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Suppressed recombination around the *MXC3* locus, a major gene for resistance to poplar leaf rust

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Abstract A positional cloning strategy is being implemented in *Populus* for the isolation of the dominant *MXC3* allele, which confers resistance to poplar leaf rust caused by *Melampsora×columbiana* (pathotype 3). AFLP markers were used to saturate the chromosomal region around the *MXC3* locus in a large (*n*=1,902) *Populus trichocarpa×P. deltoides* (T×D) mapping pedigree segregating 1:1 for rust resistance and susceptibility. The high-resolution linkage map developed around the *MXC3* locus contains 19 AFLP markers and spans a genetic distance of 2.73 cM. Of the 19 AFLP markers, seven were found to co-segregate with the locus. One co-segregating AFLP marker, CCG.GCT_01, was converted to an STS marker (*BVS1*) and used to identify a physical contig of overlapping BAC clones from the *MXC3* region. Genetic and physical mapping of markers isolated from the BAC contig failed to delimit the *MXC3* locus within a 300-kb interval defined by the overlapping BAC clones. This result indicates a >25-fold reduction in recombination frequency in the *MXC3* region compared to the average rate of recombination for the *Populus* genome.

Keywords Map-based cloning · Hybrid poplar · Plant disease resistance

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Introduction

Interspecific *Populus* hybrids are the fastest-growing trees in the temperate zone, and have been recognized as an important source of pulp, lumber, and biofuel (Zsuffa et al. 1996). Disease can have a serious impact on the growth of hybrid poplars. Leaf rusts, caused by several species of the fungal pathogen *Melampsora*, are problematic worldwide and can be extremely damaging, particularly in recently established poplar plantations (Newcombe et al. 1994).

In 1997, three newly evolved rust pathotypes were collected from hybrid poplars (*Populus trichocarpa×P. deltoides*) in Washington and Oregon, USA. These novel fungal pathotypes proved to be interspecific hybrids between *Melampsora medusae* and *Melampsora occidentalis*. The hybrid rusts have been described as pathotypes 1, 2, and 3 of *Melampsora×columbiana* (Newcombe et al. 2000), and have interesting implications for the evolution of pathogenic variation (Brasier 2000) in a 'hybrid vs. hybrid' host-pathogen interaction.

As part of routine nursery screening for disease resistance in seedlings bred by the Poplar Molecular Genetics Cooperative (PMGC), an F_1 interspecific hybrid poplar pedigree (*P. trichocarpa×P. deltoides*) was discovered apparently segregating in a 1:1 ratio for resistance: susceptibility to *M.×columbiana 3*. The putative rust resistance locus responsible for this pattern of segregation is the focus of our investigation. We have named the resistance gene *MXC3*. The immediate objectives of this study were to examine the inheritance of resistance to *M.×columbiana 3* in hybrid poplar and to construct a fine-structure genetic and physical map around the *MXC3* resistance gene.

The longer-term goal is to positionally clone *MXC3* to assist in understanding its role in both natural and plantation forests, and to genetically engineer susceptible but commercially valuable hybrid poplars for resistance to this new leaf rust pathotype. *Populus* appears to be a favorable system for positional gene cloning, with a physical genome size of 550 Mbp (approximately five

times that of *Arabidopsis*; Bradshaw and Stettler 1993a, b), and a physical:genetic distance ratio of roughly 220 kb/cM (essentially the same as *Arabidopsis*; Bradshaw et al. 1994).

Materials and methods

Genetic mapping pedigree

A female *P. trichocarpa* (clone 383–2499) from western Washington was crossed with a male *P. deltoides* (clone 14–101) from central Illinois to produce a genetic mapping pedigree of 2,028 F_1 progeny (PMGC Family 545). The *P. trichocarpa* parent is assumed to be heterozygous for the dominant resistance gene (*MXC3*/*mxc3*), while the *P. deltoides* parent is assumed to be homozygous for the recessive susceptibility allele (*mxc3*/*mxc3*). Thus, the F_1 progeny of these parents should segregate 1:1 in a testcross ratio for resistance:susceptibility to *M.×columbiana 3*. F_1 hybrid seedlings were planted at the Washington State University Farm 5 nursery near Puyallup, Wash., at a spacing of 0.7 m×1.4 m, and irrigated as necessary during establishment. Parental clones were established in a nearby stoolbed.

Dormant hardwood cuttings from the mapping pedigree, including both parental clones, were collected in January 1998 and grown in the greenhouse for determination of disease phenotypes in a controlled environment. To ensure accurate phenotypic assessment, three 30 cm cuttings were collected from each clone. Cuttings were rooted in potting soil in 2-l pots. The pots were arranged in a randomized complete block design (three blocks, with one replicate of each hybrid poplar clone per block) to minimize phenotypic scoring error due to edge effects. The cuttings were planted on a staggered schedule over a 12-day period so that the subsequent labor-intensive phases of the experiment (i.e., inoculation with the rust fungus and phenotypic scoring of resistance or susceptibility) could be performed on trees of comparable age and size. The trees were watered twice daily, fertilized with Peter's Peat-lite (Marysville, Ohio) twice weekly, and provided with 16 hours of light (natural sunlight supplemented with metal halide lamps) daily. Greenhouse temperature was maintained at 21–24°C during the day and 14–18°C at night. The trees were grown for 2 months to a height of approximately 40 cm with approximately 20 leaves prior to inoculation with *M.×columbiana 3* urediniospores.

Preparation of *M.×columbiana 3* inoculum

All inoculations were performed with a monouredinial (i.e., genetically uniform) isolate, known as 96–3, of the hybrid rust *M.×columbiana 3*. This isolate was collected from near Puyallup, Wash. Its identification as *M.×columbiana 3* was reported elsewhere (Newcombe et al. 2000).

A large number of urediniospores, sufficient to infect all of the trees in the mapping pedigree, were obtained by inoculating 50 ramets of the highly susceptible hybrid poplar (*P. trichocarpa×P. deltoides*) clone 47–174. Inoculations were performed by spreading urediniospores with a moist cotton swab onto lightly misted leaves at leaf plastochron index position (LPI) 4 or 5 (Larson and Isebrands 1971). Infected trees were covered with a plastic tent overnight to promote establishment of the rust fungus. Fourteen days after inoculation the trees were sprayed with water daily to splash-disperse the inoculum to uninfected leaves. The *M.×columbiana 3* inoculum was increased in this way over a period of 2 months prior to inoculating the trees in the mapping pedigree.

Phenotypic scoring of response to *M.×columbiana 3* infection

Inoculations of the trees in the mapping population were carried out as described above for clone 47–174. Fourteen days after infection, trees in the mapping pedigree were scored as 'resistant' or 'susceptible' on the basis of the presence or absence of necrotic flecking around the site of infection (Newcombe et al. 1996). The presence of necrotic flecking was interpreted as a hypersensitive response to rust infection by resistant tree genotypes. For 60 tree genotypes (3%) in the mapping pedigree there was an ambiguity in scoring of the rust resistance phenotype among ramets of the same clone. For each of these 60 clones, the phenotype was assigned based upon the majority score; i.e., if two of the three ramets were scored as 'resistant', and third was scored as 'susceptible', the clone phenotype was recorded as 'resistant.'

Molecular-marker analysis

Young leaf tissue was collected from the parents and F_1 offspring of the mapping pedigree. Total genomic DNA was extracted from each clone using the FastPrep homogenizer and FastDNA extraction kits (BIO 101, Vista, Calif.).

AFLP marker analysis was performed essentially as described by Vos et al. (1995). AFLP markers were named by using the three selective nucleotides in the *Eco*RI and *Mse*I primers, followed by a number representing the marker number for that primer combination. For example, CCG.AAC_01 denotes the first marker from primer combination *Eco*RI+CCG and *Mse*I+AAC.

Bulked segregant analysis to identify AFLP markers linked to *MXC3* was performed as outlined by Michelmore et al. (1991). Two DNA pools (bulks) were prepared by combining equal amounts of DNA from either nine resistant (R) or nine susceptible (S) F_1 trees from Family 545.

Tightly linked AFLP markers were converted to sequence-tagged-site (STS) (Olson et al. 1989) markers by cloning the AFLP band, sequencing the cloned fragment, and designing locus-specific PCR primers. The desired AFLP bands were sliced from the polyacrylamide gel, eluted in water, re-amplified with the appropriate AFLP primers, resolved on an agarose gel, purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, Calif.), and cloned into pGEM-T Easy (Promega, Madison, Wis.). Cloned fragments were sequenced using BigDye chemistry (PE Biosystems, Foster City Calif.) and an ABI377 Prism DNA sequencer. STS primers were purchased from Operon Technologies (Alameda Calif.).

Construction of a *P. trichocarpa* (clone 383–2499) bacterial artificial chromosome (BAC) library

Ramets of *P. trichocarpa* (clone 383–2499) were grown in the greenhouse. The trees were placed in the dark for 2 days to deplete starch reserves prior to harvesting approximately 60 g of fresh, young leaves.

Extraction of high-molecular-weight genomic DNA from leaf nuclei was performed as described in Zhang et al. (1994). Poplar genomic DNA was partially digested with *Hin*dIII followed by three rounds of size-selection from agarose gels following separation by pulsed field gel electrophoresis (PFGE). In the first two size selections, the pulsed field gel was run with a 90-s pulse at 160 V for a total run time of 18 h at 11°C. In both runs DNA ranging from 200 kb to 400 kb was excised from the gel and used in the next size selection. The final size-selection gel was run using a 6 s pulse at 150 V for 11 h at 11°C, and the compressed band representing DNA fragments greater than 150 kb was excised. Prior to ligation the final size-selected DNA was released from agarose by electroelution. The agarose-gel slice containing the >150 kb DNA was fragmented with a razor blade and the resulting pieces placed in dialysis bags (Gibco BRL, Rockville, Md.). Electroelution was carried out at 11°C for 2 h at 200 V followed by a 90-s reversed-current pulse. Eluted DNA was quantified by inspection on an agarose gel.

*Hin*dIII-digested and dephosphorylated pBeloBAC II (Shizuya et al. 1992) was used for library construction. A molar ratio of approximately 3:1 vector:insert was used for ligation. Transformations were performed by electroporation using Gene Hogs electrocompetent cells (Research Genetics, Huntsville, Ala.). Transformed

cells were plated onto LB (Difco, Sparks, Md.) agar supplemented with 12.5 µg/ml of chloramphenicol and 0.25% X-gal. White colonies (*n*=48,384) were picked and grown in 126 384-well microtiter plates.

BAC library screening

To facilitate the screening of the BAC library using PCR-based markers, a pooled DNA sample was prepared from each of the 126 microtiter plates in the *Populus* BAC library. For each 384-well plate, a VP 409 multi-blot pin replicator with 96 pins (V&P Scientific, Inc., San Diego Calif.) was used to inoculate four 96-well plates containing 1 ml per well LB supplemented with 12.5 µg/ml of chloramphenicol. The four 96-well plates were grown overnight (16–18 h) at 37°C with vigorous shaking. The bacterial cultures from the four plates were pooled and DNA was extracted using a Qiagen Plasmid Midi Kit (Qiagen, Valencia, Calif.).

Plate pool DNA preparations were screened by PCR with STS primers derived from the AFLP markers. PCR was performed in a 10-µl reaction volume containing 0.4 units of Ampli*Taq* DNA Polymerase (PERKIN ELMER, New Jersey), 1×PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, and 1.3 mg/ml of bovine serum albumin), 0.2 mM of each dNTP, 20 ng of each STS primer and 50 ng of BAC DNA. Amplification was performed in a PTC-100 thermocycler (MJ Research, Waltham, Mass.) as follows: one cycle at 94°C for 45 s, 29 cycles at 94°C for 15 s, annealing for 15 s (annealing temperatures varied according to the STS primer pair, see Tables 1 and 2), and 72°C for 1 min. A final extension of the amplified product at 72°C for 5 min completed the reaction. PCR reactions were analyzed by agarose-gel electrophoresis. BAC library plates containing the STS marker were re-screened to identify the positive BAC clone(s) in the 384-well plate. DNA was prepared from the BAC clones as described above for the plate pools. Approximately 25–50 µg of BAC DNA was isolated from a 250-ml culture. The insert size for each BAC clone was determined by pulsed-field gel electrophoresis (PFGE) using a rotating gel apparatus as described by Smith et al. (1989). BAC DNA (approximately 5 µg) was digested with *Not*I (New England Biolabs, Beverly, Mass.) for 3 h to release the insert from the vector. The digested DNA was loaded on a 0.8% low-melting-point gel and run at 5.0 V/cm, with a 12-s pulse, for 24 h at 6°C. The size of each BAC clone was determined by comparing with a λ concatamer size standard run in the same gel.

BAC sequencing and subcloning

The ends of each BAC clone were sequenced using the T7 and SP6 vector primers as described above for the development of AFLP-derived STS markers. PCR primer pairs were designed from BAC-end sequences for contig assembly and the development of STS markers. The construction of sub-libraries for each BAC clone was performed by digesting BAC DNA (approximately 1 µg) with a variety of restriction enzymes. The resulting fragments were ligated into pBluescriptII SK+ (Stratagene, La Jolla, Calif.). Transformants were picked at random and the ends sequenced for the development of STS markers. A variety of different polymorphisms were revealed with fragments amplified from BAC-end or BAC-subclone STS primers, including band presence/absence polymorphisms, length polymorphisms, or restriction fragment length polymorphisms (i.e., RFLPs or CAPS) (Southern 1975; Saiki et al. 1985).

BAC fingerprinting and automated assembly of fingerprints

Preparation of BAC DNA, restriction endonuclease digestion with *Hin*dIII, agarose-gel electrophoresis, data acquisition, and manual contig construction were performed essentially as described by Marra et al. (1997). Data acquisition was performed interactively using the program Image (Sulston et al. 1988, 1989). Contig assembly was performed using the fingerprint contigs (FPC) software (Soderlund et al. 1997). The FPC builds generated for this study were performed using a tolerance of 7 and a stringency (Sulston score) of 10^{-10}. Image and FPC have been developed and maintained at the Sanger Centre; documentation and user's manuals are available on the Sanger Centre website (http://www.sanger.ac.uk).

Fig. 1 The resistant reaction (right) to *Melampsora×columbiana 3* is characterized by a hypersensitive response resulting in necrotic flecks: this contrasts with the lack of necrosis and the high density of urediniospores observed in the susceptible reaction (left)

Results

Variation among poplar genotypes in response to *M.×columbiana* infection

An interspecific F_1 hybrid poplar pedigree, consisting of two parents and their 2,028 progeny, was tested for resistance or susceptibility when challenged with urediniospores of the leaf rust fungus *M.×columbiana 3*. The female *P. trichocarpa* parent was asymptomatic. We found no evidence of successful infection by *M.×columbiana 3* on this clone. Neither sporulation of the fungus nor necrotic flecking of the leaf tissue, which would indicate a hypersensitive response, were observed. The *P. trichocarpa* parent is thus categorized as 'resistant' to infection by *M.×columbiana 3*.

In contrast, the male *P. deltoides* parent showed heavy infection and abundant sporulation in response to inoculation, with no necrotic flecking surrounding the infected leaf tissue. The *P. deltoides* thus has a 'susceptible' phenotype. The F_1 progeny exhibited a range of responses to infection by *M.×columbiana 3*. None of F_1 clones were asymptomatic as was their *P. trichocarpa* parent. However, 1,082 of the 2,028 F_1 hybrids displayed a 'resistant' phenotype characterized by necrotic flecking (a presumed hypersensitive response) around the fungal uredinia (Fig. 1). There were 946 F_1 clones classified as

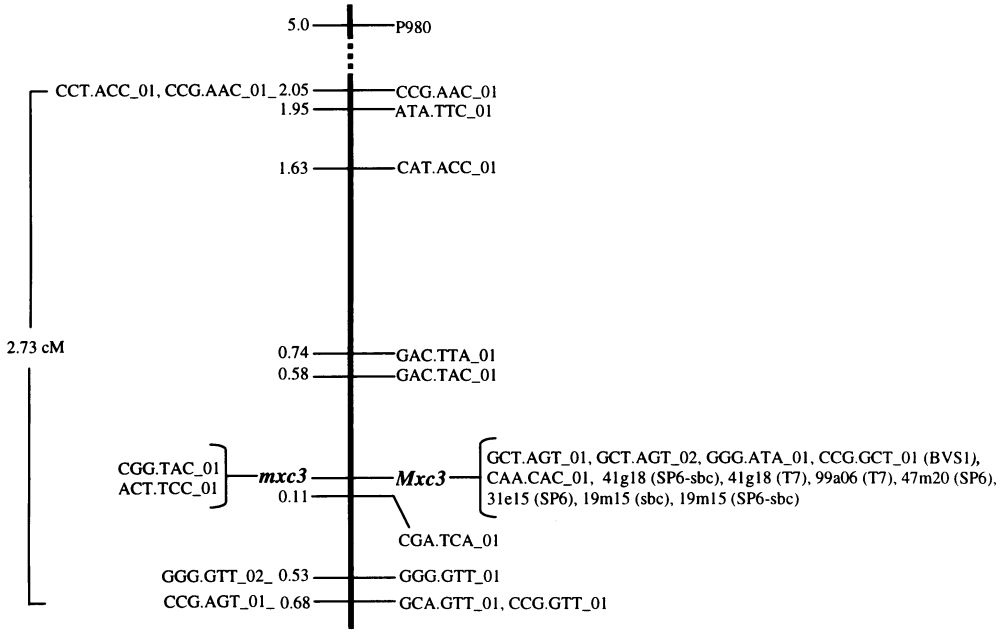

Fig. 2 High-resolution genetic map around the *MXC3* region. The map consists of 19 AFLP markers and seven STS markers derived from BAC-end sequences or BAC subclones. Markers are positioned to indicate their linkage phase with respect to the dominant allele (*MXC3*) or the recessive allele (*mxc3*). Map distances shown for each marker represent the total distance in centimorgans (cM) from *MXC3* and are based on a progeny size of 1,902. Markers derived from BAC clones are designated by plate# – row letter – column#. *SP6* and *T7* suffixes designate BAC-end markers, *sbc* designates a marker from a BAC subclone. Marker P980 is an allele-specific STS marker identified from previous mapping studies of the *Populus* leaf rust resistance locus *Mmd1* (Newcombe et al. 1996)

'susceptible' based upon the absence of necrotic flecking at the sites of infection, abundant sporulation of the rust, and moderate chlorosis of the leaves (Fig. 1).

Although there appears to be an excess of resistant phenotypes based upon the expected 1:1 segregation for resistance (χ^2=9.12; df=1; *P*=0.0025), this excess is due to the presence of triploid or aneuploid F_1 progeny carrying both maternal alleles at the *MXC3* locus. Triploidy and aneuploidy have been observed frequently in other hybrid poplar pedigrees (Bradshaw and Stettler 1993a), and can confound a study of Mendelian inheritance, which assumes a regular meiosis. For this reason, a codominant AFLP marker (CCG.AAC_01; Fig. 2) linked to the *MXC3* locus in *P. trichocarpa* (clone 383–2499) was used to identify triploid/aneuploid progeny in Family 545 so that they could be excluded from further analysis. Of the 2,028 F_1 hybrids in Family 545, 126 (6%) were found to carry both maternal alleles of the co-dominant AFLP marker. All but one of the 126 triploid/aneuploid F_1 progeny were resistant to infection by *M.×columbiana 3*.

Considering only the 1,902 diploid F_1 offspring, the observed ratio of 956 resistant:946 susceptible trees is not significantly different from the 1:1 Mendelian expectation for a cross between a *P. trichocarpa* heterozygote (*MXC3*/*mxc3*) and a *P. deltoides* recessive homozygote (*mxc3*/*mxc3*) (χ^2=0.08; df=1; *P*=0.78), suggesting that a single dominant gene is indeed responsible for the observed pattern of inheritance.

Identification of genetic markers tightly linked to *MXC3* by AFLP and bulked segregant analyses

To expedite the search for molecular markers tightly linked to *MXC3* we used bulked segregant analysis (Michelmore et al. 1991) and AFLP markers (Vos et al. 1995). A total of 2,048 AFLP primer combinations were screened on bulked (pooled) DNA samples representing nine rust-resistant (R) and nine susceptible (S) F_1 clones from the mapping pedigree. Analysis of 40 different AFLP primer combinations on the mapping parents and the F_1 clones comprising the bulks allowed us to calculate the average number of polymorphic markers inherited from the *P. trichocarpa* parent. Approximately 5.3 loci per AFLP primer pair were heterozygous in the *P. trichocarpa* parent. Thus, an estimated 10,854 polymorphic loci were screened on the bulked DNA samples, providing a nominal marker spacing of 0.25 cM in the 2,500-cM *Populus* genome (Bradshaw et al. 1994). Inspection of the AFLP autoradiograms led to the identification of 380 (3.5%) candidate polymorphisms potentially linked to the *MXC3* locus in *P. trichocarpa*.

Each candidate AFLP was tested for linkage to *MXC3* by screening an expanded set of DNA samples including the parents, the bulks, and the 18 F_1 clones that were used to make up the R and S bulks. Of the 380 candidates, 30 AFLPs had no recombination with *MXC3* in the 18 F_1 clones tested. Four of the putatively linked AFLP markers, CCG.AAC_01, CCG.GCT_01, CCG.AGT_01 and CAA.CAC_01, were tested for recombination with *MXC3* in a subset of 100 resistant and susceptible diploid F_1 clones from the mapping pedigree. We identified three recombinant F_1 clones with marker CCG.AGT_01 and three different recombinants for marker CCG.AAC_01. No recombinants were found with markers CCG.GCT_01 or CAA.CAC_01 in the 100 F_1 trees tested.

These four AFLP markers were further tested on the remaining 1,784 diploid F_1 clones of the mapping pedigree. Once again, markers CCG.GCT_01 and CAA.CAC_01 were found to be completely linked to *MXC3* (Fig. 2). Using markers CCG.AGT_01 (13 recombinants) and CCG.AAC_01 (39 recombinants), a set of 52 recombinant

Table 1 BAC clones isolated from the *P. trichocarpa* (383–2499) BAC library

BAC clones[a]	Identified by[b]	Insert size (kb)	Primers for SP6 end[c] (5′-3′)	Annealing temp. (°C)	Primers for T7 end[c] (5′-3′)	Annealing temp. (°C)
19m15	47m20 (SP6)	160	NA	NA	ATGACACGGATTCCAGCTTTG CAAACCTGAGATTCGTGTCATG	60
58c06	47m20 (SP6)	150	CACCATGAAAATCATACACCC GGGTTGTAGTTTTGTAATTATCC	55	CACTAATGAAAGTAGAGTATTTGC AATTATCTCAATAGTTTGGTGATATA	55
31e15	47m20 (SP6)	120	AATGGAAGAGAGGGACCAGC ATCGTGCTGATCACTAATCGA	55	CTTGAAGCATTTGTAAAGTTTCC AGTATCTGGGTAGAGTCAGC	55
69j01	47m20 (SP6)	110	CTAACCATCACAGCAAGGAC CCTCTCTTCCATTAAATTATGTC	55	CCATGGTTTATGGCCCTATC GATTAGAGATTCAGTTTCTTCTC	55
85a17	47m20 (SP6)	120	TCCACCATGAAAATCATACACC AAGACATTTCATGATCGGAGG	55	GCAAGCTTCATGCATCTATATC GAAGGCTTGGTACGAATTAGC	55
18o24	47m20 (SP6)	60	CATGTGTAATGACACAGACAAC AGATAATTATTCCATTATATTGTG	50	ATCCAAGTAGTGTATGTTTATAC GGTGGGTGTAATATTTGTATCC	55
47m20	BVS1	100	TAAGTGTTGACTGAATGATG ACTTAGATTTCTCATTCCTAC	50	TGCAATCCCATCTCCCTAG TTGGTGCGGATCCATGCAG	55
111j19	BVS1	110	GGGTCCTGAAGTTAACGATC TTAGAATTTGGTCCCCTCAC	55	CTTAACATAACCTCTCTAATCC GGATTCTGCGAAATTGGGTGC	55
69a07	BVS1	110	ACTCATGCTGGACAGCCTG TGACCGCCCCAACCTTTC	60	CTTAACATAACCTCTCTAATCC GGATTCTGCGAAATTGGGTGC	55
99a06	BVS1	120	NA	NA	CATGTAGATGGAAGCACCATAG CAACTTGGGAGCTCGAGAAAC	60
41g18	BVS1	130	GAGCAGCGTGACAATTGGAAC AGGCCACCAAACAGCAGGC	60	CATGCAAGCTTCGGGACTC AGTAGCAAAGGTATTGACAG	55

[a] BAC clones designated by plate# - row letter – column#
[b] STS markers from the *MXC3* region that were used to identify the designated BAC clones
[c] Primers used to amplify the SP6 or T7 ends of the designated BAC clone

F_1 clones was identified which defines a 2.73-cM window containing *MXC3* (Fig. 2). This set of 52 recombinants was used to screen other candidate markers for tight linkage to *MXC3*. None of the 52 recombinants were among the 60 clones with ambiguous rust resistance phenotypes (see Materials and methods).

Of the remaining 26 AFLP markers putatively linked to *MXC3*, 15 mapped within the 2.73-cM interval defined by CCG.AGT_01 and CCG.AAC_01. Thus, from a total of 2,048 AFLP primer combinations screened (approximately 10,854 polymorphic loci) by bulked segregant analysis, we identified 19 AFLP markers that mapped within a 2.73-cM interval surrounding the *MXC3* locus (Fig. 2).

An AFLP-derived STS marker, *BVS1*, is used to screen a *P. trichocarpa* (clone 383–2,499) BAC library

An AFLP marker co-segregating with *MXC3*, CCG.GCT_01, was converted into an STS marker, *BVS1* (Table 1), and used to screen a BAC library constructed from the *P. trichocarpa* parent of the mapping pedigree. Five BAC clones, all in coupling linkage phase with *MXC3*, were isolated and the sizes of the clones determined by PFGE (Table 1). PCR primers designed from the BAC-end sequences (Table 1) and their amplification patterns were used to resolve the order of overlap among the BACs. The degree of overlap among the five BAC clones was determined by restriction fingerprinting (see Materials and methods). The five BAC clones were assembled into an approximately 200-kb contig (Fig. 3).

STS markers developed from BAC-end sequences or BAC subclones co-segregate with *MXC3*

BAC-end specific STS primer pairs designed from the five BACs isolated with *BVS1* were tested on the mapping parents and resistant and susceptible F_1 clones to search for markers that could be mapped genetically. It was hoped that markers flanking *MXC3* could be found on the contig, narrowing the scope of the search for the *MXC3* gene itself. Three markers developed from the end sequences of BAC clones 47m20, 99a06 and 41g18, as well as a fourth marker developed by subcloning and sequencing a 2.5-kb *Not*I/*Bam*HI fragment from the SP6 end of BAC 41g18 (Table 2), were found to co-segregate with *MXC3* (Fig. 2). Two of the markers, 47m20 (SP6) and 41g18 (SP6), are located at the opposite ends of the contig (Fig. 3); thus, a physical interval spanning *MXC3* could not be defined within this set of five overlapping BAC clones. This is somewhat surprising, since the average physical:genetic distance ratio in the *Populus* genome is 220 kb/cM (Bradshaw et al. 1994). In the 1,902 diploid offspring in the mapping pedigree, a contig of 200 kb is expected to contain 18 recombinants, or approximately one recombination event every 11 kb. The

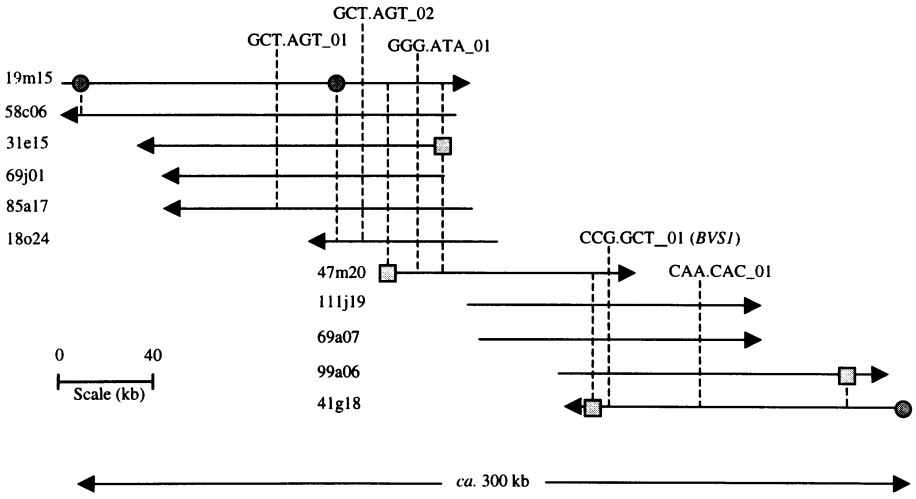

Fig 3 BAC contig of the *MXC3* region. *Arrowheads* designate the T7 end of the vector. *Solid squares*, 41g18 (T7), 99a06 (T7), 47m20 (SP6) and 31e15 (SP6), represent STS markers that were developed from BAC-end sequences and used for genetic mapping. *Solid circles*, 41g18 (SP6) 19m15 (SP6) and 19m15, represent STS markers that were developed from BAC-end or random subclones and used for genetic and/or physical mapping. The BAC contig represents the minimum physical distance (approximately 300 kb) with no recombination events

Table 2 STS markers developed from BAC-end sequences or BAC subclones. Map distances for each STS marker were determined by the number of recombinants out of 1,902 F_1 progeny in the high-resolution mapping pedigree. The order of markers was determined by the F_1 clones recombinant for each marker

Polymorphism detected	BAC clone[a] (location)	Size (bp)	Forward primer (5′-3′)	Reverse primer (5′-3′)	Annealing temp. (°C)	Map distance from *MXC3* (cM)
RFLP[b]	41g18 (subclone)	Approx. 2,500 (cut w/DraI)	CTGGCAGTAAT CTAGTTAGTC	TGTTGCATCAG CTGGTACAG	55	0
Band present/absent	41g18 (T7 end)	460	CATGCAAGCTT CGGGACTC	AGTAGCAAAGG TATTGACAG	50	0
Length	99a6 (T7 end)	432	CATGTAGATGG AAGCACCATA	CAACTTGGGAG CTCGAGAAAC	55	0
Band present/absent	47m20 (SP6 end)	432	TAAGTGTTGAC TGAATGATG	ACTTAGATTTC TCATTCCTAC	50	0
Band present/absent	31e15 (SP6 end)	360	AATGGAAGAG AGGGACCAGC	ATCGTGCTGAT CACTAATCGA	55	0
Band present/absent	19m15 (subclone)	244	CATTCACTGTG GTTTAAGGATT	CTTGATGGTTTC TAAGAGGTCG	55	0
RFLP	19m15 (subclone)	Approx. 1000 (cut w/MseI)	GAAAACTTGTA TAGTTGAAGGG	TGAGCATTGAC ATGGAGCTG	55	0

[a] BAC clone designated by plate# - row letter – column#
[b] Restriction fragment length polymorphism

complete lack of recombination events within the 200-kb contig suggests an approximately 20-fold reduction in recombination within the *MXC3* region.

A chromosome walk extends the BAC contig to 300 kb

To extend the contig in search of markers flanking the resistance gene, a chromosome walk was initiated with an STS marker derived from the SP6 end of BAC clone 47m20 (Table 2). Six additional BAC clones, all in coupling linkage phase with *MXC3*, were identified with this BAC-end STS marker and their insert sizes determined by PFGE (Table 1). Restriction fingerprinting (see Materials and methods) and PCR primers designed from the BAC-end sequences (Table 1) were used to determine the order and degree of overlap among the six additional BAC clones and to join the BACs into the existing contig (Fig. 3). The additional BAC clones extended the contig to a minimum distance of approximately 300 kb. Three new STS markers were developed, one from the SP6 end of BAC clone 31e15 and two from BAC 19m15: one from a random *Hin*dIII subclone and the other from a *Not*I/*Bam*HI fragment subcloned from the SP6 end (Table 2). When these three STS markers were mapped genetically, they were found to co-segregate with *MXC3* (Fig. 2). The marker derived from the SP6 end of 19m15 is located at the opposite end of the contig (Fig. 3); thus, the *MXC3* locus could not be defined within this 300-kb interval.

Development of integrated high-resolution genetic and physical maps around *MXC3*

A high-resolution map was constructed around *MXC3* (Fig. 2). The map consists of 19 AFLP markers, four

STS markers derived from BAC-end sequences, and three STS markers developed from BAC subclones. A 2.73-cM window around *MXC3* is defined by the CCG.AAC_01–CCG.AGT_01 marker interval. Seven AFLP markers, GCT.AGT_01, GCT.AGT_02, GGG.ATA_01, CCG.GCT_01 (*BVS1*), CAA.CAC_01, CGG.TAC_01 and ACT.TCC_01, co-segregate with *MXC3*. Five of these AFLP markers are in coupling with *MXC3*; that is, the dominant (band-present) marker allele is linked in *cis* to the dominant resistance allele *MXC3*, while the other two are in coupling-phase with the recessive allele (Fig. 2). The seven STS markers developed from either BAC-end sequences or BAC subclones also co-segregate with *MXC3*. The two closest markers flanking *MXC3* are CGA.TCT_01 (0.11 cM) and GAC.TAC_01 (0.58 cM); both of these markers are in coupling-phase with the dominant *MXC3* allele. An allele-specific STS marker, P980, also known to be linked to the *Mmd1* poplar leaf rust resistance gene (Newcombe et al. 1996), lies outside the 2.73-cM window.

Physical organization of AFLP markers closely linked to *MXC3*

The remaining four AFLP markers co-segregating in coupling-phase with *MXC3*, GCT.AGT_01, GCT.AGT_02, GGG.ATA_01 and CAA.CAC_01, were tested on the 11 BAC clones in the contig. All four of these AFLP markers mapped to the putative *MXC3* contig (Fig. 3). In addition, we also tested the closest flanking AFLP markers, CGA.TCA_01 and GAC.TAC_01, on the 11 BACs. However, neither of these AFLP markers was found on the contig.

Discussion

The development of a high-resolution map around *MXC3* provided us with tightly linked genetic markers for physical mapping and, possibly, the isolation of this rust resistance gene by positional cloning. The isolation of disease resistance genes from tomato (Martin et al. 1993), rice (Song et al. 1995) and *Arabidopsis thaliana* (Bent et al. 1994; Grant et al. 1995) by positional cloning has demonstrated that it is possible to clone plant disease resistance genes knowing only their precise location on a high-resolution linkage map. Recently, the feasibility of developing high-density genetic maps in plants makes it possible to identify markers that are physically close to a gene of interest, and can be used to screen a large-insert genomic library and land on the clone containing the gene (Tanksley et al. 1995).

When initiating this study, we believed that it would be possible isolate *MXC3* by chromosome landing. The pedigree of 1,902 F_1 hybrid poplar offspring made it possible to identify genetic markers within a nominal distance of 0.05 cM from *MXC3*. The average relationship between physical and genetic distance in *Populus*, based upon a genome size of 550 Mb (Bradshaw and Stettler 1993a) and a genetic map of 2,500 cM (Bradshaw et al. 1994), is 220 kb/cM. Thus, a marker 0.05 cM away should be within 11 kb of the gene (on average). The mean insert size of the BAC library used in this study is 120 kb, making it technically possible to land flanking markers within ±0.25 cM of the gene on a single BAC clone. Although chromosome landing on *MXC3* seemed likely to succeed, results from our physical mapping efforts demonstrate that isolating a single BAC clone containing *MXC3* was not possible despite the saturation of genetic markers around this locus. As shown in Fig. 3, a 300-kb interval was found to co-segregate with *MXC3*. Although this 300-kb contig should represent 1.4 cM of genetic distance and contain 27 recombination events in an average part of the genome, no recombination events were detected, so it was not possible to delimit *MXC3* to a physical region. This result indicates a >25-fold reduction in recombination frequency in the *MXC3* region compared to the average rate of recombination for the *Populus* genome.

The frequency of recombination varies substantially, with hot and cold spots occurring throughout the genome (e.g., Kaback et al. 1989; Schmidt et al. 1995; Nachman and Churchill 1996; Faris et al. 2000; Kunzel et al. 2000). We expected that recombination in the *MXC3* region would be equivalent to, or higher than, the average rate for the *Populus* genome, since it is thought that plant disease resistance genes have evolved by recombination events (Richter and Ronald 2000). Thus, for genomic regions surrounding plant resistance genes one might expect to find higher than average rates of recombination. High rates of recombination and unequal crossovers have been shown to be a source of new resistance specificities for the *Rp-1* rust-resistance cluster in maize (Richter 1995). A six-fold increase in recombination frequency has been demonstrated for the *Alternaria* stem canker resistance locus (*Asc*) in tomato (Mesbah et al. 1999). A ten-fold increase in recombination frequency has been observed for the I_2 resistance gene in tomato against *Fusarium oxysporum* (Segal et al. 1992).

However, suppressed recombination has also been observed around plant disease resistance loci. For example, it was recently shown that recombination is suppressed around the *Mla* (powdery mildew) resistance cluster in barley (Wei et al. 1999). Suppressed recombination has also been observed for the *Tm-2a* virus resistance gene (Ganal et al. 1989) and the *Mi* nematode resistance gene (Van Daelen et al. 1993). For *Tm-2a* and *Mi*, however, the resistance loci are physically close to the centromere, a region where suppressed recombination has been demonstrated in eukaryotic genomes (Roberts 1965; Mahtani and Willard 1998; Copenhaver et al. 1999). In addition, the *Mi* and *Tm-2a* resistance loci have been introgressed into cultivated tomato from the distantly related tomato species *Lycopersicon peruvianum*. For both loci, a severely reduced amount of recombination over the entire introgressed region has been observed in crosses

between lines that carry the introgressed gene and lines that do not (Ganal and Tankley 1996).

In the future we wish to identify the physical interval spanning the *MXC3* locus. This will require finding more genetic markers tightly linked to the *MXC3* locus and expansion of the putative *MXC3* contig by chromosome walks in both directions. Determination of the physical region spanning *MXC3* will allow us to investigate the structure of this resistance locus and hopefully provide insights into the nature of the suppressed recombination in this region, as well as leading to the isolation and manipulation of *MXC3*.

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