RESYNTHESIS OF NOVEL RAPESEED VIA PROTOPLAST FUSION

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INTRODUCTION

It has been demonstrated cytogenetically that Brassica napus(2n=38,AACC) is an amphidiploid of Brassica campestris (2n=20,AA) and Brassica oleracea(2n=18,CC)(U 1935). Early reports of artificially resynthsized B. napus involved culture techniques such as embryo rescue. The recovery of hybrids was limited, however, to crosses that generated some degree of embryo development prior to abortion. More recently, protoplast fusion has been widely used for somatic hybridization between B. campestris and B. oleracea. Since the first report of successful rapeseed resynthesis by protoplast fusion (Schenck and Röbbelen 1982), numerous somatic hybrids have been successfully recovered, increasing the germplasm base as well as the agronomic diversity and utility of this crop.

We are interested in regenerating B. napus from protoplast fusion with germplasm selected specifically for desirable extremes in fatty acid composition. Fatty acid targets include low palmitic, low linolenic and high erucic acids. In the following progress report, five of ten protoplast fusion experiments performed are discussed. In addition, the first known report of a successful sexual hybridization recovered from a precociously germinated seed is described and compared with somatic hybrids of the same parental genotypes.

MATERIALS AND METHODS

Somatic Hybridization

Seeds of 3 B. oleracea and 3 B. campestris genotypes (Table 1) were germinated and grown aseptically in MS media containing no hormones and 3% sucrose. Leaf mesophyll protoplasts were obtained by digestion of 100-150 mg of newly expanded leaf tissue in 1.5ml of an enzyme solution containing final concentrations of 2% cellulysin, 1% macerase and 0.5% driselase. In all fusions, the B. oleracea parent was treated with 5mM iodoacetate solution to prevent division of unfused protoplasts. No pretreatments were given to B. campestris protoplasts. A polyethylene glycol-based fusion technique (Thomzik and Hain 1988) was used with minor modifications. A series of regeneration media was used as previously described (Pelletier et al. 1983), with an additional medium containing 1.0 mg/l benzyladenine and .08 mg/l GA_3 as the only hormone source used to obtain shoot regeneration for fusions 1-4. A feeder layer system (Walters and Earle 1990) was used for the first 20-30 days of culture.

Shoots were removed from calli when they were at least 1.5 cm long and transferred to MS medium with no hormones. Once roots formed, the shoots were potted in a peat/vermiculite mix, acclimatized and transferred to a 24°C greenhouse with

Table 1. Parental combinations of five fusion experiments

Fusion number	*Brassica oleracea* parent ssp./LC[1]	Fatty acid[2]	*Brassica campestris* parent ssp./LC[1]	Fatty acid[2]
1	botrytis/BI	high 22:1	oleifera/AN	low 16:0
2	botrytis/BI	high 22:1	oleifera/AN	high 22:1
3	capitata/BI	low 16:0	pekinensis/AN	low 16:0
4	capitata/BI	low 16:0	oleifera/AN	low 16:0
5	italica/AN[3]	high 22:1	pekinensis/AN	low 16:0

[1] Subspecies/ life cycle: AN=annual, BI=biennial
[2] Fatty acid extremes: 16:0=palmitic acid, 22:1=erucic acid
[3] Self-compatible white-flowered broccoli selection from the cultivar 'Packman'.

supplemental light. Plants were grown to maturity and hybrids were verified on the basis of morphology, isozymes and total nuclear DNA. Total nuclear DNA was analysed with an Epics Profile Analyser(Coulter, Hialeah, FL). Leaf samples weighing 50-100 mg were chopped and stained with propidium iodide and values were calculated using chicken red blood cells as a standard with the log scale of fluorescence.

Sexual Hybridization

Ninety total crosses were made between 13 genotypes of *B. campestris* and 11 of *B. oleracea* selected for extremes in fatty acid composition in an attempt to recover sexual hybrids for comparison with somatic fusions. Bud pollinations were done 1-2 days prior to anthesis and mature seed was harvested and germinated. Seedlings were grown to maturity and hybrid verification was done as previously described.

RESULTS

Fusions 1 and 2 have produced a large population of shoots (Table 2) with most of the calli giving rise to multiple shoots. Absence of auxin stimulated the production of first observed shoots at 14 and 16 weeks from protoplast isolation for fusions 1-2 and 3-4, respectively. Fusion 5, however, produced the first observed shoots 7 weeks after isolation on medium containing auxin. The shoot production for fusions 1 and 2 occurred over a 6 week period. In contrast, fusion 5 shoots were generated over a 2 week period and the calli remain healthy and actively dividing.

Morphological observations and flow cytometric analyses revealed that an intriguing array of fusion products has been obtained thus far. Fusions 1 and 2, which have the same *B. oleracea* parent, resulted in quite different fusion products. Fusion 2 produced many plants with normal morphology which subsequently were determined to contain 2.31 pg total nuclear DNA (diploid *B. napus*). Conversely, fusion 1 produced very few plants resembling normal diploids, with a majority of the regenerates exhibiting distorted leaf morphology (Fig. 1).

Leaf samples of fusion 1 have resulted in only 1 confirmed diploid (2.34 pg) thus far. The majority of the samples showed total DNA values ranging from 3.51-3.57 pg. This suggests that the abnormal growth observed may be a direct result of fusions involving 1 B. campestris (1.1 pg DNA) and 2 B. oleracea (1.23 pg DNA each) protoplasts.

Table 2. Protoplast fusion culture efficiency and shoot regeneration summary[1]

Fusion	Protoplasts plated/ml	Calli produced (division efficiency)[2]	Calli that produced shoots (% of total)	Total shoots obtained
1	9.5×10^4	213 (35%)	27 (12.7)	68
2	2.8×10^4	167 (24%)	21 (12.6)	53
3	5.5×10^4	205 (63%)	5 (2.4)	14
4	6.0×10^4	155 (60%)	3 (1.9)	7
5	2.7×10^4	110 (37%)	10 (9.1)	18

[1] Data collection in progress for fusions 3-5.
[2] Percent of protoplasts having undergone at least 1 division over the total number of protoplasts that had resynthesized cell walls.

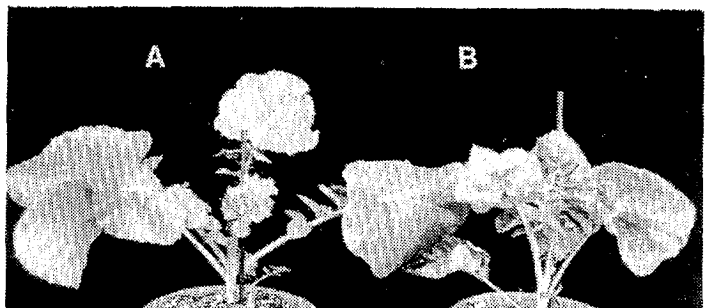

Fig. 1. Resynthesized B. napus from fusion 1. (a) normal diploid(AACC) and (b) 3-way fusion(AACCCC)

Eighty-nine of the ninety crosses performed with 50-100 flowers bud pollinated per cross were tested and found to be escapes, or self-pollinations of the female parent (data not

shown). However, in the cross of the B. oleracea and B. campestris parents listed for fusion 5 (Table 1), as the female and male, respectively, a single seed precociously germinated in the silique near the time of ovule maturation. The seedling was removed from the pod and planted in a peat/vermiculite mix. As the plant matured, leaf morphology strongly suggested the plant was a resynthesized B. napus. In a comparison of sexual versus somatic hybridization, it can be seen that the leaves of the sexual and somatic hybrids are nearly identical (Fig. 2).

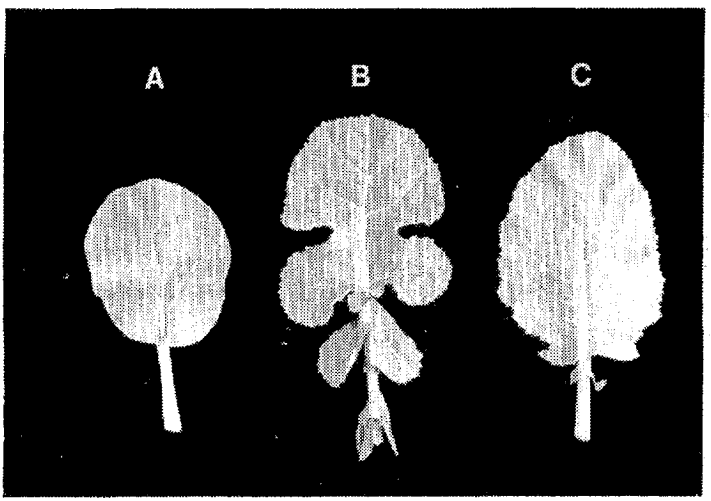

Fig. 2. Rosette leaves of (a) B. campestris ssp.pekinensis, (b) sexually resynthesized B. napus and (c) B. oleracea ssp. italica.

Further verification of the hybrids was derived from isozymes (data not shown) and flow cytometry. Flow cytometric values for the sexual hybrid through the fifth leaf stage suggested the plant was in the haploid state (1.16 pg DNA). All subsequent leaf samples prior to flowering resulted in a chimeral ploidy pattern with both haploid and diploid (2.32 pg DNA) nuclear populations in the same leaf sample (Fig. 3). The histogram in Fig. 3 is representative of 12 replicate leaf samples analysed. The right peak in Fig. 3 (4c), is actually the G2 phase peak for the 2c population. Spontaneous chromosome doubling is suspected to have occurred. Flowering was induced after a 3 week period in a 50°C growth chamber. Flow cytometric values following the vernalization period indicated that the plant had completely doubled the original chromosome number. Flower color was yellow as was the male parent, further substantiating the hybrid status. The plant was successfully self-pollinated and the F_2 progeny showed the same B. napus morphology as the F_1. Vernalization requirement and total DNA also held constant in the F_2. Flow cytometric

values for the somatic hybrids from fusion 5 resulted in a range of normal diploid values (2.31-2.33). Leaf morphology of the somatic hybrids generated thus far have ranged from a very close resemblance to the sexual hybrid (Fig. 2), to morphotypes showing some characteristics of the B. campestris parent.

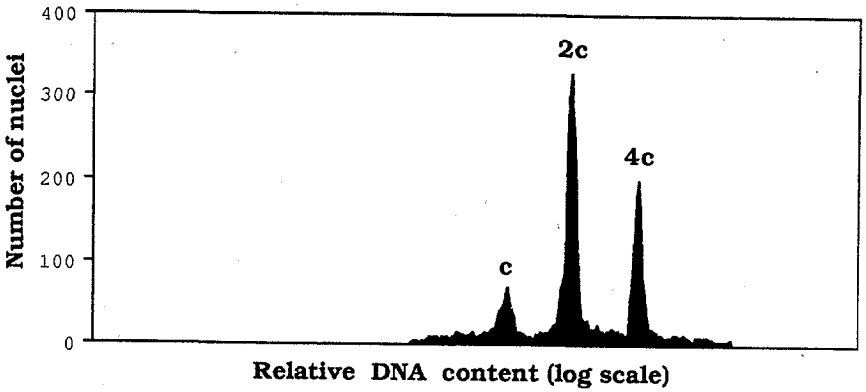

Fig. 3. Flow cytometric results from sexually resynthesized rapeseed showing multiple ploidy levels. Nuclei were isolated from the tenth mature leaf.

DISCUSSION

A large and diverse plant population was successfully obtained from fusions 1 and 2. Such large populations seem particularily desirable in cases where the majority of the regenerates exhibit abnormal growth, yet a few normal diploids are recovered. It can also be seen that fusion 1 resulted from the most dense plating of protoplasts (Table 2), which may not be optimal. Short stem length appears to have been inherited from the cauliflower parent in fusions 1 and 2. This characteristic may be useful for lodging resistance. The range of plant morphotypes generated from fusion 5 exemplify the versatility of protoplast fusion as a way of generating diversity from a single parental combination.

Flow cytometric analyses proved to be a very efficient method for screening both the sexual and somatic hybrids. Elimination of undesirable aneuploids at an early stage of plant development may be desirable, particularily for breeding programs utilizing somatic hybridization. We are particularily interested in the inheritance of the various fatty acid extremes selected for this study following protoplast fusion. Somatic hybrids from fusion 5 will be compared with the sexual hybrid, and fatty acid data from self-pollinated seed of regenerates from all fusion experiments will be compared to values obtained from selfed seed of the parents in a constant environment.

It is intriguing that the sexually resynthesized rapeseed plant required a vernalization period to initiate flowering when both parents flowered as annuals. The reasons for this and the lingering spontaneous chromosome doubling are unclear. It is also unclear why the full diploid state was generated

following the vernalization period. This does, however, provide possible evidence of how such a rare fertilization event might have occurred in nature. The flowering habit of the somatic hybrids will soon be assessed and compared with that of the sexual hybrid. The moderate cold requirement and high degree of homozygosity expected of the sexual hybrid may prove to useful in future breeding efforts.

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