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Genotypic Variability in the Frequency of Plant Regeneration from Leaf Protoplasts of Four *Brassica* spp. and of *Raphanus sativus*

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Abstract. **More than 65 different genotypes, including cultivars and inbred lines, from five cruciferous species (*Brassica oleracea* L., *B. campestris* L., *B. napus* L., *B. juncea* L., and *Raphanus sativus* L.) were tested for their in vitro response of leaf protoplasts. Protoplasts were cultured in three liquid media and the resulting colonies were placed on seven test regeneration media. Significant differences among the species were found in plating efficiency in the frequency of shoot regeneration. Two broad response groups were identified: 1) Cultivars from *B. oleracea* and *B. napus*—these generally yielded protoplasts that were able to divide, form colonies at high frequencies, and regenerate shoots at variable frequencies; and 2) cultivars of the other species evaluated, which typically exhibited low plating efficiencies and little, if any, shoot regeneration. Evaluation for the effect of the cytoplasmic constitution of a few *B. oleracea* breeding lines on in vitro performance indicated that protoplasts carrying the Ogura (R1) male-sterile cytoplasm regenerated shoots at slightly lower frequencies than the corresponding alloplasmic-fertile lines. Genotypes exhibiting high frequency of shoot formation in one medium also had efficient shoot regeneration in other media as well, while genotypes with low shoot regeneration responded consistently in the different media used. This consistency in response indicates that genotype plays a critical role in determining the success of leaf protoplast culture in the crucifers.**

Recent advances in the cellular manipulation of crucifer crops provide new tools for the improvement of horticultural traits in this important group of plants. Significant progress has been achieved in the production of haploid plants from cultured anthers and isolated microspores (Keller et al., 1987), cytoplasmic modifications by protoplast fusion (Barsby et al., 1987; Menczel et al., 1987; Pelletier et al., 1983; Robertson et al., 1987), and transformation with *Agrobacterium* (Guerche et al., 1987; Ooms et al., 1985; Pua et al., 1987). The major effort has been applied to the economically important rapeseed (*B. napus*), but there is also increasing research activity with various vegetable crops, including *B. oleracea, B. campestris, B. juncea, B. napus,* and *Raphanus sativus* (Schenck, 1984; Zee and Johnson, 1985). One potentially significant application of cellular manipulations with vegetable crucifers is the development of efficient cytoplasmic male sterility systems for hybrid seed production. A prerequisite for such an application is efficient protoplast culture and shoot regeneration procedures.

We have previously demonstrated that leaf protoplasts of broccoli and cauliflower can be readily cultured to regenerate large numbers of plants (Jourdan et al., 1985; Robertson and Earle, 1986). In these studies, there were indications that both the nuclear genotype and the cytoplasm affect the frequency of cell divisions and plant regeneration. There is ample evidence that genotype is an important determinant of in vitro behavior for such traits as callus formation (Abe and Futsuhara, 1986; Saunders and Shin, 1986), frequency of embryo development from anthers (Dunwell et al., 1985), and frequency of plant regeneration (Chen et al., 1986; Foulger and Jones, 1986; Hodges et al., 1986). In order for protoplast technology to be broadly applicable in crucifer crop improvement, it is necessary to characterize the type of response that various cultivars exhibit in culture. To this end, we have examined the behavior of leaf protoplasts from numerous commercial cultivars and breeding lines of all major vegetable forms of crucifers as well as a selected group of rapeseed forms.

Materials and Methods

Plant material. The commercial lines used in this study are listed in Table 1. The genotypes surveyed encompass a wide range of vegetative morphologies, adaptability to various environments, and horticultural qualities; they include traditional open-pollinated cultivars as well as hybrids, and they originate from various sources. Most of the cultivars are commonly grown in the northeastern United States. The breeding lines used in the comparison of different cytoplasms are listed in Table 2; they were obtained from M. Dickson, New York State Agricultural Experiment Station, Geneva. Lines that carry the R1 CMS cytoplasm (Ogura, 1968) were derived from an extensive backcross program.

Seeds were surface-sterilized by immersion in 30% (v/v) Clorox, 1% (w/v) PEX detergent for 15 to 20 min and then thoroughly rinsed three times with sterile water. Six to eight seeds were placed in a polycarbonate vessel (Magenta Corp., Chicago) containing 50 ml of MS salts and vitamins (Murashige and Skoog, 1962) without hormones, supplemented with 2% sucrose and 1% agar (Difco Bacto-agar). Seedlings were grown at 22 to 25C with a 16-hr photoperiod of 80 $\mu mol \cdot s^{-1} \cdot m^{-2}$ irradiance provided by a mixture of cool-white and Gro-lux lights. The young, fully expanded leaves of 15- to 20-day-old seedlings were used for protoplast isolation.

Protoplast isolation and culture. Protoplasts were isolated as described (Jourdan et al., 1985; Robertson and Earle, 1986).

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Briefly, the leaves were scored with parallel 1-mm cuts and placed in an enzyme solution containing 0.5% Cellulysin, 0.25% Macerozyme, and 0.12% Driselase in culture medium B. The digestion was carried out overnight at 25C on a shaking platform (50 rpm). The protoplasts were collected by flotation on a sucrose pad (0.5 M sucrose, 5 mM Mes, pH 5.8) and resuspended in SCM (0.5 M sorbitol, 10 mM $CaCl_2$, 5 mM Mes, pH 5.8). They were cultured in 24-well plates (Falcon) using 0.5 ml of

Table 1. Response of various crucifer vegetables and rapeseed to leaf protoplast culture and plant regeneration[z].

Genotype	Plating efficiency (%)[y]	Callus formation (%)[x]	Shoot regeneration (%)[w]
B. oleracea ssp. *botrytis* (heading broccoli)			
Green Mountain	33	4	<5
Green Comet	36	4	13
Early One	33	4	47
Southern Comet	33	5	14
Citation	26	5	<5
Calabrese Green Sprouting	36	5	0
B. oleracea ssp. *botrytis* (cauliflower)			
Snow Crown	20	2	5
Snowball 34	13	2	15
Snowball A	36	5	45
Self Blanche	28	3	7
White Top	22	4	<2
Early Purple Head	24	4	5
B. oleracea ssp. *capitata* (cabbage)			
Rio Verde	31	4	10
Viking	37	5	5
Chieftain Savoy	26	4	<1
Wisconsin Golden Acre	21	5	0
Late Flat Dutch	27	5	0
Red Acre	18	4	<1
Market Prize	27	3	<1
Sun Up	30	4	<1
Condor	24	5	0
B. oleracea ssp. *gemmifera* (brussels sprouts)			
Catskill (Long Island Improved)	35	4	8
Prince Marvel	42	5	30
B. oleracea ssp. *gongylodes* (kohlrabi)			
Grand Duke	24	2	<1
White Vienna	22	3	6
Early Purple Vienna	35	4	<1
B. oleracea ssp. *acephala* (kale)			
Tall Green Curled Scotch	35	6	60
Vates-Dwarf Blue Curled	35	6	65
Vates-Tall Blue Curled	36	5	50
B. oleracea ssp. *acephala* (collards)			
Vates	26	4	2
Champion	22	1	0
B. campestris ssp *chinensis* (chinese cabbage)			
Michili	0	---[v]	---
Pak-Choi Lei-Choi	0.5	0	---
Jade Pagoda	1	0	---
Early Hybrid G	0.5	0	---
B. campestris ssp *rapifera* (turnip)			
Purple Top White Globe	0	---	---
Amber Globe	0	---	---
B. campestris ssp *ruvo* (broccoli raab)			
Italian turnip	8	2	<1
B. juncea (mustard greens)			
Southern Curled	1	0	---
B. napus ssp *napobrassica* (rutabaga)			
American Purple Top	6	1	<1
Raphanus sativus (radish)			
Cherry Belle	1	<<1	0
Champion	2	1	0
White Icicle	1	<<1	0

continued on next page

Table 1 (*continued*).

Genotype	Plating efficiency (%)[y]	Callus formation (%)[x]	Shoot regeneration (%)[w]
B. campestris ssp *oleifera* (rapeseed)			
Candle(ATR)[u]	1	0	---
B. napus ssp *oleifera* (rapeseed, canola)			
Tower(ATR)	30	5	14
Altex	22	4	<1
Andor	27	5	<1
Triton	30	5	6
Topas	31	5	<1
Westar	26	4	1

[z]Data presented only for protoplasts cultured on medium B, diluted with medium C, and then transferred to medium E for callus development and shoot regeneration.
[y]Plating efficiency = percentage dividing protoplasts of total protoplasts.
[x]Callus formation = percentage initially plated protoplasts that gave rise to calluses on solid medium.
[w]Shoot regeneration = percentage of calluses on regeneration medium that gave rise to at least one shoot.
[v]Evaluation not made either because colonies or calluses were not obtained.
[u]ATR = triazine-resistant cytoplasm derived from bird's rape.

medium at a density of 2.5×10^4 protoplasts per well; eight wells were typically used for each test medium. For each genotype evaluated, protoplasts were initially cultured in three liquid media: medium B (Pelletier et al., 1983) lacking Tween 80; medium B-2, identical to B except that the growth regulator level was increased to 1.1 μM 1-naphthalene acetic acid (NAA), 9.0 μM (2,4-dichlorophenoxy)-acetic acid (2,4-D), and 4.4 μM *N*-(phenylmethyl)-1*H*-purin-6-amine (BA); and medium MSKI, which consisted of MS medium supplemented with 3% (w/v) sucrose, 5% (w/v) glucose, 1.8 μM 2,4-D, 2.3 μM kinetin, and 0.57 μM 1*H*-indole-3-acetic acid (IAA). The protoplasts were kept in darkness at 25C for 5 to 7 days and then transferred to light on a 16-hr photoperiod at 60 $\mu mol \cdot s^{-1} \cdot m^{-2}$ irradiance and at 20 to 22C. The frequency of cell wall regeneration was determined after 3 to 5 days by counting the proportion of protoplasts that had changed shape (from spherical to ovate) and had a healthy appearance (turgid; chloroplasts becoming yellow; dense cytoplasm). After 10 to 12 days, both medium B and B-2 were diluted with 0.2 ml per well of medium C (Pelletier et al., 1983), while medium MSKI was diluted with MSKI lacking glucose. The dilution was repeated twice over the next 3 to 4 days. At 12 to 15 days from the start of culture, the cultures were scored for plating efficiency (PE) by counting the number of protoplasts that had divided at least once relative to the total plated protoplasts.

Callus formation and plant regeneration. At 30 to 40 days after the start of culture, colonies that had undergone four or five cycles of division were transferred to solid medium for callus formation and shoot regeneration. The transferred colonies typically measured 0.2 to 0.8 mm in diameter. The frequency of callus formation was determined 50 to 60 days after protoplast isolation; it was calculated by counting the number of colonies that had grown to at least 2 to 5 mm in diameter (i.e., the "colonies" were now "calluses") relative to the number of protoplasts that were cultured initially. Thus, if 500 calluses were obtained from one culture well that initially contained 25,000 protoplasts, then the callus formation frequency was 2%. The seven media evaluated for callus growth and shoot regeneration were: medium E (Pelletier et al., 1983) lacking gibberellic acid (GA_3); MKG (Fu et al., 1985) MS medium + 13.8 μM kinetin and 0.29 μM GA_3; MSIB (Lu et al., 1982) MS medium + 11.4 μM IAA and 4.4 μM BA; K3G (Glimelius, 1984) K3 medium + 0.6 μM IAA and 9.1 μM zeatin; MS3G, the K3 salts and vitamins were replaced with MS salts and vitamins; C1, described by Bidney et al. (1983); and LS.2 by Schenck and Robbelen (1982). In all these media, 0.22% Gelrite (Kelco) replaced agar or agarose as the gelling agent.

After 60 to 90 days from protoplast isolation, the calluses on regeneration media were scored for shoot formation by counting the number that contained at least one shoot. All lines were evaluated in at least two independent experiments. Selected shoots regenerated from most genotypes were transferred to rooting medium (MS, no hormones, 1% sucrose, 1% agar) and plants were hardened as described for 'Green Comet' broccoli (Robertson and Earle, 1986). Some plants were later transferred to large pots and maintained in the greenhouse.

Results

Protoplasts could be readily isolated from leaves of in vitro-grown seedlings of all the genotypes surveyed. Typical yields were in the range of $1–5 \times 10^6$ protoplasts/g fresh weight. The single enzyme mixture used can probably be applied to the isolation of leaf protoplasts from most crucifer crops. Freshly isolated protoplasts from all genotypes were spherical and had chloroplasts distributed throughout the cytoplasm. Within 2 days, the protoplasts from most genotypes changed shape and became more oval as the cell wall was resynthesized.

Plating efficiency. In all the commercial vegetable cultivars of *B. oleracea* and rapeseed *B. napus* surveyed, about 50% to 60% of cultured protoplasts resynthesized walls, but, in most other vegetable cultivars (e.g., chinese cabbage, turnips, mustard greens, rutabaga, and radish) cell wall formation occurred at lower frequencies (≈5% to 30%). We observed no major differences in the response of protoplasts between the three culture media. However, we found less well-to-well variability when medium B was used; therefore, the plating efficiencies (Table 1) represent only those obtained in medium B. A high frequency of cell wall formation was a prerequisite for subsequent high PE (Fig. 1a), but numerous divisions did not necessarily follow in all cases of efficient cell wall resynthesis. For instance, ≈60% of rutabaga protoplasts regenerated cell walls, but only 10% of them subsequently divided, giving an overall plating efficiency of 6% (Table 1).

Commonly, protoplasts from *B. campestris, B. juncea, B.*

Table 2. Influence of cytoplasm on the development of protoplasts from alloplasmic pairs of *B. oleracea* lines[z].

Line	Description	Plating efficiency (%)		Callus formation (%)		Shoot regeneration (%)	
		N[y]	CMS	N	CMS	N	CMS
PI 183214	Cauliflower, Egyptian introduction with white, soft curd in full sun.	7	NA[x]	0	NA	---[w]	NA
Snowball A	Cauliflower. Good cultivar for northeastern United States.	26	NA	5	NA	45	NA
NY 7630]	Cauliflower; white	16	7	3	4	31	25
NY 7532]	curd in full sun; all	23	29	4	5	50	45
NY 7642]	derived from cross of	37	26	5	5	72	39
NY 7649]	PI 183214 x Snowball A.	40	33	5	4	49	21
NY IR 9909	Cauliflower, glossy, insect-resistant.	4	0.5	0.4	<0.1	0	---
NY IR 9941	Cauliflower, opaque, insect-resistant.	2	1	<0.1	<0.1	---	--
NY 364	Cabbage.	15	23	1	0.5	3	1
NY 548	Cabbage.	22	17	1	2	0	0
NY 1414	Cabbage.	27	22	1	1	1	1

[z]Protoplasts cultured in medium B, diluted with medium C, and transferred to medium E for callus formation and shoot regeneration.
[y]N = normal, fertile cytoplasm of maintainer lines; CMS = R1 cytoplasm of male-sterile lines.
[x]Not available; these lines only available with fertile cytoplasm.
[w]No evaluation possible because calluses were either not produced or insufficient for evaluation of shoot regeneration.

napus, and *R. sativus* accumulated a granular precipitate, presumably polyphenols (Schenck and Hoffmann, 1979), in the media (Fig. 1b). Such a process led to rapid browning and collapse of many protoplasts. In the case of 'Broccoli Raab' (*B. campestris*), rutabaga (*B. napus*), and radishes (*R. sativus*), colonies developed in spite of the browning, but at a very low frequency. Many workers have reported problems with browning during the culture of *Brassica* leaf protoplasts (Glimelius, 1984). In the chinese cabbage, turnip, and rapeseed cultivars of *B. campestris*, the production of brown exudate was so severe that all development ceased after 2 weeks in culture.

Callus formation frequency. When colonies reached ≈1 mm in diameter in medium C, they were transferred to seven different solid media, previously shown to support regeneration of shoots from *Brassica* calluses, for evaluation of callus development and subsequent shoot regeneration. Some differences in the callus formation frequency of a given genotype on the seven media were observed (data not shown). However, since medium E consistently resulted in the most efficient development of calluses for all genotypes, only data from this medium are presented (Table 1). Whenever possible, 200 to 400 colonies were initially pipeted per 10-cm plate; as the colonies grew over the next 2 weeks, they were individually transferred to fresh plates for continued growth. The highest frequency of callus formation we obtained in an experiment with any *Brassica* so far cultured was 10%. One of our most responsive genotypes, cauliflower inbred line 7642 (Dickson, 1985; Jourdan et al., 1985), had typical callus frequencies in the range of 4% to 6%. As was the case of cell divisions, most *B. oleracea* genotypes consistently exhibited high-frequency callus development in the range of 4% to 5% (Table 1). The canola rapeseed were similarly characterized by high frequencies of colony development into callus. In contrast, the various cultivars of chinese cabbage, turnips, and radish, as well as broccoli raab, mustard, and rutabaga, exhibited either a consistently low frequency of callus formation or failed to form colonies.

Shoot regeneration. Plants were regenerated from some cultivars of all subspecies of *B. oleracea*, from all *B. napus* lines, from only one *B. campestris* line, but not from *B. juncea* or *R. sativus* (e.g., Fig. 1c). However, there was a very large range in shoot regeneration frequencies among the species, and even among cultivars of the different *B. oleracea* subspecies (Table 1). Genotypes of broccoli, cauliflower, brussels sprouts, and kale exhibited the highest shoot regeneration frequencies (40% to 60%), whereas those of cabbage, kohlrabi, collard, and rapeseed showed generally lower frequencies. Among the broccoli and cauliflower group, there were some genotypes that showed frequent shoot regeneration (e.g., 50% for 'Early One') and others that showed it very infrequently (e.g., <2% for 'White Top'). Within the cabbage group, there were cultivars that failed to regenerate shoots altogether (e.g., 'Wisconsin Golden Acre'), but one, 'Rio Verde', formed shoots at a frequency of 10%. All three cultivars of kale regenerated shoots at high frequencies. A similar response for kale hypocotyl protoplasts was observed by other workers (F.S. Wu, personal communication).

Consistent with their characteristic low PE, most chinese cabbage, turnips, mustard, and radish failed to regenerate shoots. However, shoot morphogenic potential was exhibited by broccoli raab and rutabaga, albeit at a very low frequency, and it was not reproducible.

The overall in vitro response of leaf protoplasts from commercial cultivars of the five cruciferous species permits the classification of these species into two major groups. The first group consists of *B. oleracea* and *B. napus*, which showed a rather high frequency of shoot morphogenesis. The second group consists of *B. campestris, B. juncea,* and *R. sativus*, which gen-

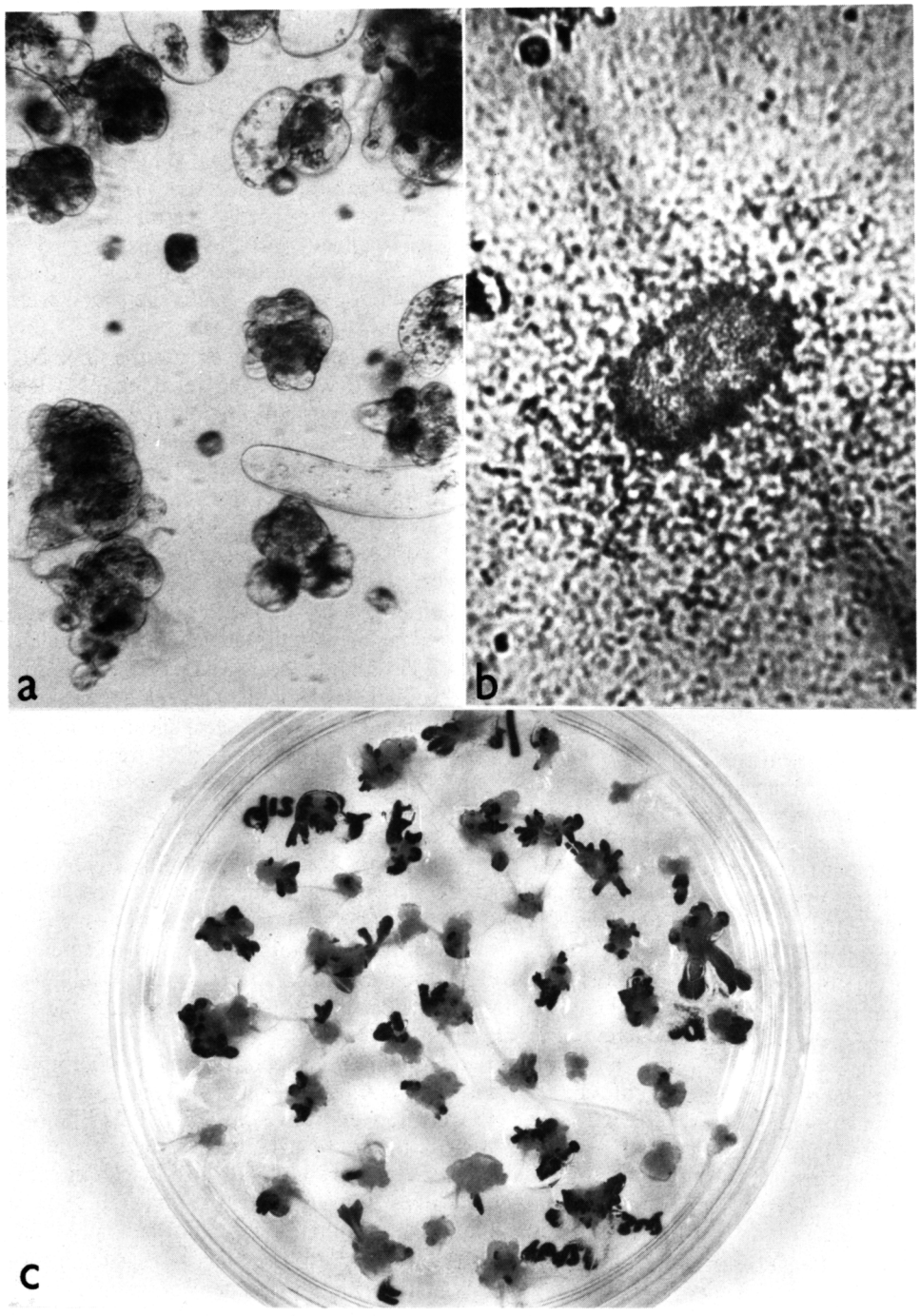

Fig. 1. Response of crucifer leaf protoplasts in vitro. (**a**) Normal development into a multicellular colony 25 days after isolation of protoplasts from cauliflower 'Snowball A'. (**b**) Severe browning response of a protoplast from turnip 'Amber Globe'. The protoplast-derived cell at the center has released a brown granular material that covers it and the surrounding surface. Contrast with the clear surface around the cells on (**a**). (**c**) High frequency of shoot regeneration from protoplast-derived calluses of cauliflower inbred line #7642B.

erally showed either no shoot morphogenesis or only sporadic shoot regeneration. In most genotypes within both groups, at least some root morphogenesis was observed even when no shoots were regenerated, but, in a few cases (radishes, 'Red Acre' cabbage, 'Champion' collard), neither roots nor any other type of morphogenesis was noted. Efficient shoot regeneration from roots of protoplast-derived callus has not yet been achieved, but an exhaustive examination has not been carried out. Guerche et al. (1987) and Lillo and Shahin (1986) have optimized a shoot regeneration system from seedling roots, and their procedure may be applicable to callus-derived roots.

The effect of cytoplasm on culture response. In addition to examining the influence of the nuclear genotype on the development of leaf protoplasts, we also examined the effect of the cytoplasm on protoplast culture. Preliminary analyses had shown that the R1 male sterile cytoplasm appeared to cause a reduction in the shoot regeneration frequency (Robertson and Earle, 1986; unpublished data). In this experiment, we tested more alloplasmic inbred pairs carrying either the R1 CMS cytoplasm (A lines) or the normal *Brassica* cytoplasm (B lines) (Table 2). The results essentially confirm the previous finding that the R1 cytoplasm has an overall depressive effect on the culture response of protoplasts and an even more pronounced effect on shoot regeneration. However, the depressive effect of the CMS varies

somewhat from line to line. For instance, line NY 7632 shows almost no difference in shoot regeneration frequency between the CMS and fertile cytoplasms.

The various inbred lines used in the analysis of cytoplasmic effects showed interesting nuclear genotype-dependent differences among themselves that complement the findings from the survey of commercial lines. For example, the insect-resistant cauliflowers (NY 9909 and NY 9941) were characterized by very low PEs, in contrast to the white curd lines (NY 7630–NY7649), which showed excellent shoot morphogenesis (Table 2). The high morphogenetic potential of these lines is probably derived from the recurrent parent 'Snowball A' in backcrosses with the PI from which the white curd trait was introduced. All the NY76 lines are related and are part of a breeding program to transfer the white curd trait to horticulturally acceptable forms.

Discussion

The role of genotype in determining the ability of plant cells to grow in vitro and regenerate whole plants has been well-documented (Tomes, 1985). Studies of various *Brassica* spp. in culture have also demonstrated the influence of genotype on the development of cells and organs (Baroncelli et al. 1973; Dietert et al., 1982; Glimelius, 1984; Murata and Orton, 1987). The culture of protoplasts in defined media represents a classical genotype by environment (G × E) interaction. This interaction can be very complex because of the innumerable components of the phenotype that reflect the genotype (e.g., different morphologies, growth rates, relative tissue composition) and the environment (kind and quantity of nutrients, osmoticum, temperature). To maximize the goal of efficient plant regeneration from protoplasts, many permutations of media components and cell types may have to be independently evaluated. In the experiments reported here, we have sought to maintain as constant an environment as possible so that the only variable was donor genotype. However, it is important to keep in mind that leaves are complex tissues containing various cell types, and, although the vast majority of protoplasts from leaves are derived from mesophyll cells, there may be a significant proportion of protoplasts from other cell types, such as the epidermis or parenchyma. This mixture of cell types could vary among cultivars depending on leaf morphology and the degree of tissue digestion. Thus, some uncontrolled variability for leaf cell types may have occurred.

The primary finding in this work is that all cultivated forms of *B. oleracea* and *B. napus* reproducibly can regenerate shoots from leaf protoplasts with the media described and under the growth conditions of these experiments, but that large differences in regeneration frequency occur among the various cultivars. In addition, sporadic shoot regeneration was obtained in one cultivar of *B. campestris*. These three species share an evolutionary relationship, where *B. oleracea* and *B. campestris* are the progenitors of *B. napus* (Prakash and Hinata, 1980). Since shoot regeneration is very common in *B. oleracea* and *B. napus* genotypes, it seems plausible that the amphidiploid species has inherited the in vitro culture behavior from the *B. oleracea* genome, even though, morphologically, *B. napus* is clearly more similar to *B. campestris*. These observations support the conclusions of Murata and Orton (1987), who examined the influence of genotype/species on the regeneration of shoots from hypocotyl-derived callus of the six major *Brassica* species and found that the highest frequencies of shoot regeneration were consistently associated with the *B. oleracea* genome.

Although procedures for efficient colony development from hypocotyl protoplasts of *B. campestris* have been established (Glimelius, 1984; Shillito et al., 1983), routine shoot regeneration of turnips, chinese cabbage, or rapeseed forms so far has not been achieved. Our results confirm the fact that regeneration in this species is sporadic at best. Since leaf protoplasts of *B. campestris* 'Broccoli Raab' exhibited some shoot regeneration, an attempt was made to culture hypocotyl protoplasts. These proved to have a higher frequency of callus formation (≈5%) than leaf protoplasts (≈2%), but, in more than 3500 calluses examined on seven different regeneration media, no shoots were observed (data not shown).

The single cultivar of vegetable *B. juncea* and the three cultivars of *R. sativus* surveyed were found recalcitrant to leaf protoplast culture. Although plant regeneration from mesophyll and hypocotyl protoplasts of oilseed *B. juncea* has been reported (Chatterjee et al., 1985; Glimelius, 1984), to our knowledge, no previous culture of radish protoplasts has been published. Since relatively few lines of these two species have been examined, it is not yet possible to generalize about the in vitro behavior of the species as a whole.

Significant effects of the cytoplasm on the callus growth rate, sensitivity to 2,4-D, and regeneration frequency have been identified for eight lines of hexaploid wheat (Mathias et al., 1986). The most noticeable parallel effects in *B. oleracea* appear to be on shoot regeneration frequency and the less clear-cut effects on the early events in culture. Lines carrying R1 are characterized by slightly lower frequencies of shoot regeneration than their corresponding normal cytoplasm maintainer lines. The R1 cytoplasm also causes a delay in the maturation of cabbage and cauliflower lines; for example, the male-sterile line 7642A typically reaches maturity ≈2 weeks after the maintainer line 7642B (M. Dickson, personal communication). The A lines are also less vigorous than the B lines; this reduced vigor is accentuated when the growing season has many cool days. Conceivably, similar physiological effects in vitro may account for the reduction in regeneration frequency.

Our studies provide additional support for the possibility of improving in vitro culturability by selective breeding. Lines NY7630–NY7649 were derived from a cross of a PI and 'Snowball A' (Dickson, 1985). The PI exhibited extremely poor in vitro response, whereas Snowball A had a high plating efficiency. The various lines derived from that cross all show a generally favorable response similar to the 'Snowball A' parent.

A significant observation derived from this work is that genotypes with high shoot regeneration frequencies in medium E showed at least some shoot morphogenesis in all the other media tested, whereas those genotypes that regenerated poorly on medium E behaved similarly in other media. Genotype, rather than culture environment, thus appears to be a more critical determinant of shoot regenerability. This suggests that recalcitrant genotypes may not be readily induced to regenerate shoots by simple medium modifications. However, whether all types of protoplasts (e.g., mesophyll, hypocotyl, suspension culture cell) from a given genotype are equally competent to develop and give rise to shoots in a specific medium has not yet been thoroughly examined.

An efficient response in culture (i.e., high frequency of shoot formation from leaf protoplasts) has been found for some cultivars of broccoli, cauliflower, kale, and brussels sprouts. These cultivars could be readily used for in vitro manipulations at the protoplast level using the procedures adapted in our laboratory. For the recalcitrant protoplasts, it is clear that alternative meth-

ods may have to be tried for successful culture. Among these methods is the use of various cell type/media combinations such as those developed for etiolated hypocotyl cells or perhaps even cultured cells.

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