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Identification of AFLP molecular markers for resistance against *Melampsora larici-populina* in *Populus*

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Abstract We have identified AFLP markers tightly linked to the locus conferring resistance to the leaf rust *Melampsora larici-populina* in *Populus*. The study was carried out using a hybrid progeny derived from an inter-specific, controlled cross between a resistant *Populus deltoides* female and a susceptible *P. nigra* male. The segregation ratio of resistant to susceptible plants suggested that a single, dominant locus defined this resistance. This locus, which we have designated *Melampsora* resistance (*Mer*), confers resistance against E1, E2, and E3, three different races of *Melampsora larici-populina*. In order to identify molecular markers linked to the *Mer* locus we decided to combine two different techniques: (1) the high-density marker technology, AFLP, which allows the analysis of thousands of markers in a relatively short time, and (2) the Bulked Segregant Analysis (BSA), a method which facilitates the identification of markers that are tightly linked to the locus of interest. We analyzed approximately 11,500 selectively amplified DNA fragments using 144 primer combinations and identified three markers tightly linked to the *Mer* locus. The markers can be useful in current breeding programs and are the basis for future cloning of the resistance gene.

Key words *Mer* · AFLP™ markers · Bulked segregant analysis · *Melampsora larici-populina* · *Populus*

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Introduction

The genus *Populus* is comprised of more than 30 species that belong to five different sections. Due to its fast growth rate, poplar has become a tree of primary economic importance. It has been estimated that poplar covers most of the artificial forest in Europe (Food and Agriculture Organization 1980). In Europe, poplar wood is used basically for the construction of boxes, pallets, soft board, and multiplex, whereas in the United States and Canada poplar wood is mainly processed to pulp and paper.

In the 17th and 18th centuries farmers in Europe, followed later by tree-breeders, selected trees for fast growth and wood quality. This breeding work has provided an abundance of information about the genetic material through multi-generation pedigrees and exhaustive screening of parents as well as respective progenies segregating for traits of scientific or commercial interest. Inter-specific hybrids, generally fertile, can be easily obtained and often display an extensive heterosis effect. Most poplar trees can be vegetatively propagated from stem or root cuttings, leading to the standardization of the genetic material for commercial or experimental use. Due to the advantageous molecular characteristics, genetic studies of the poplar genome are more accessible than in most forest trees; the nuclear genome is relatively small (approx. 550 Mb; 2C = 1.1 pg), and the chromosome number is the same for all species (2n = 38) (Bradshaw and Stettler 1993; Wang and Hall 1995). The level of polymorphism between species is high. Several species of poplar can be transformed (Leplé et al. 1992; Jouanin et al. 1993). For all these reasons poplar can be considered to be a model tree in different disciplines of forest tree biology.

The European poplar clones cultivated during the last two centuries are not immune to diseases as common and severe as leaf rusts. Therefore, tree breeders have generated and selected inter-specific hybrids that are resistant to different races of *Melampsora larici-populina* (Pinon 1992).

M. larici-populina is one of the most damaging fungi to poplar in Central and North Europe. Infection by *M. larici-populina* causes premature defoliation and can reduce growth by more than 20%. Trees defoliated early in the growing season are more susceptible to other pathogens and to environmental stress (Ostry et al. 1989). Infections over successive years can result in the complete loss of the plantation.

Since associations between genetic markers and traits of interest were first reported by Sax in 1923, much attention has been given to the potential applications of markers to improve plant breeding. This approach opens a multitude of new ways in tree improvement. It provides the basis for accelerated breeding through early selection of traits, which allows the selection within larger progenies. The use of molecular markers permits a more efficient selection of parents for subsequent breeding programs. Additionally, it should allow the selection of individuals showing a range of common traits of interest yet genetically diverse, thereby ensuring a high level of genetic polymorphism in the plantation. This high level of genetic polymorphism between individuals is expected to reduce the risk of a loss of the plantation due to new pathogens in comparison with monoclonal plantations.

Different molecular techniques, such as restriction fragment length polymorphism (RFLP)- or polymerase chain reaction (PCR)-based techniques, have been used with the aim of detecting molecular markers associated with simply inherited or complex traits (quantitative trait loci, QTLs) of poplar, especially those related to disease resistance and commercially important traits (Bradshaw and Stettler 1995a,b). The efficiency of the indirect selection will depend on the easiness of marker detection and the degree of linkage to the locus of interest. Several high-density marker technologies have recently been developed. The AFLP™ [1] technique developed by Vos et al. (1995) can be considered the most powerful.

Michelmore et al. (1991) described a screening method, the Bulked Segregant Analysis (BSA), based on bulking DNAs from individual plants to rapidly screen for molecular markers tightly linked to a locus of interest. In this paper, we report the identification of molecular markers tightly linked to the locus conferring resistance to *M. larici-populina* in *Populus* using AFLP and BSA. This strategy combines one of the most important advantages of the AFLP technology, the high number of loci that can be analyzed per experiment, with the rapid screening approach of BSA.

Materials and methods

Plant material

The three-generation *Populus* pedigree was founded in 1948 by crossing a female *P. deltoides* V5, resistant to races E1, E2 and E3 of *M. larici-populina*, and a susceptible male *P. deltoides* V1. Both trees were derived from cuttings obtained from natural populations in Iowa and Ontario, respectively. The obtained F_1 hybrid family S 9 consisted of 500 clones. A controlled cross between 1 clone of the family S 9, the female S 9-2 resistant to races E1, E2 and E3 of *M. larici-populina*, and the susceptible male *P. nigra* Ghoy (Belgium) was carried out in 1987 to produce the hybrid family 87001, which contained 262 clones.

Melampsora larici-populina resistance test

Microscopical identification of different species of *Melampsora* was based on the shape of uredospores and paraphyses following a key described by Pinon (1973). Resistance tests were carried out in the experimental nursery and by artificial infections in the laboratory. For the nursery tests, ten cuttings of all 262 seedlings were planted in the nursery in June. Plants were observed for their susceptibility to *M. larici-populina* in October. The score system used comprised 11 values ranging between 0 and 5, including half-values: 0 (rust is not present), 1 (slight presence of rust at the lower-third of the crown, but no necrosis), 2 (moderate attack on the lower-third without necrosis, but the top-third stays intact), 3 (rust throughout the crown with necrosis in the lower-third), 4 (severe attack throughout the crown, but necrosis and dead leaves affect less than 50% of the crown), and 5 (severe attack throughout and more than 50% of the crown is affected by dead leaves and necrosis). The score reflects an average for the ten cuttings per seedling.

For the laboratory tests, three cuttings of all seedlings were grown in the greenhouse. Leaf discs were cut and floated upside-down on de-mineralized water. The artificial infections were performed inoculating the leaf discs by pulverization with a suspension of uredospores (5,000 spores/ml). Three rust races, E1, E2 and E3, were used for inoculation : E1 isolated from *P. euramericana* Robusta, E2 from *P.* × *euramericana* Ogy, and E3 from a (*P. trichocarpa* × *P. deltoides*) × *P. deltoides* clone 76 004/10 (collection of IBW-Geraardsbergen). Leaf discs floating on de-mineralized water were incubated at 22 °C under continuous illumination for 14 days. All of the leaf discs were scored for the appearance (susceptibility) or absence (resistance) of uredospores. The susceptibility of the seedlings to the three races of *M. larici-populina* was scored separately. Each seedling-race interaction was studied on two leaf discs.

AFLP analysis

Genomic DNA was extracted from young frozen leaves using the procedure described by Dellaporta et al. (1983). AFLP analysis was performed according to Vos et al. (1995) with some modifications. The DNA was digested using two restriction enzymes. For poplar we used *Mse*I (frequent-cutter) and *Eco*RI (rare-cutter). Digestion was carried out in a final volume of 50 µl in 10 m*M* Tris-HAc, 10 m*M* MgAc, 50 m*M* DTT, pH 7.5, 12 U *Eco*RI (Eurogentec), 8 U *Mse*I (New England Biolabs), and 250 ng of genomic DNA for 3 h at 37 °C. Two different adaptors that had been designed to avoid the reconstruction of these restriction sites, one for the *Eco*RI sticky ends and one for the *Mse*I sticky ends, were ligated to the DNA by adding to the digestion 5 µl of a mix containing 5 pmol *Eco*RI bio-adaptor, 50 pmol *Mse*I adaptor, 8 m*M* ATP, 10 m*M* Tris-HAc, 10 m*M* MgAc, 50 m*M* DTT, pH 7.5, and 1.4 U T4 DNA ligase (Boehringer). The ligation was incubated for 3 h at 37 °C and overnight at 4 °C. The adaptor for *Eco*RI contains a 5'-biotinylated top strand, which is covalently linked by ligation to the DNA fragments containing the *Eco*RI site. The *Mse*I adaptor is not biotinylated.

All fragments containing *Eco*RI ends were subtracted from the reaction mixture using streptavidin beads (Dynal) to reduce the complexity of the DNA, which eases subsequent amplification reactions. This selection of biotinylated fragments was performed using 10 µl (100 µg) of beads per reaction. The beads were washed with 100 µl of STEX buffer containing 100 m*M* NaCl, 10 m*M* Tris-HCl, 1 m*M* EDTA, 0.1% Triton X-100, pH 8.0, and resuspended in 140 µl of STEX buffer. The beads were added to the DNA, to a final volume of 200 µl. To optimize the binding of biotinylated DNA to the beads, the mix was incubated for 30 min at room temperature with gentle agitation. The beads were collected with a magnet and, after the

[1] AFLP is a registered trademark in the Benelux

removal of the supernatant, they were washed three times with 200 µl of STEX buffer. Finally, the beads were resuspended in 200 µl of T01.E (10 m*M* Tris-HCl, pH 8.0, 0.1 m*M* EDTA) and kept at 4 °C.

A preamplification step, prior to the selective radioactive PCR, was done. The preamplification consists of a PCR reaction using primers that are complementary to the adaptors *Eco*RI and *Mse*I with an additional selective 3′ nucleotide. In this way, only 1/16 of the DNA fragments will be amplified. The PCR reactions were performed in a 50-µl volume of 10 m*M* Tris-HCl (pH 8.3), 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.2 m*M* of each dNTP, 75 ng of each primer (GENSET), 1 U *Taq* DNA polymerase (Beckman), and 5 µl of biotinylated fragments. The PCR amplifications were carried out either in a Techne PCH-3 or in a Perkin Elmer 9600 using 28 cycles, each cycle consisting of 30 s at 94 °C, 1 min at 60 °C, and 1 min at 72 °C.

The preamplification products were diluted to be used as starting material for the selective radioactive amplification; thus, 10 µl of preamplified material were diluted by adding 70 µl H_2O. For the selective radioactive amplification, only the *Eco*RI primer was labelled; both the *Eco*RI primer and the *Mse*I primer contain the same sequences as those used in the preamplification but with three selective nucleotides at the 3′ end. The PCR reaction was performed in a 20-µl volume of 10 m*M* Tris-HCl (pH 8.3), 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.08 m*M* of each dNTP, 4 ng [^{33}P]-*Eco*RI primer, 24 ng *Mse*I primer, 0.4 U *Taq* DNA polymerase (Beckman), and 5 µl of diluted preamplified DNA. The selective amplification was carried out using the following cycling parameters: 1 cycle of 30 s at 94 °C, 30 s at 65 °C, 1 min at 72 °C followed by 12 cycles in which the annealing temperature decreases 0.7 °C per cycle, followed by 23 cycles of 1 min at 94 °C, 30 s at 56 °C, and 1 min at 72 °C. The start at a very high annealing temperature allows optimal primer selectivity. By decreasing gradually the annealing temperature, we could increase the efficiency of primer binding.

At the end of the selective radioactive PCR, the samples were denatured by adding an equal volume (20 µl) of formamide buffer (98% formamide, 10 m*M* EDTA, pH 8.0, 0.05% bromo-phenol blue, and 0.05% xylene cyanol) and heating for 5 min at 95 °C. Three to 5 µl of each sample were loaded on 4.5% acrylamide/bisacrylamide 19:1, 7.5 *M* urea and 1 × TBE gels.

AFLP marker analysis

AFLP markers linked to the resistance locus were identified by BSA (Michelmore et al. 1991). The bulks were made by pooling equal amounts of 17 preamplified DNAs obtained from resistant plants and 19 from susceptible plants from the family 87001. The primer combinations yielding candidate markers were subsequently applied to each individual DNA from the family 87001 in order to confirm the linkage between the AFLP marker and the resistance locus.

Linkage analysis

Linkage estimations were based on the maximum likelihood using the appropriate formulas of Mather (1938). This recombination fraction was transformed in centiMorgans (cM) according to Kosambi (1944).

Results

Genetics of resistance to *Melampsora larici-populina* in *Populus*

The family S 9 (see Materials and methods), generated in 1948 by crossing *P. deltoides* V5 × *P. deltoides* V1, segregated in a 1:1 ratio for resistance to E1, E2, and E3, three different races of *M. larici-populina* (V. Steenackers, personal communication). Also, *P. deltoides* V5 was resistant to all three races. The susceptibility

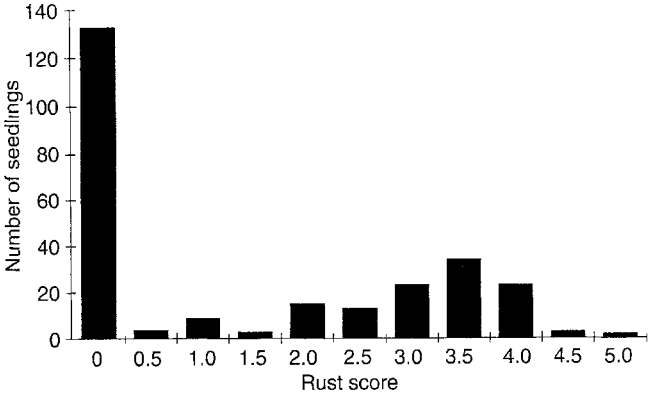

Fig. 1 Distribution of seedlings of the family 87001 (number of seedlings) as a function of the degree of susceptibility to *Melampsora larici-populina* (rust score). The total number of clones analyzed was 262. The rust score is divided into values ranging from 0 (rust not present) to 5 (severe attack) based on nursery data (see Materials and methods)

of the progeny obtained from the cross between the resistant female S 9-2 and the susceptible male *P. nigra* Ghoy to the three races of *M. larici-populina* was tested in the nursery and in the laboratory. As shown in Fig. 1, in the nursery about 50% of the seedlings were fully resistant to all three races of *M. larici-populina* (133 R:129 S: $\chi^2_{1\,1} = 0.06$, $0.75 > P > 0.90$, 1 *df*), whereas the other 50% were susceptible to various degrees. All clones that were scored as being resistant in the nursery (score 0) were also scored as resistant in the laboratory. Similarly, all clones that were susceptible in the nursery (scores 0.5–5) were also scored as susceptible in the laboratory (data not shown). The data suggest that the resistance is determined by a single dominant locus and that both females, *P. deltoides* V5 and S 9-2, were heterozygous for the resistance trait. We designated this locus *Melampsora* resistance (*Mer*). An additional oligogenic or multigenic horizontal (quantitative) resistance is suggested by the normal distribution shown by the susceptible progeny (Fig. 1).

Identification of markers linked to resistance in *Populus*

A total of 144 primer combinations was used to test parents and bulks (see Materials and methods), and these produced approximately 11,500 selectively amplified DNA fragments ranging in size from 70 to 800 nucleotides. On average, 60–100 distinguishable bands were observed after selective amplification with each primer combination, and on average 30–60 of these AFLP bands were polymorphic between both parentals, with 12–25 of these polymorphic bands usually segregating in a 1:1 ratio (e.g., markers for resistance to *Melampsora* in family 87001).

Primers E39/G01 (*Eco*RI + AGA/*Mse*I + AAG), E40/G37 (*Eco*RI + AGC/*Mse*I + TGT), and E44/G09

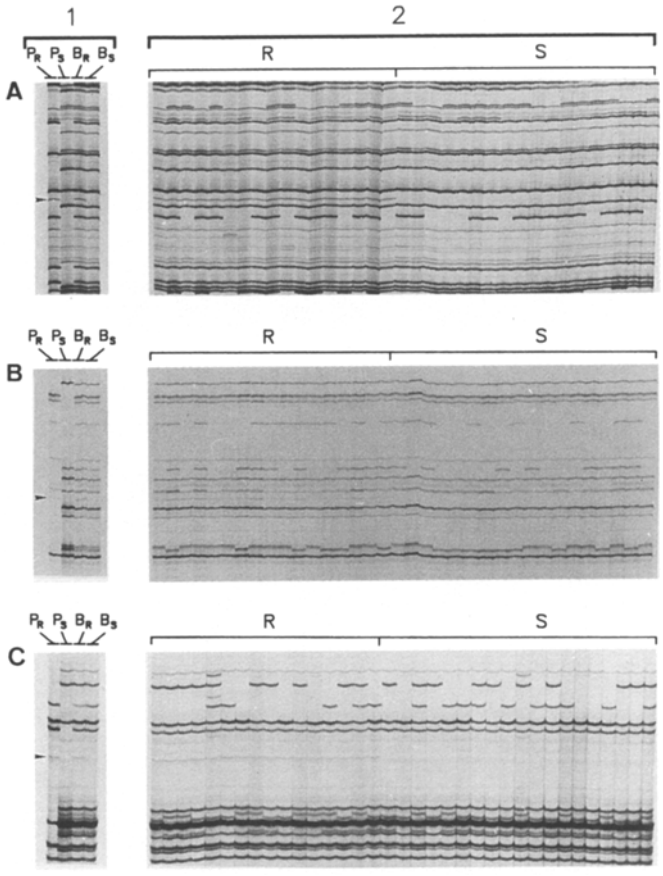

Fig. 2A–C AFLP markers linked to the *Mer* locus. *1* The Bulked Segregant Analysis (BSA) is presented as a set of four lanes: resistant parent *P. deltoides* (P_R), susceptible parent *P. nigra* (P_S), resistant bulk (B_R), and susceptible bulk (B_S). *2* The AFLP markers E39.G01.270 (**A**), E40.G37.320 (**B**), and E44.G09.100 (**C**), identified by BSA and indicated by an *arrow*, are present in the resistant (*R*) but absent in the susceptible (*S*) F_1 progeny

(*Eco*RI + ATC/*Mse*I + ACT) each revealed one AFLP marker with a size of approximately 270, 320, and 100 nucleotides, respectively. These markers were present in the resistant parent and the resistant bulk but absent in the susceptible parent and the susceptible bulk (Fig. 2,*1*).

A total of 123 DNA samples, obtained from individual clones of the family 87001, was analyzed to determine the linkage between those AFLP markers and the *Mer* allele (Fig. 2,*2*): only one recombinant tree was detected using markers E39.G01.270 and E44.G09.100, whereas the same recombinant and two more were detected with marker E40.G37.320, indicating that all three markers were tightly linked. Linkage between E39.G01.270, E40.G37.320, E44.G09.100 and the *Mer* locus was 0.81 ± 0.81, 2.44 ± 1.39, and 0.81 ± 0.81 cM, respectively.

Discussion

The results obtained indicate that resistance to three different races of *M. larici-populina* in *P. deltoides* V5 and S 9-2 is determined by a single dominant locus. The resistance might be conferred either by a single gene or by a set of closely linked genes. Three molecular markers tightly linked to the *Mer* locus were identified using a strategy that combined a BSA approach and the AFLP technique to screen a segregating family.

Recently, several high-density marker technologies have been developed, among which AFLP can be considered the most powerful (Vos et al. 1995). The AFLP technique detects polymorphisms such as the presence or absence of restriction enzyme sites, sequence polymorphisms adjacent to these sites, insertions, deletions, and rearrangements.

One of the most important advantages of the AFLP technique is the high number of markers that can be screened per experiment. Using *Eco*RI/*Mse*I and 144 primer combinations, we obtained 11,500 selectively amplified DNA fragments. Of these, 50–60% were polymorphic between both parents. Hence, about 3,000 loci were screened for each parental genome. Given that the poplar genome is 2,400-2,800 cM (Bradshaw et al. 1994), this corresponds to an average of one AFLP marker for every 0.9 cM. We have obtained three markers, two of them at $0.81 + 0.81$ cM and one at 2.44 ± 1.39 cM, from the *Mer* locus.

Another advantage of the AFLP technique compared to other PCR marker technologies is its reproducibility. RAPD analysis, based on the use of random primers, is known to suffer from a lack of reproducibility due to mismatch annealing (Neale and Harry 1994). The primers used to obtain AFLP markers combine two key characteristics: (i) they are complementary to the adaptor oligonucleotide, thus allowing a highly specific primer annealing, and (ii) they are selective, changing the 3′ nucleotides allows amplification of a different set of DNA fragments from the same population of pre-amplified fragments (Vos et al. 1995). The combination of BSA, as a strategy to rapidly screen for markers tightly linked to monogenic traits, and AFLP analysis, a high-density marker technology, enables a very efficient identification of markers adjacent to the locus of interest.

The AFLP marker system satisfies the requirements for gene introgression. The tightly linked AFLP markers identified in the resistant individuals of family 87001 can be used to select resistant individuals bred from the same progenitors or from a cross between the same resistant female and other susceptible males when the latter do not display the markers. Cloning and sequencing of the AFLP markers could help to turn them into a Sequence Characterized Amplified Region (SCAR; Paran and Michelmore 1993), which may further facilitate the screening of a progeny by a single and cheap PCR assay. SCARs might be developed to screen progenies obtained from crosses using other resistant *P. deltoides* clones that share the same resistance locus. In addition to the studied AFLP markers, the use of the AFLP technolgy to characterize other race-specific resistance loci and their subsequent mapping may help us to better understand the genetic basis of resistance

against the different races of *M. larici-populina*. We are constructing a high-density AFLP genome map of *P. deltoides* S 9-2 and *P. nigra* Ghoy that might serve in the cloning of this locus by chromosome landing when YAC or BAC libraries are available (Tanksley et al. 1995; Thomas et al. 1995).

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