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Segregation of genetic markers among plants regenerated from cultured anthers of broccoli (*Brassica oleracea* var. 'italica')

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Summary. An experiment was conducted to determine critical factors in the recovery of embryos from cultured anthers of broccoli (*Brassica oleracea* var. 'italica') and to unambiguously distinguish whether embryos were of gametophytic origin. Among factors tested, genotype, genotype × anther developmental stage, and method of anther culture had a distinct impact on embryo recovery, whereas length of anther exposure to the culture medium did not. However, extreme heterogeneity of embryo emergence within and among replications precluded statistical contrasts. Among 762 plants derived from embryos of four independent cultivars, only one was determined to be of sporophytic origin by use of heterozygous codominant isozyme markers. Two of the cultivars tested were heterozygous at two or more loci. While segregation among loci was consistent with previously published linkage data, segregation of alleles was consistently non-random. In all of seven separate cases involving four cultivars, a significant over-representation of the fast-migrating class was observed. It appears, therefore, that populations of plants derived from microspores within cultured anthers of broccoli do not necessarily represent a random gametic array, and that care must be exercised in breeding and genetic applications.

Key words: Haploids – Isozymes – Segregation distortion – Broccoli – *Brassica oleracea*

Introduction

The increasing availability of haploids in crop plant species in recent years has led to speculation regarding their potential uses in plant breeding programs (Kasha 1974; Wenzel 1980). Part of this potential has already been realized in the pedigree breeding method where haploids greatly reduced the time necessary to produce a true breeding line from a polyheterozygote. Successful haploid breeding programs to date include tobacco (Collins and Legg 1980), barley (Kasha and Reinbergs 1980), wheat (Hu Han et al. 1978), and rice (Zhen-Hua 1982). Haploids also afford the potential opportunity for rapid production of parental inbred lines for hybrid varieties. Haploids arising from pollen and anther culture have also been shown to be a potential source of variant germplasm due to mutations that arise during the culture period (Hoffman et al. 1980; DePaepe et al. 1981; Schaeffer 1982). However, the value of haploids with novel mutations in a breeding program is currently only speculative.

The limiting step in the application of this technology is the absence of methods to efficiently obtain and confirm haploids. Although the list of species from which haploids can be obtained is slowly growing, most major crop species have not yielded to empirical research. Among the most successful approaches are wide hybridization followed by uniparental chromosome elimination (Kasha 1974) and anther or pollen culture (Bajaj 1983). Anther culture has been developed as a tool for obtaining haploids in a number of crop species including tobacco (Nitsch and Nitsch 1969; Burk and Matzinger 1976), rice (Chu et al. 1978), barley (Sunderland 1983), maize (Brettel et al. 1981), oilseed rape (Keller and Armstrong 1978), turnip rape (Keller and Armstrong 1979), marrow stem kale (Keller and Armstrong 1981), and broccoli (Keller and Armstrong 1983). However, since the anther is a complex structure consisting of both sporophytic and gametophytic tissues, the source of regenerating structures must be verified. Chromosome numbers have been used extensively for this purpose, but this approach is extremely tedious. Moreover, where cytological studies have been conducted, they have sometimes shown the existence of diploids among regenerates but lacked the capability of differentiating between diploids of sporophytic origin and doubled haploids (Keller et al. 1975; Keller and Armstrong 1977). Expression of divergent phenotypes sug-

gested that diploid regenerates arose from haploids by spontaneous chromosome doubling. Scholl and Amos (1980) and Zamir et al. (1981) advanced the use of allozymes as tools to determine the tissue origin of plants regenerated from cultured anthers.

The present study was undertaken to 1) test the utility of heterozygous isozyme markers for distinguishing the origin of plants regenerating from cultured anthers of broccoli, and 2) to identify critical factors in haploid production using previously determined methods of anther culture (Keller et al. 1975; Keller and Armstrong 1977; Keller, personal communication).

Materials and methods

Seeds of broccoli (*Brassica oleracea* var. 'italica') hybrid cultivars were obtained from Dr. Alan Johnson (Keystone Seed Co., Hollister, CA, USA). Twenty plants of each cultivar were grown in a greenhouse in Golden, Colorado between the months of September and November, 1983. The seed was sown in foam plugs (Techniculture, Watsonville, California) and seedlings were transplanted to a 5:1 peat: perlite mixture in 18 cm^2 plastic pots. Plants were fertilized twice weekly with 20-20-20 (N-P-K) fertilizer.

Approximately one month following transplantation, plants were surveyed for heterozygosity at the isozyme loci described by Arus and Orton (1983). All plants not exhibiting at least one heterozygous locus were discarded. Remaining plants within cultivars headed more or less asynchronously. Three representative plants of each cultivar which were at a similar stage were chosen as the anther donor plants. The isozyme phenotypes of these plants, which were the same for all plants within a cultivar, are listed in Table 1.

Anthers were prepared and cultured using procedures modified slightly from those reported by Keller et al. (1975); Keller and Armstrong (1977) and Keller (personal communication). Immature racemes were removed from heads as they began to elongate, but well before the first buds opened. Racemes were immersed in 20% W:V household bleach for 15 min under agitation, with one drop of detergent per 100 ml as a surfactant. They were then washed three times with sterile distilled water, 10 min per wash. Anthers were removed by carefully peeling back the immature calyx and corolla and gently rupturing the point of filament attachment to the anther axis. Extreme care was taken not to damage the anther tissue. Anthers were placed into liquid anther culture medium of Keller and Armstrong (1977) as specified in the experimental design below. They were then cultured at 35 °C for 36 h, and transferred to 25 °C (all in the dark) for various periods. Anthers were then transferred to the embryo culture medium (agar solidified in 100×15 mm petri dishes) specified by Keller and Armstrong (1977) and moved to continuous light (approximately 10,000 lux cool white fluorescent). As embryo-like structures emerged from anthers, they were removed individually, tallied, and transferred to fresh embryo culture medium.

The design of the empirical culture experiment was as follows: four factors tested were: genotype (5 hybrid cultivars: Table 1), developmental stage (3–4, 4–5, and 5–6 mm bud length), duration of exposure to the anther culture medium of Keller and Armstrong (1977) (15 or 30 days), and method of

Table 1. Summary of the number of putative haploid-derivative embryos recovered from cultured anthers according to treatment[a]

Cultivar[b]	No. days exposure to anther culture medium	Culture mode flask (F) or dish (D)	No. embryos from anthers from buds of length (mm)		
			3–4	4–5	5–6
'Futura'	15	F	18	45	485
(Pgm-1)		D	34	210	693
	30	F	0	63	18
		D	44	441	878
'Bravo'	15	F	5	3	0
(Pgi-3)		D	0	0	0
	30	F	10	0	13
		D	0	8	0
'Green Duke'	15	F	73	107	0
(Pgm-1, Pgm-2,		D	783	817	0
Lap-1)	30	F	504	24	0
		D	1,332	25	0
'Premium Crop'	15	F	0	0	0
(Pgm-1)		D	0	0	0
	30	F	2	0	0
		D	0	0	0
'Emperor'	15	F	2	63	15
(Pgm-1, Pgm-2)		D	66	1,395	30
	30	F	92	102	11
		D	9	10	1

[a] $n=96$ anthers per treatment
[b] loci in parentheses heterozygous (FS)

culture (3.5 ml of medium in stationary 60 × 15 mm sealed petri dishes or 50 ml of medium in 125 ml Bellco flasks at 90 rpm on a rotary shaker). Since cultivars matured at different rates, different genotypes were used at different dates (see Table 1). Each treatment consisted of four replications of 24 plated anthers.

Electrophoretic procedures were as specified by Arus and Orton (1983). Approximately 50 mg FW of leaf or cotyledon tissue of putative haploid regenerates (chosen randomly) was sampled such that viability was not impaired. Histochemical stains used were as specified by Vallejos (1983) for phosphoglucomutase (PGM), leucine aminopeptidase (LAP), and phosphoglucoisomerase (PGI).

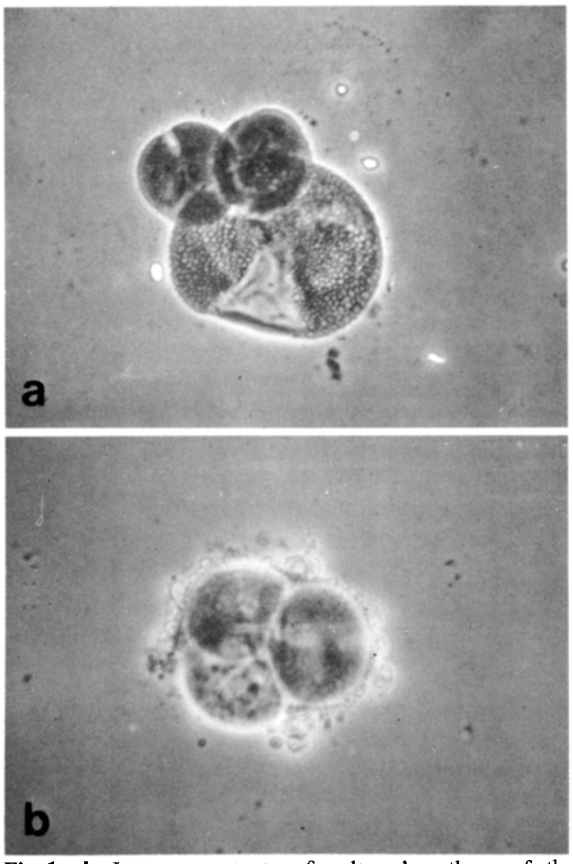

Fig. 1 a, b. Lumen contents of cultured anthers of the cv. 'Empereor'. **a** Seven days after explanting. Some of the the pollen grains exhibited marked swelling and wall rupture (*lower grain*), while most did not respond (*upper two grains*); **b** Ten days after explanting. Multicelled structures such as the one shown were observed. × 3,750

Results

Contents of cultured anthers were examined from excess materials in order to discern early morphogenic events. Approximately seven days after explanting, certain of the pollen grains within certain of the anthers began to swell, while most of the grains showed no change (Fig. 1 a; actual proportions not determined). After 10 days, swollen pollen and multicelled (2–10 cells) structures were seen (Fig. 1 B), which presumably proceeded to develop into embryos.

The results of the empirical culture experiment described in the "Materials and methods" section are presented in Table 1. A total of 8,419 embryo structures were obtained from 5,760 anthers plated. Embryos were first observed approximately 16 days after original plating, and continued to emerge for up to 50 days. During this period, the stage of development ranged from small globular to more mature cotyledon-like forms, consistent with the observations of Keller and Armstrong (1983).

Based on the patterns of appearance of embryos (i.e. highly inconsistent among and within treatments), it was decided that the value of statistics would be tenuous. Hence, the total number of embryo-like structures appearing across all replications was summed (96 total anthers per treatment). The most striking apparent factor was that of genotype: the cultivars 'Futura', 'Green Duke', and 'Emperor' were highly productive whereas few embryo-like structures were obtained from the cultivars 'Bravo' or 'Premium Crop' (Table 2). When considering cultural factors across cultivars, the only striking pattern was that culture in stationary small volumes gave consistently higher yields than higher volumes under agitation. The cultivar × bud

Table 2. Summary of the grouped number of putative haploid-derivative embryos from cultured anthers

Cultivar	Total	No. days exposure to anther culture medium		Culture mode		Bud length (mm)		
		15	30	Flask	Dish	3–4	4–5	5–6
'Futura'	2,927	1,483	1,444	627	2,300	96	759	2,072
'Bravo'	29	8	21	21	8	15	11	13
'Green Duke'	3,665	1,780	1,885	708	2,957	2,692	973	0
'Premium Crop'	2	0	2	2	0	2	0	0
'Emperor'	1,796	1,571	225	285	1,511	169	1,570	57

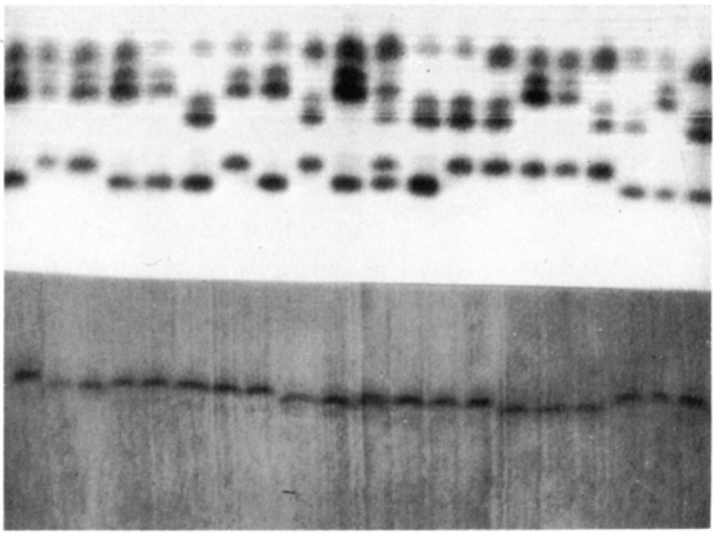

Fig. 2. Isozyme phenotypes among embryo-like structures regenerated from cultured anthers of cv. 'Green Duke'. Phenotypes are as follows: *Pgm-1:* F, F, F, F, F, S, F, F, S, F, FS (control), S, S, S, F, F, S, S, F, S; *Pgm-2:* S, F, F, S, S, S, F, S, F, S, FS (control), S, F, F, F, F, F, S, S, S; *Lap-1:* F, S, S, F, F, F, F, F, S, S, FS (control), F, F, F, S, S, S, F, F, F

length interaction was interesting to note: respectively 3–4, 4–5, and 5–6 mm buds of cvs. 'Green Duke', 'Emperor', and 'Futura' responding best to the applied treatments. Since statistical treatment of the data was not possible, however, these results can only be considered as highly suggestive.

Among 762 putative haploid plantlets tested for departures from the originally heterozygous isozyme phenotype (Fig. 2), only one was identified (cv. 'Green Duke') which was apparently of sporophytic origin.

Among the cultivars from which significant numbers of haploid embryos was obtained were cv. 'Green Duke', which was originally heterozygous at *Pgm-1, Pgm-2,* and *Lap-1* (Table 1). Joint segregation data were tested to determine whether class ratios agreed with those reported by Arus and Orton (1983). The expectation from these previous findings was that these loci should have segregated independently and, hence, equal representation would have been expected in each of the eight phenotypic classes. However, this was not the case, as the Chi-square value for homogeneity was highly significant (Fig. 2, Table 3). Moreover, the individual segregation ratios were significantly different from 1 : 1 at all three loci, and the distortion was always in favor of over-representation by F segregants (Table 4). The Chi-square value for independence of the three loci was not significant, in agreement with previous observations that they are not linked (Arus and Orton 1983).

The cultivar 'Emperor' was jointly heterozygous at *Pgm-1* and *Pgm-2* (Table 1). As with 'Green Duke' (above), the Chi-square value for homogeneity was highly significant (Table 5), and over-representation by F segregants at both loci was responsible for this distortion (Table 6). Likewise, haploid-derivative regenerates from anthers of cv. 'Futura', originally heterozygous only at *Pgm-1,* exhibited a significant distortion in

Table 3. Joint isozyme phenotypes of haploid-derivative plants from cv. 'Green Duke'

Phenotype at locus			No.
Pgm-1	*Pgm-2*	*Lap-1*	
F	F	F	66
		S	62
	S	F	57
		S	40
S	F	F	57
	S	F	37
		S	27

$n = 395$; χ^2 (homogeneity) = 26.21; $P < 0.001$

Table 4. Individual isozyme phenotypes of haploid-derivative plants from cv. 'Green Duke'

Locus	No. of phenotype			
	F	S	χ^2 [a]	*P*
Pgm-1	225	170	7.66	< 0.01
Pgm-2	234	161	13.49	< 0.001
Lap-1	217	178	3.85	< 0.05

[a] Homogeneity

Table 5. Joint isozyme phenotypes of haploid-derivative plants from cv. 'Emperor'

Phenotype at locus		No.[a]
Pgm-1	*Pgm-2*	
F	F	74
	S	40
S	F	36
	S	20

[a] $n = 170$; $\chi^2 = 36.4$; $P < 0.001$

Table 6. Individual isozyme phenotypes of haploid-derivative plants from cv. 'Emperor'

Locus	No. of phenotype			
	F	S	χ^2	P
Pgm-1	114	56	19.79	<0.001
Pgm-2	110	60	14.71	<0.001

favor of F segregants. (86 F: 57 S; $\chi^2 = 5.88$; $P < 0.02$). Finally, regenerates from cultured anthers from the cv. 'Bravo' were 41 *Pgi-3* F: 13 *Pgi-3* S, also significantly different from expected ($\chi^2 = 14.52$; $P \ll 0.001$).

Discussion

W. A. Keller and his associates have developed procedures for the successful recovery of haploid plants via embryogenesis from cultured anthers of several *Brassica* species. Recovery rates as high as 2.7 embryos per anther plated were reported from the broccoli (*B. oleracea* var. 'italica') cv. 'Green Mountain' (Keller and Armstrong 1983). Following the optimal procedures described in this paper with minor changes (i.e. culturing anthers in 3.5 ml liquid anther culture medium in 60 × 15 mm petri dishes as suggested by W. A. Keller; personal communication), we and at least one other group (R. L. Gabrielson and W. C. Anderson; personal communication) have successfully repeated this work and extended the range of amenable cultivars. We are able to obtain "efficiency" (embryo yield from 1,000 anthers; Keller and Armstrong 1983) values from one treatment as high as 1.45×10^4 but it is likely that replication over time would produce variable results. Ten replications over time of one particular treatment by Keller and Armstrong (1983; see Table 2) gave widely disparate embryo yield values. Similarly, we obtained much lower values in a comparable experiment to that described herein. Hence, it would appear that one or more factors critical to microscpore embryogenesis have not yet been identified. Stringent control of environment and genotype should be employed to enhance the reproducibility of empirical anther culture experiments.

Uneven patterns of embryogenesis within treatments and cultivar asynchrony precluded a meaningful statistical treatment of the data. However, it appears quite likely that genotype of the anther donor plant contributed significantly to embryo yield. This is consistent with the speculation by Keller and Armstrong (1983) that striking variation among experiments replicated over time (above paragraph) were probably a consequence of "inter-plant genetic variation". The cultivar they used, 'Green Mountain', is an open-pollinated type, and probably contains considerable genotypic variation. Since F_1 hybrid varieties were used in the present study, and anthers from three representative plants were bulked, such individual genotype effects should have been minimized.

Another factor which appeared to contribute significantly to embryo yield was culture mode. Among the three cultivars exhibiting high embryo yields, culturing in 3.5 ml of anther culture medium in stationary 60 × 15 mm petri dishes gave higher embryo yields than when anthers were cultured in 50 ml medium in 125 ml Erlenmeyer flasks on a rotary shaker. Further, there appeared to be a striking genotype × developmental stage interaction (Table 2). Bud length was used as the operational parameter to separate stages, and it would be interesting to see whether actual microspore developmental stage can be used as a more accurate predicter of embryogenic potential. The duration of exposure to anther culture medium had no discernible effect.

A recurring observation among studies in which embryos have been successfully regenerated from *Brassica* sp. anthers is that a significant proportion fail to develop normally (Keller and Armstrong 1977, 1983; Loh and Ingram 1982). It was presumed that the majority of these abnormalities were due to persistent culture effects since previous work indicated that normal plants emerged from recultured embryos. An undetermined small proportion, however, exhibited gross phenotypic alterations of pigment (white, green and purple), suggesting genetic segregation or mutation. Perhaps the failure to develop normally among certain embryos was a consequence of specific genetic sublethals in addition to persistent cultural effects.

Genetic markers have been used in several previous studies to efficiently and unambiguously distinguish regenerates from anther cultures of sporophytic vs. gametophytic origin. Zamir et al. (1981) used heterozygous isozyme markers to demonstrate unambiguously that approximately 500 regenerates from callus derived from anther cultures of a tomato line bearing the male sterile mutation *Ms-10*^{35} were all of sporophytic origin. Chen et al. (1982) used heterozygous morphological markers and progeny tests to show that 100% of plants they generated from rice anthers were of gametophytic origin. Keathley and Scholl (1983) obtained 169:24 plants respectively of sporophytic and gametophytic origin from cultured *Arabidopsis thaliana* anthers of plants heterozygous at the *Aps-B* locus. Of 762 regenerates from the three amenable cultivars tested, only one regenerate of sporophytic origin was detected. The source of this and presumably other diploids among the population of regenerates was probably filament tissue. Despite efforts to remove the filament, small remnants were sometimes inadvertantly introduced into the culture along with the anther, and such tissues were observed to exhibit a broad array of morphogenic responses, including root and shoot organogenesis. Isozyme markers can further be

used to verify the independent origin of haploid-derivative plants. For example segregation of multiple markers (e.g. 'Green Duke') among regenerates from a single anther is primary evidence for multiple events (Fig. 2).

Anther culture may emerge in *Brassica* and other species as a tool for efficient gene mapping studies. Test-cross analysis to obtain co-segregation data in diploid *Brassicas* suffers from the disadvantages that hand emasculation is tedious, seed maturation is time consuming, and the process requires simultaneous flowering of a putative polyheterozygote and a fixed counterpart. Selfing introduces more phenotypic classes and, hence, complicates the analysis. Moreover, bud pollination is frequently necessary to circumvent self-incompatibility. To conduct mapping studies via anther culture, one needs only the polyheterozygote. Haploid-derivative plants emerging from anthers should be a direct reflection of the segregation products from meiosis.

Analysis of marker segregation among haploid regenerates from 'Green Duke' permitted a test of the above assertion. Extensive segregation data presented previously for 'Green Duke' ('H_2'; Arus and Orton 1983) showed that *Pgm-1, Pgm-2*, and *Lap-1* behaved as independent Mendelian co-dominant loci. Hence, equal representation of each of the eight phenotypic classes would have been expected. However, the Chi-square value for homogeneity was highly significant, and a primary cause of departures from homogeneity was segregation distortion at all three loci in favor of F segregants. This was not an isolated observation, since departures from homogeneity among regenerates from cv. 'Emperor' (jointly heterozygous at *Pgm-1* and *Pgm-2*) were also found to be a consequence of segregation distortion favoring F embryos. Finally, segregation distortion, again favoring F segregants, was also observed among haploid regenerates from cvs. 'Futura' and 'Bravo' (heterozygous respectively at *Pgm-1* and *Pgi-3* only). Hence, we conclude that these observations are representative of a phenomenon which differentiates male gametophyte from microspore embryogenic development. Most pollen grains within cultured anthers did not exhibit any signs of morphogenesis (Fig. 1), an observation perhaps related to this phenomenon. The reasons for association of the slow-migrating isozymes and an apparent antagonistic effect on microspore embryogenesis as compared to male gametophyte development and growth remain obscure. Segregation distortion among microspore-derived plant populations has been observed previously (Collins, personal communication), although normal assortment patterns have been reported from rice anthers by Chen et al. (1982). In addition to segregation distortion, high mutation frequencies are sometimes observed among anther culture-derived plants (Hoffman et al. 1980; DePaepe et al. 1981; Schaeffer 1982). Hence, caution should be exercised when attempting to use anther culture as an alternative to traditional methods to study inheritance and linkage.

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