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Trichothecene biosynthesis in *Gibberella pulicaris:* inheritance of C-8 hydroxylation

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SUMMARY

Naturally occurring strains of *Gibberella pulicaris (Fusarium sambucinum)* produce different kinds and levels of trichothecene toxins. Progeny from crosses between strains which produce trichothecenes with an oxygen-containing group at C-8 (C8+) and those that do not (C8−) can segregate in a 1:1 ratio for this trait. These results define a genetic locus, which we have designated *Tox*1. The segregation patterns observed for progeny obtained from crosses between high-toxin producers and low-toxin producers indicate that the level of toxin production is determined by several loci. One gene which controls quantitative aspects of toxin production segregates independently of both the *Tox*1 locus and another locus which controls toxin levels. These results suggest that multiple, unlinked nuclear loci are involved in the control of trichothecene biosynthesis.

INTRODUCTION

The trichothecenes are a group of sesquiterpenoid mycotoxins produced by various species of *Fusarium* and have been implicated in mycotoxicoses of man and animals [13]. Genetic analysis of trichothecene biosynthesis has recently become possible due to the ability to produce in the laboratory the sexual stage of a trichothecene-producing species, *Gibberella pulicaris* (Fries.) Sacc. (anamorph, *Fusarium sambucinum* (Funkel)) [1]. It has been shown for this species that traits for sex, pigmentation and mating type segregate in a normal Mendelian fashion. High fertility and high ascospore viability facilitated both random ascospore and tetrad analysis [1,3–6]. In a previous report, we demonstrated that natural variants of *G. pulicaris* can be used to investigate the genetic control of trichothecene production [1]. Those studies have been extended in the present work in which the main objective was to identify genes affecting trichothecene biosynthesis.

In this report, we define a locus, designated *Tox*1, which shows 1:1 segregation for a biochemical step

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Table 1

Phenotypes of *G. pulicaris* strains used in this study

Strain No.	Trichothecene production[a]		Hydroxylation at C-8	Red pigmentation	Mating type	Source
	level	major toxins				
R-583	low	DAS	C8−	Red+	Mat1	P.E. Nelson
R-5390	high	DAS	C8−	Red−	Mat1	P.E. Nelson
R-5455[b]	very low	DAS+8-AcNeo	C8+	Red+	Mat2	P.E. Nelson
R-6380	high	DAS	C8−	Red−	Mat1	P.E. Nelson
R-7843	low	8-AcNeo	C8+	Red+	Mat2	P.E. Nelson
NRRL-13500	high	DAS	C8−	Red−	Mat1	R. Caldwell

[a] As determined after 7 days growth in YEPD-5G liquid shake cultures incubated at 28°C and 200 rpm. High ≥ 70 μM; low = 10–30 μM; very low < 2 μM. DAS = diacetoxyscirpenol; 8-AcNeo = 8-acetylneosolaniol.

[b] This strain is derived from *F. roseum* strain V-18 which has been previously reported to produce DAS and 8-AcNeo [7]. Although it has also been reported to make Neo and T-2 toxin [7,10], our isolate does not accumulate these two trichothecenes under the growth conditions used in this study.

(hydroxylation at C-8) in the trichothecene biosynthetic pathway. The results from several crosses also indicate the occurrence of at least four other nuclear genes which affect trichothecene production.

MATERIALS AND METHODS

Cultures

Strains of *G. pulicaris* used in this study (Table 1) were kindly supplied by P.E. Nelson from the *F. sambucinum* collection at the Fusarium Research Center (FRC), The Pennsylvania State University (strains with the prefix R are from this collection) and by R. Caldwell, University of Wisconsin-Madison. All strains were reisolated from single conidia prior to use. Cultures were routinely grown on V-8 agar medium [18] slants or plates, on an alternating 12 h, 25°C light/12 h, 20°C dark schedule. For long-term storage, strains were maintained on V-8 agar slants at 4°C, as conidial suspensions in 10–15% (v/v) glycerol at −90°C, and as lyophilized conidial suspensions in the Agricultural Research Service Collection, Peoria, IL. For all assays, fresh transfers of the strains were obtained from stock cultures stored at 4°C. Cultures were scored for production of red pigment following 10–14 days growth on potato-dextrose agar [11].

Genetic crosses: random ascospore and tetrad analysis

Crosses were made between strains of opposite mating types, designated Mat1 and Mat2; crosses between strains of the same mating type are infertile. Techniques for crossing and random ascopore isolations have been described previously [1]. Mulberry twigs on water agar slants were inoculated with the female parent and grown for approximately 1 month before the addition of conidia from the male parent. After fertilization, crosses were incubated at 15°C until perithecia were mature. Crosses used in this study demonstrated good fertility and a high rate of ascospore germination (Table 2).

For tetrad dissections, mature intact perithecia were placed on a Buchner funnel lined with LABX 123 (Berkshire Paper Co., Great Barrington, MA) and washed under vacuum with sterile water to remove mycelial debris and conidia. The washed perithecia were ruptured in a pool of 0.1 M KCl on a plate containing tetrad agar (glucose 2 g, peptone 1 g, agar 11 g, M-2 salts solution 2 ml and water

220 ml). The M-2 salts solution was KH_2PO_4 10% (v/v) and $MgSO_4 \cdot 7H_2O$ 5% (v/v). Tetrad dissections were conducted under a Wild stereomicroscope at 200×. Ascospores from an individual ascus were separated by hand using an eyelash attached to a wooden rod and each ascospore was immediately transferred to an individual V-8 agar slant; the slants were incubated for 8–10 days and stored at 4°C.

Ascospore progeny were catalogued by a series of three numbers: the cross number, the tetrad number (or *R* for random ascospore progeny) and the ascospore number (1–8 for tetrads and consecutive numbers for random ascospore progeny) [20].

Analysis of toxin production

Trichothecene production was measured in liquid shake cultures incubated for 7 days at 28°C on a rotary shaker operating at 200 rpm. These cultures were inoculated at a starting density of 1×10^5 conidia per ml YEPD-5G medium (yeast extract (Difco) 0.1%; peptone (Difco) 0.1%, glucose 5.0%) [19] with conidia washed from strains grown on V-8 agar plates for 7–10 days. The liquid cultures were grown in Erlenmeyer flasks (50 or 300 ml) in a volume of medium equal to one-half the volume of the flask.

Each whole culture was extracted twice with an equal volume of ethyl acetate. The two extracts were pooled and passed through a charcoal column (Romer Labs, Inc., Washington, MO) that was prewashed with 20 ml ethyl acetate. The eluent was combined with a subsequent wash (20 ml ethyl acetate) and evaporated to dryness on a rotary evaporator. The residue was resuspended in 1 ml of ethyl acetate. An aliquot (50–100 μl) was evaporated to dryness at 80°C under nitrogen, reacted with 100 μl trimethyl silating reagent (Tri-Sil/TBT, Pierce Chemical Company, Rockford, IL) for 1 h at 80°C and brought to 1 ml with hexane. Measurements were made by flame ionization detection on either a Spectro-Physics (model SP 7110) or a Hewlett Packard (model 5890) gas chromatograph. Purified samples of T-2 toxin, diacetoxyscirpenol (DAS) and neosolaniol (Neo) were purchased from Sigma (St. Louis, MO); 8-acetylneosolaniol (8-AcNeo)

Table 2

Segregation of C-8 hydroxylation in five crosses between different C8+ and C8− wild type strains

Cross	Parents	Phenotype	Random ascospore progeny		Ascospore germination[a]
			C8+	C8−	(%)
279	R-5455 R-5390	(C8+) (C8−)	6	14	100
287	R-5455 R-6380	(C8+) (C8−)	8	7	75
297	R-5455 R-583	(C8+) (C8−)	11	29	92
712	R-5455 NRRL-13500	(C8+) (C8−)	10	10	89
1104	R-7843 R-6380	(C8+) (C8−)	10	10	95

[a] The ascospore germination percent refers to the total number of random ascospore progeny picked from a given cross: 54, 80, 48, 54, and 54 from crosses 279, 287, 297, 712, and 1104, respectively. For the study of C-8 hydroxylation, a subgroup of ascospores was randomly selected from each cross.

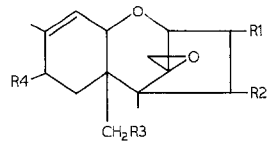
was kindly supplied by Richard Cole (USDA, National Peanut Research Laboratory, Dawson, GA). Structures for DAS, Neo and 8-AcNeo are shown in Fig. 1.

RESULTS

Random ascospore analysis

Crosses were made between strains of *G. pulicaris* that produced different levels and types of trichothecenes (Table 1). The high-toxin-producing strains, R-5390, R-6380 and NRRL-13500, and the low-toxin-producing strain, R-583, produced primarily DAS. The low-toxin-producing strain, R-7843, produced primarily 8-AcNeo, and the very low-toxin-producing strain, R-5455, produced both DAS and 8-AcNeo. DAS differs from 8-AcNeo in that it contains a hydrogen at C-8 instead of an acetate moiety (Fig. 1). The acetate moiety results from the subsequent acetylation of a hydroxyl group. Thus strains R-5390, R-6380, and NRRL-13500 do not add a hydroxyl group to C-8 (C8−) while strains R-5455 and R-7843 do (C8+).

In each cross made between a C8+ strain and a C8− strain, the progeny showed segregation for hydroxylation at C-8 (Table 2). The five crosses involved two C8+ parents (R-5455 and R-7843) and four C8− parents (R-6380, NRRL-13500, R-5390, and R-538). Three of the crosses (R-5455, C8+ × R-6380, C8−; R-7843, C8+ × R-6380, C8−; and R-5455, C8+ × NRRL-13500, C8−) displayed a 1:1 segregation for C-8 hydroxylation. These results suggest that the C8–strains R-6380 and NRRL-13500 each carry a single gene difference which is responsible for their C8− phenotype. Crosses R-5455 (C8+) × R-5390 (C8−) and R-5455 (C8+) × R-538 (C8−) showed a 1:3 segregation ratio for C8+:C8− which suggests that strains R-5390 and R-583 are each carrying two, unlinked loci which can independently produce the C8− phenotype.



Trichothecene	R1	R2	R3	R4
DAS	OH	OAc	OAc	H
Neo	OH	OAc	OAc	OH
8-AcNeo	OH	OAc	OAc	OAc

Fig. 1. Structure of trichothecenes DAS, Neo and 8-AcNeo.

Toxin analysis data for cross 1104 between R-7843, a C8+, low-toxin producer, and R-6380, a

Table 3

Random ascospore analysis of inheritance of trichothecene production in cross 1104 between a C8+, low-toxin producer (R-7843) and a C8−, high-toxin producer (R-6380)

Strain	Trichothecene production (μM)				
	DAS	8-AcNeo	Neo	C8	total
Parents					
R-6380	76	0	0	−	76[a]
R-7843	0	17	0	+	17[a]
Progeny spore No.					
1104-R-04	9	0	0	−	9
1104-R-14	17	0	0	−	17
1104-R-19	35	0	0	−	35
1104-R-03	37	0	0	−	37
1104-R-17	48	0	0	−	48
1104-R-01	61	0	0	−	61
1104-R-10	70	0	0	−	70
1104-R-13	73	0	0	−	73
1104-R-07	84	0	0	−	84
1104-R-11	96	0	0	−	96
1104-R-12	5	5	0	+	10
1104-R-09	10	6	0	+	16
1104-R-06	0	19	0	+	19
1104-R-08	32	4	0	+	36
1104-R-05	9	28	5	+	42
1104-R-15	22	46	0	+	68
1104-R-16	37	57	16	+	110
1104-R-20	39	87	25	+	151
1104-R-02	15	121	17	+	153
1104-R-18	0	114	60	+	174

[a] The ranges of variation for R-6380 and R-7843 are 174 ± 92 μM and 16 ± 13 μM, respectively, and are based on four separate experiments conducted over a period of several months. The toxin data presented in this table for these two strains are those obtained when they were grown and assayed simultaneously with the 20 ascospore progeny.

C8−, high-toxin producer, are presented in Table 3. The results illustrate that a complex pattern of toxin production is superimposed on the simple 1:1 segregation observed for C-8 hydroxylation. This complexity indicates that there are additional genes segregating which affect trichothecene production. For example, one-half of the 8-AcNeo-producing (i.e., C8+) progeny also make Neo, which is identical to 8-AcNeo except that the C-8 moiety is OH instead of OAc. This pattern of inheritance is consistent with the presence of a gene, unlinked to the 'C-8' gene, which is responsible for the subsequent acetylation state of the C-8 oxygenated moiety. Finally, the rate of production of recombinant progeny which are either C8+, high-toxin producers or C8−, low-toxin producers indicates that at least one gene which determines the level of toxin production is segregating in this cross and that this gene is not tightly linked to the gene responsible for C-8 hydroxylation. The lack of a simple 1:1 Mendelian ratio for high-toxin production:low-toxin production (>70 μM:10–30 μM) may further suggest that two genes are segregating which affect the level of toxin production. However, it should be noted that these data represent a small number of random progeny which are derived from two parents that both show some variability in their level of toxin production (Table 3). Accordingly, questions regarding the inheritance of genes which control the amount of toxin produced could be better addressed by conducting tetrad analysis and by using a cross where the two parents display a greater difference between their levels of toxin production. This was done in the following experiment.

Tetrad analysis

The pattern of inheritance of genes affecting toxin production was examined in greater detail by selecting cross 287 between the very low-toxin producer, R-5455 (Red+ (red pigmentation), C8+), and the high-toxin producer, R-6380 (Red−, C8−), for tetrad analysis. The data presented in Table 4 show that, in each case, the seven or eight ascospore progeny derived from a single ascus could be grouped into four pairs based on pigmentation, C-8 hydroxylation, and toxin levels. The typical range of variability in measuring toxin production is demonstrated by the data shown in Table 4. Since each spore pair is derived from the same meiotic product, their analysis is equivalent to conducting each assay in duplicate. While there is some variability, in no case did we observe any overlap between toxin levels determined for intermediate-toxin (>10 and <70 μM) producers and those determined for either very high-toxin (>150 μM) or extremely low-toxin (<2 μM) producers. Representative gas chromatograms of extracts from strains R-6380 and R-5455, and of eight tetrad progeny from a single ascus from a cross between these two strains, are shown in Fig. 2. These data are presented to illustrate the similarity of trichothecene toxin production profiles between ascospore twin strains from the same tetrad.

The results shown in Table 4 confirm the 1:1 segregation for C8+:C8−. The gene which is responsible for C-8 hydroxylation in this cross was designated *Tox*1. The data are also consistent with the segregation of at least two loci which affect the level of trichothecene production. The recovery of tetratype tetrads (15, 11, and 3; Table 4 and Fig. 2) with respect to level of toxin production is possible only if two or more loci are involved.

The high percentage of recombinant progeny that are intermediate toxin producers and either *Tox*1$^+$ or *Tox*1$^-$ suggests that at least one of the loci which affect toxin levels is unlinked to *Tox*1. In addition, the *Red* locus and the *Tox*1 locus appear to be unlinked. The segregation pattern of *Red*$^-$*Tox*1$^-$:*Red*$^-$ *Tox*1$^+$:*Red*$^+$ *Tox*1$^-$: *Red*$^+$ *Tox*1$^+$ has an overall ratio of 5:6:5:4. This near 1:1:1:1 Mendelian ratio is indicative of two independently segregating loci.

DISCUSSION

Genetic data pertaining to trichothecene production have been obtained from this study and a previous study [1], both of which employed sexual crosses between *G. pulicaris* strains that accumulate different kinds and/or amounts of trichothecenes. All crosses to date indicate that multiple, unlinked

Table 4

Tetrad analysis of inheritance of pigmentation and trichothecene production in cross 287 between a Red+, C8+, very low-toxin producer (R-5455) and a Red−, C8−, high-toxin producer (R-6380)

Progeny strain[a]	Red pigmentation[b]	C-8 hydroxylation	Trichothecene production (μM)[c]			
			DAS	8-AcNeo	Neo	total
287-15-2	−	−	177.4	0	0	177.4
287-15-5	−	−	163.5	0	0	163.5
287-15-7	+	−	12.8	0	0	12.8
287-15-8	+	−	11.2	0	0	11.2
287-15-6	−	+	12.8	6.4	0	19.2
287-15-1	−	+	8.2	4.2	0	12.4
287-15-4	+	+	0.5	0.9	0	1.4
287-15-3	+	+	0.8	0.9	0	1.7
287-11-1	(+)	−	173.3	0	0	173.3
287-11-4	(+)	−	238.3	0	0	238.3
287-11-7	−	−	16.9	0	0	16.9
287-11-8	−	−	42.6	0	0	42.6
287-11-2	−	+	10.9	6.4	0	17.3
287-11-3	−	+	10.1	5.4	0	15.5
287-11-5	+	NS	NS[d]	NS	0	NS
287-11-6	+	+	Tr[e]	Tr	0	Tr
287-5-6	+	−	57.0	0	0	57.0
287-5-7	+	−	34.4	0	0	34.4
287-5-2	(+)	−	11.5	0	0	11.5
287-5-8	(+)	−	13.4	0	0	13.4
287-5-3	−	+	59.2	62.2	13.9	135.3
287-5-4	−	+	36.3	42.5	8.9	87.7
287-5-1	−	+	4.1	Tr	NS	4.1
287-5-5	−	+	9.3	Tr	NS	9.3
287-3-3	−	−	123.4	0	0	123.4
287-3-1	+	−	12.0	0	0	12.0
287-3-6	+	−	7.4	0	0	7.4
287-3-7	−	+	24.8	28.9	0	53.7
287-3-4	−	+	4.4	2.9	0	7.3
287-3-2	−	NS	4.4	NS	0	4.4
287-3-5	−	NS	3.5	NS	0	3.5
287-1-7	−	−	70.4	0	0	70.4
287-1-8	−	−	108.5	0	0	108.5
287-1-3	−	−	33.0	0	0	33.0
287-1-5	−	−	31.2	0	0	31.2
287-1-1	+	+	47.6	32.6	6.8	87.0
287-1-4	+	+	33.9	32.6	3.9	70.4
287-1-2	+	+	18.1	6.9	NS	25.0

[a] The first portion of the strain number indicates the cross number, the second portion indicates the ascus number and the third portion indicates the ascospore.

[b] + = dark red, (+) = light red.

[c] Trichothecene production from 7 day liquid shake cultures prepared as described in text.

[d] NS = not scoreable.

[e] Tr = trace amounts (< 1 μg/ml).

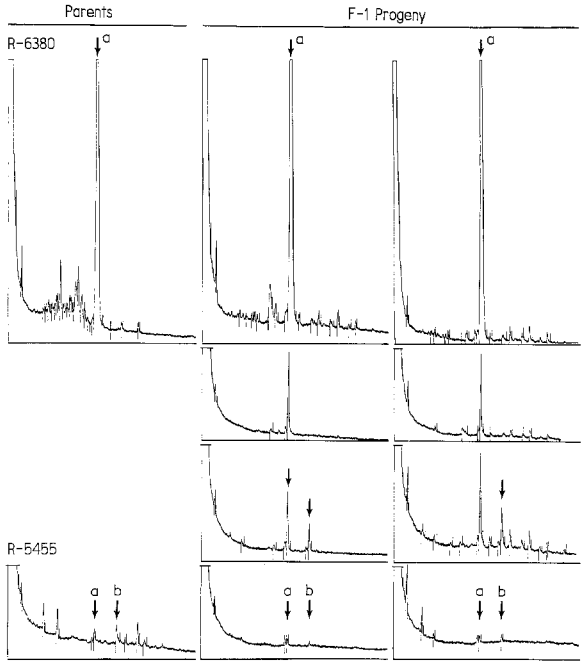


Fig. 2. GC tracing from the analysis of trichothecene production in parental and progeny strains. This figure compares the patterns of toxin production in the parents and the resulting progeny from a 'tetratype' tetrad where the true parental types appear. This figure also demonstrates (1) the ease with which twin spores could be paired on the basis of toxin production, (2) the distinct appearance of recombinant C8+ and C8− progeny that produce intermediate levels of toxins, and (3) the degree of reproducibility observed for 'duplicate' samples as represented by the twin pairs. The F1 progeny pairs shown in the set of panels to the right are in descending order: 287-15-2 and 287-15-5; 287-15-7 and 287-15-8; 287-15-6 and 287-15-1; and 287-15-4 and 287-15-3. 'a' denotes the DAS peaks and 'b' denotes the 8-AcNeo peaks.

chromosomal loci are involved in the control of toxin production. In this paper, we have documented the segregation of at least two genes which affect the quantity of trichothecene production. We have also observed the segregation of genes which affect the type of trichothecenes produced. One of these genes, *Tox*1, controls C-8 hydroxylation. Formation of C-8 oxygenated trichothecenes was inherited as a single gene difference in crosses involving the C8− strain R-6380 and the C8+ strains R-5455 and R-7843. Therefore, by our conventions, R-6380 is *Tox*1$^-$ and R-5455 and R-7843 are both *Tox*1$^+$. When three other C8− strains (NRRL-13500, R-583 and R-5390) were crossed to R-5455, only NRRL-13500 displayed a 1:1 segregation pattern for C-8 hydroxylation; both R-583 and R-5390 displayed a 1:3 segregation pattern for C8+:C8− progeny. These results indicated a single gene difference in NRRL-13500 and double gene differences in R-583 and R-5390 where the two loci are unlinked. The allelic relationships among the loci determining the C8− phenotypes in strains R-6380, R-583, R-5390 and NRRL-13500 remain to be determined. Also, in all crosses in this study, every C8+ progeny made some 8-AcNeo. Therefore a gene for acetylation of the C-8 hydroxyl group is either present in all the C8− strains or it is linked to the C8+ allele(s). In addition, some progeny produced Neo as well as 8-AcNeo. This suggests the segregation of a gene which is responsible for the subsequent acetylation state of the C-8 hydroxyl group.

The biochemical basis for the inherited qualitative differences observed in trichothecene biosynthesis could not be elucidated from this study. Additional research is required to determine whether the *Tox*1 gene affects precursor pools or the enzyme responsible for the C-8 hydroxylation.

It is interesting to note that recombinant progeny were recovered that simultaneously produced high toxin levels and trichothecenes with C-8 oxygenated moieties. To date, this combination of traits has not been observed by us in nearly 70 strains isolated from nature. In the wild type isolates, the gene or genes for C-8 hydroxylation appear to be associated with one or more genes that lead to a low level of trichothecene production in cultures grown under the conditions employed in this study.

Information about the genetics of trichothecene production reported here parallels the occurrence and organization of genes that qualitatively and quantitatively govern the production of other fungal secondary metabolites. Two distinct, and probably unlinked, loci have been identified for gibberellin biosynthesis in *F. moniliforme* [16,17]. One locus affects gibberellin synthesis quantitatively while the other affects it qualitatively. In *Aspergillus flavus*, three independently isolated aflatoxin mutants have been mapped to three linkage groups [13,14].

Similarly, four genetic loci affecting penicillin synthesis are located on four separate chromosomes in *A. nidulans* [2,8,9].

Clustering of genes required for secondary metabolites has also been revealed. In *P. chrysogenum* the organization of five loci involved in penicillin synthesis represents a combination of apparently clustered and dispersed loci [12], as do the seven genes which have been defined for the biosynthesis of fusarubin, a naphazarin pigment in *Nectria haematococca* [15]. In the latter case, three loci control the biosynthesis of fusarubin; two of these are tightly linked. The other four appear to be regulatory genes; three of these are loosely linked. It is not yet known whether any of the genes for trichothecene biosynthesis are similarly clustered.

In conclusion, natural variants of *G. pulicaris* are amenable to the genetic analysis of trichothecene biosynthesis. Further characterization of these wild type variants will aid in understanding the genetic and biochemical mechanisms governing trichothecene biosynthesis.

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