Evidence of Cytoplasmic Inheritance of Virulence in *Cronartium ribicola* to Major Gene Resistance in Sugar Pine

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ABSTRACT

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Tests for Mendelian segregation of virulence and avirulence in *Cronartium ribicola*, causal agent of white pine blister rust, to a major gene (R) for resistance in sugar pine were made using haploid basidiospore progenies from single diploid telia as inoculum on resistant genotypes. The telia were sampled from a small deme in the Siskyou Mountains of northern California, where a few mature sugar pines known to be Rr genotypes had become infected after withstanding the chronic blister rust epidemic for several decades and where intermediate frequencies of virulence in the ambient basidiospore population were subsequently measured. Infection type on inoculated seedlings with R was qualitative: all progenies of 81 single telia tested over 3 different years were either virulent (compatible) or avirulent (inducing hypersensitive necrosis), never a mixture of both reactions. The complete absence of heterozygotes in the telia population is strong evidence that virulence is not controlled by a nuclear gene. The data are consistent with earlier tests showing that basidiospore inoculum derived from aeciospores isolated from infected Rr trees produced mostly (>90%) virulent reactions on R– seedlings. The evidence indicates that transmission of virulence is uniparental via the cytoplasm of aeciospores. Exchange of spermatia between haploid thalli does not appear to be involved.

Additional keywords: *Pinus lambertiana*.

A major gene (R) for resistance to white pine blister rust in sugar pine (*Pinus lambertiana* Douglas) is neutralized by a virulent race of the rust that appeared suddenly on resistant pines in an experimental plantation in the Siskyou Mountains near Happy Camp, CA (8). We have assumed that pathogen interaction with this resistance allele is controlled at a single locus with alternate alleles for virulence (vR) and avirulence (AVR) (10), because the pathological and epidemiological attributes of resistance and virulence in this pathosystem resemble those of classical gene-for-gene relationships. For example, basidiospores with the putative vR allele induce high infection type (HIT), characterized by bright yellow or red spots on needles, the primary infection courts, on both R– (i.e., RR or Rr) and rr host genotypes. In vivid contrast is the low infection type (LIT), a hypersensitive necrosis that results from interaction with most other inoculum sources fixed for AVR (hereafter, wild type) (11) on R– hosts. Also, vR is specific to R: it does not affect other resistance factors in either sugar pine (9) or a major resistance gene in western white pine (B. B. Kinloch, Jr., R. A. Sniezko, G. D. Barnes, and T. Greathouse, *unpublished data*). For convenience in this paper, we will use the genotypic convention, vR and AVR, to indicate virulence and avirulence, even though they remain only phenotypic designations in the absence of segregation data from controlled crosses.

There is a limited distribution for vR; for many years, it was found only at the sugar pine progeny test site at Happy Camp, where it rapidly approached fixation (11). In 1987, it was detected on a mature sugar pine known to be Rr on Thompson Ridge, a site ~4 km east and downwind of Happy Camp with a long history of chronic and heavy infection on old growth sugar pine (10). Subsequently, vR became detectable at low to intermediate frequencies in inoculum from alternate host *Ribes* spp. immediately surrounding this tree. More recently, it appeared at Mountain Home State Demonstration Forest, ~700 km south of Happy Camp in the southern Sierra Nevada, on known R– sugar pines in two plantations (7).

Reciprocal crosses between virulent and wild-type isolates could be used to determine the genetic basis of virulence but are difficult to make artificially in a caulicolus, perennial rust like *Cronartium ribicola* (2). However, natural matings are available in abundance from diploid telia on infected leaves of different *Ribes* spp. In a single telium, all teliospores are clonal (2). Upon germination, individual teliospores undergo meiosis and produce four basidiospores, and many hundreds of basidiospores may be produced by the matrix of teliospores in a single telial column. Basidiospores infect pine needles and, because they are haploid, the IT on R– host genotypes denotes the genotype of the spore that induced it. Thus, telia heterozygous for vR should segregate in a 1:1 ratio (HIT/LIT) among basidiospore progenies if virulence is conditioned by a single nuclear gene. The small subpopulation of the rust on Thompson Ridge has had intermediate frequencies of virulence and avirulence to R in bulk inoculum since sampling started in 1990 (7,10). *C. ribicola* is highly outcrossing and in Hardy-Weinberg equilibrium for each of 15 polymorphic molecular markers tested (2,5). Accordingly, heterozygotes at the virulence locus should be at a frequency of 2 *pq* (in which *p* and *q* represent the putative alternate alleles for virulence and avirulence) in the Thompson Ridge deme, and segregate in the 1:1 ratio expected upon inoculation of known R– sugar pine genotypes with basidiospores of single telia. Here we report a test of this hypothesis.

MATERIALS AND METHODS

Inoculum. To test transmission of virulence through different spore stages, inoculum from both sugar pine and *Ribes* spp. hosts was used. Aeciospores ($n + n$), produced in perennial cankers on white pines, develop after dikaryotization of haploid mycelium in the hymenial layer of the aecium. Dikaryotization presumably is effected by spermatia (2). The dikaryon persists through vegeta-

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tive reproduction until karyogamy and meiosis take place in mature teliospores on *Ribes* spp. Basidiospores, the haploid meiotic products of teliospore germination, infect white pine needles. Mycelium then grows down into shoot tissues, where pycnia usually develop the following year.

Telia. Telia were obtained from *R. sanguineum* (Pursh.) leaves on Thompson Ridge near an infected, mature sugar pine ('TR 5042') known from routine screening tests to be heterozygous (Rr) for resistance to wild-type inoculum. To determine the frequency of vR in the latent basidiospore population surrounding this tree, infected leaves were sampled when telia were mature (late summer to early autumn) within an approximate 30-m radius around the tree. Usually, one leaf was sampled from each of 20 to 50 bushes. Leaves were kept fresh until used (within 3 days), when they were well mixed and then suspended over recently germinated R– sugar pine seedlings in a dew chamber for 48 h at 16°C, inducing basidiospore production and cast (11). From 10 to 44 seedlings were used in different trials. Yellow (HIT) and necrotic (LIT) spots on cotyledon leaves appeared within a few weeks; the IT of each infection was recorded when well resolved and before HIT spots began to coalesce. Each spot was presumed to result from infection by a single haploid basidiospore. Counts of the two phenotypic classes thus provided direct estimates of the putative allele frequencies in the sample population.

Genotypes of individual diploid telia in the rust population near 'TR 5042' were inferred from phenotypic ratios (HIT/LIT) resulting from infection of R– sugar pines by their basidiospore progeny. Single telia were detached from the *Ribes* spp. leaves and incubated over one or two R– sugar pine seedlings to induce basidiospore cast. The single telium and test seedlings were enclosed in individual subchambers, such that basidiospores could not drift from one subchamber to another. A more even distribution of basidiospores casting from a single telium over foliage of target seedlings was enhanced by placing seedling containers on a Bellco rocker platform (Bellco Glass, Vineland, NJ) and rotating them 7° from vertical on either side every minute, thereby exposing more needle surface. Only parental telia that resulted in two or more needle spots (i.e., the minimum number required to detect a heterozygote) could be genotyped, but telia that produced only one spot were still useful for estimating allele frequencies in the single telia population subsample. Single telia collections and inoculations were repeated in a similar manner in 3 different years, from 1993 to 1995.

Inoculum from telia on infected *Ribes* spp. leaves at all three sites where vR has been detected were sampled from time to time to monitor the frequency of vR (11; B. B. Kinloch, Jr. and G. E. Dupper, *unpublished data*). Happy Camp has been monitored almost continuously from 1982 to 1997; Thompson Ridge, in 1984 and from 1990 to 1997; and Mountain Home State Demonstration Forest, in 1982 and from 1996 to 1997.

Aeciospores. Aeciospores were isolated from known R– sugar pines (making these infections virulent phenotypes by definition) in the three locations where virulence was known to exist: in 1979, from 15 sporulating cankers on R– trees at Happy Camp (8); in 1989, from the only two sporulating cankers on Rr tree 'TR 5042' on Thompson Ridge (10); and in 1996, from two groups of sporulating cankers on R– sugar pines in two plantations at Mountain Home State Demonstration Forest (eight cankers in the first group and six in the second, all from different trees). Spores from both Happy Camp and Thompson Ridge, as well as each group from Mountain Home, were pooled. Aeciospores from all pools were inoculated onto a single clone of *R. nigrum* L. in a greenhouse to produce telia and basidiospore inoculum for testing R– sugar pine seedlings.

Analysis. Phenotypic frequencies of HIT and LIT spots were estimated for both bulked and single telia inocula on Thompson Ridge in each year sampled. Tests for differences between the two kinds of inocula were made by the chi-squared (likelihood ratio) test. Observed allele and genotype frequencies in the single telia inocula were compared with expected frequencies based on Hardy-Weinberg equilibria. For the expected allele frequencies and calculation of genotype frequencies, we used putative allelic frequencies from the bulked inocula, because they represented a larger sample size.

RESULTS

Incipient symptoms appeared on sugar pine cotyledons within 2 weeks of inoculation, and their reaction types were diagnostic within 4 weeks. HIT spots were bright yellow and continued to enlarge. In contrast, LIT spots ceased to enlarge after onset of necrosis, which usually began at spot margins and progressed inward. The two phenotypes were readily distinguishable from each other on R– sugar pine genotypes (Fig. 1).

vR was not detected in bulked inocula around 'TR 5042' in 1984 (11), before infection was observed on this tree; but from 1993 to 1995, frequencies fluctuated between 0.32 to 0.71, with 95% confidence limits of approximately ±7% in each case (Table 1). No significant differences in estimates of vR frequency between bulked and single telia inocula were found in any of the 3 years surveyed.

Amount of infection with single telium inoculations was much lower compared with bulk inoculations that used many (random) telia, as expected. Numbers of spots produced were more variable, ranging from 0 to 18 per telium (Fig. 2). Only telia with basidiospore progeny producing two or more spots (i.e., the minimum number for detecting segregation) could be used to compute genotype frequencies. Telia that produced only two spots were the most frequent class, and the total number with two or more spots was 81. While the probability of misclassifying the genotype of any individual telium with only a few progenies was relatively high, estimates of overall genotype frequencies were not affected.

Although allele frequencies from bulked and single telia inocula were not significantly different, genotype frequencies deviated greatly from expectation, because there were no heterozygotes (vR/AVR) in any of the 81 single telia that had two or more progeny (Table 2). All telia produced basidiospore progenies that had either HIT (vR/vR) spots or LIT (AVR/AVR) spots on R– sugar pine seedlings. No single telia produced basidiospore progenies with mixed ITs.

Inocula derived from aeciospores isolated from virulent infections on R– trees at three locations had mostly HITs. The four different aeciospore bulks, representing a total of 31 cankers on R– genotypes, averaged 97.2% HIT (range, 93 to 100%) (Table 3). In contrast, bulked inocula derived from *Ribes* spp. leaves at the same sites had intermediate frequencies of HIT reactions (Table 3).

DISCUSSION

The contrast between phenotypic expression of virulence and avirulence and other marker loci in *C. ribicola* is paradoxical. On one hand, the lack of segregation among basidiospore progeny of any of the single telium families from a population with intermediate frequencies of vR and AVR is consistent with an earlier hypothesis that *C. ribicola* might be obligately inbreeding and be fixed for alternate alleles at this putative locus (8). That hypothesis was based on lack of segregating phenotypes in inocula derived from bulked aeciospores from infections on 15 different sugar pines growing in a plantation at Happy Camp known to have R. Over 96% of the resulting foliar symptoms on test seedlings were HIT (8) (Table 3). We speculated at that time that these collections were products either of self matings or of outcrosses among like genotypes for virulence. The latter possibility seemed unlikely because many susceptible sugar pine genotypes remained in the population at the time the natural matings occurred and should have harbored some proportion of avirulent rust genotypes (8). Similar results were subsequently obtained with aeciospores collected and bulked from two different virulent infections on sugar pine 'TR 5042', an Rr genotype on Thompson Ridge, and 14 virulent infections from R– trees at Mountain Home State Demonstration Forest (7) (Table 3). On the other hand, recent data from basidio-

spore progenies of single telia showed Mendelian segregation in 15 molecular marker loci, including three isozymes, eight random amplified polymorphic DNAs (RAPDs), and four restriction fragment length polymorphisms (RFLPs), and a high degree of outcrossing (2,5). This evidence falsified the inbreeding hypothesis.

An alternative interpretation is that vR is not a nuclear gene. Evidence from aeciospore inocula strongly indicates uniparental transmission of virulence from the maternal thallus residing in infected pine tissue. For example, frequencies of vR derived from aeciospores bulked from different cankers on Rr sugar pine parent 'TR 5042' on Thompson Ridge was 100% versus inocula from bulked telia in the immediate vicinity, which ranged from 0.11 to 0.89. At Mountain Home, corresponding frequencies were 93 to 100% for aeciospores bulked from different R– parents versus inocula from bulked telia inside the same plantation that ranged from 18 to 90% (Table 3). Although the low frequency of LIT reactions from some of the aeciospore isolates (up to 7%) (Table 3) is problematic, they do not appear to represent isolates that are at intermediate frequencies, much less segregating, at any of the sites sampled. A simple explanation for these low frequencies of LIT reactions is contamination with wild-type aeciospores or urediospores on *Ribes* spp. used to generate basidiospore inoculum in the greenhouse. Cytoplasmic transmission is consistent with the relatively much greater amount of cytoplasm in aeciospores than pycniospores (spermatia); these cells are filled almost entirely by the nucleus and have little cytoplasm.

Our observations of telia genotype frequencies are from natural crosses of *C. ribicola* in a wild population, and our inferences are based on the Hardy-Weinberg principle that allelic and genotypic frequencies should reach equilibrium in the absence of strong selection, drift, migration, or inbreeding. The absolute selection against heterozygous telia genotypes that would have to be invoked to rationalize their complete absence in this deme, where frequencies of the putative virulence and avirulence alleles were intermediate in all of the 3 years sampled, is extremely improbable. Drift is equally unlikely, by similar reasoning. While recent migration from Happy Camp is the most likely origin of vR on Thompson Ridge, the high degree of outcrossing that prevails in this species (2,5) would tend to bring the migrant founders to this long-established population into early equilibrium. An alternative explanation is that, in each sample year, we actually measured two different but sympatric populations (Wahlund effect) on Thompson Ridge: one endemic and the other consisting of annual migrants from Happy Camp, where vR is nearly fixed (11). This also seems unlikely, especially because vR was never detected on Thompson Ridge prior to 1987.

Our data show that putative allele frequencies of vR on Thompson Ridge fluctuated greatly over the 3 successive years of measurement, typical of a founder population, but that genotypic equilibrium was not approached in any. We have already shown that 15 polymorphic molecular marker loci of three different types (isozymes, RAPDs, and RFLPs) are at intermediate frequencies at Thompson Ridge, Happy Camp, and Mountain Home, the three

Fig. 1. Needle lesion infection types of sugar pine seedlings with a major gene for resistance (R) challenged with virulent, avirulent, and mixed virulent/avirulent inocula of *Cronartium ribicola*.

TABLE 1. Frequency of the putative vR allele in the basidiospore population of *Cronartium ribicola* near sugar pine 'TR 5042'

	Bulk inocula		Single telia	
Year	*n*[a]	vR (%)[b]	*n*[a]	vR (%)[b]
1993	170	31.8	62	40.3
1994	226	70.8	69	63.7
1995	227	39.2	65	43.5

[a] Total number of individual needle spots on R– seedlings.
[b] Approximate confidence limits ($P = 0.95$) ± 7%.

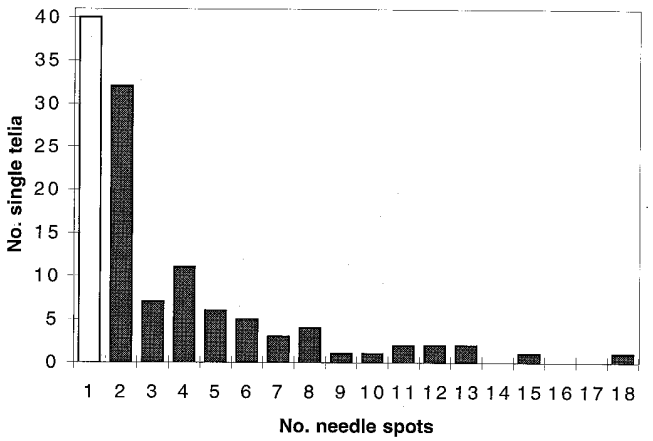

Fig. 2. Distribution of single telium basidiospore progenies of *Cronartium ribicola* into classes of needle spot numbers. Solid bars are progeny classes (≥2) usable for testing Mendelian segregation.

TABLE 2. Observed and expected numbers of alleles and genotypes at the putative vR locus in 81 single telia of *Cronartium ribicola* sampled near Rr sugar pine 'TR 5042' in 1993 to 1995

	Allele[a]			Genotype[c]			
Year	vR	AVR	P[b]	vR/vR	vR/AVR	AVR/AVR	P
1993							
Observed	25	37	0.227	10	0	12	0.000
Expected[d]	54	176		3.6	10.6	7.8	
1994							
Observed	44	25	0.613	19	0	10	0.000
Expected	160	66		11.8	13.4	3.8	
1995							
Observed	28	37	0.984	13	0	17	0.000
Expected	210	279		5.6	14.7	9.7	

[a] vR and AVR are putative alternate alleles inducing high infection type (yellow) and low infection type (hypersensitive necrosis) symptoms, respectively, on R– seedlings of sugar pine.
[b] Likelihood ratio χ^2 test.
[c] Genotypes of individual telial columns inferred from infection types induced by their basidiospore progeny on R– seedlings of sugar pine.
[d] Expected allele numbers are based on frequency data of infection types from bulked inocula collected at 'TR 5042' (cf. Table 1); expected genotype numbers on single telia inocula collected at 'TR 5042'.

sites where vR is known to exist, and that the average heterozygosity for these marker loci is virtually the same at all sites (12). Since heterozygosity for virulence and avirulence is completely lacking, we conclude that virulence to major gene resistance in sugar pine is not under control of a nuclear gene and that vR and AVR are most likely alternate forms of the same plasmagene.

Cytoplasmic inheritance of virulence is apparently rare, but not unprecedented. In his extensive study on the inheritance of pathogenic characters of *Puccinia graminis* f. sp. *avenae*, Johnson (6) stated "cytoplasmic influence affecting the infection types on . . . two varieties [of oats] persists to the F2 generation." This was based on reciprocal crosses between defined rust races, in which only the "maternal" parent infection type was expressed in spore progenies. A similar situation obtained for certain wheat stem rust races on the wheat cultivar Marquis. Johnson's conclusion, however, was more equivocal: "the type 4 and type 1 infection may be governed by cytoplasmic factors. [But] the results of selfing studies indicate that in some clones, at least, nuclear factors may affect inheritance." The issue was revisited by Green and McKenzie (3), who confirmed lack of segregation and the expression of maternal phenotypes in F2 cultures of a reciprocal cross between two races (also reconfirmed recently by Sock et al. [16]). Although the host variety challenged was different in Green and McKenzie's (3) study from the one Johnson (6) used, they shared the same resistance gene (Pg-3). In this case, however, infection phenotypes showed a wider range of expression (including the mesothetic × reaction), and pooling of some phenotypic classes was necessary to demonstrate the point. Green and McKenzie (3) speculated that variation in the types of pustules in the mesothetic × reaction may be associated with variation in the number of "cytoplasmic particles" conferring virulence. In neither study was cytoplasmic inheritance of virulence a general phenomenon—it was anomalous, observed only in interaction with a small number of specific host varieties.

In contrast to the specific, inbred host lines used in the *Avena* spp.-*P. graminis* f. sp. *avenae* pathosystem (3,6,16), our data for screening rust populations for virulence over the years derives from challenging open-pollinated progenies of wild sugar pine seed parents from widely distant locations. These seed parents have in common only that they are homozygous for R (so that all offspring unambiguously detect virulence or avirulence when challenged, irrespective of the pollen parent). Yet, phenotypes are precise, in spite of different genetic backgrounds; different progenies have consistently shown the same basic reaction to both virulent and avirulent inocula (8,11).

Candidate genomes for this putative plasmagene include mitochondrial DNA, associated plasmids, and double-stranded RNA (dsRNA), but there are interpretive problems with each of these alternatives. Mitochondria are known to be present in spermatia of *Cronartium* spp. (13) and, thus, also might be transmitted along with the nucleus during mating. A large number of fungal plasmids have been discovered recently that vary greatly in size, construction (linear and circular), and function (4). Sock et al. (16) were unable to detect plasmids in either parental isolate of *P. graminis* f. sp. *avenae* virulent and avirulent to Pg-3 in oats. They also found no differences between the two isolates either in the organization of the circular mitochondrial genome or in length polymorphisms at 18 restriction sites. dsRNAs are also widespread in fungi, but usually have no obvious effect on virulence or growth rate (14,15). Notable exceptions are hypovirulence effects in *Cryphonectria parasitica*, cause of chestnut blight, and *Ophiostoma novo-ulmi*, cause of Dutch elm disease. Hypervirulence (1) may be even more infrequent. In all cases of observed hypo- or hypervirulence, expression has been variable and quantitative. We are unaware of any instance of cytoplasmically transmitted virulence of the qualitative nature and specificity we describe here.

The epidemiological consequence of uniparental inheritance may be some restriction to gene flow. Basidiospores carry vR to pine hosts, but vR apparently is not vectored to other haploid thalli on pines via spermatia, the presumed gametes. It is still not entirely clear whether the small proportion of avirulent phenotypes sometimes expressed from bulked aeciospore inoculum derived from R– sugar pines resulted from contamination with ambient wild type or from some other mechanism such as cytoplasmic "leakage" through paternal gametes (4). Whatever the mechanism is, it is clear is that maternal transmission is predominant.

TABLE 3. Comparison of putative vR allele frequency in aeciospore inocula of *Cronartium ribicola* derived from aeciospores bulked from infected R– sugar pines in year indicated with inocula derived from bulked telia on infected *Ribes* spp. at three sites over the range of years indicated

	Aeciospores			Telia
Site[a]	Year	*n*[b]	vR (%)	vR (%)[c]
HC	1979	133 (15)	96.2	83–98 (1990–1997)
TR	1987	42 (2)	100.0	11–89 (1990–1997)
MH1	1997	852 (8)	99.9	18–52 (1996–1997)
MH2	1997	579 (6)	92.7	86–90 (1996–1997)

[a] HC = Happy Camp, TR = Thompson Ridge, and MH = Mountain Home Demonstration State Forest (groups 1 and 2).

[b] Number of individual needle spots on R– seedlings from (number of cankers from which aeciospores were derived and pooled).

[c] Range of vR frequency in bulked inoculum from infected *Ribes* spp. over years indicated in parentheses.

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