GENETIC ALTERATIONS OF ASCUS DEVELOPMENT IN *NEUROSPORA TETRASPERMA*

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THE ascus of Neurosopra has proved to be a favorable system for developmental genetic studies. One aspect of its experimental utility is that the genotype of the zygote contributes to ascus shape in a straightforward fashion such that standard relationships of dominance and recessiveness apply. In *N. crassa*, MURRAY and SRB (1959, 1962) reported a mutant gene, peak-2 (*pk-2*), which when homoallelic in a cross produces abnormal asci. Asci initiated by +/*pk-2* zygotes are phenotypically normal. At present, abnormal ascus mutants with similar effects have been found to map to at least seven different sites in the genome of *N. crassa*. The mutants fall into complementation groups, and dominants as well as recessives have been found (SRB and BASL 1969). Comparative cytological studies of the mutant and of normal asci have enlightened the basis of their differences and revealed some of the potential for variability in the sexual reproductive apparatus of the species (PINCHEIRA and SRB 1969).

Additional insight into the genetic control of the development of the ascus could be expected to emerge from studies of mutants affecting a system in which the sexual reproductive apparatus is homologous to that of *N. crassa* but differs from it. Such a system is available in the pseudohomothallic species *N. tetrasperma*. The typical ascus of this species contains four bisexual spores, in contrast to the eight unisexual spores normally found in the ascus of *N. crassa*. The functional differences in the two kinds of ascospores and the difference in spore numbers are demonstrably related to the orientation of meiotic and mitotic spindles in the developing ascus (DODGE 1927). Thus the two species have evolved a marked difference in their sexual reproductive apparatus.

PINCHEIRA and SRB (1969) have studied the effects of mutant *pk-2* in *N. tetrasperma* after hybridizing and backcrossing were used to transfer the gene from *N. crasssa*. The effect of *pk-2* on ascus development in *N. tetrasperma* is not simply recessive. Although the effects of homozygous *pk-2* are similar in the two species, heterozygotes in *N. tetrasperma* do not show simple dominance of the wild-type allele and do follow a unique developmental pattern. The lack of predictability for the action of a mutation transferred from one species to another emphasizes the desirability of a study of ascus mutants obtained directly in *N. tetrasperma*. This report concerns such a study.

MATERIALS AND METHODS

Culture techniques: For mapping and other routine purposes where a crossing medium was desired, slants containing the medium of WESTERGAARD and MITCHELL (1947) were used. For cytological purposes, crosses were made on Difco cornmeal agar because the perithecia are more easily collected from among the reduced vegetative growth on that medium. Stocks were kept on agar complete medium (BEADLE and TATUM 1945). Ascospores were transferred to this medium after manual isolation under a stereoscopic microscope.

Strains: The wild-type heterothallic *N. tetrasperma* strains *T-7A* and *T-7a* were derived from pseudohomothallic strain *T-220* provided by J. H. WARCUP, who isolated the strain in Borneo. The mutant peak-2 (*pk-2*), isolated in *N. crassa*, is a recessive abnormal ascus mutant allelic to biscuit in linkage group V of that species. In the *pk-2* cultures utilized for these studies, the mutant is in a genome primarily that of wild-type *N. tetrasperma*, to which *pk-2* was transferred by more than ten generations of backcrossing. The transferred *pk-2* is designated here as *pk-2-cra* to distinguish its species of origin from that of an allelic dominant, *Pk-1-tet*, induced in wild-type *N. tetrasperma*. A recessive and nonallelic abnormal ascus mutant induced in *N. tetrasperma* is designated *asc-1*. The DODGE strain carrying dominant gene *E* is 340.6*aE* (Fungal Genetics Stock Center #605).

Mutagenesis and selection technique: Both of the new ascus mutants described in this report were obtained following mutagenic treatment of conidia of wild type *T-7A* with dimethyl sulfate (0.1% solution in water for 13 min). Following mutagenesis, the method of selecting for presumptive ascus mutants was essentially that described by SRB and BASL (1969). Since most of the ascus mutants they obtained in *N. crassa* were originally recognized as mutants on the basis of colonial morphology of the vegetative mycelium, colonial phenotype was used as a starting point in the search for ascus mutants in *N. tetrasperma*. The particular hope was to obtain an ascus mutant at the peak-2 locus since such a mutant could be compared meaningfully with *pk-2-cra*. In the first instance dominant mutants were sought, because of the relative simplicity of the procedure for detecting them; one needs only to cross a colonial mutant to wild type and observe the asci produced.

Cytology: Perithecia were scraped from crossing tubes and fixed in absolute ethanol, chloroform, and acetic acid (6:3:1). Initial fixation was for 8 hr at 20°C, after which the material was placed at 4°C. Fixed perithecia were placed on a slide in a drop of 20% acetic acid and their contents squeezed out through the ostiole with forceps. A drop of 1% aceto-carmine was used for staining. Photographs were taken with a Zeiss WL microscope on 35mm Kodak Panatomic-X film. The film was processed in Kodak Microdol-X developer diluted (1:3) with water.

RESULTS

From twenty different experiments employing a variety of mutagens, over 400 colonial mutants were selected and tested before a dominant ascus mutant (*Pk-1-tet*) was found following dimethyl sulfate treatment of wild type. After a cross of the mutant to wild type, the perithecia contained asci as shown in Figure 1A. Note that dominance is not complete and that a few normal asci are produced. In the absence of a gene–centromere crossover, heterokaryotic spores derived from crosses to wild type produced wild-type mycelia; that is, in the vegetative heterokaryotic mycelium the colonial phenotype is recessive. The potential to form abnormal asci always segregates with the colonial phenotype. Thus colonial morphology and abnormal asci are two effects of the same mutation. When *Pk-1-tet* is homoallelic, virtually every ascus is abnormal. The asci tend to disintegrate easily when expressed from a perithecium, and intact perithecial squashes of the kind shown in Figure 1B are hard to find.

TABLE 1

Results of the cross* Pk-1-tet A × pk-2-cra a *showing evidence for allelism of* Pk-1-tet *and* pk-2-cra

Class of ascospore isolated	Number germinated from 100 isolates	Wild type	Colonial		
			A	*A,a*	*a*
small (homokaryotic)	66	0	26	2†	38
normal (heterokaryotic)	81	0	2	78	1‡

* Progeny were scored for wild-type *vs.* colonial phenotype and for mating type.
† Progeny listed as bisexual *A,a* arose from small spores that were expected to give rise to unisexual (homokaryotic) progeny.
‡ Progeny arising from presumptively heterokaryotic (bisexual) spores but proving to be unisexual can be accounted for in a number of ways as discussed by HOWE (1964).

When *Pk-1-tet* is crossed to *pk-2-cra*, all spores produced colonial cultures (Table 1), and the asci are similar to those shown in Figure 1B. The absence of wild-type progeny among the cultures derived from homokaryotic spores means that recombination is rare or does not occur. In addition, functional allelism of the mutant genes is designated by the fact that heterokaryotic spores produce colonial cultures, inasmuch as nonallelic recessives, whether linked or unlinked, should complement and produce wild-type mycelium. To the authors' knowledge the present report is the first to show directly an allelism of two mutations obtained independently in *N. crassa* and *N. tetrasperma*.

The phenotypic effect of *Pk-1-tet* is much different from that observed with *pk-2-cra*. Briefly, *pk-2-cra*, when heterozygous with wild type in *N. tetrasperma*, produces a high frequency of linear asci that contain more than four spores (Figure 1C), in contrast to the results of homozygous wild-type crosses, where 98–99% of all asci contain only four spores (Figure 1D). When *pk-2-cra* is homoallelic, the result is a high frequency of nonlinear (balloon-shaped) seven- and eight-spored asci. The consequence is that the pseudohomothallism of *N. tetrasperma* is virtually eliminated (PINCHEIRA and SRB 1969). The genetic although not the morphological consequences are similar to those of DODGE's gene, dominant *E*, which when heterozygous with wild type gives essentially all eight-spored linear asci in *N. tetrasperma* (DODGE 1939).

TABLE 2

Evidence that Pk-1-tet *and* pk-2-cra *segregate independently from* E

Cross	Number germinated from 100 isolates‡	Genotype			
		+ +	*E* +	+ *col**	*E col*
a E × *Pk-1-tet A*	31†	18	2	11	0
a E × *Pk-2-cra A*	26	17	0	9	0

* In both cases *col* is used to designate the colonial mutant segregating in the cross.
† Ascospores resulting from crosses involving the *N. tetrasperma* stocks used in our laboratory and *E* always yield low germination frequencies. DODGE (1939) reported that spores carrying *E* usually die at the germling stage.
‡ Presumptively homokaryotic spores were isolated in each case.

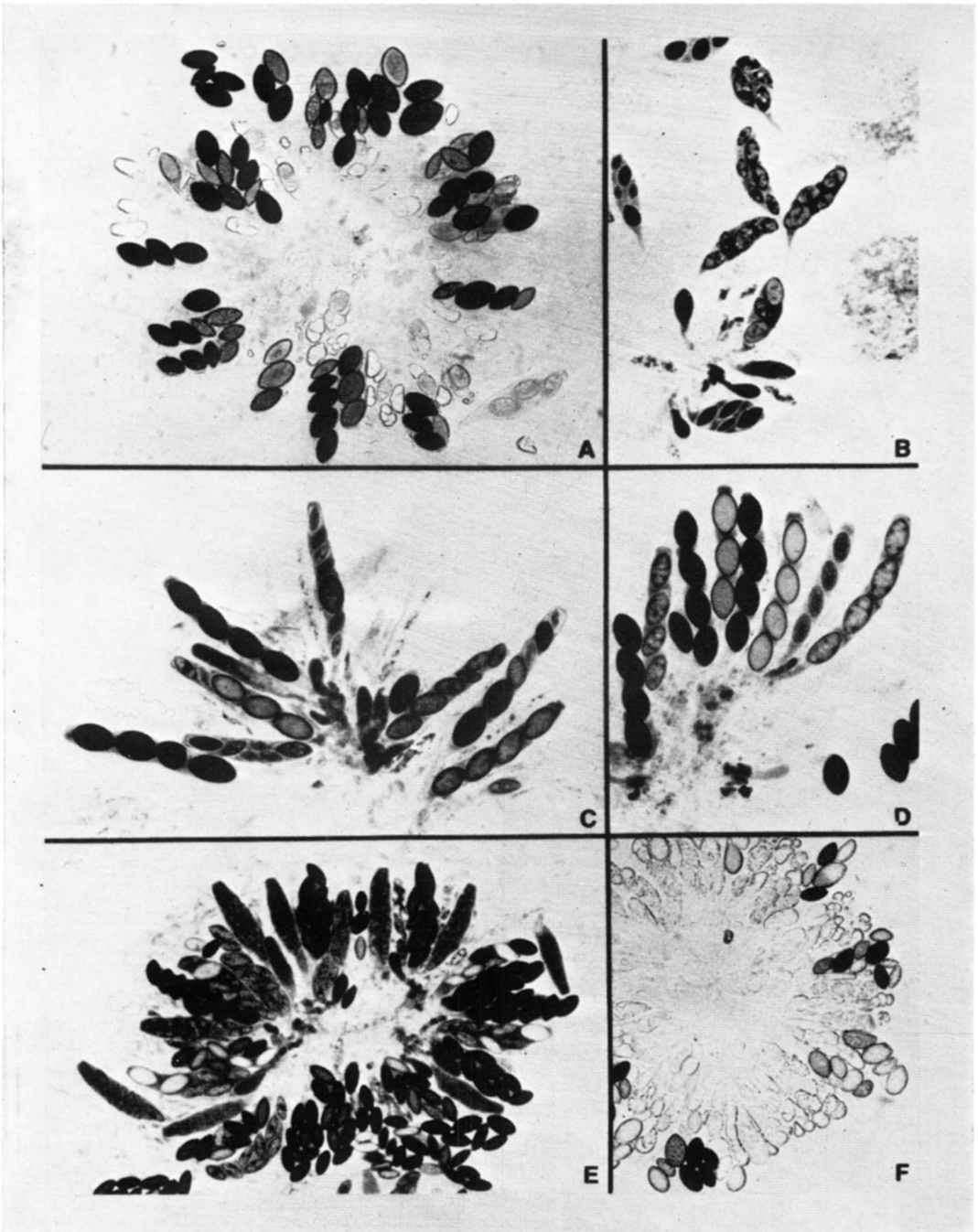

FIGURE 1.—Perithecial squashes showing typical asci produced in six different crosses involving wild type and/or mutants described in the text. A: *Pk-1-tet A* × *T-7a*. B: *Pk-1-tet A* × *Pk-1-tet a*. C: *pk-2-cra A* × *T-7a*. D: *T-7A* × *T-7a*. E: *Pk-1-tet A* × *a E*. F: *asc-1 A* × *asc-1 a*.

By contrast with its allele in *N. crassa*, *Pk-1-tet*, both when homozygous and heterozygous, gives rise to a number of spores per ascus that rarely exceeds six. The frequency of five- and six-spored asci, however, is much higher than observed in homozygous wild-type crosses. Nevertheless, the number of small spores in the spore print of a *Pk-1-tet* cross is even higher than expected from the observed frequency of five- and six-spored asci. Cytological examination reveals that *Pk-1-tet*, either when heterozygous or homozygous, produces asci with combinations of ascospores that are not usual even among exceptional asci of wild-type *N. tetrasperma*. Normally, asci of wild-type *N. tetrasperma* have four heterokaryotic, normal-sized spores, each with four nuclei. (Each nucleus originally included in a spore undergoes a simple mitosis as shown in Figure 2.) As shown by DODGE (1942), if spindles fail to orientate properly, spores other than the usual four homothallic ones may be produced in a given ascus. Five-spored asci are the most frequent exceptions. In such cases, three spores are heterokaryotic and contain four nuclei each, while the remaining two spores are smaller, homokaryotic, and contain two nuclei each (Figure 3). A three-spored ascus has one spore with eight nuclei and two spores with four nuclei each, etc. Spores are usually cut out to contain a final nuclear constitution of two, four, or some multiple of four nuclei (Figure 4A–D1). In a wild-type cross, an unlikely combination is, for example, a four-spored ascus in which one spore has six nuclei, two spores have four nuclei, and one spore has two nuclei. Asci containing spores with unusual nuclear constitution of this kind are routinely produced, however, by *Pk-1-tet*. The result is a higher percentage of small spores than would be expected simply on the basis of the frequency of five- and six-spored asci (Figure 4E–F1).

Since DODGE's *E* gene gives rise to eight-spored asci routinely, *Pk-1-tet* was crossed with *E* to study the effect of both dominant genes in a common cytoplasm. The result showed both *E* and *Pk-1-tet* to retain their dominance; mostly eight-spored nonlinear asci are produced (Figure 1E). In the case of asci with fewer than eight spores, no tendency to form spores containing more than four nuclei each was observed. When crossed to *E*, *pk-2-cra* acts purely as a recessive. Progeny tests indicate that both *Pk-1-tet* and *pk-2-cra* segregate independently of *E* (Table 2), a result consistent with *E* having been mapped to linkage group VI (HOWE and HAYSMAN 1966).

Of the several hundred colonial mutants isolated in *N. tetrasperma*, only a few have yet been tested in the homoallelic crosses that would reveal recessive ascus aberrancy. As found in *N. crassa* (SRB and BASL 1969), many homoallelic mutant crosses are not fertile. However, in *N. tetrasperma* if a crossover occurs between a colonial mutant gene and its centromere, and if a heterokaryotic bisexual spore homoallelic for the colonial marker is recovered, the spore upon germination usually gives rise to perithecia even when the corresponding cross between two unisexual homokaryotic cultures is infertile.

One of the colonials tested to date (*asc-1*) yields abnormal asci when homoallelic in a cross but not when crossed to wild type. The homoallelic crosses are

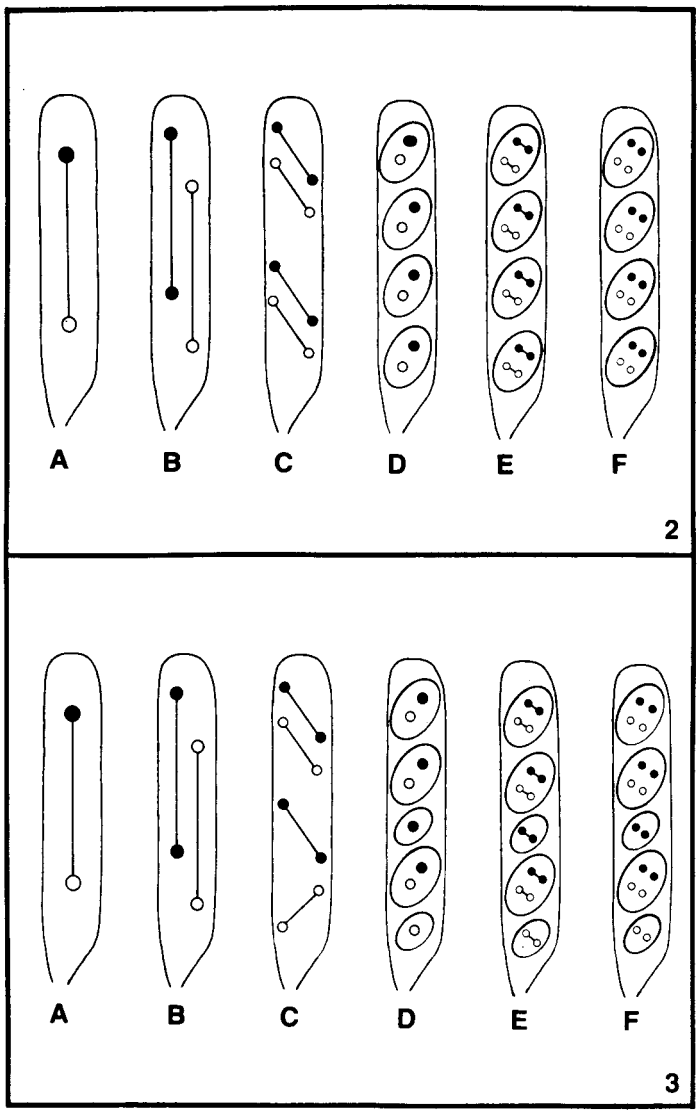

FIGURE 2.—Normal meiosis in *N. tetrasperma* resulting in a four-spored ascus. Within asci, the shaded and unshaded circles represent nuclei containing one or the other member of an allelic pair undergoing first-division segregation. Nuclear division spindles are designated by straight lines. A: Meiosis I. B: Meiosis II. C: Mitosis. D: Formation of binucleate spores heterokaryotic for the allelic pair. E: Mitosis within the spores. F: Maturation of tetranucleate spores.

FIGURE 3.—Spindle misalignment resulting in exceptional asci in *N. tetrasperma*. The sequence of nuclear events corresponds to that in Figure 2.

only slightly fertile and few asci per perithecium mature (Figure 1F). The behavior of *asc-1* mimics that of *Pk-1-tet* rather than of *pk-2-cra* in that the aberrant asci include spores with unusual nuclear numbers. Also in contrast to *pk-2-cra*, when *asc-1* is crossed to wild type, the frequency of asci with more than

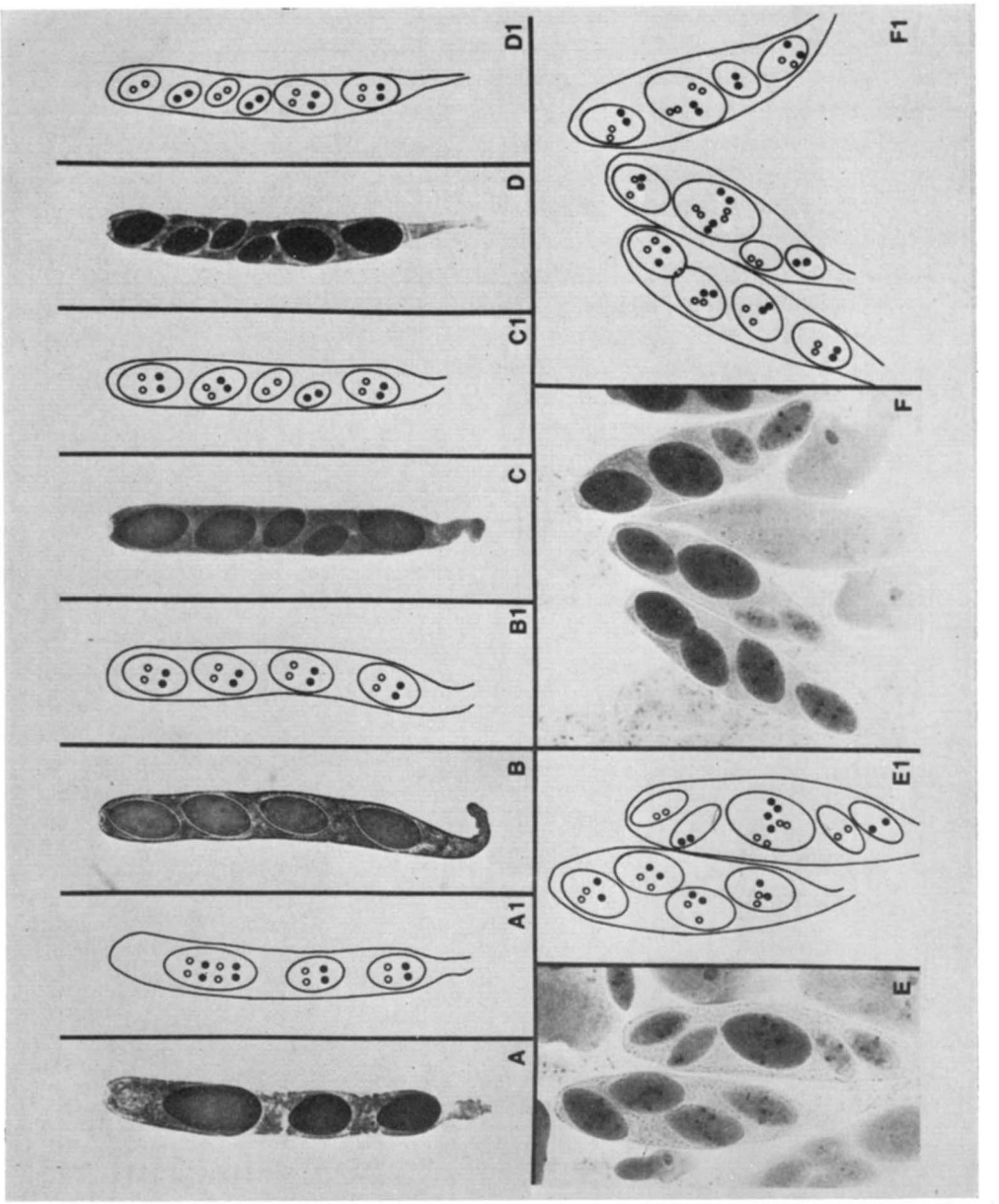

FIGURE 4.—Nuclear constitution of ascospores produced in wild-type and *Pk-1-tet* crosses. Solid *versus* hollow circles represent nuclei with one or the other members of a segregating allelic pair completely linked to the centromere. In the diagrams the number of circles drawn in each spore is based on a cytological count of the stained nuclei. The numbers of the two kinds of circles in each spore are interpretational but are very probably correct, since in all cases when asci with similar patterns of large and small spores were dissected and the spores analyzed genetically, results of the analysis were consistent with the distributions of nuclei shown here. The relative positions of particular kinds of circles within a spore are arbitrarily assigned. FIGURES A–D1: Normal four-spored and exceptional asci produced in the wild-type cross, *T-7A* × *T-7a*. FIGURES E–F1: Asci produced in the cross, *Pk-1-tet A* × *T-7a*.

TABLE 3

Evidence for close centromere linkage of asc-1 *and of* Pk-1-tet

Cross	Number germinated from 100 isolates‡	Wild type			Colonial		
		A	*A,a*	*a*†	*A*	*A,a*	*a*
Pk-1-tet A × *T-7a**	95	2	89	0	0	4	0
asc-1 A × *T-7a*	93	1	87	1	1	3	0
Pk-1-tet A × *asc-1a*	54	1	53	0	0	0	0

* In crosses involving *Pk-1-tet*, one cannot be certain of the nuclear constitution of ascsopores (see text).
† Unisexual progeny unexpected (see Table 1).
‡ Presumptively heterokaryotic spores were isolated in each case.

four spores is no greater than is produced by crosses of wild type × wild type. Crosses of wild type either to *Pk-1-tet* or to *asc-1* yield few self-fertile colonial progeny, indicating in each instance fairly close centromere linkage of the mutant locus (Table 3). However, because crosses involving *Pk-1-tet* give irregular numbers of nuclei per spore, a critical distance between that mutant locus and the centromere of its chromosome cannot be obtained by the methods originally mentioned by COLSON (1934) and reviewed and outlined in detail by HOWE (1963). Crosses of *asc-1* to *Pk-1-tet* or *pk-2-cra* (the phenotypes of all three mutant parent cultures are tightly colonial) yield heterokaryotic ascospores which, in the absence of crossovers, give rise to wild-type cultures. Therefore, *asc-1* is not functionally allelic with representatives of the *pk* locus. In addition, a high frequency of homokaryotic wild-type cultures is derived from *Pk-1-tet* × *asc-1* crosses, indicating that *asc-1* segregates independently of *Pk-1-tet* (Table 4). The possibility that *Pk-1-tet* and *asc-1* are in the same linkage group even though they segregate independently is deemed unlikely in view of the preliminary analysis of gene–centromere relationships mentioned earlier. The present indication is that *asc-1* is not in linkage group V.

TABLE 4

Evidence for independent segregation of Pk-1-tet *from* asc-1

Cross	Number germinated from 100 isolates*	Wild type			Colonial		
		A	*A,a*†	*a*	*A*	*A,a*	*a*
asc-1A × *T-7a*	55	10	5	20	15	0	5
Pk-1-tet A × *T-7a*	75	19	4	22	12	0	18
Pk-1-tet A × *asc-1 a*	63	8	1	10	27	0	17‡

* Small presumptively homokaryotic spores were isolated in each case. Scoring of progeny was for wild *vs.* colonial phenotype and for mating type.
† Unexpected progeny (see Table 1).
‡ Colonial mutants were not distinguished from each other or from double colonial mutants because of problems with fertility.

DISCUSSION

Several considerations are suggested by the observation that, in contrast to its behavior in *N. crassa*, *pk-2-cra* acts as a partial dominant in *N. tetrasperma* and that its phenotypic effects differ from those of ascus mutants derived directly in that species. An argument can be made that cultures of *pk-2-cra* may retain additional genetic material from *N. crassa* that accounts for the tendency to produce more than four spores when the mutant gene is heterozygous with a wild allele. DODGE (1928) reported that in interspecific crosses between *N. tetrasperma* and *N. sitophila* eight-sporedness is dominant to four-sporedness of the ascus. However, after progeny from these interspecific crosses were backcrossed to *N. tetrasperma*, the dominance of eight-sporedness disappeared, a strong indication of effects deriving from more than one locus. On the other hand, cultures of *pk-2-cra*, being progeny from an 11th backcross of *pk-2* into *N. tetrasperma*, represent a much more intensive effort at introgression, and relatively little of *N. crassa* genome in addition to *pk-2* is likely to have been retained. Many wild-type isolates from *pk-2-cra* × *T-7* have been backcrossed to *T-7* and none has produced other than four-spored asci in high frequency. If genetic material other than that of the *pk-2-cra* locus is causing a high frequency of asci with more than four spores in heterozygotes, it must be closely linked.

Although *Pk-1-tet* is allelic to *pk-2-cra*, the mutant genes may derive from wild-type genes that are isoallelic rather than identical. The *N. tetrasperma* wild-type allele of *pk-2* has been transferred into *N. crassa* (SRB and JAROLMEN 1967), and in that species it is completely dominant to *pk-2* in a heterozygous cross, where only linear eight-spored asci are produced. Furthermore, after this wild-type allele had been transferred into *N. crassa*, it was subjected to mutagenesis and gave rise to several recessive *pk-2* alleles that behaved similarly to the recessive *pk-2* mutants of *N. crassa*. These results do not provide firm proof that the wild-type alleles of peak in *N. crassa* and *N. tetrasperma* are identical. An observation possibly consistent with such a conclusion, however, is that asci produced by *Pk-1-tet* × *E* include spores with the kinds of nuclear constitution typical of spores produced in nonlinear asci of *N. crassa*. Results of the *Pk-1-tet* × *E* cross indicate that crossing *Pk-1-tet* into *N. crassa* may not be rewarding. On the other hand, a transfer of the wild-type allele of *E* into *N. crassa*, although obviously difficult to achieve, might enlighten our understanding of fundamental differences in ascus development in the two species, particularly in reference to nuclear spindle orientation.

The finding that the recessive ascus mutant (*asc-1*) does not map at the *Pk-1-tet* locus is not surprising since phenotypically similar recessive ascus mutants in *N. crassa* have been mapped to at least seven loci (SRB and BASL 1969). A more interesting aspect of *asc-1* and *Pk-1-tet* is the production of asci with arrays of ascospores with unusual nuclear numbers.

That *Pk-1-tet* and *asc-1*, but not *pk-2-cra*, regularly give rise to asci containing spores with other than one, two, or some multiple of two nuclei (prior to the

mitosis within the spore) is novel but not unanticipiated. DODGE (1927) showed how in normal *N. tetrasperma* two nuclei seem to cooperate in cutting out a spore and how sometimes a failure to cooperate results in the production of small unisexual spores. He also showed how, in theory, four to eight nuclei could cooperate in cutting out large spores. A very specific orientation of nuclear spindles leads to four-spored asci, two nuclei in the normal case being close enough together to cooperate in spore delimitation. A slight shift in spindle orientation during the second or third nuclear division during ascus development may result in other than four-spored asci (DODGE 1942). Because in the case of abnormal (balloon-shaped) asci, spindles at the second and third divisions lose their correct orientation (PINCHEIRA and SRB 1969), one expects that by chance two or more nuclei may be near enough to each other following the third division to give the results obtained with *Pk-1-tet* and *asc-1*.

That proximity is not the sole requirement for nuclei being incorporated together into spores is indicated by the kinds of asci produced by the crosses *Pk-1-tet* × *E* and *pk-2-cra* × *pk-2-cra*. These asci, although nonlinear, include spores of normal nuclear constitution. Perhaps attractive and/or repelling forces account for the cooperation or the failure to cooperate by nuclei in spore delimitation, and such forces may be those affected in the mutants being studied.

In search for abnormal ascus mutants of *N. crassa*, SRB and BASL (1969) isolated 2,372 genetic variants with abnormalities of the vegetative mycelium. Most, if not all, crossed readily with wild type and other mutants. In 65% of the instances, however, initial attempts to observe the effects of a mutant on ascus development failed because of infertility of crosses where the parents were homoallelic for the mutation being tested. The present work with *N. tetrasperma* has provided similar observations. But in this species, mutants that are sterile in ordinary homoallelic crosses may cross successfully in cultures derived from a bisexual ascospore that is homoallelic for the mutant gene as a consequence of crossing over between the mutant locus and the centromere of its chromosome. Because of its significance for the biology of Neurospora, the phenomenon needs an explanation. One sort of hypothesis rests on the possibility that bisexual spores homoallelic for the mutant gene may represent recombination at different loci. Mycelia derived from different spores of this kind may offer diverse genetic backgrounds to a cross, some of them relatively favorable to fertility. Another possibility is that, in contrast to the case of crosses made by inoculating homokaryons simultaneously onto a common medium, the bisexual mycelia initiated from a single ascospore may have more regular nuclear ratios and that this latter nuclear relationship is more favorable to fertility. The two lines of explanation are not mutually exclusive, but resolution of the situation requires additional studies. One needs to know whether or not ready establishment of balanced heterokaryons follows the artificial mixture of homokaryons carrying the same mutant gene but different mating types. Studies are needed also to determine whether the failures in fertility are pre- or postzygotic.

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SUMMARY

A large number of colonial mutants was screened in order to find mutants affecting the development of the ascus in *Neurospora tetrasperma*. Both a dominant and recessive ascus mutant were recovered. The dominant mutant (*Pk-1-tet*) is allelic to a recessive abnormal ascus mutant (*pk-2-cra*) originally isolated in *N. crassa*, mapped to linkage group V, and then transferred into *N. tetrasperma*. In spite of their allelism, *Pk-1-tet* and *pk-2-cra* differ in their effects on the sexual reproductive apparatus. A recessive mutant (*asc-1*) isolated in *N. tetrasperma* is not allelic to the dominant mutant and is in a different linkage group. Evidence is presented to show that the effect of the ascus mutants of *N. tetrasperma* is to distort the shape of the ascus, with the consequence that spores of unusual nuclear constitution are delimited.

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