TRANSFORMATION OF FRESHLY ISOLATED *BRASSICA* MICROSPORES AND REGENERATION TO FERTILE HOMOZYGOUS PLANTS

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ABSTRACT

We have developed an *Agrobacterium*-mediated transformation system for freshly isolated microspores of *Brassica rapa* line "CV2" and *B. napus* cv. "Topas". The production of up to 15 independent putative transformants per 1 x 10^7 microspores can be achieved. Putative transformed plants carrying the PAT gene have been selected *in vitro* using phosphinothricin (syn. glufosinate ammonium). Southern analysis confirmed the integration of single copies of the PAT gene. R_0 plantlets were treated with colchicine and transferred to soil two to three months after microspore culture initiation. Selfed R_1 and crossed seeds were obtained. Progeny plants survived spray treatment with phosphinothricin demonstrating the sexual transmission of the transferred gene.

INTRODUCTION

Microspore embryogenesis is well suited for genetic transformation. Single celled microspores with high regenerative potential can be easily handled in large numbers and the introduced trait can be duplicated during chromosome doubling for doubled haploid production. Where an efficient tissue culture system is established, the vector of choice for plant transformation is *Agrobacterium* (Potrykus 1993). The *A. tumefaciens* system is simple, inexpensive and the plants obtained have a limited number of gene insertions (DeBlock 1993).

Several attempts have been made to transform plant microspores with *Agrobacteria* (Pechan 1989, Huang 1992, Sangwan *et al.* 1993). However these reports have indicated that it is extremely difficult to obtain transgenic plants by this method. Integration of foreign DNA and regeneration into fertile plants has never been achieved in a reproducible manner. The report by Pechan (1989) shows only the cocultivation of *B. napus* microspores with *Agrobacteria* but did not demonstrate DNA integration or sexual transmission of the transgene. This is the first report of successful and reproducible *Agrobacterium*-mediated transformation of microspores, their regeneration to fertile homozygous plants and the sexual transmission of the transferred trait.

MATERIALS & METHODS

The transformation system for microspores was developed using the *B. rapa* line "CV2" (Ferrie *et al.* 1995). The microspore culture protocol described by Baillie *et al.* (1992) was modified as follows. Microspores 0 - 2 days after isolation were infected with *A. tumefaciens* carrying the PAT or GUS gene. Cellulolytic enzymes added during the cocultivation period enhanced transformation frequency dramatically. After two to three days of cocultivation the bacteria were washed out using B_5 medium (Gamborg *et al.* 1968) containing 500 mg/l carbenicillin or 200 mg/l timentin (SmithKline Beecham,

Oakville). Washing at least once with lysozyme increased recovery of embryos substantially. The above transformation system was also applied to *B. napus* cv. "Topas" using the protocol as described in Keller *et al.* (1987).

After the transformation procedure, embryo development usually occurred one week later than the control. Embryos three to four weeks in culture were placed on a shaker (75 rpm at 22°C) under continuous light for one week. They were then kept in the cold (4°C) for an additional week. Embryos were subsequently cultured and treated with colchicine as described in Baillie *et al.* (1992).

Rooting assays were conducted on the young plantlets. Roots were trimmed off and shoots were transferred to B_5 medium with 20 mg/l phosphinothricin (l-PPT) for five days. The shoots were then transferred to B_5 medium and observations were taken one week later. GUS staining was conducted according to Jefferson (1987). DNA extractions (Dellaporta *et al.* 1983), Southern analyses (Sambrook *et al.* 1989) and PAT assays (Hoechst AG, Frankfurt) were carried out on the plantlets. Greenhouse spray tests were performed on young plants by applying Liberty™ herbicide (3 g/l) followed by observations after one week.

RESULTS & DISCUSSION

Initial experiments using *A. tumefaciens* carrying the GUS gene demonstrated the early transformation events of microspores (data not shown). In the experiments where infection occurred two days after culture initiation, we observed partial GUS staining in approximately 25% of the embryos and complete GUS staining in 1% of the embryos after four weeks. The observations in these early experiments indicate the potential of the microspore transformation method and that it may be more desirable to infect earlier.

In experiments with the PAT gene only, we have regenerated more than 100 putative PAT positive *B. rapa* and *B. napus* transformants. In a typical experiment with infection occurring at day 0 and from infecting 1×10^6 microspores, we can recover up to 40 embryos. The embryos on solid medium develop into normal plantlets. From these plantlets up to two (or up to 15 using 1×10^7 microspores) survived the rooting assay for l-PPT tolerance and are considered putative transformants. These R_0 plants were treated with colchicine prior transfer to soil. Transgenic plants can be transferred to soil two to three months after microspore culture initiation.

So far several transformants have been confirmed using PAT and Southern analyses. The PAT assay shows a strong acetylated l-PPT band and the Southern analysis shows the integration of a single copy of the PAT gene. The R_0 plants produced fertile pollen and have been selfed and crossed in the greenhouse to other agronomically important cultivars. Progeny plants have been sprayed in the greenhouse and have survived demonstrating the sexual transmission of the transferred gene.

Experimental variability among microspore batches has been observed in the present study as has also been reported by Jähne *et al.* (1994). Results show that high frequency embryogenesis is necessary for high transformation frequencies.

We have shown molecular and genetic evidence of stable gene integration using a novel haploid transformation method. Although Pechan (1989) showed cocultivation of microspores, he did not provide evidence of gene integration. The only reports of reproducible haploid transformation via *Agrobacterium* used multicellular embryo-derived explants (Swanson & Erickson 1989, Oelck *et al.* 1991, Huang 1992).

The advantage of our method is the use of unicellular microspores which reduce the occurrence of chimeras as well as shortens the time for the production of transformants.

Future improvements presently being studied include the use of colchicine *in vitro* and the use of l-PPT in an early selection strategy for transformed embryos.

ACKNOWLEDGEMENTS

The authors thank Dr. Kevin Falk (Agriculture and Agrifood Canada, Saskatoon) for providing the *B. rapa* line "CV2" as well as Brigitte Weston and Colleen Bennett for the Southern blot analyses.

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