STUDIES WITH PURPLE ADENINE MUTANTS IN NEUROSPORA CRASSA. II. TETRAD ANALYSES FROM A CROSS OF AN AD-3A MUTANT WITH AN AD-3B MUTANT[1]

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PREVIOUS studies (DE SERRES 1956a) with a series of purple-adenine mutants in *Neurospora crassa* have demonstrated that these mutants can be divided into two groups on the basis of physiological (heterokaryon) and genetic (crossing) analyses. These studies indicated that the two groups arose from independent mutations at two closely linked loci in the *ad-3* region. The genetical analyses were based on random ascospore platings from intergroup crosses of adenine mutants carrying closely linked markers on both sides of the *ad-3* region. In most instances, adenine-independent segregants were associated with a regular recombination of markers, suggesting that orthodox crossing over was taking place. However, in a few instances, exceptional adenine-independent segregants (with parental combinations of markers) were obtained. Because of these results, and in view of recent evidence for unorthodox segregation in tetrads of certain crosses in Neurospora (MITCHELL 1955a) and Saccharomyces (LINDEGREN 1955), it seemed desirable to perform a tetrad analysis on an intergroup cross of purple adenine mutants to determine whether the expected double mutant could be obtained, and whether tetrad segregations would prove to be regular in other respects.

MATERIALS AND METHODS

The following mutants carrying the markers indicated were crossed to provide asci for serial ascospore isolations and subsequent tetrad analysis: *a hist-2* (C94) *ad-3A* (mutant A2) *ad-3B+* *nic-2* (43002) *al-2+* (stock no. 74-YU192-la) crossed with *A hist-2+* *ad-3A+* *ad-3B* (35203) *nic-2+* *al-2* (15300) (stock no. 35203-15300A). All the markers used are in linkage group I (BARRATT *et al.* 1954), and the order is as indicated. Previous evidence obtained by DE SERRES (1956a and unpublished) indicated that the *ad-3* region was located two to three crossover units to the right of the *hist-2* locus and two to three units to the left of the *nic-2* locus.

Crosses were made on the media described by DE SERRES (1956a). Serial ascospore isolations were made into individual tubes containing Fries minimal medium supplemented with adenine, histidine and niacin. The genotypes of individual isolates from all asci were determined subsequently on appropriately supplemented media. Random ascospore analyses were made utilizing the overplating methods of NEWMEYER (1954).

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EXPERIMENTAL RESULTS

In all, 646 asci were obtained in which at least one member of each spore pair germinated. In this total were six asci containing one adenine-independent segregant, presumably derived from crossing over between the two adenine mutants, and three adenine-dependent segregants. All the asci were classified with respect to the segregation of markers and these results are summarized in table 1. Since the original two parental mutants were not in heterokaryon-positive (compatible) stocks and would not form heterokaryons with one another, classification for crossing over in the *hist-2—ad-3A* and *ad-3B—nic-2* intervals was not feasible. The percentage crossing over between *ad-3A* and *ad-3B* is similar to that calculated previously from random spore platings of this cross, but that between *hist-2* and *nic-2* is greater than the previous value reported by DE SERRES (1956a).

In addition to the results given in table 1, data were also obtained for second division segregation frequencies for the *hist-2* and *nic-2* markers. The percentage crossing over between the centromere and these two genes was determined as 0.154 and 8.66, respectively. Segregation of the other available markers in the single ascus analyzed having a second division segregation for *hist-2*, places this mutant in the right arm of linkage group I.

Since the main purpose of this investigation was to determine whether regular tetrad segregation occurred in instances of apparent crossing over between the two types of purple adenine mutants, the next problem was to examine in detail the six asci in which such regular crossing over had been assumed to occur.

The phenotypes of these asci are given in column two of table 3. In each of the six asci, recombination had occurred between the *hist* and *nic* markers such that the adenine-independent segregant was a crossover type (*hist*$^{+}$ *nic*), one of the three adenine segregants represented the reciprocal crossover type (*hist nic*$^{+}$), and the other two adenine segregants had the parental combinations of markers. These results were consistent with the interpretation that in each ascus a single crossover had occurred at the four strand stage between the two adenine mutants and that the adenine crossover type was the expected double mutant. In order to prove this, however, it was necessary to identify the double mutant, distinguishing between it and single mutants of similar phenotype.

The initial test to distinguish the double mutant was a genetic one involving back-

TABLE 1

Summary of tetrad analyses of 646 complete asci from a cross of an ad-3A *with an* ad-3B *mutant. Mutants crossed:* a hist-2 ad-3A nic-2 al-2^{+} × A hist-2^{+} ad-3B nic-2^{+} al-2 (*See text for further details*)

Ascus classification	No. of asci with recombination in interval			
	hist-2—nic-2	*hist-2—al-2*	*nic-2—al-2*	*ad-3A—ad-3B*
Parental ditype	538	115	125	640
Nonparental ditype	0	45	34	0
Tetratype	108	486	487	6
Percent crossing over	8.35	44.58	42.95	0.46

TABLE 2

Results of back crosses of adenine segregants in six asci having one adenine-independent segregant (see table 1)

Asco-spore no.	Crossed with *ad-3A*					Crossed with *ad-3B*				
	No. spores plated	Percent viability	No. viable spores tested	No. ad.-indep. colonies	Percent ad.-indep. colonies	No. spores plated	Percent viability	No. viable spores tested	No. ad.-indep. colonies	Percent ad.-indep. colonies
61.4	23,000	40.6	9,350	62	0.66	31,500	10.8	3425	1*	0.03
61.5	64,660	2.8	1,835	0	0.00	27,750	13.9	4200	60	1.42
61.7	22,600	0.3	72	0	0.00	46,580	1.7	816	0	0.00
101.1	38,400	2.9	1,128	0	0.00	90,520	25.1	22,724	0	0.00
101.4	111,750	15.8	17,750	0	0.00	45,000	64.4	29,000	148	0.51
101.5	58,320	50.8	29,650	139	0.47	32,760	4.0	1470	1*	0.07
182.1	43,250	7.4	3,375	0	0.00	113,500	17.3	19,700	0	0.00
182.4	84,750	5.6	4,725	0	0.00	67,500	50.0	33,750	242	0.72
182.7	20,000	66.7	13,325	63	0.47	70,320	9.0	6,888	0	0.00
321.1	119,790	3.7	4,430	0	0.00	50,250	9.1	4,600	0	0.00
321.4	25,650	1.3	325	0	0.00	47,600	24.7	11,785	71	0.62
321.5	52,675	32.4	20,251	144	0.71	36,800	5.9	2,169	0	0.00
322.1	17,860	3.2	574	0	0.00	54,280	38.6	21,000	188	0.89
322.4	99,220	3.4	3,300	5*	0.15	24,500	13.0	3,100	0	0.00
322.6	27,600	23.1	6,371	22	0.34	101,840	4.3	4,392	0	0.00
523.1	14,400	2.5	360	0	0.00	52,450	11.6	6,125	0	0.00
523.3	21,315	0.4	98	0	0.00	--	—	15,875	66	0.41
523.5	55,000	29.4	16,200	69	0.42	15,360	15.0	2,400	0	0.00

* For further discussion of these isolates, see text.

crosses of all three adenine segregants in a single ascus to both an *ad-3A* mutant (A2) and an *ad-3B* mutant (35203). Random spore platings were made to determine whether any adenine-independent segregants occurred. In the first ascus tested, no. 61, the results were contrary to expectation, since the presumptive double mutant, culture no. 61.5, yielded a substantial number of adenine-independent colonies when crossed with *ad-3B*, but none with *ad-3A* (table 2). Thus this isolate appeared to be an *ad-3A* mutant. Culture no. 61.4 behaved as an *ad-3B* mutant, yielding a large number of adenine-independent isolates when crossed to *ad-3A* and none with *ad-3B*. (The single colony obtained here probably represented a contaminant). Culture 61.7, however, appeared to be a double, since no adenine-independent isolates were obtained from a cross with either *ad-3A* or *ad-3B* (although the number of viable spores tested was rather small in both crosses). Although the results with ascus 61 were anomalous with respect to markers, those obtained with the other five asci were as expected, since in all instances (with one exception noted below) the presumptive double mutants gave no adenine-independent isolates when crossed with either *ad-3A* or *ad-3B*, whereas the presumptive singles behaved as expected on the basis of

their phenotypes as either *ad-3A* or *ad-3B* (table 2). In one instance, culture 322.4, a few unexpected adenine-independent isolates were obtained. The origin of these isolates can not be proven; they could have arisen as a result of a reverse-mutation or wild type contaminant in the population of *ad-3A* conidia used in this cross.

In view of the unexpected results with ascus 61, and in order also to confirm the conclusions with the other five asci, it seemed particularly desirable to utilize other

TABLE 3

Summary of results of tests used to characterize adenineless cultures in asci having one adenine-independent segregant

Culture	Phenotype	Backcross results		Heterokaryon response			X-ray-induced reversions*	*ad-3* genotype indicated	
		To *ad-3A*	To *ad-3B*	*ad-5*	*ad-3A*	*ad-3B*			
Parent 1	*a hist ad-3A nic* +	no ad^+	ad^+	+	0	+	+ (361)		
Parent 2	*A* + *ad-3B* + *al*	ad^+	no ad^+	+	+	0	+ (162)		
Ascus 61.1	*A* + + *nic al*	—	—	—	—	—		+	+
Ascus 61.4	*a* + *ad* + +	ad^+	no ad^+†	+	+	0	+ (145)	+	*ad-3B*
Ascus 61.5	*a hist ad* + *al*	no ad^+	ad^+	+	0	+	+ (101)	*ad-3A*	+
Ascus 61.7	*A hist ad nic* +	no ad^+	no ad^+	+	0	0	0 (0)	*ad-3A*	*ad-3B*
Ascus 101.1	*a hist ad* + +	no ad^+	no ad^+	+	0	0		*ad-3A*	*ad-3B*
Ascus 101.4	*a hist ad nic al*	no ad^+	ad^+	+	0	+		*ad-3A*	+
Ascus 101.5	*A* + *ad* + *al*	ad^+	no ad^+†	+	+	0		+	*ad-3B*
Ascus 101.7	*A* + + *nic* +	—	—	—	—	—		+	+
Ascus 182.1	*a hist ad* + *al*	no d^+	no ad^+	+	0	0	0 (0)	*ad-3A*	*ad-3B*
Ascus 182.4	*a hist ad nic* +	no ad^+	ad^+	+	0	+	+ (201)	*ad-3A*	+
Ascus 182.5	*A* + + *nic al*	—	—	—	—	—		+	+
Ascus 182.7	*A* + *ad* + +	ad^+	no ad^+	+	+	0	+ (109)	+	*ad-3B*
Ascus 321.1	*a hist ad* + +	no ad^+	no ad^+	+	0	0		*ad-3A*	*ad-3B*
Ascus 321.4	*A hist ad nic al*	no ad^+	ad^+	+	0	+		*ad-3A*	+
Ascus 321.5	*A* + *ad* + +	ad^+	no ad^+	+	+	0		+	*ad-3B*
Ascus 321.7	*a* + + *nic al*	—	—	—	—	—		+	+
Ascus 322.1	*a hist ad nic* +	no ad^+	ad^+	+	0	+		*ad-3A*	+
Ascus 322.4	*a hist ad* + *al*	no ad^+‡	no ad^+	+	0	0		*ad-3A*	*ad-3B*
Ascus 322.6	*A* + *ad* + *al*	ad^+	no ad^+	+	+	0		+	*ad-3B*
Ascus 322.7	*A* + + *nic* +	—	—	—	—	—		+	+
Ascus 523.1	*a hist ad* + +	no ad^+	no ad^+	+	0	0		*ad-3A*	*ad-3B*
Ascus 523.3	*a hist ad nic al*	no ad^+	ad^+	+	0	+		*ad-3A*	+
Ascus 523.5	*A* + *ad* + +	ad^+	no ad^+	+	+	0		+	*ad-3B*
Ascus 523.7	*A* + + *nic al*	—	—	—	—	—		+	+

* Numbers in parentheses indicate total number of revertants obtained; these values do not indicate reversion frequencies.

† One ad^+ colony obtained (see table 2 and text).

‡ A few ad^+ colonies obtained (see table 2 and text).

methods to distinguish the presumptive double mutant. Two such methods have been used: one involves heterokaryon tests with appropriate tester stocks, and the other makes use of the frequency of X-ray-induced reversions to adenine-independence. Since the original parental stocks did not carry appropriate genetic factors permitting heterokaryon formation with one another, it was necessary to cross these cultures, as well as all the adenine-dependent F_1 segregants, to stocks carrying such factors and to select heterokaryon-positive isolates. Such *ad-3* isolates were first tested for heterokaryon formation against a nonpurple, biochemically distinct mutant mutant(*ad-5*), and then checked for their response with both *ad-3A* and *ad-3B* testers. The results of this analysis are given in table 3. All the F_1 isolates testing genetically as double mutants also test as doubles by heterokaryon analysis, since they respond to neither the *ad-3A* nor the *ad-3B* testers. Additionally, all the genetic tests for single mutants are confirmed by the heterokaryon tests.

Tests for X-ray-induced reversions to adenine-independence were performed by exposing macroconidia of appropriate adenine cultures to 35,000r of 250 KV X-rays and plating onto appropriately supplemented media with and without adenine. Further details of the procedures used are given in GILES (1951). In general, platings were made simply to determine whether reversions did or did not occur, and no rigorous attempts were made to obtain quantitative comparative data. The results of these tests are summarized in table 3 for the two asci tested in this manner. Again, it will be noted that cultures testing by crossing and heterokaryon analyses as double mutants also behave as such by the mutation (reversion) test, since such cultures fail to yield revertants, whereas the single mutants, both parental and segregants, do revert. All previous genetic tests have indicated that reversions in these purple adenine mutants occur by reverse mutation and not by suppressor mutation (GILES 1956).

On the basis of the three types of tests performed—genetic (crossing), physiological (heterokaryon), and mutational (induced-reversions)—the *ad-3* genotypes inferred in each ascus are indicated in the last column of table 3. In each instance the expected segregation pattern is obtained, yielding the two parental single mutants and the two doubles. In five asci, the behavior of the linked markers is also in agreement with these results, confirming a proximal position for the *ad-3A* mutant. The anomalous results

TABLE 4

Tests of adenine-independent isolates from random ascospore platings from back crosses of F_1 adenine segregants (cf. tables 2 and 3)

Culture crossed	No. adenine indep. isolates tested	No. pseudo-wild types	Genotypes of remaining ad.-indep. isolates			
			$hist^{+}$ *nic*	*hist* nic^{+}	$hist^{+}$ nic^{+}	*hist nic*
101.4 *hist ad-3A + nic*	142	1	137	0	1	3
101.5 + + *ad-3B* +	139	0	139	0	0	0
182.7 + + *ad-3B* +	62	3	58	0	0	1
321.4 *hist ad-3A + nic*	67	0	65	0	1	1
321.5 + + *ad-3B* +	133	2	127	0	3	1
Totals	543	6	526	0	5	6

with markers in ascus 61 can be most easily explained on the basis of a three strand double crossover in the *hist-2—nic-2* interval, one crossover having occurred in the *ad-3A—ad-3B* interval and the second in the *ad-3B—nic-2* interval.

The occurrence of an additional crossover in a closely adjacent region in one out of six asci exhibiting crossing over in the *ad-3A—ad-3B* interval was unexpected unless some type of negative interference was operating. Although this single instance could not establish such a phenomenon, it did suggest the desirability of obtaining additional data on this point. Certain earlier results (DE SERRES, 1956a) also suggested that negative interference might be operating in certain crosses of purple adenine mutants. Additionally, results in other crosses of allelic and closely linked mutants suggest the possibility of negative interference (PRITCHARD 1955; GILES 1956). Consequently, tests for chromosome interference were performed by an extensive analysis of adenine-independent recombinants obtained from crosses of selected *ad-3A* and *ad-3B* isolates (from the six tetrads) made to complementary adenine mutants carrying appropriate linked markers.

The results of these tests are summarized in table 4. In each of the five crosses analyzed, the vast majority of isolates were of the expected *hist*$^+$ *nic* genotype. All exceptional types were first tested, using conidial plating techniques, to determine whether they were heterokaryotic and hence presumably pseudowild types (MITCHELL, PITTENGER and MITCHELL 1952). After plating, a number of the exceptional isolates (predominantly of genotype *hist*$^+$ *nic*$^+$) proved to be pseudowild types as indicated in table 4. The genotypes of the remaining isolates are as indicated. If one assumes that these exceptional isolates have arisen as a result of double crossing over, the second crossover must have occurred in either the *hist-2—ad-3A* or the *ad-3B—nic-2* interval. In order to calculate interference values for these crosses, the *hist-2—nic-2* interval has been taken as 8.35 crossover units (table 1). Random ascospore isolations from crosses of mutants derived from the F_1 generation of the cross under consideration gave an average value of 2.9 crossover units for the *hist-2—ad-3A* interval leaving a value of 5.0 crossover units for the *ad-3B—nic-2* interval (DE SERRES, unpublished). Consequently, the expected number of double crossovers involving either the *hist-2—ad-3A* or the *ad-3B—nic-2* intervals, if there is no interference, should be 2.9 and 5.0 percent, respectively, of the recovered adenine-independent isolates. The total number of homokaryotic adenine-independent isolates from these five crosses is 537; 2.9 and 5.0 percent of this number equal 15.6 and 26.9, respectively. The actual numbers of exceptional (presumptive double crossover) isolates of genotype *hist nic* and *hist*$^+$ *nic*$^+$ are 6 and 5, respectively.

DISCUSSION

The results of the present serial ascospore isolations from an intergroup cross of two purple adenine mutants (*ad-3A* × *ad-3B*) support previous conclusions based on random ascospore isolations that adenine-independent segregants from such crosses arise by recombination mechanisms involving orthodox crossing over. Of 646 complete tetrads tested, six had one adenine-independent segregant. In each of these asci, crossing over had occurred between the two markers on either side of the *ad-3* region such that the six adenine-independent isolates were all of the same crossover

genotype. The other three segregants consisted of three adenine cultures—two non-crossover presumptive parental types and one reciprocal crossover presumptive double mutant. In each of the six asci the expected double and two parental single mutants were demonstrated to be present by three types of tests: genetic (back crosses to both parents), physiological (heterokaryon tests with compatible parental strains), and mutational (tests for induced reverse mutations to adenine independence). In one of the six asci the genotypes of the double and one of the single mutants indicated that a second crossover had occurred in the *ad-3B—nic-2* interval.

Thus, on the basis of the present sample, there is no evidence that gene conversion is involved in the origin of adenine-independent segregants in crosses of these two purple adenine mutants. Whether conversion occurs in the opposite direction (from a wild type to a mutant allele) at either the *ad-3A* or *ad-3B* locus could not be easily tested in the present cross because the parental types were not in heterokaryon-positive (compatible) stocks. It may be noted, in passing, that no irregular tetrads were encountered involving any of the markers used in this cross—*hist-2*, *nic-2*, and *al-2*.

The occurrence of one double crossover in the sample of six asci tested suggested that negative chromosome interference might be characteristic of the intervals adjacent to the *ad-3* region of linkage group I. This possibility was tested by an examination of further intergroup crosses, carrying appropriate markers, of two *ad-3A* and three *ad-3B* segregants from the six complete asci already discussed. In the total of 537 homokaryotic adenine-independent isolates obtained by plating of ascospores from the five crosses, all but 11 were of the genotype expected on the basis of single crossing over between the *ad-3A* and *ad-3B* loci with the *ad-3A* locus located proximally. As already indicated on the basis of the crossover distances between the two markers, assuming no interference, the expected doubles in the two regions were 15.6 and 26.9, whereas the observed numbers were 6 and 5. Hence these data indicate the existence of positive interference and certainly provide no evidence for negative interference. Furthermore they agree very closely with previous data obtained in a similar single cross by DE SERRES (1956a).

These results are of further interest in conjunction with the recent analysis of HOWE (1956) which indicates that there is no interference across the centromere in linkage group I, and positive interference on the left arm. The present tetrad analyses have confirmed the results of previous crosses involving random isolations (DE SERRES 1956a) which showed the order of loci used to be *sex*, *hist-2 ad-3A ad-3B nic-2* and have also shown that the *hist-2* locus is on the right arm. Hence, these studies indicate that positive interference occurs in the proximal portion of the right arm as well as on the left arm.

It may be noted that in the present tetrad analysis, the recombination frequency for the *hist-2—nic-2* interval is considerably greater than that previously reported by DE SERRES (1956a). However, it is now clear from the results of random ascospore analyses on various crosses involving linkage group I markers (DE SERRES unpublished) that wide variations in recombination frequencies for a given interval can be obtained with the same mutant strains in different genetic backgrounds, and the

present results can be attributed to such differences. Similar results, from a more elaborate analysis of crosses of markers in linkage group IV, have been reported by STADLER (1956). However, the rather wide range of values obtained for the *ad-3A*—*ad-3B* interval (table 2) probably results from a combination of differential survival of adenine-independent and adenine-dependent segregants in these crosses under plating conditions which resulted in low viability, as well as from heterogeneity in the genetic backgrounds of the isolates of mutants A2 and 35203 tested from each ascus.

The present crosses (table 2) also confirm previous tests (DE SERRES 1956a) indicating that, in general, spore viability is much higher in intergroup than in intragroup crosses. This is the case whether the latter involve single or double mutants. The occasional occurrence in these tests of adenine-independent isolates from selfings is of interest. In two crosses, where only single colonies were observed, each was of completely wild type genotype and these may well have been contaminants. However, in one cross involving a double mutant (ascospore 322.4, table 2), five colonies were obtained, of which two carried the *al-2* marker. Although contamination cannot be completely excluded in such instances, this appears to be an unlikely explanation. Reverse mutation in the conidia of the *ad-3A* parent may of course have occurred. However, the possibility of some type of unexpected interaction at meiosis, even in cases of selfings, must be kept in mind.

Although the present tetrad results demonstrate that in the six asci studied from a cross of an *ad-3A* with an *ad-3B* mutant, the origin of adenine-independent segregants is associated with regular crossing over and the recovery of the expected double mutant, this does not exclude the possible presence of other mechanisms. It may well be that gene conversion occurs with a low frequency in such crosses. Of particular interest in this connection is the recent evidence of DE SERRES (1956b, and unpublished) from random ascospore platings of intragroup crosses (within both *ad-3A* and *ad-3B*), that in such crosses the origin of adenine-independent segregants shows little, if any, relation to recombination of markers on either side of the locus. Furthermore, there is some evidence (DE SERRES 1956a) that in intergroup crosses in which the apparent percentage recombination is quite low, suggesting a relatively closer approximation of the mutational sites in the two loci than in the present cross, the frequency of exceptional "noncrossover" adenine-independent isolates is increased. Such exceptional types may represent instances of gene conversion. However, it is also possible that they arise from multiple crossover events, provided the assumption is made that strong negative interference is operating in such instances. The evidence for positive interference in the two adjacent marker intervals from the cross of mutants A2 and 35203 argues against this view. Alternatively, it is possible to postulate that such multiple crossing over is confined to very much shorter chromosomal segments, as has been suggested by PRICHARD (1955) and SANSOME (1956). Critical evidence to distinguish between these possibilities can only be obtained from tetrad data and such data would be of particular interest in intergroup crosses involving more closely linked mutants, although these studies involve considerable technical difficulties because of the low recombination frequencies.

SUMMARY

A tetrad analysis has been performed in order to obtain further evidence on the mechanism of origin of adenine-independent isolates from crosses between purple adenine mutants at the two closely linked loci, *ad-3A* and *ad-3B* in *Neurospora crassa*. The cross involved the *ad-3A* mutant, A2, carrying closely linked markers on either side of the *ad-3* region and the *ad-3B* mutant, 35203.

In a total of 646 complete tetrads examined, six contained one adenine-independent segregant. In each of these asci, crossing over had occurred between the two markers on either side of the *ad-3* region such that the six adenine-independent isolates were all of the same crossover genotype. The other three segregants consisted of three adenine cultures—two noncrossover presumptive parental types and one reciprocal crossover presumptive double mutant. In each of the six asci, the expected double and two parental single mutants were demonstrated to be present by three types of test: genetic (backcrosses to both parents), physiological (heterokaryon tests with compatible parental strains), and mutational (tests for induced reverse mutations to adenine-independence).

These results support previous conclusions based on random ascospore isolations that adenine-independent segregants from intergroup crosses of purple adenine mutants arise by recombination mechanisms involving orthodox crossing over, rather than by a mechanism such as gene conversion.

In addition, an analysis of over 500 adenine-independent isolates derived from random ascospore platings of five intergroup crosses involving suitably marked F_1 segregants from the six asci provide evidence for positive rather than negative chromosome interference in this region of linkage group I.

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