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Three Genes for Metabolism of the Phytoalexin Maackiain in the Plant Pathogen *Nectria haematococca*: Meiotic Instability and Relationship to a New Gene for Pisatin Demethylase

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Some isolates of the plant-pathogenic fungus *Nectria haematococca* mating population (MP) VI metabolize maackiain and medicarpin, two antimicrobial compounds (phytoalexins) synthesized by chickpea (*Cicer arietinum* L.). The enzymatic modifications by the fungus convert the phytoalexins to less toxic derivatives, and this detoxification has been proposed to be important for pathogenesis on chickpea. In the present study, loci controlling maackiain metabolism (*Mak* genes) were identified by crosses among isolates of *N. haematococca* MP VI that differed in their ability to metabolize the phytoalexin. Strains carrying *Mak1* or *Mak2* converted maackiain to 1a-hydroxymaackiain, while those with *Mak3* converted it to 6a-hydroxymaackiain. *Mak1* and *Mak2* were unusual in that they often failed to be inherited by progeny. *Mak1* was closely linked to *Pda6*, a new member in a family of genes in *N. haematococca* MP VI that encode enzymes for detoxification of pisatin, the phytoalexin synthesized by garden pea. Like *Mak1*, *Pda6* was also transmitted irregularly to progeny. Although the unusual meiotic behaviors of some *Mak* genes complicate genetic analysis, identification of these genes should afford a more thorough evaluation of the role of phytoalexin detoxification in the pathogenesis of *N. haematococca* MP VI on chickpea.

The heterothallic ascomycete *Nectria haematococca* Berk. et Br. mating population (MP) VI (asexual state; *Fusarium solani*) causes stem and root rots in many plant species, including some which produce antimicrobial compounds (phytoalexins) (1) in response to challenge by microorganisms. The pathogenicity of this fungus on pea (*Pisum sativum* L.) is determined in part by its ability to detoxify (+)-pisatin, an isoflavonoid phytoalexin synthesized by pea (22). Detoxification is accomplished by a cytochrome P-450 monooxygenase-catalyzed demethylation of pisatin (10) that produces the less toxic compound (+)-6a-hydroxymaackiain (25) (Fig. 1). These monooxygenases are encoded by the *Pda* gene family of *N. haematococca* MP VI. High virulence of the pathogen on pea is associated with *Pda* genes that encode moderate to high enzyme activity and are rapidly induced.

Maackiain and medicarpin are isoflavonoid phytoalexins from chickpea (*Cicer arietinum* L.) that are structurally similar to pisatin but have opposite stereochemistry at positions 6a and 11a (6). Three reactions, all mechanistically consistent with catalysis by monooxygenases, are known for metabolism of the chickpea phytoalexins by *N. haematococca* MP VI (Fig. 1), and for those examined, the same set of reactions are performed on both maackiain and medicarpin (4). All conversion products appear to be less toxic than the parent compounds, as determined by direct bioassays of these metabolites or similar products from analogous reactions on other phytoalexins (3).

A survey of 130 field isolates of *N. haematococca* MP VI (8) showed differences in whether they could perform none, one, two, or all three modifications of maackiain; interestingly, most isolates which metabolized maackiain also metabolized pisatin, leading to the speculation that some *Pda* genes might also control maackiain metabolism (8, 21). The correlation that the most virulent field isolates were Mak$^+$ and highly tolerant of maackiain strongly suggested that pathogenesis on chickpea, as on pea, could be determined by whether an isolate of *N. haematococca* MP VI could detoxify a host species' phytoalexins. However, there was one Mak$^-$ isolate that was moderately tolerant and moderately virulent on chickpea. A more critical evaluation of the importance of the different metabolic conversions in these processes would be possible if the loci encoding the enzymes were defined and their effects on virulence and tolerance were assayed in a variety of genetic backgrounds. Therefore, the purpose of the present study was to identify the genes controlling the metabolism of maackiain in *N. haematococca* MP VI, with particular note of their relationship to the genes for pisatin demethylation.

MATERIALS AND METHODS

Fungi. Table 1 summarizes the characteristics of the *N. haematococca* MP VI strains used in this study. Field isolates used as primary parents were selected by three criteria: (i) expression of different Mak phenotypes, (ii) ability to give fertile crosses, and, where possible, (iii) moderate to high virulence on chickpea. T-126 was the most virulent field isolate that did not metabolize maackiain (Mak$^-$) identified in the previous survey (8), and T-161 was one of the few maackiain-metabolizing (Mak$^+$) isolates which crossed with T-126. The phenotype of T-161 is more specifically called 1aHM$^+$ because it metabolizes maackiain to (−)-1a-hydroxymaackiain (1aHM) (Fig. 1). T-161 has been used in other genetic studies on pisatin demethylation (9, 16). T-200 was the most virulent isolate that made (−)-6a-hydroxymaackiain (6aHM) as a conversion product (8); the

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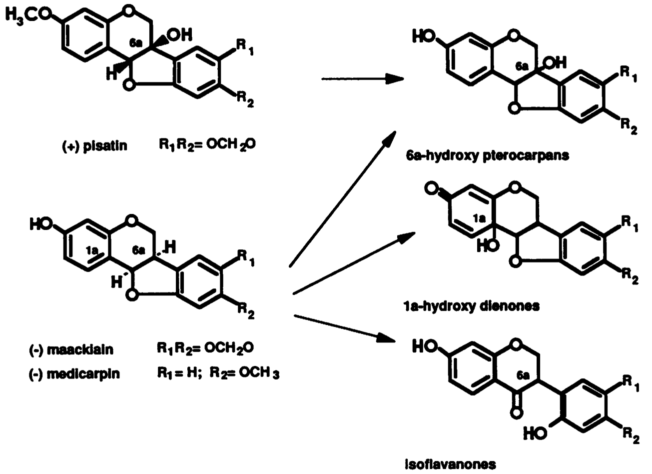

FIG. 1. Metabolism of the phytoalexins pisatin, maackiain, and medicarpin by *N. haematococca* MP VI.

Mak^+ phenotype of T-200 is more specifically called $6aHM^+$.

The techniques used for culture maintenance on V8 Juice agar, performing crosses, and ascospore isolation are described in detail elsewhere (16). In general, the mycelium of the female parent is grown for 7 to 10 days and then fertilized by a conidial suspension of a male parent. Dikaryotic hyphae derived vegetatively from the initial fusion cell formed at fertilization proliferate inside perithecia (fruiting bodies made by the female parent) that appear 5 to 7 days after crossing and mature within 3 weeks. A dikaryotic cell which undergoes karyogamy (fusion of its two nuclei) and meiosis becomes an ascus, and the meiotic products become ascospores. Asci of *N. haematococca* MP VI contain eight ascospores due to a postmeiotic division of each of the four immediate products of meiosis; these unordered progeny are recoverable as a set (a tetrad) by dissection of the ascus. Individual (random) ascospores were sometimes collected instead of or in addition to tetrad ascospores. Cultures derived from ascospores are haploid, allowing direct inference of genotypes from phenotypes. When fewer than eight spores are isolated from an ascus, results are reported as if all eight spores were recovered if marker segregation indicated that all four products of meiosis were represented. Whenever possible, progeny in the tables are grouped by female parent and perithecium of origin. Tables 2 and 4 summarize the crosses done for this study.

Nomenclature of strains. Progeny were assigned numbers specifying the cross, ascus of origin (if known), and ascospore; e.g., strain 156-30-6 is tetrad ascospore 6 from ascus 30 of cross 156, while strain 6-36 is random ascospore 36 from cross 6. The information in Table 2 thus allows the pedigree of most strains (excluding previously described reference strains and strain 260-1-1) to be traced back to field isolate T-126, T-161, or T-200; the parents of 260-1-1 were 156-30-6 and a Pda^- sibling from cross 156 (strain 156-30-5).

Chemicals. Maackiain was extracted from red clover plants and (−)-1a-hydroxymaackiain and (−)-6a-hydroxy-

TABLE 1. Strains of *N. haematococca* MP VI

Strain type	Strain no.	Phenotype[a]	Standard or proposed genotype[a]	Reference(s)
Field isolates	T-126	Mak^- Pda^+		8, 24
	T-161	$1aHM^+$ Pda^+	*Mak1 Mak2 Pda6-1*	8, 24
	T-200	$6aHM^+$ Pda^+	*Mak3 Pda6-2*	8, 24
Reference strains	6-36	Mat^+	*Mat1*	20
	6-94	Mat^-	*Mat2*	20
	77-2-3	Pda^+	*Pda1*	7
	96-67	Pda^+	*Pda2*	7
	62-1	Pda^+	*Pda3*	7
	196-10-7	Pda^+	*Pda4*	9
	55-5-1	Pda^+	*Pda5*	10a
Progeny strains[b]	156-2-1	Mak^- Pda^-		This study
	156-30-6	$1aHM^+$ Pda^+	*Mak1 Pda6-1*	This study
	156-31-3	$1aHM^+$ Pda^-	*Mak2*	This study
	230-25-7	Mak^- Pda^-		This study
	230-27-7	Mak^- Pda^-		This study
	230-29-4	$1aHM^+$ Pda^+	*Mak1 Pda6-1*	This study
	230-30-6	$1aHM^+$ Pda^+	*Mak1 Pda6-1*	This study
	230-31-1	Mak^- Pda^-		This study
	241-1-1	$6aHM^+$ Pda^+	*Mak3 Pda6-2*	This study
	241-26-7	$6aHM^-$ Pda^+	*Pda6-2*	This study
	241-35-7	$6aHM^-$ Pda^+	*Pda6-2*	This study
	241-36-1	$6aHM^-$ Pda^+	*Pda6-2*	This study
	241-36-2	$6aHM^+$ Pda^+	*Mak3 Pda6-2*	This study
	241-36-6	$6aHM^+$ Pda^-	*Mak3*	This study
	245-2-6	$6aHM^+$ Pda^+	*Mak3 Pda6-2*	This study
	260-1-1	$1aHM^+$ Pda^+	*Mak1 Pda6-1*	This study
	269-13-1	$1aHM^+$ Pda^-	*Mak2*	This study

[a] Designations are explained in Materials and Methods. Genotypes for reference strains follow the system of VanEtten and Kistler (20); proposed genotypes refer to the *Mak1*, *Mak2*, *Mak3*, and *Pda6* loci based on evidence in this study. Isolate T-161 is now thought to carry *Pda6-1* rather than *Pda3-2* (8) (see text).

[b] Progeny of isolate T-200 are noted only as $6aHM^-$ rather than Mak^- because a novel 1aHM-like metabolite discovered in these crosses could not be differentiated from authentic 1aHM by TLC (see text).

TABLE 2. Crosses

Cross no.	Parent strains	Phytoalexin metabolism phenotype(s) scored
156	T-161 × T-126	1aHM, Pda
230	156-30-6 × 156-2-1	1aHM, Pda
241	T-200 × 230-25-7	6aHM, Pda
245	T-200 × 230-29-4	6aHM, Pda
261	241-36-2 × 241-36-6	6aHM, Pda
263	260-1-1 × 230-27-7	1aHM, Pda
264	156-31-3 × 241-36-1	Pda
268	156-31-3 × 230-30-6	1aHM, Pda
269	156-31-3 × 230-31-1	1aHM
272	156-30-6 × 230-30-6	1aHM, Pda
279	156-31-3 × 269-13-1	1aHM
284	156-31-3 × 241-1-1	6aHM, Pda
285	230-29-4 × 245-2-6	6aHM, Pda
287	230-25-7 × 241-35-7	Pda
288	T-200 × 241-26-7	Pda

maackiain were obtained by in vitro metabolism of (−)-maackiain (4). (+)-Pisatin was extracted from pea seedlings and (+)-[3-*O-methyl*-^{14}C]pisatin was prepared by fungal demethylation of pisatin to (+)6aHM followed by methylation of (+)6aHM with $^{14}CH_3I$ (15, 24).

Metabolism of maackiain. Mak phenotypes were scored by a modified thin-layer chromatography (TLC) procedure (4). Conidia or mycelial fragments from cultures on V8 Juice agar slants were transferred with a bacteriological loop to test tubes containing 3.5 ml of GA, a glucose-and asparagine-based liquid medium (4). Cultures were shaken at room temperature for 18 to 24 h before maackiain (100 μg in 10 μl of ethanol) was added; the culture was shaken for another 18 to 24 h, during which maackiain was metabolized. The contents of each tube were extracted with 6 ml of ethyl acetate, filtered through Whatman 1PS filters, and dried under reduced pressure. The residue was dissolved in 50 μl of ethanol, and 15 μl of the solution was chromatographed on silica gel plates (Analtech Inc.; GHLF, 250 μm) with toluene-ethyl acetate (1:1, vol/vol). The compounds were detected by UV quenching. Approximately 70 μg of maackiain could be recovered by this method from control media inoculated with strains that did not metabolize the phytoalexin. Isolates whose extracts contained little or no maackiain but significant amounts of compounds comigrating with 1aHM or 6aHM were designated 1aHM$^+$ or 6aHM$^+$, respectively. Isolates with substantial maackiain but no maackiain metabolites in the extract were designated Mak$^-$.

Pisatin-demethylating ability (Pda). Pda was scored by a minivial assay (9) in which fungi were grown at 25°C in 7-ml plastic scintillation vials containing 250 μl of a peptone-glucose agar medium (18) amended with 4 μg of [3-*O-methyl*-^{14}C]pisatin (approx. 5 × 10^5 dpm/μmol). After 8 to 10 days, 4.5 ml of toluene containing 0.55% 2,5-diphenyloxazole was added to each vial. The amount of phytoalexin remaining could be determined by scintillation counting because only unmetabolized pisatin partitions into the organic phase; labeled degradation products either remain in the aqueous phase or are lost as $^{14}CO_2$. Isolates that decreased the toluene-partitionable radioactivity by 75% or more were designated Pda$^+$, while those which produced changes of 20% or less were considered Pda$^-$. An intermediate level of demethylation was observed in some progeny of crosses 263, 264, 268, and 284. This phenotype was attributable to segregation at an additional locus modifying Pda expression (10b); these strains were considered Pda$^+$ in this study.

Other markers. Mating type (Mat) was determined by crosses to reference strains (20). Mat consistently segregates as two alleles at a single locus, *Mat*, and was used to verify that all meiotic products were represented in a tetrad (16). A diffusible bluish-black pigment whose production was probably not under simple genetic control was occasionally used to identify ascospore twins within a tetrad.

Statistical analysis. Random ascospore data, except for cross 156, were analyzed by the chi-squared test. Results were not significantly different at the $P = 0.05$ level from the models proposed unless indicated.

RESULTS AND DISCUSSION

The study began with crosses between field isolates differing in Mak phenotypes to determine how many genes were responsible for that trait and was continued with crosses among progeny strains to characterize each locus. Two genes, *Mak1* and *Mak2*, were identified from field isolate T-161. They conferred the 1aHM$^+$ phenotype and were unusual in their high level of instability; *Mak1* was further notable for its tight linkage to a new gene for pisatin demethylase, *Pda6*.

***Mak1* and *Pda6-1* from isolate T-161.** Field isolate T-161 (1aHM$^+$ Pda$^+$) probably carried several *Mak* genes because most of the 30 random progeny from a cross between this isolate and T-126 (Mak$^-$ Pda$^+$) were 1aHM$^+$ (cross 156, Table 3); however, this cross had low fertility, so tetrads could not be obtained to verify the number of *Mak* genes deduced. Mat segregated 15:15 Mat$^+$:Mat$^-$, as expected for two alleles at one locus, and the presence of the recombinant class of Pda$^-$ progeny suggested that the genes for Pda in T-161 and T-126 were nonallelic.

In order to identify individual *Mak* genes from T-161, strain 156-30-6, a 1aHM$^+$ Pda$^+$ progeny from cross 156, was crossed to a Mak$^-$ Pda$^-$ sibling (cross 230). All 14 tetrads analyzed segregated in a 4+:4− ratio for both 1aHM and Pda, indicating single-gene control for each trait. The locus controlling Mak in strain 156-30-6 was designated *Mak1*. The locus for Pda was recognized as a new *Pda* gene, *Pda6*, after significant numbers of Pda$^-$ progeny were observed in crosses of strain 156-30-6 to reference strains for each of the

TABLE 3. Segregation of Mak (1aHM) and Pda among random ascopores derived from isolates T-161 and T-126

Cross no.	Parent strains[a]	Phenotype or genotype	No. of progeny of phenotype: 1aHM$^+$ Pda$^+$	1aHM$^+$ Pda$^-$	1aHM$^-$ Pda$^+$	1aHM$^-$ Pda$^-$	Total
156	T-161 (F), T-126	1aHM$^+$ Pda$^+$, Mak$^-$ Pda$^+$	13	12	1	4	30
268	230-30-6 (F), 156-31-3	*Mak1 Pda6-1*, 1aHM$^+$ Pda$^-$	10	10	0	7	27
268 (R)[b]	230-30-6, 156-31-3 (F)	*Mak1 Pda6-1*, 1aHM$^+$ Pda$^-$	12	6	0	12	30

[a] F, female parent.

[b] R, reciprocal cross.

TABLE 4. Segregation for Pda in crosses between strain 156-30-6 and the *Pda* reference strains

Cross no.	Reference strain[a]	Genotype	No. of random progeny		Expected ratio for 2 unlinked genes (Pda^+:Pda^-)	χ^2
			Pda^+	Pda^-		
249	77-2-3	*Pda1*	52	55	3:1	32.04[b]
250	96-67	*Pda2*	63	26	3:1	0.84
251	62-1	*Pda3*	84	21	3:1	1.40
252	196-10-7	*Pda4*	74	34	3:1	2.41
253	55-5-1	*Pda5*	48	26	3:1	2.70

[a] Reference strains carry an active allele at only one *Pda* locus, e.g., strain 77-2-3 carries an active allele only at *Pda1*.
[b] Rejected at $P = 0.05$.

previously characterized *Pda* genes (Table 4); there were more Pda^- progeny than expected in cross 249 against the *Pda1* reference strain (discussed below). The *Pda6* allele in strain 156-30-6 was designated *Pda6-1*. Similar results were observed in cross 263, in which these genes were derived from strain 156-30-6 through other crosses (Table 2): all seven tetrads had a 4:4 $1aHM^+$ Pda^+:Mak^- Pda^- segregation. Approximately equal numbers of parental ditype (PD) and nonparental ditype (NPD) tetrads with respect to Mat and 1aHM or Pda were recovered in crosses 230 (5PD: 6NPD) and 263 (4PD:2NPD), so no linkage was indicated between *Mat* and either *Mak1* or *Pda6*.

Recovery of exclusively PD tetrads for Mak and Pda from crosses 230 and 263 indicated that *Mak1* and *Pda6* were linked. This linkage is interesting because it has been suggested that *Mak* and *Pda* belong to a family of detoxification genes in *N. haematococca* MP VI (21). An evolutionary relationship between the *Mak* and *Pda* genes might exist, because the enzymatic reactions they control are similar mechanistically, involve recognition of structurally related substrates, and possibly fulfill analogous roles during pathogenesis. The lack of recombination between *Mak1* and *Pda6* raises speculation that they might belong to a cluster of related genes with recently diverged functions or that they might even be the same gene. The linkage per se precluded testing *Mak1* for homology to *Pda6* with a cloned *Pda* gene (26). However, DNA from isolates carrying the other two *Mak* genes identified in this study (described below) and additional Pda^- field isolates that metabolize maackiain did not hybridize to the *Pda* gene in Southern analysis (11; unpublished), suggesting that *Mak1* and *Pda6* are probably different genes. Resolving this issue requires either further crosses that succeed in breaking the linkage or molecular cloning of these genes.

Aberrant transmission of *Mak1* and *Pda6*. The first instance of unusual behavior by *Mak1* and *Pda6* was observed in cross 272, a backcross of progeny strain 230-30-6 (*Mak1 Pda6-1*) to the *Mak1 Pda6-1* source strain, 156-30-6. In this cross, only $1aHM^+$ Pda^+ progeny were expected, because there would be no segregation of traits when both parents carried the same alleles and there was no previous indication that *Pda6* (and presumably *Mak1*) was unstable; 95 mitotically derived single-spore subcultures of 156-30-6 all were Pda^+ upon testing. However, when 156-30-6 served as the female parent (i.e., produced the perithecia from which tetrads were collected), the progeny from 5 of the 11 tetrads were all $1aHM^+$ Pda^+, but the other 6 tetrads had the unexpected segregation of 4:4 $1aHM^+$ Pda^+:Mak^- Pda^-, where both phenotypes were absent simultaneously. In addition, there was one Pda^- representative among 68 random ascospores scored just for Pda. When strain 230-30-6 was the female parent, segregation in tetrads was normal; four tetrads from one perithecium scored for both Mak and Pda consisted of only $1aHM^+$ Pda^+ progeny, and 12 tetrads (4 from each of three other perithecia) scored just for Pda were exclusively Pda^+. Nonetheless, unusual segregation must have occurred in these perithecia, albeit at a much lower frequency, because 1 of 70 random ascospores was Pda^-. The significance of the observed maternal influence is presently unclear, as other crosses (described below) did not appear to share this feature.

Irregular meiotic transmission of *Pda* genes has been noted previously, but its cause was unknown (1a, 7, 9, 9a, 10a, 16, 17). In principle, excess null phenotypes could arise as a result of epistatic suppression by a modifying gene. If so, then the modifier might be separated from a functional *Pda* gene by recombination, resulting in Pda^+ progeny from crosses between Pda^- parents; however, Cowling and VanEtten (2) failed to find such recombinants in their screen of crosses between Pda^- isolates from a variety of sources.

That both 8:0 and 4:4 $1aHM^+$ Pda^+:Mak^- Pda^- tetrads were found in the same perithecium provides a clue regarding the period during which the novel null phenotypes were created. Because all tetrads in a perithecium are presumably derived from the same pair of parental nuclei, occurrence of both normal and abnormal 4:4 tetrads in the same perithecium argues that gene loss in this cross occurred premeiotically. This premeiotically associated loss of phenotype in cross 272 superficially resemble the premeiotically active gene-silencing processes ("RIP" or "MIP") of *Neurospora crassa* (12, 13) and *Ascobolus immersus*, respectively (5). However, the situation in *N. haematococca* is distinct because other studies show that the Pda^- phenotype arises not by point mutation to a dysfunctional state, as in *N. crassa*, nor via DNA methylation-associated silencing of genes, as in *A. immersus*, but by a chromosome deletion that encompasses *Pda6* and *Mak1* (11, 11b). Also, RIP and MIP both require a sequence duplication, but there is no evidence that this is required for loss of phenotype in *N. haematococca*. Furthermore, crosses with other genes (below) suggest that gene loss in *N. haematococca* is not restricted to the premeiotic period but can occur during meiosis, and possibly even postmeiotically.

***Mak2* from isolate T-161.** Since *Mak1* appeared to be very closely linked to *Pda6*, progeny from cross 156 which were $1aHM^+$ but Pda^- most likely would have a different *Mak* gene. When strain 156-31-3 ($1aHM^+$ Pda^-) was crossed to a *Mak1 Pda6-1* strain (cross 268, Table 3), about 25% of the random progeny were Mak^-. This result is consistent with segregation for two unlinked, independently sufficient genes for the $1aHM^+$ phenotype in cross 268. Also, all Pda^+ progeny were $1aHM^+$, while one-third of the $1aHM^+$ progeny were Pda^-. These data suggest that there are two *Mak* genes, one linked to a *Pda* locus, the other not linked.

TABLE 5. Segregation for *Mak2* in crosses 269 and 279

Cross no.[a]	Parent strains[b]	Phenotype or genotype	Perithecium no.	No. of tetrads of each segregation type (Mak$^+$:Mak$^-$)
269	156-31-3 (F), 230-31-1	*Mak2*, Mak$^-$	1	5 (4:4) 1 (2:6)
			2	3 (4:4) 2 (0:8)
269 R	156-31-3, 230-31-1 (F)	*Mak2*, Mak$^-$	1	4 (4:4) 1 (6:2)
			2	5 (0:8)
279	156-31-3 (F), 269-13-1	*Mak2*, *Mak2*	1	1 (8:0) 1 (4:4)
			2	1 (6:2) 1 (4:4)
			3	4 (4:4)
279 R	156-31-3, 269-13-1 (F)	*Mak2*, *Mak2*	1	2 (4:4)
			2	1 (8:0) 1 (4:4)

[a] R, reciprocal cross.

[b] F, female parent.

The hypothesis of a second *Mak* gene (*Mak2*) unlinked to *Pda6* in strain 156-31-3 was tested by crossing it to a Mak$^-$ strain (cross 269, Table 5). Many tetrads segregated as expected for a single locus (4:4 1aHM$^+$:Mak$^-$), but others segregated 2:6, 0:8, and 6:2. One interpretation of the 4:4, 2:6, and 0:8 ratios is that Mak in this background required the interaction of two unlinked genes. However, the predominance of 4:4 tetrads is also consistent with the idea that *Mak2* is a single locus and that 2:6 and 0:8 tetrads result from gene loss, as encountered at *Mak1* in cross 272. A 6:2 tetrad in either case could have arisen by gene conversion. In this cross, 4:4 tetrads occurred in the same perithecium with tetrads of the other ratios, regardless of which strain was the female parent. *Mat* was observed to segregate normally in several tetrads representing each type of segregation for Mak, so transmission irregularities did not extend to all genes.

To examine whether *Mak2* was also meiotically unstable, a 1aHM$^+$ progeny strain from a 4:4 1aHM$^+$:Mak$^-$ tetrad of cross 269 was backcrossed to strain 156-31-3 (cross 279, Table 5). Only 1aHM$^+$ progeny were expected, regardless of the number of genes required for the 1aHM$^+$ phenotype in this lineage, because both parents must carry the same gene(s) for this trait. Instead, many tetrads showed 6:2 or 4:4 segregation. On first inspection, such results from a 1aHM$^+$ × 1aHM$^+$ cross could indicate control of Mak by two independently sufficient genes; however, knowledge of the pedigree and that there is potential for aberrant transmission, as shown in the analysis of *Mak1*, suggest that loss of *Mak2* during meiosis is most likely involved. If this is so, then the occurrence of 6:2 tetrads (and 2:6 tetrads in cross 269 above) is significant, because it signifies that the process responsible for loss of *Mak2* is not constrained to a premeiotic window. The cross seemed normal in other respects; five representative tetrads showed 4:4 segregation for Mat, and there was no bias in Mak ratios that could be associated with maternal parentage.

***Mak3* from isolate T-200.** In contrast to the unusual transmission of *Mak1* and *Mak2*, the gene responsible for the 6aHM$^+$ phenotype of field isolate T-200, *Mak3*, was conventionally inherited in crosses of T-200 with a 6aHM$^-$ strain. All 22 tetrads from cross 241 and 11 of 12 tetrads from cross 245 showed 4:4 6aHM$^+$:6aHM$^-$ segregation; the exceptional tetrad in cross 245 had an extra 6aHM$^-$ member as well as an extra Pda$^-$ member (discussed below). Progeny *Mak3* strains also showed approximately equal numbers of 6aHM$^+$ and 6aHM$^-$ progeny when crossed against 6aHM$^-$ strains (crosses 284 and 285, Table 6), and all progeny from a *Mak3* × *Mak3* cross were 6aHM$^+$, as expected (cross 261, Table 6). *Mak3* was not linked to *Mat*, as there were equal numbers of PD and NPD tetrads in crosses 241 (3PD:4NPD) and 245 (4PD:3NPD).

TABLE 6. Segregation for 6aHM among progeny derived from isolate T-200

Cross no.[a]	Parent strains[b]	Phenotype or genotype	Perithecium no.	No. of random progeny		No. of tetrads of each segregation type (6aHM$^+$:6aHM$^-$)
				6aHM$^+$	6aHM$^-$	
284	241-1-1, 156-31-3 (F)	*Mak3*, 6aHM$^-$		9	19	
284 R	241-1-1 (F), 156-31-3	*Mak3*, 6aHM$^-$		10	10	
285	245-2-6, 230-29-4 (F)[c]	*Mak3*, 6aHM$^-$	1			6 (4:4)
			2			1 (4:4)
			3			1 (4:4)
261	241-36-2 (F), 241-36-6	*Mak3*, *Mak3*	1	9	0	1 (8:0)
			2	4	0	1 (8:0)
			3	6	0	1 (8:0)
			4	7	0	1 (8:0)

[a] R, reciprocal cross.

[b] F, female parent.

[c] Strain 230-29-4 (*Mak1*) makes 1aHM but not 6aHM. However, some progeny derived from T-200 produced a 1aHM-like metabolite in crosses 245 and 285 which precluded reliable scoring for *Mak1*; thus, only 6aHM production was analyzed.

TABLE 7. Segregation for Pda among progeny derived from isolate T-200[a]

Cross no.	Parent strains[b]	Phenotype or genotype	Perithecium no.	No. of random progeny		No. of tetrads of each segregation type (Pda^+:Pda^-)
				Pda^+	Pda^-	
245	T-200, 230-29-4	Pda^+, *Pda6-1*				17 (8:0) 1 (7:1)
241	T-200, 230-25-7	*Pda6-2*, Pda^-				8 (4:4) 24 (0:8)
261	241-36-2 (F), 241-36-6	*Pda6-2*, Pda^-	1	5	3	2 (4:4)
			2	1	1	3 (4:4)
			3	2	2	3 (4:4)
			4	2	3	3 (4:4)
264	241-36-1, 156-31-3 (F)	*Pda6-2*, Pda^-	1			1 (4:4)
			2			6 (4:4) 1 (2:6)
			3			1 (0:8)
			4			6 (4:4)
284	241-1-1 (F), 156-31-3	*Pda6-2*, Pda^-		10	10	
284 R	241-1-1, 156-31-3 (F)	*Pda6-2*, Pda^-		22	31	
288	T-200 (F), 241-26-7	*Pda6-2*, *Pda6-2*	1			1 (8:0)
			2			6 (8:0) 1 (7:1) 1 (6:2)
			3			4 (8:0) 1 (4:4)
285	245-2-6, 230-29-4 (F)	*Pda6-2*[c], *Pda6-1*	1			5 (4:4) 3 (0:8)
			2			1 (8:0)
			3			1 (4:4)
287	241-35-7, 230-25-7 (F)	*Pda6-2*, Pda^-	1	0	18	1 (0:8)
			2	8	27	8 (0:8)
			3	0	16	2 (4:4)

[a] Some of these crosses were also listed in Table 6. The number of progeny listed here may exceed that in Table 4 because more progeny were scored for Pda than for Mak. See also Table 6, footnotes *a* and *b*.

[b] Which of the two parents served as the female in crosses 241 and 245 was not recorded.

[c] Strain 245-2-6 probably contains *Pda6-2*, based on karyotype analysis showing that this strain inherited a *Pda6*-carrying chromosome characteristic of strain T-200 (10b).

Mak3 strains were crossed to *Mak1* and *Mak2* strains to test for epistasis among the genes controlling alternate routes of maackiain metabolism. Mak^- progeny were obtained, suggesting that the genes for the $6aHM^+$ and $1aHM^+$ phenotypes were different. However, more detailed tests were complicated by the finding that isolate T-200 also had the ability to metabolize maackiain to an unidentified compound with mobility on TLC and high-pressure liquid chromatography (HPLC) similar to that of 1aHM but with a different UV spectrum (10b). Little or none of this metabolite was produced by T-200 itself, but some $6aHM^-$ progeny from crosses of T-200 with $6aHM^-$ strains produced it; this metabolite's similarity to 1aHM in TLC precluded reliable scoring of $1aHM^+$ in crosses with a T-200 background. Production of the new metabolite appeared to be controlled by a single locus, and *Mak3* was most likely epistatic to it, because progeny predicted by tetrad analysis to have both genes consistently made 6aHM as the predominant product. The unknown compound was not investigated further.

An additional unexpected result from *Mak3* crosses was recovery of auxotrophic progeny in nearly half the tetrads of crosses 241 and 245. These exhibited a typical nitrogen starvation phenotype on maintenance medium (normal radial growth but sparse branching and little aerial mycelium) but did not grow on minimal medium unless it was supplemented with ornithine cycle intermediates. The phenotype probably arose from a novel combination of genes, because both parents were prototrophic. The auxotrophic condition neither influenced the viability of fungi upon isolation as ascospore cultures or on routine transfer, nor did it interfere with Mak or Pda assays, because none of these procedures involved minimal medium. Among the 23 auxotrophs (not including twins), 10 were $6aHM^+$ and 13 were $6aHM^-$. Interestingly, 18 of 23 were Mat^- and all but 1 failed to function as a female parent, even on supplemented medium.

***Pda6-2* from isolate T-200.** Isolate T-200 was proposed to have *Pda6-2*, an allele of *Pda6-1*, because with the exception of one Pda^- strain from a tetrad that also had an extra Mak^- member (different from the aberrant Pda^- strain), there were no other Pda^- progeny when isolate T-200 was crossed to a *Pda6-1* strain (cross 245, Table 7). Like *Pda6-1*, *Pda6-2* was stable mitotically but unstable meiotically; 140 single conidial subcultures of T-200 were Pda^+, in contrast to the majority of tetrads in cross 241 (T-200 with a Pda^- strain), which were devoid of Pda^+ members (Table 7). The results of subsequent crosses involving *Pda6-2* progeny from cross 241 and Pda^- tester strains were generally consistent with the model that *Pda6-2* was the only gene responsible for the Pda^+ phenotype of T-200 (crosses 261, 264, and 284, Table 7). *Pda6-2* showed no linkage to *Mat* (3PD:6NPD in cross 264, consistent with the previous conclusion that *Pda6-1* was not linked to *Mat*) or to *Mak3*. First, cross 284 had approximately equal numbers of parental and recombinant progeny, indicating independence of *Pda6* and *Mak3*, and second, loss of *Pda6* in many tetrads of crosses 241 and 285 was not associated with unusual behavior by *Mak3*.

Further crosses involving one or both *Pda6* alleles provided more information pertaining to the gene loss process in *N. haematococca*. A rare 7:1 Pda$^+$:Pda$^-$ tetrad in a *Pda6-2* × *Pda6-2* cross (cross 288, Table 7) and another in a *Pda6-2* × *Pda6-1* cross (cross 245) are noteworthy because they suggest that loss of *Pda6* is not confined to the premeiotic or meiotic periods but could also occasionally occur in the mitotic divisions immediately after meiosis.

The difference in inheritance data observed from tetrads and that from random ascospore samples in cross 287 (Table 7) was striking. While it may simply derive from inadvertent selection during tetrad collection for certain types of "allowed" meioses which generate no lethal genotypes (15) (only asci with a full complement of ascospores are collected for tetrad analysis), analysis of asci containing less than the full complement of ascospores may provide clues to the interaction of lethal genes and the transmission of *Pda6*. The magnitude of gene loss that is possible was highlighted in cross 285 (Table 7), where only 1 of 10 tetrads inherited both *Pda* alleles as expected (8:0 segregation); remarkably, three tetrads appeared to have lost both *Pda6-1* and *Pda6-2*.

The results from this study help delineate the concern about reliance on the segregation ratio for distinguishing among loci, such as those for Pda in *N. haematococca*. Loss of a gene at such high frequencies could lead to misleading inflation of the presumed recombinant class of Pda$^-$ progeny in crosses between non-allelic *Pda* genes, e.g., perhaps in cross 249 (Table 4), or yield results inconsistent with the pedigree of the strain (cross 272). A *Pda* gene in isolate T-161 was proposed as a new locus in this study because allelism tests against each known *Pda* gene showed 25% Pda$^-$ progeny (50% in cross 249) among approximately 100 random ascospores. However, Mackintosh et al. (9) concluded that the single *Pda* gene in T-161 was most likely an allele of *Pda3*, based on the recovery of five tetrads with 8:0 Pda$^+$:Pda$^-$ segregation and partial tetrads totaling nine spores, two of which were inexplicably Pda$^-$. One interpretation is that Pda$^-$ progeny in the *Pda3* allelism tests of both studies arose by new deletions rather than by recombination; if so, then the *Pda* gene of T-161 is really an allele at *Pda3*. Alternatively, the sample from the previous study may simply have been too small for detection of a sufficient number of conventional Pda$^-$ recombinants; in that case, the T-161 gene could be distinct from *Pda3*. We tentatively assign the T-161 gene as a new gene, *Pda6*, until the implications of gene loss on standard allelism tests in *N. haematococca* MP VI are fully evaluated and solutions are devised.

An analysis of *Mak* genes as factors in the disease-causing ability of *N. haematococca* MP VI is described in the accompanying article (11a). Meiotic instability of the genes should not a priori influence their performance during the pathogenic phase. The physical loss of genes might be considered fortuitous, as it creates additional "null" genotypes for testing the relationship between specific genes and the biological phenomenon they are thought to influence. For example, a conclusive result that would rule out a causal role for *Mak* genes in pathogenicity would be a loss of these genes without an accompanying loss of pathogenicity.

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