Identification and characterization of the WRKY transcription factor family in *Pinus monticola*

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Abstract: The *WRKY* gene family represents an ancient and highly complex group of transcription factors involved in signal transduction pathways of numerous plant developmental processes and host defense response. Up to now, most WRKY proteins have been identified in a few angiosperm species. Identification of *WRKY* genes in a conifer species would facilitate a comprehensive understanding of the evolutionary and function-adaptive process of this superfamily in plants. We performed PCR on genomic DNA to clone WRKY sequences from western white pine (*Pinus monticola*), one of the most valuable conifer species endangered by white pine blister rust (*Cronartium ribicola*). In total, 83 *P. monticola* WRKY (*PmWRKY*) sequences were identified using degenerate primers targeted to the WRKY domain. A phylogenetic analysis revealed that *PmWRKY* members fell into four major groups (1, 2a+2b, 2c, and 2d+2e) described in *Arabidopsis* and rice. Because of high genetic diversity of the *PmWRKY* family, a modified AFLP method was used to detect DNA polymorphism of this gene family. Polymorphic fragments accounted for 17%–35% of total PCR products in the AFLP profiles. Among them, one WRKY AFLP marker was linked to the major resistance gene (*Cr2*) against *C. ribicola*. The results of this study provide basic genomic information for a conifer *WRKY* gene family, which will pave the way for elucidating gene evolutionary mechanisms in plants and unveiling the precise roles of *PmWRKY* in conifer development and defense response.

Key words: genetic map, phylogenetic analysis, transcriptional factor, western white pine, WRKY protein.

Résumé : La famille de gènes *WRKY* représente un groupe à la fois ancien et très complexe de facteurs de transcription qui sont impliqués dans de nombreux processus du développement et des réactions de défense chez les plantes. À ce jour, la plupart des protéines WRKY ont été identifiées chez quelques espèces d'angiospermes. L'identification de gènes *WRKY* chez un conifère contribuerait à une connaissance plus complète des processus évolutifs et adaptatifs menant à l'acquisition de fonctions au sein de cette superfamille chez les plantes. Les auteurs ont réalisé des amplifications PCR sur de l'ADN génomique afin de cloner des séquences WRKY chez le pin argenté (*Pinus monticola*), l'une des espèces de conifères les plus importantes et qui est menacée par la rouille vésiculeuse du pin blanc (*Cronartium ribicola*). Au total, 83 séquences WRKY du *P. monticola* (*PmWRKY*) ont été identifiées à l'aide d'amorces dégénérées ciblant le domaine WRKY. Une analyse phylogénétique a montré que les membres de la famille *PmWRKY* logent au sein des quatre groupes majeurs (1, 2a+2b, 2c et 2d+2e) décrits chez *Arabidopsis* et le riz. En raison de la grande diversité génétique au sein de la famille des *PmWRKY*, une technique AFLP modifiée a été employée pour détecter du polymorphisme au sein de cette famille. Entre 17% et 35% de tous les amplicons PCR obtenus dans ces profils AFLP étaient polymorphes. Parmi ceux-ci, un marqueur WRKY-AFLP était lié à un gène majeur de résistance (*Cr2*) au *C. ribicola*. Les résultats de cette étude fournissent les informations génomiques de base pour une famille de gènes *WRKY* chez un conifère. Cette avancée pave la voie en vue de l'élucidation des mécanismes d'évolution des gènes chez les plantes et de la découverte des rôles précis des *PmWRKY* dans le développement et les réactions de défense chez les conifères.

Mots-clés : carte génétique, analyse phylogénétique, facteur de transcription, pin argenté, protéines WRKY.

Introduction

As transcription factors, WRKY proteins play extensive roles in plant growth and development as well as in plant–pathogen interactions. They contain one or two copies of a DNA-binding domain that is composed of about 60 amino acids with the N-terminal sequence WRKYGXK followed by a zinc finger motif (C-X4-5-C-X22-23-H-X-H or C-X5-8-C-X25-28-H-X1-2-C) at the C-terminus (Eulgem et al. 2000; Zhang and Wang 2005). WRKY proteins regulate a number of defense-related genes, including pathogenesis-related (PR) genes, through interaction with the W-box (C/T)TGAC(T/C) in the promoter regions of these genes (Rushton et al. 1996; Du and Chen 2000; Eulgem et al. 2000; Ülker and Somssich 2004). *WRKY* genes appear to be unique to plants and comprise a large gene family with 74 members in *Arabidopsis thaliana*, 105 members in rice (*Oriza sativa*), and at least 109 members in soybean (*Glycine max*) (Wu et al. 2005, Zhang and Wang 2005). All of the angiosperm plants analyzed to

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date have numerous members classified into three groups (Eulgem et al. 2000). Recently, these three groups were subdivided into five major groups (groups 1, 2a+2b, 2c, 2d+2e, and 3) based on the number of WRKY domains, intron positions, and the structural features of the zinc finger motif (Zhang and Wang 2005).

The roles of *WRKY* genes in regulating host defense response against pathogen infection have been elucidated in several investigations. In *Arabidopsis*, more than two thirds of *AtWRKY* members are regulated by bacterial infection and treatment with salicylic acid, suggesting a key role of this family in biotic stress (Dong et al. 2003; Kalde et al. 2003; Eulgem 2005). Ectopic expression of the *AtWRKY18* gene led to enhanced expression of PR genes and resistance to the bacterial pathogen *Pseudomonas syringae* (Chen and Chen 2002). Overexpression of OsWRKY45 also enhanced resistance to rice blast fungus (Shimono et al. 2007). In contrast, analysis of both T-DNA insertion mutants and transgenic overexpression lines showed that AtWRKY7 and AtWRKY25 function as a negative regulator of salicylic acid mediated defense responses to *P. syringae* (Kim et al. 2006; Zheng et al. 2007). Pathogen-induced AtWRKY33 regulates the antagonistic relationship between defense pathways mediating responses to *P. syringae* and necrotrophic pathogens (Zheng et al. 2006). *Arabidopsis* protein (AtWRKY52 or RRS1), which has a nucleotide-binding site and leucine-rich repeats in addition to the WRKY domain, confers genetic resistance to bacterial wilt (*Ralstonia solanacearum*) by its physical interaction with PopP2, a type III pathogenic effector targeted to the plant nucleus (Deslandes et al. 2003). Recently, Shen et al. (2007) found a molecular mechanism of barley R (MLA) protein resistance to barley powdery mildew where the MLA protein directly interacts with a WRKY transcription factor in the nucleus, derepressing basal defense genes.

In addition to regulating biotic stresses in plants, a microarray study demonstrated that some AtWRKYs play important roles in *Arabidopsis* responses to cold, drought, and salt stresses (Seki et al. 2002). A barley gene (*HvWRKY38*) is also involved in cold and drought response (Maré et al. 2004). RNAi silencing analysis revealed that AtWRKY75 plays a modulator role in phosphate acquisition and root development in *Arabidopsis* (Devaiah et al. 2007).

Western white pine (*Pinus monticola*) is a very important forest species in western North America. This conifer is tolerant to frost and drought and is especially valuable because of its nonhost resistance to laminated root rot. These biological characteristics make *P. monticola* a good candidate species for forest restoration in western North America. Despite its economic and ecologic importance and long-term efforts over the past 50 years in breeding programs, western white pine is still not used for timber production because of its susceptibility to *Cronartium ribicola*, the causal agent of white pine blister rust (WPBR). To search for genetic resistance materials and to maintain the diversity of the genetic resources of WPBR resistance in western white pine populations is a difficult task in breeding programs. The most promising breeding strategy is involved in a major dominant resistance gene (*Cr2*) against WPBR (Kinloch et al. 1999). In the defense response triggered by WPBR infection, a variety of families of PR proteins are regulated in western white pine (Liu et al. 2004). One western white pine PR10 promoter contains W-boxes and directs a pathogen-induced gene expression (Liu et al. 2005). A W-box has also been found in the western white pine gene promoter for an antimicrobial peptide (PmAMP1) (Ekramoddoullah et al. 2006). Being transcription factors, WRKY proteins may take part in the white pine defense response against *C. ribicola* infection. Characterization of *WRKY* genes and analysis of their association with disease resistance will be significant for our understanding of host resistance mechanism in the WPBR pathosystem.

Although *WRKY* genes have been reported in more than 10 angiosperm species, little is known about the presence and function of this family in a gymnosperm species (Zhang and Wang 2005). To further understand the functions and evolution of the WRKY family in higher plants, here, we carried out a sequence search of *WRKY* genes in a conifer genome. The aim of this study was to characterize gene organization and polymorphism in the western white pine WRKY family (*PmWRKY*). A comparative phylogenetic analysis was performed on WRKY members from western white pine, loblolly pine (*Pinus taeda*), *Arabidopsis*, and rice to reveal genomic organization of gene family.

Materials and methods

Plant materials

Pinus monticola seed lot Nos. 3277, 3278, and 3566 were used in the present study, all of which are known to possess the *Cr2* gene conferring resistance to *C. ribicola*. Seed lot Nos. 3277 and 3278 were obtained from the Dorena Genetic Resources Center, USDA Forest Service (Liu and Ekramoddoullah 2007). Both of these seed lots were open-pollinated seeds from two mother resistant trees with USDA registration Nos. 119-15045-845 and 119-15045-845 × 15045-841, respectively (Kinloch et al. 1999). Seed lot No. 3566 was open-pollinated seeds from one mother tree, originally from a bulked seed orchard collection from the Dorena Genetic Resources Center. This mother tree was previously identified as heterozygous resistance (*Cr2/cr2*) (Liu et al. 2006).

Genomic DNA searching of *PmWRKY* sequences

Genomic DNA was extracted from needles of family Nos. 3277 and 3278 resistant seedlings using a Qiagen DNeasy kit (Qiagen Inc., Mississauga, Ontario). A targeted PCR approach was used to clone WRKY domain sequences from the western white pine genome. The conserved WRKY domain was used to design degenerate PCR primers for *WRKY* gene cloning and molecular marker development (Borrone et al. 2004). Degenerate primers were designed based on conserved motifs in the WRKY domain of different groups of WRKY proteins (Table 1). PCR was performed using a PCR Master Mix kit (Qiagen) in a final volume of 50 μL containing 100 ng of genomic DNA on a Perkin-Elmer themocycler (Perkin-Elmer Applied Biosystems, Foster City, California). Thermal cycling conditions consisted of an initial denaturation step at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 40 s, primer annealing at 42 °C for 1 min, and primer extension at 72 °C for 1.5 min with a final 10-min extension at 72 °C.

DNA sequence analysis

The DNA fragments were amplified by PCR and resolved

Table 1. PCR primers for WRKY cloning and WRKY AFLP polymorphism detection in *Pinus monticola*.

Primer name	Targeted amino acid motif	WRKY group	Nucleotide sequence (5′ to 3′)[a]	Reference/accession No.[b]
WRKY-F1	WRKYGQK	All groups	TGGMGIAARTAYGGNCARA	Borrone et al. 2004
WRKY-R1	TTYEG(Q/V)H(N/T)H	Group1, 2	TGRKTRTGYWSICCYTCRTAIGT	Borrone et al. 2004
WRKY-R2	TY(T/E)G(E/D)HNH	Group2d+2e	TGRTTRTGYTCICCIKYRTAIGT	NM_122748, DAA05104
WRKY-R3	SYLGRHNH	Group2c	TGRTTRTGYCTNCCNAGRTANCT	DR024229
WRKY-R4	TYXGEHTC	Group3	CAIGTRTGYTCICCIIIRTAIGT	DAA05110, ABF95809
WRKY-R5	TYIGEHTC	Group3	CANGTRTGYTCNCCNATRTANGT	DAA05110
WRKY-R6	TYYGHHTC	Group3	CANGTRTGRTGNCCRTARTANGT	CK109883
WP-W-R	TEIVYKG	Group1	CCYTTGTAMACWAT TTCMGT	AK226301

[a]Degenerate IUB group codes: R = A+G, Y = C+T, M = A+C, K = G+T, W = A+T, S = G+C, D = G+A+T, B = T+C+G, N = A+T+C+G.

[b]Primers were synthesized according to the reference or designed in the present study based on amino acid sequences as indicated by GenBank accession numbers.

by agarose gel electrophoresis. The DNA bands with expected sizes were excised and purified using a MinElute gel extraction kit (Qiagen) and cloned into the pGEM-T easy vector (Promega). Insert DNA sequences of recombinant clones were determined on both strands on an ABI310 DNA sequencer (Applied Biosystems) using a Thermo-cycle sequence kit (Amersham, Baie d'Urfe, Quebec). Nucleotide sequence data were compiled and analyzed using ExPASy Proteomics tools (Swiss Institute of Bioinformatics, Berne, Switzerland) and BLAST programs (National Center for Biotechnology Information, Bethesda, Maryland) (Altschul et al. 1997).

Phylogenetic analysis

Alignment analysis of nucleotide or putative amino acid sequences was performed online with the Clustal W network service at the European Bioinformatics Institute (Cambridge, UK). Based on the sequence alignment analysis, phylogenetic trees were constructed using the neighbor-joining method in the MEGA3 software package (Kumar et al. 2004). The reliability of each tree was established by conducting 1000 neighbor-joining bootstrap sampling steps. The *PmWRKY* sequences reported here have been registered in the GenBank database under accession Nos. EU69775 to EU69857 that correspond to *PmWRKY1* to *PmWRKY83* in number order.

DNA polymorphism of WRKY sequences in western white pine

To detect WRKY polymorphism, a modified AFLP method was used following a method described by Hayes and Maroof (2000). Megagametophyte genomic DNA from seed lot No. 3566 was digested with the restriction enzymes *Eco*RI and *Mse*I and ligated with adaptors according to standard AFLP protocol (Vos et al. 1995). Primers *Mse*I-CC and *Eco*RI-AC were used for preamplification in a PCR mixture of 25 μL total volume with 5 μL of diluted restriction/ligation DNA mixture as template, 0.4 μmol/L E-AC and M-CC primers, and 12.5 μL of 2× *Taq* Master Mix (Qiagen). Three WRKY primers, WRKY-F1, WRKY-R1, and WP-W-R (Table 1), were coupled with one of 16 *Eco*RI-AC + 2 selective primers, respectively, in the AFLP selective amplification. AFLP marker cloning was performed as described previously (Liu and Ekramoddoullah 2008).

To detect WRKY AFLP polymorphic markers in the *Cr2* linkage, WRKY AFLP primer combinations were screened using bulked segregant analysis and haploid segregation analysis as performed previously (Liu et al. 2006). WRKY AFLP amplification products were separated in 7 mol/L urea – 6% polyacrylamide gels and the DNA fragment patterns were visualized by silver staining. Each marker was tested for Mendelian segregation by χ^2 ($\alpha = 0.05$) using the locus genotype frequency analysis of JoinMap version 3.0 software (Van Ooijen and Voorrips 2001) and mapped on the *Cr2* linkage using JoinMap version 3.0 with the Kosambi mapping function provided by the software.

Results

Characterization of WRKY genomic DNA sequences

Six combinations of degenerate primers (F1/R1, F1/R2, F1/R3, F1/R4, F1/R5, and F1/R6) were used to clone *PmWRKY* genomic sequences (Table 1). These primer pairs produced different amplicon patterns under the same PCR conditions. In total, five genomic DNA fragments were amplified to sizes of about 0.27, 0.43, 0.49, 0.64, and 1.0 kb. Following purification and ligation, these DNA fragments were cloned into the pGEM-T easy vector. Based on restriction enzyme analysis, 246 genomic clones were selected for nucleotide sequence analysis. DNA sequences encoding for the WRKY domains were obtained. In case of possible PCR integration errors, only genomic sequences with nucleotide identities of 98% or less were considered as distinct members of the *PmWRKY* family for further analysis. Genomic clones without significant similarity to angiosperm WRKY domains in a BLAST search were excluded from this study. Of *PmWRKY* genes, the cloned genomic sequences ranged from 250 to 1001 bp. Taken together, 83 genomic DNA sequences (*PmWRKY1* to *PmWRKY83*) were identified as different members of the *PmWRKY* family.

The genomic sequences of 83 *PmWRKY*s were highly divergent, with nucleotide identities ranging from 6% to 98%. In a phylogenetic analysis based on nucleotide sequence alignment, these *PmWRKY* genomic clones were grouped into four monophyletic clusters with general nucleotide identity below 18% between groups (Fig. 1). These monophyletic clusters corresponded to angiosperm WRKY groups 1, 2a+2b, 2c, and 2d+2e (Zhang and Wang 2005). *PmWKRY* group 2a+2b was the most complex and consisted of 74 members (*PmWRKY1* to *PmWRKY74*) with nucleotide identities ranging from 28% to 98%, and it was subdivided into 13 classes (I to XIII) with a general nucleotide identity

Fig. 1. Phylogenetic analysis of *PmWRKY* genomic sequences. The phylogenetic tree was generated based on Clustal W alignment analysis of 83 *PmWRKY* genomic nucleotide sequences, including intron sequences, using a neighbour-joining method. A bootstrap of 1000 replications was employed to evaluate the reliability of the tree branching. The numbers I–XIII indicate classification of *PmWRKY* sequences into 13 similarity classes with an 82% identity threshold. The scale bar at the bottom indicates genetic distance proportional to the nucleotide substitutions per site.

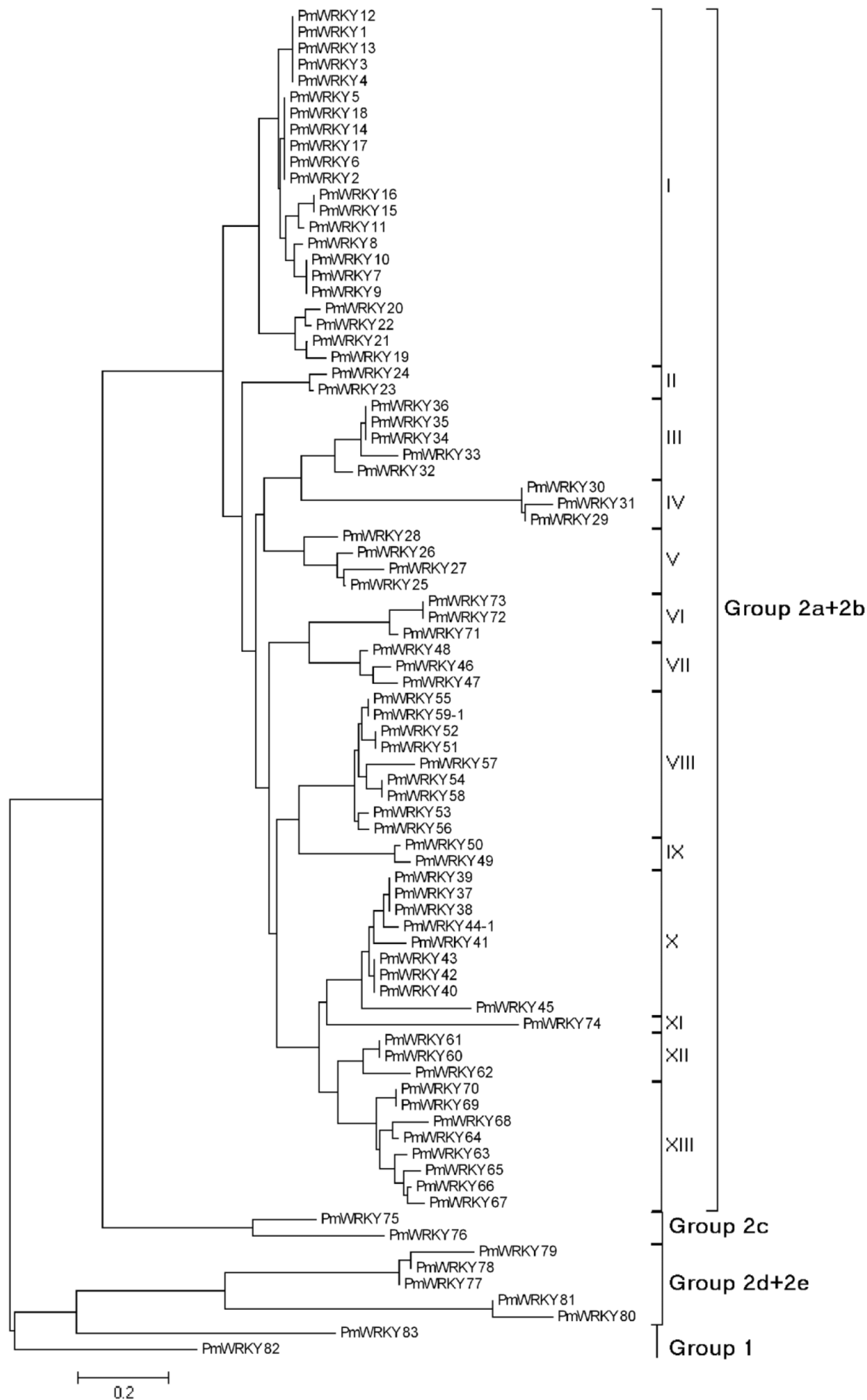

Fig. 2. WRKY domain comparison of PmWRKY proteins with AtWRKY and OsWRKY proteins. Alignment analysis was performed using the Clustal W program. The conserved motif (WRKYGQK) and cysteine (C) and histine (H) are highlighted in grey. The amino acid residues whose codons are interrupted by introns are highlighted in black and indicated by an arrow (phase 0 intron) or arrowheads (phase 2 introns). Gene annotation of *Arabidopsis* and rice homologues was described by Zhang and Wang (2005). Among 83 *Pinus monticola WRKY* genes, 11 representatives were included in the comparison analysis: *PmWRKY19* (EU269793), *PmWRKY23* (EU269797), *PmWKY25* (EU269799), *PmWRKY40* (EU269814), *PmWRKY55* (EU269829), *PmWKY62* (EU269836), *PmWRKY71* (EU269845), *PmWRKY75* (EU269849), *PmWRKY80* (EU269854), *PmWRKY82* (EU269856), and *PmWRKY83* (EU269857). Four *Pinus taeda* WRKY sequences were also included in the comparison analysis (TC67599, CF478959, AW437880, and DR694646).

Group 1 C-terminal WRKY domain

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PmWRKY82     WRKYGQKVVKGNPHPRSYYKCTNVGCSVRKHVERASNDVKAVITTYEGKHTH 52
Pt-TC67599   WRKYGQKVVKGNPNPRSYYKCTNAGCSVRKHVERASHDPKAVITTYEGKHNH 52
PmWRKY83     WRKYGQKVVKGNPYPRSYYKCTSLKCAVRKHVERASDDPKAVITTYEGKHTH 52
AtWRKY20     WRKYGQKVVRGNPNPRSYYKCTAHGCPVRKHVERASHDPKAVITTYEGKHDH 52
AtWRKY1      WRKYGQKSVKGSPYPRSYYRCSSPGCPVKKHVERSSHDTKLLITTYEGKHDH 52
             ******* * * * ***** *    * * ***** * * *  ******** *
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Group 2a+2b

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PmWRKY25     WRKYGQKKTKSNPLPRAYYKCAWGPGCPVKKQVQRSAEDPTIVITTYEGKHTH 53
PmWRKY55     WRKYGQKSTRNNPRPRSYYRCAMAPGCPVKKQVQRCAEDPTIVRTTYEGKHAH 53
PmWRKY71     WRKYGQKMTRNNPRPRSYYRCAMAPSCSVKRQVQRCAEDPTIVVTTYEGKHTH 53
PmWRKY62     WRKYGQKSTKNNPCPRSYYRCAMAPSCPAKKQLQRCAQDPTIVLTTYEGKHTH 53
PmWRKY40     WRKYGQKMTKNNKRPRSYYKCSLAPGCPVKKQVQRCAQDPTIVLTTYEGKHTH 53
PmWRKY19     WRKYGQKMTRNNPLPRSYYKCAGAPACPVKKQVQRCAQDPTIVITTYEGKHAH 53
PmWRKY23     WRKYGQKMTRNSTLPRSYYKCAMVPGCPVKKQVQRCAEDPTIVITTYEGKHSH 53
AtWRKR6      WRKYGQKMAKGNPCPRAYYRCTMATGCPVRKQVQRCAEDRSILITTYEGNHNH 53
AtWRKR18     WRKYGQKVTRDNPSPRAYFRCSFAPSCPVKKKVQRSAEDPSLLVATYEGTHNH 53
             *******       ** *  *     *     *** * *      **** * *
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Group 2c

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PmWRKY75     WRKYGQKSVKNRPNPRNYYRCSDSNCDVKKTVERDARDKGIVITTYEGKHTH 52
AtWRKY23     WRKYGQKAVKNSPFPRSYYRCTTASCNVKKRVERSFRDPSTVVTTYEGQHTH 52
OsWRKY49     WRKYGQKAVKNSPFPRSYYRCTTQKCPVKKRVERSYQDAAVVITTYEGKHTH 52
Pt-CF478959  WRKYGQKVVKNTHHPRSYYRCTQNNCRVKKRVERLADDPRMVITTYEGRHTH 52
Pt-AW437880  WRKYGRKLLRSSPHPRSYYRCSESNCNVKKRVEREVSDPGLLITTYEGTHNH 52
             ***** *        ** ****    * *** ***    *      *** * * *
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Group 2d+2e

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PmWRKY80     WRKYGQKPIKGSPHPRGYYRCNSVRGCPARKHVERSLDDPSMLIVTYDGEHNH 53
AtWRKY15     WRKYGQKPIKGSPHPRGYYKCSSVRGCPARKHVERAADDSSMLIVTYEGDHNH 53
OSWRKY6      WRKYGQKPIKGSPHPRGYYRCSSKKDCPARKHVERCRSDPAMLLVTYENEHNH 53
PT-DR694646  WRKYGQKPIKGSPYPRGYYRCSSCKGCPARKQVERSHTDPTKLVITYTAEHNH 53
             ************* ***** *      ***** ***    *    *  **    ***
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threshold of 82% between classes. In contrast, only two members were identified in group 1 (*PmWRKY82* and *PmWRKY83*) and group 2c (*PmWRKY75* and *PmWRKY76*), respectively. There were five members in group 2d+2e (*PmWRKY77* to *PmWRKY81*) that were further divided into two classes with a nucleotide identity threshold of 65% between themselves.

Comparison of western white pine and angiosperm WRKY domains

Alignment analysis of genomic DNA sequences of *PmWRKY*s with WRKY sequences from *Arabidopsis* and from rice demonstrated that the intron positions in WRKY domains are conserved between gymnosperms and angiosperms (Fig. 2). The positions and phases of the introns in the WRKY domains of *PmWRKY* genes were the same as those identified in WRKY groups 1, 2a+2b, 2c, and 2d+2e from *Arabidopsis* and rice (Xie et al. 2005; Zhang and Wang 2005). In group 2a+2b, the phase 0 intron was found after the fifth codon downstream of CX_5C, and in group 2c and 2d+2e, the phase 2 intron was localized after the second nucleotide of the fifth codon upstream $CX_{4–5}C$. As in the angiosperms, the intron position in the C-terminal WRKY domain of *PmWRKY* group 1 members was also conserved and localized at the same position and phase as those in groups 2c and 2d+2e. All 5′-exon/intron and 3′-intron/exon boundaries conformed to the well-known GT/AG donor/acceptor site rule except in the case of *PmWRKY80* where a rare

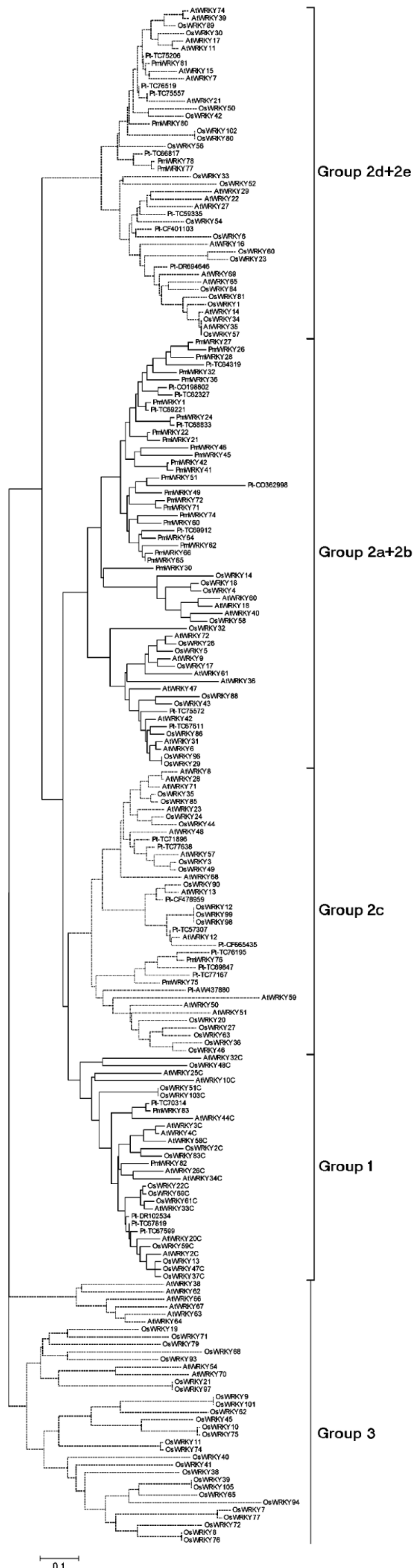


Fig. 3. Phylogenetic analysis of PmWRKY proteins. The phylogenetic tree was constructed based on the WRKY domain peptide sequences. Thirty-two representative *Pinus monticola* WRKY proteins and 29 *Pinus taeda* homologues (Pt series) were clustered into four groups (1, 2a+2b, 2c, and 2d+2e). One hundred and forty-three WRKY proteins from *Arabidopsis* and rice were included in the analysis, and annotation of these *Arabidopsis* and rice genes was described by Zhang and Wang (2005). The scale at the bottom indicates genetic distance proportional to the amino acid substitutions per site.

donor/acceptor site GT/AA was found (data not shown). Further investigation is needed to confirm this rare donor/acceptor site GT/AA because of possible PCR error.

After putative pre-mRNA splicing, 81 of 83 genomic DNA clones contain continuous open reading frames (ORF) encoding WRKY domains with homologies to those from angiosperms (Fig. 2). *PmWRKY28* had a site mutation that resulted in a premature stop codon just ahead of the intron, and *PmWRKY62* had two single base insertions, leading to ORF shifts, suggesting that these two WRKY sequences may be pseudogenes or PCR artifacts.

Translation of 83 *PmWRKY* DNA sequences produced 62 distinct WRKY domain sequences with amino acid identities from 40% to 98%. Fourteen *PmWRKY* DNA sequences shared the same amino acid sequence as the following: *PmWRKY2*, *PmWRKY9*, *PmWRKY13*, and *PmWRKY16*; *PmWRKY3*, *PmWRKY8*, *PmWRKY10*, and *PmWRKY11*; *PmWKY6* and *PmWRKY18*; *PmWRKY30* and *PmWRKY31*; *PmWRKY33* and *PmWRKY35*; *PmWRKY37*, *PmWRKY39*, and *PmWRKY40*; *PmWRKY46*, *PmWRKY47*, and *PmWRKY48*; *PmWKY52* and *PmWRKY56*; *PmWRKY53*, *PmWRKY54*, and *PmWRKY55*; *PmWRKY57* and *PmWRKY58*; *PmWRKY60* and *PmWRKY61*; *PmWRKY67* and *PmWRKY70*; *PmWRKY68* and *PmWRKY69*; and *PmWRKY77* and *PmWRKY79*. The WRKY domain is the dominant structural feature of WRKY proteins and is used to distinguish groups of this superfamily (Fig. 2). Generally, *PmWRKY* members shared higher identities inside the group (63%–98%) than among groups (40%–64%). The amino acid replacements in the WRKY domain in each group may reflect evolutionary divergence of individual family members. Those distinct DNA sequences sharing identical WRKY domains were either different gene members or alleles of the same gene members.

To comprehensively understand the relationship between angiosperm and gymnosperm WRKY proteins, alignment analysis of the putative amino acid sequences was performed to construct a phylogenetic tree (Fig. 3). Apart from representative members from *Arabidopsis* (*AtWRKY*) and rice (*OsWRKY*), this phylogenic analysis also included 29 *P. teada WRKY* genes that we searched out from the DFCI Pine Gene Index (release 6.0, 19 July 2005, a total of 45 557 output sequences) provided by the Computational Biology and Functional Genomics Laboratory at the Dana-Farber Cancer Institute and Harvard School of Public Health (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi) (Table 2). PmWRKY proteins were assigned to different groups based on phylogenetic analysis with well-classified WRKY proteins from *Arabidopsis* (Eulgem et al. 2000)

Table 2. Expressed members of the WRKY families in *Pinus teada*.

PtWRKY[a]	*P. teada* EST sequence[b]	Group[c]
1	TC67599, TC10778, TC25556, TC31987, TC49174, TC5325	1N, 1C
2	TC67819	1C
3	TC70314, TC11712, TC19803, TC37973, TC44417, TC6838	1C
4	DR102534	1C
5	TC67611, TC29306, TC42772	2a+2b
6	TC69912	2a+2b
7	TC69221	2a+2b
8	CO198802	2a+2b
9	TC64319, TC45579	2a+2b
10	TC75572, TC49622	2a+2b
11	TC62327	2a+2b
12	TC68833, TC48794	2a+2b
13	CO362998	2a+2b
14	TC77638, TC44174	2c
15	TC76195	2c
16	TC69847	2c
17	TC77167	2c
18	TC71896	2c
19	AW437880, ST73F02	2c
20	CF478959,	2c
**21*	TC57307, TC20439, TC35508, TC47838	2c
22	CF665435	2c
23	DR694646, EST1084738	2d+2e
24	TC59335, TC53663	2d+2e
***25*	CF401103	2d+2e
26	TC66817, TC16416, TC26277, TC28002, TC41928, TC5870	2d+2e
27	TC75206, TC36489, TC39130, TC48810, TC56850	2d+2e
28	TC75557	2d+2e
29	TC76519, TC55982	2d+2e

[a]*ORF interrupted by not processed intron sequence; **ORF interrupted by single nucleotide deletion.
[b]GenBank accession numbers or DFCI Pinus Gene Index numbers (TCxxxxx).
[c]Phylogenetic classification according to Zhang and Wang (2005).

and rice (Zhang and Wang 2005). According to the WRKY classification in *Arabidopsis* and rice, *PmWRKY*s were clustered into four WRKY groups: 1, 2a+2b, 2c, and 2d+2e (Fig. 3). The WRKY proteins were clustered in a similar pattern to that of the gene nucleotide sequences. The phylogenetic tree indicates that a common gene may have duplicated to give rise to the ancestor genes of each group before the split of gymnosperms and angiosperms. In groups 1 and 2d+2e, most conifer genes were diversely distributed in the clades containing angiosperm members. In groups 2a+2b and 2c, most conifer sequences formed monophyletic clades separable from *Arabidopsis* and rice homologues, suggesting that in large part, these *WRKY* genes might have evolved independently in the conifers.

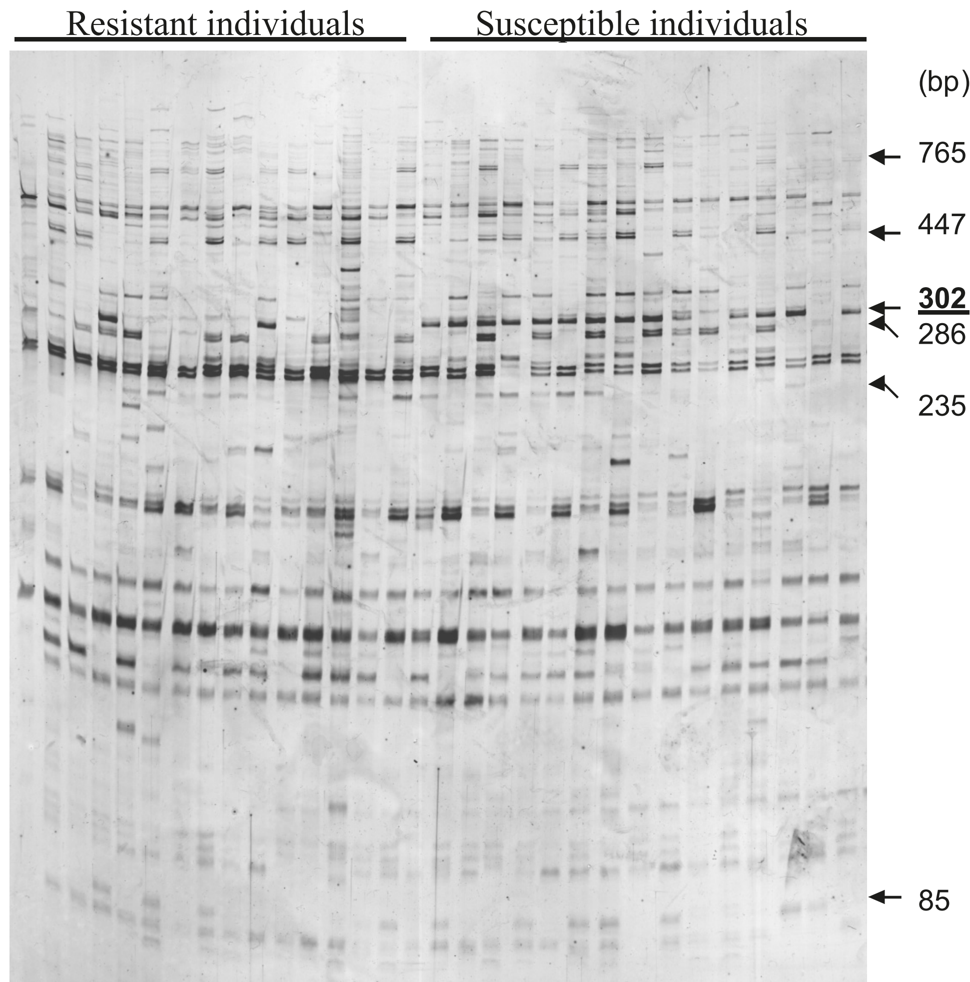
Polymorphism of WRKY-related sequences

Three WRKY primers (WRKY-F1, WRKY-R1, and WP-W-R) were combined with 16 *Eco*RI-AC+2 selective primers to perform selective PCR for WRKY AFLP genotyping. The detectable number of DNA fragments amplified by each of 48 primer combinations ranged from 53 (WRKY-R1/*Eco*RI-ACCG) to 67 (WRKY-R1/*Eco*RI-ACAC) with an average of 60. The sizes of amplified bands ranged from 60 to 800 bp. The polymorphic bands accounted for 17%–35% of the total bands derived from these primer combinations.

To explore potential WRKY markers linked to the *Cr2* gene against WPBR (*C. ribicola*), bulked segregant analysis was used to reveal polymorphism between resistant and susceptible bulks. Among the 48 primer combinations for WRKY AFLP amplification, only one pair of primers (WP-W-R/*Eco*RI-ACTC) detected a polymorphic DNA fragment of 302 bp linked to *Cr2* in the repulsion phase (Fig. 4). Segregation of this *Cr2*-specific WRKY AFLP marker, W/E13-302r, was tested in family No. 3566 and was found in a 1:1 ratio for the presence and absence of the marker. This WRKY AFLP marker was mapped onto the *Cr2* linkage with the five RAPD loci that we identified previously (Liu et al. 2006). In this genetic map of the *Cr2* gene, W/E13-302r was 2.9 cM away from *Cr2* (Fig. 5).

The DNA marker W/E13-302r and the other 12 WRKY AFLP polymorphic fragments were cloned and sequenced (Table 3). Sequence analysis demonstrated that those 12 fragments contained WRKY-specific and AFLP selective primers at their 5′ and 3′ ends. Thus, all but one of the markers contained amino acid sequences corresponding to WRKY primers. The polymorphic DNA fragment W-E5-283 was a nonspecific AFLP fragment amplified from one single AFLP selective primer, *Eco*RI-ACAC (Table 3). The BLASTP program of "search for short, nearly exact matches" found that eight DNA fragments, including W/E13-302r, encoded addi-

Fig. 4. WRKY AFLP polymorphic profiles. AFLP selective amplification was performed using one WRKY primer coupled with one AFLP selective primer. The arrows point to the polymorphic DNA fragments amplified by primer pair WP-W-R/*Eco*RI-ACTC. One WRKY AFLP marker with size 302 bp (W/E13-302r) was linked to *Cr2* in repulsion. Samples of 15 resistant individuals and 16 susceptible individuals are shown here.



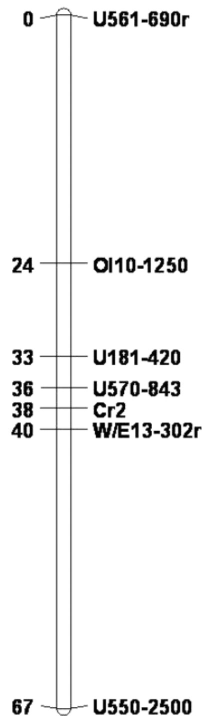
tional amino acid sequences extended from corresponding WRKY primers that were significantly similar to WRKY sequences, whereas the other five fragments showed no homology with any WRKY proteins (Table 3). The low level of WRKY AFLP marker homology with known *WRKY* genes may result from short sequences with limited ORFs and the presence of introns in the WRKY domains. Long-distance genomic DNA walking is necessary to definitively identify western white pine WRKY AFLP polymorphic fragments.

Discussion

A superfamily of WRKY proteins has been characterized in numerous angiosperm species. However, database search revealed only a few members in lower plants (alga, moss, and fern) and conifers (Eulgem et al. 2000; Ülker and Somssich 2004; Wu et al. 2005; Zhang and Wang 2005). A previous survey of *WRKY* genes from 81 802 *Pinus* ssp. ESTs revealed only four sequences (three in group 1 and one in group 2d+2e), suggesting a lower WRKY member abundance in conifers than in angiosperms (Zhang and Wang 2005). This led to questions about when and how evolutionary expansion has occurred for this family in the plant kingdom. The present study presents the first comparative genomic study and evolutionary analysis of the WRKY family of genes in a gymnosperm species. Through extensive searching of its genome, we identified 83 members of the *PmWRKY* family in western white pine. Based on the phylogeny of these sequences at both the nucleotide and amino acid levels, *PmWRKY* genes were clustered into four WRKY groups (1, 2a+2b, 2c, and 2d+2e). Our search of a *P. teada* EST databank with 45 557 output sequences found only 29 *PtWRKY* genes, and these were distributed in a similar grouping pattern as those in *P. monticola*. No group 3 member was found in the *P. taeda* transcriptome.

The distribution of the cloned sequences demonstrated that not all *WRKY* genes and alleles are amplified equally in western white pine by the primer sets that we used. Although reverse primers R4, R5, and R6 (Table 1) were based on group 3 WRKY members from *Arabidopsis*, rice, and tree species (*Populus*), respectively, no member was isolated from group 3 in western white pine. Borrone et al. (2004) reported a preferential amplification of group 1 and group 2 WRKY genes in *Theobroma cacao*. We characterized about 83 members of the *PmWRKY* family using six

Fig. 5. Genetic map of the *Cr2* linkage. The genetic distance unit is centimorgan (cM) defined by the Kosambi mapping function. The location of the WRKY AFLP marker (W/E13-302r) is shown in the *Cr2* linkage map with a genetic distance of 2.9 cM from *Cr2*. This marker adds to the five other RAPD loci described and mapped previously by Liu et al. (2006).



combinations of degenerate primers targeted at the conserved WRKY domain. Compared with gene numbers in *Arabidopsis* and rice, the true number of *WRKY* genes in western white pine may be much higher and not completely represented in the current study because of the absence of group 3. Although group 3 members comprise about 20% of the family in *Arabidopsis* and 36% in rice (Wu et al. 2005), they are not found in fern (Zhang and Wang 2005) and *Chlamydomonas reinhardtii* (Rensing et al. 2008). Our recent search of the EST database of spruce (release 3.0, 11 July 2008) also did not find an EST hint of a group 3 sequence. Analysis of rapid amplification of the cDNA end (3′-RACE) using WRKY-F1 and oligo d(T) primers detected only mRNA expression of group 1 and group 2 genes in more than 300 clones from various western white pine tissues (root, stem, needle, shoot, and immature male and female cone) (data not shown). All of these data suggest a low expression level of group 3 genes in conifer species. Although group 3 *WRKY* genes have been identified in the genomes of *Physcomitrella* and *Ostreococcus* (Rensing et al. 2008), their WRKY domain identities to rice and *Arabididopsis* homologous genes ranged only from 26% to 51%, indicating a high variation of group 3 *WRKY* genes during plant evolution. The difficulty in identifying conifer group 3 *WRKY* genes may result from longer introns in WRKY domain, a lower level of gene expression, and higher variation from identified homologous members. Further efforts are needed to confirm the presence of group 3 *WRKY* genes in conifers.

The WRKY superfamily experienced a significant expansion during evolution of the plant kingdom. The evolution of the WRKY family has undergone gene duplication events and subsequent divergences (Eulgem et al. 2000; Dong et al. 2003; Zhang and Wang 2005). Intron number and position in the WRKY domain are important gene structural characteristics for gene classification in rice and *Arabidopsis*. The intron positions of gene members of the *PmWRKY* family in each group were the same as their homologs in angiosperms. Zhang and Wang (2005) proposed that genes of group 1 with only a C-terminal WRKY domain are ancestors of other descendant genes in the other four groups (2a+2b, 2c, 2d+2e, and 3). In each group, *PmWRKY* genes showed higher identities with their corresponding *WRKY* genes from *Arabidopsis* and rice than western white pine members in other groups, suggesting that the duplication event for group differentiation might have occurred before the separation of gymnosperms and angiosperms. However, the ancestor gene numbers of each group should be limited. With a databank search, Zhang and Wang (2005) found one gene of group 1 in a green alga (*C. reinhardtii*) and fern (*Ceratopteris richardii*). The moss *Physcomitrella* genome contains only 38 *WRKY* genes (three in group 1, seven in group 2a+2b, 17 in group 2c, five in group 2d+2e, five in group 3, and one other) (Rensing et al. 2008). The later evolution of ancestor genes of each group may have given rise to different members, leading to a rapid and great expansion of each group in seed plants.

In each group, the subgrouping pattern into classes showed that in general the *PmWRKY* members were more similar to each other and to *P. taeda* members than to those in *Arabidopsis* and rice (Fig. 3). Most conifer *WRKY* genes were clustered together, phylogenetically distinct from the homologous members in *Arabidopsis* and rice. During evolutionary processes, orthologs are usually under selective pressure to maintain gene functions, whereas paralogs tend to accumulate mutations for deviation of gene function or regulation. In the transcription factor MADS-box gene family, an orthologous relationship was detected between angiosperms and gymnosperms (Liu et al. 2003). Here, we did not detect orthologous relationship between angiosperm and gymnosperm WRKY members, suggesting that the members in each WRKY group might have expanded independently in gymnosperms and angiosperms. This type of gene divergence between angiosperms and gymnosperms was previously observed in another western white pine superfamily related to disease/pest resistance, the nucleotide-binding site – leucine-rich repeat (NBS-LRR) gene family (Liu and Ekramoddoullah 2003, 2007). Both tandem duplications and large-scale duplications were implicated in the expansion of the NBS-LRR gene family (Meyers et al. 2003). It awaits further investigation to determine whether a similar expansion pattern has happened to the WRKY family after the split of gymnosperms and angiosperms. It is believed that the intra- and intergenic recombination or structural chromosome rearrangements play an important role in the gene divergence of the angiosperm WRKY family (Xie et al. 2005; Zhang and Wang 2005).

Several WRKY members were found to play a variety of developmental and physiological roles in plants, including plant immune responses to abiotic and biotic stresses (Ülker and Somssich 2004; Eulgem 2005), regulating embryogenesis (Lagace and Matton 2004), senescence (Robatzek and Somssich 2002), and nutrient starvation response and root

Table 3. WRKY AFLP polymorphic DNA fragments in *Pinus monticola* detected using a modified AFLP procedure.

Marker name[a]	PCR primers	Putative amino acid sequence[b]	Homologous WRKY (species) (GenBank accession No.)[c]	Homology score (bits)
W-E1-275	WRKF/*Eco*RI-ACCC	WRKYGQIKVTVSDT GARIRVDSKCRVQGC	WRKY25 (*Arabidopsis thaliana*) (NP_180584.1)	29.9 (63)
W-E2-266	WRKF/*Eco*RI-ACAC	KYGQREK*	NS	—
W-E3-408	WRKF/*Eco*RI-ACCT	WRKYGQTTNLEIHQ	WRKY46 (*Arabidopsis thaliana*) (AAK96020.1)	29.5 (62)
W-E4-175	WRKR/*Eco*RI-ACAC	NNLMQATTYEGKHT	WRKY33 (*Arabidopsis thaliana*) (NP_181381.2)	27.8(58)
W-E5-283	WRKR/*Eco*RI-ACAC	AFLP primer at both ends	NS	—
W-E6-345	WRKR/*Eco*RI-ACCG	ASQFRRLISNLF KTHTTYEGKH	WRKY3 (*Nicotiana attenuata*) (AAS13439.1)	27.4 (57)
W-E7-376	WRKR/*Eco*RI-ACCG	ERYAPIIHPSRTTYEGKH	WRKY2 (*Nicotiana benthamiana*) (AAS55706.1)	27.4 (57)
W-E8-173	WRKR/*Eco*RI-ACGG	*NLTTYEGKHT	NS	—
W-E9-376	WRKR/*Eco*RI-ACGG	ERYAPIIHPSRTTYEGKH	WRKY2 (*Nicotiana benthamiana*) (AAS55706.1)	27.4 (57)
W-E10-286	WPWR/*Eco*RI-ACTC	GTIISKYENKLTEIVYKG	NS	—
W-E11-765	WPWR/*Eco*RI-ACTC	KIESFITEIVYK	WRKY1 (*Pimpinella brachycarpa*) (AAC31956.1)	28.2 (59)
W-E12-447	WPWR/*Eco*RI-ACTC	*KNFTEIVYKG	NS	—
W-E13-302r	WPWR/*Eco*RI-ACTC	GEKQVKVRRSERIKKT SQGPQFIDLGTEIVYKG	WRKY30 (*Oryza sativa*) (AAW63719.1)	31.2 (66)

[a]The second number in the marker indicates the DNA fragment length (bp).
[b]A stop codon is represented by an asterisk.
[c]NS, no homologous WRKY was detected using the "search for short, nearly exact matches" program in PLASTP.

development (Devaiah et al. 2007). These WRKYs belonged to different classes based on structural features. In each of 13 *PmWRKY* classes of group 2a+2b, nucleotide sequence identities between members generally ranged from 82% to 98%, and some of them were identical at the amino acid level. Amino acid replacement in the conserved WRKY domain of closely related *WRKY* genes in each class suggests that paralogous evolution might have given rise to those related members, contributing to functional diversity in this superfamily. The results of the current study provide the necessary genomic information for further investigations into the molecular functions of *PmWRKY*s and their evolutionary expansion in a conifer genome. Future research is required to reveal the full complement of *PmWRKY* sequences. Identification of full-length and additional genes in conifers would facilitate a more comprehensive understanding of the evolutionary and function-adaptive process of this gene superfamily in plants.

Because of the large and divergent family of *WRKY* genes with multiple regulatory functions in different plant processes, it is possible to develop functional markers of candidate genes to understand their roles. *WRKY* genes are potential regulatory candidates for plant defense response against pathogen attack and other environmental stresses (Cheong et al. 2002). A ligation-mediated PCR revealed that genetic loci related to *WRKY* regulatory genes and other defense genes were significantly associated with potato quantitative disease resistance (Trognitz et al. 2002). The candidate gene approach is an effective method to investigate molecular mechanisms underlying complex plant traits such as disease resistance. For conifer species with large genomes (>10^7 kb), the association of a functional marker with a special phenotype could provide an accessible pathway leading to the identification of key genetic components.

Characterization of PCR products amplified by conserved motif primers has led to the isolation of WRKY sequences. Furthermore, the abundance and diversity of the *WRKY* gene family have been used for gene linkage mapping (Borrone et al. 2004). One WRKY-related primer in combination with a linker-specific primer was used to generate WRKY AFLP markers and map WRKY-related loci in potato (Trognitz et al. 2002). The present study developed WRKY AFLP markers in western white pine using one WRKY primer coupled with one typical AFLP selective primer. This modified AFLP strategy has been used for genetic mapping of disease resistance gene analogs (Hayes and Maroof 2000; Liu and Ekramoddoullah 2007) and transposable elements (Teunissen et al. 2003). The proportion of polymorphic bands revealed in WRKY AFLP (17%–35%) was similar to that of the standard AFLP (26%–40%) in western white pine. The PmWRKY-related DNA markers will help in understanding gene organization and evolution of this transcription factor family in a conifer species.

In conclusion, we characterized 83 sequences of the *PmWRKY* family through searching the western white pine genome. Our investigation further demonstrated that the diversity and variations of the *PmWRKY* family could provide potential candidate markers for conifer breeding.

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