to WPBR, and to further investigate the genetic contribution and functional role in western white pine QDR by in vitro anti-fungal assay and association study. Functional characterization of a PmPR10 protein with antimicrobial activity facilitates identification of QDR candidate genes and biomarkers with application in conventional tree breeding, speeding up selection of host resistance for the control of WPBR in western white pine and other related five-needle pines.

MATERIALS AND METHODS

Identification of *PmPR10s* expressed in western white pine defense response

To gain a global view of *PmPR10s* expressed in western white pine, PmPR10 proteins identified in a previous study (Liu and Ekramoddoullah 2004) were used as queries for a Blastx search with the lowest E-value < e-05 and minimum length of 100 amino acids in needle and stem transcriptomes in response to infection by *C. ribicola* (Liu et al. 2013b; 2014). To classify novel putative PmPR10s and analyze their phylogenetic relationship with other members, putative PR10 sequences were mined out from transcriptomes of limber pine (*P. flexilis*) and whitebark pine (*P.*

albicaulis) (Liu et al. 2016a; 2016b) and the sugar pine (*P. lambertiana*) genome sequence (v1.5) encoding putative 85,053 proteins (Steven et al. 2016). The proteins with full-length amino acid sequences were aligned by Clustal Omega with default parameters. An unrooted phylogenetic tree was constructed using the minimum evolution method in MEGA5 software (Tamura et al. 2011) with bootstrap test of 1000 times. *Nicotiana tabacum* polyketide cyclase-like (Uniprot: Q53HY7) was included as an outgroup (Radauer et al. 2008).

Protein over-expression in *Escherichia coli*

Because of its upregulation in *P. monticola* defense response (Liu and Ekramoddoullah 2004; Liu et al. 2013b), *PmPR10-3.1* was further selected for protein over-expression in *E. coli*. Based on its cDNA sequence (GenBank acc. AY596273), two PCR primers were designed and used to introduce the full-length coding sequence into the protein expression vector pET-3a (Novagen): forward primer (5'-CGT CGC CAT ATG GTG A GT GGG ACT GCA ACA AT-3') and reverse primer (5'-GCG GGA TCC TTA GCA GTA TAA GCT AAA GTT GGA GA-3'). The PCR fragment was first cloned into the pGEM plasmid (Promega, Madison, WI, USA). After verification of plasmid inserts by Sanger sequencing, the gene was further inserted into pET-3a expression plasmid using Nde I and BamH I restriction sites.

The expression vector pET-3a-PmPR10-3.1 was introduced in *E. coli* cells, strain BL21 (DE3) pLysS. The transformed cells were grown at 37°C in LB culture supplemented with 100 µg/ml ampicillin. Once *E. coli* culture reached the logarithmic growth phase (OD₆₀₀ ~1.0), protein expression was induced by 1.0 mM isopropyl β- d-1-thiogalactopyranoside (IPTG). After 4 hours of growth at 37°C, the cells were harvested by centrifugation, resuspended in lysis buffer, and sonicated. Cell debris was pelleted by centrifugation at 15,555g and 4°C for 1 hour. Most of the PmPR10-3.1 protein was found in the insoluble fraction; therefore the precipitate was treated with 7.2 M urea. Renaturation of the protein was conducted by dialysis against 20 mM Tris buffer, pH 8.0 containing 5% glycerol and 5 mM β-mercaptoethanol. The dialysed fraction was clarified by centrifugation and applied to further purification on a HiPrep S300 (GE Healthcare) column. Two rounds of size exclusion chromatography were performed in 20 mM Tris buffer, pH 8.0, 5% glycerol, and 5 mM β-mercaptoethanol. To confirm whether PmPR10-3.1 recovered from inclusion bodies was properly folded, circular dichroism (CD) experiments were carried out in the far-UV region (205-265 nm). The CD spectra obtained were recorded with 100 nm/min scan and the scan was repeated five times for an average spectra.

The protein was spun down using Millipore ultrafree-0.5 10K spin columns (Millipore Inc.) for removal of any trace chemicals that might interfere with antifungal assays. Through this process, the protein solution was also concentrated in the desalt buffer (20 mM Tris-HCl, pH7.0, 0.15 mM NaCl). Purified recombinant PmPR10-3.1 protein was applied to antifungal assays in concentrations varying from 10 µg/mL to 100 µg/mL.

Analysis of fungal spore germination rate

The ascomycete *Phoma exigua* and *P. argillacea* were used to test PmPR10's effect on fungal spore germination. Fungal agar plugs were propagated on fresh potato dextrose agar (PDA; Difco) for at least 14 days, at 20°C, 50% humidity, and continuous light to stimulate sporulation. Mature conidiospores were harvested by flooding the culture plate with sterile distilled H₂O. The spore suspension was quantified using a haemocytometer and adjusted to approximately 1000 spores in 45 µL volume of 0.1% water-agar.

To assess spore germination, the PmPR10-3.1 protein was applied directly to microcentrifuge tubes containing 45 µl of each spore suspension in 0.1% agar. The PmPR10-3.1 concentrations was adjusted to final concentrations of 10 µg/mL or 42 µg/mL. Water and desalt buffer were used as negative controls for each spore suspension. Tubes containing a mixture of spore suspension and each of the treatments were incubated at room temperature and spore germination rates were observed at the same time for both controls and protein treatment at 10, 12, 14, 16, 18 and 20 hours post-incubation.

To quantify germination rates, a small aliquot of the spore suspension was pipetted onto a glass slide at each time point and the number of germinating fungal spores per 100 count were determined using a Nikon, light microscope. An inhibition rate was calculated as $I = [(C-T)/C]*100$, where I = inhibition over control; C = the highest count of germination (%) in control; T = count of germination (%) in treatment. The experiment was run on two different occasions in triplicate for each time point.

Assessing the effect of PR10 on hyphal tip growth and morphology

Urediniospores of *C. ribicola* and conidia of *P. exigua* were used for the assessment of hyphal tip morphology and growth rate. Spore suspensions of each fungal species were diluted in 0.1% water-agar, and dispensed into 1.5 mL microcentrifuge tubes for each treatment. In separate tubes, the PmPR10-3.1 protein was applied to the *P. exigua* spore suspension at final concentrations of 10

168 $\mu\text{g/mL}$, 42 $\mu\text{g/mL}$, 75 $\mu\text{g/mL}$, and 100 $\mu\text{g/mL}$, and to *C. ribicola* spore suspension at 100 $\mu\text{g/mL}$.
 169 Desalt buffer and double distilled H_2O were used as negative controls. The spore suspensions were
 170 incubated for 18 hrs and 24 hrs for *P. exigua* and *C. ribicola*, respectively. Following this incubation
 171 period, hyphal tip length was measured and morphology was observed for each spore suspension
 172 using a Zeiss Axio Imager A1 (Jena, Germany) with a Lumenera Infiniti 3 camera using software to
 173 capture images and measure fungal hyphal length.

174 One-way ANOVA (Analysis of Variance) with post-hoc Tukey HSD (Honestly Significant
 175 Difference) was performed to determine the effect of PmPR10-3.1 on spore germination and hyphal
 176 growth across all tested fungal isolates.

178 **Detection of DNA variations and association study**

179 Eighteen open-pollinated families with a total of 160 seedlings (Table S4) were used to
 180 explore DNA variations and potential association of *PmPR10-3.1* genotypes with *P. monticola* QDR to
 181 *C. ribicola*. Phenotyping seedlings was performed for their traits related to QDR against WPBR as
 182 reported previously (Liu et al. 2011). Briefly, seeds were sown in 2003 and the seedlings were
 183 inoculated by basidiospores under controlled conditions in fall 2004 at the United States Department
 184 of Agriculture Forest Service, Dorena Genetic Resource Center (Cottage Grove, OR). Disease
 185 symptoms were assessed on a yearly basis for the next five years. QDR levels were ranked as: rust
 186 dead (0), normal canker with aecia development (1), normal canker only without aecia (3), normal
 187 canker and partial bark reaction without aecia (4), normal canker and bark reaction without aecia (5),
 188 partial bark reaction without aecia (6), bark reaction and partial bark reaction without aecia (7), bark
 189 reaction only without aecia (8), and complete resistance as stem symptom-free (10).

190 Genomic DNA was extracted from needle tissues for each seedling using a Qiagen DNeasy kit
 191 (Qiagen Inc., Mississauga, ON, Canada). Based on PmPR10-3.1 cDNA sequence (Liu and
 192 Ekramoddoullah 2004), a pair of gene-specific primers, forward 5'-ACA ACA AGC AGC TTA CGT TTC-3',
 193 and reverse 5'- GTT TGG AGT CTG GAC ATA TAG-3', were designed to amplify genomic DNA.
 194 Following gel purification using a MinElute gel extraction kit (Qiagen), the amplified PCR fragments
 195 were cloned into the pGEM-T easy vector (Promega), and recombinant plasmids were sequenced on
 196 both strands using an ABI3130xl Genetic Analyzer (Applied BioSystem). Genomic DNA sequences of
 197 representative *PmPR10-3.1* alleles were deposited in GenBank (Acc no. MH444691-MH444693).

198 DNA variations and genotype-trait association analyses were performed as described
 199 previously (Liu et al. 2011). Briefly, DNA variations of single nucleotide polymorphisms (SNPs) were

determined using the program DNASP5.0 (Librado and Rozas 2009). To exclude possible PCR errors, SNPs at a frequency below 5% were excluded for association analysis. A set of SSR polymorphic fragments, genotyped by using 19 pairs of simple-sequence repeat (SSR) primers developed in eastern white pine (Echt et al. 1996), were scored for the calculation of both K and Q matrixes as population structure. Significance of trait-genotype associations was computed using a mixed linear model (MLM) ($y = \text{marker} + Q + K + e$) using the software TASSEL (Bradbury et al. 2007). Significance of phenotypic difference among genotypes was tested using one way ANOVA.

RESULTS

Identification of PmPR10 genes expressed in western white pine

A Blastx search revealed that western white pine primary needle and stem transcriptomes in response to WPBR infection, each contained 26 transcripts encoding putative proteins with significant sequence homology to typical PR10 proteins. After removal of repetitive and partial sequences, 10 unique PmPR10 proteins were identified with full-length amino acid sequence (Table S2). PmPR10 proteins varied significantly throughout the total lengths, and the amino acid identities among PmPR10s ranged from 18.67% to 94.44% (Table S3). Only seven amino acid sites were identical across all sequences, including three glycine residues in a glycine-rich P-loop motif (G-X-G-X-G) (Fig. S1). Of the 10 unique PmPR10s, SN176002-c0-seq1, S172011-c0-seq1, and S170694-c1-seq1 encoded PmPR10-1.10, PmPR10-2.1, and PmPR10-3.1 proteins, respectively; which were reported previously (Liu and Ekramoddoullah 2004). The other seven members were reported here for the first time in the PmPR10 family, including two typical PR10s encoded by N141673-c1-seq1 and S170013-c0-seq2 and five PR10-like proteins encoded by SN168113-c0-seq1, S162003-c0-seq2/seq3, and N143454-c0-seq1/seq2. According to usual nomenclature, the proteins encoded by N141673-c1-seq1 and S170013-c0-seq2 have been named as PmPR10-4.1 and PmPR10-4.2, respectively. PmPR10.4.1 and PmPR10-4.2 shared 82.17% amino acid identity. As compared to other known PmPR10s, they showed the highest amino acid identities of 67.95% and 66.46% to PmPR10-1.13 (GenBank Acc no AAL50006), respectively.

Phylogenetic analysis of putative PR10 proteins in closely related five-needle pines

To explore the evolutionary relationship of the PR10 families in five-needle pines, we conducted a search for PR10 sequences in other related five-needle pine species within the available

genomic resources. PR10 members were found in the whitebark pine and limber pine transcriptomes and in the sugar pine genome by Blastx analysis using PmPR10 proteins as query. A total of six, six, and 45 unique PR10 homologs with approximate full-length sequences were unveiled in whitebark pine, limber pine and sugar pine, respectively. Phylogenetic analysis showed three main clusters well supported from bootstrap-tests in the tree (Fig. 1); one grouped all genuine PR10 proteins while the other two clusters (PR10-like I and II) represented PR10-like sequences. Four subclusters (I to IV) were detected in the PR10 group. The first three subclusters were represented by PmPR10-1, PmPR10-2, and PmPR10-3. The fourth subcluster of PR10s contained two novel PmPR10s (PmPR10-4.1 and PmPR10-4.2 encoded by N141673-c1-seq1 and S170013-c0-seq2) and their orthologs and homologs from limber pine and sugar pine. Across four species of five-needle pines, PR10s contained a typical P-loop motif (G-D-G-G-V-G) while a much less conserved sequence (G-x-G-x-x-G) was revealed in the PR10-like proteins. Remarkably, family members in the same clusters or subclusters from different species had protein sequence identities much higher than those in different clusters/subclusters from the same species, which implied a relatively high synteny of the same clusters/PR10 subclusters across different five-needle pine species.

Recombinant PmPR10-3.1 protein showed anti-fungal activity

Recombinant PR10-3.1 protein was purified and used for an anti-fungal assay (Fig. S2). Overall, PmPR10-3.1 significantly inhibited spore germination of *P. exigua* and *P. argillacea* over a 20-hour (hr) time trial (Fig. 2). This was particularly evident at 10 hr, 12hr, and 14 hr post-treatment where the germination rates of treated spores were significantly lower than that of the control for both *P. exigua* and *P. argillacea* (One-way ANOVA with post-hoc Tukey HSD, $p < 0.05$). The inhibitory effect showed a dosage effect, which lasted longer time at higher protein concentration (42 $\mu\text{g}/\text{mL}$ vs. 10 $\mu\text{g}/\text{mL}$), even up to 20 hr for *P. argillacea*. From the 16 hr time point onwards to 20 hr, the PmPR10-3.1 protein was still revealed with significantly inhibitory effect on *P. exigua* using 10 $\mu\text{g}/\text{mL}$ or on *P. argillacea* using 42 $\mu\text{g}/\text{mL}$ compared to the controls by t-test ($p < 0.05$) (Fig. 2).

The effect of PmPR10-3.1 on *P. exigua* and *C. ribicola* hyphal length was significant both in terms of morphological changes observed under the microscope as well as in absolute differences in hyphal length. As shown in Fig. 3, one way ANOVA on ranks revealed a significant difference in *P. exigua* hyphal length between treatments and controls ($p < 0.001$). The hyphal growth further decreased significantly ($p < 0.0001$) when PmPR10-3.1 concentration increased from 42 $\mu\text{g}/\text{mL}$ to 75 $\mu\text{g}/\text{mL}$ (Fig. 3a). At the morphological level, germinating spores treated with PmPR10-3.1 showed

much more hyphal tip abnormalities (Fig. 4a-f). The control spores (treatments by water and desalt buffer) grew long hyphal strands which appeared normal (Fig. 4a-b). With the PmPR10-3.1 treatments (Fig. 4c-f) we observed swelling of the hyphal tips and branching of the very end of the hyphal tips. These abnormal growth patterns indicate a potential weakening of the cell wall due to exposure to the antifungal protein. Taken together, all of these results demonstrated that PmPR10-3.1 delayed fungal spore germination and hyphal tip growth, and caused morphological changes in hyphal tips.

Similar inhibitory effects were observed during germination of *C. ribicola* urediniospores (Fig. 3b, Fig. 4g-i). Following treatment of 100 µg/mL of PmPR10-3.1, *C. ribicola* hyphae growth decreased about three times with swelling of the hyphal tips compared to negative water control (t-test, $P < 0.001$).

PmPR10-3.1 DNA variations are associated with western white pine QDR to WPBR

Survival rates post rust infection varied greatly among seed families. WPBR killed about 80% of total *C. ribicola*-infected seedlings within five years post inoculation, ranging from 11.8% up to 96.3% among different seed families. For all seedlings used in the association study, 15.4%, 20.5%, 38.5%, and 25.6% were grouped into four phenotypic categories: rust dead (QDR level at 0), normal canker (QDR level at 1~4), bark reaction (QDR level at 6~8), and stem symptom-free (QDR level at 10), respectively (Fig. S3).

DNA variations of *PmPR10-3.1* were detected by sequencing genomic DNA fragments in a breeding collection of 18 seed families. A total of 21 SNPs were found and further used in association analysis. The *PmPR10-3.1* SNPs included four synonymous SNPs in the exons and intron and 17 nonsynonymous SNPs (ns-SNP) that cause amino acid changes in the putative proteins. MLM-based association analysis indicated that eight ns-SNPs were significantly associated ($p < 0.05$) with QDR levels. The SNP-based coefficient of relationships explained proportions of phenotypic variation (R^2) in a range from 4.04% to 8.71% (Table 1).

The ns-SNP locus t178g showed trait-genotype association at the highest level of statistical significance ($R^2 = 8.71\%$, $p = 0.0013$). The ns-SNP loci t178g and t180a caused amino acid changes at position 44 (Phe44/Val44/Leu44), as counting from the start codon (Table 1), located at the beginning of the conserved P-loop region (Fig. S1). Trees, with genotypes for different protein isoforms derived at the AA44 position, showed significant difference of QDR to *C. ribicola* (one way ANOVA test $p = 0.0021$). The genotypes for Phe44/Leu44 and Val44/Val44 showed resistance levels significantly

higher than the genotypes Phe44/Phe44 and Phe44/Val44. The genotype Leu44/Leu44 exhibited medium QDR levels, with no significant difference from the other four genotypes (Fig. 5).

Discussion

This study characterized the PmPR10 family and revealed genetic and functional contributions of the gene member to tree QDR against *C. ribicola* in western white pine. Pyramiding of QTLs or combining QDR with MGR is becoming an important strategy for durable resistance to pathogens and pests in plant breeding (Pilet-Nayel et al. 2017). Accordingly, the highest QDR is considered to protect MGR from *C. ribicola* virulent strains by combining QDR and MGR mechanisms in elite seed orchards in breeding programs of five-needle pines (Kinlock et al. 2012; Sniezko et al. 2014). Given the importance of PR genes in the plant defense response, identification of novel PR genes and characterization of their genetic and functional contributions to QDR would help application of this genetic pyramiding strategy in forest breeding programs for long-term WPBR management. Causal genes underlying plant QDR have been characterized with molecular roles in initial perception of pathogens (such as NBS-LRR and RLK genes), signal transduction, and downstream defense and host metabolism (Pilet-Nayel et al. 2017). As one group of defense components, PR10s have been identified in a number of plant species from moss, gymnosperms, to higher angiosperm plants (Castro et al. 2016). Differential expression of PR10 genes in responses to biotic and abiotic stressors implied their potential roles in plant immunity systems. To date, several studies were reported on the genomic organization, evolution, and functions of the PR10 family (Kosuth et al. 2013), but such analyses for conifers are scarce (Liu and Ekramoddoullah 2006).

The transcriptome-wide identification of PmPR10 genes allowed a further global gene expression analysis to profile expression patterns of the family members in response to WPBR infection using available RNA-seq data. Differential expression patterns suggested distinct biological roles in response to rust infection for different members of the PmPR10 family (Liu et al. 2013b). Host QDR to *C. ribicola* usually displayed as a late defense response occurring in the stem tissues, showing different levels of canker damage and severity, ranging from normal canker, partial bark reaction with incomplete inhibition of fungal growth, complete bark reaction without fungal activity inside stem tissues, to symptom-free stems (Sniezko et al. 2008; 2014). Consistent with that, PmPR10-3.1 orthologs were upregulated in whitebark pine upon treatment of methyl jasmonate, suggesting its role in conifer induced systematic resistance (Liu et al. 2017). Differential expression to members of the PmPR10 family suggested various roles of PmPR10 proteins in the plant immune system and

other systems (Liu and Ekramoddoullah 2006; Liu et al. 2013b; 2013c). Evidence from evolution and regulation of the PmPR10 family allows inclusion of them as candidates to explore genetic association with QDR in molecular breeding programs of five-needle pines.

In this study, we performed a comprehensive search in available genomic resources for the expressed genes that belong to the PR10 family in western white pine and related five-needle pine species. In addition to those PmPR10 genes reported previously (Liu and Ekramoddoullah 2004), we identified seven novel PR10 and PR10-like genes. It is worth noting that the PmPR10 family members may be more numerous because partial transcripts encoding incomplete ORFs were excluded for further analysis. Based on alignment analysis of the PR10 amino acid sequences retrieved for four species of five-needle pines, a phylogenetic analysis grouped them into three main clusters, one consisting of typical PR10s with four subclusters and two PR10-like groups (Fig. 1). Genetic distances were much longer among intraspecific paralogs than interspecific orthologous members in the subgenus *Strobus*, providing new insights into the evolutionary relationship among members of the PmPR10 family. This syntenic pattern detected between western white pine and closely related five-needle pines suggested that at least six common progenitors of the PR10 family arose before the divergence of these species (Fig. 1).

Genetic contribution of PmPR10-10-3.1 to tree QDR to *C. ribicola* was further investigated by a functional analysis using in-vitro anti-fungal arrays. Several PR10 genes have been reported with in-vitro anti-microbial activities or enhanced transgenic resistance against pathogens by gene-over-expression studies (Fernandes et al. 2013; Agarwal and Agarwal 2014; Jain and Kumar 2015). Our confirmation of delayed spore germination as well as reduced hyphal tip growth and morphological changes of *C. ribicola* and two ascomycete species indicated PmPR10-3.1 to be an antifungal component in *P. monticola* QDR against a broad spectrum of pathogens. We observed that the *in-vitro* anti-fungal activity of the recombinant PmPR10 protein was reduced as time progressed, and became less effective without replenishment of the protein extract. Additionally fungal proteases may have been released to counteract the effect of the host protein.

As plant PR10s are now well recognized as versatile binders of various small molecules, including phytohormones (Pasternak et al. 2006; Ruszkowski et al. 2014) and other metabolites (Mogensen et al. 2002; Kofler et al. 2012; Sliwiak et al. 2018), it might be argued that the anti-fungal activity of our PmPR10-3.1 preparations was connected with some unknown molecular cargo delivered in complex with the protein. This argument can be firmly dismissed because,

serendipitously, the recombinant protein was purified from inclusion bodies using urea solubilization and refolding steps.

Furthermore, an association study was performed to test whether PmPR10s are among candidates for participating in the QDR mechanism for *P. monticola* to fight against *C. ribicola*. Several previous studies investigated candidate genes for host QDR to *C. ribicola* by SNP genotyping in populations of a few five-needle pines species, suggesting that variants of defense-related genes were main contributors of some resistance genotypes (Liu et al. 2005, 2011, 2013b; Vázquez-Lobo et al. 2017). In the present study, based on the patterns of differential gene expression, PmPR10-3.1 was selected and scanned for its genomic variations. Mapping multiple SNP loci with small phenotypic effects suggests that PmPR10-3.1 may be one of the QDR components. In particular, we were able to link the most significant ns-SNP locus to an amino acid change site close to the conserved P-loop motif. This potential functional change might present a causal variant as a beneficial allele to resistance, making PR10 a source of genetic factors for genotypic selection and QDR enhancement to decrease infection probability and WPBR severity in high biohazard regions.

In previous studies, most QDR causal genes showed partial phenotypic effects with molecular functions in the downstream defense mechanisms; the proteins encoded by these genes included PR proteins of various families as well as enzymes from synthetic pathways of different secondary metabolites (Kump et al. 2011; Belcher et al. 2012). In addition to PmPR10-3.1, previous studies revealed anti-fungal activity of an anti-microbial peptide (Zamany et al. 2011; Verma et al. 2012), and association of a specific chitinase isoform with host QDR in the WPBR pathosystem (Liu et al. 2011). Although each gene of the polygenic QTLs usually contributes a small phenotypic effect, the concerted action of several genes may explain a large amount of phenotypic variations of the QDR traits against pathogen attacks (Roux et al. 2014). Thus, PmPR10-3.1 might be working synergistically with other PR classes in enhancing *P. monticola* QDR to *C. ribicola*. Although QTLs and the regulatory networks that confer QDR to *C. ribicola* await further molecular dissections in the subgenus *Strobus*, our results suggest that components of different PR families integrate together, conferring synergistic effect in the WPBR pathosystem. Both resistance mechanisms and the number of genes controlling the trait are critical factors affecting QDR durability (Mundt 2014). As advanced genomics approaches are used to dissect QDR mechanisms in non-model plants, more conifer genes will be identified for functional verification in the WP-BR interactions. A genomics-based platform derived from them would provide effective and efficient tools for genotypic screening of elite germplasm with expected resistance traits of adaptive significance.

Because population size was limited and percentage of phenotypic variations explained by *PmPR10-3.1* SNPs were low in our association analysis, more research is required to confirm whether there truly is a genetic contribution of PR genes to *P. monticola* QDR against WPBR. Allelic differential expression analysis would provide an efficient method for precise prediction of QDR-causing genes. A future in-planta functional validation of PR10 will be performed using an experimental system of five-needle pines.

Acknowledgements

We thank G. Roke and G. Sumampong for providing fungal isolates; P. Rajkumar, G. Ross, K. Acharya, L. Baerg, G. Sumampong, M. Girard-Martel, and T. Holmes at CFS for their technical supports; and the technicians and staff at DGRC for seedling phenotyping and tissue sampling. This study was funded in part by the CFS Pest Risk Management Project.

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References

- Agarwal, P., and Agarwal, P. K. 2014. Pathogenesis related-10 proteins are small, structurally similar but with diverse role in stress signaling. *Mol. Biol. Rep.* 41:599–611.
- Agarwal, P., Bhatt, V., Singh, R., Das, M., Sopory, S. K., and Chikara, J. 2013. Pathogenesis-related gene, JcPR-10a from *Jatropha curcas* exhibit RNase and antifungal activity. *Mol. Biotechnol.* 54:412–425.
- Agarwal, P., Dabi, M., More, P., Patel, K., Jana, K., and Agarwal, P. K. 2016. Improved shoot regeneration, salinity tolerance and reduced fungal susceptibility in transgenic tobacco constitutively expressing PR-10a gene. *Front. Plant Sci.* 7:217.
- Belcher, A. R., Zwonitzer, J. C., Cruz, J. S., Krakowsky, M. D., Chung, C. L., Nelson, R., Arellano, C., and Balint-Kurti, P. J. 2012. Analysis of quantitative disease resistance to southern leaf blight and of multiple disease resistance in maize, using near-isogenic lines. *Theor. Appl. Genet.* 124:433–445.
- Biesiadka, J., Bujacz, G., Siorski, M. M., and Jaskolski, M. 2002. Crystal structure of two homologous pathogenesis-related proteins from Yellow Lupine. *J. Mol. Biol.* 319:1223–1234.
- 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.
- Castro, A., Vidal, S., and de León, I. P. 2016. Moss pathogenesis-related-10 protein enhances resistance to *Pythium irregulare* in *Physcomitrella patens* and *Arabidopsis thaliana*. *Front. Plant Sci.* 7:580.
- Chadha, P., and Das, R. H. 2006. A pathogenesis related protein, AhPR-10 from peanut: an insight of its mode of antifungal activity. *Planta* 225:213–222.
- Echt, C. S., May-Marquardt, P., Hseih, M., and Zahorchak, R. 1996. Characterization of microsatellite markers in eastern white pine. *Genome* 39:1102–1108.
- Fan, S., Jiang, L., Wu, J., Dong, L., Cheng, Q., Xu, P., and Zhang, S. 2015. A Novel pathogenesis-related class 10 protein Gly m 4I, increases resistance upon *Phytophthora sojae* Infection in soybean (*Glycine max* [L.] Merr.). *PLoS ONE* 10:e0140364.
- Fernandes, H., Michalska, K., Sikorski, M., and Jaskolski, M. 2013. Structural and functional aspects of PR-10 proteins. *FEBS J.* 280:1169–1199.
- Gómez-Gómez, L., Rubio-Moraga, A., and Ahrazem, O. 2011. Molecular cloning and characterisation of a pathogenesis-related protein CsPR10 from *Crocus sativus*. *Plant Biol (Stuttg)* 13:297–303.

- 434 Han, X., He, X., Qiu, W., Lu, Z., Zhang, Y., Chen, S., Liu, M., Qiao, G., and Zhuo, R. 2017. Pathogenesis-
435 related protein PR10 from *Salix matsudana* Koidz exhibits resistance to salt stress in transgenic
436 *Arabidopsis thaliana*, Environmental and Experimental Botany 141:74-82.
- 437 Jain, S., and Kumar, A. 2015. The Pathogenesis related class 10 proteins in plant defense against biotic
438 and abiotic stresses. Adv. Plants Agric. Res. 2:00077.
- 439 Kinloch, B. Jr., Snieszko, R., and Dupper, G. (2004. Virulence gene distribution and dynamics of the
440 white pine blister rust pathogen in western North America. Phytopathol. 94:751–758.
- 441 Kinloch, B. Jr., Burton, D., Davis, D. A., Westfall, R., Dunlap, J., Vogler, D. R., Snieszko, R. A., Yanchuk, A.
442 D., Kliejunas, J. T., Palmieri, K. M., Alexander, J. M., and Frankel, S. J. 2012. Strong partial
443 resistance to white pine blister rust in sugar pine, Proceedings of the fourth international
444 workshop on the genetics of host-parasite interactions in forestry: Disease and insect resistance in
445 forest trees, 2012 (tech. coords.) Gen. Tech. Rep. PSW-GTR-240. US Department of Agriculture,
446 Forest Service, Pacific Southwest Research Station, Albany, CA, pp. 80–91.
- 447 Kofler, S., Asam, C., Eckhard, U., Wallner, M., Ferreira, F., and Brandstetter, H. 2012.
448 Crystallographically mapped ligand binding differs in high and low IgE binding isoforms of birch
449 pollen allergen bet v 1. J. Mol. Biol. 422:109-123.
- 450 Kosuth, J., Hrehorova, D., Jaskolski, M., and Cellarova, E. 2013. Stress-induced expression and
451 structure of the putative gene hyp-1 for hypericin biosynthesis. Plant Cell Tiss. Organ Cult.
452 114:2007-2016.
- 453 Kump, K. L., Bradbury, P. J., Wisser, R. J., Buckler, E. S., Belcher, A. R., Oropeza-Rosas, M. A.,
454 Zwonitzer, J. C., Kresovich, S., McMullen, M. D., Ware, D., Balint-Kurti, P. J., and Holland, J. B.
455 2011. Genome-wide association study of quantitative resistance to southern leaf blight in the
456 maize nested association mapping population. Nat. Genet. 43:63–168.
- 457 Librado, P., and Rozas, J. 2009. DnaSP v5: A software for comprehensive analysis of DNA
458 polymorphism data. Bioinformatics doi: 10.1093/ bioinformatics/btp187
- 459 Liu, J.-J., and Ekramoddoullah, A. K. 2004. Characterization, expression and evolution of two novel
460 subfamilies of *Pinus monticola* cDNAs encoding pathogenesis-related (PR) -10 proteins. Tree
461 Physiol. 24:1377–1385.
- 462 Liu, J-J, and Ekramoddoullah, A. K. 2006. The family 10 of plant pathogenesis-related proteins: their
463 structure, regulation, and function in response to biotic and abiotic stresses. Physiol. Mol. Plant
464 Pathol. 68:3–13.

- 465 Liu, J.-J., Snieszko, R. A., and Ekramoddoullah, A. K. 2011. Association of a novel *Pinus monticola*
 466 chitinase gene (*PmCh4B*) with quantitative resistance to *Cronartium ribicola*. *Phytopathol.* 101:
 467 904-911.
- 468 Liu, J.-J., Hammet, C., and Snieszko, R. A. 2013a. *Pinus monticola* pathogenesis-related gene *PmPR10-2*
 469 alleles as defense candidates for stem quantitative disease resistance against white pine blister
 470 rust (*Cronartium ribicola*). *Tree Genet. Genom.* 9:397–408.
- 471 Liu, J.-J., Sturrock, R. N., and Benton, R. 2013b. Transcriptome analysis of *Pinus monticola* primary
 472 needles by RNA-seq provides novel insight into host resistance to *Cronartium ribicola*. *BMC*
 473 *Genomics* 14:884.
- 474 Liu J-J, Ekramoddoullah AK, Hawkins B, Shah S (2013c) Overexpression of a western white pine PR10
 475 protein enhances cold tolerance in transgenic Arabidopsis. *Plant Cell Tiss Organ Cult.* 114: 217-
 476 223.
- 477 Liu, J.-J., Snieszko, R. A., Sturrock, R. N., and Chen, H. 2014. Western white pine SNP discovery and
 478 high-throughput genotyping for breeding and conservation applications. *BMC Plant Biology*
 479 14:380.
- 480 Liu, J.-J., Snieszko, R., Murray, M., Wang, N., Chen, H., Zamany, A., Sturrock, R., Savin, D., and Kegley,
 481 A. 2016a. Genetic diversity and population structure of whitebark pine (*Pinus albicaulis* Engelm.)
 482 in western North America. *PLoS ONE* 11:e0167986.
- 483 Liu, J.-J., Schoettle, A. W., Snieszko, R. A., Sturrock, R. N., Zamany, A., Williams, H., Ha, A., Chan, D.,
 484 Danchok, D., Savin, D. P., and Kegley, A. 2016b. Genetic mapping of *Pinus flexilis* major gene (*Cr4*)
 485 for resistance to white pine blister rust using transcriptome-based SNP genotyping. *BMC*
 486 *Genomics* 17:753.
- 487 Liu, J.-J., Williams, H., Li, X. R., Schoettle, A. W., Snieszko, R. A., Murray, M., Zamany, A., Roke, G., and
 488 Chen, H. 2017. Profiling methyl jasmonate-responsive transcriptome for understanding induced
 489 systemic resistance in whitebark pine (*Pinus albicaulis*). *Plant Mol. Biol.* 95:359-374.
- 490 Mogensen, J. E., Wimmer, R., Larsen, J. N., Spangfort, M. D., and Otzen, D. E. 2002. The major birch
 491 allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. *J. Biol. Chem.*
 492 277:23684-23692.
- 493 Mundt, C. C. 2014. Durable resistance: A key to sustainable management of pathogens and pests.
 494 *Infect. Genet. Evol.* 27:446-455.

- 495 Park, C. J., Kim, K. J., Shin, R., Park, J. M., Shin, Y. C., and Paek, K. H.. 2004. Pathogenesis-related
496 protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. *Plant J.*
497 37:186–198.
- 498 Parlevliet, J. E. 2002. Durability of resistance against fungal, bacterial and viral pathogens; present
499 situation. *Euphytica* 124:147–156.
- 500 Pasternak, O., Bujacz, G. D., Fujimoto, Y., Hashimoto, Y., Jelen, F., Otlewski, J., Sikorski, M. M., and
501 Jaskolski, M. 2006. Crystal structure of *Vigna radiata* cytokinin-specific binding protein in complex
502 with zeatin. *Plant Cell* 18:2622–2634.
- 503 Pilet-Nayel, M.-L., Moury, B., Caffier, V., Montarry, J., Kerlan, M.-C., Fournet, S., Durel, C. E., and
504 Delourme, R. 2017. Quantitative resistance to plant pathogens in pyramiding strategies for
505 durable crop protection. *Front. in Plant Sci.* 8:1838.
- 506 Pungartnik, C., da Silvan, A. C., de Melo, S. A., Gramacho, K. P., de Mattos Cascardo, J. C., Brendel, M.,
507 Micheli, F., and da Silva Gesteira, A.. 2009. High-affinity copper transport and Snq2 export
508 permease of *Saccharomyces cerevisiae* modulate cytotoxicity of PR-10 from *Theobroma cacao*.
509 *Mol. Plant Microbe Interact.* 22:39–51.
- 510 Radauer, C., Lackner, P., and Breiteneder, H. 2008. The Bet v 1 fold: an ancient, versatile scaffold for
511 binding of large, hydrophobic ligands. *BMC Evolutionary Biology* 8:286
- 512 Roux, F., Voisin, D., Badet, T., Balagué, C., Barlet, X., Huard-Chauveau, C., Roby, D., and Raffaele, S.
513 2014. Resistance to *phytopathogens* e tutti quanti: placing plant quantitative disease resistance on
514 the map. *Mol. Plant Pathol.* 15:427–432.
- 515 Ruszkowski, M., Sliwiak, J., Ciesielska, A., Barciszewski, J., Sikorski, M., and Jaskolski, M. 2014. Specific
516 binding of gibberellic acid by cytokinin-specific binding proteins: a new aspect of plant hormone-
517 binding proteins with PR-10 fold. *Acta Cryst.* D70:2032–2041.
- 518 Sliwiak, J., Dauter, Z., and Jaskolski, M. 2016. Crystal structure of Hyp-1, a *Hypericum perforatum* PR-
519 10 protein, in complex with melatonin. *Front. Plant Sci.* 7:1–10.
- 520 Sliwiak, J., Sikorski, M., and Jaskolski, M. 2018. PR-10 proteins as potential mediators of melatonin-
521 cytokinin cross-talk in plants: crystallographic studies of LIPR-10.2B isoform from yellow lupine.
522 *FEBS J.* 285:1907–1922.
- 523 Sniezko, R. A., Kegley, A. J., and Danchok, R. 2008. White pine blister rust resistance in North America,
524 Asian and European species—results from artificial inoculation trials in Oregon. *Ann. For. Res.*
525 51:53–66.

- 526 Sniezko, R. A., Smith, J., Liu, J.-J., and Hamelin, R. C. 2014. Genetic resistance to fusiform rust in
 527 southern pines and white pine blister rust in white pines—A Contrasting tale of two rust
 528 pathosystems—Current status and future prospects. *Forests* 5:2050-2083.
- 529 Stevens, K. A., Wegrzyn, J. L., Zimin, A., Puiu, D., Crepeau, M., Cardeno, C., Paul, R., Gonzalez-Ibeas, D.,
 530 Koriabine, M., Holtz-Morris, A. E., Martínez-García, P. J., Sezen, U. U., Marçais, G., Jermstad, K.,
 531 McGuire, P. E., Loopstra, C. A., Davis, J. M., Eckert, A., de Jong, P., Yorke, J. A., Salzberg, S. L.,
 532 Neale, D. B. , and Langley, C. H. 2016. Sequence of the sugar pine megagenome. *Genetics*
 533 204:1613-1626.
- 534 Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular
 535 evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum
 536 parsimony methods. *Mol. Biol. Evol.* 28:2731-2739.
- 537 Van Loon, L. C., Rep, M., and Pieterse, C. M. J. 2006. Significance of inducible defense related proteins
 538 in infected plants. *Annu. Rev. Phytopathol.* 44:135-162.
- 539 Vázquez-Lobo, A., De La Torre, A. R., Martínez-García, P. J., Vangestel, C., Wegrzyn, J. L., Čalić I.,
 540 Burton D., Davis, D., Kinloch, B., Vogler, D., and Neale, D. B. 2017. Finding loci associated to partial
 541 resistance to white pine blister rust in sugar pine (*Pinus lambertiana* Dougl.). *Tree Genet. Genom.*
 542 13:108.
- 543 Verma, S. S., Yajima, W., Rahman, M. H., Shah, S., Liu, J.-J., and Ekramoddoullah, A. K., Kav N. N. V.
 544 2012. A cysteine-rich anti-microbial peptide from *Pinus monticola* (PmAMP1) confers multiple
 545 disease resistance in canola (*Brassica napus*). *Plant Mol. Biol.* 79:61–74.
- 546 Wu, F., Yan, M., Li, Y., Chang, S., Song, X., Zhou, Z., and Gong, W. 2003. cDNA cloning, expression, and
 547 mutagenesis of a PR-10 protein SPE-16 from the seeds of *Pachyrrhizus erosus*. *Biochem. Biophys.*
 548 *Res. Commun.* 312:761–766.
- 549 Xie, Y. R., Chen, Z. Y., Brown, R. L., and Bhatnagar, D. 2010. Expression and functional characterization
 550 of two pathogenesis-related protein 10 genes from *Zea mays*. *J. Plant Physiol.* 167:121–130.
- 551 Zamany, A., Liu, J.-J., Ekramoddoullah, A. K., and Sniezko, R. 2011. Antifungal activity of a *Pinus*
 552 *monticola* – antimicrobial peptide 1 (Pm-AMP1) and its accumulation in *Cronartium ribicola*-
 553 infected western white pine. *Can. J. Microbiol.* 57:667–679.
- 554 Zhou, X. J., Lu, S., Xu, Y. H., Wang, J. W., and Chen, X. Y. 2002. A cotton cDNA: GaPR-10, encoding a
 555 pathogenesis-related 10 protein with in vitro ribonuclease activity. *Plant Sci.* 162:629–636.

Table 1: Association analysis of *PmPR10-3.1* SNPs with quantitative disease resistance to *Cronartium ribicola*.

Figure Captions

Figure 1: Phylogenetic analysis of putative PR10 and PR10-like proteins from four five-needle pines. Phylogenetic analysis was performed based on alignment of amino acid sequences using program MEGA (v5). The graphic representation shows the results by the minimum evolution method. The scale indicates genetic distance proportional to the substitutions per site. Here, six, six, ten, and forty-five putative proteins sequences were included for *Pinus albicaulis* (Pa), *P. flexilis* (Pf), *P. monticola* (Pm), and *P. lambertiana* (Pl), respectively. *Nicotiana tabacum* polyketide cyclase (Q53HY7_TOBAC) was shown as an outgroup.

Figure 2: Inhibitory effect of PmPR10-3.1 on spore germination of fungal pathogens. (a) *Phoma exigua*; (b) *P. argillacea*. Control samples contained spore suspension with desalt buffer. Bars represent means and standard error of the mean (SEM) of two trials, each with three repeats at different time points. Significance of difference between treatments was tested using one-way ANOVA with post-hoc Tukey HSD, * $p < 0.05$, ** $p < 0.01$. From 16 hr up 20 hr, the differences between PmPR10-3.1 treatments and control were still significant by t-test ($p < 0.05$)

Figure 3: Effect of PmPR10-3.1 on hyphal growth of fungal pathogens. (a) *Phoma exigua* hyphal length (μm) was measured at early spore germination stage (18 Hrs) with PmPR10-3.1 treatment at four different concentrations. (b) *Cronartium ribicola* hyphal length (μm) was measured at early spore germination stage (24 Hrs) with PmPR10-3.1 treatment at 100 $\mu\text{g/mL}$. Bars represent means and standard error of the mean (SEM) of 20 *C. ribicola* or 30 *P. exigua* samples for each treatments. The hyphal strands of germinating spores were measured under the microscope. Different letters above the bars indicate statistically significant difference among multiple PmPR10-3.1 treatments (one way ANOVA, $p < 0.001$).

Figure 4: Micrographs showing the effects of PmPR10-3.1 on spore germination of fungal pathogens. Conidiospores of *Phoma exigua* (isolate PFC 2705) were treated by pure PmPR10-3.1 protein for 18 hours and photographs were taken at 63 X magnification DIC. (a) Desalt buffer control; (b) 10 $\mu\text{g/mL}$

PR10-3.1; (c) 42 $\mu\text{g/mL}$ PR10; (d) 75 $\mu\text{g/mL}$ PR10; (e) 100 $\mu\text{g/mL}$ PR10-3.1. Urediniospores of *Cronartium ribicola* were treated with pure PmPR10-3.1 protein for 24 hours and photographs were taken at 200X using Nimarsky filter: (f) desalt buffer control; (g) 100 $\mu\text{g/mL}$ PR10-3.1. Bars in insets represent 100 μm . Arrows indicated reduced hyphal growth and swelling at hyphal tips due to PmPR10-3.1

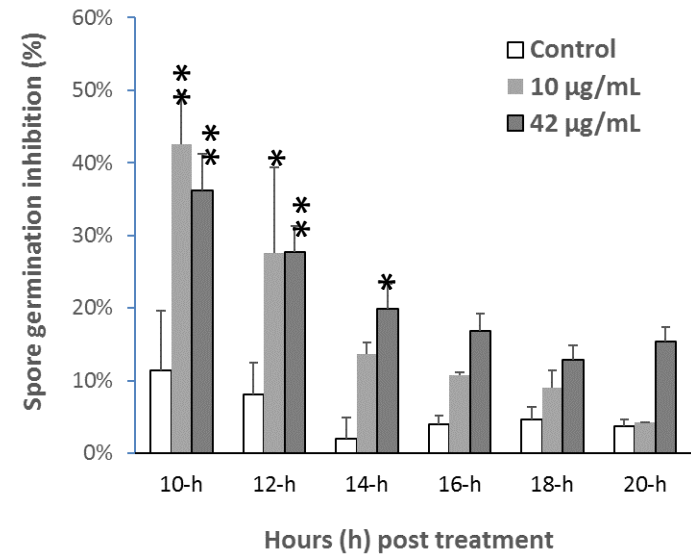
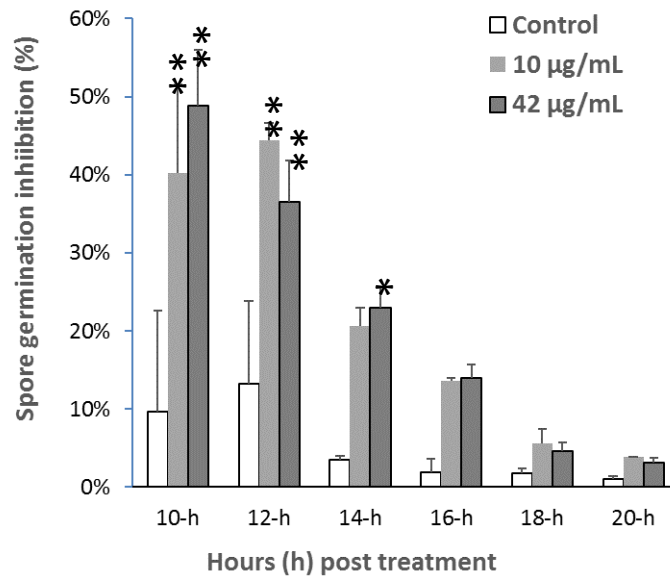
Fig. 5. Association of *PmPR10-3.1* genotypes with relative levels of quantitative disease resistance (QDR). Genotypes were determined based on the SNPs of the genetic codon for amino acid at position 44. Correlation of genotype-phenotype was depicted by the box plot. A horizontal dark line indicates the median of QDR levels. Box represents the lower and upper quantile for each genotype, but the genotypes Phe/Phe and Val/Val had no the upper quantile. The genotype Leu/Val was excluded because it had only one tree. Significant statistical differences between genotypes are labeled with different letters (one way ANOVA test $p < 0.001$).

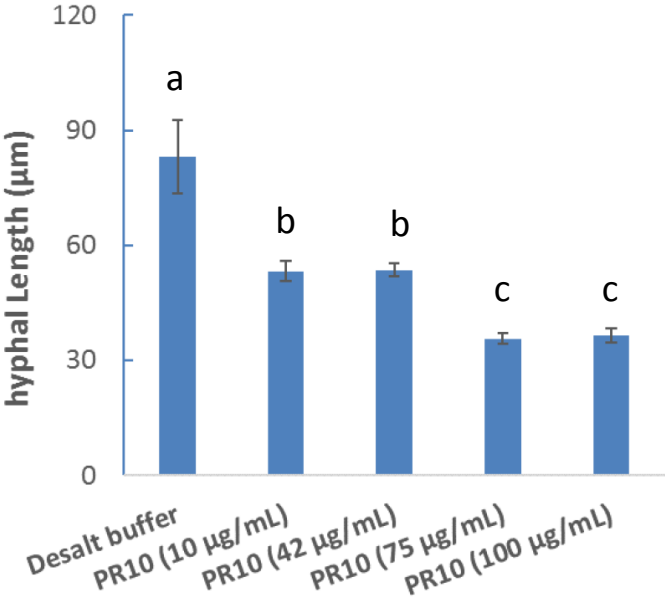
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Table 1: Association analysis of *PmPR10-3.1* SNPs with quantitative disease resistance to *Cronartium ribicola*.

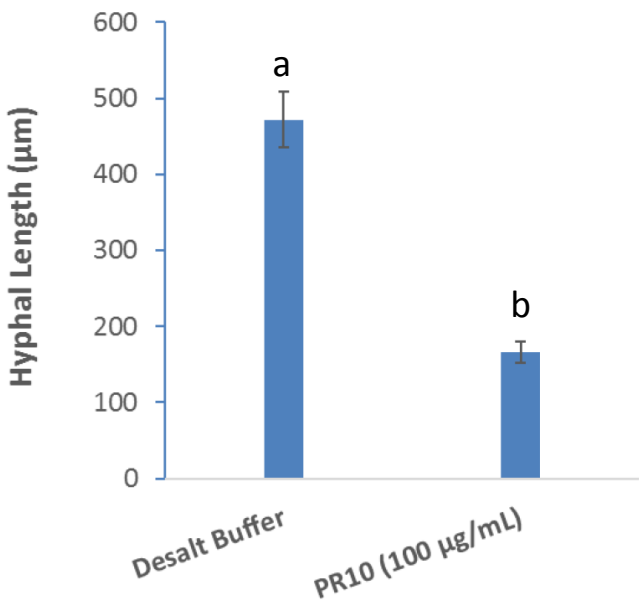
SNP locus	Gene region	Codon	Amino acid	Df-Error	R ² -Marker	p-Marker
a78t	exon1	GAA/GAT	Glu10/Asp10	150	0.0508	0.0185 *
a103g	exon1	ATA/GTA	Ile19/Val19	150	0.0531	0.0153 *
g117t	exon1	CTG/CTT	Leu23	148	0.0732	0.0032 **
a127g	exon1	AGC/GGC	Ser27/Gly27	150	0.0283	0.111
c173t	exon1	GCG/GTG	Ala42/Val42	150	0.0146	0.3254
c177g	exon1	ACC/ACG	Thr43	149	0.0172	0.2672
t178g	exon1	TTT/GTT	Phe44/Val44	144	0.0871	0.0013 **
t180a	exon1	TTT/TTA	Phe44/Leu44	150	0.0278	0.1156
c206g	exon1	ACC/AGC	Thr53/Ser53	150	0.0547	0.0134 *
c218t	exon1	ACA/ATA	Thr57/Ile57	150	0.0023	0.8404
a219c	exon1	ACA/ACC	Thr57/Ile57	147	0.0187	0.2407
a293c	intron	---	---	146	0.0078	0.5614
a374t	exon2	GAA/GAT	Glu65/Asp65	149	0.0312	0.184
a384g	exon2	ACC/GCC	Thr69/Ala69	148	0.0298	0.1013
a396g	exon2	ATC/GTC	Ile73/Val73	148	0.0012	0.9112
c397t	exon2	GCC/GTC	Ala73/Val73	150	0.0326	0.0791
a426t	exon2	ATC/TTC	Ile83/Phe83	150	0.0055	0.6563
c439t	exon2	ACC/ATC	Thr87/Ile87	150	0.0404	0.0425 *
a529t	exon2	AGT/ATT	Ser117/Ile117	150	0.052	0.0167 *
a599g	exon2	GAA/GGA	Glu140/Gly140	149	0.0478	0.0579
c651t	exon2	CCT/TCT	Pro157/Ser157	150	0.0479	0.0062 **







Treatment by PmPR10-3.1 protein



Treatment by PmPR10-3.1 protein

