A Major Gene for Resistance to *Melampsora medusae* f. sp. *deltoidae* in a Hybrid Poplar Pedigree

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ABSTRACT

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A three-generation *Populus trichocarpa* × *P. deltoides* (T×D) hybrid poplar pedigree, comprising backcross and F_2 progenies, was used to investigate the genetic control of resistance to an isolate of *Melampsora medusae* f.sp. *deltoidae*. Necrotic flecking and rust severity were evaluated in two successive years in different field locations, in three growth-room experiments, and in a leaf-disk assay in the laboratory. Necrotic flecking in the field and growth-room experiments was found to be governed by a single, dominant gene inherited from the *P. trichocarpa* parent. This was shown in both the Mendelian analysis of the full pedigree and a genome map-based analysis of qualitative and quantitative trait loci (QTL) focused in the F_2 population. Nonparametric and QTL analyses of rust severity in the field and growth-room experiments indicated that the single gene for necrotic flecking, *Mmd1*, played a major role in resistance to *M. medusae* f.sp. *deltoidae* in the Pacific Northwest. *Mmd1* was mapped to linkage group Q, approximately 5 centimorgan (cM) from a restriction fragment length polymorphism (RFLP) marker, P222. In contrast, expression of necrotic flecking in the leaf-disk assay was low, did not indicate a role for necrotic flecking in resistance, and did not support a simple genetic interpretation.

Additional keywords: disease introduction, forest tree, leaf rust, natural pathosystem.

Interspecific hybrids of *Populus* have growing economic importance, in North America and elsewhere, as a source of pulp, lumber, and biofuel. Their importance hinges upon their extremely fast growth in short-rotation intensive culture (pulp and biofuel) and in longer rotations (lumber) (1,35). Disease can seriously impact their growth, however. In particular, leaf rusts caused by *Melampsora* spp. are problematic worldwide (25). *M. medusae* Thuem. f.sp. *deltoidae* Shain (30), native to eastern North America, with an original range presumably coincident with that of the eastern cottonwood, *P. deltoides* J. Bartram ex Marsh., first appeared in hybrid poplar plantations in the Pacific Northwest in 1991 (20). It is currently the most widespread and common disease of hybrid poplar in the region, and has been linked to significant reductions in growth elsewhere (40). In the Pacific Northwest, mortality in a newly established stool bed was highly correlated with the severity of leaf rust caused by *M. medusae* f.sp. *deltoidae* during the preceding fall (22).

Poplar F_1 hybrids can be selected for disease resistance and other traits and then clonally propagated (23). Estimates of the broad-sense heritability of resistance to Melampsora leaf rust in poplars in the field have tended to be relatively high, indicating strong genetic control (14,38). In other studies, broad-sense heritabilities were not computed; nevertheless, it was clear that most of the variation in rust severity was among clones, rather than within them (9). In contrast, in some laboratory studies employing the leaf-disk assay, environmental factors (particularly temperature and light intensity) were found to be more important than genetic factors in disease expression (5,6).

A variety of resistance mechanisms may operate during colonization of the host, depending upon the particular combination of poplar clone and rust isolate. Necrotic flecking has been commonly observed in poplars inoculated with rust fungi and may result from hypersensitive necrosis of invaded host cells (10) or from early plasmolysis developing more slowly into necrosis (31). These two responses, characterized by a difference in timing, have also been described in other plant-rust combinations (37). Typically, necrotic flecking in other better-studied plant species, in response to invasion by rust fungi, has been found to be under major gene control; thus, it is scored in a qualitative fashion (19). In spite of the common occurrence and observation of necrotic flecking associated with poplar leaf rust, attempts to determine its genetic control have been inconclusive (24).

This study is part of a larger one employing a genome-mapped, three-generation hybrid poplar pedigree, including backcross progenies, developed by the University of Washington/Washington State University Hybrid Poplar Project. The genetic basis of resistance to three *Melampsora* spp. is but one component of the larger study (2,3,4). Preliminary phytopa-

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thological work demonstrated that resistance to *M. occidentalis* H. Jacks., *M. larici-populina* Kleb., and *M. medusae* f.sp. *deltoidae* segregated within this pedigree. Easy clonability of hybrid poplar allowed us to evaluate this pedigree for Mendelian patterns of resistance to *M. medusae* f.sp. *deltoidae*, both in the field and in repeated growth-room and leaf-disk studies. Linkage mapping of this same pedigree provided a second method of genetic analysis of resistance to *M. medusae* f.sp. *deltoidae*. Our objective was to determine the genetic control of necrotic flecking and its relationship to resistance to leaf rust caused by *M. medusae* f.sp. *deltoidae*.

MATERIALS AND METHODS

The mapping pedigree. Development of the qualitative and quantitative trait loci (QTL) mapping pedigree began in 1981 by interspecific hybridization between *P. trichocarpa* Torr. & A. Gray (female clone 93-968 from western Washington) and *P. deltoides* (male clone ILL-129 from central Illinois) (4). *P. trichocarpa* is the native black cottonwood of the Pacific Northwest, while *P. deltoides* is the eastern cottonwood of eastern North America. Two F_1 (T×D) siblings (i.e., 53-246 and 53-242) were crossed in 1988 to produce the inbred F_2 family 331 (TD×TD). In field evaluations of rust and necrotic flecking and in inoculation studies, we focused on 20 F_1 clones and 68 clones in the F_2 family which were genotyped using restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers (3). Known triploids were excluded from analyses (2).

Backcross progenies. The F_1 female clone 53-246 was also crossed to ILL-129 to produce backcross family 342 (TD×D). The female *P. trichocarpa* parent, 93-968, was crossed with male F_1 offspring, 53-239, to produce the backcross family 353 (T×TD).

Field plantings. All above-mentioned clones were established in seedling stool beds at WSU-Farm 5 near Puyallup, WA, in the years of their breeding. Stools were grown at a spacing of 0.6 m within rows and 1.2 m between rows, and coppiced each spring. The pedigree planting near Clatskanie, OR, was established in the spring of 1993 on 1.7 ha made available by the James River Corporation. It was designed for the study of growth and development of T×D hybrid poplars in an environment in which a number of poplar pests and pathogens, including leaf rust due to *M. medusae* f.sp. *deltoidae*, had become problematic. Trees were planted at a spacing of 1.5 m within rows and 3.0 m between rows in a modified randomized complete block design. The planting contains three blocks of two-tree plots of 376 genotypes or clones of F_2 family 331, both F_1 offspring, and the parental clones 93-968 and ILL-129.

Plant propagation and care for growth-room and leaf-disk assays. Hardwood cuttings were cut from coppiced stools in the field in January or February and stored in sealed plastic bags at –5°C until needed. Cuttings were rooted in potting soil mix in a greenhouse with periodic misting. Biological control of mite and insect pests was practiced to avoid the use of pesticides which might interact with rust inoculations.

Isolate selection and inoculum increase. All inoculations were performed with a monouredinial isolate, 139-91, of *M. medusae* f.sp. *deltoidae*. This isolate was obtained from a collection of rusted leaves of a T×D clone, 11-11, growing in Woodburn, OR, in the fall of 1991. This same isolate was used previously and found to be similar in pathogenicity to two other monouredinial isolates of *M. medusae* f.sp. *deltoidae* (20). Inoculum was increased for experiments by inoculating detached leaves and leaf pieces of a susceptible T×D clone, 47-174, as previously described (20).

Inoculation procedures. Leaf-disk assay. Either the fourth or fifth leaf, depending on the clone, from the "index leaf" (i.e., the leaf, the lamina of which was closest to 2 cm in length) (17) was detached in order to punch 16-mm-diameter disks from it, as previously described (11). In each of three separate experiments, a given clone was represented by a total of six disks partitioned in three randomized two-disk replicates. Each 9-cm-diameter petri plate equipped with filter paper, moistened with a 100-ppm solution of gibberellic acid, had eight leaf disks as well as two agar (i.e., 1.5% water agar amended with 100 μg/ml each of streptomycin and chloramphenicol) disks. The agar disks were used to quantify urediniospore deposition and germination 24-h postinoculation.

A urediniospore suspension (approximately 3×10^4 spores per milliliter), with 0.05% Tween 20 at final volume, was sprayed into a metal settling tower (1-m tall and 25.4-cm in diameter) at the base of which were four open petri plates containing the disks. A thin-layer chromatographic (TLC) reagent sprayer operating at 0.014 MPa (2 psi) was allowed to spray the suspension for 6 s. Spores were allowed to continue to deposit on the disks for another 3 min after the sprayer was shut off. This inoculation procedure resulted in uniform deposition for a given spray (coefficients of variability for each spray were always less than 10%, $n = 8$). Variation among sprays resulted in a range of deposition from 100 to 400 spores per agar disk. Inoculated leaf disks were incubated at 20°C under constant illumination at 35 $\mu E\ m^{-2}\ s^{-1}$.

Inoculation procedures. Growth-room assay. Plants were initially started and grown in a rust-free greenhouse until they reached a 10-leaf stage. They were then transferred to, and randomized on, benches covered with plastic tents in a 540-m^3 growth-room. Light intensity was approximately 70 $\mu E\ m^{-2}\ s^{-1}$ under the plastic tent at plant height, with 16-h daylength and average day and night temperatures of 20 and 15°C, respectively. A spore suspension, as described for leaf-disk assays, was sprayed onto the plants. The plastic tent was then sealed to maintain leaf wetness for 18 h. Growth-room experiment 1 (GRI) was inoculated only once, GRII was inoculated twice, and GRIII was inoculated daily simply by misting the benches, after the initial appearance of rust so as to mimic a field epidemic. A susceptible control, T×D hybrid 47-174, was used on each bench in all three growth-room experiments. GRI included uninoculated controls of 10 clones.

Identification of *Melampsora* spp. causing leaf rust in the field. Ratings of hybrid poplar leaf rust in the field in the Pacific Northwest must currently be accompanied by sampling to identify causal *Melampsora* spp., since three species (*M. medusae* f.sp. *deltoidae*, *M. larici-populina*, and *M. occidentalis*) may now occur (21). Sampling and identification on the basis of urediniospore morphology were as previously described (20,21).

Evaluation of leaf-rust severity. Field. Rust severity was rated according to the method of Schreiner (29) and consisted of rating the most severely rusted leaves of a given plant as lightly (i.e., arbitrary numerical rating of 1), moderately (i.e., rating of 5), or heavily rusted (i.e., rating of 25). The percentage of rusted leaves of the plant was estimated at ≤ 25, 26 to 50, 51 to 75, or > 75, and given a numerical value of 1, 2, 3, or 4, respectively. Multiplication of the two numerical values gave a Schreiner rating from 0 (highly resistant) to 100 (highly susceptible). In October 1993, in the stool beds of Field-93 near Puyallup, WA, single ramets of each of 93-968, ILL-129, F_1, F_2, and the two above-mentioned backcross progenies were rated for rust. In late September 1994, in the replicated planting near Clatskanie, OR, F_2 clones were rated (Field-94). Whether coppiced in spring (Field-93) or not (Field-94), there was considerable, mostly clonal, variation in plant size at the time of rust rating, as expected (3).

Evaluation of leaf-rust severity. Growth-room. Schreiner's method as outlined above was again used, except that the percentage of leaves in a given severity category was calculated by counting all leaves and the number in the most severe category, rather than being visually estimated as it was in the field. GRI

consisted of F_2 clones only, while GRII and GRIII included the full extended pedigree (i.e., 93-968, ILL-129, F_1, F_2, and backcross progenies). In each experiment, each clone was represented by three ramets.

Evaluation of necrotic flecking. Necrotic flecking was rated in 1993 in the stool beds at WSU-Farm 5 near Puyallup, WA, in the three growth-room experiments and in the leaf-disk assay. To score necrotic flecking qualitatively it was necessary to focus on the youngest rusted leaves, since older rusted leaves often had developed some necrosis associated with old uredinia and formative telia. Flecking phenotypes were qualitatively either "fleck+", if the mean number of young leaves bearing uredinia but no flecks was less than or equal to two, or "fleck–", if the mean number was greater than two. Thus, most fleck+ clones had rusted leaves on which flecking accompanied the uredinia, although some had rust without flecking on the youngest rusted leaf. Clones showing flecking without uredinia were also scored as fleck+. Clonal means were of three branches in the case of the 1993 field data, and of three ramets in GRI, GRII, and GRIII.

With the leaf-disk assay, it was not possible to score flecking in the above manner. Since preliminary work had made it clear that incidence of flecking in the leaf-disk assay was low, clones were considered fleck+ if any necrosis occurred in any one of the three replicates. Since there were six disks per replicate, clones were considered fleck+ even if only one disk of a total of 18 bore necrosis, even necrosis contiguous with uredinia. Fleck– clones bore no necrosis. Evaluation was performed 14 days after inoculation.

Broad-sense clone mean heritabilities (H^2). Values of H^2 for rust severity were estimated, as described previously (3), from the following model using the type III mean squares (MS) calculated by the SAS GLM procedure:

$$\sigma^2_{\text{trait}} = \sigma^2_{\text{rep}} + \sigma^2_{\text{clone}} + \sigma^2_{\text{rep}\cdot\text{clone}} + \sigma^2_{\text{error}}$$

$$H^2 = (\text{MS}_{\text{clone}} - \text{MS}_{\text{rep}\cdot\text{clone}}) / \text{MS}_{\text{clone}}$$

in which σ^2_{trait} is the total phenotypic variance, σ^2_{rep} is the phenotypic variance among blocks, and σ^2_{clone} is the phenotypic variance among clones.

Statistics. Sigmastat (Jandel Scientific Software, San Rafael, CA) was used to calculate Pearson correlation coefficients to assess the degree of similarity of growth-room assays and field evaluations. Chi-square tests, with Yates correction for continuity, were used to test the single-gene hypothesis in the extended pedigree. Normality of data distributions was tested with the Kolmogorov-Smirnov (K-S) procedure. Owing to nonnormality of rust-severity data, especially in the fleck+ class, a nonparametric Kruskal-Wallis analysis of variance (ANOVA) on ranks of mean rust scores for fleck+ and fleck– clones, followed by Dunn's multiple comparison method, was used to determine the effect flecking had with respect to rust severity. To test whether necrotic flecking results from the leaf-disk assay predicted susceptibility to *M. medusae* f.sp. *deltoidae* in the field, a nonparametric Mann-Whitney rank sum test in Sigmastat (Jandel Scientific Software) was used to compare mean rust severities (1993 field data) of fleck+ versus fleck– scores (as determined by the leaf-disk assay).

Linkage map construction. A linkage map composed of 343 RFLP, RAPD, and sequence-tagged site (STS) markers was constructed for the F_2 family 331, as described previously (3,4). The map covered approximately half the length of the *Populus* genome. Qualitative data for necrotic flecking were coded as having the *P. trichocarpa* (93-968) allele dominant to that of *P. deltoides* (ILL-129). MAPMAKER 3.0 (16) was used to calculate the distance from the nearest linked RFLP marker (P222) to the flecking locus scored in the field and growth-room. Quantitative data on rust severity were analyzed using MAPMAKER/QTL 1.1 (18).

RESULTS

Identification of *Melampsora* spp. in the field evaluations. In the field at WSU-Farm 5 near Puyallup, WA, in 1993, eight clones in the 353 family (T×TD backcross progeny) were found to be infected with *M. occidentalis*. These were not included in the data. In the field near Clatskanie, OR, in 1994, only *M. medusae* f.sp. *deltoidae* was found on the evaluated F_2 clones in the 331 family. No samples of *M. larici-populina* were found.

Appearance of necrotic flecking in response to *M. medusae* f.sp. *deltoidae*. Necrotic flecking was first observed in the mapping pedigree in the field in 1993. Since other causal factors could operate in the field, it was only in GRI that it became clear that flecking occurred in response to *M. medusae* f.sp. *deltoidae* alone. Uninoculated plants in GRI did not show flecking, while inoculated ramets of the same clones did. Since necrotic flecking is often the manifestation of a hypersensitive response (HR) (10), we made observations in all three growth-room experiments on the timing of the appearance of necrotic flecking. Necrotic flecking became evident macroscopically within the same time frame as uredinia (i.e., from 7 to 14 days, depending on the clone). Fleck size, relative extent of association of flecks with uredinia, and fleck number per unit leaf area all varied depending on the clone, but no attempt was made in these experiments to measure these characters.

A measure of necrotic flecking. Scoring clones qualitatively for flecking in terms of number of young leaves bearing uredinia without accompanying flecking is illustrated in Figure 1 (results of GRIII). The frequency distribution was bimodal in tendency and was divided into fleck+ (i.e., less than two, and most often no, young leaves bearing uredinia without flecking) and fleck– (i.e., greater than two leaves bearing uredinia without accompanying flecking). This threshold seemed justified since it divided the bimodal distribution at the point of low frequency between the two peaks. It also divided the overall, nonnormal distribution into a nonnormal fleck+ class and a normal fleck– class in GRIII (Table 1). In general, dividing F_2 into two phenotypic classes, fleck+ and fleck–, divided the overall nonnormal distribution into nonnormal and normal subdistributions (Table 1), in keeping with expectations for a single major gene. The dominant allele skewed the distribution of the fleck+ class, while its absence resulted in normal or near-normal distributions for the fleck– class.

Mendelian inheritance of necrotic flecking. In the field, in 1993, and in growth-room experiments GRII and GRIII, the parents, F_1, F_2, and the two backcross progenies were all scored for necrotic flecking (Table 2). In all cases, 93-968, the *P. trichocarpa* parent, displayed necrotic flecking (i.e., it was fleck+), while ILL-129, the *P. deltoides* parent, did not. Necrotic flecking

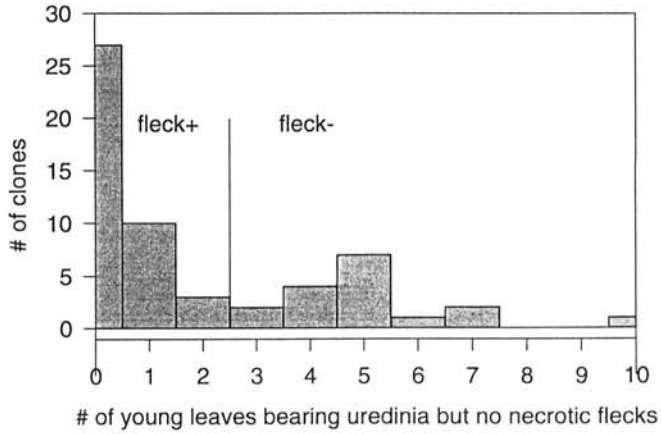

Fig. 1. Frequency distribution for the number of young leaves bearing uredinia of *Melampsora medusae* f.sp. *deltoidae* but no flecks, based on F_2 clonal means. Line divides fleck+ (zero to two leaves) from fleck– (> two leaves).

while ILL-129, the *P. deltoides* parent, did not. Necrotic flecking (fleck+/fleck–) segregated 1:1 in F_1, 3:1 in F_2, and 1:1 in each of the backcross progenies. The simplest explanation was that 93-968 was heterozygous for a single dominant gene governing necrotic flecking, while ILL-129 was homozygous recessive. The two F_1 parents of F_2, 53-242 and 53-246, were fleck+ phenotypically and heterozygous genotypically. The F_1 parent of the backcross family 353 (T×TD), 53-239, was fleck– phenotypically and homozygous recessive genotypically.

In the leaf-disk assay, both 93-968 and ILL-129 were fleck– phenotypically, while both 53-242 and 53-246, the F_1 parents of F_2, were fleck+. In F_2, flecking segregated 1:1 as there were 29 clones in each of the flecking classes. This pattern of inheritance was not only distinct from that in the growth-room experiments and the field, but it also could not be explained by a single gene. Although the F_1 results might initially suggest that a single recessive gene controls necrotic flecking, there would then be an expectation of no segregation in F_2, with all clones being fleck+, which was not observed.

Evaluating necrotic flecking in the field, growth-room, and leaf-disk assays. Since clones were categorized as either fleck+ or fleck–, data from separate experiments were tabulated in a matrix of percentage of agreement (Table 3). Agreement in evaluations of flecking in the field and in the three growth-room experiments did not differ significantly from 100%. However, the evaluation of flecking in the leaf-disk assay did not agree ($P < 0.005$) with any of the other four evaluations.

We were interested in determining whether flecking as expressed in the leaf-disk assay might predict rust resistance in the field. Clones in the fleck+ and fleck– classes from the leaf-disk assay were compared with respect to their rust-severity scores from the 1993 field evaluation. Again owing to nonnormality, a nonparametric Mann-Whitney rank sum test was used to compare the fleck+ mean of 35.8 (standard deviation [SD] = 40.2) with the fleck– mean of 28.1 (SD = 36.1). The two means did not differ significantly ($P = 0.54$). The two distributions were both nonnor-

TABLE 1. The importance of necrotic flecking to resistance of F_2 poplars to *Melampsora medusae* f.sp. *deltoidae*, as evidenced by a comparison of rust-severity scores for fleck+ and fleck– phenotypic classes in field and growth-room experiments[t]

	Fleck+[u]				Fleck–[v]			
	Mean rust score[w]	SD	CV[x] (%)	K-S distance[y]	Mean rust score	SD	CV(%)	K-S distance
Field93	10.4 bc[z]	17.4	167	0.3 ($P << 0.001$)	66.7 a	35.6	53	0.28 ($P < 0.001$)
Field94	8.4 c	14.1	168	0.3 ($P << 0.001$)	41.3 a	24.2	59	0.16 ($P = 0.3$)
GRI	0.9 d	1.5	167	0.3 ($P << 0.001$)	21.7 a–c	23.9	110	0.23 ($P = 0.02$)
GRII	3.8 cd	8.7	229	0.33 ($P << 0.001$)	24.9 ab	18.9	76	0.22 ($P = 0.03$)
GRIII	3.3 cd	3.4	103	0.17 ($P = 0.008$)	26.6 a	15.3	58	0.15 ($P = 0.4$)

[t] 1993 and 1994 field data were collected in plantings near Puyallup, WA, and Clatskanie, OR, respectively. Growth-room data were from studies of potted plants inoculated with *M. medusae* f.sp. *deltoidae* and maintained with a 16-h daylength of approximately 70 μE m^{-2} s^{-1} at plant height and temperatures of 20 and 15°C during day and night.

[u] Clones were fleck+ if the mean number (of three branches [in the field] or of three plants [in the growth room, GR]) of young leaves bearing uredinia but no flecks was less than two.

[v] Clones were fleck– if the mean number (of three branches [in the field] or of three plants [in the growth room, GR]) of young leaves bearing uredinia but no flecks was two or greater.

[w] Mean rust score was based on a scale of 0 to 100.

[x] CV = coefficient of variability.

[y] Kolmogorov-Smirnov test for normality. Distance values with $P > 0.01$ indicate normal distributions.

[z] Means followed by the same letter are not significantly different ($P < 0.05$), according to Dunn's method of multiple comparison, following a nonparametric Kruskal-Wallis analysis of variance on ranks of mean rust scores.

TABLE 2. Mendelian analysis of a dominant, single gene governing a necrotic flecking response to *Melampsora medusae* f.sp. *deltoidae* in a three-generation hybrid poplar pedigree[v]

Parentals	Progeny family number	Origin	Flecking[w]; postulated genotypes at *Mmd1* locus	Observed fleck+/fleck– ratio[x,y]	Expected fleck+/fleck– ratio	χ^2 probability[y,z]
Ill-129 (59-129-17)		*P. deltoides*, Illinois	No (–); *mmd1mmd1*			
93-968		*P. trichocarpa*, Washington	Yes (+); *Mmd1mmd1*			
	53	F_1 (93-968 × Ill-129)	*Mmd1mmd1* × *mmd1mmd1*	(a) 8:11	1:1	(a) $0.5 > P > 0.25$
				(b) 10:10		(b) $0.9 > P > 0.75$
				(c) 12:8		(c) $0.5 > P > 0.25$
	331	F_2 (53-246 × 53-242)	*Mmd1mmd1* × *Mmd1mmd1*	(a) 48:20	3:1	(a) $0.5 > P > 0.25$
				(b) 37:17		(b) $0.5 > P > 0.25$
				(c) 35:16		(c) $0.5 > P > 0.25$
	342	Backcross (53-246 × Ill-129)	*Mmd1mmd1* × *mmd1mmd1*	(a) 32:28	1:1	(a) $0.75 > P > 0.5$
				(b) 18:19		(b) $0.9 > P > 0.75$
				(c) 21:12		(c) $0.25 > P > 0.1$
	353	Backcross (93-968 × 53-239)	*Mmd1mmd1* × *mmd1mmd1*	(a) 27:22	1:1	(a) $0.5 > P > 0.25$
				(b) 9:12		(b) $0.5 > P > 0.25$
				(c) 12:10		(c) $0.75 > P > 0.5$

[v] Further information on this pedigree is found in the University of Washington/Washington State University Poplar Program's "Pedigree Clone Register", Nov. 1992.

[w] Flecking phenotypes were scored as either fleck+ or fleck–. Clones were fleck+ if the mean number (of three branches [in the field] or of three plants [in the growth room, GR]) of young leaves bearing uredinia but no flecks was less than two, and fleck– if the mean number was two or greater.

[x] Field data: Puyallup, 1993. Separate growth-room studies (i.e., GRII and GRIII) were conducted with potted plants, using a 16-h daylength of approximately 70 μE m^{-2} s^{-1} at plant height. Day and night average temperatures were 20 and 15°C, respectively.

[y] Location of study: (a) field, (b) GRII, and (c) GRIII.

[z] With Yates correction for continuity.

mal (K-S distance = 0.26, $P < 0.0001$ for fleck–; K-S distance = 0.24, $P < 0.001$ for fleck+), unlike the growth-room experiments and field data in which fleck– distributions were generally normal (Table 1).

Role of necrotic flecking in resistance. The first analysis which demonstrated an important role in resistance for necrotic flecking was of the 1993 field data. A 2 × 2 contingency table for resistance and necrotic flecking was constructed. Resistant clones were defined as clones with mean rust-severity scores below 25 on the 0 to 100 Schreiner scale, a cut-off value originally suggested by Schreiner (29).

Of the 68 F_2 clones, 48 were fleck+ (44 of which were resistant) and 20 were fleck– (16 of which were susceptible) (Table 2). The analysis indicated a significant relationship between resistance and necrotic flecking ($\chi^2 = 24.8$, $P < 0.001$).

Rust-severity data for F_2 from the field in 1993 and 1994 and from the three growth-room experiments were all nonnormal. Mean rust scores for fleck+ and fleck– phenotypic classes were analyzed with a nonparametric Kruskal-Wallis ANOVA on ranks (Table 1). Within each field evaluation or growth-room experiment, fleck+ was significantly more resistant than fleck– ($P < 0.05$). There were no significant differences among the five experiments and evaluations for fleck–, but there were among those for fleck+ in that GRI fleck+ was significantly different from fleck+ in both field evaluations. Rust severity among fleck+ clones in GRI was both very low and variable (Table 1). Generally low and variable rust infection in GRI also explained why its fleck– class did not differ significantly from the fleck+ class in GRIII and the two field evaluations. Similarly, somewhat low and variable rust infection in GRII explained the fact that its fleck– class did not differ significantly from the fleck+ class in the 1993 field evaluation. In GRIII, in which an attempt was made to mimic field conditions by maintaining leaf wetness each night, rust severity was somewhat higher and much less variable (i.e., much lower coefficient of variability in Table 1) than in GRI and GRII. Thus, GRIII was like both field evaluations in that its fleck– and fleck+ classes were significantly different from all fleck+ and fleck– classes, respectively, in all other experiments and evaluations.

The 1993 field rust evaluation differed from that of 1994 and GRIII in one respect. Rust severity in the fleck– class of the former was greater, even though insignificantly, because it was evaluated relatively late in the epidemic. The greater severity in 1993 in the fleck– class undoubtedly explained its nonnormality, while the same class in 1994 and GRIII was normally distributed.

Rust severity in growth-room experiments and field evaluations. In Table 4, we ignored the role of necrotic flecking to find the extent of correlation for rust severity among the three growth-room experiments and the two field evaluations. All growth-room experiments and field evaluations were significantly correlated to varying extents. The highest correlations (Spearman $r = 0.87$, $P < 0.01$) were found between the two field evaluations, carried out in successive years in different locations, and GRII and GRIII. GRI, characterized by a single inoculation and relatively low and variable rust, was different from GRII and GRIII in this analysis as well ($r = 0.67$, $P < 0.01$).

Broad-sense clone H^2 for rust resistance. H^2 (Table 5) was actually higher in the field in 1994 (i.e., 0.95) than in any of the growth-room experiments (i.e., 0.82, 0.85, and 0.87 in GRI, GRII, and GRIII, respectively). This likely resulted from the higher severity and uniformity of rust in the field, causing low variance within clones. The high H^2 values in this study are similar to some previous studies of resistance to *M. medusae* f.sp. *deltoidae* (14,38).

Linkage analysis of necrotic flecking. The qualitative data for necrotic flecking allowed it to be coded as marker data. In keeping with the generally good agreement in rating F_2 clones for flecking in the field (1993) and in three growth-room experiments (Table 3), all of the flecking data mapped to roughly the same position on linkage group Q (Fig. 2). Best agreement in rating flecking was achieved in GRII and GRIII (Table 3), and flecking explained more of the variance in rust severity in GRIII than in GRI or GRII (see next paragraph). Thus, GRIII flecking analysis was used to locate the *Mmd1* locus 5.1 cM from the mapped RFLP marker P222 (Fig. 2).

QTL analysis of necrotic flecking and rust severity. The QTL analysis of the rust-severity data from the three growth-room experiments and the field evaluations consistently identified

TABLE 3. Percentage of agreement in rating F_2 poplar clones for flecking response to *Melampsora medusae* f.sp. *deltoidae* in the field, and in growth-room (GR) and leaf-disk assays

	GRII[x]	GRIII	Field, 1993	Leaf-disk assay
GRI	81.6 (40/49)[y], $0.25 > P > 0.1$[z]	83.0 (39/47), $0.25 > P > 0.1$	92.6 (50/54), $0.75 > P > 0.5$	38.0 (19/50), $P < 0.005$
GRII		98.0 (51/52), $0.9 > P > 0.75$	76.8 (43/56), $0.1 > P > 0.05$	34.6 (18/52), $P < 0.005$
GRIII			77.8 (42/54), $0.1 > P > 0.05$	38.5 (20/52), $P < 0.005$
Field, 1993				36.8 (21/57), $P < 0.005$

[x] Flecking phenotypes were scored as either fleck+ or fleck–. Clones were fleck+ if the mean number (of three branches, or plants, in the field and in the growth room, respectively) of young leaves bearing uredinia but no flecks was less than two, and fleck– if the mean number was two or greater. In contrast in the leaf-disk assay, clones were fleck+ if any necrotic flecking was observed in any of three replications in time, each replication consisting of six leaf disks per clone.

[y] Number of clones in agreement/total number of clones shared by the two tests being compared.

[z] χ^2 test of the null hypothesis that percentage of agreement did not differ significantly from 100%.

TABLE 4. A matrix of Spearman rank correlation coefficients for severity of leaf rust caused by *Melampsora medusae* f.sp. *deltoidae* on F_2 poplar clones, rated in growth-room and field environments[y]

	Growth room I	Growth room II	Growth room III	Field, 1994
Field, 1993	0.81*[z]	0.68*	0.66*	0.87*
Growth room I		0.67*	0.67*	0.79*
Growth room II			0.87*	0.77*
Growth room III				0.69*

[y] 1993 and 1994 field data were collected in plantings near Puyallup, WA, and Clatskanie, OR, respectively. Growth-room data were from studies of potted plants inoculated with *M. medusae* f.sp. *deltoidae* and maintained with a 16-h daylength of approximately 70 $\mu E\ m^{-2}\ s^{-1}$ at plant height, and temperatures of 20 and 15°C during day and night. Correlations are of clonal means, with n varying from 47 to 63.

[z] Asterisk indicates significance at $P < 0.01$.

TABLE 5. Genome map-based analysis of severity of rust caused by *Melampsora medusae* f.sp. *deltoidae* on F_2 poplar clones in field evaluations and growth-room experiments

	LOD[v]	Phen%[w]	H^2[x]	Gen%[y]
FLD93[z]	10.95	83	ND	ND
FLD94	8.16	73	0.95	77
GRI	3.09	36	0.82	44
GRII	2.64	27	0.85	32
GRIII	8.51	87	0.87	100

[v] The logarithm of the odds ratio at the point of maximum likelihood.

[w] The proportion of the total phenotypic variance explained by the *Mmd1* locus.

[x] Broad-sense heritability; ND = not determined.

[y] The proportion of the total genotypic variance explained by the *Mmd1* locus.

[z] GRI, II, and III = Growth-room-inoculation experiments I, II, and III, respectively. FLD93 = Puyallup, 1993; and FLD94 = Clatskanie, 1994.

part of phenotypic variance (Table 5), although logarithm of the odds ratio at the point of maximum likelihoos (LOD) scores varied. As in the previous analysis (Table 1), GRIII was much more similar to the field data than GRI or GRII. In this case, necrotic flecking appeared to play a more important role in resistance in the field in 1993 (percentage of phenotypic variance explained by the *Mmd1* locus: 83%) and 1994 (73%) and in GRIII (87%), than in GRI (36%) and GRII (27%).

It should be noted that the nonnormality of rust severity violated an assumption of the parametric QTL analysis. However, with respect to the fundamental conclusion of this study (i.e., that the single gene for necrotic flecking was a major gene for resistance) the QTL analysis of rust severity was congruent with the nonparametric analysis (Table 1). The QTL and nonparametric analyses were also congruent with respect to the clustering of GRIII with field data, distinct from GRI and GRII.

DISCUSSION

Necrotic flecking in hybrid poplar, in response to infection by *M. medusae* f.sp. *deltoidae*, can be governed by a single dominant gene, just as in other plant species (19). In this study, both the Mendelian and the genome mapping analyses indicated that a single, dominant allele inherited from the *P. trichocarpa* parent governed necrotic flecking. The dominant allele, at what we are calling the *Mmd1* locus, was not only effective against a monouredinial isolate in growth-room experiments, but was also effective in a similar way in two field locations in two successive years. Correlation and QTL analyses suggested that the *Mmd1* locus played a prominent role in determining disease incidence and severity in the field. Support for this conclusion consisted of the following: i) in all field and growth-room experiments, fleck+ clones, on average, were significantly more resistant than fleck– clones; ii) distributions of rust-severity means of fleck+ clones were nonnormal, while those of fleck– clones tended to be normal (provided that they were evaluated at the time when the most susceptible clones were becoming maximally rusted); and iii) the fact that a QTL on linkage group Q coincident with the necrotic flecking locus (established by coding the qualitative necrotic-flecking data as marker data) accounted for a large percentage of phenotypic variance in rust severity in all experiments, but especially so in the field and the growth-room experiment (i.e., GRIII) which simulated field conditions.

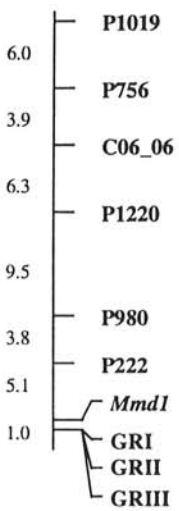

Fig. 2. Mapping of gene for necrotic flecking of poplar in response to infection by *Melampsora medusae* f.sp. *deltoidae*. Position of *Mmd1* flecking locus on linkage group Q 5.1 cM from the mapped restriction fragment length polymorphism (RFLP) marker, P222. Interval estimated from growth-room (GR) experiments I, II, and III.

The inheritance of resistance to *M. medusae* Thuem. has previously been studied in the F_1 progeny of a *P. deltoides* J. Bartram ex Marsh. cross (26). For Mendelian analysis, infection types 0 and 1 (including necrotic flecking) were classed as resistant, while 2 to 4 were susceptible. Depending upon the race employed, the genetic basis of resistance was hypothesized to vary from that of a single, dominant gene to that of three to four additive genes. However, interpretation of results was tentative owing to the exclusive study of F_1 material in the absence of backcross and F_2 progenies.

Inheritance of three quantitative measures of resistance (i.e., latent period, uredinial infection frequency, and sporulation capacity) in the same F_1 progenies of the above-mentioned cross of eastern cottonwood, *P. deltoides*, to two Australian races of *M. medusae*, has also been studied (27). Continuity of the resulting frequency distributions for the three characters was interpreted as evidence of polygenic control. Other studies have focused on the above, and other, quantitative parameters of poplar leaf rust with accompanying speculation as to the nature of their genetic control (32). However, with the advent of genetic linkage maps, it is now frequently possible to distinguish between polygenic and oligogenic control of continuously distributed quantitative characters (36).

In this study, 93-968, the *P. trichocarpa* parent, was infected by *M. medusae* f.sp. *deltoidae* in addition to displaying necrotic flecking. The literature, as reviewed by Pinon (25), indicates that the susceptibility of *P. trichocarpa* to *M. medusae* f.sp. *deltoidae* is poorly understood. This is, in part, because the formae speciales of *M. medusae* have only recently been described (30), and, in part, because authors have regarded *M. medusae* and *M. albertensis* as synonymous (42). We found that *M. medusae* f.sp. *deltoidae* was able to infect and sporulate on many, but not all, *P. trichocarpa* individuals in both the growth room and the field (G. Newcombe, *unpublished data*). Thus, 93-968 was not unusual in its susceptibility to *M. medusae* f.sp. *deltoidae*.

The assay method was critical in the analysis of genetic control of necrotic flecking in this study. Unlike qualitative scoring of necrotic flecking in both the field and all growth-room experiments which yielded marker data placing the *Mmd1* locus at the same approximate map position on linkage group Q, qualitative scoring of flecking in the leaf-disk assay of the same host material did not result in successful mapping. There was also significant deviation from the expected Mendelian ratio of 3:1 fleck+/fleck– in the leaf-disk assay. In fact, the observed 1:1 ratio overestimated the expression of necrotic flecking in this assay, since it represented a relaxed standard for scoring a clone as fleck+. The flecking results from the leaf-disk assay also deviated significantly from phenotyping of flecking in the field or growth room (Table 2). Flecking results from the leaf-disk assay also did not predict susceptibility to *M. medusae* f.sp. *deltoidae* in the field, even though infection efficiency, latent period, or some other quantitative parameter may. It is quite possible that a QTL analysis of quantitative data from the leaf-disk assay would confirm the importance of the *Mmd1* gene, which, assuming one had performed nothing but the leaf-disk assay, would leave the researcher with the diagnosis of a major gene for resistance, but without the corresponding disease-resistance phenotype. Similarly, previous attempts to analyze genetic control of necrotic flecking (24) expressed in the leaf-disk assay have been inconclusive.

As expected for a single gene of major effect, there was apparently little effect of environment on phenotypic expression of *Mmd1*. Flecking results from the growth-room experiments agreed with those from the field, even though the light intensity of the growth room was low (i.e., 70 $\mu E\ m^{-2}\ s^{-1}$) relative to the field. Other abiotic and biotic factors of the field environment were also likely poorly duplicated in the growth room, without significant effect.

The leaf-disk assay constituted an environment that was not conducive to the expression of necrotic flecking by the poplar clones of this study in response to *M. medusae* f.sp. *deltoidae*. Although light intensities employed in the leaf-disk assay of this study (i.e., 35 $\mu E\ m^{-2}\ s^{-1}$) and others (e.g., 50 versus 200 $\mu E\ m^{-2}\ s^{-1}$ – 6; 100 $\mu E\ m^{-2}\ s^{-1}$ – 23) have also been low in relation to light intensities commonly encountered in the field, our growth-room results indicated that low light intensity was unlikely to be the explanation. Temperature during the day was the same in the growth-room and leaf-disk studies (i.e., 20°C). Higher temperatures might suppress disease expression (34), and low and variable disease incidence and severity in the leaf-disk assay already pose problems in interpretation of data (11). Wounding during detachment of leaves and excision of disks from them may be responsible for poor expression of flecking in the leaf-disk assay. It is only in studies in which the leaf-disk assay was employed that environmental factors have been more important than genetic factors in poplar leaf rust (6,33).

Since we used only one monouredinial isolate, we do not know whether the *Mmd1* gene is race-specific with respect to *M. medusae* f.sp. *deltoidae*. In view of the race specificity of single, major genes for resistance to rust in other plants, it is likely that inoculations of the pedigree with monouredinial isolates of *M. medusae* f.sp. *deltoidae* from elsewhere in the world (e.g., France, New Zealand, and eastern North America) will reveal the race-specificity of the *Mmd1* gene. Pathogenic variation in *M. medusae* f.sp. *deltoidae* is apparently common (28) in its native range on *P. deltoides* in eastern North America.

The dominant *Mmd1* allele segregating in the pedigree was derived from the *P. trichocarpa* parent. The gene was polymorphic in *P. trichocarpa*, since 93-968 was heterozygous at the locus, and many, but not all, *P. trichocarpa* individuals were fleck+ phenotypically when inoculated with the monouredinial isolate of this study (G. Newcombe, *unpublished data*). Until 1991, *M. medusae* f.sp. *deltoidae* was apparently absent from the maritime Pacific Northwest west of the Cascades Mountains (20). Its principal host species in eastern North America, *P. deltoides*, also does not occur naturally in the maritime Pacific Northwest. *P. trichocarpa*, on the other hand, is distributed well east of the Cascade Mountains into western Montana (8), but 93-968, the female *P. trichocarpa* parent of the pedigree, is a "west-side" clone from the vicinity of Granite Falls, in Snohomish County, WA. West-side *P. trichocarpa*, like 93-968, can apparently harbor single, resistance genes of major effect against a rust species such as *M. medusae* f.sp. *deltoidae*, even though the two organisms do not share a recent evolutionary past.

A congeneric rust species, *M. occidentalis* Jacks., commonly attacks west-side *P. trichocarpa* and can cause significant damage to its wild host (12,39). Resistance in *P. trichocarpa* to *M. occidentalis* is known, however, to be race-nonspecific (11,13). We are currently exploring means of investigating the possibility that the *Mmd1* gene has a significant effect against *M. occidentalis*, which might explain its occurrence and probable polymorphism in *P. trichocarpa*.

Mmd1 may or may not govern a true HR (10). Our observations on timing of macroscopic appearance suggested that in this host-pathogen relationship necrotic flecking may be more like what has been described as a slow or moderate development from early plasmolysis into necrosis (31) than a true HR (10). In any case, characterization of the response which *Mmd1* governs remains to be done.

Although *Mmd1* accounted for most of the phenotypic and genotypic variance in resistance, minor genes for resistance must also occur in the pedigree. These genes most likely derived from ILL-129, the *P. deltoides* parent, since this clone was fleck– and yet consistently had low rust-severity scores.

No pathogenic variation has been noted in *M. medusae* f.sp. *deltoidae* in the Pacific Northwest since 1991 on hundreds of hybrid clones of diverse parentage. *M. medusae* f.sp. *deltoidae* has not been observed infecting conifers on which sexual recombination would occur to give rise to pathogenic variation.

Polyclonal hybrid poplar plantations are not necessarily diverse with respect to genes for resistance; thus, disease incidence and severity may be uniform across the planting. In this study, rust severity was highly uniform (e.g., $H^2 = 0.95$) across the randomized, replicated planting of roughly 600 clones near Clatskanie, OR. It seems unlikely that polyclonal plantings will reduce rust if they are largely monogenic with respect to resistance to a prevailing pathogenic variant.

RFLP markers for major genes for resistance to fungal disease in tree species are relatively new. Our finding (i.e., P222 5.1 cM from the *Mmd1* flecking locus) followed a study which identified RAPD markers linked to the *Vf* gene for scab resistance in apples (41) and the white pine blister rust resistance gene in sugar pine (7,15).

In summary, the implications of this study are on two levels. First, the *Mmd1* gene is currently of epidemiological significance in conferring resistance to rust to the hybrid poplar plantations of the Pacific Northwest. Second, in a general sense, it seems likely that with mapped, pedigreed host material, such as that used in this study, and appropriate inoculation assays the genetics of disease resistance in *Populus* will likely be as amenable to analysis as that in other plants.

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