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Nrs1, a Repetitive Element Linked to Pisatin Demethylase Genes on a Dispensable Chromosome of *Nectria haematococca*

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We have identified a repetitive DNA element in *Nectria haematococca* mating population VI, isolate T-2. This repetitive sequence has been called Nrs1. DNA hybridization analysis indicates the sequence is found in several isolates of the fungus pathogenic to *Pisum sativum*. A 2,027-bp clone containing the Nrs1-2 allele contains a long polyA sequence, imperfect RNA polymerase III promoter sequences, multiple inverted repeats, and the potential for extensive secondary structure similar to known RNA polymerase III transcripts and related retroelements. Ten of the 11 *Hin*dIII restriction fragments from isolate T-2 DNA that hybridize to Nrs1-2 segregate in a manner consistent with a 1:1 ratio for random ascospore progeny. The 10 restriction fragment length polymorphism (RFLP) loci define three linkage groups and correspond to three chromosome-sized DNAs from T-2 separated by pulsed field gel electrophoresis. Three RFLP loci defined by hybridization to the gene for pisatin demethylase and localized on the 1.6 million base pair (Mb) chromosome were genetically linked to each other and to several Nrs1 loci. These sequences recombined despite the fact that no obvious homolog exists for the 1.6-Mb chromosome in one parent strain. Allelic RFLPs corresponding to the gene sequence of cutinase were unlinked to Nrs1 loci.

Additional keywords: chromosome polymorphism, *Fusarium solani*, SINEs, supernumerary chromosome.

Dispersed repetitive DNA sequences have been useful for the genetic analysis of plant pathogenic fungi. For example, the MGR sequence from *Magnaporthe grisea,* a short repetitive element found dispersed in about 50 copies per haploid genome of rice-infecting strains of the fungus (Hamer et al. 1989; Hamer and Givan 1990) has been used to DNA fingerprint individual strains of the fungus and has been used to infer phylogenetic relationships among strains (Borromeo et al. 1993; Levy et al. 1991). Additionally, MGR has been used for genetic mapping; for example, to map a mutation in the morphologically altered Smo$^-$ strains (Hamer and Givan 1990). The sequence of MGR suggests that it may be a retrotransposon (Hamer et al. 1989). Likewise, dispersed repetitive sequences and transposable elements isolated from other plant pathogenic fungi have proven to be useful for DNA fingerprinting strains (Daboussi and Langin, 1994; Goodwin et al. 1992; Kistler et al. 1991; Kohli et al. 1992; McDonald and Martinez 1991; Milgroom et al. 1992; Rodriguez and Yoder 1991) and for genetic mapping (Goodwin et al. 1992; Milgroom et al. 1992).

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Nectria haematococca mating population VI (*Fusarium solani* f. sp. *pisi*) has been extensively studied as a genetic model for soilborne fungal pathogens. Perhaps the most significant finding with respect to the pathogenic specialization of the fungus was the discovery that pathogenicity determinants are located on dispensable supernumerary chromosomes (Miao et al. 1991a; VanEtten et al. 1994). This work began as the study of genes involved in metabolic detoxification of the pea phytoalexin pisatin. Pathogenicity to common pea (*Pisum sativum*) was absolutely linked to the presence of genes for the detoxifying enzyme pisatin demethylase in sexual crosses segregating for pathogenicity and the enzyme (Kistler and VanEtten 1984a, 1984b; Mackintosh et al. 1989). Genes for pisatin demethylase, a cytochrome P-450 monooxygenase (Desjardins and VanEtten 1986), are part of a multigene family (Maloney and VanEtten 1994) and sequences at three distinct pisatin demethylase loci have been cloned and characterized (Reimmann and VanEtten 1994; Straney and VanEtten 1994; Weltring et al. 1988). However, pisatin demethylase genes are unstable during meiosis (Miao and VanEtten 1992; VanEtten et al. 1994) and their loss has been explained by their position on dispensable chromosomes (Miao et al. 1991a; VanEtten et al. 1994).

A causal relationship between pisatin demethylase and pea pathogenicity had been inferred from linkage data derived from standard genetic crosses. However, when the pisatin demethylase gene of a virulent isolate was specifically disrupted by a transformation-based method (VanEtten et al. 1994), resulting mutants with no pisatin demethylase activity were only slightly reduced in virulence compared with wild-type. Other mutants of *N. haematococca* that lose the entire dispensable chromosome containing the gene for pisatin demethylase are essentially nonpathogenic on pea (VanEtten, unpublished). These cumulative results are consistent with the model that additional genes conferring pathogenicity reside on the dispensable chromosome. These putative pathogenicity

factors have been called PEP (*pea pathogenicity*) genes (VanEtten et al. 1994).

We have sought to identify repetitive DNA sequences in *N. haematococca* that would be useful for genetic mapping and, specifically, for study of genetic transmission of dispensable chromosomes involved in pathogenicity. Described here is one small repeated element called *Nectria* repetitive sequence 1 (Nrs1) found on supernumerary chromosomes in strains of the fungus pathogenic to pea. We have sequenced a clone containing Nrs1, mapped it to multiple genetic loci, and have tested for genetic linkage between Nrs1 loci and genes for pisatin demethylase.

RESULTS

Repetitive DNAs were identified from a partial library made from *Hin*dIII-digested total DNA from *N. haematococca,* mating population VI (MPVI), isolate T-2. In order to select clones containing repetitive DNAs, total DNA from T-2 was labeled and used to probe Southern blots of 95 arbitrarily chosen genomic clones. Several clones hybridized strongly to T-2 DNA, and one, designated pT2-62, was examined in greater detail. The insert of pT2-62 appeared to be 1.75 kb and hybridized to multiple restriction fragments in *Hin*dIII-digested total DNA from isolates T-2 and isolate T-9 (Fig. 1). At low stringency conditions for hybridization, DNA from several other isolates of *N. haematococca* MPVI (Table 1) also contained multiple bands with sequence similarity to the probe. Hybridization was not detected to DNA from strains of MPVI nonpathogenic to peas (44-100, 171-3), nor to DNA from isolates from MPI, MPV, homothallic *N. haematococca*, *F. oxysporum,* or *F. sambucinum.* At higher hybridization stringency, 11 distinct *Hin*dIII fragments from T-2 hybridized to pT2-62, while no fragments hybridized to DNA from strain 6-36, a laboratory-derived strain with moderate virulence to pea.

Also at these conditions, the repetitive sequence contained on pT2-62 hybridized strongly to DNA from field isolates that were pathogenic to pea (T-2, T-9), or strains derived from these isolates. Two derived strains, 77-13-5 and 96-2, are single-ascospore isolates originating from a T-2 genetic background. Both 77-13-5 and 96-2 were obtained by backcrossing strains that produce the enzyme pisatin demethylase to the maternal strain 44-100, which lacks genes for pisatin demethylase (Kistler and VanEtten 1984a). DNA from 44-100 also does not hybridize to pT2-62 (Fig. 1). Paternal parents in the backcross series were selected only for the presence of pisatin demethylase; distinct pisatin demethylase loci were identified in strain 77-13-5 (pisatin demethylase locus *Pda*1) and 96-2 (*Pda*2). Strains 77-13-5 and 96-2 also contain three and two *Hin*dIII fragments, respectively, that hybridize at high stringency to pT2-62. These observations suggested that Nrs1 sequences might be genetically linked to the pisatin demethylase loci.

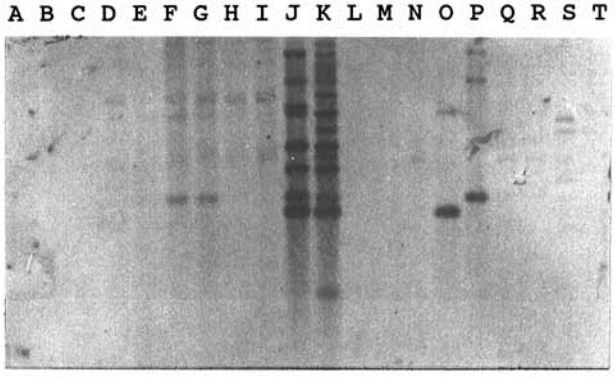

Fig. 1. Southern hybridization of pT2-62 to total DNA from 20 isolates of *N. haematococca* and related species. DNA is from isolates (A) 3299, (B) PHW777, (C) PF-16, (D) PF-3, (E) 171-3, (F) 156-30-6, (G) 34-18, (H) 6-94, (I) 6-36, (J) T-9, (K) T-2, (L) S-66, (M) S-1, (N) C-5, (O) 96-2, (P) 77-13-5, (Q) 58B5, (R) 44-100, (S) 44-46, and (T) 25. Approximately 5 µg of *Hin*dIII-digested DNA was added to each lane. Low stringency hybridization and washing conditions (see Materials and Methods) were used.

To test for linkage between Nrs1 and Pda loci, chromosome-sized DNAs from a few *Nectria haematococca* MPVI strains were separated by pulsed field gel electrophoresis (PFGE) under conditions that resolved the two largest chromosomes of *Saccharomyces cerevisiae* and the three chromosomes of *Schizosaccharomyces pombe*. Chromosome-sized bands hybridized to a clone containing a telomere consensus sequence from *Fusarium oxysporum* and thus were assumed to be authentic chromosomes (data not shown). To determine the distribution of Nrs1 in strains T-2, 6-36, 44-100, 77-13-5, 96-2, 156-30-6, and 171-3, Southern blots were probed with pT2-62. Hybridization in T-2 was primarily to a 1.6-Mb band, although bands of approximately 1.28 and 1.8 Mb also hybridized to pT2-62 (Fig. 2A). No hybridization was seen to 6-36, 44-100, or 171-3 chromosomes. Hybridization in 96-2, 77-13-5, and 156-30-6 was to a single 1.6-Mb band; hybridization to DNA from 156-30-6, a laboratory strain with low virulence toward pea, was weak (data not shown). This indicates that the repeated sequences detected by pT2-62 are primarily located on a 1.6-Mb chromosome. Curiously, pT2-62 also hybridized to the 2.2-Mb chromosome XII from *S. cerevisiae* but not to *Sc. pombe* DNA (data not shown).

The gene(s) for pisatin demethylase were localized, by Southern hybridization, to a 1.6-Mb chromosome in T-2, and

Table 1. Strains used in this study

Strain	Species	Reference or source
T-2	*N. haematococca* MPVI[a]	Kistler and VanEtten, 1984a
T-9	*N. haematococca* MPVI	Kistler and VanEtten, 1984a
6-36	*N. haematococca* MPVI	Tegtmeier and VanEtten, 1982
6-94	*N. haematococca* MPVI	Tegtmeier and VanEtten, 1982
44-46	*N. haematococca* MPVI	Kistler and VanEtten, 1984a
44-100	*N. haematococca* MPVI	Kistler and VanEtten, 1984a
77-13-5	*N. haematococca* MPVI	Kistler and VanEtten, 1984a
96-2	*N. haematococca* MPVI	Kistler and VanEtten, 1984a
34-18	*N. haematococca* MPVI	H.D. VanEtten
171-3	*N. haematococca* MPVI	H.D. VanEtten
156-30-6	*N. haematococca* MPVI	H.D. VanEtten
25	*N. haematococca* MPI	S. Leong
C-5	*N. haematococca* MPI	Samac and Leong, 1989
S-66	*N. haematococca* MPV	H. D. VanEtten
S-1	*N. haematococca* homothallic	Daboussi-Bareyre and Parisot, 1989
PF-3	*N. haematococca* homothallic	R. Ploetz
PF-16	*N. haematococca* homothallic	R. Ploetz
PHW 777	*Fusarium oxysporum*	Kistler et al. 1987
3299	*F. sambucinum*	M. Beremand

[a] MP are mating populations as defined by Matuo and Snyder (1973).

to a 5.0-Mb band in 6-36 (Fig. 2B). Isolates 77-13-5, 96-2, and 156-30-6 also contain a 1.6-Mb chromosome that hybridizes to the pisatin demethylase gene (data not shown). These results are consistent with those previously reported (Miao et al. 1991b) for the same or similar isolates.

To test for recombination, a cross (cross K1) was made between the strains T-2 and 6-36. *Hin*dIII-digested DNAs from 100 random ascospore cultures were individually probed with pT2-62. The probe hybridized to 11 bands in strain T-2 and no bands in 6-36. The 11 bands were designated Nrs1-1 to Nrs1-11, from smallest to largest; the insert of clone pT2-62 appeared to be Nrs1-2. Ten of 11 bands, including Nrs1-2, segregated in Mendelian proportions in progeny (Fig. 3 and Table 2). Nrs1-8 did not fit a 1:1 ratio ($X^2 > 36$) but fit a 3:1 ratio. The 3:1 ratio is consistent with two comigrating restriction fragments, each with sequence similarity to pT2-62, present at unlinked genetic loci.

Clones containing the *N. haematococca* gene for cutinase or pisatin demethylase were used to detect restriction fragment length polymorphism (RFLP) markers for cross K1. The cutinase clone hybridized to one monomorphic and seven polymorphic *Hin*dIII fragments. The polymorphic fragments were designated C1 through C7, according to increasing size. Fragments C1 to C4 appear to be allelic to fragments C5 and C7 since they are found in different parents and segregate in the ascospore progeny. Fragment C6 is found in parent T-2 and in half of the ascospore progeny but hybridized poorly and was difficult to score. C6 appears, at most, distantly linked to the locus defined by C1–C4/C5+C7 that was considered to be the authentic cutinase locus.

The pisatin demethylase clone pUCH1-pda hybridized to five polymorphic and two monomorphic *Hin*dIII fragments within strains T-2, 6-36 and random ascospore progeny of cross K1. The *Hin*dIII fragments, from smallest to largest, were designated P1 through P7. These fragments do not segregate as alleles and indeed only the three polymorphic *Hin*dIII fragments derived from T-2 (P2, P4, and P6) are transmitted in proportions consistent with a 1:1 ratio (Table 2). P2 and P4 are closely linked (Table 3) and both show some linkage to P6. Fragments P3 and P7, derived from parental strain 6-36, are represented in progeny at frequencies inconsistent with simple Mendelian inheritance. Fragments P6 and P4 are thought to correspond to the genes *Pda*1 and *Pda*2 since they are present in DNA from strains 77-13-5 and 96-2 respectively (data not shown). These strains have been shown previously (Kistler and VanEtten 1984a) to contain these single genes for pisatin demethylase.

Three linkage groups were formed by the Nrs1 loci (Table 3). Linkage group 1 consists of pisatin demethylase loci derived from T-2 (P2, P4, P6), and Nrs1-2, Nrs1-3, Nrs1-10, and Nrs1-11. Group 1 corresponds to the 1.6-Mb chromosome since the pisatin demethylase gene hybridizes only to this chromosome in T-2. Linkage groups 2 and 3 are defined by the other Nrs1 loci and each must correspond to either the 1.28 or 1.8 Mb chromosome of T-2 (i.e., the two other chromosomes that hybridize to pT2-62). Therefore the number of chromosome bands that hybridize to pT2-62 equals the number of linkage groups defined by Nrs1 loci.

For linkage group 1, the 1.6-Mb chromosome of T-2, the implied order of genes was Nrs1-2, P4, P2, P6, (Nrs1-10, Nrs1-11) Nrs1-3. We had anticipated that, since no apparent homolog for the 1.6-Mb chromosome exists in 6-36, the markers on this chromosome would be transmitted in an all-or-nothing fashion or in a manner consistent with simple de-

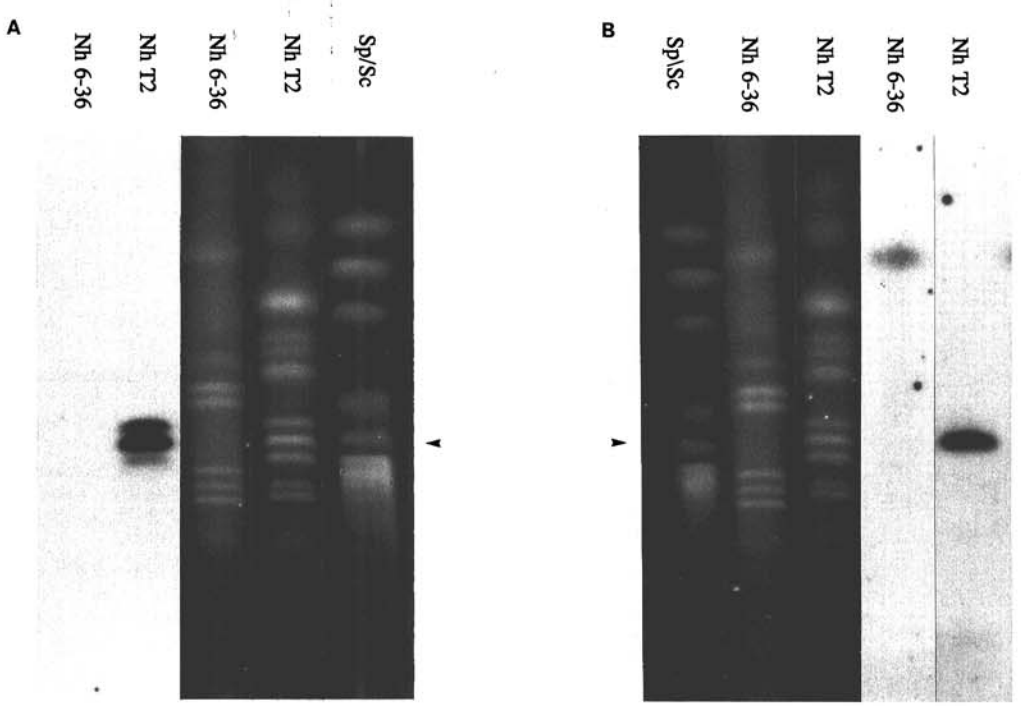

Fig. 2. CHEF gel separation of *Nectria haematococca* chromosomes of strains T-2 and 6-36 and their corresponding Southern hybridizations. **A,** Hybridization signals obtained using the 2.0 kb insert of pT2-62 as the probe. **B,** shows hybridization obtained using a 2.1-kb pisatin demethylase fragment as the probe. Arrows indicate the 1.6-Mb chromosome. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were used as size standards. High stringency hybridization and washing conditions were used.

letion or translocation. Surprisingly, more extensive recombination among markers was seen. For example, three of the 100 ascospores analyzed had recombinant genotypes that could be explained only by 3 or more recombination events or a minimum of 2 deletions each. The parental combination of markers [Nrs1-2, P4, P2, P6, (Nrs1-10, Nrs1-11) Nrs1-3] was ++++(++)+ for T-2 and ----(--)- for 6-36. The three aforementioned recombinant ascospore progeny had +-+-(--)-, +--+(--)-, and -+--(++)+ genotypes.

The insert of pT2-62, Nrs1-2, was sequenced and found to contain a 2,027-bp *Hin*dIII fragment. Little similarity was found between Nrs1-2 and other sequences in nucleotide sequence databases except that due to a large A/T-rich region including a perfect 35-nucleotide stretch of polyadenylic acid (Fig. 4A). Hybridization of Nrs1-2 to other Nrs1 loci does not appear to be due solely to the polyA region of the clone. When an internal *Sst*I fragment (position 445 to 1,272, Fig. 4A) was used to probe genomic digests of strain T-2 DNA at high stringency, Nrs1-2 through Nrs1-10 hybridized. However, a subclone containing the polyA tract (the *SstI*/*Hin*dIII fragment from position 1,269 to 2,026, Fig. 4A), hybridized only to Nrs1-1, Nrs1-2, Nrs1-4, Nrs1-6, and Nrs1-11 (data not shown).

The entire Nrs1-2 DNA sequence including that immediately adjacent to the polyA has a considerable number of short direct repeats as well as numerous perfect and imperfect inverted repeats. The latter repeats have the potential for generating cruciform structures and may account for the anomalous electrophoretic mobility of the Nrs1-2 sequence: the 2,027-bp fragment migrates as if it were 1.75 kb during agarose gel electrophoresis. A predicted complementary RNA also may be involved in elaborate, and potentially stable, stem and loop formation (Fig. 5). Two short stretches 450 bp upstream of the polyadenine region are similar to promoter consensus sequences for the RNA polymerase III (Fig. 4B).

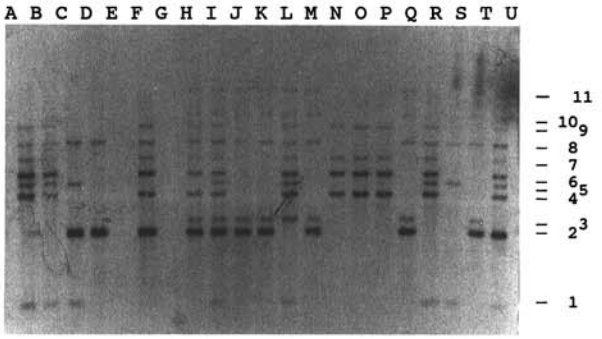

Fig. 3. Southern hybridization of pT2-62 to *Hin*dIII-digested DNA from 21 random ascospore progeny (A through U) of cross K1. The positions of the 11 restriction fragment length polymorphism bands are indicated at the right. Band sizes are listed in Table 2. High stringency hybridization and washing conditions were used.

Table 2. Segregation of polymorphic restriction fragments in random ascospore progeny of cross K1

Probe	*Hin*dIII fragment (size-kb)	T-2	6-36	Progeny scored	X^2
pT2-62	Nrs1-1 (0.8)	43[a]	57[a]	100	1.98
	Nrs1-2 (1.75)	52	48	100	0.16
	Nrs1-3 (2.0)	46	54	100	0.64
	Nrs1-4 (2.65)	46	54	100	0.64
	Nrs1-5 (2.95)	42	58	100	2.56
	Nrs1-6 (3.3)	47	53	100	0.36
	Nrs1-7 (3.9)	46	54	100	0.64
	Nrs1-8 (4.5)	80	20	100	1.33[b]
	Nrs1-9 (5.5)	49	51	100	0.04
	Nrs1-10 (6.5)	42	58	100	2.56
	Nrs1-11 (9.6)	42	58	100	2.56
pUCH1-pda (pisatin demethylase)	P2 (3.3)	41	50	91	0.89
	P3 (4.8)	19	72	91	30.9
	P4 (5.2)	41	50	91	0.89
	P6 (7.6)	37	55	92	3.52
	P7 (8.7)	3	88	91	79.4
pU5-11 (cutinase)	C1-C4	58	40	98	3.31
	C5+C7	58	40	98	3.31
	C6	40	40	80	...

[a] Number of progeny with restriction fragment length polymorphisms corresponding to parental strains.

[b] Chi-squared test for a 1:1 segregation ratio, except for Nrs1-8, for which a 3:1 ratio was tested. Values greater than 3.84 indicate significant ($P \leq 0.05$) deviation from the expected ratio.

DISCUSSION

We have identified a DNA sequence from *Nectria haematococca* isolate T-2 with structural features suggestive of a short interspersed repeated DNA element (SINE). These features include the presence of RNA polymerase III promoter sequences, a 3′ A-rich region and the potential for complex and stable secondary structure (Deininger 1989). Similar features have been noted for apparent SINE-like retroelements from the plant pathogenic fungi *Erysiphe graminis*

Table 3. Linkage relationships of RFLPs in cross K1[a]

Marker A	Marker B	Map Units	X^2	95% C.I.	Progeny scored
Nrs1-1	Nrs1-5	3	84	0.8 - 9.2	100
Nrs1-2	P6	35	7.5	25.6 - 46.1	91
Nrs1-2	P4	12	50	6.6 - 21.3	90
Nrs1-2	P2	14	44	8.2 - 23.9	90
Nrs1-2	Nrs1-11	38	5.3	28.6 - 48.4	100
Nrs1-2	Nrs1-10	38	5.3	28.6 - 48.4	100
Nrs1-2	Nrs1-3	38	5.0	28.6 - 48.4	100
Nrs1-3	P6	25	21	17.0 - 35.8	91
Nrs1-3	P2	34	7.4	24.9 - 45.4	90
Nrs1-3	Nrs1-11	4	81	1.3 - 10.5	100
Nrs1-3	Nrs1-10	4	81	1.3 - 10.5	100
Nrs1-4	Nrs1-9	3	85	0.8 - 9.2	100
Nrs1-4	Nrs1-7	0	96	0.0 - 4.6	100
Nrs1-4	Nrs1-6	1	92	0.0 - 6.2	100
Nrs1-6	Nrs1-9	4	81	1.3 - 10.5	100
Nrs1-6	Nrs1-7	1	92	0.0 - 6.2	100
Nrs1-7	Nrs1-9	3	85	0.8 - 9.2	100
Nrs1-10	P6	24	22	16.1 - 34.6	91
Nrs1-10	P4	38	4.1	28.0 - 48.8	90
Nrs1-10	P2	33	8.2	24.0 - 44.3	90
Nrs1-10	Nrs1-11	0	96	0.0 - 4.6	100
Nrs1-11	P6	24	22	16.1 - 34.6	91
Nrs1-11	P4	38	4.1	28.0 - 48.8	90
Nrs1-11	P2	33	8.2	24.0 - 44.3	90
P2	P6	33	8.3	23.7 - 43.8	91
P2	P4	7	65	2.7 - 14.4	91
C1-C4	C6	36	5.1	26.0 - 48.0	80

[a] The likely order of markers on linkages groups are: Group 1: Nrs1-2, P4, P2, P6, (Nrs1-10,Nrs1-11), Nrs1-3; Group 2: Nrs1-9, (Nrs1-4,Nrs1-7), Nrs1-6; Group 3: Nrs1-1, Nrs1-5; Group 4: C1-C4, C6. C1–C4 and C5+C7 are allelic. Other linkage relationships were not statistically significant. The order of markers in parentheses is ambiguous.

(Rasmussen et al. 1993) and *Magnaporthe grisea* (Skinner et al. 1993). Unlike highly repeated SINEs and similar dispersed repetitive elements from fungi, only a relatively few copies of Nrs1, at most, are found in isolates of *N. haematococca*. These copies are localized on one or a small number of chromosomes. By intensity of hybridization, many copies in T-2 are concentrated on the 1.6-Mb band. Hybridization to DNA from other strains is limited to the 1.6-Mb band. A 1.6-Mb chromosome previously has been shown to be meiotically unstable and dispensable (Miao et al. 1991a), and to carry genes for pisatin demethylase (VanEtten et al. 1989; VanEtten et al. 1994).

The exact meaning of linkage relationships for markers on a dispensable chromosome is not known. The 1.6-Mb chromosome from T-2 has no obvious homolog, based on size or Southern hybridization, in strain 6-36 yet, clearly, reassortment of markers on this chromosome appears to be taking place. There may be a low frequency of meiotic loss associated with this chromosome, as markers derived from it, in this and other crosses (Kistler and VanEtten 1984a), are found consistently in <50% of random ascospore progeny. A false apparent linkage of markers thus could result from "co-segregation" due to meiotic loss of an entire chromosome/linkage group. However the frequency with which this occurs is not high enough to significantly skew X^2 values for 90 to 100 random ascospores and cannot explain recombination patterns consistent with single and multiple crossing-over events.

Another possibility is that isolate T-2 itself may contain two homologous copies of a 1.6-Mb chromosome with heterozygous alleles at loci that can recombine. The intensity of ethidium bromide staining of the 1.6-Mb band indeed suggests a doublet. Further study will be required to determine whether recombination of markers was due to recombination

A

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   1  AAGCTTCCAC TCTTTGGAGG TGCTCGACGA CGCCCATAAC TGACTTCACT
  51  ATCCAAACTC GATGTCGTCG CAGAGACTAG GCAGAAGAAA TGATATGACG
 101  CAGCGTGATC GGTAGTATAC ACATGGTGGA GGACAAATTC ACGCAGTAGA
 151  ACTTGAATTC GTACTTATCT CACTACATCA ATGTCAGAGA AATGATGGGC
 201  AATTAAGCCC CATCCTCACT CTAATCTTCC CCTCGTAGTT ATGTGTGATT
 251  TGGAGTTTTA CCTACCCTAC AGTTGGTAAT CTACCATTTT GTACCTCCAT
 301  CTAGGTCTGG AAAAGTTTAT TCTTCCCCGC CGCTGTTAGC GGATTGCTAG
 351  CGGGCGGGGA TTTTTCTGGC TACCCCGACC ATGCTTTTTG CCTTTGAGAA
 401  ACCGTCGGGG CCTTAACCGG GCACGGACAA AAGGACTTTA GGAGAGCTCA
 451  TGTCGCCGGA CGTCGCGACT GCCTTTCAGA ACCAAGACAG ATCGGGAGTA
 501  TCGGAAGGCG CGCAAGGCCC TCCAAACAAC GACGGCTAGT CGCAGATGCT
 551  TTTCTCTCTG ACGGCAAGCT GCACGCCAGG CTTGGATTCC TTCCAAGGCA
 601  AGATTCGTCC AGCAGCGTAG CTAGTCTAAC ACGCCCTCCA GCGCCACCGA
 651  AAAGGGGCCT CGCGCTTCTG CAGGTACGGT TCATCACGTG ATAAGTTTCG
 701  TTCCATGTGC CTGCTCGTTC GCTCCTCCTG TGGCAGAGAA GACGTCCAAA
 751  ACAGATCCAG AGCGATAGGA TAACGCGTCA GCGCTTCGGG GAGAAAACGT
 801  AAAATTCCCG CCACGAGTAT CCACCGACCC ACTGCTGAAA ATTTCCGTCG
 851  AAAAAAGGAT GCTAACGTCA TTTGCCGACG CCCACCGCTT CTTCCGGCCG
 901  CCTCAATTGA AGATGCGAAT GGTGGGTCCG CTCGTACAGA AAATTGGCGG
 951  CCGATTTGGC GGTTATCCTC CCACTCCTCA GACGGGAATG TTGGGCTCAT
1001  TTGGGACGTT TGGGACGTTC TGTGCACTCG GCCAGAAGGT CGCGCTAGGC
1051  CAGAAAATTG GTGGCCGATT TGGCCGATTT CCCTCCGTCT CCTCTTGGCG
1101  TCATGAATGT AATTAAATAT CTCCCGCATT TACCTTGGCC TAAATTGGTA
1151  TAGTGCCTCC TGGTAAAATC TCATCACGTG ATGAGCTTAT TTCAACGTCA
1201  TCAGAGGCCG GTTAAGAAAG AAGAGAAATT TGAGTCTGGT GTTCGAGTAT
1251  CGAATCATGC GATCGCAGAG CTCAAATGGG ACCGAAATGT GAGGTGAAAA
1301  GTTGACAGAA CATGCGGAAC GTACAAAGTG AACAAACAAA ACAGATACCG
1351  AGGCAATGAA GGCCAAAATG GGCCAGAAAG TATGAGTCGT TGAAATGGGC
1401  TCCGCTCGCA GAGCACGGGA GAGGTGAAAC AACCCCCCCA CTCCCCCCCC
1451  AAACCCAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AGGCTAAAAA
1501  AAAGACGCAA AAGCGAAAGT CAAACCGGGT TGGCCGATCT CTACTTTCTG
1551  CTCGACATCA ACATCGGCTT GAGACCCGAA AATTTCGACG GCAATCTTAG
1601  CCGTAACCTT TAGAGCCCAC TCTGTTCAAG CTCCGCCTTG GTTACTAAAC
1651  GCTTGCCGAA GCAATTTTCG TTTGCCGAGG CTAAACATCA CCCATGCCTT
1701  CAGTGCCAAG ACGCAGAAGG ACAGTAGGCT TGATTAGACT GAACGATGGG
1751  GAACAATTCC GCCAAATTGC TTTCTACCTC CCACCCTCTC ATTAAACAAA
1801  GCTCCATCAA CCCCCACTCG CTGAACCTTG CGTACATATT GACGTTACAT
1851  ACTACGTAGT ACGGCTGAAA CGGGCATCAT GCTAGTGACC ATCGGCCATC
1901  ATCAGTCGCG TGAAAAGATG GTGCATCCCC ACCATGTGGG CGCTTCTTGC
1951  GCACGGTCGA AATTCAATGT AACAGCGGGC GCGCACCGTG CTTGAAGAAA
2001  TGGATATTGG CTTTCAAATG CAAGCTT
```

B

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pol III          A-box              B-box
consensus      gGGCGCaGTGGC       aGTTCtgGGCt
               ||||  ||  | |       ||||||  ||
Nrs1           GGGCTCATTTGG       CGTTCTGTGCA
```

Fig. 4. A, DNA sequence of pNRS1-2 (GenBank accession number: U20433). The 35-bp poly-A region is in bold. Restriction sites *Sst*I and *Hin*dIII are shown in italics. **B,** The A-box and B-box motifs (underlined and double underlined, respectively, in **A** compared with the consensus RNA polymerase III promoter for the 7SL RNA gene (Deininger 1989).

between chromosomes acting as homologs but carried by a single parental strain, or was perhaps due to translocation between the 1.6-Mb chromosome and other chromosomes. Only parental-size restriction fragments hybridizing to Nrs1-2 were observed, indicating that translocation/recombination among these loci did not occur at an observable frequency.

Although most RFLP loci studied here segregated in Mendelian ratios, two exceptions were noted. These were loci P3 and P7, which were derived from strain 6-36 and apparently located on a 5.0-Mb chromosome (Fig. 2B). A chromosome of similar size is not found in isolate T-2. Most random ascospore isolates of the cross between T-2 and 6-36 have P3 and P7 restriction fragments corresponding to the 6-36 parent. We postulate that other genes on this chromosome also may be preferentially transmitted in crosses.

These results are the first step in the endeavor to study transmission of pathogenicity determinants on the 1.6-Mb chromosome in *Nectria haematococca*. Recent gene disruption experiments indicate that the *Pda*1 gene on the 1.6-Mb chromosome is not required for high virulence to pea. Rather, pea pathogenicity determinants appear to be closely linked to the pisatin demethylase locus (VanEtten et al. 1994). In this regard, Nrs1 may be helpful for mapping and isolating the PEP gene(s) residing on this chromosome.

MATERIALS AND METHODS

Strains.

The strains of fungi used for this investigation are shown in Table 1. Strains of three genetically distinct mating populations (MP) of *Nectria haematococca* were represented. Also *Fusarium oxysporum* and *F. sambucinum* isolates were included in the survey.

DNA isolation, procedures and sources.

Procedures for DNA isolation and cloning were described previously (Momol and Kistler 1992). A partial genomic library of *Hin*dIII-digested DNA from isolate T-2 was made in pUC119 (Vieira and Messing 1987) and transformed into *Escherichia coli* strain DH5α (GIBCO BRL, Gaithersburg, Md.). The enzyme *Hin*dIII was chosen in order to exclude the major repeated sequence containing the genes for ribosomal RNA (rRNA). The 8-kb rDNA tandem repeat does not contain a restriction site for *Hin*dIII and thus is contained on a very large DNA fragment (Kistler and Benny, unpublished). The average size of the fungal DNA insert (n = 95) was 2.20 ± 1.55 kb. To identify potential clones carrying repetitive DNAs, a Southern blot of genomic clones was probed with labeled total DNA from strain T-2. It was reasoned that clones hybridizing more strongly to this probe would more likely contain repeated DNA sequences.

The cloned cutinase gene was obtained from Martin Dickman. The pUC8 (Vieira and Messing 1982) clone called pU5-11 contains a 5.5-kb *Sst*I fragment from genomic DNA of *N. haematococca* strain T-8 containing the cutinase coding sequence, a 51-bp intron, as well as 1.7-kb 5′ and 1.0-kb 3′ flanking DNA (Soliday et al. 1989). The pisatin demethylase DNA sequence (Weltring et al. 1988; Maloney and VanEtten 1994) was obtained from Hans VanEtten. The pUCH1 (Schäfer et al. 1989) clone called pUCH1-pda contains a 3.2-kb *Xho*I genomic fragment from *N. haematococca* strain T-9 containing the pisatin demethylase coding sequence and 1.4 kb of flanking DNA. The clone pNLA17 contains the telomere sequence $[TTAGGG]_{18}$ on a 126-bp *Nla*III fragment (Powell and Kistler 1990) cloned in the *Sph*I site of pUC118.

DNA sequencing and sequence comparison.

The DNA sequences were obtained through the University of Florida DNA Sequencing Core facility using automated, fluorescence-based procedures (Applied Biosystems, Perkin-Elmer Corp., Foster City, Calif.). Sequence similarities were tested through the National Center for Biotechnology Information (NCBI) using the BLAST network service. Primary DNA sequence comparisons were made using the BLASTN

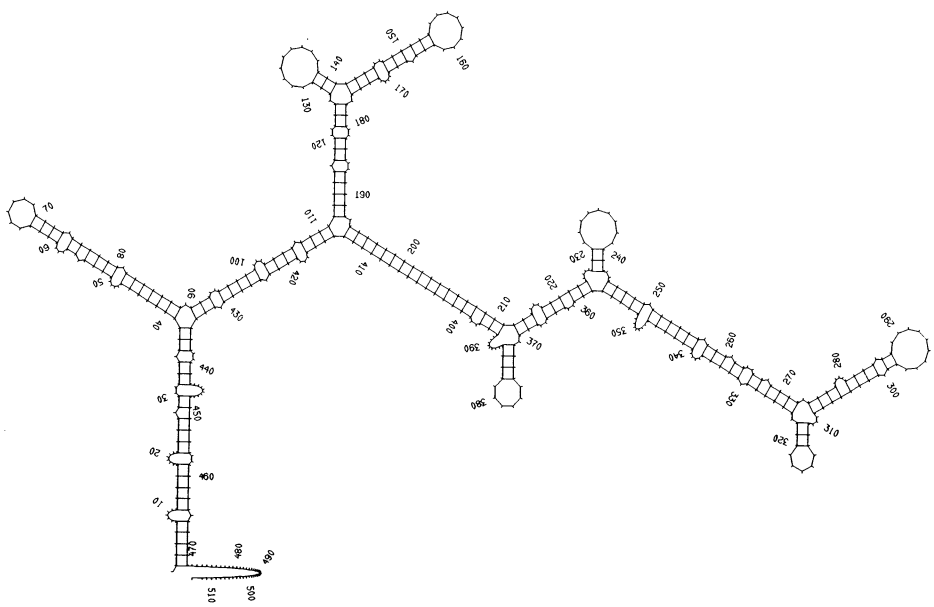

Fig. 5. Predicted secondary structure calculated with GCG programs FOLD and SQUIGGLES (Devereux et al. 1984). The predicted free energy of this structure including the 468 nucleotides immediately 5′ to the poly (A) region is –121.9 kcal per mol.

algorithm (Altschul et al. 1990). Open reading frames were converted to inferred amino acid sequences by the TRANSLATE or PEPDATA programs of the Sequence Analysis Software Package (Devereux et al. 1984) and compared with sequence databases by the BLASTP program (Altschul et al. 1990).

Southern hybridizations.

Transfer of DNAs from 0.7% agarose gels to nylon membranes was by capillary action essentially as described previously (Sambrook et al. 1989). Probes of plasmids and genomic DNAs were made using random-primed labeling and nonradioactive detection protocols (GENIUS, Boehringer Mannheim, Indianapolis, Ind., or RENAISSANCE, DuPont - NEN, Wilmington, Del.). Hybridization and washes were carried out at 68°C for high stringency, or at 65 and 55°C respectively, for low stringency conditions.

Pulsed field gel electrophoresis.

Contour-clamped homogeneous electric field electrophoresis was used to resolve chromosome-sized DNA molecules (hereafter called chromosomes) from *Nectria haematococca*. DNAs were prepared as described previously (Boehm et al. 1994). Electrophoresis was performed using a commercially available apparatus (BioRad CHEF DRII) and highly purified agarose (SeaKem Gold, FMC BioProducts, Rockland, Maine). Larger (>2 Mb) DNAs were separated on 0.6% agarose gels at 40 V using 0.25X Tris-borate-EDTA (TBE) buffer (Sambrook et al. 1989) at 4°C. Pulse time was ramped from 20 to 100 min; total run time was 240 h. Smaller DNAs were separated on 1% agarose gels at 200 V using 0.25X TBE buffer at 4°C. Switching was ramped from 60 to 90 s or 60 to 120 s over the 24-h running time.

Strain T-2 appears to have at least 16 chromosomes. The sizes of bands were >5.7, 5.4, (3.75), 3.10, 2.85, (2.55), 1.80, (1.60), 1.28, 1.22, 0.70, 0.60, and 0.57 Mb. Strain 6-36 appears to have at least 10 chromosomes; sizes were (5.0), 3.20, 2.75, 2.35, 2.10, 1.05, 0.66, 0.54, and 0.48 Mb. Values in parentheses indicate doublet bands suggested by relative fluorescence intensity of ethidium bromide–stained gels.

Genetic analysis.

A genetic cross between strains 6-36 (♀) and T-2 (♂) was obtained as described previously (VanEtten and Kistler 1988). Segregation of DNA polymorphisms was analyzed by the HAPMAP program (Bronson et al. 1989) provided by C. Bronson, Dept. Plant Pathology, Iowa State University. The null hypothesis of independent segregation was tested by a X^2 test for pair-wise comparison of markers. Pairs of loci deviating from independence were assigned a map distance and 95% confidence intervals for distance.

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