Fine Mapping and Identification of Nucleotide Binding Site/Leucine-Rich Repeat Sequences at the *MER* Locus in *Populus deltoides* 'S9-2'

J. Zhang, M. Steenackers, V. Storme, S. Neyrinck, M. Van Montagu, T. Gerats, and W. Boerjan

First, third, fifth, sixth, and seventh authors: Vakgroep Moleculaire Genetica, Departement Plantengenetica, Vlaams Interuniversitair Instituut voor Biotechnologie (VIB), Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium; and second and fourth authors: Instituut voor Bosbouw en Wildbeheer, Gaverstraat 4, B-9500 Geraardsbergen, Belgium.
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ABSTRACT

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Melampsora larici-populina is the most damaging leaf pathogen for poplar in Europe. Previous genetic analyses have revealed both qualitative and quantitative resistance to this fungus. As a starting point for positional cloning of the gene or genes conferring qualitative resistance to *M. larici-populina* races E1, E2, and E3, a local genetic map of the *Melampsora* resistance (*MER*) locus was constructed based on amplified

fragment length polymorphism (AFLP) markers. Eleven AFLP markers were identified by bulked segregant analysis. These markers were used to identify 17 recombinants at the *MER* locus, from a total of 512 progenies derived from three interspecific crosses involving the same resistant female parent, *Populus deltoides* 'S9-2'. The local genetic map covered a 3.4-centimorgan interval encompassing the target locus. Sequence analysis of these AFLP markers revealed similarities to the nucleotide binding site/leucine-rich repeat class of disease resistance genes.

Additional keyword: bulked segregant analysis.

Species of the genus *Populus* (poplars and aspens) grow primarily in the temperate and cold regions of the northern hemisphere and are among the most important species for plantation forestry worldwide. During the past decades, selection and breeding of *Populus* spp. have resulted in highly productive clones that have been deployed in many countries (30). Thanks to its small genome size (550 Mb, approximately 220 kb/centimorgan [cM]) and amenability to genetic transformation, *Populus* spp. have also become organisms of choice for research on trees, especially for genetics and functional genomics (10,12,31).

Leaf rust fungi are the major class of pathogens that affect poplar all over the world. They can cause premature defoliation, shoot and root growth reduction, and even plant death (28,29). In Europe, *Melampsora larici-populina* is the most damaging species (26). This Eurasian fungus has been introduced into other continents and has become one of the major pathogens for poplar in Australia and New Zealand as well (24,25,27,36).

In Europe, poplar breeding has led to the selection of interspecific hybrid cultivars that were completely resistant to *M. larici-populina* (30). However, due to the fast evolution of this pathogen, new races have emerged and have overcome the resistance (23,26). Genetic studies have revealed two kinds of interaction between *Populus* spp. and *Melampsora* spp.: qualitative and quantitative resistance (15,16). Mendelian segregation for qualitative resistance against different physiological races of *M. larici-populina* (E1, E2, and E3) has been observed in interspecific *P. deltoides* × *P. nigra* and *P. deltoides* × *P. trichocarpa* hybrid progenies, suggesting that a dominant gene or genes in the *P. del-*

toides genome confers the resistance (5,14,15,33). Moreover, a major quantitative trait locus for resistance to race E2 is associated with the locus controlling incompatibility in a *P. deltoides* × *P. trichocarpa* interspecific hybrid pedigree (14). Therefore, this locus might contain a cluster of disease resistance genes for both qualitative and quantitative resistance.

Disease resistance genes identified to date can be classified into five classes, based on the primary structure of the corresponding proteins (3,8,9,11,18). Most of them, classified as the nucleotide binding site/leucine-rich repeat (NBS/LRR) disease resistance genes, seem to be members of an ancient gene family that encodes nucleotide binding proteins. These proteins are possibly involved in the signal transduction pathway that leads to the resistance response in plants (18). They can be classified further into two groups: the Toll/interleukin-1-receptor (TIR)/NBS/LRR class, which is represented by the *Nicotiana glutinosa* TMV resistance gene *N* (35), and the non-TIR/NBS/LRR class, which is represented by the *RPS-2* gene from *Arabidopsis thaliana*, which confers resistance to *Pseudomonas syringae* pv. *tomato*, which carries *avrRpt2* (4). Both groups share conserved domains with distinguishable features (18). In poplar, no resistance genes have been cloned and characterized at the molecular level so far, although several resistance loci have been genetically described (5,14,20–22,24,32,33).

Our work ultimately aims at the positional cloning of the gene or genes that confer qualitative resistance to *M. larici-populina* in poplar. Previously, three amplified fragment length polymorphism (AFLP) markers closely linked to the *Melampsora* resistance (*MER*) locus have been identified (5). Here, we report the construction of a local genetic map of the *MER* locus and the sequence analysis of closely linked flanking AFLP markers. Our results indicate that the *MER* locus is linked to a cluster of genes that share similarity to the NBS/LRR class of disease resistance genes.

Corresponding author: W. Boerjan; E-mail address: woboe@gengenp.rug.ac.be

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MATERIALS AND METHODS

Plant material. Five full-sib populations segregating for qualitative resistance to *M. larici-populina* races E1, E2, and E3 were analyzed. Populations 87001 (139 individuals) and 95001 (77 individuals) were generated from an interspecific cross between the female parent *Populus deltoides* 'S9-2' and the male parent *P. nigra* 'Ghoy' (5). Populations 87002 (106 individuals) and 95002 (120 individuals) were generated from an interspecific cross between the same female parent *P. deltoides* S9-2 and the male parent *P. trichocarpa* 'V24'. The female parent *P. deltoides* S9-2 is resistant to *M. larici-populina* races E1, E2, and E3 and heterozygous for the dominant resistance gene or genes (5), whereas the two male parents, *P. nigra* Ghoy and *P. trichocarpa* V24, are susceptible to all three races of this pathogen. Population 95003 (70 individuals) was generated from a backcross (*P. deltoides* 'S9-2' × *P. nigra* 'Ghoy') 'Ghoy' × *P. nigra* 'Ghoy'. Individuals of a given family are designated by a letter ("A" for 87001, "B" for 87002, "C" for 95001, "D" for 95002, and "E" for 95003) followed by a number for a given individual.

***M. larici-populina* resistance test.** The parents and progenies from the populations described above were evaluated for resistance or susceptibility to *M. larici-populina* races E1, E2, and E3, as described (5). Per clone, resistance and susceptibility were evaluated on two leaf disks of three ramets.

TABLE 1. List of amplified fragment length polymorphism (AFLP) primer combinations generating markers linked to the *Melampsora* resistance (*MER*) locus^a

Selective nucleotides (<i>Eco</i> RI)	Code	Selective nucleotides (<i>Mse</i> I)	Code	Markers
AAC	E32	TTC	G43	E32G43
AGA	E39	AAG	G01	E39G01
AGA	E39	CCA	F39	E39F39r
AGC	E40	TGT	G37	E40G37
ATC	E44	ACT	G09	E44G09
ATA	E43	CTA	G28	E43G28
ATG	E45	CTG	G29	E45G29
CAA	E47	GAC	G14	E47G14
CAC	E48	GAC	G14	E48G14
CCA	E51	AGG	G05	E51G05
CTG	E61	TGC	G36	E61G36

^a Primers that were used in the selective amplification and that generated markers closely linked to the *MER* locus in the *Populus deltoides* 'S9-2' genome are shown. The sequence for *Eco*RI and *Mse*I AFLP primers has been reported before (34).

AFLP analysis. Genomic DNA was prepared from lyophilized young leaves with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). AFLP analysis was performed essentially according to Vos et al. (34), with some modifications for the poplar genome according to Cervera et al. (5). To generate closely linked markers, 556 primer combinations (*Eco*RI + Axx or +Cxx combined with *Mse*I + xxx) were analyzed. For adaptors and primers used in the ligation, preamplification, and radioactive polymerase chain reaction (PCR), the reader is referred to Vos et al. (34). Primer combinations that generated AFLP markers closely linked to the *MER* locus are listed in Table 1.

Bulked segregant analysis. Bulk segregant analysis (BSA) was performed essentially as described (5,19). Preamplification reactions were carried out on genomic DNA. Equal amounts of preamplified PCR products were pooled in bulks.

Sequence analysis of AFLP markers from different genotypes. AFLP markers were named with combined primer codes (Table 1). An "r" was given to the marker linked in repulsion phase. Purification and cloning of AFLP markers were carried out essentially as described (7) with the following modifications: reamplified PCR products were further purified with a QIAquick Gel Extraction Kit (Qiagen) before ligation into the p-GEMT vector (Promega, Madison, WI). Sequencing was accomplished with the "Big Dye Terminator Sequencing kit" and performed on the ABI Prism DNA 377 automated sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). GCG software was used for sequence analysis (version 10.1; Genetics Computer Group, Madison, WI). BLASTx and tBLASTx were carried out with 11 AFLP marker sequences against the nonredundant protein and DNA databases, respectively (1).

RESULTS

Resistance to races E1, E2, and E3 of *M. larici-populina* is tightly linked in the *P. deltoides* S9-2 genome. For all five populations analyzed (512 plants), resistance to E1, E2, and E3 cosegregated and not a single recombinant could be identified. In four populations, the segregation for resistance followed a Mendelian ratio (1:1; $P > 0.05$); in population 87002, the segregation did not fit such a ratio ($P < 0.05$). This discrepancy was due to the fact that several clones of the family were lost over the years because of susceptibility to rust.

Identification of AFLP markers linked to the *MER* genes. Three markers (E40G37, E39G01, and E44G09) linked to the *MER* locus have previously been identified (5). To identify a larger number of markers closely linked to the *MER* locus, BSA

TABLE 2. Phenotypes of recombinants and their genotypes for 11 amplified fragment length polymorphism (AFLP) markers closely linked to the *Melampsora* resistance (*MER*) locus

Marker	Recombinant number ^a																
	A58	A141	A172	A173	A183	A184	A185	A268	E64	E199	B127	B170	B213	B229	B253	B256	D82
E40G37	0	1	1	1	0	0	1	1	0	0	1	1	0	1	1	1	0
E39G01	0	0	1	0	0	0	1	1	0	0	1	1	0	1	1	1	0
E44G09	0	0	1	0	0	0	1	1	0	0	1	1	0	1	1	1	0
E32G43	0	0	1	0	0	0	1	1	0	0	1	1	0	1	1	1	0
E45G29	0	0	1	0	0	0	1	1	0	0	1	1	0	1	1	1	0
E47G14	0	0	1	0	0	0	1	1	0	0	1	1	0	1	1	1	0
<i>MER</i>																	
E39F39r	1	0	1	0	0	0	1	1	1	1	1	1	0	1	0	0	0
E48G14	1	0	0	0	1	0	1	0	1	1	1	1	1	0	0	0	1
E61G36	1	0	0	0	1	0	1	0	1	1	1	0	1	0	0	0	1
E51G05	1	0	0	0	1	0	1	0	1	1	0	0	1	0	0	0	1
E43G28	1	0	0	0	1	1	0	0	1	1	0	0	1	0	0	0	1
Phenotype	S	S	R	S	S	S	R	R	R	R	R	R	S	R	S	R	S

^a Seventeen recombinants at the *MER* locus identified in interspecific hybrid populations (A, 87001; B, 87002; D, 95002; E, 95003); phenotype of recombinants: R = resistant and S = susceptible; 0 and 1 correspond to presence or absence of AFLP marker, respectively (except for the marker in repulsion E39F39r, for which 1 and 0 correspond to absence and presence, respectively). Markers are listed according to their position on the local genetic map. Recombinants generated from the population 87001 have been used in the bulked segregant analysis.

was carried out on family 87001 with additional primer combinations. Two bulks were made by pooling preamplified PCR products of 10 resistant progeny plants ("resistant bulk") and 10 susceptible progeny plants ("susceptible bulk"). The resistant female parent *P. deltoides* S9-2 and the susceptible male parent *P. nigra* Ghoy were analyzed in parallel. For markers linked in coupling to the *MER* locus, AFLP fragments were searched for that were present in the resistant parent *P. deltoides* S9-2 and the resistant bulk and absent in the susceptible parent *P. nigra* Ghoy and the susceptible bulk. For markers linked in repulsion, AFLP fragments were searched for that were present in the resistant parent and the susceptible bulk and absent in the susceptible parent and the resistant bulk.

One AFLP marker (E43G28) was identified with BSA by analyzing 46 AFLP primer combinations (Table 1). Fingerprint analyses of 139 individuals of population 87001 led to the identification of six recombinants for E43G28 (A58, A172, A183, A184, A185, and A268) (Table 2). Two recombinants (A141 and A173) were identified for E40G37, a previously identified linked marker (5) (Table 2). To limit the marker hunting region to the interval between E40G37 and E43G28, BSA was carried out with another "susceptible bulk" made by pooling preamplified PCR products from seven susceptible progenies and three susceptible recombinants (A141, A173, and A184). Inspection of an additional 510 primer combinations (*Eco*RI + Axx or Cxx/*Mse*I + xxx) led to the identification of six new AFLP markers in coupling phase and one marker (E39F39r) in repulsion phase within the interval between E40G37 and E43G28. Thus, a total of 11 AFLP markers closely linked to the *MER* locus have been identified.

Construction of a local genetic map. A high-resolution genetic map was constructed by ordering the markers with recombinants that had crossovers adjacent to the target locus. Individuals from five full-sib populations were fingerprinted for two flanking markers, E40G37 and E43G28. Seventeen recombinants that had recombination between these two markers were identified from four populations (87001, 87002, 95002, and 95003) (Table 2). No recombinants were found from the 95001 population (77 individuals). For confirmation, these recombinants were phenotyped a second time for resistance or susceptibility to E1, E2, and E3 by artificial infections of leaf disks. Subsequently, genotyping of these recombinants for all linked AFLP markers allowed us to order them and to construct a local genetic map, which spanned a 3.4-cM interval encompassing the *MER* locus (Fig. 1). The phenotypes of the recombinants and their genotypes for the 11 linked AFLP markers are presented in Table 2.

BLAST analysis of AFLP markers. Subsequently, the 11 AFLP markers were cloned and sequenced. BLASTx and tBLASTx analyses of 11 AFLP marker sequences showed that 7 out of 11 markers were significantly similar to the putative coding sequences in the database, indicating that they were probably derived from coding sequences. Four markers had no significantly homologous hit in the BLASTx analysis, whereas tBLASTx analysis showed that they all had homologs in the noncoding sequences in the *Arabidopsis* genome. For each marker, the length as well as the best homologs from BLASTx and tBLASTx analyses are presented in Table 3. Three flanking AFLP markers E47G14 (0.6 cM), E48G14 (1.6 cM), and E61G36 (1.8 cM) (Fig. 1) exhibited significant similarities at the amino acid level to the LRR, the TIR, and the NB-ARC domains of the NBS/LRR class of plant disease resistance proteins, respectively.

DISCUSSION

BSA and AFLP were used to identify 11 AFLP markers within a 3.4-cM interval encompassing the *MER* locus in the *P. deltoides* S9-2 genome, which contains a gene or genes conferring qualitative resistance to three races of *M. larici-populina* (5). The markers were integrated into a fine-scale local genetic map, a prerequisite

to initiate a positional cloning of the *MER* allele or alleles. Sequence analyses of flanking AFLP markers revealed the presence of disease resistance gene analogs near the target gene or genes.

The average number of scorable AFLP fragments, which are heterozygous in *P. deltoides* S9-2 and absent in *P. nigra* Ghoy, is 8.8 per primer combination (6). Thus, by assuming an equal polymorphism level all over the genome, by analyzing 556 primer combinations, approximately 5,000 markers are expected from the haplotype, which contains the *MER* locus. Our effort led to the identification of 10 markers in coupling and 1 in repulsion phase in a 3.4-cM interval. A significant imbalance (10:1) between the number of markers in coupling phase and that in repulsion phase was found, which might imply that the syntenic locus at the homologous chromosome is smaller or even absent. In several genomes of cereals, the copy number of the NBS/LRR class of *R* genes is highly variable at syntenic map positions of the genome between cultivars of the same species and can range from absence to high copy numbers; in addition, interspecific analyses of *R*-like genes frequently revealed nonsyntenic map locations between cereal species in which colinearity in gene order is a hallmark (17). The marker density of this local genetic map is theoretically sufficient to initiate positional cloning in the poplar genome, for which, on average, 1 cM corresponds to 220 kb. As a next step in positional cloning, a physical map of this locus needs to be constructed based on overlapping bacterial artificial chromosome (BAC) clones.

Disease resistance genes are commonly clustered in the genome. For example, the *M* locus in flax (*Linum usitatissimum*),

Fig. 1. Local genetic map of the *Melampsora* resistance (*MER*) locus. A local genetic map with 11 markers located within a 3.4-centimorgan (cM) interval encompassing the *MER* locus in the *Populus deltoides* 'S9-2' genome is shown. Markers linked in coupling phase are listed on the right and that in repulsion phase on the left. Map distances are given relative to the *MER* locus.

TABLE 3. BLASTx and tBLASTx analyses of amplified fragment length polymorphism markers closely linked to the *Melampsora* resistance (*MER*) gene or genes in *Populus deltoides* 'S9-2'^a

Marker	Accession ^b	Size (bp)	E value		Putative function of the gene ^c
			BLASTx	tBLASTx	
E40G37	AF393731	315	4e-036	2e-039	<i>Copia</i> -type polyprotein/retroelement (<i>Arabidopsis thaliana</i>)
E39G01	AF393732	209	2e-020	1e-018	Unknown protein (<i>Arabidopsis thaliana</i>)
E44G09	AF393733	139	1e-012	7e-014	DNA-directed RNA polymerase (subunit) (<i>Arabidopsis thaliana</i>)
E32G43	AF393734	120	>0.1	6e-004	...
E45G29	AF393735	334	>0.1	0.002	...
E47G14	AF393736	471	2e-027	4e-030	NBS/LRR disease resistance protein (<i>Arabidopsis thaliana</i>)
E39F39r	AF393737	378	>0.1	0.001	...
E48G14	AF393738	449	3e-019	3e-023	NBS/LRR disease resistance protein (<i>Solanum tuberosum</i>)
E61G36	AF393739	379	1e-012	2e-012	NBS/LRR disease resistance protein (<i>Arabidopsis thaliana</i>)
E51G05	AF393740	338	1e-030	1e-036	Zn finger protein, programmed cell death gene (<i>Arabidopsis thaliana</i>)
E43G28	AF393741	390	>0.1	0.002	...

^a Markers are listed according to their position on the genetic map. Size of markers includes the *Eco*RI and *Mse*I sites without adaptors; ... indicates no annotation. Only the best hit is given.

^b GenBank accession number.

^c NBS/LRR = nucleotide binding site/leucine-rich repeat.

which confers resistance to *M. lini*, is a complex locus composed of an array of tightly linked genes (2). In contrast, the *L* locus in flax consists of a single gene with 13 alleles in different cultivars (13). Qualitative resistance to three races of *M. larici-populina* co-segregates in all five populations analyzed, indicating that either one single gene or a set of tightly linked genes confers resistance to different races of this pathogen. In the interspecific *P. deltoides* × *P. trichocarpa* progenies described (14), resistance to races E1, E2, and E3 was also tightly linked; no recombinants could be detected. Nevertheless, because qualitative resistance to E3 was lost in F1 family 83B, and to E2 in F1 family 54B, a digenic model was proposed for resistance to these races. Therefore, it was postulated that a cluster of several tightly linked genes controls resistance to different races of this pathogen. In strong support to this hypothesis, sequence analyses of the AFLP markers flanking the *MER* locus showed the presence of three disease resistance gene analogs (Table 3). The similarities for these markers were significant to more than 20 homologs of the same class of disease resistance proteins in the BLAST analyses. The hypothesis of a gene cluster containing disease resistance genes is further supported by the presence of three DNA segments that share similarity to the TIR/NBS/LRR class of disease resistance genes in a 95-kb sequence of a BAC mapped to this region. Based on annotation, at least one of these three segments encodes a functional protein (M. Lescot, unpublished data).

Conclusions. Poplar exhibits qualitative and quantitative disease resistance to several pathogens. However, the corresponding disease resistance genes have not been isolated and characterized at the molecular level. Here, we constructed a fine-scale local genetic map around the gene or genes that confer resistance to *M. larici-populina* in the *P. deltoides* S9-2 genome. We have demonstrated, for the first time, the presence of genes sharing similarity to the NBS/LRR class of disease resistance genes in the poplar genome. This fine map is a starting point towards the positional cloning of the *MER* gene or genes from poplar.

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