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Genetics

Random amplified polymorphic DNA markers tightly linked to a gene for resistance to white pine blister rust in sugar pine

(bulked segregant analysis/megagametophytes/linkage map/gymnosperm)

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ABSTRACT **We have genetically mapped a gene for resistance to white pine blister rust (*Cronartium ribicola* Fisch.) in sugar pine (*Pinus lambertiana* Dougl.) by using an approach which relies on three factors: (*i*) the ability to assay for genetic markers in the haploid stage of the host's life cycle, using megagametophyte seed tissue; (*ii*) a simple and clearly defined pathosystem; and (*iii*) the use of random amplified polymorphic DNA (RAPD) markers that can be quickly and efficiently evaluated. Resistance to white pine blister rust in sugar pine is known to be controlled by a single dominant gene (*R*). Maternal segregation of *R* and dominant RAPD markers were scored simultaneously following collection of megagametophytes for DNA assays and seedling inoculation with *C. ribicola*. Bulked samples of haploid megagametophyte DNA from resistant and susceptible offspring of segregating full-sib and half-sib families were used to evaluate 800 random decanucleotide primers. Ten loci linked with the gene for resistance to white pine blister rust were identified and segregation data were obtained from five families. Six of the linked markers were within 5 centimorgans of the gene, and one marker was 0.9 centimorgan from *R*. These and other markers derived by this approach may provide starting points for map-based cloning of this important gene.**

Major genes for resistance to plant disease occur in diverse taxa and are important in protecting agricultural crops, as well as in regulating endemic disease in natural populations. Cloning and sequencing of resistance genes has helped to elucidate their mode of action, as shown recently with *Pto* in tomato (1) and *HM1* in maize (2). *Pto* was isolated by a map-based cloning approach, the techniques of which are extremely laborious, particularly in species with large genomes.

Forest trees are generally considered to be difficult subjects for genetic analysis. Long generation time, outbred mating systems, and lack of genetic linkage information are all limitations associated with these species. Genome size in pines is perhaps the greatest hindrance to gene mapping and cloning. Sugar pine (*Pinus lambertiana* Dougl.) has a 1C DNA content of 31.7 pg or about 30,000 megabases (3), which is >40 times the size of the tomato genome (4). However, one advantage that pines and other gymnosperms have is that the megagametophyte tissue of their seeds is haploid (Fig. 1). DNA extracted from this tissue can be used to efficiently map genetic markers. Each megagametophyte represents a single meiotic product identical to the maternal contribution to the embryo, and direct analysis of inheritance of genetic loci is therefore possible without the use of controlled crosses, which are costly and often unavailable. Most isozyme inheritance studies in conifers have been based on marker segregation in megagametophytes from heterozygous seed trees (5, 6).

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White pine blister rust ranks as one of the catastrophic disease epidemics in history (7). Sugar pine, largest member of the genus and one of the most valuable, is highly susceptible, and the impact of the disease on it has been severe. Surprisingly, although the pathogen was introduced into western North America only early in the present century (8), a simple gene-for-gene relationship exists. A major gene in the host that confers virtual immunity to the disease (9, 10) is complemented by a corresponding gene in the rust with specific virulence to it (11). Both genes are at low frequencies in natural populations (12, 13).

The primary infection courts are leaves (needles), which are entered through stomata by germinating basidiospores. The rust establishes in mesophyll tissue, where it causes a bright yellow spot to form in susceptible genotypes (Fig. 2 *Lower*). Mycelium then grows down the needle into the living bark at the base of branch or stem, where it eventually girdles and kills the shoot. In contrast, sugar pines with the dominant allele (*R*) for resistance respond to infection by the wild-type race of rust with a hypersensitive reaction, wherein cells immediately surrounding invading hyphae collapse and become necrotic, arresting further development of the pathogen. Visual distinction between the two phenotypes is clear (ref. 10 and Fig. 2).

The development of random amplified polymorphic DNA (RAPD) markers (14) enabled efficient mapping of the *R* gene in sugar pine. RAPD marker assays are based on the polymerase chain reaction (PCR) and use single oligonucleotide primers, of arbitrary sequence, to direct the amplification of discrete loci. RAPD markers do not distinguish between heterozygous and homozygous dominant loci in diploid material; however, they are fully informative when haploid megagametophyte tissue is assayed (15). Segregation of heterozygous loci in haploid tissue is defined by presence/absence of amplified product in a 1:1 ratio, thus greatly simplifying data analysis. We present here a method that has been used in sugar pine to quickly identify flanking markers to a gene for resistance to white pine blister rust, caused by *Cronartium ribicola* Fisch.

MATERIALS AND METHODS

Plant Material. Control crosses in an arrangement suitable for this study were limited to a single cross, 5042 × K-36-SAR, in which the female is heterozygous (*Rr*) for resistance and the male is homozygous recessive (*rr*). Approximately 200 seeds from this cross were obtained, but only 37 of these germinated. Four open-pollinated families were used to obtain better estimates of recombination and position of linked markers. Heterozygous (*Rr*) seed trees, as determined from progeny

Abbreviation: RAPD, random amplified polymorphic DNA. Genetic mapping data have been deposited in the Dendrome database, Institute of Forest Genetics, P.O. Box 245, U.S. Department of Agriculture/Forest Service, Berkeley, CA 94701. Email: dendrome@s27w007.pswfs.gov.

FIG. 1. Sugar pine seed (*Left*, ≈10 mm in length) and dissected seed (*Right*) showing embryo and surrounding megagametophyte tissue. In conifers, the megagametophyte and female egg cell are derived from a single meiotic event; both are haploid and genetically identical.

tests, were chosen from areas in Northern California where the *R* gene frequency is known to be <1% (13). Four seed trees were selected from Klamath, Six Rivers, and Mendocino National Forests: seed trees 5003, 5701, 6000, and 11300. Approximately 2000 seeds from each seed tree were obtained, and initially 200 from each were germinated and inoculated under routine conditions with blister rust spores of known avirulence to *R* (16). To eliminate the possibility of error resulting from *R* pollen, only susceptible individuals were used for RAPD segregation analysis, except for 8–14 resistant individuals from each seed tree which were included to determine phase and to ensure that the PCR product was amplified as expected.

DNA Extraction. Megagametophytes were collected following seedling germination and stored at −20°C prior to DNA extraction. Yields of 10–20 μg of DNA were obtained with extraction according to Dellaporta *et al.* (17).

RAPD Assays. PCR template consisted of 10 ng of megagametophyte DNA from either an individual megagametophyte or a bulked sample. Reactions were run in 10 mM Tris (pH 8.3 at 25°C) containing 50 mM KCl, 2.0 mM $MgCl_2$, 0.33 μM primer, 200 μM each dNTP, and 0.9 unit of Ampli-*Taq* DNA polymerase (Perkin–Elmer/Cetus) in a total volume of 15 μl. Forty-three cycles of PCR were done on a Perkin–Elmer 9600 machine: 3 cycles of 2 min at 94°C, 2 min at 35°C, and 2 min at 72°C and 40 cycles of 1 min at 92°C, 1 min at 35°C, and 2 min at 72°C. RAPD primers were obtained from Operon Technologies (Alameda, CA). PCR amplification products were electrophoresed in 1.5% agarose gels in 45 mM Tris/45 mM boric acid/1 mM EDTA; nucleic acids were visualized by ethidium bromide fluorescence or Southern blot hybridizations.

Bulked Segregant Analysis. Bulked samples from 5042 × K-36-SAR consisted of DNA from 11 susceptible or 20 resistant individuals. Thirty individuals were used for each of the resistant and susceptible bulks in seed tree 5701. Primers OPA-1 to OPZ-20 and OPAA-1 to OPAN-20 were screened with bulked samples from 5042 × K-36-SAR and 5701, respectively.

Southern Hybridizations. Some of the RAPD loci could not be visualized with ethidium bromide staining in families other than the one in which they were originally identified. For these loci, PCR amplification products were electrophoresed and transferred to Zetaprobe GT (Bio-Rad) membrane. Twenty nanograms of the appropriate fragment from 5042 × K-36-SAR or 5701, which had been either cloned or cut out of an agarose gel and reamplified, was labeled with 10 μCi (370 kBq) of [α-^{32}P]dCTP. The blots were hybridized and washed at 65°C; washings were in 15 mM NaCl/1 mM sodium phosphate, pH

FIG. 2. The resistant reaction to white pine blister rust (*Upper*) is characterized by a hypersensitive response resulting in small necrotic flecks; this contrasts with bright yellow areas formed in a susceptible reaction (*Lower*).

7.4/1 mM EDTA. Exposures were from 1 hr to overnight at −80°C.

Linkage Analysis. Data were combined over the five families and analyzed with the CRI-MAP linkage program (18). Scoring errors were checked by recoding repulsion phase markers to coupling and then sorting marker data by locus order (with EXCEL). Errors appeared as double recombinants, and DNA from these individuals were reamplified with the appropriate RAPD primer for verification.

RESULTS AND DISCUSSION

We were able to simultaneously score maternal gametic segregations of both the *R* locus and RAPD loci by using a test cross (*Rr* × *rr*) between a heterozygous female and a homozygous recessive male. Segregation of *R* occurs in the maternal parent and can be scored following seedling inoculation with *C. ribicola*. The megagametophytes corresponding to each seedling were collected at germination for DNA extraction and RAPD assays. Linkage analysis of *R* and RAPD loci was based on gametic segregation in the maternal parent only.

The region surrounding the *R* gene was specifically targeted using bulked samples of megagametophyte DNA from each phenotypic class, resistant and susceptible, to screen for polymorphic RAPD loci (19). Presence of a RAPD fragment in one phenotypic bulk and absence in the other indicate a putatively linked marker (Fig. 3*A*), and subsequent cosegregation analysis with DNA from individual megagametophytes verifies linkage (Fig. 3*B*). Both coupling and repulsion phase linkages can be detected with haploid megagametophytes. Five hundred twenty RAPD primers (Operon Technologies) were evaluated in megagametophytes from the controlled cross 5042 × K-36-SAR; subsequently an additional 280 primers were screened by using open-pollinated seed from the seed tree 5701. Six linked loci were found by using bulked samples from 5042 × K-36-SAR, and four loci were found in seed tree 5701. Screening and identification of linked markers were completed within a 3-month period.

Finding a given RAPD locus in individuals from different segregating populations can be a problem, possibly more so in pines than in other species, due to genome size. Heun and Helentjaris (20) refer to "epistatic" effects on RAPD markers which occur when fragments are amplified from different genetic backgrounds, presumably a result of competition from other target sites. Reproducibility within and among families

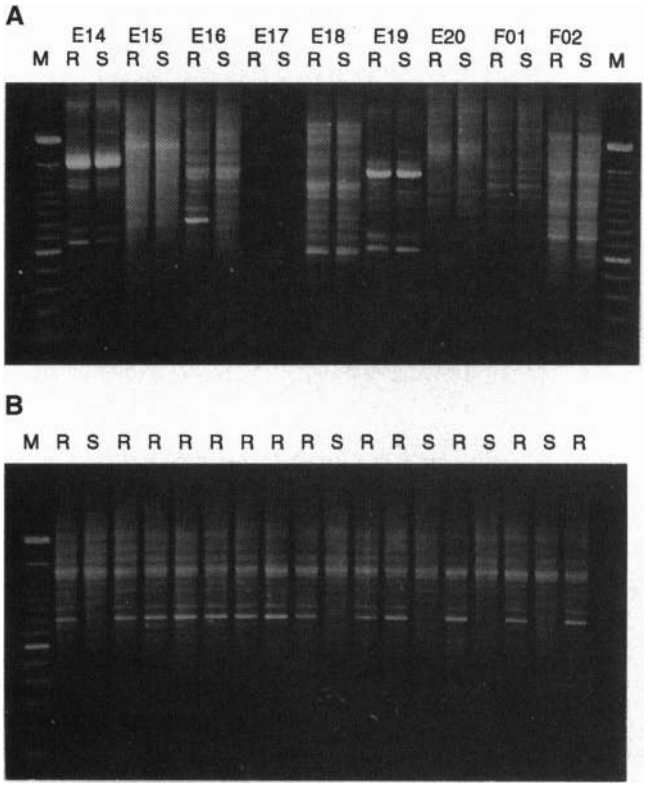

FIG. 3. (*A*) Bulked samples of DNA from resistant (R) and susceptible (S) individuals were used to screen 800 RAPD primers. Shown here are amplification products from nine primers, OPE-14 through OPF-02, on R and S bulked DNAs. Any difference between the two samples identifies a putatively linked marker, such as with OPE-16, where two fragments (800 and 900 bp) are amplified in the resistant bulked sample but not in the susceptible sample. Lanes M, size markers (100-bp ladder; BRL). (*B*) Subsequent cosegregation analysis with OPE-16 and DNA from individual megagametophytes verifies linkage.

seems to be related to intensity of the ethidium-stained fragment. RAPD fragments of loci OPE-16/800 (primer name/fragment length in base pairs), OPK-01/1110, OPT-15/650, OPAI-03/650, and OPF-07/950 were bright bands and readily visualized in all families on ethidium-stained agarose gels. The

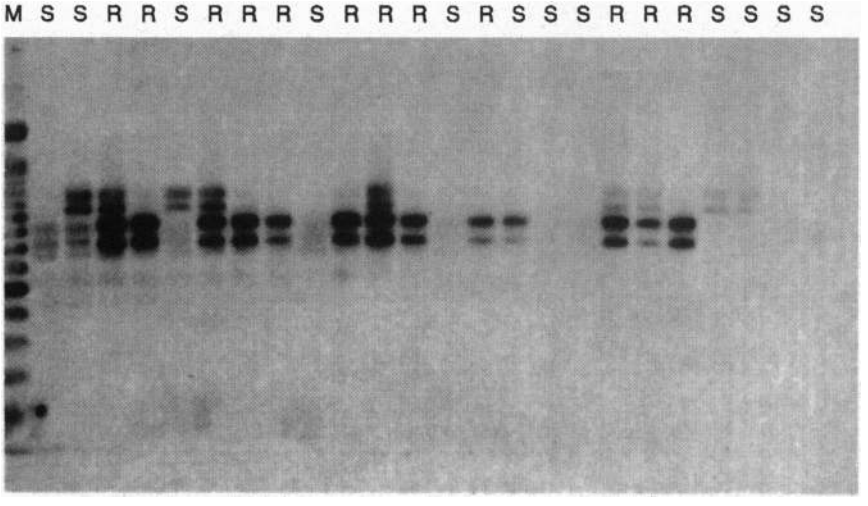

FIG. 4. Southern blot of the PCR products from 24 susceptible (S) or resistant (R) individuals from seed tree 5003 amplified with primer OPF-03 and hybridized with the 810-bp OPF-03 fragment from controlled cross 5042 × K-36-SAR. This fragment was not amplified sufficiently to allow visualization on ethidium-stained agarose gels in families other than 5042 × K-36-SAR. An amplification product of the same size as that observed for the linked RAPD locus in 5042 × K-36-SAR was detected in resistant and recombinant individuals, along with other linked and unlinked fragments. Lane M, size markers as in Fig. 3.

Table 1. Phase of segregating RAPD markers with respect to *R* in five sugar pine families, and two-point linkage analysis between RAPD and *R* loci

Locus*	Two-point recombination†		Phase with respect to *R*				
	Rec. fraction	LOD score	5003 (n = 50)	5042 (n = 37)	5701 (n = 82)	6000 (n = 68)	11300 (n = 87)
OPD-19/1120	0.06	23.4	NS	Repulsion	Coupling	NS	NS
OPE-16/800	0.05	24.6	NS	Coupling	Coupling	NS	NS
OPF-03/810	0.01	63.6	Coupling	Coupling	Coupling	Coupling	Coupling
OPF-07/950	0.14	4.5	NS	Coupling	NS	NS	NS
OPK-01/1110	0.14	27.4	Coupling	Coupling	Coupling	Coupling	NS
OPT-15/650	0.08	33.3	NS	Repulsion	Coupling	Coupling	NS
OPAD-09/920	0.16	8.6	NS	NS	Coupling	NS	NS
OPAG-05/610	0.05	47.6	Repulsion	Coupling	Coupling	Coupling	Repulsion
OPAI-03/650	0.03	53.5	Repulsion	Repulsion	Coupling	Coupling	NS
OPAN-10/590	0.04	40.5	NS	Repulsion	Coupling	Repulsion	NS

NS, nonsegregating.
*Designated as primer name/fragment length in base pairs.
†Data were combined over the five families (5003, 5042, 5701, 6000, and 11300) for estimates of recombination fractions and logarithm-of-odds (LOD) scores.

other loci (OPD-19/1120, OPF-03/810, OPAD-09/920, OPAG-05/610, and OPAN-10/590) were difficult or impossible to visualize by ethidium bromide staining in families other than the one in which they were originally identified. For these loci, Southern blots of progeny PCR products were hybridized with the appropriate RAPD fragment, cloned or cut out and reamplified, from controlled cross 5042 × K-36-SAR or seed tree 5701. Hybridization always detected a fragment of length identical to that observed in the original family, and often one or more additional fragments, either linked or unlinked (Fig. 4). With longer exposures, some hybridization could be observed in all lanes, resistant and susceptible, although signal intensity of the positive allele was many times that of the null. Southern hybridizations indicate that the target sequence for amplification is still present in the other families, but was not amplified to an extent which allowed visualization on ethidium-stained gels. Amplification products from RAPD primers often contain dispersed repetitive DNA sequences, which may explain why more than one fragment was detected on Southern blots.

RAPD loci were consistently linked with the *R* gene in all five families (Table 1). Because some of the families were homozygous for the presence or absence of the marker, the number of informative individuals varies from 37 to 324. With natural populations in linkage equilibrium, both coupling and repulsion phase of the marker and *R* loci can be found, even for tightly linked markers, as was observed for loci OPAI-03/650 and OPAN-10/590. Position and distances of RAPD markers were derived from data combined over one full-sib (5042 × K-36-SAR) and four open-pollinated families (5003, 5701, 6000, and 11300) (Fig. 5). Except for a possible inversion of OPK-01/1110 and OPAD-09/920, the order shown has a logarithm-of-odds score that is at least 1000 times better than that of the next-best order. The nearest flanking loci, OPF-03/810 and OPT-15/650, are 0.9 and 7.6 centimorgans, respectively, on either side of the *R* locus. Six of the 10 linked loci are within 5 centimorgans of the gene.

A long-term objective of this project is to use these markers as anchors for physical mapping and starting points for chromosome walking. RAPD markers corresponding to interspersed multicopy DNA would not be immediately useful for hybridization and identification of cloned DNA corresponding to unique genomic locations. It will, therefore, be necessary to convert them to locus-specific markers (21), which would then allow the use of PCR to screen genomic libraries (22). We will also need to identify more closely linked loci. Because of the size of the sugar pine genome, physical distance between markers and the *R* gene is expected to be substantial even for closely linked markers, although this assumes a homogeneous relationship between physical distance and map units in different parts of the genome.

Pine stem rusts in *Cronartium* and *Periderium*, including white pine blister rust and fusiform rust (*Cronartium quercuum* f. sp. *fusiforme*) of Southern pines, are among the most damaging pathogens of forest trees. The *R* gene of sugar pine is genetically and otherwise well characterized and clearly

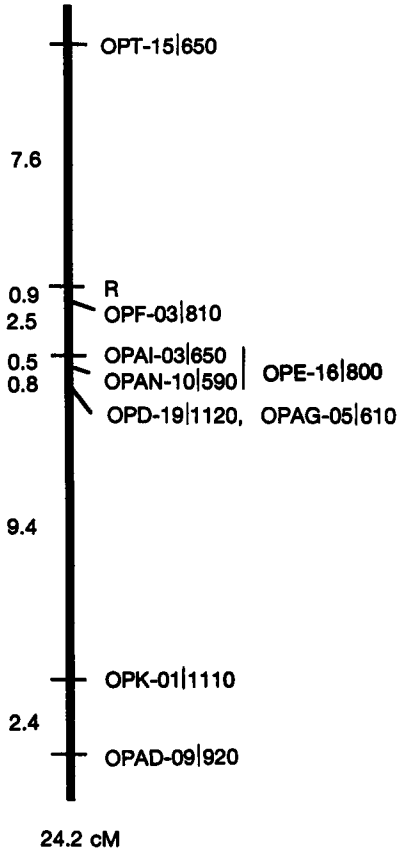

FIG. 5. RAPD map of the region surrounding the gene for resistance to white pine blister rust (*R*) in sugar pine, based on segregation data combined over five families. OPF-07/950 (not shown) maps 13.9 centimorgans (cM) above OPT-15/650, with segregation in only one family. Distances (cM) are given to the left of the vertical line.

represents the best possibility for cloning a disease-resistance gene from pine. The successful cloning of this gene will increase our understanding of the sugar pine–blister rust pathosystem and may also lead to the identification and cloning of rust resistance genes from other pines.

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