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Two Additional Genes for Pisatin Demethylation and Their Relationship to the Pathogenicity of *Nectria haematococca* on Pea

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The ability of the fungus *Nectria haematococca* to demethylate pisatin is required for its tolerance to this phytoalexin and for its virulence on *Pisum sativum*. Pisatin demethylase activity (Pda) is inducible by pisatin, and among Pda^+ *N. haematococca* isolates there are large differences in the level of activity observed after induction. Previous analysis of two field isolates identified three genes controlling pisatin demethylation. *Pda1* confers a high level of demethylase activity (Pda^{SH} phenotype), while *Pda2* and *Pda3* encode low rates of demethylation (Pda^{LL}). High virulence on pea was associated only with *Pda1*. The present study was an analysis of the control of pisatin demethylation in field isolate T-23, which expressed an intermediate level of activity (Pda^{SM}), and in another Pda^{LL} field isolate, T-161. A fourth gene, *Pda4*, was found to control the Pda^{SM} phenotype, while the *Pda* gene in isolate T-161 may represent another allele at the *Pda3* locus. In crosses segregating for *Pda4* or *Pda3*, only progeny with the Pda^{SM} phenotype were virulent; both Pda^{LL} and Pda^- progeny were nonvirulent. In a cross involving *Pda1* and *Pda4*, Pda^{SH} progeny were more virulent than Pda^{SM} progeny. Apparently the amount of phytoalexin-detoxifying enzyme produced by this fungus can directly affect its virulence on pea.

Additional keywords: cytochrome P-450, *Fusarium solani*, phytoalexin detoxification, virulence gene regulation.

Nectria haematococca Berk. and Br. mating population (MP) VI (anamorph: *Fusarium solani*) is a fungal pathogen of garden pea (*Pisum sativum* L.). Pea plants accumulate high concentrations of the phytoalexin pisatin when challenged by virulent or avirulent isolates of *N. haematococca* (Pueppke and VanEtten 1976; Tegtmeier and VanEtten 1982b). However, all virulent isolates of *N. haematococca* are able to demethylate pisatin to a less toxic compound (Tegtmeier and VanEtten 1982b; VanEtten *et al.* 1980; Kistler and VanEtten 1984b). These isolates are also highly tolerant of pisatin *in vitro*. Isolates unable to demethylate pisatin are low in virulence and are sensitive to pisatin. These correlations suggest that the demethylation of pisatin by *N. haematococca* is an important mechanism by which this fungus tolerates its host's phytoalexin and is one factor required for its pathogenicity on pea.

However, not all isolates of *N. haematococca* MP VI with pisatin demethylating activity (Pda^+) are virulent (Tegtmeier and VanEtten 1982b). A survey of 59 Pda^+ field isolates revealed extensive variation in the rate of pisatin demethylation observed after induction (VanEtten and Matthews 1984). Genetic analysis of two field isolates, T-2 and T-219, which represented extremes in the quantitative expression of pisatin demethylase, showed that each phenotype was under separate single-gene control. These phenotypes were assigned the names Pda^i and Pda^n, for "inducible" and "noninducible" (Kistler and VanEtten 1984a). Here they will be referred to as Pda^{SH} (Short lag period, High enzyme activity) and Pda^{LL} (Long lag, Low activity) to better relate them to the new phenotype reported in this paper. Three independent *Pda* genes were identified in isolates T-2 and T-219. An active allele at the *Pda1* locus conferred the Pda^{SH} phenotype and was epistatic to the active alleles of *Pda2* and *Pda3*, either of which alone conferred the Pda^{LL} phenotype. *Pda1* was associated with high virulence toward pea and tolerance to pisatin, whereas isolates with only *Pda2* or *Pda3* possessed very low virulence and generally lower tolerance to pisatin, although they were more tolerant than Pda^- isolates (Kistler and VanEtten 1984b). These results suggested that natural differences in the regulation of pisatin demethylation might affect pisatin tolerance and thereby influence the relative virulence of Pda^+ isolates.

In the survey of *N. haematococca* field isolates, a continuous range of pisatin demethylation rates was found, including rates intermediate between those of the Pda^{SH} and Pda^{LL} phenotypes. Some highly virulent isolates, such as T-23, showed demethylation rates an order of magnitude lower than that of isolate T-2 and barely above that of isolate T-219, which was below the limit of accurate quantitation under the experimental conditions used (VanEtten and Matthews 1984). The high virulence of these isolates suggests that the level of enzyme activity conferred by *Pda1* is in excess of what is required for virulence on pea. The activity expressed by an isolate such as T-23 might represent the minimal level of expression that is sufficient for virulence. The current study was undertaken to test this hypothesis and to analyze the genetic control of isolate T-23's Pda phenotype. The approach was to determine whether this phenotype is reliably distinguishable from Pda^{LL}, and if so whether this regulatory difference is 1) heritable, 2) associated with a characteristic level of tolerance to pisatin, and 3) sufficient for virulence.

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MATERIALS AND METHODS

Fungal isolates. *N. haematococca* MP VI is a heterothallic ascomycete that forms asci in perithecia (VanEtten and Kistler 1988). Parent isolates used in this study are described in Table 1. Isolate names beginning with "T-" designate field isolates from natural habitats. Progeny isolates from crosses are named with a sequence of two numbers for random ascospores or three numbers for tetrad ascospores. The first number indicates the particular cross. For random ascospores the second number identifies the particular ascospore from that cross. For tetrads the second number designates the ascus and the third specifies which ascospore of that ascus.

Chemicals. Pisatin specifically labeled with ^{14}C at the 3-*O*-methyl position ("[^{14}C]pisatin," 2–20 × 10^4 dpm·μmol) and unlabeled pisatin were obtained by published procedures (VanEtten *et al.* 1980; Sweigard and VanEtten 1987). Pisatin was dissolved in dimethylsulfoxide (DMSO) and added to cultures to give a final DMSO concentration of 0.5 to 2.0%.

Crosses. Isolates were maintained on V-8 agar (medium 29; Stevens 1974) under continuous fluorescent lighting at 20° C. Techniques for crossing *N. haematococca* and for isolating ascospores have been described previously (Tegtmeier and VanEtten 1982a; VanEtten 1978). Asci of *N. haematococca* contain a maximum of eight ascospores, but frequently fewer are produced or some fail to grow. If segregation of markers in such asci indicated that all four meiotic products were represented, these tetrads were used for genetic analysis; for clarity, the segregation ratios are reported as if all eight spores were viable except where more detailed information is needed. When the four meiotic products could not be identified but the segregation pattern gave useful information, the result is reported as a partial tetrad. For example, a ratio reported as 2:3 represents a partial tetrad. "Random" ascospores were isolated from ascospore masses emerging from the ostioles of mature perithecia.

Several markers in addition to pisatin demethylation were scored to identify the four meiotic products in each ascus and to assess the possible occurrence of irregular genetic phenomena. The most useful marker was mating type (*MAT*) (Tegtmeier and VanEtten 1982a), which segregated 4:4 in every ascus but one. Female fertility (Fem) was not simply inherited, indicating that more than one gene was controlling this trait, as has been observed previously (Tegtmeier and VanEtten 1982a); this trait was mainly used to identify mitotic twins. In some crosses an esterase isozyme marker existed, because isolate 44-46 possessed an isozyme with low electrophoretic mobility (Est^s) absent from isolates T-23 and T-161 (Est^f); this marker helped to distinguish the meiotic products in one ascus from cross 196 and in one from cross 225. In addition, many of the progeny showed distinctive morphological forms or colorations that were not inherited simply but could be used for identification of mitotic twins.

In all asci except one from cross 221, segregation of markers corresponded to what would be expected for the presence of four or fewer distinguishable products of meiosis in the ascus. Another ascus from the same cross segregated 5 *MAT-1*:2 *MAT-2*. Although such exceptional asci have been observed before, they occur so rarely that the possibility of errors in the isolation of the ascospores cannot be excluded. Therefore these two asci were disregarded in the analysis of this cross.

Assay of pisatin demethylation: Pda^+ versus Pda^-. A modification of the previously described "[^{14}C]pisatin mycelial growth bioassay" (Tegtmeier and VanEtten 1982b) was used to determine if isolates had the ability to demethylate pisatin. Conidia were inoculated onto 0.25 ml of M-2 agar (VanEtten 1973) amended with 161 $\mu g \cdot ml^{-1}$ [^{14}C]pisatin, in 7-ml plastic scintillation vials, and the cultures were incubated for 1 wk in the dark at 20° C. Scintillation fluid, 4.5 ml of 0.55% 2,5-diphenyloxazole in toluene, was added to each vial, and the two-phase mixtures were allowed to stand overnight before measuring toluene-extractable radioactivity with a Beckman LS355 scintillation spectrometer. Pda^- cultures retained greater than 90% of the added pisatin whereas Pda^+ cultures retained less than 45%.

"Time course" assay of pisatin demethylation. The primary method used in this study to measure pisatin demethylation rates was to monitor the time course of [^{14}C]pisatin disappearance from cultures in which pisatin demethylase had not been previously induced. Conidia of fungal isolates were suspended in 200 ml of a glucose, casein hydrolysate, yeast extract liquid medium (VanEtten and Barz 1981) in 500-ml Erlenmeyer flasks. Cultures were incubated on a reciprocal shaker at 100 strokes per minute at 25° C for 24 hr. Resulting mycelium was collected on a cotton cloth filter (pore size approximately 50 μm) and

Table 1. Parent isolates used in this study

Isolate[a]	Traits				Proposed *Pda* genotype[b]	Reference
T-23	Pda^{SM}	*MAT-1*[c]	Fem^-	Est^f	*Pda4*	VanEtten 1978
T-161	Pda^{LL}	*MAT-2*	Fem^+	Est^f	*Pda3-2*	VanEtten 1978
179-1-1	Pda^{SM}	*MAT-1*	Fem^+	Est^f	*Pda4, Pda3-2*	This study
196-10-7	Pda^{SM}	*MAT-2*	Fem^+	Est^s	*Pda4*	This study
196-10-2	Pda^{LL}	*MAT-1*	Fem^+	Est^f	*Pda3-2*	This study
44-46	Pda^-	*MAT-2*	Fem^+	Est^s		Kistler and VanEtten 1984a
44-100	Pda^-	*MAT-1*	Fem^+	Est^f		Kistler and VanEtten 1984a
179-2-1	Pda^-	*MAT-1*	Fem^+			This study
184-6-2	Pda^-	*MAT-1*	Fem^+			This study
189-5-1	Pda^-	*MAT-1*	Fem^+			This study
225-1-4	Pda^{LL}	*MAT-1*	Fem^+		*Pda3-2*	This study
225-3-6	Pda^{LL}	*MAT-2*	Fem^+		*Pda3-2*	This study
233-3-5	Pda^{LL}	*MAT-1*	Fem^+		*Pda3-2*	This study
77-13-6	Pda^{SH}	*MAT-1*	Fem^-		*Pda1*	Kistler and VanEtten 1984a
96-17	Pda^{LL}	*MAT-2*	Fem^+		*Pda2*	Kistler and VanEtten 1984a
62-1	Pda^{LL}	*MAT-2*	Fem^+		*Pda3-1*	Kistler and VanEtten 1984a

[a] Isolate T-23 was obtained from diseased pea. The original habitat of T-161 is unknown. All other isolates are laboratory strains.

[b] Only active alleles are indicated, that is "*Pda4*" means the isolate has an active allele at the *Pda4* locus and negative alleles at *Pda1*, *Pda2*, and *Pda3*.

[c] The gene for mating type has been renamed in accord with the recommendations of Yoder *et al.* (1986). *MAT-1* is the allele previously referred to as MAT^+, and *MAT-2* is the new name for MAT^- (VanEtten and Kistler 1988).

washed with sterile water. Mycelium was resuspended in potassium phosphate buffer (50 mM, pH 6.5) at a ratio of 30 mg (fresh weight) per milliliter in 25-ml Erlenmeyer flasks. At time zero [^{14}C]pisatin was added to each culture to give a final pisatin concentration of 31 $\mu g \cdot ml^{-1}$ (100 μM), a concentration that is not toxic to this fungus. At intervals up to 54 hr, 0.5-ml samples were transferred to 4.5 ml of scintillation fluid, and the remaining [^{14}C]pisatin was measured by scintillation counting of the two-phase mixture (Kistler and VanEtten 1984a).

The concentration of [^{14}C]pisatin, P, was plotted against time, t, and used to fit a logistic equation:

$$P = A / (1 + e^{B \ln (t / T_{50})}).$$

The NLIN procedure for nonlinear regression (SAS Institute, Cary, NC) was used to find the values of A, B, and T_{50} that best fit the data for each time course. In this equation, T_{50} is the time required for 50% demethylation. The slope at this time point equals $A\ B\ /\ (4\ T_{50})$ in units of micromoles per liter per hour. This slope, converted to units of picomoles per minute per milligram fresh weight of mycelium, is reported as R_{50}, the rate of demethylation at T_{50}. A, the y-intercept of the curve, was always found to be close to the intended initial concentration of pisatin, 100 μM. Other equations were tested but this one fit the data best, as determined visually and by residual sums of squares. Although T_{50} appears in the expression for R_{50}, these two parameters are free to vary independently. Tests with sample data showed that the equation and the curve-fitting procedure could generate good fits to data with long lags followed by high rates, or short lags and low rates, in addition to the two combinations actually found in our experiments. Thus, the inverse correlation between T_{50} and R_{50} observed in this study is a biological relationship rather than an artifact of the mathematical model.

"Preinduction" assay of pisatin demethylation. This assay of demethylation rate was performed as described previously (Kistler and VanEtten 1984a). Mycelial suspensions in phosphate buffer were treated with 100 μM unlabeled pisatin. Cultures were incubated with shaking for 6 hr at 25° C, then 31 μg of [^{14}C]pisatin was added per milliliter of culture. At 4- or 5-min intervals over 20 to 35 min, 0.5-ml samples were transferred to 4.5 ml of scintillation fluid. The rate of pisatin demethylation was calculated by linear regression of the decrease in toluene-extractable radioactivity with time.

Pisatin sensitivity. Inhibition of growth of *N. haematococca* isolates by pisatin was determined as previously described (VanEtten *et al.* 1980; Kistler and VanEtten 1984b). Radial growth on agar medium in the presence and absence of 161 $\mu g \cdot ml^{-1}$ pisatin was measured to the nearest 0.1 mm daily up to 7 days. Pisatin-treated and control cultures both contained 2% DMSO.

Virulence toward pea. The previously described "test-tube assay" (VanEtten *et al.* 1980) was used to determine virulence of fungal isolates. Six days after inoculation of wounded epicotyls, virulence was measured as the average length of the brown lesions on eight replicate plants. Wounded but uninoculated control plants displayed little (<1 mm) or no discoloration at the site of the wound.

Esterase isozyme assay. Isolates were grown in 75 ml of liquid medium in 125-ml flasks as described for the time course assay. Mycelium was then collected by vacuum filtration, washed with distilled water, and stored at −80° C until needed. Samples were prepared and starch gels run according to previously described methods (Tooley *et al.* 1985). The gel buffer was 0.046 M Tris, 0.0068 M citric acid (pH 8.4), and a 1:10 dilution of tray buffer. Tray buffer was 0.029 M LiOH and 0.38 M boric acid. The resulting gel was stained for esterase activity according to Conkle *et al.* (1982).

RESULTS

Description of Pda phenotypes by the time course of pisatin demethylation. The time course of pisatin demethylation by field isolate T-23, in the absence of preinduction by pisatin, was compared with those of T-219 and five other field isolates (T-61, T-161, T-201, T-203, and T-223) that had shown very low demethylation rates when measured by the preinduction assay (VanEtten and Matthews

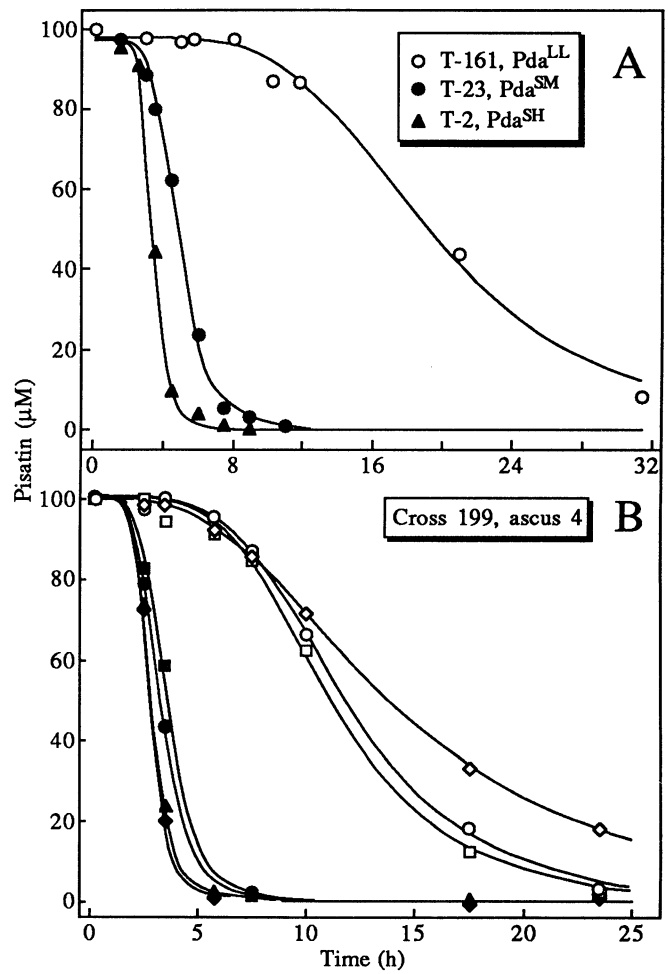

Fig. 1. Time course of pisatin demethylation by (**A**) field isolates T-2, T-23, and T-161. Isolates T-61, T-201, T-203, T-219, and T-223 showed time courses similar to that of T-161. **B**, Progeny from ascus 4 of cross 199. Curves represent the equation $P = A / (1 + e^{B \ln (t / T_{50})})$, using the fitted values of the parameters A, B, and T_{50}.

1984). Representative time courses for isolates T-23 and T-161 are given in Figure 1A. The time course of the Pda^{SH} isolate T-2 is also shown for comparison. Using this assay, isolate T-23 showed reproducible differences from the Pda^{LL} field isolates both in the length of lag period before pisatin demethylation was detectable and in the maximum rate of demethylation. The length of lag was somewhat difficult to quantify however, because demethylation began very gradually, especially for Pda^{LL} isolates. The parameters that appeared to be most reliably estimated by the fitted equation were the time for half-demethylation of the pisatin supplied (T_{50}) and the rate of demethylation at that time (R_{50})

The conclusion from this initial comparison was that isolate T-23 had a shorter T_{50} and a faster R_{50} than the Pda^{LL} isolates, and repeated assays of T-23 and T-161 verified this difference (Table 2). The phenotype of T-23 therefore appeared to be different from that of the Pda^{LL} isolates. Previous studies had already established that T-2 (Pda^{SH}) has a demethylation rate about 10-fold higher than that of T-23 when compared by the preinduction assay (VanEtten and Matthews 1984). Therefore, the Pda phenotype of T-23 was given a new designation, Pda^{SM} (Short lag period, Moderate enzyme activity).

Based on the distribution of T_{50} and R_{50} values of T-23 and T-161 from repeated experiments (Table 2), quantitative values for these Pda regulatory classes were tentatively defined. The difference between the longest T-23 T_{50} and the shortest T-161 T_{50}, and the difference between the lowest T-23 R_{50} and the highest T-161 R_{50} were relatively small, suggesting a significant risk of scoring some progeny phenotypes incorrectly due to experimental variation. Therefore, both parameters were used as criteria. Isolates were considered Pda^{SM} (or Pda^{SH}) if they had a $T_{50} < 8$ hr and an $R_{50} > 30$ pmol·min^{-1}·mg^{-1} fresh weight, and Pda^{LL} if they had a $T_{50} > 8$ and $R_{50} < 30$. Only four of the progeny examined in this study were found to meet one criterion and not the other; these progeny are pointed out below. Within a tetrad, demethylation time courses commonly fell into two discrete classes so that the Pda phenotypes could be identified easily (for example, Fig. 1B). However, the actual T_{50} and R_{50} values for these classes differed somewhat between tetrads and between experiments. Examples of the range of values obtained for several crosses are given in Table 2.

Inheritance of Pda^{SM} and Pda^{LL} phenotypes. The sequence of crosses described below is summarized in Figure 2. Segregation of Pda phenotypes in these crosses is shown in Table 3.

Crosses between field isolates. Isolate T-23 was crossed

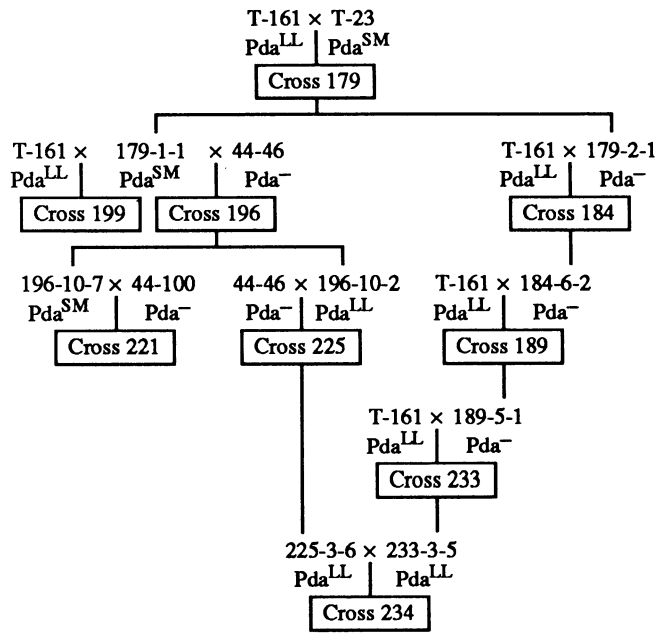

Fig. 2. Principal crosses used in this study.

Table 2. Time course of pisatin demethylation by field isolates T-23 and T-161, and by progeny derived from them

	n[a]	Phenotype	T_{50}[b]		R_{50}[c]	
Isolate						
T-23	14	Pda^{SM}	4.9 ± 1.3	(3.6–7.1)	61 ± 18	(34–93)
T-161	14	Pda^{LL}	24 ± 10	(10–44)	7 ± 5	(1–20)
Cross						
184	14	Pda^{LL}	11 ± 3	(8.3–17)	13 ± 5	(5–24)
199	24	Pda^{LL}	14 ± 4	(10–27)	14 ± 7	(3–29)
199	27	Pda^{SM}	4.2 ± 1.4	(2.0–6.8)	72 ± 19	(40–112)
221	27	Pda^{SM}	5.4 ± 1.3	(3.1–7.8)	62 ± 11	(35–76)
225	21	Pda^{LL}	28 ± 15	(10–59)	5 ± 4	(1–14)

[a] Number of times the isolate was assayed, or number of progeny from the indicated cross assayed.
[b] Time (hr) for 50% demethylation of 100 μM pisatin, ± standard deviation, range in parentheses.
[c] Rate of demethylation (pmol·min^{-1}·mg^{-1} fresh weight) at T_{50}, ± standard deviation, range in parentheses.

Table 3. Segregation of pisatin demethylation phenotypes in progeny derived from T-161 × T-23

		Tetrads					Single ascospores[b]	
		Segregation ratio						
Cross	Parents[a]	Pda^{SM}	Pda^{LL}	Pda^-	Pda^+	Number	Pda^+	Pda^-
179	T-161 × T-23 Pda^{LL} × Pda^{SM}						16	6
199	T-161 × 179-1-1 Pda^{LL} × Pda^{SM}	4	4	0		6		
		4	2	0	2[c]	2		
		2	2	4		1		
196	179-1-1 × 44-46 Pda^{SM} × Pda^-	4	4	0		1	22	19
		4	0	4		1		
221	196-10-7 × 44-100 Pda^{SM} × Pda^-	4	0	4		3	7	4
		6	0	2		1		
225	196-10-2 × 44-46 Pda^{LL} × Pda^-	0	4	4		5	8	6
		0	2	6		1		
233	T-161 × 189-5-1 Pda^{LL} × Pda^-	0	4	4		5		
		0	2	6		1		
234	233-3-5 × 225-3-6 Pda^{LL} × Pda^{LL}			0	8[d]	5	24	0

[a] Parent isolates, female parent listed first.
[b] Spores from partial tetrads, except for cross 179 also includes six random ascospores (2 Pda^+:4 Pda^-).
[c] Phenotype did not fit Pda^{LL} or Pda^{SM} category.
[d] Not assayed for Pda regulation.

to the Pda^{LL} field isolates T-61, T-161, T-201, T-203, and T-223 to test whether the regulatory phenotype of T-23 is heritable. (Crossing to T-219 is not possible because it is of the same mating type as T-23.) Of these isolates, only T-161 produced a fertile cross with T-23 (cross 179). Although many perithecia were produced in this cross, most were barren and no complete tetrads were obtained. Of the 22 ascospores isolated (random and partial tetrad, Table 3), 6 were Pda^-, suggesting that the *Pda* genes of T-23 and T-161 are nonallelic. The two partial tetrads obtained segregated 3:2 and 2:4 into the parental phenotypes Pda^{SM} and Pda^{LL}.

To overcome the problem of ascospore inviability to permit tetrad analysis of the Pda^{SM} phenotype, one of the progeny of this cross that showed the same traits as T-23 (high virulence, Pda^{SM}), isolate 179-1-1, was tested as a parent for further crosses. This approach was successful in producing much higher fertility, and T-23 itself was not used further.

When 179-1-1 was crossed to T-161 (cross 199), six of nine tetrads segregated as parental ditypes for Pda, that is 4 Pda^{SM}:4 Pda^{LL} (Table 3). Two asci contained two progeny each with intermediate Pda phenotypes (Table 3); these progeny had high values for both T_{50} and R_{50}, the most extreme case being a T_{50} of 12.3 hr with an R_{50} of 47 $pmol \cdot min^{-1} \cdot mg^{-1}$ fresh weight. These were the only isolates encountered in this study that could not be classified as Pda^{SM} or Pda^{LL} by the criteria used.

The ninth ascus of cross 199 segregated 4 Pda^+:4 Pda^- (Table 3). The existence of Pda^- progeny, even though few in number, could indicate that isolates 179-1-1 and T-161 lack common *Pda* genes. However, evidence that they possess a common *Pda* gene will be presented below.

Type and number of *Pda* genes in isolate T-161. To determine how many *Pda* genes T-161 contains, three generations of backcrosses of Pda^- progeny to T-161 were performed (crosses 184, 189, and 233, Fig. 2). Crosses 184 and 189 produced a majority of asci with only four spores, so that tetrad analysis was not possible. Overall, progeny from these crosses segregated 28 Pda^{LL}:24 Pda^- and 7 Pda^{LL}:10 Pda^-, respectively. In the third backcross (cross 233), five of six tetrads segregated 4 Pda^{LL}:4 Pda^-, and the remaining ascus was 2 Pda^+:6 Pda^-. Except for the 2:6 ascus, these results are consistent with the segregation of a single *Pda* gene.

One ascus with six or eight Pda^- progeny was also obtained in crosses 184 and 189 (data not included in ratios above). In this laboratory, asci with 2:6 or 0:8 Pda^+:Pda^- ratios have appeared at a low frequency in crosses that otherwise appear to involve only one *Pda* gene. Such asci can apparently arise from non-Mendelian processes in *N. haematococca*, as described in the Discussion. Taking this phenomenon into account, we interpret the results of crosses 184, 189, and 233 as being consistent with the existence in isolate T-161 of a single positive *Pda* allele that confers the Pda^{LL} phenotype.

Type and number of *Pda* genes in isolate 179-1-1. Isolate 179-1-1 was crossed with the Pda^- isolate 44-46 (cross 196) to continue analysis of the genetic control of the Pda^{SM} phenotype. One of the two complete tetrads obtained segregated 4 Pda^{SM}:4 Pda^{LL}:0 Pda^- (Table 3). This tetrad suggests that 179-1-1 contains positive alleles at at least two *Pda* loci, either of which can confer pisatin demethylating ability. As a test of this hypothesis, Pda^{SM} and Pda^{LL} siblings from this tetrad (ascus 196-10) were crossed with Pda^- isolates (Fig. 2).

When the Pda^{SM} isolate 196-10-7 was crossed with a Pda^- isolate (cross 221), progeny in three tetrads segregated 4 Pda^{SM}:4 Pda^-; one tetrad was 6 Pda^{SM}:2 Pda^-. The 6:2 tetrad suggests that 196-10-7 might actually contain two Pda^{SM} genes. If so, the two genes are linked, because only one such tetrad was obtained among the 15 relevant asci (those of crosses 221, 196, and 199). Alternatively, the 6:2 ratio (actually 5:2, this ascus having produced an odd number of viable ascospores) might have arisen by a mistake in ascospore isolation. A larger number of progeny from this background are needed to test whether there are really two linked Pda^{SM} genes. However the primary question in this cross, whether Pda^{SM} is genetically independent on Pda^{LL}, was resolved: all of the Pda^+ progeny (including progeny from incomplete tetrads as well as the four full tetrads just mentioned) were Pda^{SM}. The absence of Pda^{LL} progeny indicates that the Pda^{SM} phenotype is not controlled by a regulatory gene acting on an independently segregating Pda^{LL} gene. Rather, the regulatory phenotype was inherited along with the ability to demethylate pisatin at all, as was observed in previous work on the Pda^{SH} and Pda^{LL} phenotypes (Kistler and VanEtten 1984a).

When the Pda^{LL} isolate 196-10-2 was crossed with a Pda^- isolate (cross 225), five of six tetrads segregated 4 Pda^{LL}:4 Pda^-. One tetrad segregated 2 Pda^{LL}:6 Pda^-. With the exception of this 2:6 tetrad and the 4 Pda^- progeny in one tetrad of cross 199, the results are consistent with the hypothesis that 179-1-1 contains active pisatin demethylation genes at two separate loci, one conferring the Pda^{SM} phenotype and the other the Pda^{LL} phenotype.

Allelism test of Pda^{LL} genes from T-161 and 179-1-1. To test whether the Pda^{LL}-conferring gene in 179-1-1 was derived from T-161, one of the Pda^{LL} progeny from cross 225 was crossed to one from cross 233 (cross 234, Fig. 2). Of the nine tetrads isolated, five segregated 8 Pda^+:0 Pda^- and four were 6 Pda^+:0 Pda^-. The absence of Pda^- progeny strongly suggests that these Pda^{LL} genes are one and the same.

Relation of these *Pda* genes to *Pda1*, *Pda2*, and *Pda3*. Isolates carrying a single active allele of the Pda^{SM} or the Pda^{LL} gene were crossed to tester isolates possessing each of the three previously identified *Pda* genes (Kistler and VanEtten 1984a) (Table 4). The high frequencies of Pda^- progeny in crosses 212, 239, and 235 indicate that the Pda^{SM} gene is not at the *Pda1*, *Pda2*, or *Pda3* locus. We therefore designate it as a new gene, *Pda4*.

The high frequencies of Pda^- progeny in crosses 238 and 237 indicate that the Pda^{LL} gene is not at the *Pda1* or *Pda2* locus. The existence in cross 236 of only two Pda^- progeny (both from one partial tetrad) suggests that this gene is at or very closely linked to the *Pda3* locus. Because anomalous Pda^- progeny were found at low frequency in some of the other crosses in this study, as noted above, we are reluctant to propose a new locus for the Pda^{LL} gene based on these data alone. Rather, we tentatively designate the Pda^{LL} gene from T-161 as a new allele of

Pda3, *Pda3-2*. The allele in isolate 62-1, originally obtained from field isolate T-219 (Kistler and VanEtten 1984a), will be called *Pda3-1*. This would be the first instance in which *Pda* genes from different *N. haematococca* field isolates have been found to reside at the same locus.

Pda phenotypes versus tolerance to pisatin. In previous work, the pisatin tolerance of $Pda^{L.L.}$ isolates, taken as a class, was found to be intermediate between the high tolerance of Pda^{SH} isolates and the low tolerance of Pda^{-} isolates. However, the tolerance of individual isolates of these three classes overlapped somewhat when the pisatin effect was assessed only by a one-time measurement of colony radius at the time that the control culture reached the edge of the petri dish ("end-point" inhibition, I_{end}). Larger differences were found when the two components of the growth curve, the lag period and the final linear growth phase, were examined. Pisatin inhibited the growth rate in the linear phase (I_{rate}) by only about 10% for both Pda^{SH} and $Pda^{L.L.}$ isolates, but it markedly lengthened the lag period (ΔLag) of $Pda^{L.L.}$ isolates. Pda^{-} isolates, on the other hand, showed high values of I_{rate} but little or no ΔLag (Kistler and VanEtten 1984b).

Progeny from crosses 199, 221, 225, and 233 were tested to compare the effects of Pda^{SM}, $Pda^{L.L.}$, and Pda^{-} phenotypes on the growth of *N. haematococca* in the presence of 0.5 mM pisatin. All Pda^{+} progeny, whether $Pda^{L.L.}$ or Pda^{SM}, were more tolerant than Pda^{-} progeny, as measured by I_{end} or I_{rate} values (Table 5). However, no definite difference between Pda^{SM} and $Pda^{L.L.}$ progeny was observed. Results from the previous study indicated that experiment-to-experiment variation in measured pisatin tolerance could interfere with the detection of significant differences between Pda phenotypes (Kistler and VanEtten 1984b). Therefore the data for cross 199, in which $Pda^{L.L.}$ and Pda^{SM} progeny were compared in the same experiment, are the most reliable. There was a trend for Pda^{SM} progeny to have shorter lag periods and lower end-point inhibitions than $Pda^{L.L.}$ isolates, but the differences within cross 199 were not statistically significant.

Pda phenotypes versus virulence on pea. Figure 3 shows the relationship between T_{50} values and virulence in the progeny of cross 199. Figure 4 shows the same relationship for the Pda^{+} progeny of crosses 221 and 225. All highly virulent isolates had T_{50} values less than 8 hr, that is the Pda^{SM} phenotype. When the R_{50} values, the other parameter used to distinguish the Pda^{SM} and $Pda^{L.L.}$ phenotypes, and virulence values for the same progeny were compared, all highly virulent isolates had R_{50} values greater than 30 $pmol \cdot min^{-1} \cdot mg^{-1}$ fresh weight, that is the R_{50} of a Pda^{SM} phenotype (data not shown). Pda^{-} progeny from all crosses were also tested for virulence. There was no significant difference in virulence between Pda^{-} and $Pda^{L.L.}$ progeny: neither were even moderately virulent (data not shown). These results suggest that in infected tissue the rate of pisatin demethylation by Pda^{SM} isolates, but not $Pda^{L.L.}$ isolates, is rapid enough to permit expression of high virulence.

Comparison to the Pda^{SH} phenotype. With regard to the relationship between the Pda^{SM} and Pda^{SH} phenotypes, the results presented above establish only that *Pda4* and *Pda1* are at different loci. Further interesting questions include whether the demethylase phenotypes conferred by these two genes are heritably distinguishable and whether they differ in their effects on virulence. Although a thorough study of these questions is beyond the scope of this work, some of the progeny obtained in cross 212 made it possible to obtain some relevant information.

This cross, between the Pda^{SM} isolate 196-10-7 and the Pda^{SH} isolate 77-13-6, gave rise to two asci that segregated 8 Pda^{+}:0 Pda^{-} (Table 4). The simple interpretation is that these asci are parental ditypes, 4 Pda^{SM}:4 Pda^{SH}. To test this hypothesis, the progeny were assayed for their rate of demethylation after a 6-hr preinduction with pisatin, conditions which previous work (VanEtten and Matthews 1984; Kistler and VanEtten 1984a) had suggested might

Table 4. Allelism tests of the Pda^{SM} and $Pda^{L.L.}$ genes from T-23 and T-161 with *Pda1*, *Pda2*, and *Pda3*

					Single ascospores			
		Tetrads			Partial tetrads		Random ascospores	
Cross	Parents[a]	Pda^{+}	Pda^{-}	Number	Pda^{+}	Pda^{-}	Pda^{+}	Pda^{-}
212	196-10-7 × 77-13-6	8	0	2	4	1		
	Pda^{SM} × *Pda1*	4	4	4				
239	221-5-4 × 99-18				3	2	39	9
	Pda^{SM} × *Pda2*							
235	62-1 × 221-3-1	8	0	1	8	5	9	0
	Pda3-1 × Pda^{SM}	4	4	1				
238	225-3-6 × 77-13-6	8	0	1	20	10		
	$Pda^{L.L.}$ × *Pda1*	6	2	3				
237	225-1-4 × 96-17						32	11
	$Pda^{L.L.}$ × *Pda2*							
236	233-3-5 × 62-1	8	0	5	7	2		
	$Pda^{L.L.}$ × *Pda3-1*							

[a] Parent isolates, female parent listed first.

Table 5. Inhibition by pisatin of the growth of progeny from crosses 199, 221, 225, and 233

Cross	Pda phenotype	n[a]	I_{end} (%)[b]	I_{rate} (%)[c]	ΔLag (hr)[d]
199	Pda^{SM}	5	16 ± 3[e]	9 ± 3[e]	12 ± 2[e]
	$Pda^{L.L.}$	6	20 ± 3[e]	10 ± 4[e]	17 ± 6[e]
221	Pda^{SM}	25	14 ± 2[e]	8 ± 3[e]	9 ± 4[e]
	Pda^{-}	8	37 ± 3[f]	35 ± 3[f]	5 ± 3[f]
225	$Pda^{L.L.}$	20	15 ± 3[e]	8 ± 4[e]	11 ± 4[e]
	Pda^{-}	5	40 ± 4[f]	37 ± 3[f]	3 ± 5[f]
233	$Pda^{L.L.}$	10	25 ± 5[e]	10 ± 3[e]	22 ± 7[e]
	Pda^{-}	4	45 ± 1[f]	42 ± 5[f]	4 ± 7[f]

[a] Number of progeny assayed.

[b] I_{end} = end-point inhibition, the percent decrease in radius of the pisatin-treated culture relative to the control culture at the time the latter reached 25 to 30 mm (6 to 7 days).

[c] I_{rate} = inhibition of the final, linear growth rate of the pisatin-treated culture relative to the control, measured by linear regression of observations beginning at 72 hr.

[d] ΔLag = increase in length of the lag phase of growth due to pisatin treatment, measured by extrapolating the linear phases of the pisatin-treated and control cultures back to their abscissa-intercepts.

[e,f] For each parameter, means within a cross followed by the same letter are not significantly different at the 0.05 level, based on t tests with pooled estimates of variance.

differentiate these two phenotypes. Pda^{SH} progeny had shown rates of 31 to 138 pmol·min^{-1}·mg^{-1} fresh weight, whereas Pda^{LL} progeny's rates were less than 11 (Kistler and VanEtten 1984a). Using 30 pmol·min^{-1}·mg^{-1} as the boundary value for distinguishing the Pda^{SM} and Pda^{SH} phenotypes, these asci from cross 212 segregated 4 Pda^{SM}:4 Pda^{SH} (actually 4:3 and 3:4), and in both asci the segregation of Pda was consistent with that of the *MAT* and Fem markers. We therefore classify the progeny that demethylated at rates less than 30 pmol·min^{-1}·mg^{-1} under these conditions as Pda^{SM} (Fig. 5).

The Pda^{SH} progeny in each of these tetrads were higher in virulence than their Pda^{SM} siblings (Fig. 5). This correlation is based on only eight independent meiotic products, because the progeny in Figure 5 include mitotic twins. Also, some Pda^{SM} progeny from other crosses (Figs. 3 and 4) were more virulent than those from this cross. Nonetheless, the results of cross 212 suggest that when Pda^{SH} and Pda^{SM} phenotypes are segregating in a common genetic background, the virulence of an isolate can be correlated with its rate of pisatin demethylation.

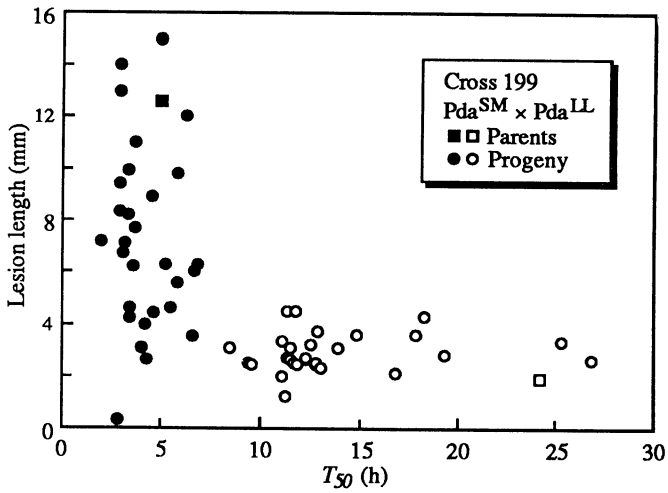

Fig. 3. Time required for half-demethylation of 100 μM pisatin (T_{50}) versus virulence of progeny from cross 199. Filled symbols denote Pda^{SM} isolates and open symbols Pda^{LL} isolates.

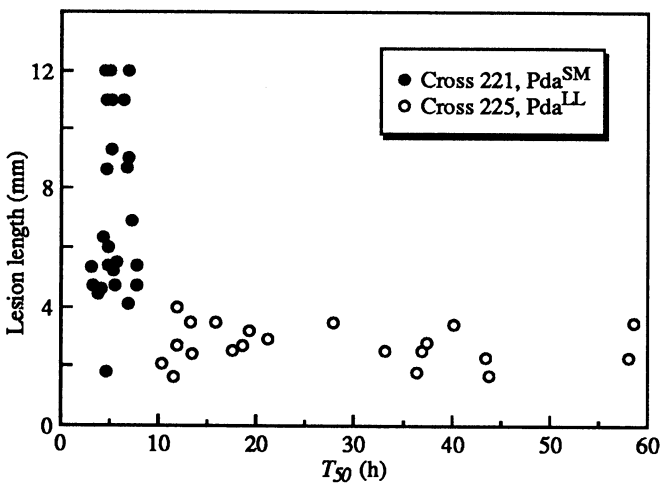

Fig. 4. Time required for half-demethylation of 100 μM pisatin versus virulence of Pda^{+} progeny from crosses 221 and 225.

It should be noted that Pda^{SM} isolates' R_{50} values, measured by the time course assay, were always greater than 30 pmol·min^{-1}·mg^{-1} (Table 2). Why rates measured after preinduction (Fig. 5) should be lower than R_{50} values is not clear. For isolate T-23, which was tested at least six times by each method, the preinduction assay gave values from 7 to 67 pmol·min^{-1}·mg^{-1} fresh weight (VanEtten and Matthews 1984) while the time course assay showed R_{50}s of 34 to 93 (Table 2).

DISCUSSION

By measuring the time course of pisatin demethylation over a 24-hr period, it was possible to distinguish consistently between the Pda phenotypes of field isolates T-23 (Pda^{SM}) and T-161 (Pda^{LL}). Subsequent genetic analyses indicated that these two phenotypes are encoded by different genes. Allelism tests with previously identified *Pda* genes indicated that the gene responsible for the Pda^{SM} phenotype is at a new locus, *Pda4*, whereas the Pda^{LL} gene may represent another allele (*Pda3-2*) at a known locus. In both cases, the regulatory phenotype was coinherited with the gene for demethylation itself. To date we have not identified any separable genes that control the level of expression of a *Pda* gene.

The results of this and previous studies (Kistler and VanEtten 1984b; VanEtten and Matthews 1984) indicate that it is necessary to use two parameters, T_{50} (or R_{50}) and demethylation rate after preinduction, to distinguish all the phenotypes of the known *Pda* genes. Isolates that show high rates in the preinduction assay are Pda^{SH}; those that do not may be Pda^{SM} or Pda^{LL}. The time course assay distinguishes Pda^{LL} isolates from the other two kinds.

A remarkable feature of the Pda^{LL} phenotype is the long lag before appreciable demethylation occurs (Fig. 1). In addition, the maximum rate of demethylation during the time course (R_{50}) is very low for Pda^{LL} isolates. However it is not as low as the values we previously reported for such isolates, 0.2 to 1 pmol·min^{-1}·mg^{-1} fresh weight (Kistler and VanEtten 1984a). Those values were rates calculated from a few measurements of pisatin concentrations over

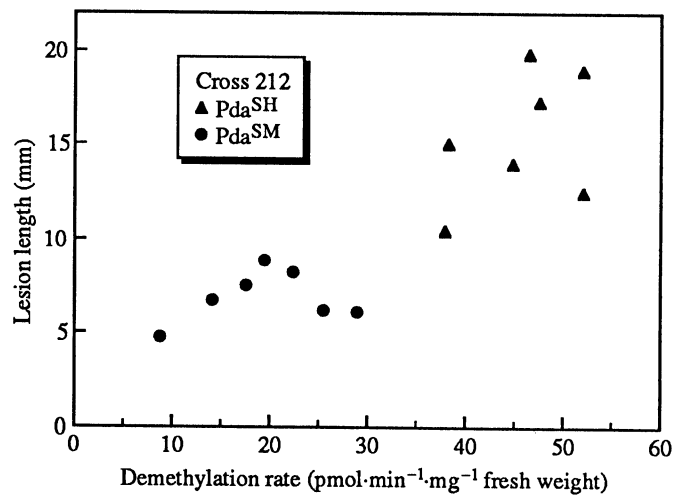

Fig. 5. Demethylation rate versus virulence of progeny from asci 1 and 5 of cross 212. Demethylation was measured over a 25-min interval after a 6-hr preinduction with 100 μM pisatin.

a 50- to 70-hr interval; for lack of sufficiently detailed time course experiments, the rates were assumed to be approximately constant over this period. At that time we also referred to this phenotype as "Pda^n," for "noninducible." Now that the nonlinearity of the demethylation time course is apparent, it seems likely that some kind of induction process is occurring, though why it is so much slower than for Pda^{SM} and Pda^{SH} isolates is currently unknown.

In several Pda^{LL} × Pda$^-$ crosses (184, 189, 233, and 225), one tetrad contained more than four Pda$^-$ progeny. Such tetrads have been found occasionally by nearly everyone who has studied the *Pda* genes of *N. haematococca* (Tegtmeier and VanEtten 1982b; Kistler and VanEtten 1984a; Miao and VanEtten 1988; P. Matthews and D. Matthews, unpublished). One interpretation would be that the Pda$^-$ parent lacks two genes, both of which are required for pisatin demethylation; the two genes should be linked, because the recombinant 2:6 and 0:8 Pda$^+$:Pda$^-$ tetrads are relatively infrequent. A second interpretation would be that gene conversion is occurring. Both of these hypotheses predict that 0:8 tetrads should be much less frequent than 2:6 tetrads, which in general is not the case.

As a direct test of the first hypothesis, all possible combinations of Pda$^-$ field isolates and some of the Pda$^-$ progeny from crosses that had produced excess Pda$^-$ progeny were intercrossed (Cowling and VanEtten 1986). The progeny were screened for the presence of Pda$^+$ recombinants, whose existence would imply that the Pda$^-$ phenotype can result from a deficiency in any one of two or more functional classes of genes. No Pda$^+$ recombinants were found, suggesting that all Pda$^-$ isolates share the same biochemical deficiency.

Pisatin demethylation is catalyzed by a microsomal monooxygenase composed of two proteins, NADPH-cytochrome P-450 reductase and cytochrome P-450 (Desjardins *et al.* 1984; Desjardins and VanEtten 1986; Matthews and VanEtten 1983). The reductase has been purified and appears to be the same in Pda$^+$ and Pda$^-$ isolates of *N. haematococca* (Scala *et al.* 1988). Thus it seems likely that the *Pda* genes are a family of structural genes for cytochrome P-450 isozymes, and recent results further support this suggestion. A gene that confers on *Aspergillus nidulans* the ability to demethylate pisatin has been cloned from *N. haematococca* field isolate T-9 (Weltring *et al.* 1988). This gene shows sequence homology to known cytochrome P-450 genes (A. Maloney, unpublished) and hybridizes to DNA from all Pda$^+$ isolates of *N. haematococca* examined thus far.

When two 0:8 tetrads from a Pda^{LL} × Pda$^-$ cross were probed with this cloned *Pda* gene, no homologous DNA was found in them (Miao and VanEtten 1988). This finding does not support the two-required-genes hypothesis described above, but rather indicates a non-Mendelian phenomenon involving physical loss of the *Pda* DNA during meiosis.

The cloned *Pda* gene has also provided information about the significance of ascus 199-8, the 2 Pda^{SM}:2 Pda^{LL}:4 Pda$^-$ ascus from cross 199, T-161 × 179-1-1 (Table 3). Whereas the absence of Pda$^-$ progeny in cross 234 (Fig. 2) indicates that the Pda^{LL} gene in isolate 179-1-1 is at the same locus as the one in T-161, this conclusion is contradicted by the existence of Pda$^-$ progeny in ascus 199-8. However it is supported by recent studies using the *Pda* clone to probe genomic DNA from isolates of interest. DNA from isolate 179-1-1 contains two restriction fragments that hybridize to the cloned gene. One of these bands is also found in isolates T-23 and 196-10-7 but not T-161, and presumably corresponds to *Pda4*; the other is present in isolate T-161 but not T-23 or 196-10-7, and therefore appears to represent *Pda3-2* (unpublished results).

Because both T-161 and 179-1-1 are thus presumed to contain *Pda3-2*, all four meiotic products of ascus 199-8 should have inherited it, but in fact at least two of them did not (Table 3). In this case, gene conversion cannot account for the result, because both parents possess the same positive allele of *Pda3*. The behavior of *Pda4* in this ascus was also irregular: two of the meiotic products should have been Pda^{SM} but only one was. Remarkably, in this ascus two unlinked genes were lost in the course of a single meiosis.

Currently we do not know the mechanism underlying this occasional failure of transmission of *Pda* genes to ascospore progeny. Until this phenomenon is better understood, we must be conservative in drawing conclusions from the presence of only a few Pda$^-$ progeny in a cross. For this reason the fact that two of the progeny of cross 236 were Pda$^-$ (Table 4) has been deemed insufficient to warrant proposing that the *Pda* gene derived from isolate T-161 is at a locus separate from *Pda3*.

Previous work had indicated that isolate T-161 actually possesses two *Pda* genes. Of eight tetrads obtained from a cross between T-161 and the Pda$^-$ isolate T-110, two had segregated 6:2 and one 8:0 Pda$^+$:Pda$^-$ (Tegtmeier and VanEtten 1982b). This previous result is not easily reconciled with the current findings. First, none of the six tetrads examined in cross 233 contained more than four Pda$^+$ progeny. Further, to explain the 2:6 and 0:8 (Pda$^+$:Pda$^-$) tetrads in crosses 233, 184, and 189 would require the simultaneous loss of both *Pda* genes. An alternate hypothesis is that isolate T-161 lost one of these genes in the course of subculture and storage since the earlier study. Future work may help to resolve this question. Fortunately, it does not appear to complicate the analysis of the Pda^{SM} gene of isolate T-23, which was the focus of this study.

The non-Mendelian inheritance pattern that occasionally occurs in *N. haematococca* forces us to be cautious in our interpretation of the genetic control of the Pda phenotypes. Future studies using cloned *Pda* genes should resolve the uncertainties and allow us to determine not only the number of genes controlling demethylation rates, but also whether the phenotypic differences are primarily due to different structural genes for cytochrome P-450 isozymes with different activities toward pisatin, or due to *trans* or *cis* acting factors affecting the expression of similar structural genes.

Regardless of how many genes are eventually shown to control the pisatin demethylase phenotypes, the consistent association of the different phenotypes with different levels of virulence is persuasive evidence that the level of activity of this phytoalexin detoxifying enzyme directly affects the virulence of this fungus. Among hundreds of ascospore progeny representing numerous recombinational events, all Pda$^-$ and Pda^{LL} ascospore isolates have been essentially

nonpathogenic on pea (Tegtmeier and VanEtten 1982b; Kistler and VanEtten 1984b; this study). This correlation was observed even in tetrads showing non-Mendelian inheritance of *Pda* genes. No combination of genes has been obtained that can replace the dependence of this fungus on a gene conferring the Pda^{SH} or Pda^{SM} phenotype for pathogenicity on pea. The most straightforward conclusion is that adequate production of this enzyme is required for pathogenicity, and the genes conferring this property are therefore pathogenicity genes.

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