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p-Fluorophenylalanine-induced mitotic haploidization in *Ustilago violacea*

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SUMMARY

Haploid segregants from vegetative diploids of *Ustilago violacea* appear as spherical colonies (papillae) growing above a background of dead diploid cells on complete medium containing DL-*p*-fluorophenylalanine (PFP). Most mutants segregate normally but one-third of the mutants were expressed infrequently in 0–5% papillae only. These mutants, designated 'missing-markers', were found to be on either of two chromosomes that remained disomic after treatment with PFP. When cells from a disomic papillum were streaked on complete medium, monosomics in which 'missing-markers' were expressed segregated spontaneously at a low frequency. Thus, of 10–12 linkage groups identified in *U. violacea*, two remain disomic after PFP treatment. Possible reasons for these differences between chromosomes in the same genome are discussed.

Haploid and diploid stock cultures did not differ either in resistance to PFP, or in the production of papillae on PFP medium. Haploid segregants from a diploid were slightly more resistant to PFP than the wild-type haploid cultures under some conditions, but were very different in that they no longer produced papillae on PFP medium. These haploid segregants resembled one of three PFP-resistant mutants (*pfp-A*) isolated from a wild-type haploid stock grown on PFP medium. The significance of these results to the mechanism of haploidization in *U. violacea* is discussed.

1. INTRODUCTION

During haploidization of vegetative diploid cells of fungi one member of each chromosome pair is lost, and a haploid nucleus is formed. With the segregation of whole chromosomes, genes on the same chromosome segregate together, whereas genes on different chromosomes recombine randomly. This provides an easy and efficient way of assigning new markers to a linkage group which has been used in the mapping of *Aspergillus nidulans* and certain other fungi (Käfer, 1958; McCully & Forbes, 1965; Dorn, 1967). Initially, spontaneous haploid segregants were selected, but following the discovery of Morpurgo (in Lhoas, 1961) most workers now use DL-*p*-fluorophenylalanine (PFP) to induce a high frequency of haploid segregants.

The type of haploidization found in *Aspergillus nidulans* has also been reported for the related species *A. niger* (Lhoas, 1967) and *A. amstelodami* (Lewis, 1969), for *Schizosaccharomyces pombe* (da Cunha, 1970) and for the smut fungus *Ustilago*

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violacea (Day & Jones, 1966, 1968, 1969), but most investigations of the parasexual cycle in other fungi have revealed deviations from this expected 'normal' type of haploidization, whether or not PFP has been used to induce segregation. Little is known of the mechanism of this process, although Käfer (1961) has suggested that it occurs by a stepwise series of non-disjunctions in *A. nidulans*.

The purpose of this paper is to describe haploidization in *U. violacea* and to investigate the action of PFP on this process. The genetical data obtained during these studies have been summarized previously (Day & Jones, 1969).

2. METHODS AND MATERIALS

The life-cycle, stocks, media, general techniques and the technique for PFP-induced haploidization of diploids of *U. violacea* have been described in detail in earlier papers (Day & Jones, 1968, 1969).

3. THE EFFECT OF PFP ON DIPLOID CELLS

About 70% of the papillae are haploid or near haploid (Day & Jones, 1969). The remaining papillae are probably aneuploid and vary greatly in shape and texture and cell size.

About 1×10^{-5} to 10^{-6} of the papillae are diploid as judged by the criteria for diploid cells published previously (Day & Jones, 1968). The diploid papillae do not produce further papillae when they are reinoculated to PFP medium and therefore are different from the original diploid. The significance of this observation is discussed later.

An approximate calculation suggests that only one diploid cell in 10^5 to 10^6 produces a viable haploid or near haploid line after treatment with PFP. About 30% of the genes did not segregate normally in these haploid lines (Day & Jones, 1969). These genes failed to appear or appeared very rarely (less than 5%) in the haploid segregants, and were called 'missing-markers'. These markers are discussed in detail below.

4. THE MISSING-MARKER PHENOMENON

Thirteen of the 33 mutants that were used regularly in mapping experiments did not segregate normally, but were rarely represented (0 to 5%) in the haploid segregants from any diploid which contained them. Other markers in the same diploid segregated normally and had allele ratios of about 50% (see Table 1). The group of missing-markers included mutants with a wide variety of requirements as follows (see table 1 of Day & Jones (1969) for a full description of the isolates):

cit_1	isolates 115, 143*
pro_1	isolate 121
his_3	isolate 112
lys_3	isolates 84, C 3, 184, 195, 197*
met_1	isolates 75, 145
nir_1	isolate 125 } not tested for allelism
nir_2	isolate 126 } not tested for allelism

* Other isolates mutant at these loci have not yet been tested.

Table 1. *Haploidization results involving 'missing-markers'*

Diploid no.	Diploid genotype*	Phenotypes of haploid segregants recovered†				Allele frequency (%)	Recombination (%)	Remarks
		Yellow (y)		Pink (+)				
H12	C C2 75 $\frac{y}{+}\ \frac{his_1}{+}\ \frac{+}{met_1}\ \frac{a_1}{a_2}$	y	25	+	12	y = 59	y-h = 63	met_1 did not reappear in haploid progeny. Other markers segregated randomly
		y h	10	h	12	h = 37	y-m = ?%	
						m = 0	m-h = ?%	
		Total 59						
GS	112 716 7161 $\frac{his_3}{+}\ \frac{+}{lys_2}\ \frac{+}{arg_3}\ \frac{a_1}{a_3}$			+	40	l = 26	a-l = 48	his_3 did not reappear in haploid progeny. Other markers segregated randomly
				a	34	a = 46	a-h = ?	
				l	14	h = 0	l-h = ?	
				a l	12			
				Total	100			
E 31	C C1 15 84 $\frac{y}{+}\ \frac{met_3}{+}\ \frac{+}{orn_1}\ \frac{+}{lys_3}\ \frac{a_1}{a_2}$	y	7	+	4	y = 54	y-o = 57	lys_3 segregated rarely amongst the haploid progeny. Other markers segregated randomly
		y o	6	o	8	o = 50	y-m = 46	
		y m	7	m	4	m = 43	o-m = 54	
		y o m	5	o m	3	l = 4	y-l = ?	
				l	1		o-l = ?	
				o m l	1		m-l = ?	
		Total 46						
C 3	C C3 15 729 $\frac{y}{+}\ \frac{lys_3}{+}\ \frac{+}{orn_1}\ \frac{+}{inos_1}\ \frac{a_1}{a_2}$	y +	34	+	25	y = 61	y-o = 51	lys_3 segregated rarely amongst the haploid progeny. Other markers segregated randomly
		y o	22	o	12	o = 38	y-i = 50	
		y i	25	i	13	i = 44	o-i = 43	
		y l	1	l	1	l = 4	y-l = ?	
		y o i	17	o i	10		o-l = ?	
		y o l	0	o l	0		i-l = ?	
		y i l	0	i l	2			
		y o i l	2	o i l	0			
		Total 164						

* The top line represents the genotype of the first haploid parent, and the bottom line that of the second haploid parent. The number of the isolate used is shown above each gene symbol (see Day & Jones, 1969).

† The genotypes of the haploid segregants are described only by the recessive characters that they express, and an abbreviated form of the gene symbol is used.

These markers did not segregate normally either in different isolates of the same diploid or in any of the many diploids that contained them. Some explanations of this phenomenon can be eliminated immediately. Back mutation of the marker or mitotic crossing-over in the diploid would not explain the regularity of the loss from all diploids containing the marker. Limited genetic exchange between nuclei seems to occur in *Schizophyllum* (Ellingboe, 1964), but this explanation is unlikely to account for the 'missing-markers' in *U. violacea* as it would be expected to affect both wild-type and mutant alleles equally.

Differential viability of different strains was not the explanation as haploids carrying missing-marker alleles were found to be no less viable on PFP medium than strains with other marker alleles.

Analysis of sexual segregation in diploids containing missing-markers showed that these markers segregated normally at meiosis. It appeared likely therefore that the missing-marker genes were on chromosomes which remained disomic following PFP treatment. This was confirmed by an analysis of single cells from a single papillum.

Papillae were isolated from a diploid containing a missing-marker and each papillum, now called a whole papillum to distinguish it from the isolates obtained from it, was streaked out on complete medium to obtain monosporidial isolates. The colonies in these monosporidial isolates were of two types; the great majority were small, creamy-white, wrinkled, and sectored; a few were larger, pink, smooth, and non-sectored. The small sectoring colonies had cells that were approximately haploid-sized, but which varied greatly in shape. The sectors in them grew much faster than the rest of the colony and closely resembled the few pink non-sectoring colonies. The cells in the sectors and in the pink colonies were uniform and typically haploid. When the genotype of these colonies was analysed, it was found that about 50 % of the sectors and of the pink colonies expressed a missing-marker, but that the rest of the sectoring colony invariably did not express these markers. These results indicate that the majority of the cells in a papillum form slow-growing colonies because they are disomic for one or more of the chromosomes. These disomic colonies occasionally produce more vigorous completely haploid sectors, expressing either the mutant gene or its wild-type allele. A few of the cells in a papillum are already completely monosomic or haploid before they are streaked on complete medium and so produce the large non-sectoring haploid colonies.

This analysis of monosomic segregants made it possible to map the missing-markers. The segregation of the missing-markers lys_3 and met_1 in diploid F_1 provides an example of this (Table 2).

The whole papillae from diploid F_1 required either inositol or histidine, or were prototrophic. No whole papillum required either lysine or methionine. Small sectoring colonies from each of these papillae had the same requirements as the parental papillum, and none required either lysine or methionine. The larger, non-sectoring colonies had the same requirement as the parental papillum but in addition required either lysine or methionine. These colonies never required either both lysine and methionine, or neither of these amino acids. That is, there were no phenotypes resulting from recombination between the two markers. These results indicate that

Table 2. *The analysis of monosomic segregants from disomic papillae of diploid* F_1

Genotype of diploid F_1 ...	729 *inos* / +	C 3 *lys*$_3$ / +	75 + / *met*$_1$	113 + / *his*$_4$	*a*$_1$ / *a*$_2$

A. Analysis of segregants from individual papillae

	Pap. 1	Pap. 2	Pap. 3
Genotype* of whole papillum...	*i l* + / + *m* + *a*$_2$	+ *l* + / + *m* + *a*$_2$	*i l* + / + *m* *h a*$_1$
Genotypes of monosporidial isolates from each papillum			
(i) Small sectoring colonies†	15 *i l* + / + *m* + *a*$_2$	2 + *l* + / + *m* + *a*$_2$	6 *i l* + / + *m* *h a*$_1$
(ii) Large non-sectoring colonies	39 *i* + *m* + *a*$_2$ 42 *i l* + + *a*$_2$	3 + + *m* + *a*$_2$ 10 + *l* + + *a*$_2$	4 *i* + *m h a*$_1$ 4 *i l* + *h a*$_1$

B. Analysis of the sectors produced in three small sectoring colonies from papillum 1‡

Sectoring colony no. ...	1	2	3
Sector A	*i l* + + *a*$_2$	*i* + *m* + *a*$_2$	*i l* + + *a*$_2$
Sector B	*i l* + + *a*$_2$	*i* + *m* + *a*$_2$	*i* + *m* + *a*$_2$
Sector C	*i* + *m* + *a*$_2$	*i* + *m* + *a*$_2$	*i l* + + *a*$_2$

* For simplicity an abbreviated form of each gene symbol is used.

† The genotype shown for these sectoring colonies is that of the original part of the colony. The ratio of sectoring colonies to non-sectoring colonies shown here is not meaningful. In practice about 99 % of the colonies were sectoring.

‡ Sectors were chosen at random from each sectoring colony.

Table 3. *The segregation of monosomics from disomic cells plated on complete medium*

	Genotype of diploid plated on PFP medium	Monosomic segregants	Linkage data
Diploid S 5	75 121 *met*$_1$ / + + *pro*$_1$ / *a*$_1$ / *a*$_2$	*met* + 14 + *pro* 2	*met*$_1$ and *pro*$_1$ linked
Diploid S 8	75 143 *met*$_1$ / + + *cit*$_1$ / *a*$_1$ / *a*$_2$	*met* + 14 + *cit* 14	*met*$_1$ and *cit*$_1$ linked
Diploid S 21	112 C 3 *his*$_3$ / + + *lys*$_3$ / *a*$_1$ / *a*$_2$	+ + 4 *his* + 14 + *lys* 6 *his lys* 2	*his*$_3$ and *lys*$_3$ not linked
Diploid S 31	112 121 *his*$_3$ / + + *pro*$_1$ / *a*$_1$ / *a*$_2$	+ + 11 *his* + 3 + *pro* 5 *his pro* 2	*his*$_3$ and *pro*$_1$ not linked
Diploid S 37	C 3 143 *lys*$_3$ / + + *cit*$_1$ / *a*$_1$ / *a*$_2$	*lys* + 13 + *cit* 4	*lys*$_3$ and *cit*$_1$ linked
Diploid S 44	143 121 *cit*$_1$ / + + *pro*$_1$ / *a*$_1$ / *a*$_2$	*cit* + 15 + *pro* 4	*cit*$_1$ and *pro*$_1$ linked

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lys$_3$ and *met*$_1$ are on homologous chromosomes and were linked in repulsion in diploid F_1.

Similar results obtained from the diploids shown in Table 3, and from several others, proved that most of the missing-markers were linked. Thirteen missing-markers were shown by complementation tests to be at seven loci (Day & Jones, 1969), and four of these loci, *cit*$_1$, *pro*$_1$, *lys*$_3$ and *met*$_1$, were linked. The histidine mutant 112 (*his*$_3$) was clearly not linked to this group of mutants, and is therefore on a separate chromosome. The other two mutants, 125 and 126, which both require ammonium ions, were not investigated in detail and have not yet been assigned to a linkage group. As both are missing-markers and have similar requirements (table 1, Day & Jones, 1969), it seems probable that they are allelic and situated either on the chromosome carrying *cit*$_1$, *pro*$_1$, *met*$_1$ and *lys*$_3$, or on the *his*$_3$ chromosome. These results are included in the summary of linkage data presented previously (Day & Jones, 1969).

A few papillae (0–5%) become uniformly monosomic for a chromosome carrying a recessive missing-marker early in their development, but it was not possible to distinguish the other type of papillum that was monosomic for the chromosome carrying the wild-type allele. It is estimated therefore that the total frequency of papillae that become uniformly monosomic for a missing-marker chromosome on PFP medium may reach 10%.

5. PFP RESISTANCE IN HAPLOID AND DIPLOID STRAINS

Papillae are produced on a medium because the cells which initiate them have a growth advantage over the rest of the population. PFP is markedly inhibitory to growth of wild-type sporidia. It seemed likely therefore that this chemical not only induces haploidization but also selects for haploid genotypes, as the majority of the papillae on PFP medium were haploid or near-haploid. Experiments were therefore devised to determine whether haploid cells are intrinsically more resistant to PFP than diploid cells or whether recessive PFP resistant mutants are selected during haploidization which would be expressed in haploids only.

When wild-type haploid cells are streaked on PFP medium most of the cells die and papillae form just as with the diploid strains. Three main types of haploid papillae classified as A, B and C were isolated from wild-type haploids on PFP medium. Papillae of types A, B and C occurred in an approximate ratio of 5:3:1. Type A papillae were spherical, soft-textured and cream in colour. The sporidia were spherical on PFP medium and ellipsoid on complete medium, and were usually in chains. Type B papillae were hemispherical, firm in texture, and were orange in colour. The cells were distorted with collapsed walls on PFP medium and when they were transferred to complete medium. The type C papillae were flattened with a central dome, soft in texture and were off-white in colour. The cells of these papillae were spherical on PFP medium and on complete medium.

Suspensions of cells from (i) diploid VX, (ii) two haploid segregants obtained from this diploid by PFP treatment (VX 3, VX 7), (iii) the wild-type haploid a_1 stock, and (iv) the three types of papillae, A, B and C, isolated from the wild-type haploid

on PFP medium, were plated on PFP medium. The concentration of PFP in complete and minimal media which suppressed growth of these six stocks is shown in Table 4.

The wild-type haploid stock and the diploid VX were identical in their resistance to PFP (Table 4) and in their growth rates on sublimiting levels of PFP. The difference in the limiting level of PFP in complete medium from that in minimal medium is probably due to the presence of analogues of PFP in complete medium. Tests on minimal medium which showed that the toxic effect of PFP is competitively inhibited by phenylalanine and by tyrosine support this conclusion. There was a slight

Table 4. *Growth of haploid, diploid, haploid segregant, and haploid PFP-resistant strains on media containing PFP*

Culture	Origin	Limiting concentration of PFP (ppm)		Growth habit on sublimiting concentrations of PFP
		In complete medium	In minimal medium	
a_1	Wild-type haploid	600	1·0	Papillae form of types A, B, C
VX	Freshly synthesized diploid	600	1·0	Papillae form similar to VX 3, VX 7
VX 3	Haploid segregants from diploid VX	600	2·5	No papillae form
VX 7		600	2·5	No papillae form
Type A	Papillae obtained from a_1 wild-type haploid stock after growth on PFP	600	2·5	No papillae form
Type B		1200	2·0	Papillae of types A, B, C form
Type C		1200	1·0	Papillae of types A and B form

increase in the tolerance of VX 3, VX 7 and type A to increased levels of PFP in minimal medium, but this was not measurable in complete medium. However, these three strains *were* very different from the other strains in that they did not produce papillae on PFP medium. It seems likely that strains VX 3 and VX 7 are mutant in the same gene as the type A strain, as they have identical growth characteristics on PFP media, and they are also very similar in cell and colony morphology.

All of the three types of papillae isolated from the wild-type haploid were found to be prototrophic. None responded to tyrosine, phenylalanine, tryptophan, anthranilic acid or *p*-aminobenzoic acid, as do some PFP-resistant mutants in other species (Sinha, 1967).

6. DISCUSSION

(i) *The induction of haploidization by PFP*

Unusual segregations following treatment with PFP have been reported for *Aspergillus nidulans* (Millington-Ward, 1967), *A. amstelodami* (Lewis & Barron, 1964), *A. fumigatus* and *Penicillium* sp. (Strømnaes & Garber, 1963; Strømnaes, Garber & Beraha, 1964; Fjeld & Strømnaes, 1966) and in this paper for *U. violacea*. These irregularities contrast markedly with the regular segregation reported in *A.*

nidulans (Käfer, 1961) and *A. niger* (Lhoas, 1967) and with the segregation of most of the markers in *U. violacea* (Day & Jones, 1969). It is not clear why PFP induces haploidization in *U. violacea* and *Schizosaccharomyces pombe* (Gutz, 1966; da Cunha, 1970) but not in *U. maydis* (Dr R. Holliday, personal communication) and *Saccharomyces cerevisiae* (Strømnaes, 1968). Strømnaes' (1968) suggestion that PFP cannot induce haploidization in unicellular species is disproved by the results with *U. violacea* and *S. pombe*.

In *A. nidulans* (McCully & Forbes, 1965) and *A. niger* (Lhoas, 1968) haploids have an inherent growth advantage over diploids on PFP medium. A different type of haploid growth advantage involving a PFP-resistance gene has been reported in this paper for *U. violacea*. PFP has therefore two important effects on diploid cells of *U. violacea*, *A. nidulans* and *A. niger*, which combine to make it such a useful haploidizing agent. First, it interferes with nuclear division in cells and induces chromosome loss. Second, it selects haploid cells from the background of diploid growth. Both of these effects will be examined in turn.

(ii) *The effect of PFP on nuclear division*

The papillae from diploids which did not contain missing-markers were usually of uniform genotype. If the process of haploidization occurred in a gradual stepwise manner, it is reasonable to assume that some of the intermediate aneuploid cells ($2n-1$, $2n-2$, etc.) would divide to produce cells with the same chromosome number. In this case, the random loss of chromosomes from identical aneuploid cells would produce several different haploid clones. Consequently either papillae of mixed genotype or aggregations of papillae with different genotypes would occur. As neither of these types of papillae were found, reduction probably occurs either in one step or in a few steps. However, the reduction in some cases is not complete, for about 30% of the papillae seem to be stable aneuploids.

Sisken & Wilkes (1967) have shown that PFP appears to be incorporated into proteins in place of phenylalanine, possibly into spindle microtubules (fibres) or in chromosomal proteins. Inactivation of some microtubules or parts of microtubules by PFP might induce cells to lose several chromosomes simultaneously.

Alternatively, if the chromosomes in somatic cells of fungi are connected in chains as has been suggested recently (W. V. Brown, in preparation), PFP might disrupt the connexions between chromosomes and so induce loss of chromosomes during division. If this model of somatic cell division is correct, then haploidization would not be caused by non-disjunctions, but by breakdown of chromosomal chains, and PFP would act by increasing the rate of breakage.

Da Cunha (1970) performed a pedigree analysis on diploid cells of *Schizosaccharomyces pombe* on PFP medium. He showed that PFP produced haploid cells at least as early as the fourth consecutive cell division. As the genetical data indicated a haploid chromosome number of at least six, the haploidization process must have occurred rapidly with several if not all chromosomes being lost in a single nuclear division.

(iii) *The selection of haploid cells by PFP*

The mechanism by which PFP selects haploid cells from diploid cells in *U. violacea* differs from that reported for *A. nidulans* and *A. niger*. In these species of *Aspergillus*, haploid strains, whether they are PFP-induced segregants from a diploid, or haploid strains that have not been exposed to PFP, grow more rapidly in PFP medium than diploid cells (McCully & Forbes, 1965; Lhoas, 1967, 1968). Lhoas suggested that PFP induced chromosome losses in both diploid and haploid cells. He argued that loss of chromosomes from a haploid nucleus would stop it dividing further and would not disturb the growth of the rest of the colony, while loss of chromosomes from a diploid would produce aneuploid nuclei which might accumulate in the mycelium and produce a slow-growing colony.

In *U. violacea* haploid colonies have no inherent growth advantages over diploid colonies, but the PFP-induced haploid segregants obtained as papillae from a diploid culture are slightly more tolerant to PFP than wild-type haploid cells which have not been exposed previously to PFP. Evidence has been presented that these haploid segregants are identical to one (type A) of three strains selected directly from the wild-type haploid for their vigorous growth on PFP medium. The distinctive phenotype of this strain, a two- or threefold increased tolerance to PFP in minimal medium and a marked resistance to the disruptive effect of PFP on nuclear division, was heritable and it seems likely that it is determined from one locus, which we call *pfp-A*.

We explain haploidization in *U. violacea* in the following way. The haploid papillae are thought to originate after mutations to PFP tolerance at the *pfp-A* locus have occurred. The frequency of haploidization (1×10^{-5} to 10^{-6}) is of the right order for mutation. These mutants are recessive and there will therefore be a strong selection favouring the survival of cells that have become hemizygous for the mutant allele through PFP-induced loss of the homologous chromosome. When the resistance allele becomes hemizygous before chromosome reduction has proceeded very far, the aneuploids formed are probably resistant to the nuclear effects of PFP, and therefore tend to remain aneuploid or to haploidize at a slower rate. This would account for the 30% aneuploid papillae which were obtained during haploidization. Such cells, although slow growing, would have a selective advantage over the original diploid because of the resistance gene, and would therefore form papillae. The diploid papillae that were obtained at the low frequency of 1×10^{-5} to 10^{-6} probably originated from a mutation to resistance in diploid cells which were already heterozygous for resistance through some previous mutation. Such diploids, which were presumably homozygous for *pfp-A*, were unaffected by PFP and did not haploidize.

(iv) *The missing-marker phenomenon*

A likely explanation for the missing-markers may be that these chromosomes are the only ones that produce a viable phenotype when they are disomic and other chromosomes are monosomic. Another possibility is that the microtubules responsible for the disjunction of these chromosomes may be low in phenylalanine content

relative to the remainder of the chromosomes. This presumes specific binding between a particular chromosome and a particular microtubule or other proteinaceous attachment site. Specificity of this sort seems unlikely.

An alternative explanation of the missing-marker phenomenon involving the *pfp-A* resistance gene is possible if PFP reduces chromosome pairs in a definite order rather than in a random manner. In this case, these two chromosomes may not be reduced until after the chromosome pair carrying the *pfp-A* allele becomes monosomic and may therefore be left disomic when the cell becomes resistant to the effects of PFP. Another explanation is possible if the chromosomes are connected in chains in somatic cells. These chromosomes may be near an attachment point of the chain to the nuclear membrane, and may therefore rarely be lost through breakages of the chain.

Further work on this interesting phenomenon is necessary to determine what is unusual about these chromosomes, and to correlate the results with cytological observations. Thus the possibility that chromosome loss occurs in a definite order may not be very likely if mitosis follows the classical scheme, but may be much more feasible if the chromosomes are associated in unusual arrangements during somatic nuclear division (see Burnett, 1968).

This work was carried out while one of us (A.W.D.) was the holder of a Postgraduate Studentship from the Ministry of Agriculture, Fisheries and Food.

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