INTERGENERIC CYTOPLASMIC HYBRIDIZATION IN CRUCIFERAE BY PROTOPLAST FUSION.

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I. INTRODUCTION

Alloplasmic lines (the nucleus of one species in a foreign cytoplasm) are generally obtained by sexual crosses and backcrosses with the same pollinator. They are known to sometimes lead to abnormalities in plant development (1). Among these, chlorophyll deficiency and male sterility are the most common (2, 1).

Starting from a sexual cross between cytoplasmic male sterile Raphanus sativus (3) as female and Brassica oleracea as male, the Brassica genome was introduced by repeated backcrosses in radish cytoplasm (5). B. napus and B. campestris alloplasmic lines have been derived from the same cytoplasm by crosses and backcrosses with these species. In all alloplasmic combinations, resulting plants exhibit yellowing at low temperature (below 15° C) and although green at higher temperature, they always maintain a low level of chlorophyll (6). In all cases, plants are cytoplasmic male sterile (cms).

In addition, the flowers of these plants have less developed nectaries and a reduced production of nectar. This defect may be of importance in hybrid seed production, since the cms plants are less attractive for honey bees, the most important vectors in cross pollination (7).

Another type of alloplasmic situation is of interest for Brassica crops. An atrazine-resistant B. campestris biotype has been discovered (8, 9). Indeed alloplasmic B. napus lines with B. campestris atrazine-resistant cytoplasm have been shown to be fully resistant to this herbicide (10). Atrazine-resistance could be useful as a weed control strategy.

Two kinds of fusion experiments have been performed using these alloplasmic lines with the rapeseed nucleus.

In the first type, the two cytoplasms were Raphanus sativus and Brassica napus, in the second, Raphanus sativus and Brassica campestris atrazine-resistant.

We describe here, for both cases, the creation of "cybrid" plants combining desirable traits in a new "hybrid" cytoplasm.

II. MATERIAL AND METHODS

- Table 1 summarizes the plant material and genetic markers used :

Table 1

Name of cultivars	Nuclear markers		Cytoplasmic markers			
	Erucic acid	Petals	S/F	N^+/N^-	G/Y	Atr^R/Atr^S
"Brutor"	0	narrow	F	N^+	G	Atr^S
"C"	+	large	S	N^-	Y	Atr^S
"Tower"	0	large	F	N^+	G	Atr^R

S/F : male sterility / male fertility
N^+/N^- : developed nectaries / underdeveloped nectaries
G/Y : green leaves / yellow leaves
Atr^R/Atr^S : atrazine resistance / atrazine susceptibility

- Protoplasts were isolated from leaves of these plants, by enzymatic treatment and fused with P.E.G 6000 (25 %) in 3 % $CaCl_2$ 2 H_2O.

- Plants regenerated from colonies were grown in soil and put in the cold for two weeks (temperature between 12 and 15° C). Under these conditions, it is possible to detect plants showing chlorophyll deficiency (yellow young leaves and pale green well expanded leaves). After this cold treatment, plants were cultivated in the greenhouse until flowering in order to test male sterility/fertility.

- Chloroplast types have been characterized by endonuclease restriction analysis of DNA and two dimensional separation of thylakoïd polypeptides (see VEDEL & al. these proceedings).

- Meiosis were analysed on pollen mother cells (metaphase I). Chromosomes in mitosis were counted on root tip cells treated by Feulgen method.

- Dosages of erucic acid in cotyledons were performed according to (11).

III. RESULTS

A. Plant regeneration from leaf protoplasts of Brassica napus

Early reports on plant regeneration from rapeseed mesophyll protoplasts showed that the frequency of regeneration was very low (12, 13, 14). Three recent reports using different procedures and different starting materials (15, 16, 17) have shown improved regeneration rate from stem embryos, roots or leaf protoplasts. Rapeseed itself has been resynthesized from its parental species by protoplast fusion and selection based on its better ability to regenerate (18).

To obtain plant regeneration from protoplasts, a sequence of four different media has been used from protoplast isolation until bud regeneration of colonies. Our procedure is relatively efficient since it allowed us to regenerate several hundred plants.

We first have regenerated plants from normal and from cms rapeseed as controls. We examined 63 plants at flowering, regenerated from "Brutor" protoplasts. All of them possessed normal anthers, although some plants displayed morphological alterations (crisped leaves, large flowers). In the later case, some chromosome number modifications had occured (results not shown). On the other hand, 48 plants regenerated from "cms" protoplasts were all male sterile and chlorophyll deficient.

B. Phenotypes of plants produced by protoplast fusion

1. Fusion of rapeseed protoplasts containing R. sativus cytoplasm with protoplasts containing B. napus cytoplasm

Table 2 - Phenotype of regenerated plants (1st experiment)

Nuclear markers		Cytoplasmic markers			Number of plants
Erucic Acid	Petals	S/F	N^+/N^-	G/Y	
not tested	large	S	-	Y	48
+	large	S	+	G	5*
-	narrow	1/2 F	+	G	2*
not tested	narrow	F	+	G	76

* Plants displaying a new combination of S/F and Y/G characters.

Among about 7.000 colonies derived from a fusion experiment, 176 regenerated buds. We have now grown 131 of these plants to the flowering stage. Table 2 gives the number of different phenotypes obtained according to morphological markers. Seven plants coming from different colonies are of particular interest because they display a

new combination of the two different cytoplasmic markers. Plants n° 23, 27, 58, 85, 118 are fully male sterile but with normal color of leaves. Plants n° 41 and 122 have green leaves and produce little pollen and no seeds without manual intervention (classed as half-fertile.

2. Fusion between rapeseed protoplasts with R. sativus and B. campestris atrazine-resistant cytoplasm

Table 3 - Phenotypes of regenerated plants (2nd experiment)

Nuclear markers	Cytoplasmic markers			Number of plants
Erucic Acid	S/F	N^+/N^-	G/Y	
not tested	S	N^-	Y	83
+	S	N^+	G	1*
not tested	F	N^+	G	1

* Atrazine resistance/susceptibility tests are in progress particularly for this line.

On about 16.000 colonies resulting from five fusion experiments, only 199 regenerated buds (more than half of the colonies did not grow and controls showed that our "Tower" parent regenerated very poorly under our conditions). We now have 85 plants at the flowering stage. Table 3 shows that only one plant is of interest. It corresponds to the colony number 77^{II} : this plant is male sterile and of normal green color at low temperature.

Plants number 23, 27, 58, 85, 118 and 77^{II} derived from the above fusion experiments, which show clearly a new combination of cytoplasmic morphological markers, i.e. full male sterility, and green leaves were considered to be cybrids. Plants 41 and 122 need confirmation because they are only half fertile.

C. Analysis of cp DNA from regenerated plants by restriction fragments electrophoresis

A total of 85 regenerated plants from both experiments were analyzed for cp DNA. We found in the first experiment only B. napus or R. sativus patterns, without any changes in patterns or mixtures between two restriction profiles. Green plants possess B. napus cp CNA, yellow ones always R. sativus cp DNA. In the second experiment, the two green plants obtained show the B. campestris chloroplast DNA pattern.

Among these, cybrids, which are green and cms, have B. napus (in the first experiment) or B. campestris (in the second experiment) chloroplast DNA.

D. Identification of chloroplast by two dimensional separation of thylakoid polypeptides

It has been confirmed by this method that cybrids 23, 27, 58, 85, 118 and 77^{II} possess B. napus or, for the last one, B. campestris (undistinguishable) chloroplast ATPase.

E. Chromosomes counting

Chromosomes of cybrids obtained in the first experiment were counted at meiosis (metaphase I). Cybrid 23 possesses 76 chromosomes ; cybrid 27, 63 chromosomes ; cybrid 58, 76 chromosomes ; cybrid 118, 76 chromosomes. Chromosomes of line 77^{II} coming from the second experiment have been counted on root tips of cuttings. It possesses 38 chromosomes. 6 plants of its progeny have also the normal chromosome number of rapeseed (38).

F. Dosage of erucic acid

Cybrids 23, 27, 58, 118, pollinated by "Brutor" variety (no erucic acid) give seeds containing erucic acid. This result proves that cybrids tested possess at least one "C" genome from the cms parent. Cybrid 77^{II} pollinated by "Brutor" variety gives seeds with erucic acid. It is very likely in this case that this line possesses only "C" nucleus.

G. Progeny of cybrids plants

We have obtained now several plants after two successive backcrosses from cybrids. These progenies keep stably their new cytoplasm (plants are male sterile and normally green).

IV. DISCUSSION

Using somatic hybridization we obtained plants containing novel combinations between chloroplasts of B. napus or B. campestris and male sterility from a R. sativus cytoplasm.

Our results show that cytoplasmic hybridization through protoplast fusion may be useful in plant improvement. We were able to obtain cms plants with functional chloroplast. This new cytoplasm is now presumably usable for hybrid rapeseed production, and after transfer to cabbage and turnip, it might become a useful agronomical tool in these species as well. Moreover, in the plant obtained in the second experiment, two cytoplasmic characters belonging to two different genera, Raphanus sativus, for male sterility and Brassica campestris for atrazine resistance, are associated with the nucleus of a third

species, B. napus. This kind of plant might allow the commercialisa- of hybrid seeds of atrazine-resistant Brassica. Cuttings of this plant and its progeny are now under investigation for atrazine-resistance.

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