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Chromosomal Variability in Tissue Cultures and Regenerated Plants of *Hordeum**

T.J. Orton
Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan (USA)

Summary. Spontaneous polyploidy, aneuploidy, and chromosomal rearrangements were observed in callus and suspension cultures of *Hordeum vulgare*, *H. jubatum*, and their interspecific hybrid. The extent to which each class of chromosomal variability was present in a culture depended upon differentiated state, age, and history. Cytological and isozymic analysis of subdivided callus cultures revealed spatial segregation of chromosomal variability. Cytogenetic analyses were performed to determine the expression of this in vitro chromosomal variability in corresponding regenerated plant tissues. A complete loss of polyploidy and a decrease in aneuploidy and chromosomal rearrangements were observed. Analyses of specific isozyme activities in regenerates suggested that a quantitative segregation of *H. vulgare* and *H. jubatum* genomes had occurred in tissue cultures of their interspecific hybrid. Possible uses of in vitro chromosomal variability for plant breeding and genetical studies are discussed.

Key words: Plant tissue culture – Cytogenetics – Plant breeding – Barley

Introduction

Variability of chromosome number and morphology in cultured plant cells is a common phenomenon (Partanen 1965; D'Amato 1975; Sunderland 1977). However, no patterns are discernible in the generation and distribution of chromosomal variability among and within plant groups. Even callus cultures derived from different tissues of the same plant are often karyologically different (D'Amato 1975). This phenomenon has some undesirable consequences. For example, genetic and biochemical studies generally assume that somatic tissues are genetically uniform. Therefore, the conclusions of such studies are often weakened by the possibility of unstable chromosome constitution. Potential advantages include the production of novel gene associations and chromosomal mutations useful in basic science and plant breeding (Sunderland 1977).

The accumulation of in vitro variability of chromosome numbers has been associated with a loss of regenerative potential (Muir 1965; Murashige and Nakano 1965; Torrey 1957). Also, regenerating plants are chromosomally less variable than precedent tissue cultures, suggesting that regeneration selects for cells with specific chromosomal constitutions (Sacristan and Melchers 1969; Novak and Vystot 1975). In other reports, however, populations of regenerated plants exhibited variability comparable to that of source cultures (Ogura 1975, 1976; Heinz and Mee 1971). Thus, differences in chromosomal plasticity after regeneration exist between species, explants, and culture systems.

Few reports exist concerning chromosomal variability in cell and tissue cultures of cereal grains. Plants regenerated from scutellum-derived callus of maize (Green and Phillips 1975) and shoot tip of barley (Cheng and Smith 1975) were karyotypically identical to the original explants, although no counts were taken from the source callus. Such variability has been reported, however, in cultures of wheat (Kao et al. 1970; Shimada 1971), rice (Nishi and Mitsuoka 1969), and rye (Asami et al. 1976) where conditions are established for callus induction, growth, and regeneration. Saalbach and Koblitz (1977) regenerated plants with highly variable chromosome numbers from haploid-derived callus of barley.

Hybrids of *Hordeum vulgare* × *H. jubatum* are sterile due to a genome incompatibility leading to a breakdown in microsporogenesis (Murry 1975; Orton unpublished). Preliminary observations of aneuploidy and polyploidy

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(Orton 1979) in tissue cultures of *Hordeum* suggested their use as potential sources of genetic variability. In cultures of sterile intertaxon hybrids, such as *H. vulgare* × *H. jubatum*, it may be possible to isolate and regenerate fertile or novel genetic variants.

The present study will examine the occurrence, degree, distribution, and classes of chromosomal variability in tissue/cell cultures and regenerated plants of *H. vulgare* × *H. jubatum*. The possible significance of these phenomena as resources for studies in plant genetics and breeding will be discussed.

Materials and Methods

Plants or seed stocks of *Hordeum vulgare* (HV, 2n = 2x = 14), *H. jubatum* (HJ, 2n = 4x = 28), and their interspecific hybrid (HV × HJ, 2n = 3x = 21, HV as female parent), were provided by Dr. J.E. Grafius, Dept. of Crop and Soil Sciences, Michigan State University. The isolation, maintenance and nomenclatural designation of callus and suspension cultures were described previously (Orton 1979). All tissue was maintained on B5 basal medium (Gamborg et al. 1968) supplemented with 4 mg/l 2,4-D and 3% sucrose (W:V) at pH 6.0. Cultures derived from immature ovarian tissues were used exclusively in this study.

The following cytological techniques were employed: (1) Root tip and type A callus tissues were pretreated optionally with .05% aqueous colchicine for 2 hours and fixed in fresh acetic ethanol (1:3) under vacuum at 25°C for 24 hours. Callus and root tips were hydrolyzed in 1N HCl at 60°C for 25 to 35 min. and 10 to 15 min. respectively, stained with Feulgen reagent for 1 hour, and squashed with a coverslip in 45% acetic acid. (2) Tissues of type E callus and suspension cultures were pretreated optionally with .05% aqueous colchicine. Fixing and staining were performed concomitantly by immersing live tissue in modified carbol fuschin stain (Kao 1975) for 5 minutes. The preparation was then macerated and squashed with a coverslip. (3) Immature ovary wall tissues and pollen mother cells were prepared by removing spikes of the proper stage and fixing as described above, using no pretreatments. Whole ovaries were excised, stained in aceto carmine for 30 seconds, and squashed. Anthers were macerated to liberate pollen mother cells, stained in aceto carmine and squashed following the removal of excess debris.

It was more difficult to make accurate counts on cells with progressively higher chromosome numbers. Thus, if all cells with uncertain metaphase chromosome counts had been excluded, the data may have become biased in favor of lower chromosome numbers. To alleviate this possibility, the following procedure was adopted: Each cell was tallied three times. If the difference of the highest and lowest of three counts exceeded 10% of the mean, the datum was discarded. Means and ranges, to the nearest whole number, were recorded for each cell within tolerance. For each experiment, a coefficient of the degree of certainty (DC) was calculated as follows: $DC = 1 - \Sigma(\text{high} - \text{low count})/\Sigma(\text{mean})$. With three exceptions, this value ranged from .9 to 1.0.

Analyses of variance of chromosome numbers were performed to examine the path of in vitro chromosomal variability into regenerated plants, using a nested design. For regenerating root tips, counts were partitioned to callus and root as follows: (1) root tips from primary callus; 4 calli × 3 roots per callus × 3 counts per root, (2) root tips from secondary type E-derived type A callus; 5 calli × 3 roots per callus × 3 counts per root. Counts from ovary wall tissue were partitioned as follows: 8 plants × 2 tillers per plant × 3 florets (ovaries) per tiller × 3 counts per ovary.

A method was devised to demonstrate the existence of chromosomal rearrangements based on comparative length ratios of longest to shortest (L/S) chromosomes. This procedure was first suggested by Shimada and Tabata (1967). It was assumed that the extent of chromosomal contraction may have varied from cell to cell, but was consistent over all chromosomes within a given cell (L/S ratio should be invariant in tissues of invariant karyotype). Chromosome lengths were measured with an ocular micrometer. The control ratio, determined from metaphase plates of 20 root tip cells in the original HV × HJ F_1 hybrid, was 2.04 ± 0.46 (mean ± standard error, S.E.). If the L/S ratio was significantly less than 2.04 in a sampled population, it could be concluded that either chromosomal rearrangements or whole chromosome loss were responsible (the loss of a longest or shortest chromosome would depress this ratio). If the L/S ratio was significantly greater than 2.04, however, it could be concluded that chromosomal rearrangements (translocations, deletions, duplications, etc.) had occurred.

To determine the possibility of isolating and propagating karyotypic variability, subcalli of 200 to 400 cells each were physically removed from a lawn of HV × HJ type E callus with a pair of fine forceps and propagated separately. Smaller aliquots of cells appeared to result in a depression of callus growth. After six weeks, cells were removed from each of the 35 subcalli and prepared for cytological examination as described above. Twenty chromosome counts were taken from each culture. From the remaining cells of each subcallus, six subsubcalli of 200 to 400 cells each were isolated, and cultured separately for another six weeks. Of the 210 subsubcalli, equal aliquots of tissue were removed and prepared for starch gel electrophoresis as described below (Fig. 1).

Crude extracts of type E subcalli were prepared, electrophoresed, and stained for peroxidase (PRX) and esterase (EST) isozymes to detect isozymic variability among subsubcalli. Similarly, crude extracts of young leaf tissue were electrophoresed and stained for EST and glutamate-oxaloacetate transaminase (GOT) activities among plants regenerated from HV × HJ type A callus. Equal amounts of tissue were crushed and the crude extract taken up into Beckman electrophoresis paper wicks cut into equal sizes. Using a pair of fine forceps, the wicks were inserted into a transverse cut through a 12% starch gel in .015M tris + 0.03M citrate. The tray buffer, .3 M boric acid at pH 7.8, was conducted onto the gel through pellon. For sample elution, a potential of 100V was applied to the wicks for 30 minutes. The wicks were then removed and electrophoresis continued at 8 watts per gel until the anodal borate front had migrated 10 cm from the origin. Gels were maintained at 0 to 1°C with ice water and circulated air in a Paige Instruments electrophoresis chamber. After running, gels were removed immediately, sliced in half horizontally and stained: PRX; the bottom cathodal slice was immersed in .5 mg/ml 3-amino-9-ethyl carbazole, .045% H_2O_2, and .03 M $CaCl_2$ in .05 M sodium acetate buffer at pH 4.5 for one hour. EST; the bottom anodal slice was placed in a solution of .03% α napthylacetate, .03% β napthylacetate, .03% α napthyl valerate, and 1.5 mg/ml fast blue RR salt in .1 m tris buffer at pH 7.0 for 2 hours. GOT; the top anodal slice was immersed in 2 mg/ml L-aspartic acid, 1 mg/ml L-glutamic acid, .1 mg/ml pyridoxal phosphate, and 1.5 mg/ml fast blue BB salt in .1 M tris buffer at pH 8.0 for 2 to 3 hours.

Results

No variation from the expected 21 was observed among 20 chromosome counts of HV × HJ root tip cells. In

previous studies on microsporogenesis of this hybrid, however, chromosome numbers were observed to vary from 12 to 22 (Rajathy and Morrison 1959; Murry 1975) and exhibited 12% aneuploidy (Murry 1975). It is hence conceivable that in vitro variability of chromosome numbers was, in part, pre-existent in the original explants of HV x HJ. Chromosome counts from root tips of HV and HJ were invariably 14 and 28 respectively.

Differences in mean chromosome number and coefficient of variation (V) were found to exist between cultures and over time (Table 1). Figure 2 illustrates the dynamics of chromosome numbers over time in cultures of HV x HJ. The mean chromosome number of type A callus remained stable at approximately 18 to 21 over the period studied (16 months, starting from callus induction). Type E callus was spontaneously generated from type A nodes after six months of subculture. Mean chromosome numbers in this type E callus increased drama-

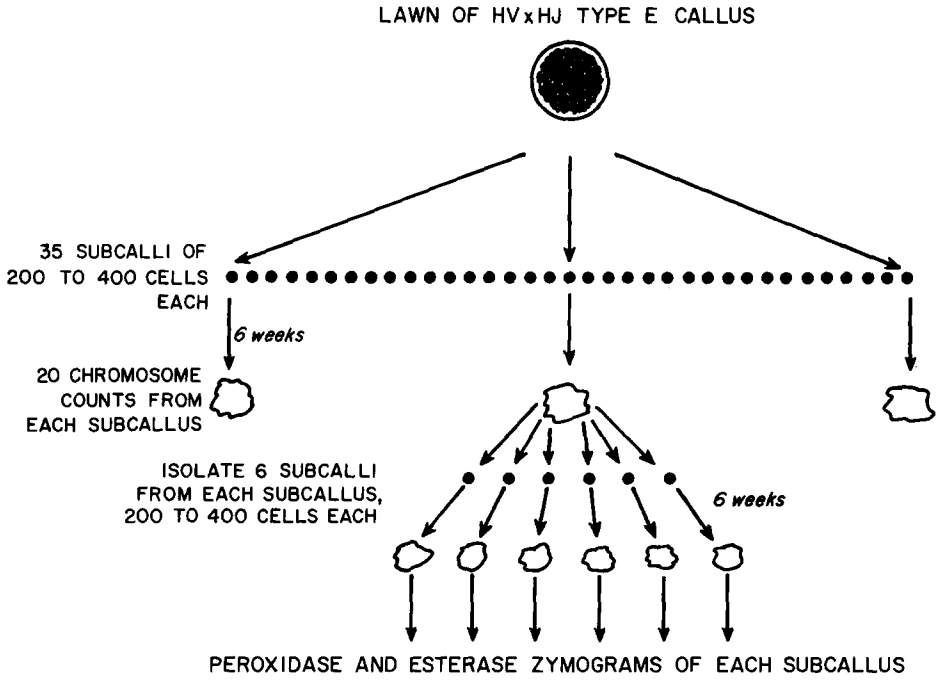

Fig. 1. Procedure used for isolating and propagating karyotypic variability

Table 1. A comparison of means and distributions of chromosome numbers among cultures derived from HV X HJ

Culture	Age months	Mean chromosome number	DC	Standard error	V	N
Type A	6	19.03	.92	.317	.090	30
Type A	10	21.19	.92	.748	.154	20
Type A	15	18.10	.93	2.579	.767	30
Type E, primary	10 (6)[a]	54.56	.92	6.260	.538	23
Type E, primary	15.5(6)	34.95	.93	.710	.541	710
Suspension	8 (7,6)[a]	48.00	.88	5.564	.478	18
Suspension	10 (7,6)	50.38	.92	3.758	.365	25
Suspension	12 (7,6)	38.43	.94	1.004	.131	25
Type E, secondary	11.5(9,7,6)[a]	57.12	.95	5.565	.390	16
Type E, secondary	13 (9,7,6)	52.67	.92	4.795	.490	30

[a] Age of initial callus induction, followed in parentheses by the ages at which previous transformations were induced or occurred spontaneously [11.5 (9,7,6) implies type E secondary callus 11.5 mo. old from original callus induction, transformation to type E callus at 6 months, to suspension at 7 months, and to secondary type E at 9 months]

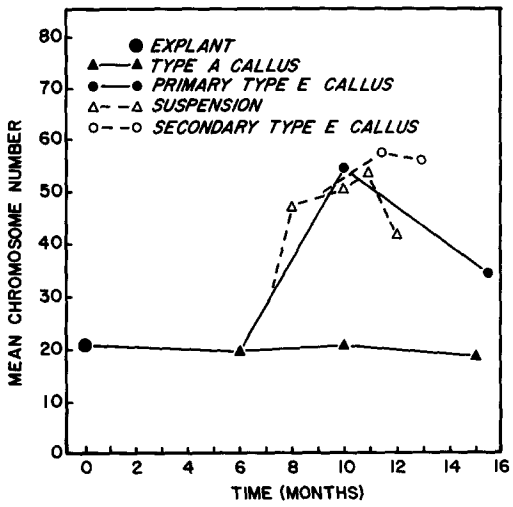

Fig. 2. Dynamics of mean chromosome number from callus induction (time = 0) to 16 months

tically to approximately 54, then decreased and stabilized at 35 to 36. Initially, mean chromosome number increased even more dramatically over time in suspension cultures generated from type E calli. This rapid increase was followed by a plateau, and rapid decrease as in type E callus. When these suspension cultures were plated onto solid media, secondary type E calli were formed. The mean chromosome number increased to approximately 58, levelled off, and then decreased gradually. Examples of typical metaphase plates are pictured in Fig. 3.

A gradual, exponential increase in the coefficient of variability of chromosome numbers V was observed over the 16 month period in type A calli (Fig. 4). Type E callus exhibited a rapid increase in V that levelled off and stabilized after 4 months. In suspension cultures derived from type E callus the pattern was similar, only more rapid, as with respective mean chromosome numbers (Fig. 2). Unlike primary type E callus, however, the coefficient of variation decreased rapidly. In secondary type E calli, V decreased at a slower rate than the source suspension culture, followed by a gradual increase.

The distributions of chromosome counts of type A callus were approximately normal and in the euploid range (Fig. 5). A comparison of the distributions at 6 and 15 months indicated that while the mean was stable, variability tended to increase. Type A callus, therefore, exhibited aneuploidy, but no polyploidy. The distribution of chromosome numbers for type E callus at 16 months, however, was not normal and appeared to be three to four modal (Fig. 5). An approximately equal number of counts were centered on clusters at 20 to 22 and 38 to 40 with minor clusters at 70 to 75 and 140 to 150. The pattern of these clusters approximated a hypoeuploid series with flanking aneuploidy. Polyploidy and aneuploidy were also prevalent in suspension cultures, with major clusters at 30 to 40 and 60 to 75 (Fig. 5D). These data were taken from a relatively young culture which had probably not reached steady state (see Discussion section for explanation). In secondary type E callus, polyploidy and aneuploidy, and clusters of cells with chromosome numbers of 20 to 25 and 54 to 63 were observed.

It is possible that variability of chromosome numbers in cultured cells of HV × HJ was a consequence of parental genome interactions in the hybrid nucleus and not

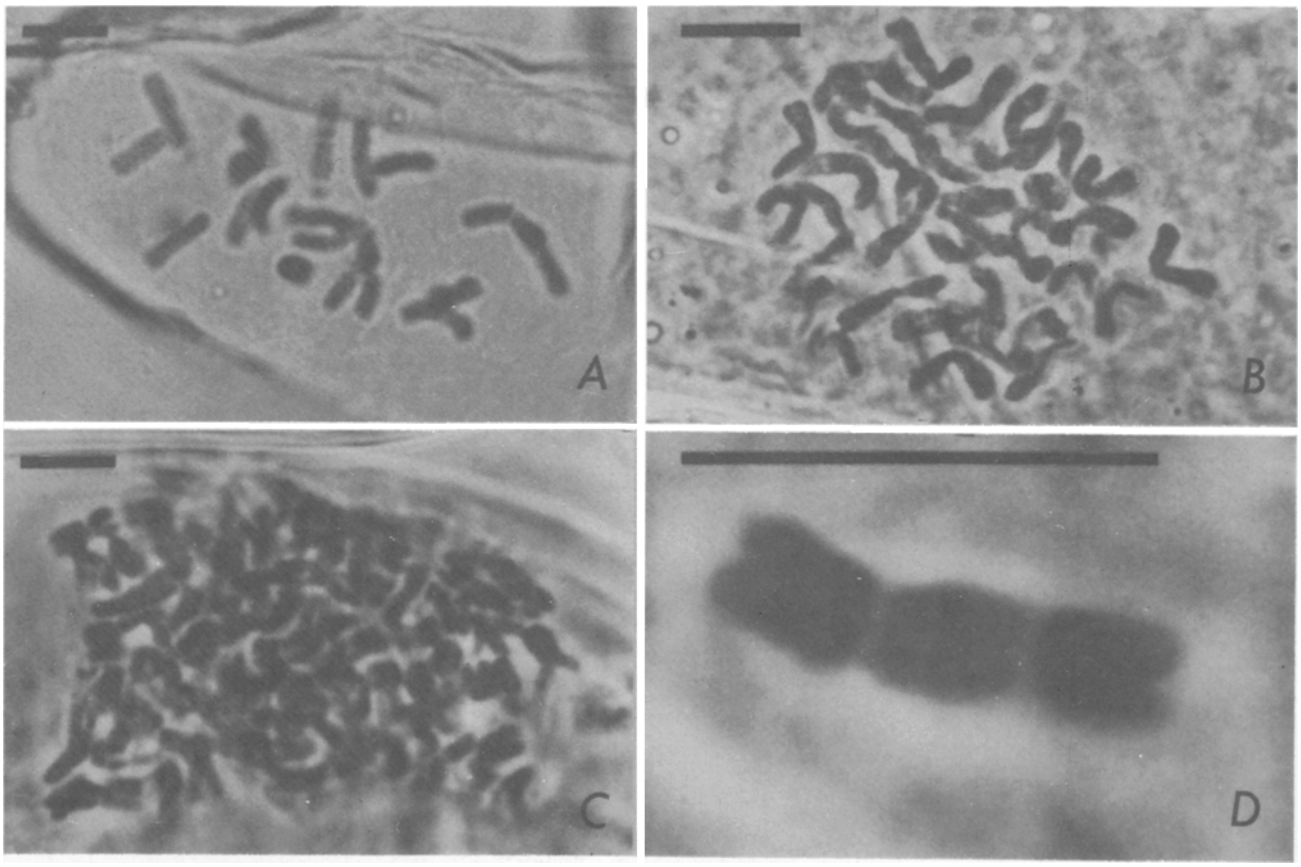

Fig. 3A-D. Karyotypic variability in tissue cultures of *Hordeum*: **A** type E callus cell of HV × HJ with 19 chromosomes; **B** suspension culture cell of HV × HJ containing approximately 36 chromosomes; **C** highly polyploid cell of HV × HJ type E callus; **D** Dicentric chromosome (Bar = 10 μm)

of the cultured state itself. Variation in chromosome numbers was observed in pollen mother cells of HV X HJ (Rajathy and Morrison 1959; Murry 1975), but not in tissues of HV or HJ. Immature ovaries of HV and HJ were introduced into culture in a manner identical to HV X HJ, yielding callus types described previously (Orton 1979).

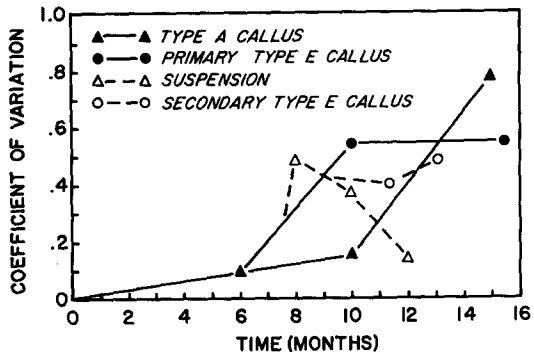

Fig. 4. Dynamics of the coefficient of variation (V) of chromosome numbers from callus induction (time = 0) to 16 months, vs. culture state

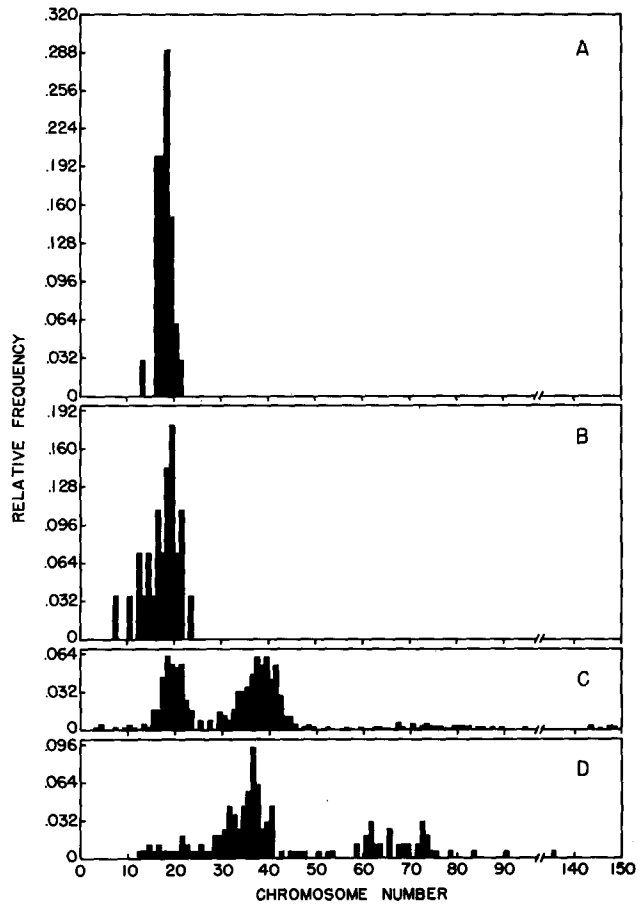

Fig. 5A-D. Composite distributions of chromosome numbers in tissue cultures of HV X HJ. A type A callus, 6 months; B type A callus, 15 months; C primary type E callus, 15 months; D suspension culture, 11 months

Chromosome numbers for cultures of HV and HJ are presented in Table 2. All callus types and suspension cultures exhibited aneuploidy and/or polyploidy.

Preliminary examinations of chromosomal morphology in cultured cells revealed an array of novel types, particularly of increased size differentials and multicentrics (Fig. 3D, Table 3). The frequency of their occurrence in the karyotypes of cultured cells (over 90% of HV X HJ suspension cells with one or more multicentrics) suggested that chromosomal rearrangements might constitute another source of karyotypic variability. For all cultures, i.e. type A and E callus and 11 and 27 month old suspension cultures, mean L/S ratios were significantly greater than the control (Table 3)[1]. The magnitude and variability of L/S was also greater in both type E callus and suspension cultures than in type A callus.

Table 2. Summary of chromosome numbers in tissue cultures of HV and HJ

Source plant	Culture type	Mean chromosome number	DC	Standard deviation	Range	N
HV	B	35.44	.89	–	26 - 51	9
HV	B	31.46	–	–	12 - 44	13
HV	Suspension	60.12	.95	33.45	24 - 171	25
HJ	A	27.56	–	2.17	25 - 30	24
HJ	A	26.37	–	2.54	20 - 30	19
HJ	C	23.40	.89	5.69	21 - 28	15
HJ	Suspension	43.26	.91	14.44	19 - 83	23

Table 3. Comparison of L/S ratios in cultured tissues of HV X HJ

Tissue	Mean chromosome number	DC	Mean L/S	V L/S	Mean no./cell: dicentrics	tricentris	fragments
Root meristem, origin HV X HJ hybird	21	1	2.04	.10	0	0	0
Type A callus	18.10	.93	2.15[a]	.28	.17	0	0
Type E callus	34.95	.93	2.30[a]	.24	1.12	.06	.47
Suspension 11 months	57.74	.92	3.04[a]	.52	1.87	.06	.35
Suspension 27 months	32.35	.94	2.86[a]	.41	.91	.15	.11

[a] Significant at p = .001

1 The distribution of L/S for the original HV X HJ hybrid was used in all contrasts

Among the 35 subcalli isolated from HV × HJ type E callus, mean chromosome numbers ranged from 19.50 to 59.95 and standard deviations from 2.66 to 34.70. A single factor analysis of variance was performed to test the hypothesis that all of the means were equal. Variance attributable to 'between subcalli' was highly significant at P = .001. Specific isozyme bands from crude extracts of subsubcalli were completely missing in certain cases, but most of the variability of activity could be attributed to comparative quantitative differences in band intensity. These differences in intensity were probably genetic and not epigenetic in origin, since type E callus is stable and uniform. No variability of band expression was observed among zymograms of replicate controls (bulk subculture of original callus lawn).

Roots regenerating from callus cultures exhibited a mean of 16.94 compared to 18.10 in the source callus and 21.00 in the original explant (Table 4, Fig. 7). Chromosome counts ranged from 13 to 20 among 36 cells and the statistical variance was significantly less than that of the source callus. The distribution of chromosome numbers from immature ovary wall tissues of mature plants regenerated from the same culture was approximately normal with a mean of 20.47 (Fig. 1C). Counts ranged from 12 to 28 in this sample of 144 cells, and the statistical variance was significantly greater than that of regenerating root tips (Table 4).

Spontaneous type A-like nodes arose at a very low frequency in lawns of secondary type E callus, generated from rapidly growing, (doubling time = 48 hrs.), finely divided, long-term suspension cultures. Types E and A callus differed dramatically in karyology; type E callus cultures exhibited extensive polyploidy, aneuploidy, and chromosomal rearrangements whereas type A callus exhibited only, and to a less extent than type E callus, aneuploidy and chromosomal rearrangements. Complete karyotypic analysis of callus tissue was not possible due to the rarity of the event but three counts obtained ranged from 18 to 21. Chromosome counts of root tips regenerated from these nodes clustered around a mean of 20.00 with no polyploidy (Fig. 8). Longest: shortest chromosome length ratios (L/S) were calculated from cells of root

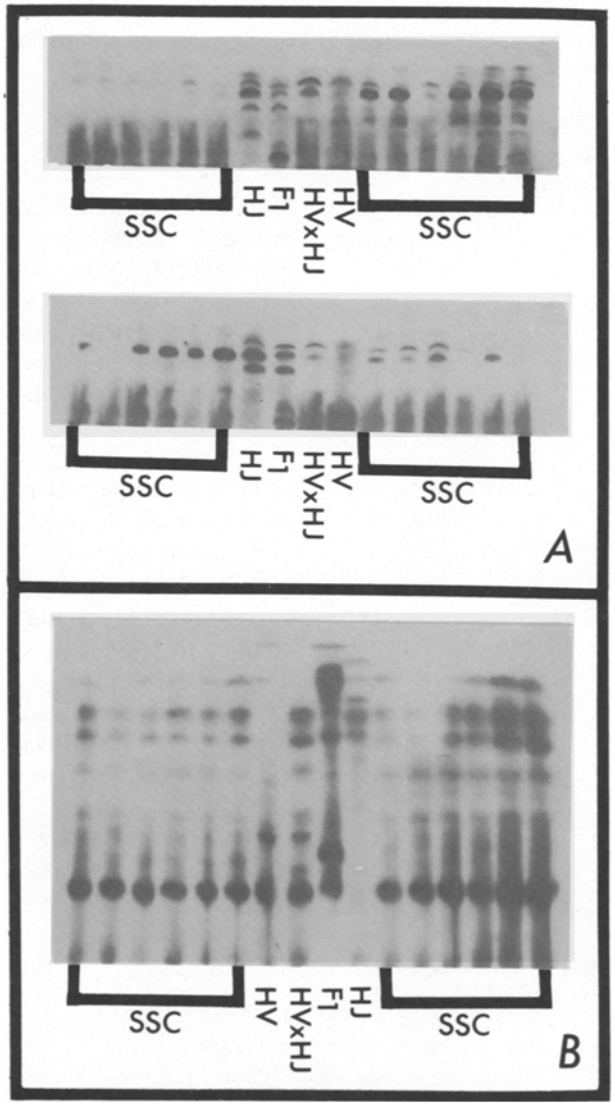

Fig. 6A and B A Cathodal PRX and B anodal EST zymograms showing quantitative and qualitative differences in band expression among subsubcalli of HV X HJ type E callus. Explanation of labels: SSC; subsubcalli grouped according to subcallus source, HV; type E callus of HV, HV X HJ; type E callus of HV X HJ (control), F_1; Plumule extract from the original HV X HJ F_1 hybrid, HJ; type E callus of HJ

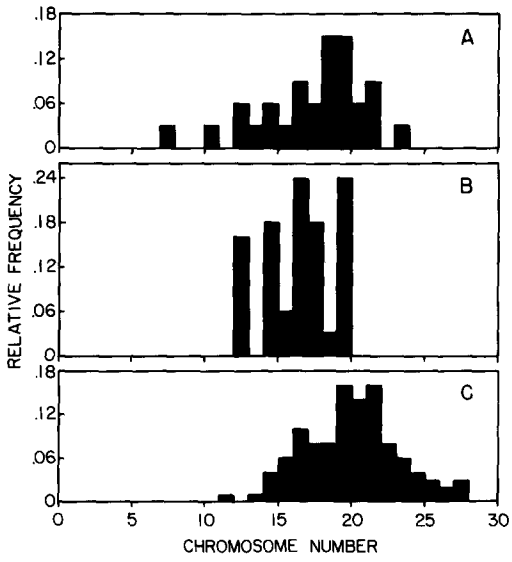

Fig. 7A-C. Composite of distributions of chromosome numbers from A HV X HJ type A callus; B roots regenerating from HV X HJ type A callus; C and immature ovary wall tissue in HV X HJ regenerates

Table 4. Comparison of chromosomal rearrangements between in vitro and corresponding regenerated tissue

Tissue	Mean chromosome number	DC	V chromosome number	Mean L/S	V L/S	Mean no./cell		
						dicentric	tricentric	fragment
Root meristem, source plant	21	1	0	2.04	.10	0	0	0
Type A callus	18.10	.93	.77	2.15[a]	.28	.17	0	0
Regenerated root, primary type A	16.94	.96	.13	2.03	.25	0	0	0
Suspension, 11 months	57.74	.92	.66	3.04[a]	.52	1.87	.06	.35
Regenerated root, secondary type A	20	.94	.16	2.14[a]	.27	.13	.08	0

[a] Significant at P = .001

tips regenerated from primary and secondary callus as previously described to determine if rearranged chromosomes were present in the cells of regenerates (Table 4). The mean L/S ratio had decreased slightly from 2.15 to 2.03 among root tips regenerated from primary type A callus, suggesting possible selection for cells without rearranged chromosomes. Mean L/S in root tips regenerated from secondary type E-derived type A callus decreased from 3.04 to 2.14. The mean of this sample was significantly greater than 2.04 (the L/S distribution of the original HV × HJ hybrid), and multicentric chromosomes were observed, suggesting that chromosomal rearrangements were present in some of the cells of regenerated root tips. Thus, the process of regeneration was manifested by intense, but not always complete, selection for cells with unrearranged chromosomes.

To gain an insight into the pathway of chromosomal variability into regenerated plant tissues, the variances of chromosome numbers were partitioned and tested for significance. The variances attributable to between calli and within calli between roots were highly significant for roots regenerated from primary and secondary type A callus. Likewise, for ovary wall tissues of mature regenerated plants, all partitioned variances (i.e. between plant, within plants between tillers, and within tillers between ovaries) were highly significant. Variability of chromosome numbers within tissue was observed in all cases.

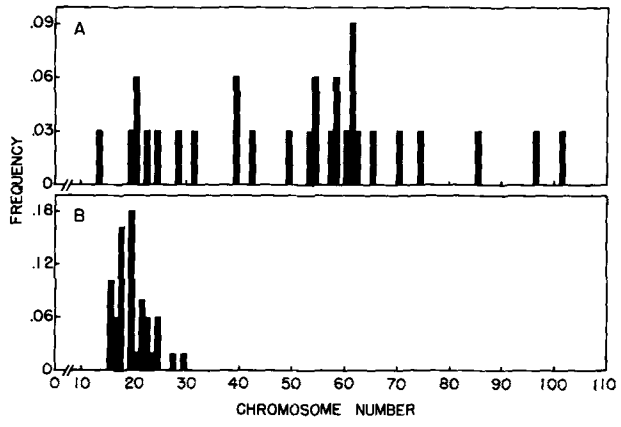

Fig. 8A and B. Distributions of chromosome numbers in **B** root tips regenerating from secondary type A callus and **A** in the HV × HJ progenitor suspension culture

Certain regenerates from HV × HJ type A callus exhibited meiotic configurations with a high degree of chromosome pairing (Fig. 9, Table 5). Studies of microsporogenesis in the original HV × HJ F_1 hybrid found nearly complete asynapsis (average .5 to 1.1 bivalents per cell, Rajathy and Morrison 1959). A comparison of metaphase I pairing configurations between selected regenerates of HV × HJ type A callus and the HV × HJ F_1 hybrid is presented in Table 5. Generally, increased pairing was manifested by an increase in bivalents, trivalents, etc., and a decrease in univalents as compared with the original hybrid.

Comparisons of anodal EST and GOT zymograms among HV, HJ, and HV × HJ from crude extracts of

Table 5. Comparisons of chromosome associations among four regenerated HV × HJ plants

Plant	No. cells	Mean chromosome number	Mean no./cell				
			I	II	III	IV	V
HV × HJ	–	20.99	19.85	1.08	.06	0	0
1	13	17.46	15.77	.62	.15	0	0
2	6	21.50	9.33	3.67	.67	.50	.17
3	6	21.50	12.17	4	.67	0	0
4	5	16.20	10.40	.80	1.40	0	0

young leaves yielded 5 HV-specific, 3 HJ-specific, 1 comigrating (present in both parents), and 1 hybrid band (present in neither parent) (Fig. 10). The observation of three GOT bands in the hybrid and only one in each parent suggested that this particular isozyme is a homodimer. Forty-three plants regenerated from HV × HJ type A callus culture were visually scored for relative EST and GOT isozyme activities as compared to those of the HV × HJ F_1 hybrid (control). Replicated samples did not generally differ from each other (Fig. 11). For each plant, the genome constitution was estimated by calculating a ratio of (ΣHJ relative isozyme activities: ΣHJ relative isozyme activities). In control plants, this ratio was .6 (3/5). The distribution of this ratio was bimodal in a continuum from 0 to greater than 1.0 (Fig. 12).

Morphological variability in phenotypes such as growth habit, growth rate, size, head morphology, and auricle expression was observed among these regenerated plants. Variability in head morphology was observed in awns (length and spines), number of rows per rachis (from 2 to 6), and average palea length, and auricle morphology ranged from HV-like, through the entire spectrum, to HJ-like. HV-like plants were observed in a higher frequency than HJ-like plants.

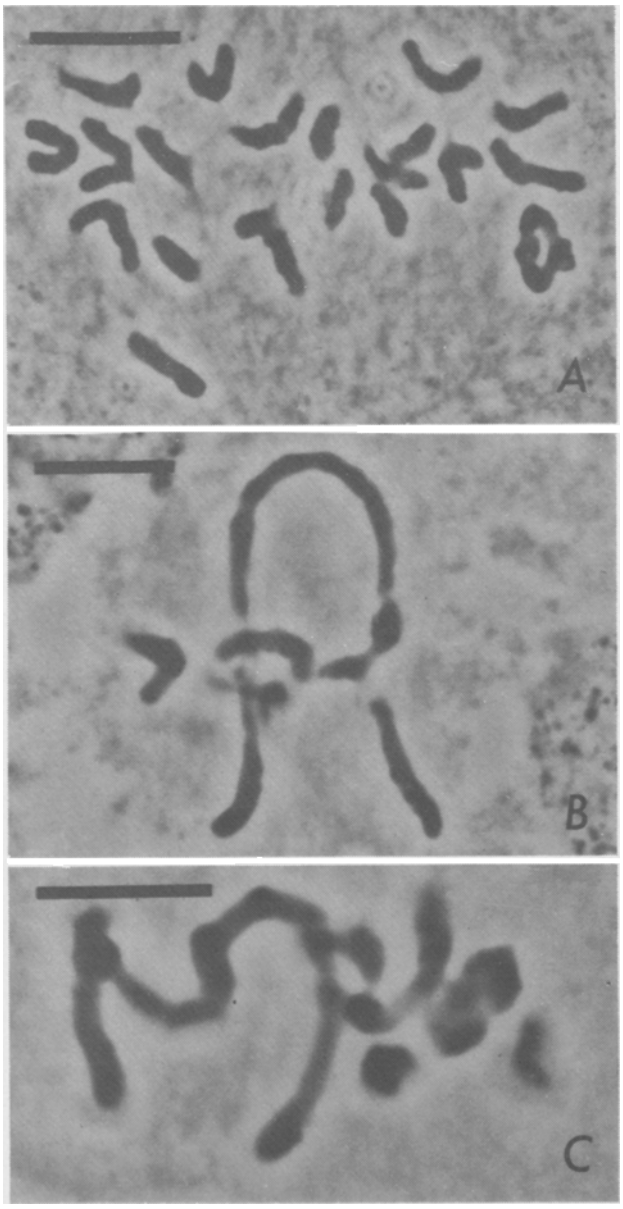

Fig. 9A-C. Comparison of metaphase I chromosome associations between the original HV × HJ hybrid and HV × HJ regenerates: A metaphase I in the original HV × HJ hybrid with one bivalent; B and C HV × HJ regenerates exhibiting multivalents (Bar = 10 μm)

Discussion

Callus cultures of *Hordeum* formed stable 'types' shortly after callus induction (Orton 1979), which expressed dif-

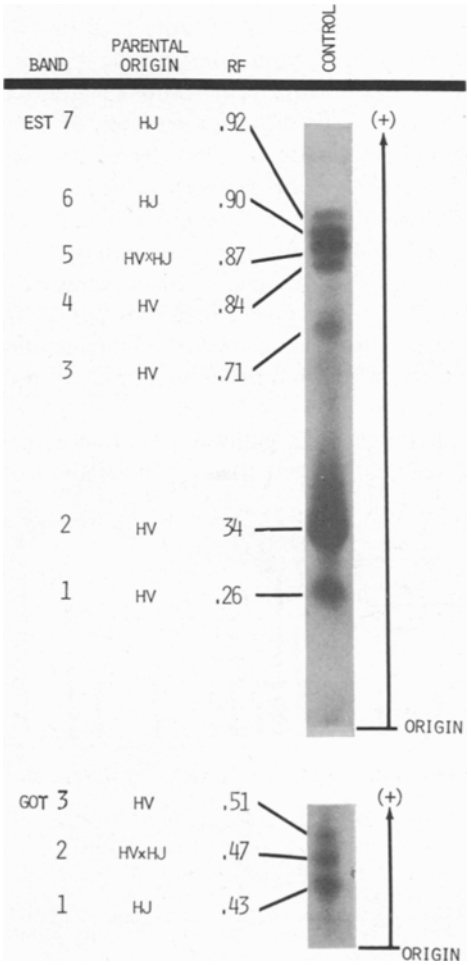

Fig. 10. Nomenclature for GOT and EST zymograms of crude extracts from young leaf tissues of HV × HJ regenerates

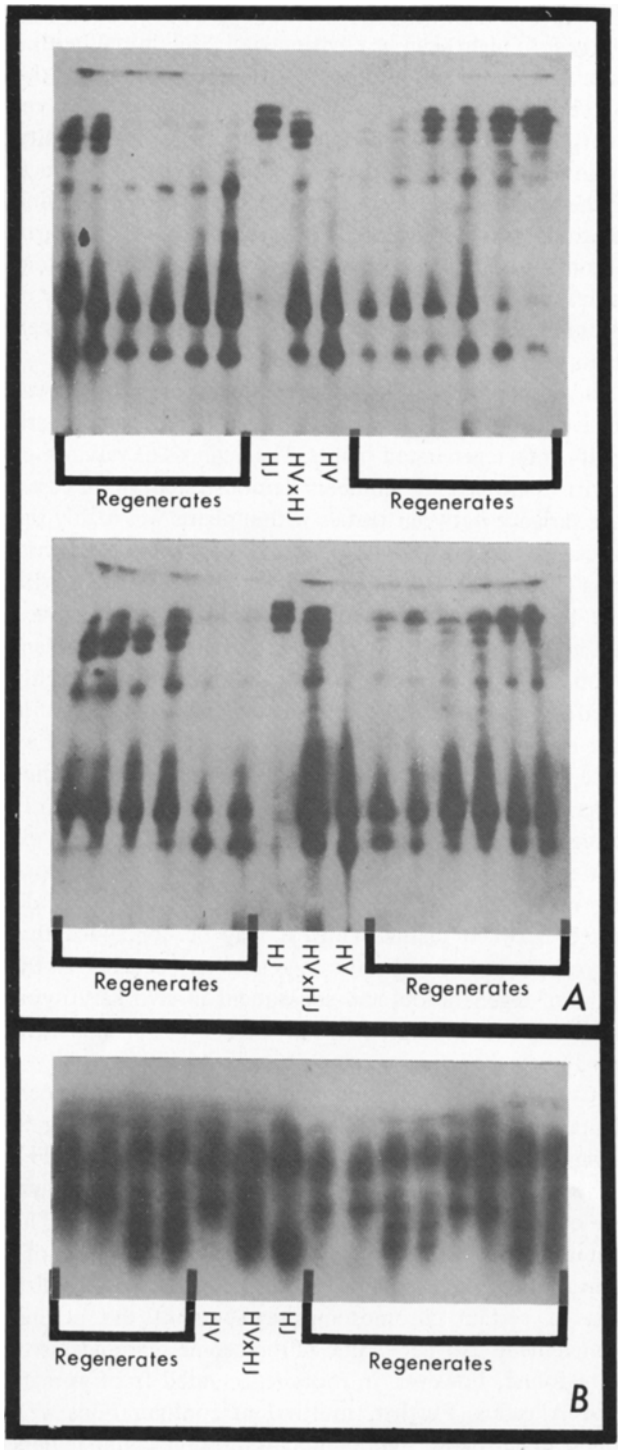

Fig. 11A and B. Typical examples of A EST and B GOT zymograms among HV X HJ regenerates showing quantitative and qualitative differences in isozyme activities. Zymograms of regenerates are replicated in adjacent pairs

ferences in the mean and variance of chromosome numbers independent of explant source. The observation of variable chromosome numbers in cultures of HV and HJ suggested that genome interactions were not responsible for chromosomal variability in HV x HJ cultures. Hence, chromosomal variability was spontaneously generated as a consequence of the interaction of explanted *Hordeum* tissues with culture conditions and was manifested in three distinct classes: polyploidy, aneuploidy, and chromosomal rearrangements.

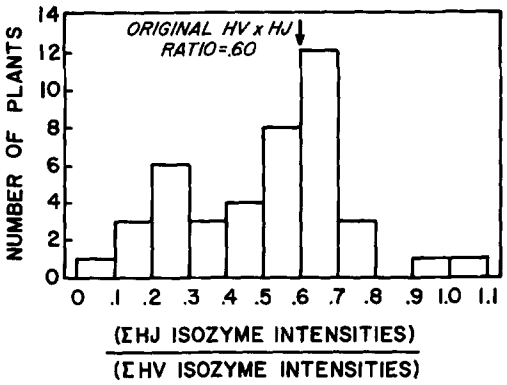

Fig. 12. Histogram of the distribution of ΣHJ:ΣHV relative isozyme band activities, grouped as follows: 0 to .1, .1 to .2, etc.

Type A callus cultures were characterized by a stable mean chromosome number over time, usually diploid or slightly hypodiploid, in an approximately normal distribution. Aneuploidy accumulated in these cultures over time (Figs. 5, 6) and a significant degree of chromosomal rearrangements was detected at 16 months (Table 3). Primary type E calli frequently arose spontaneously on the periphery of type A callus. Concomitant with this transformation was a dramatic change in the mean, coefficient of variation, and the distribution of chromosome numbers of primary type E callus. After increasing and reaching a plateau, mean chromosome numbers attenuated, and stabilized at 35 to 36 while the coefficient of variation increased to a plateau. A distribution of clusters in a hypoeuploid series was generated (hypo-3x, hypo-6x, hypo-12x, etc.) and hypo-3x and hypo-6x clusters predominated (Fig. 6C). Chromosomal rearrangements in primary type E callus, as manifested by mean L/S ratios, increased slightly in comparison to type A callus (Table 3). The dynamics of mean and V of chromosome numbers for suspension and secondary type E callus cultures were similar to primary type E callus, from which they were derived. The rate of the process, however, appeared to have been increased or decreased. Cytological and isozymic analyses of type E subsubcalli revealed spatial segregation of chromosomal variability.

Differences between types A and E callus in tissue organization and growth rate (Orton 1979) were compatible

with corresponding differences in karyology. Cells of type A callus were highly associated by an intercellular matrix and retained some tissue organization. It is possible that this developmental state favored the proliferation of cells in a very narrow range of chromosomal variability. Conversely, the cells of type E callus were not associated and exhibited no apparent organization, thus permitting the generation a much expanded array of chromosomal variability.

The observed distribution of chromosome numbers in these cultures could be visualized as an interaction of cell or tissue growth potentialities, as described above, with a challenging environment. Assume that chromosomally variant cells were generated by the culture at a constant rate per cell division, the rate being an intrinsic property of the callus/culture type. The constant rate of production of chromosomally variant cells was balanced by the constant rate of selection for proliferative cell types. A steady-state distribution of chromosome numbers was generated after a period of time at a constant growth rate. Selection for specific constituents of a variable cultured plant cell population has been demonstrated previously (Demoise and Partanen 1969; Singh et al. 1975; Bayliss 1975; Singh and Harvey 1975; Kallak and Jarvekulg 1977).

A comparison of chromosome numbers between tumorous and non-tumorous callus cultures of tobacco and corresponding regenerated plants showed that selection played an important role during regeneration (Sacristan and Melchers 1969). Reports on the expression of chromosomal variability in regenerated plants are at variance, ranging from very little (Ogura 1975, 1976) to intermediate (Sacristan and Melchers 1969; Novak and Vystot 1975), to complete selection (Shimada et al. 1967; Yamane 1974) for euploidy. Ogura (1978) recently investigated the progeny of crosses between chromosomally variable and normal tobacco plants and concluded that the generation of chromosomal variability is under genetic control. Moreover, the accumulation of altered karyotypes in vitro may be responsible for the observed loss of regenerative potential in plant cell and tissue cultures with increasing age (Reinert and Backs 1967; Smith and Street 1974). In the present study, an attenuation of in vitro polyploidy, aneuploidy, and chromosomal rearrangements was observed in regenerated HV × HJ tissues, suggesting that constituents of a chromosomally variable population of cultured cells expressed different potentials for regeneration. Regeneration selected completely against polyploid cells/tissues and to an intermediate degree against those exhibiting aneuploidy and chromosomal rearrangements.

The regenerated pool of karyotypic variability must be expressed among plants, and not within plants, to realize the full potential of genetic studies and plant breeding. Three processes for the pathway of chromosomal variability into plants can be envisioned: 1) Cell initials with a lesser degree of chromosomal variability relative to the source culture give rise to chromosomally 'uniform' plants, 2) cell initials exhibiting chromosomal variability approximately equal to the source culture give rise to each regenerative entity, which then propagates clonally, giving rise to mosaic plants, and 3) after regeneration, new chromosomal variability is generated, giving rise to mixoploid tissues in conjunction with cases 1 or 2. Differences in chromosome constitution should be detected between plants for case 1, but not for case 2.

It was unlikely that any one of the above processes was involved in the pathway of in vitro chromosomal variability into regenerated plants. The analysis of variance of in vitro chromosome numbers demonstrated, in all cases, that variance between tissues within plants was highly significant. Variability was expressed between root meristems at the point of initial regeneration, consistent with case 1 above. However, within-root variance was observed and at a later point in development, variances within-plant among tillers and among ovaries within tillers were highly significant. These findings implicated the generation of in vivo chromosomal variability subsequent to regeneration (case 3). Mitotic anomalies, such as multipolar cell divisions and lagging chromosomes, were observed frequently in regenerated tissues. Although all regenerated plants exhibited variability within tissue, cytogenetic, morphological, and molecular (i.e. isozymic) differences were detected between plants. Thus, it may be concluded that karyotypic/putative genetic segregations occurred at the point of regeneration, and subsequent in vivo karyotypic variability was generated within the context of that segregated in regenerates.

Cytogenetic observations and analysis of meiotic configurations in certain regenerated HV × HJ plants (Fig. 3) deviated from the data of Murry (1975) on the original F_1 HV × HJ hybrid (Table 5). In three such cases, the number of pairing configurations increased dramatically. This increase could have resulted from loss or inactivation of a homoeologous pairing inhibitor or the selective diploidization of certain chromosomes/segments of the original complement. No detectable chromosome rearrangements were found, however, in roots regenerated from primary type A callus. Further, multivalent configurations were found in cells with hypoploid chromosome complements. Thus, the loss of a homoeologous pairing inhibitor, possibly via chromosomal variability, appeared the most likely explanation of this phenomenon.

The loss or gain of single chromosomes from HV × HJ implied a quantitative change in the ratio of the number of HV to HJ chromosomes. Taking this notion of parental genome imbalance further, it was hypothesized that continuous mixtures of parental genomes existed in cultured

populations of chromosomally variable HV × HJ cells. Independently, each parental genome possesses all of the necessary functions for life, making the existence of pure parental types feasible. The most direct test of the hypothesis would have been a comparison of predicted with actual distributions of parental chromosomes in the karyotypes of regenerated HV × HJ plants. All attempts to develop consistent chromosome banding techniques, however, were unsuccessful. The analysis of allozymes was previously shown to be an effective tool for elucidating genetic differences in barley, such as cultivar identification (Fedak 1975, Bassiri 1976) and the quantification of genetic variability and isozyme polymorphism (Allard et al. 1970; Babbel and Wain 1977). In other studies, isozymes were used to distinguish somatic and sexual hybrids of *Nicotiana glauca* × *N. langsdorfii* (Wetter and Kao 1976). Tang and Hart (1975) effectively used isozymes to identify wheat-rye addition lines, and to determine the genetic identity of triticale lines. The isolation and propagation of genetic variability was demonstrated earlier by comparing specific isozyme activities in crude extracts of test calli. Isozyme patterns differed markedly between different callus types from the same source plant, making the identification of specific HV and HJ isozymes in callus cultures quite difficult. It was much easier to make comparisons between HV and HJ in whole plants, however, because such uncontrolled tissue differences were obviated. A direct correlation has been observed between chromosome dose and corresponding isozyme activity in most isozyme systems, although contrary cases have been reported (Nakai 1977, Fobes 1977). Autotetraploids of HV and HJ generally exhibited increased activities for all EST and GOT isozymes, as compared to diploids (Orton, unpublished data). Further, karyotypically variable populations of chromosomally doubled plants regenerated from colchicine-treated HV × HJ calli showed increases in specific isozyme activities as compared to the original hybrid (Orton et al. 1979, in preparation). Hence, activities of specific HV and HJ isozymes in regenerated plants were useful as an indirect measure of quantitative HV and HJ genome mixtures as a source of chromosomal variability.

Ratios of HV:HJ isozyme activities and variability in the expression of parental morphological characteristics among HV × HJ regenerates supported the hypothesis of an in vitro continuum of parental genome mixtures (Fig. 6). A bimodal distribution was observed, with a large cluster of ratios centered on HV × HJ values (approximately .6) and a smaller cluster in the direction of HV (i.e. less than .6). The use of horizontal starch gel electrophoresis is a relatively crude technique for such analyses. More rigorous techniques, employing controlled extractions, polyacrylamide gel electrophoresis, and quantitative determinations of enzyme activity, are currently being developed for confirmation and extension of these results. The segregation of genomes in interspecific hybrids has been previously observed in oats (Ladizinsky and Fainstein 1978). Further, meiosis-like reduction divisions have been observed in somatic tissue (Wilson and Cheng 1949). Perhaps this phenomenon is related functionally to directional chromosome elimination, as observed in embryos from certain interspecific crosses in *Hordeum* (Kasha 1974). If the chromosomes of parental genomes segregate randomly, the occurrence of pure parental cells should be defined by a probability function. Alternatively, multipolar anaphase separations could have segregated whole parental genomes in a directed, non-random fashion, as postulated by Tai (1970).

This study demonstrated that regeneration selected for a certain range from chromosomally variable tissue cultures of HV × HJ. This selection, however, was incomplete, allowing variability to be expressed within regenerates. Further, significant differences in mean chromosome number and isozyme expression suggested that in vitro chromosomal variability was transmitted to regenerated plants. Chromosomal variability may have been further manifested as a continuum of parental genome mixtures, from pure HV to pure HJ. Conventional introgressive breeding programs require a great deal of time and space for their perpetration and are often plagued by hybrid sterility, chromosome elimination, or incompatibilities. The production of regenerated plants from callus cultures, however, requires very little input beyond the establishment of conditions for induction, maintenance, and regeneration. Finally, in vitro chromosomal variability may generate entities unobtainable by conventional plant hybridization, which may be of potential use in genetics and breeding.

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Dr. T.J. Orton
Department of Vegetable Crops
University of California
Davis, California 95616 (USA)