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Selective Chromosomal Elimination during Haploid Formation in Barley Following Interspecific Hybridization

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Abstract. Cytological observations were made on embryo and endosperm tissues with different genome combinations that were produced by crossing the diploid and tetraploid cytotypes of *Hordeum vulgare* and *H. bulbosum*. The high frequency of barley haploids results from hybridization followed by the selective elimination of *bulbosum* chromosomes during the early development of embryos which initially contained a ratio of 1 *vulgare* to 1 *bulbosum* genomes. Elimination is gradual as indicated by the increase in the percentage of cells with the gametic chromosome number. However, the balance between genetic factors of the two parents appears to regulate the stability or elimination of chromosomes. Triploid embryos containing 1 *vulgare* to 2 *bulbosum* genomes are relatively stable. The most stable endosperm tissues examined had a ratio of 1 *vulgare* to 4 *bulbosum* genomes. Evidence of genetic control in both the *vulgare* and *bulbosum* chromosomes and their interaction is discussed. As has been suggested by Lange (1971) and also found in mammalian somatic cell hybrids, the most probable basis for selective chromosome elimination relates to mitotic rhythm and the duration of cell cycle phases.

Introduction

There are a number of recent reports dealing with the production of haploids in cultivated barley (*Hordeum vulgare* L.) following the interspecific cross between *H. vulgare* (2n = 14) and *H. bulbosum* (2n = 14) (Kao and Kasha, 1969; Kasha and Kao, 1970; Kasha and Sadasivaiah, 1971; Lange, 1969, 1971a, b; Symko, 1969). These reports agree that chromosome elimination leads to haploid formation, whereas Davies (1958), who obtained diploid *vulgare* progeny from the cross between tetraploids (2n = 28) of *H. bulbosum* and *H. vulgare*, suggested that these progeny resulted from male parthenogenesis. Fertile dihaploids of *H. vulgare* can also be produced from reciprocal crosses between autotetraploid (2n = 28) cytotypes of *H. vulgare* and *H. bulbosum* whereas triploid hybrids (2n = 21) result from the cross of diploid *H. vulgare* by tetraploid *H. bulbosum* (Kasha and Sadasivaiah, 1971).

Kasha *et al.* (1970) proposed that the balance of the parental genomes in the hybrid tissue was an important factor in chromosome stability and secondly, that selective elimination of *bulbosum* chromosomes occurred during haploid production. Their proposal was strengthened by the data of Kasha and Sadasivaiah (1971). However, Lange (1971) suggested that the chromosomes of either *bulbosum* or *vulgare* may be eliminated although the *bulbosum* chromosomes are eliminated more frequently.

This study was undertaken to reveal the pattern of chromosome elimination in tissues (embryo and endosperm) with different genomic constitutions. Our results provide evidence for the occurrence of fertilization, followed by selective elimination of *bulbosum* chromosomes leading to the formation of *vulgare* haploids. Comparisons are drawn with chromosome elimination phenomenon from the somatic cell hybrids between different mammals.

Material and Methods

For embryological studies, both diploid and induced autotetraploid forms of *Hordeum vulgare* L. cultivar York were used in crosses with diploid *H. bulbosum* L. (Acc. GBC-77, which is PI 318649 from D. A. Reid, USDA, Beltsville, Md.) as well as tetraploid *H. bulbosum* L. (Accs. B-816 and B-830 from T. Rajhathy, C.D.A., Ottawa). Caryopses to be examined cytologically were removed at different day intervals after pollination. Additional embryos were cultured according to the techniques of Kao and Kasha (1969) to obtain seedlings.

Cytological examinations were made of embryos and endosperm with the expected different possible genomic combinations listed in Table 1. The order of the symbols V and B reflects the direction of the cross. Freshly harvested caryopses were pretreated in cold water (0–2° C) for 24 hours and fixed in Carnoy's (6:3:1) solution under vacuum for 15 min and left for 2 days at room temperature. (The cold water pretreatment blocks the division of most cells at metaphase and permits the accumulation of cells with chromosomes in the shortened visible stage.) Caryopses were washed in 2 changes of 70% ethanol and then stored in 70% ethanol under refrigeration (0° C) until used. For staining, caryopses were

Table 1. Cross combinations and expected genomic constitutions (assuming fertilization) of embryos and endosperm used for cytological examinations

Female parent	Male parent	Expected genomic constitution	
		Embryo	Endosperm
VV[a]	BB	VB (1V:1B)	VVB (2V:1B)
BB	VV	BV (1V:1B)	BBV (1V:2B)
VV	BBBB	VBB (1V:2B)	VVBB (1V:1B)
BBBB	VV	BBV (1V:2B)	BBBV (1V:4B)
BBBB	VVVV	BBVV (1V:1B)	BBBBVV (1V:2B)

[a] *Hordeum vulgare* VV (2n = 14), VVVV (2n = 28); *H. bulbosum* BB (2n = 14), BBBB (2n = 28).

submerged in a mixture of Snow's 2% alcoholic hydrochloric acid carmine and a 4% aqueous iron alum solution (at the rate of 3 or 4 drops of mordant to 1 ml of stain) and left for at least 3 days at room temperature. After staining the caryopses were transferred into 70% ethanol. For slide preparations, the individual caryopses were placed in a drop of 45% acetic acid and the embryo and endosperm were removed with sharp-pointed tweezers under a dissecting microscope. Carefully separated embryo and endosperm tissues were transferred onto separate slides and squashed under coverslip. Slides of embryo tissues were then scored for the chromosome numbers in dividing cells and other abnormalities in both dividing and non-dividing cells. Detailed chromosome counts were mainly restricted to embryos as technical difficulties, particularly with the higher chromosome numbers and starch grain formation in endosperm cells, made it difficult to obtain reliable counts in such tissues. However, general observations on the range of chromosome numbers and the extent of endosperm and starch development were recorded in such instances.

Results

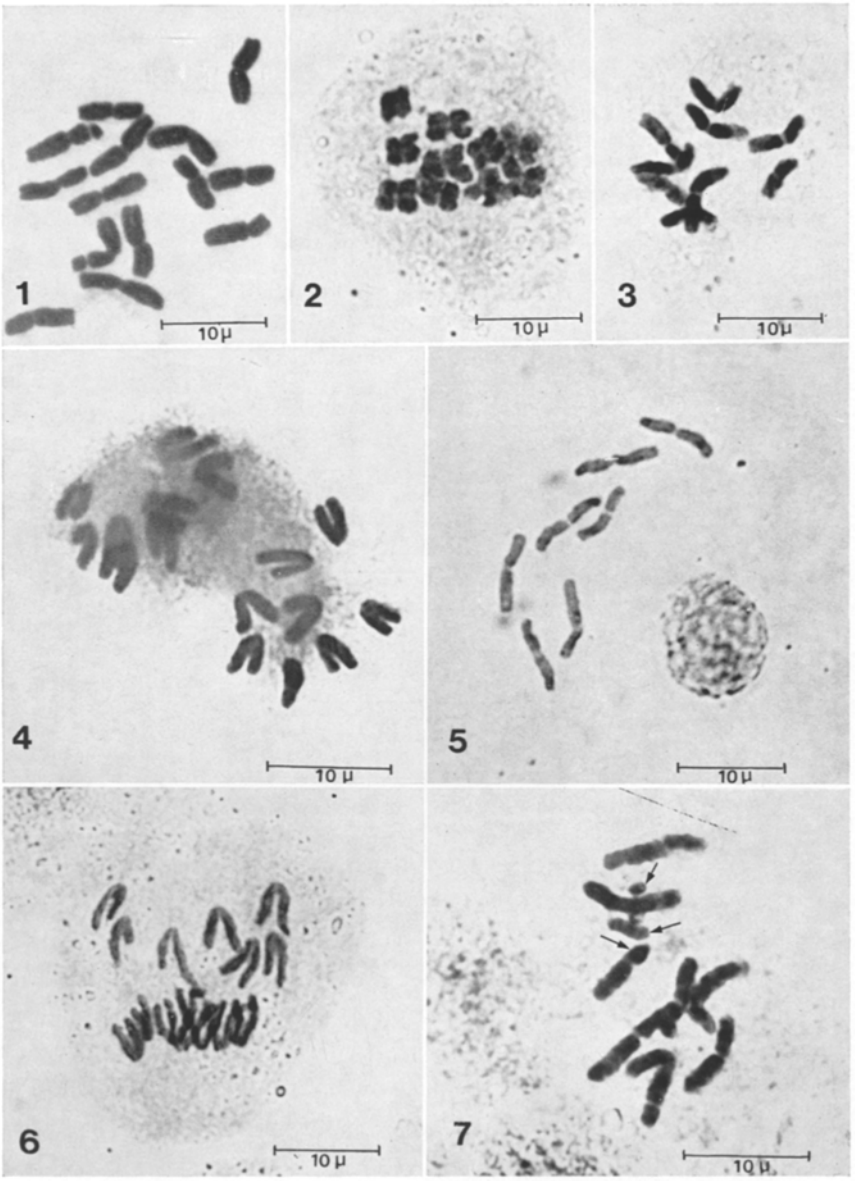
Embryos

The results for embryos with the VB genomic constitution are presented in Table 2. Chromosome numbers varied from 14 down to

Table 2. Chromosome variations and abnormalities in embryos with VB constitutions

Age in days	No. of embryos scored	No. of countable cells with chromosome numbers of								Total No. of countable cells	% of cells with extra-chromatin material	Mean No. of cells per embryo
		7[a]	8	9	10	11	12	13	14			
3	6	3 (42.85)		1		2		1		7	50.30	37
4	13		3		2	2	1	2	1	11	30.26	75
5	14	10 (37.03)	6	4	4	1	1	1		27	16.70	199
6	15	26 (52.00)	14	5	3			1	1	50	9.81	370
7	17	68 (68.68)	16	10	3	1			1	99	9.70	772
8	17	160 (90.90)	11	2	2		1			176	7.98	1178
9	10	177 (77.29)	41	11						229	4.54	2306
10	5	218 (90.45)	13	7	2	1				241	3.39	4710
11	4	431 (93.69)	22	7						460	2.36	7430

[a] Percentages given in parenthesis.



Figs. 1—7. Cells of VB embryos. Figs. 1, 2, and 3. Cells with 14, 10, and 9 chromosomes respectively. Fig. 4. Anaphase disjunction in an 8 chromosome cell. Fig. 5. 7 chromosome cell. Fig. 6. Anaphase distribution of 7 chromosomes. Fig. 7. Cell with 7 chromosomes and 3 fragments (arrows)

7 (Figs. 1 to 6) with an increase in the proportion of cells carrying 7 chromosomes with the advancement of age (3 to 11 days after pollination). The frequency of cells containing 7 chromosomes (the gametic number) changed from 37% at 5 days to 93.7% at 11 days after pollination. Abnormalities such as chromosome fragments (Fig. 7), micronuclei (Fig. 9), a degraded type of chromatin with a granular appearance (Fig. 10) and condensed bodies were frequently found. Different cells in the same embryo showed different abnormalities. All these abnormalities are grouped together in Table 2 under "extrachromatin material". The frequency of cells in which they were observed decreased from 50.3% at 3 days to 2.4% at 11 days. As the age of the embryo advanced, the size of the embryo (*i.e.* the number of cells) increased. Although there were wide variations in embryo size at the same age from the cross, it was apparent that all embryos were continuing to grow.

The changes in chromosome number in dividing cells and the extent of extrachromatin material (predominantly from non-dividing cells) of VB embryos are illustrated in Fig. 8. The initial fluctuation in the percent of cells with 7 chromosomes is likely a reflection of the small numbers of countable cells.

Embryos from the reciprocal cross (BB by VV) showed a similar trend in all aspects of chromosome elimination (data are not included). In embryos with the VBB or BBV genomic constitution most cells contained 21 chromosomes and a few contained fewer than 21 chromosomes (Table 3). Unlike the data on crosses between the diploids, the percentages of cells with 21 chromosomes and of cells with extrachromatin material remained relatively constant in embryos examined from 3 to 9 days after pollination (Fig. 8).

Observations on embryo cells with the VVB and VVBB genomes were made at 5 and 10 days after pollination and abnormalities similar to those of VB embryos were found. In 10 day old VVBB embryos, most cells contained only the gametic chromosome number of 14. Exact chromosome counts were difficult to obtain in such embryos and the actual percentage of cells with various chromosome numbers were not recorded.

Endosperm

Endosperm tissues from crosses (Table 1) giving the genomic constitutions of VVB, VVBB, and BBV, BBBBVV and BBBBV were examined. In endosperm cells with VVB constitution chromosome numbers were quite variable. Some cells with 14 chromosomes (Fig. 11) were seen while others contained up to 28 chromosomes. Endosperm

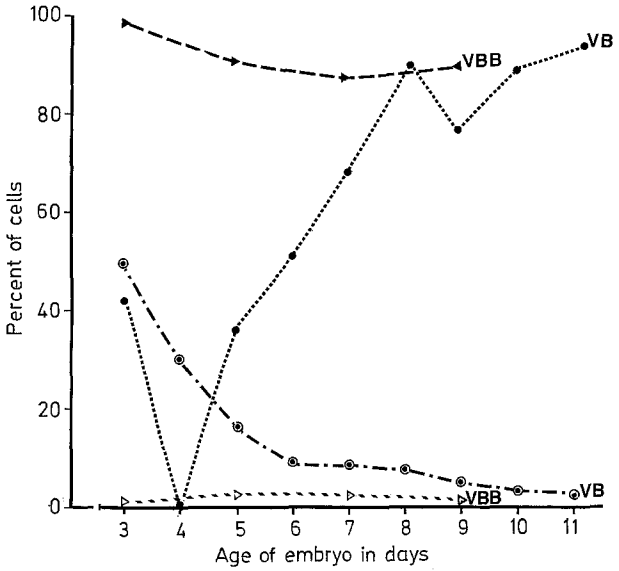


Fig. 8. Percentage of cells from VB embryo with 7 chromosomes (●-----●) and extrachromatin material (⊙-·-·-⊙); and from VBB embryos with 21 chromosomes (▸---▸) and with Extrachromatin material (▷----▷)

Table 3. Chromosome variations and abnormalities in embryos with *VVB* constitution:

Age in days	No. of embryos scored	No. of cells with chromosome numbers of			Total No. of countable cells	% of cells with extra-chromatin material	Mean No. of cells per embryo
		21[a]	20	19			
3	10	12 (100)	—	—	12	0.93	32
5	15	66 (91.67)	6	—	72	2.30	208
7	15	110 (88.70)	12	2	124	3.51	894
9	5	148 (91.36)	13	1	162	1.83	1895

[a] Percentages given in parenthesis.

cells with VVBB constitution showed a similar trend in chromosome number and abnormalities. The endosperm was very poorly developed and possibly depleted by the growing embryo in both of these types.

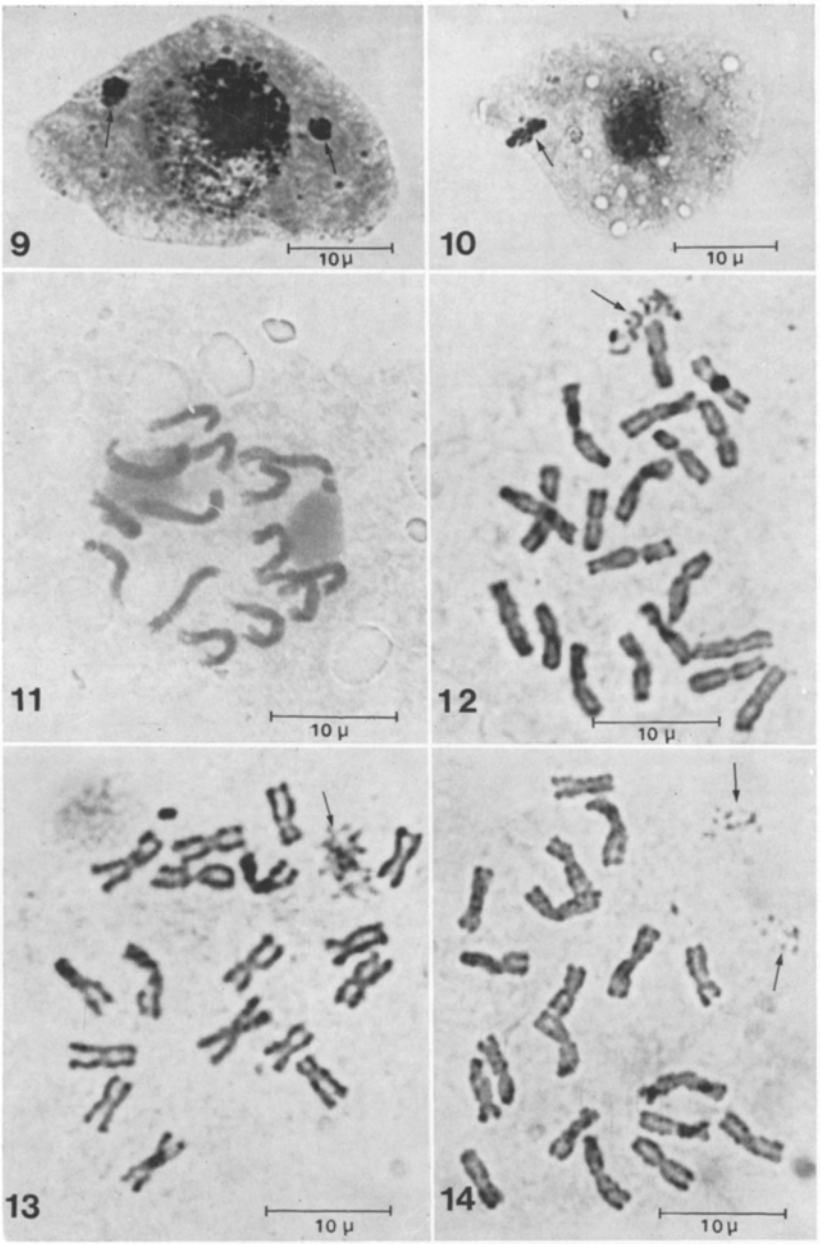
In endosperm with BBV constitution examined 7 days after pollination, accurate chromosome counts were made on 63 cells and 68% of these cells showed 21 chromosomes (Fig. 12), although the range was from 16 to 22. Chromosome fragments and degraded types of chromosomes (Figs. 12–14) were the usual abnormalities and the extent of cells with extrachromatin material was 33.9%. Thus, stability was less than in embryos with the BBV constitution. However, endosperm development was much better than in caryopses with the VVB and VVBB endosperm constitution.

Interesting features of BBV endosperm were the differences in relative chromosome stability and the extent of endosperm development obtained in different caryopses from the same cross. Out of 29 caryopses examined, 7 showed very few chromosomal abnormalities or micronuclei and had well developed endosperm and starch grains, 14 were relatively stable and intermediate in endosperm and starch development, while 8 were quite unstable and poorly developed as in endosperm of the VVBB constitution.

Endosperms with the BBBBVV constitution were examined at 3, 5, and 7 days after pollination and development resembled that of BBV endosperm. Although cells in the dividing stage were visible, the chromosome spread was poor and most cells were broken during squashing because of the presence of starch grains.

Endosperms with the BBBBV constitution were examined at 3, 5, and 7 days after pollination and found to be very well developed compared to any of the other endosperm tissues studied. Even at 3 days after pollination, caryopses were full of starch grains which gradually increased in size (and probably number) with the advancement of age. Development of the endosperm (BBBBV) in 3 day old caryopses was equal to or better than 5 day old endosperm of BBBBVV or BBV genomic constitution. Again, the numerous starch grains rendered chromosome counts impossible.

Abnormalities, such as in Fig. 15 were more frequent and extensive in the VVB and VVBB endosperm than in BBV and BBBBVV endosperm. Endosperm development in different genomic combinations could be rated BBBBV > BBV, BBBBVV > VVBB > VVB. Giant nuclei with several nucleoli (Fig. 16) and nuclei with different sizes (Fig. 17) were common features in endosperm tissue regardless of genomic constitution.



Figs. 9 and 10. Nondividing cells from VB embryos showing micronuclei (arrows Fig. 9) and a degraded type of chromatin at the cell boundary (arrow Fig. 10). Fig. 11. Endosperm cell from VVB constitution showing only 14 chromosomes (note 2 nucleoli)

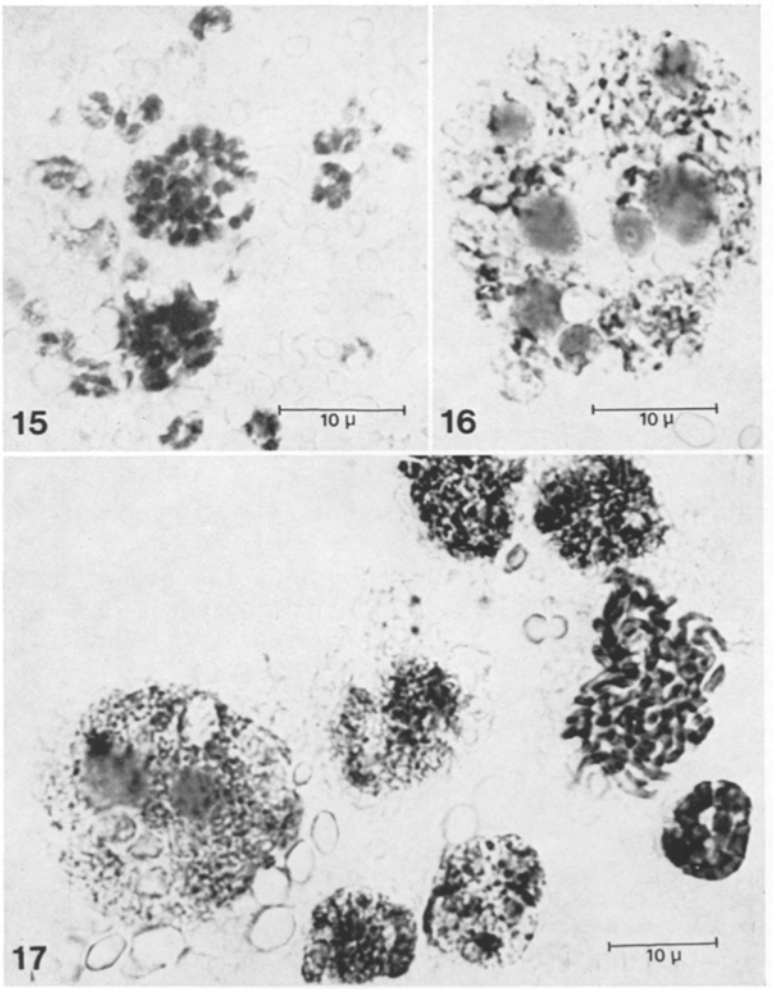


Fig. 15. Endosperm tissue from VVB constitution showing micronuclei

Fig. 16. Giant nucleus of an endosperm cell from VVBB constitution

Fig. 17. Endosperm tissue at expected BBBBVV constitution showing nuclei of different sizes

Figs. 12—14. Endosperm cells from BBV constitution. Fig. 12. 21 chromosomes with 1 chromosome being degraded (arrow). Fig. 13. Showing 17 chromosomes + 1 degraded (arrow). Fig. 14. Cell with 19 chromosomes and 2 degraded fragments (arrows)

Table 4. Progeny obtained from crosses between diploid (2n = 14) and tetraploid (2n = 28) cytotypes of *Hordeum vulgare* and *H. bulbosum*

Cross combination	Seed set		Embryos cultured	Progeny obtained	Genotype and chromosome number			
	%	No.			V (7)	VV (14)	VB (14)	VBB (21)
VV × BB	62	2402	2073	356	355		1	
VV × BBBB	50	301	262	79				79
VVVV × BBBB	31	47	42	8		8		
VVVV × BB	25	38	21	6		6		

Progeny

The progenies obtained from the cultured embryos of different combinations are presented in Table 4. Their chromosome numbers are consistent with earlier reports (Kasha and Sadasivaiah, 1971).

Discussion

The results obtained in this study, where by both embryos and endosperm were examined cytologically at various days after pollination, clearly indicate that double fertilization occurs in all possible cross combinations between diploid and tetraploid cytotypes of *Hordeum vulgare* and *H. bulbosum*. After fertilization there is a gradual loss or elimination of chromosomes in certain genomic combinations as illustrated in Table 2. Here we find cells with 7 to 14 chromosomes and the percentage of cells with 7 chromosomes increases with the age of the embryo. Contrary to the suggestion of Davies (1958), we found no evidence of parthenogenesis in over 100 embryos examined from the cross between diploid forms nor in the embryos examined from crosses involving other ploidy levels. Thus, as was first suggested by Schooler (1963) and Rajhathy (1967), chromosome elimination subsequent to fertilization occurs in certain cross combinations between these two species.

The question of whether elimination involves only *bulbosum* chromosomes or whether both *bulbosum* and *vulgare* chromosomes are being eliminated as suggested by Lange (1969, 1971b) deserves some discussion. During various studies at Guelph we have observed about 4000 progeny from the diploid *H. vulgare* by diploid *H. bulbosum* cross. Roughly 99% were haploids resembling *H. vulgare* and the other 1% are diploid interspecific hybrids. Haploids of *H. bulbosum* have not been obtained even when *H. bulbosum* had been used as the female parent. If the *H. vulgare* as well as *H. bulbosum* chromosomes are being

eliminated we might expect to find haploids of *H. bulbosum* and somatic cells with fewer than 7 chromosomes. We have not observed embryo cells with fewer than 7 chromosomes from crosses between diploid cytotypes or fewer than 14 chromosomes from crosses involving tetraploid *H. vulgare*. Plants with such deficiencies are not expected to survive whereas embryo cells with fewer than 7 chromosomes might be seen if both *vulgare* and *bulbosum* chromosomes were being eliminated.

Although only chromosomes 5, 6, and 7 have been distinguished from each other in these species (Kasha and Sadasivaiah, 1971) somatic cells of haploid and dihaploid seedlings exhibit only the *vulgare* type for these chromosomes. In studies (unpublished) on the production of haploids using marker genes on the *vulgare* chromosomes, the markers are consistently expressed in the haploids.

The observations of Lange (1971a, b) could be interpreted as being consistent with our findings that only *bulbosum* chromosomes are being eliminated. However, both Symko (1969) and Lange (1971b) have indicated that *vulgare* chromosomes might also be eliminated in somatic cells. Symko (1969) obtained monoploids from a cross between diploids of *Hordeum bulbosum* and *H. vulgare* some of which showed characteristics of both *vulgare* and *bulbosum*. He suggested the latter might have originated by nuclear gene hybridity followed by somatic reduction or by male parthenogenesis with differences in plasmagene activity. Lange (1971a) obtained dihaploids and "diploid hybrids" (his designation) from tetraploid crosses and concluded that vegetative segregation resulted in such "diploid hybrids". Although Symko (1969) and Lange (1971a) checked root-tips and sporocytes, neither mentioned shoot-tip chromosome counts or differences in morphology of tillers on the same hybrid. Occassionally, some of the diploid hybrids produced at Guelph from the VV by BB cross have 7 chromosomes in root-tips but 14 in their hybrid shoot-tips (determined by leaf-tip chromosome counts). Furthermore, occassional tillers on some hybrids have been found to be monoploid shoots of the *vulgare* type. Sporocytes from such tillers have been found to be haploid and exhibit the variations in chromosome numbers typical of pollen mother cells as reported by Sadasivaiah and Kasha (1971). It may be suggested that sporocytes of such origin could have led Symko (1969) and Lange (1971a) to erroneous conclusions since these chimeras are difficult to detect at that stage. Our results do not comply with the explanations of Symko (1969) and Lange (1971a, b). We would conclude that selective elimination of *bulbosum* chromosomes leads to haploid progeny of the *vulgare* parent and that there is no conclusive evidence for the elimination of any *vulgare* chromosomes.

A comparable situation may be the selective chromosome elimination in mammalian somatic cell hybrids. Such reports include the loss

of rat chromosomes from rat-mouse hybrids (Weiss and Ephrussi, 1966); human chromosomes from man-mouse hybrids (Weiss and Green, 1967); mouse chromosomes from mouse-hamster hybrids (Handmaker, 1971); and of human chromosomes from human-hamster cell hybrids (Westerveld *et al*,. 1971; Rao and Johnson, 1972).

The stability of chromosomes in hybrids between *H. vulgare* and *H. bulbosum* is influenced by the balance of parental genomes as has been proposed earlier (Kasha *et al.*, 1970; Kasha and Sadasivaiah, 1971). The embryos with a ratio of 1 *vulgare* to 2 *bulbosum* genomes are relatively stable as we have shown in this report while the most stable endosperm had a 4B:1V ratio. This could mean that either a different proportion of *bulbosum* to *vulgare* chromosomes is most stable in embryos as compared to endosperm tissues or that embryos with a higher proportion of *bulbosum* genomes might be even more stable. The different endosperm tissues could be placed in order of chromosome stability and development as follows: BBBBV > BBBBVV, BBV > VVBB > VVB.

More conclusive evidence for genic control comes from Ho (unpublished; Kasha *et al.*, 1972) and Barclay *et al.* (1972) where they have shown that apparent genetic factors on chromosomes 2 and 3 of the *vulgare* parent are controlling the chromosome stability in hybrids of *H. vulgare* and *H. bulbosum*. Our observations on the variation in the extent of stability of BBV endosperm in different caryopses indicate that the genotype of *H. bulbosum* may also be important. Each caryopsis would contain a different but homozygous genotype in the secondary nucleus from the *bulbosum* parent while the genotype from the *vulgare* cultivar York should be consistent in each endosperm. Thus, if the variability is genic it would likely be from the *bulbosum* parent At this stage we would suggest that the control over selective chromosomal elimination resides in genetic factors and their balance in *vulgare* and *bulbosum* chromosomes. The required balance of such factors need not be exactly the same for embryo and endosperm tissues. Further studies on embryos with a higher proportion of *bulbosum* genomes are required, although suitable parents for such crosses are not available.

Further experimentation is also required to determine the mechanism(s) of chromosome elimination and the basis for its genetic control. Based on the observations (both ours and Lange's, 1971 b) of chromosome fragmentation (Fig. 7), micronuclei (Fig. 9), chromatin degradation (Figs. 12–14) and/or extrusion of degraded material (Fig. 10), chromosome elimination is a gradual and somewhat variable process under the genetic control. The mechanism could be related to mitotic disturbances such as genic disharmony between parental genomes as suggested by Lange (1971 b). He studied 3 types of hybrid tissue; endo-

sperm, vegetative and generative and found differences between them which could have been caused by differences in their mitotic rhythms. Differences in cell cycle times have also been proposed as the cause for selective chromosome elimination in somatic cell hybrids in mammals (Rao and Johnson, 1972) and could be a logical reason for different rates of elimination in different tissues in *H. vulgare* by *H. bulbosum* hybrids. For example, although endosperm of the BBV consitution was relatively stable it was much less stable than embryo tissue of the BBV constitution. The early development of endosperm is usually much more rapid than the embryo and it is expected to have a correspondingly faster cell generation time. The 4C nuclear DNA content (considered to have a linear correlation with minimum cell cycle time) of *H. vulgare* and *H. bulbosum* are not different (Benett and Smith, 1971). However, investigations of the relative mitotic cycle time and S-phase duration are required.

Gupta (1969) has shown differences in the presynthetic, synthetic and postsynthetic periods of *Nicotiana plumbaginifolia* and the hybrid derivative of *N. tabacum* by *N. plumbaginifolia* where instability of *plumbaginifolia* chromosomes is exhibited. However, the total durations of the mitotic cycle did not differ. He concluded that the late DNA replication in certain heterochromatic regions of *N. plumbaginifolia* were the cause of such instability. The actual genetic control may involve the timing of the cell cycle processes such as DNA replication or stage initiation. Studies of Plaut *et al.* (1966) on polytene chromosomes of *Drosophila melanogaster* have indicated that all regions of chromosomes are under some central control and that their individual replicative processes are probably phased so as to bring them all into a common synthetic period at sometime during the total replicative cycle. The synthetic period for the entire genome and its regulation by a central mechanism should presumably coincide in time, otherwise it will lead to irregularity in DNA replication of different regions.

Rhoades *et al.* (1967) reported elimination of certain segments with knobs in maize induced by supernumerary B chromosomes. Subsequently Rhoades and Dempsey (1972) have shown genetic evidence of such losses and advanced an argument that faulty replication of heterochromatic segments in the presence of 2 or more B chromosomes leads to nondisjunction or breakage of chromosomes at the second microspore division. Perhaps during mitosis certain regions of the *bulbosum* chromosomes fail to replicate while *vulgare* chromosomes have a precocious replication. Such asynchrony could lead to bridges and breakage in the unreplicated regions of *bulbosum* chromosomes during division, resulting in the failure of these chromosomes to be included in daughter nuclei following cell division. However, such upsets are probably over-

come by a compatible balance of genetic factors of *vulgare* and *bulbosum* as found in triploid embryos.

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