Towards *in situ* androgenesis in *Brassica napus*

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An efficient way to create hybrid cultivars is to use cytoplasmic male sterile (cms) lines. In *Brassica napus*, various cytoplasms inducing male sterility exist, some of them originating from somatic fusions (1). To transfer these cytoplasms into fertile rapeseed cultivars, time consuming backcrosses are required.
In situ androgenesis is a natural phenomenon leading to the development of a haploid plant bearing the maternal cytoplasm and only the haploid paternal genome; after doubling the set of chromosome of the haploids, an homozygous line is obtained. This provides a means to directly transfer a specific cytoplasm to a cultivar in one generation.
However the occurance of androgenic plants is rare, as estimated in tobacco for example (2). Therefore, to increase this efficiency it is necessary to establish a counter-selection system with a conditional marker gene. This will maximize the probability of recovering haploids among a large population of F1 seedling at the earliest stage.

Methods

a) Estimation of the androgenetic haploid production frequency:
The strategy of this estimation is based upon the recovery of haploids among the F1 seedlings resulting from a cross between a homozygous NPTII cms line 1 and a fertile line 2. The F1 seeds are heterozygous for NPTII, thus resistant to kanamycin. When sown on the antibiotic, they will all grow normally except the sensitive paternal haploids.To identify the genome of these putative haploids, they are then tested for isoenzymes (line 1 and 2 show distinct patterns) and analyzed cytologically.

b) Counter-selection:
A conditional marker gene is provided by the "*aux2*" gene found in the TR region of the Ri plasmid of *Agrobacterium rhizogenes* (agropine strain A4). This gene encodes an enzyme converting an auxin precursor (NAM, naphthalene acetamide) into an auxin (NAA, naphthalene acetic acid). It has been characterized and cloned in a transformation vector, associated with the 19S NPTII gene confering kanamycin resistance to plant cells. The gene "*aux2*" was inserted under the control either of its own promoter or of the CaMV 35S promoter with a double enhancer sequence (4).
"*aux2*" transformed plant tissues are able to overproduce auxin when in presence of the precursor. Consequently, "*aux2*" seeds germinated in presence of NAM cannot develop normal roots, and it is easy to distinguish them from non-transformed seedlings.
The counter-selection strategy is to pollinate a cms line, homozygous for the "*aux2*" gene, with the cultivar and to germinate seeds in presence of NAM. Only the androgenetic haploid will grow normally.

Transformed Material

a) Cms lines of *Brassica napus* :
The lines used are:
- the fertile spring line "Brutor"(A), and the "cms Brutor"(B) bearing the cybrid cytoplasm 27 (coming from somatic fusion between rapeseed and an allopasmic rapeseed having the cms cytoplasm of Japanese radish found by Ogura (1)).
- the rapid cycling CRGC5 (C) and this line bearing the cms Ogura cytoplasm(D).

b) Kanamycin marker:
Previously, the NPTII gene confering kanamycin resistance was introduced into the fertile line B by electroporation (3). From it, the homozygous cybrid cms line 1

(Brutor) was developed and crossed with a different fertile line 2, (Drakkar) at the Plant Beeding Station at Le Rheu . These two lines were chosen because it is possible to discriminate them by isoenzyme analysis. A large amount of seeds is now available.

c) Transformation with the entire Ri T-DNA:
Floral axis segments of (B) plants were inoculated with wild *Agrobacterium rhizogenes* (A4). Plants regenerated from the hairy roots contain the TR-DNA and exhibit the sensitivity to NAM, so they can be used in this program. Although most of the transformed plants show a deleterious phenotype (wrinkled leaves, short internodes and low fertility) some of them grow normally, have a good fertility, still react to the NAM. Two Ri T-DNA homozygous lines have been developed, a cms 27 "Ri T-DNA" line, and its fertile maintainer.

d) Transformation with the "*aux2*" gene:
The construct "*aux2*, NPTII" is available in a disarmed *A . tumefaciens strain* (C58). By co-inoculation with the two bacteria, wild-type A4 and C58 "*aux2*", (C) plants were transformed and "*aux2*" plants were regenerated. Homozygotes for "aux2" at either one or two separate loci have been obtained.
Floral axis segments of (A) plants were also co-inoculated. Besides hairy roots, shoots were obtained directly . One shoot was co-transformed. This technique is an easy way to transform rapeseed, provided sufficient transformants are obtained to ensure independant integrations. Segregation of the non desired wild-type Ri T-DNA from the "*aux2*, NPTII" genes was achieved through backcrossing on the cms line, with selection on kanamycin of the offsprings. Here again, the two homozygous "*aux2*, NPTII" lines have been developed, the 27 cms line and its maintainer.

Preliminary experiments.

a) First, protocols have been developed to recover sensitive plants after a kanamycin treatment of the seedlings resulting from a cross between the homozygous NPTII cms line 1 and the sensitive fertile line 2. A thousand resistant seeds mixed with a few seeds of the sensitive parent are germinated in vitro on a medium containing 100mg/l kanamycin . After 5 days, white hypocotyl and yellowish cotyledon plantlets can be recovered. Subsequently, they can survive in a medium free of kanamycin.
b) On a medium containing 2mg/l NAM, a clear distinction is possible between the normal and transformed Ri-T-DNA or "*aux2*" seedlings.
c) Greenhouse conditions will also be established to examine a larger population of seeds to recover the androgenetic haploids.

Conclusion

To date, we have established conditions to: - determine the frequency of the production of androgenetic haploids, - produce them in a plant breeding program. The results are presented on the transformation of rapeseed as a means of introducing different markers into cms lines and their maintainers.

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