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Saturation mapping of a major gene for resistance to white pine blister rust in sugar pine

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Abstract The molecular basis of resistance to diseases in plants can be better understood if the genes coding for resistance can be cloned. The single major dominant gene (R) that confers resistance to the white pine blister rust fungus (*Cronartium ribicola* Fisch.) in sugar pine (*Pinus lambertiana* Dougl.) has been previously mapped. The objectives of the present study were to saturate the region flanking R with tightly linked markers and to construct genetic maps for each of four individual seed trees. Bulked segregant analysis (BSA) and haploid segregation analysis were employed to identify random amplified polymorphic DNA (RAPD) markers linked to R. Automated PCR analysis was used to assay 1115 primers with susceptible and resistant DNA pools from each of four seed trees (8920 PCR reactions). Thirteen RAPD loci were identified that were linked to R. The linkage analyses programs JoinMap 1.4 and Mapmaker 2.0 were used to order RAPD loci relative to R and to construct maps for each of the individual seed trees. Two seed trees, 5701 and 6000, had a large number of tightly linked markers flanking R. These trees will be used in subsequent high-resolution mapping experiments to identify very tightly linked markers to facilitate the eventual cloning of R.

Key words *Pinus lambertiana* · *Cronartium ribicola* · Bulked segregant analysis · RAPD mapping

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Introduction

A diverse array of plant disease resistance genes have been molecularly cloned in recent years. Some of the genes that have been cloned are those coding for resistance to bacterial speck in *Arabidopsis* (Bent et al. 1994; Grant et al. 1995) and tomato (Martin et al. 1993; Salmeron et al. 1996), rust fungus in flax (Lawrence et al. 1995; Anderson et al. 1997) and wheat (Feuillet et al. 1997), tobacco mosaic virus in tobacco (Whitham et al. 1994), fusarium wilt in tomato (Ori et al. 1997), leaf mold in tomato (Jones et al. 1994; Dixon et al. 1996), bacterial blight in rice (Song et al. 1995), downy mildew in *Arabidopsis* (Parker et al. 1997), and powdery mildew in barley (Buschges et al. 1997). Nearly all of these reports were preceded by the initial low-resolution mapping of the resistance gene using molecular markers such as restriction fragment length polymorphisms (RFLPs) or random amplified polymorphic DNA (RAPD) markers.

We have followed a similar strategy towards eventually cloning a major dominant gene for resistance (R) to the white pine blister rust fungus (*Cronartium ribicola* Fisch.) in sugar pine (*Pinus lambertiana* Dougl.) using bulked segregant analysis (BSA) and haploid segregation analysis to identify RAPD markers linked to R (Devey et al. 1995). Disease phenotypes (susceptible or resistant) were determined from needle reactions on seedlings following inoculation with the blister rust fungus. Because the seed trees were heterozygous for R and the effective pollen pool was almost entirely

susceptible (r), linkage between R and the RAPD markers could be estimated on the basis of haploid segregation analysis. We identified ten RAPD markers linked to R; the most tightly linked marker, OP_F03_810, was 0.9 cM from R (Devey et al. 1995). The final map resulted from combining segregation data from five mapping populations; thus maps for individual trees were not obtained.

White pine blister rust is an introduced fungal pathogen that infects and threatens many North American white pines and has been described as one of the most devastating diseases of forest trees (Klinkowski 1970; Bega and Scharpf 1993). Sugar pine is a major component of the Sierra Nevada mixed conifer forest and is one of the most highly valued timber species (Kinloch and Scheuner 1990). Resistance to white pine blister rust in sugar pine is controlled by a single dominant gene (Kinloch et al. 1970; Kinloch and Littlefield 1977).

The objectives of the present study were to saturate the region flanking R with tightly linked markers and to construct genetic maps for each of four individual trees. RAPD markers and bulked segregant analysis were used as before (Devey et al. 1995), but for this study the number of RAPD primers as well as the sample sizes of individual megagametophyte DNAs from each seed tree were increased. This resulted in the identification of 13 new markers, three of which were more closely linked than any markers previously identified, and the construction of separate maps for each of four different seed trees.

Materials and methods

Strategy for identifying RAPD markers linked to R

The strategy for identifying RAPD markers linked to R was essentially as described in Devey et al. (1995). Bulked segregant analysis (Michelmore et al. 1991) was conducted in a partially automated laboratory at the USDA Forest Service, Southern Research Station, Southern Institute of Forest Genetics, in Saucier, Mississippi. The use of robotics greatly increased the throughput of the assay and the consistency from run to run. Bulk DNA pools of resistant and susceptible phenotype were constructed by combining equal amounts of megagametophyte DNA from 30 individuals from each of the four families, for a total of eight bulks. A total of 1115 primers were assayed on the BSA samples (see Table 1). Primers that revealed obvious RAPD band differences between the susceptible and resistant bulks were used in secondary screens. Secondary screen RAPD-PCR reactions were performed on putative positives using eight individual susceptible and eight individual resistant megagametophyte DNAs. Any loci that exhibited three or fewer recombinants during the secondary screen were used in tertiary screens, where RAPD-PCR reactions were performed on the full mapping population. This level of stringency was applied to ensure that no tightly linked markers were missed, while preventing further mapping of loosely linked markers.

Plant materials and DNA isolation

Open-pollinated seeds from each of the four heterozygous (Rr) seed trees employed in the previous study (Devey et al. 1995) were again used in this study (see Table 2). No controlled-pollinated seeds of appropriate genotype were available. DNA was extracted from seed megagametophytes essentially as described by Dellaporta et al. (1983).

Determination of disease phenotypes

Seeds were sown in Ray Leach Cone-Tainers™ (Stuewe and Sons; Corvallis, Ore., USA) and germinants were grown in a greenhouse under standard conditions (Kinloch and Comstock 1980). Approximately 3–4 weeks after sowing, the megagametophyte was rescued from each germinant and uniquely identified with its corresponding seedling. These megagametophytes were stored in microfuge tubes at -80°C prior to DNA extraction. The seedlings were then inoculated with an avirulent rust source (Kinloch and Comstock 1981) to identify susceptible and resistant segregating phenotypes. Disease reactions were observed on cotyledons and primary needles 6–8 weeks after inoculation (Kinloch and Comstock 1980).

RAPD assays

RAPD analyses were performed following the method of Williams et al. (1990). RAPD primers were obtained from Operon Technologies (Alameda, Calif., USA and The University of British Columbia (Vancouver, BC, CA). All lyophilized primers were dissolved in a Tris-EDTA buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Bulked segregant analysis reactions were set up using a Hamilton Microlab ATPLUS Robotic bath sample processor (Hamilton Co.; Reno, Nev., USA). PCRs consisted of 3 ng of template DNA amplified in a reaction mixture containing 1×PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$) (Boehringer Mannheim; Indianapolis, Ind., USA), 133 μM of each dNTP (Promega Corp.; Madison, Wis., USA), 0.8 units of *Taq* Polymerase (Boehringer Mannheim; Indianapolis, IN, USA), and 0.2 μM of primer. Total reaction volumes were 24 μl. Reaction mixtures were overlaid with 50 μl of light mineral oil (Sigma Chemical Co.; St. Louis, Mo., USA) before amplifications were done in MJ-Research PTC-100-96 Programmable Thermal Controller (MJ-Research; Watertown, Mass., USA) under the following cycling parameters:

Step	Min:s	Temp. (C°)
1	0:05	95
2	1:55	92
3	0:05	95
4	0:55	92
5	1:00	35
6	2:00	72
7	Go to Step 3, 44 times (45 cycles total)	
8	7:00	72

Subsequent amplification products were separated by electrophoresis (3.0 V/cm) in 2.0% SeaKem LE agarose (FMC BioProducts; Rockland, Me., USA) in 1×TAE (40 mM Tris-Acetate, 1.0 mM EDTA), and then visualized by ethidium bromide fluorescence. All secondary and tertiary screens were performed at the USDA Forest Service, Institute of Forest Genetics, Pacific Southwest Research Station, Placerville, California. Amplifications for secondary and tertiary screens were performed under the same reaction conditions used for the primary reactions. All measures were taken to simulate the robotic procedure. The only notable differences were in the use of MJ-Research PTC-100-96V Programmable Thermal Controller (MJ-Research; Watertown, Mass.,

Table 1 Results from applying large numbers of RAPD primers for identifying linked markers to a gene for resistance to white pine blister rust fungus in sugar pine

Set	Primers in reaction set	Primary screens	Secondary screens	Tertiary screens	Number mapped
A	BC_577 through BC_721	144	36	4	1
B	BC_722 through OP_D06	144	18	1	0
C	OP_D07 through OP_K10	132	26	10	6
D	OP_K11 through OP_X14	144	21	0	0
E	OP_X15 through BC_098	144	30	6	1
F	BC_099 through BC_244	144	35	5	1
G	BC_245 through BC_393	144	48	7	1
H	BC_394 through BC_538	144	32	4	3
I	BC_539 through BC_574	35	7	0	0
Totals		1115	253	37	13

USA), and electrophoresis in 1×TAE (3.0 V/cm) in 2.0% Molecular Biology Reagent Grade agarose (Gibco BRL; Gaithersburg, Md., USA).

Linkage analysis

Data were compiled for each individual family, and two-point linkages between all RAPD markers and R were estimated (Adams and Joly 1980). JoinMap 1.4 (Stam 1993) and Mapmaker Macintosh 2.0 (Lander et al. 1987) programs were used to construct maps. Segregation data were analyzed as a backcross population for JoinMap analyses using a critical LOD of 3.0 for linkage and 0.05 for mapping. Likewise, segregation data were analyzed as an F_2 backcross population in Mapmaker analyses using a LOD of 3.0 for linkage and 0.4 for the recombination fraction. The Kosambi mapping function was used both with JoinMap and Mapmaker.

Results

Identification of linked RAPD markers

Of 1115 primers used in the primary screen, 253 revealed polymorphic loci that were assayed in secondary screens (Table 1). Secondary screens identified 37 putatively linked loci that met the criterion of three or fewer recombinants among the 16 individuals. Tertiary screens for these 37 loci yielded a total of 13 RAPD markers that were linked to R (Fig. 1). The remaining loci were unlinked.

Two-point linkage analysis and construction of maps

Two-point recombination frequencies were calculated for all pairwise combinations of RAPD markers and R (Table 2). Estimates were obtained from segregation data of individual seed trees instead of combining data across trees as in Devey et al. (1995). One new linked marker, BC_105_575, was identified for seed tree 5003, but it was not closely linked to R [$r = 0.13$ (0.05)]. Seven new RAPD markers were identified for seed tree 5701. Four markers (OP_E12_1500, OP_E12_1700, OP_G16_950, and BC_432_1110) were tightly linked, although none was closer than $r < 0.03$. Marker OP_F03_810, which had previously been mapped (Devey et al. 1995), had no recombinants in a sample of 81 megagametophytes. Although additional megagametophytes were available for assay, this marker could be revealed only by hybridization (Devey et al. 1995), an approach not used in the present study. Five new markers were obtained for seed tree 6000, one of which (OP_F19_1300) was tightly linked to R [$r = 0.03$ (0.01)]. No new markers were identified for seed tree 11300.

All of the new and previously obtained RAPD markers were ordered on linkage groups using both Mapmaker and JoinMap linkage-analysis programs (Fig. 1). Individual maps were constructed for all four seed trees. The maps show the saturation of RAPD markers on both sides of R for seed trees 5701 and 6000.

Discussion

Saturation mapping of RAPD markers in the region surrounding the R gene in sugar pine identified markers more tightly linked to R than those previously found and enabled the construction of individual genetic maps for each of four seed trees. Many more markers were mapped in seed trees 5701 and 6000 than in 5003 and 11 300, presumably because of higher levels of heterozygosity in these trees. Subsequent, high-resolution mapping efforts will concentrate on seed trees 5701 and 6000. Flanking genetic markers OP_G16_950 and BC_432_1110 is seed tree 5701, each less than 2 cM from R, are good candidates for screening large samples for recombinants in this interval. Likewise, flanking markers OP_AI03_650 and OP_F19_1300 are good candidates for seed tree 6000. Samples of approximately 1000 new seedlings from both 5701 and 6000 will be screened with the flanking markers to identify recombinants for high-resolution mapping.

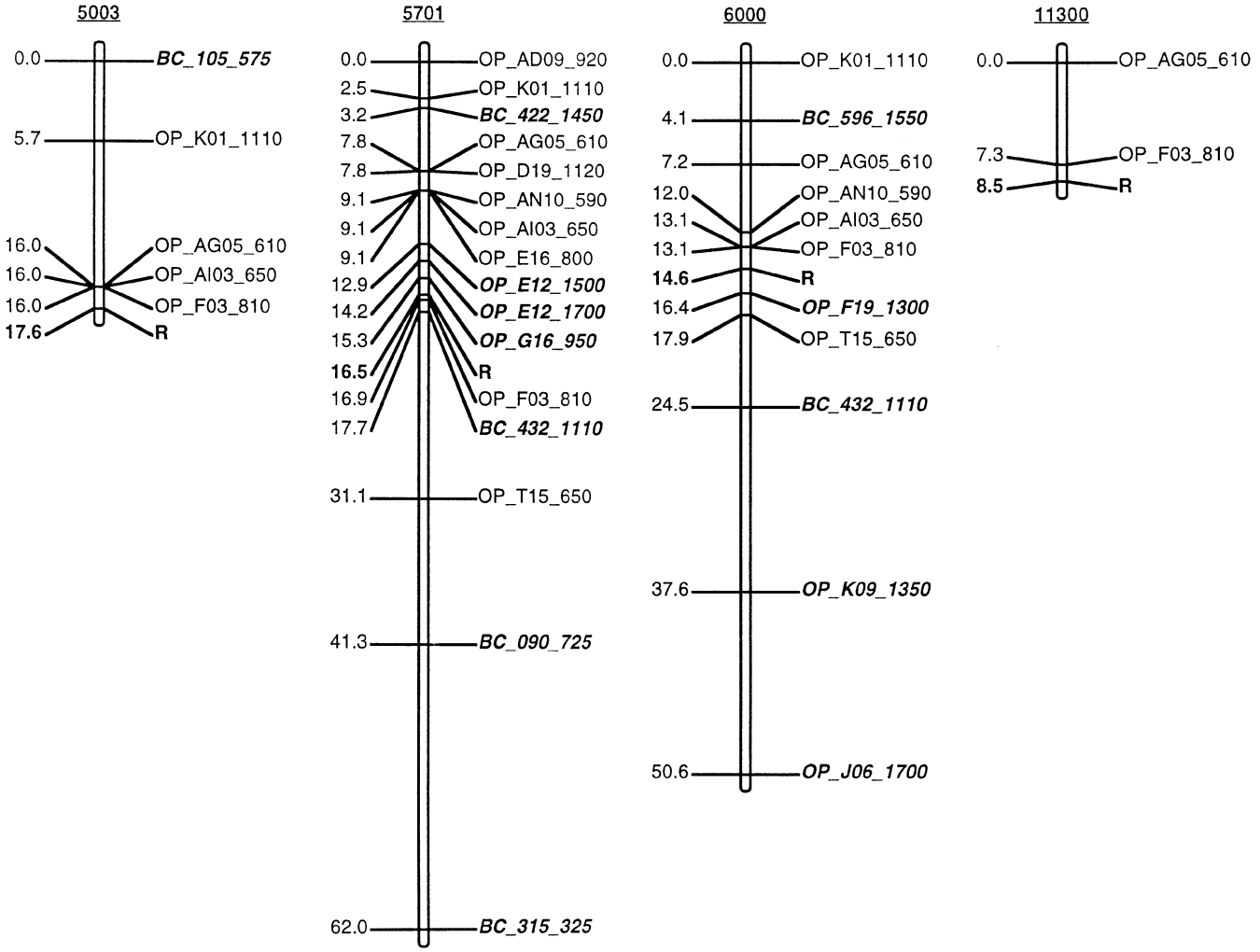

Fig. 1 RAPD maps of the region flanking the gene for resistance (R) to the white pine blister rust fungs (*C. ribicola* Fisch.) in sugar pine (*P. lambertiana* Dougl.) from four heterozygous (Rr) open-pollinated seed trees. RAPD markers identified in the present study are shown in *bold italics*; others are from Devey et al. (1995). Map distances (at the left of map verticals) are in centiMorgans (cM)

The expected minimum distance [E(d)] between a RAPD marker and a target gene was given by Martin et al. (1991) as:

$$E(d) = c/2(nx + 1),$$

where
c = genome size in cM
(assume 2000 cM for sugar pine),
n = number of primers (1115 in this study),
x = average number of PCR products per primer
(assume four per primer).

An E(d) = 0.22 cM would have been expected in this study. However, it would be impossible to detect markers at distances less than about 1 cM, given the sample sizes used. No recombinants in a sample of 81 megagametophytes were detected for marker OP_F03_810 in seed tree 5701 (Devey et al. 1995); however, additional samples could not be obtained for this marker because of the difficulty, and lack of repeatability, associated with detecting RAPD markers by hybridization. Possibly converting marker OP_F03_810 to a SCARs marker (Paran and Michelmore 1993) would enable additional meioses to be assayed with this marker.

The estimated physical size of the sugar pine genome is 1C = 31.7 pg (Wakimaya et al. 1993), one of the largest plant genomes reported. Assuming a genetic distance of 2000 cM for the sugar pine genome, this leads to an estimate of 15 000 kb/cM. Assuming that this estimate applies to the genomic region surrounding the R gene, there would be little realistic opportunity for cloning R by a chromosome-landing strategy (Tanksley et al. 1995). Alternative strategies are being pursued to clone R, such as cloning resistance gene-homologus by PCR (Staskawicz et al. 1995; Michelmore 1996). Nevertheless, high-resolution mapping of the R gene will facilitate this or other strategies, especially if resistance genes are clustered in linked arrays on chromosomes (Witsenboer et al. 1995).

Table 2 Cosegregation of RAPD marker bands (presence, +; absence, −) with R gene phenotypes (resistant, susceptible); linkage phase (c, coupling; r, repulsion); and estimates of two-point recombination distance (Adams and Joly 1980) in each of four seed trees (5003, 5701, 6000, 11 300). Markers identified in the present study in italics; others from Devey et al. (1995)

Tree	Locus	Resistant +	Resistant −	Susceptible +	Susceptible −	Total	Phase	2-Point recombination frequencies	Standard error
5003	*BC_105_575*	6	3	3	34	46	c	0.13	0.05
	OP_K01_1110	6	3	3	35	47	c	0.13	0.05
	OP_AG05_610	0	9	37	1	47	r	0.02	0.02
	OP_AI03_650	0	9	37	1	47	r	0.02	0.02
	OP_F03_810	9	0	1	37	47	c	0.02	0.02
5701	OP_AD09_920	5	3	10	63	81	c	0.16	0.04
	OP_K01_1100	7	1	10	63	81	c	0.14	0.04
	BC_422_1450	7	1	10	63	81	c	0.14	0.04
	OP_AG05_610	7	0	7	66	80	c	0.09	0.03
	OP_D19_1120	8	0	7	66	81	c	0.09	0.03
	OP_E16_800	8	0	6	67	81	c	0.07	0.03
	OP_AN10_590	8	0	6	67	81	c	0.07	0.03
	OP_AI03_650	8	0	6	67	81	c	0.07	0.03
	OP_E12_1500	8	0	3	68	79	c	0.04	0.02
	OP_E12_1700	8	0	2	70	80	c	0.03	0.02
	OP_G16_950	0	8	241	7	256	r	0.03	0.01
	OP_F03_810	8	0	0	73	81	c	0.00	0.00
	BC_432_1110	8	0	7	239	254	c	0.03	0.01
	OP_T15_650	7	1	10	63	81	c	0.14	0.04
	BC_090_725	3	4	57	13	77	r	0.21	0.05
	BC_315_325	3	5	48	25	81	r	0.35	0.05
6000	OP_K01_1100	6	4	5	53	68	c	0.13	0.04
	BC_596_1550	10	0	7	50	67	c	0.10	0.04
	OP_AG05_610	8	2	2	56	68	c	0.06	0.03
	OP_AN10_590	2	8	58	0	68	r	0.03	0.02
	OP_AI03_650	9	1	0	58	68	c	0.01	0.01
	OP_F03_810	9	1	0	58	68	c	0.01	0.01
	OP_F19_1300	2	8	201	5	216	r	0.03	0.01
	OP_T15_650	10	0	3	55	68	c	0.04	0.02
	BC_432_1110	9	1	16	200	226	c	0.08	0.02
	OP_K09_1350	4	6	48	8	66	r	0.18	0.05
	OP_J06_1700	1	9	40	16	66	r	0.26	0.05
11 300	OP_AG05_610	2	10	66	5	83	r	0.08	0.03
	OP_F03_810	13	0	1	73	87	c	0.01	0.01

There is considerable justification for continuing with the challenging efforts to clone the R gene from sugar pine. Successful cloning of R would lead to a deeper understanding of the molecular basis of resistance to pine rusts and might also shed light on the evolution of resistance genes in plants in general. Its practical significance would lie in diagnostic tools that could be developed to screen large sugar pine populations for the presence of R without expensive and time-consuming progeny testing. Furthermore, R might be introduced into genomes of other susceptible species of white pines where no, or only weak, genetic resistance has been identified.

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