APPLICATION OF MICROSPORE CULTURE TO CANOLA IMPROVEMENT

B.Huang, E.B.Swanson, C.L.Baszczynski, W.D.Macrae, E.Barbour, V.Armavil, L.Cooke, M.Arnoldo, S.Rozakis, M.Westecott, R.F.Keats and R.Kemble

Allelix Crop Technologies, 6850 Goreway Dr. Mississauga, Ontario, Canada L4V 1P1

INTRODUCTION

Conventional plant breeding has brought about major improvements in yield and quality of many crops, including oilseed Brassica species. An excellent example is the breeding of low erucic acid (≤ 2%), low glucosinolate (≤ 30uM) (canola quality)rapeseed cultivars in Canada. The rapidly emerging biotechnological approaches, including in vitro plant cell and tissue culture, in vitro induction of haploid plants as well as gene transfer technology, have also contributed to genetic modifications towards crop improvement. In Brassica species, particularly B.napus, the isolated microspore culture system (Keller et al. 1987) and its application to canola improvement have been investigated extensively in recent years. As a result, microspore culture is now used by many canola breeders to rapidly achieve homozygosity in conventional breeding programmes. Genetically modified canola plants can also be readily produced by mutagenesis of microspores (Swanson et al. 1989) or transformation of microspore-derived embryos (Swanson and Erickson, 1989).

The isolated microspore culture in B.napus is single-celled, haploid, and yields large numbers of microspore-derived embryos. Both microspores and embryos are easily manipulated in culture. In many cultivars of B.napus, including Topas, Westar, Delta, Bounty, Winfield, Corvette and many others, thousands to millions of microspore-derived embryos can be produced from one isolation (one person/day). Many embryos develop into double-haploid plants that are morphologically normal, making the isolated microspore culture in Brassica one of the most efficient system for plant regeneration in vitro.

This paper discusses approaches to canola improvement through the use of microspore culture in conjunction with conventional plant breeding and mutagenesis, and in particular, with gene transfer techniques. Procedures for isolation, culture and mutagenesis of microspores, as well as transformation of microspore-derived embryos, are described in detail.

MATERIALS AND METHODS

Seeds of Brassica napus cultivars were sown and seedlings germinated and grown at 15° day/ 5° night. Plants were watered and fertilized daily. For winter cultivars, seedlings bearing 2-4 true leaves were vernalized at 4°C for 10-12 weeks before transfer to 15°/5°C.

Microspore isolation and culture. Flower buds 2.5-3.5mm in length, green to yellow green in colour, were harvested from plants, surface sterilized for 10 min in commercial

bleach (6% hypochlorite) and rinsed three times in sterile water. Buds were transferred to B5 medium (Gamborg et al. 1968) supplemented with 13% sucrose (B5-13), and then either macerated with a glass plunger from a syringe against a glass beaker (Huang et al. 1989) or blended in a cool microblender (Swanson et al. 1987). The microspores were filtered through 44um nylon mesh, centrifuged at 100 x g for 5min. The supernatant was discarded, the pellet resuspended in B5-13, and the suspension centrifuged at 100 x g for 5min. This step was repeated two more times, and the washed microspores were resuspended at 10,000 to 50,000 per ml modified Lichter (Lichter,1981) medium supplemented with 13% sucrose. Potato extract and growth regulators were omitted from Lichter's medium. Microspores were plated in 100 or 60mm Petri dishes, cultured in darkness at 32°C for 7 days and then transferred to 25°C (shaking at 50RPM).

Plant regeneration. Approximately 21 days after microspore isolation, microspore-derived embryos (3-5mm in length) were transferred to B5 medium supplemented with 2% sucrose and 0.2% gelrite, at a density of 10 embryos per 100mm Petri dish. Embryos were cultured at 20-25°C, 16h light for 2-6 weeks with a transfer interval of 2 weeks. Plants were regenerated either directly from the embryos or from shoots and secondary embryos developed on the primary embryos. Plants with healthy roots were immersed in 0.2% aqueous colchicine solution and left under strong light for 2-4 hours before they were thoroughly rinsed and potted in soil.

Microspore mutagenesis. Mutagenesis was conducted 1d after microspore isolation with either 0.5Krads gamma irradiation or 20uM ethyl nitrosourea (ENU) (Swanson et al. 1988). For ENU mutagenesis, microspores were washed with modified Lichter medium 3 days after the addition of ENU.

Agrobacterium-mediated transformation of microspore-derived embryos. A series of binary vectors containing various promoter, gene and terminator cassettes were constructed and introduced into the Agrobacterium tumefacience nopaline-producing strain, C58, and its disarmed derivatives. Using the prototype vector pALLTKREP (Fig.1), various useful promoter or gene cassettes in front of, or in place of the GUS gene, respectively, were introduced and used for canola transformation. All transformation vectors contained the NPTII gene within the t-DNA border which confers resistance to kanamycin when expressed in plant cells.

The Agrobacterium strains were grown overnight in liquid L-Broth medium supplemented with 20uM acetosyringone and the appropriate antibiotics. Bacterial culture was centrifuged at 1000 x g for 20 minutes, the bacterial pellet resuspended in a small volume of L-Broth and added to liquid cultures of microspore-derived embryos (2-5mm in length, 21-35 days after isolation) to a final concentration of about 10^8 per ml or OD_{600} of 0.1 (about 100 embryos per ml culture). Embryos were wounded with forceps or crushed glass during or after the 30 min co-cultivation period. The embryo/Agrobacterium mixture was filtered onto a 1mm mesh nytex filter and the embryos (together with the filter) were incubated on B5-cocultivation medium (B5 medium supplemented with 2% sucrose, 0.1ppm 2,4-D, 0.05ppm BAP, 20uM acetosyringone, 10mM MES pH 6.0 and 0.8% agar) for 2 days. After Agrobacterium co-cultivation, embryos, still on filter, were transferred onto BC medium (B5 + 0.05ppm BAP + 250ppm carbenecillin) and cultured for 5 days.

Individual embryos were then transferred onto BC medium containing 50ppm kanamycin for the period of the experiments (2-5 transfers to fresh medium with 14 day intervals). Only one shoot was retained from each embryo. Green shoots formed and rooted on kanamycin-containing medium were colchicine treated and potted.

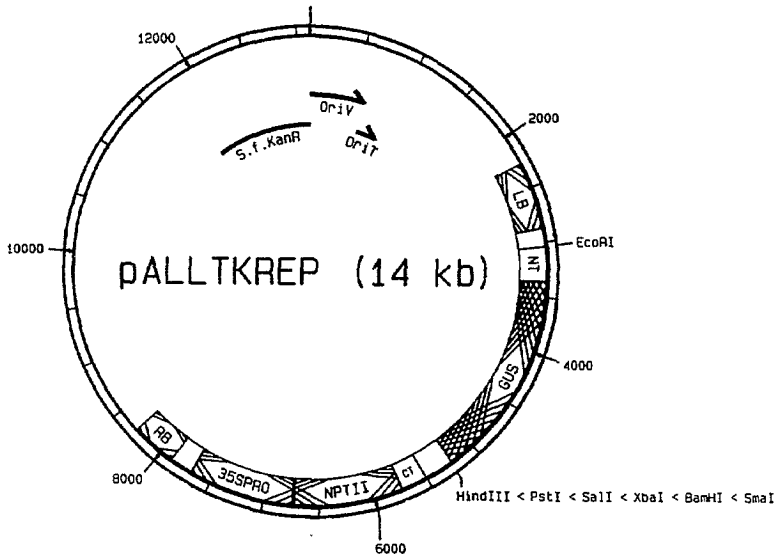

Figure 1. Restriction map of the binary vector pALLTKREP.

Analysis of transformed and mutant plants Stable integration and expression of the foreign genes in the haploid Brassica genome were confirmed by the NPTII dot blot assay (McDonnell et al. 1987), Southern analysis (Maniatis et al. 1982) and seed germination on medium containing 100ppm kanamycin. Protocols for molecular analysis of mutant plants were based on those described by Maniatis et al (1982). Fatty acid composition of seeds were analysed using gas chromatography.

RESULTS

Herbicide tolerant mutants from microspores mutagenesis In an earlier report (Swanson et al. 1989), five plants that were tolerant to Pursuit, a sulfonylurea herbicide (up to 10 times the field application level, 50-500g/ha) were obtained from the microspore mutagenesis of B.napus cv. Topas microspores. Pursuit (5-ethyl-2-[-isopropyl-4-methyl-5 oxo-2-imidazolin-2-yl]nicotinic acid) is a broad spectrum herbicide that was considered environmentally safe. It was provided by American Cyanamid Company of Princeton, New Jersey. Plants tolerant to Pursuit were regenerated from microspore-derived embryos formed in the presence of 40-50ug/l Pursuit. Crosses between these homozygous, Pursuit-tolerant plants and unaltered B.napus cv. Topas produced progeny showing intermediate tolerance. Crosses between plants of two of the Pursuit tolerant lines, P1 and P2, however, produced plants that were superior in Pursuit tolerance to either of

the parental mutants.

Acetohydroxyacid synthase (AHAS), the first enzyme in the pathway leading to the biosynthesis of leucine, valine and isoleucine, is the primary site of action for the inhibitory effects of the sulfonylureas. The genes coding for the AHAS enzyme have been cloned from one of the Pursuit tolerant lines P2. One of the two transcriptionally active AHAS genes from P2 had at least two mutations that appeared functional (sequence not shown).

Mutants having altered fatty acid profiles Hundreds of double-haploid plants(DHs) were regenerated from mutagenized microspores and seeds from these plants analysed for fatty acid composition. Alterations in fatty acids of nutritional value include lower saturated fatty acid (palmitic and stearic acids) and higher oleic acid. Both were achieved to a certain degree in several populations of DHs. For instance, in one population of 250 DHs derived from mutagenized microspores of spring canola Topas, 8 plants contained less than 6% saturated fatty acids in their seeds (Topas control was 7.8%) and 2 plants contained more than 75% oleic acid (Topas control was 65.8%).

Transformation of microspore-derived embryos Embryos from Topas untransformed control produced only white/purple shoots on medium containing 50ppm kanamycin whereas green shoots developed from virtually all the embryos on kanamycin free medium (100% survival rate). After co-cultivation with Agrobacterium, the survival rate was not reduced and up to 10% of embryos produced shoots that remained green on 50ppm kanamycin for over 10 weeks. Approximately 50% of these shoots were found to have neomycin phosphotransferase activity using a dot blot assay. All shoots that tested positive consistently in this assay showed foreign DNA integration in Southern analysis.

When the oncogenic Agrobacterium strain C58 was used, 60-80% of the green shoots surviving kanamycin selection were teratomas. These were discarded.

The frequency of transformation in Topas (% of embryos giving rise to NPTII positive shoots) varied from 0 to 5% with different Agrobacterium/vector combinations and averaged 1-2% (Table 1). Transformation frequencies in other cultivars were similar to those in Topas, although the yield of microspore-derived embryos was much lower. Genes involved in disease resistance, meal quality and oil content modification have been transferred into canola using the Agrobacterium-mediated transformation of microspore-derived embryos.

Table 1. Transformation frequency (% microspore-derived embryos giving rise to plants that tested positive in NPTII dot blot assay, teratomas excluded) in B.napus cv Topas.

Experiment #	No. embryos co-cultivated	Agrobacterium Vector	Transformation frequency
1	100	dC58/pALL70	3.0
2	200	C58/pALL21	2.5
3	160	C58/pALL21	2.5
4	242	C58/pBI121.2	0.8

Note: dC58 was disarmed C58.

Approximately 50% of the transformants had integration of a single copy of vector DNA in the plant genome while others had two or more integrations. With the exception of one plant which was possibly chimeric, all transgenic plants produced seeds of which 100% were kanamycin-resistant in seed test, indicating homozygosity of the plants. Small scale field tests showed that transformed plants were not different from the original cultivar in their morphology, maturity date and oil quality.

DISCUSSION

The microspore culture system in Brassica The value of haploidy in breeding has been realized for many years. Homozygosity can be achieved in one step by doubling the haploid genome. Many attempts have been made to produce haploid individuals in various crops. In canola, our ability to efficiently recover homozygous diploid plants from cultured microspores has enabled us to utilize this system in various aspects of plant breeding and biotechnology projects.

At Allelix, microspore culture is being conducted routinely on hybrid breeding lines to recover homozygous diploid plants. One such example is a cross between the canola quality Topas that's highly responsive in microspore culture and a genotype of poorer quality (e.g. high erucic) but highly resistant to Sclerotinia. Hundreds of plants have been produced and are being tested in the field. Since the fatty acid composition of seeds has been shown to be correlated with that of microspore-derived embryos, many embryos were screened and only the embryos having low erucic content (2%) have been grown to maturity. The significant saving in field test efforts is obvious.

Microspore mutagenesis The combination of haploidy and mutagenesis has yielded valuable mutants in canola. Haploidy is particularly advantageous when recessive or multiple genes are involved. A recessive mutation in a diploid genome will not produce a phenotype and will easily be lost. For traits controlled by more than one gene, the use of haploidy significantly reduces the time required to achieve homozygosity and increases the chance of combining all mutations in homozygous state.

The mutants produced at Allelix described here are of two categories: (a) herbicide tolerance and (b) altered fatty acid composition. While the Pursuit tolerant lines proved satisfactory in field tests over several years, lower levels of saturated fatty acid are desired from the other type of mutants (current lowest just under 5%). The level of saturated fatty acid in canola is likely to be controlled by many genes. Currently various lines containing low levels of saturated fatty acids are being inter-crossed. Homozygous diploids will be recovered from these crosses and fatty acid composition will be analysed.

Haploid transformation The use of haploid explants for transformation leads to the production of homozygous diploid transformants in one step. In addition to microspore-derived embryos, other haploid explants such as stem segments and leaf discs have also been used for transformation. Transformed canola plants have been produced by Agrobacterium-mediated transformation of haploid stem segments at a frequency similar to that of diploid stem segments (1-10%). When the number of

embryos is limited, they can be micropropagated to produce many haploid plants from which stem segments can be used for transformation.

Co-cultivation of freshly isolated, uninucleate microspores with Agrobacterium has produced transformed canola plants at very low frequencies. Microinjection of microspores and microspore-derived embryos (Neuhaus et al. 1987) has also been attempted but produced no transfomed canola yet in our hands.

Microspore culture is also being used to recover homozygous diploid plants from transformed canola plants which are heterozygous for the foreign gene(s). If the transformant has more than 1 insertions of the foreign gene, microspore culture can be used to produce plants homozygous for all, or any combination of, the foreign gene insertions.

Conclusions Microspore culture of B.napus is a very efficient system for regeneration of homozygous diploid plants, and is being used to achieve homozygosity in conventional breeding programmes. Microspore culture has also been utilized successfully for the production of mutant lines and for gene transfer.

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