Gene transfer by *Agrobacterium tumefaciens* to microspores and microspore-derived embryos of winter oilseed rape (*Brassica napus* L.).

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Abstract

Embryogenic microspores and microspore-derived embryos of *Brassica napus* are very suitable materials for introduction of foreign genes. Both microspores and microspore-derived embryos from winter oilseed rape homozygous line DH-O-120 and cultivar Bor were used in our study of *Agrobacterium*-mediated transformation. Those androgenic forms were cocultivated with strains EHA105 or LBA4404, each of them containing the binary plasmid pP35SGIB with the *GUS* gene with intron under the 35SRNA CaMV promoter and the *bar* gene as a selective marker.

To optimize the transformation system based on microspores and microspore-derived embryos, highly efficient and reproducible embryogenesis is essential. Recently, we have developed an efficient system for microspore embryogenesis and double-haploid production in winter oilseed rape. The transformation of microspores and microspore-derived embryos yields green plants without the complication of chimeras.

Up to now all regenerated plants were haploids. PCR analysis was made to determine the presence of the *pat* gene in the regenerated plants and confirm that transformation was effective.

Key-words: *Agrobacterium tumefaciens,* androgenesis, *Brassica napus*, microspore, microspore-derived embryos, transformation, winter oilseed rape.

Introduction

Microspores, haploid embryos, or cells derived from such embryos, are potentially useful as recipients of foreign genes, especially if the frequency of embryogenesis is high. A high frequency of plant regeneration is required for successful gene transfer and recovery of the transgenics. Microspore-derived embryos generally exhibit a high regeneration potential. Also, as a result of chromosome duplication, the introduced trait can be evaluated in the homozygote. To obtain fertile homozygous transgenic plants the technique of the *Agrobacterium*-mediated gene is used the most frequently (Huang 1992). One of the many attractive features of the microspore embryogenic system is the possibility of transformation

of individual cells. Because embryos are derived from individual cells, transformants can be recovered without the complication of chimeras.

Because of the many variables (especially those related to plant materials, *Agrobacterium* strains, and gene constructs with limited access), it is difficult to reproduce published results exactly.

The goal of this study was to develop an efficient method for delivery of foreign genes to winter oilseed rape microspores and microspore-derived embryos with the use of *A. tumefaciens.*

Materials and methods

Bacterial strains and plasmids

Agrobacterium strains LBA4404 and EHA105, containing the binary plasmid pP35SGIB with the *GUS* gene with intron under the 35SRNA CaMV promoter and the *bar* gene as a selectable marker were used.

Plant material

Microspores and microspore-derived embryos (MDEs)of winter oilseed rape *(Brassica napus)* homozygous line DH-O120 and cultivar Bor were used. Microspores were isolated as in our earlier study (Cegielska-Taras et al. 1999), while microspore-derived embryos were prepared as in another study (Cegielska-Taras et al. 2001).

Transformation of microspores

Microspores of oilseed rape have thick walls, which make them difficult to inoculate with foreign DNA. Forty-eight hours after isolation, microspores were used for *Agrobacterium*-mediated transformation. For this specific inoculation we used liquid NLN medium, pH 5.7, with 10 mg/l acetosyringone and 1M glucose for 20 min. After one day of cocultivation the bacteria were washed out with fresh NLN-medium containing 13% of sucrose and 100 mg/l timentin, and then incubated in the dark at 30°C for 7 days and then at 24°C. When the microspore-derived embryos became visible to the naked eye, they were placed on an illuminated rotary shaker at 24°C. When the embryos began to turn green, the medium was changed to NLN medium with 8% of sucrose and 100 mg/l timentin. Green embryos were cultured on solidified B_5 medium containing 0.1 mg/l GA_3, 300 mg/l timentin, 10 mg/l Basta and 2% of sucrose at 1°C for 2 weeks, to stimulate shoot development. Next, the petri dishes with green embryos were transferred to a growth chamber (24°C, photoperiod 16D/8N) for 2 weeks. Survived green explants were cultured on MS medium supplemented with 0.1 mM

kinetin, 300 mg/l timentin, 10 mg/l Basta and 2% of sucrose. The shoots that remained green on this medium were considered to be transformants and were transferred to root induction medium and then planted in the soil for further development.

Transformation of microspore-derived embryos

The 21-day-old microspore-derived embryos growing on B_5 medium with 0.1 mg/l GA_3 were exposed to low temperature for 2 weeks. Next, they were put for a few days in the growth chamber and used for transformation. Cotyledons and hypocotyls were punctured with a sawing needle dipped in *A. tumefaciens* suspension for 2 hours. After infection with *A. tumefaciens*, the explants were placed immediately in B_5 medium (basic) without antibiotics. The explants were cocultivated with *A. tumefaciens* for two days and transferred to B_5 medium with antibiotics (to inhibit bacterial growth) and 10 mg/l Basta for 4 weeks. After that period the still green explants were transferred to shoot induction media (MS medium containing 0.1 mM kinetin) with antibiotics and Basta. The explants were transferred to fresh medium every two weeks. Shoots that remained green on this medium were considered to be transformants and were placed in root induction medium. Plantlets were transferred to soil as above.

All plants obtained were haploids, but chromosome doubling in these plants was quite easy. We used the method of colchicine treatment of secondary auxiliary shoots. The putative transgenic plants developed under greenhouse conditions to the fruiting stage and their seeds were collected.

Confirmation of transformation: plant DNA isolation and PCR analysis

Genomic DNA was isolated from leaf tissues by the modified CTAB method of McGarvey and Kaper (1991). The isolation procedure was modified by additional phenol and chloroform extraction of crude preparations before nucleic acid precipitation. DNA preparations were quantified with agarose gel electrophoresis by visual comparison of the samples with pUC18 DNA digested with *Eco*R I as a quantity standard.

Polymerase chain reactions (PCR) were performed in a volume of 20μl containing about 10ng of plant genomic DNA, 10mM Tris-HCl pH 8.8, 50mM KCl, 0.1% Triton X-100, 1.5mM $MgCl_2$, 0.2mM dNTP, 50μM of each primer (primer F: 5'- TGA GCC CAG AAC GAC GCC-3'; primer R: 5'-GGA CTT CAG CCT GCC GGT AC-3'; internal primers of the *pat* gene were designed to amplify the 516bp DNA fragment) and 1 unit *Taq* DNA polymerase (Finnzymes). The PCR programme was set as follows: initial denaturation at 94ºC for 4 min,

next 35 cycles of denaturation at 94ºC for 1 min, annealing at 65ºC, elongation at 72ºC for 1 min, and a final extension at 72ºC for 5 min.

Screening of plant genomic DNA samples for *Agrobacterium* DNA residual contamination was done by PCR analysis. Approximately 0.5 ng of *Agrobacterium* DNA was used in control reactions. Total bacterial DNA was extracted as follows: a sample of bacteria was suspended in 200µl of water, next boiled for 10 min and centrifuged. The supernatant was used as a PCR template. Amplification reactions were performed under similar conditions as above, except primers and PCR programme. Internal primers (primer F: 5'-TTG ACC TTG TTT CAG GTT TAC ACA-3'; primer R: 5'-GAC GAG GAT AAT CAT CAT CGA AAC-3') of agrobacterial, chromosomal *chvA* gene were designed to amplify the 777bp DNA fragment. The PCR programme has been performed as previously, except annealing at 62ºC.

Polymerase chain reactions were performed in a PTC-200 MJ Research thermal cycler. The amplification products were analysed by agarose gel electrophoresis. Molecular weights were estimated with the use of 1kb ladder (Sigma) as a standard.

Results and discussion

Over the last 30 years a great deal of effort has gone into improving the quality of *Brassica napus* by both classical breeding and several tissue culture techniques, such as haploids and doubled haploids. Genetic engineering potentially can be used as a method for adding specific characteristics to existing varieties.

Brassica microspore embryogenesis is a very desirable system for the introduction of foreign genes (Huang, 1992). The transformation of microspores directly yields green haploid embryos which can be recovered without the complication of chimeras. To optimize a microspore-based transformation system, highly efficient and reproducible embryogenesis is essential. Transformed embryos developed 1-2 weeks slower than controls conducted at the same time. Recently, we have developed a protocol for efficient microspore embryogenesis and doubled haploid production for winter oilseed rape (Cegielska-Taras et al. 1999, Cegielska-Taras et al. 2001). The most common herbicide resistance genes used for transformation of *Brassica* are *bar* and *pat*, which confer resistance to phosphinothricin, the active ingredient of the herbicide Basta.

In experiments with microspores and the *bar* gene we regenerated more than 20 putative transformants. In a typical experiment with infection by *Agrobacterium tumefaciens* on the third day of microspore culture we can recover up to 30 embryos. The embryos on solid medium with the selective agent did not develop normally. Approximately five to ten embryos from each series survived in selective medium. Shoots were regenerated from

survived embryos through secondary embryogenesis (Cegielska-Taras, Szała 1997). After rooting, normal plants developed. All the plants were haploids. After chromosome doubling, seeds from each putative transformant were obtained. The results show that a high frequency of microspore embryogenesis is necessary for high transformation frequencies.

Cocultivation of *A. tumefaciens* with microspore-derived embryos is a very convenient procedure because more than one hundred independent transformants have been produced thanks to the possibility of handling a great number of embryos in one experiment. The efficiency of the procedure was improved through using microspore-derived embryos after cold treatment. The cold treatment stimulates shoot regeneration very efficiently. Shoots developed readily from embryos that survived in the selective medium. The method is attractive also because of the high regenerative potential of the embryos.

To detect the presence of the *bar* gene in the regenerated plants and confirm that transformation was effective, PCR analysis was made. In putative transgenic plants it indicated amplification of a 516bp fragment of the *bar* gene (Fig. 1). The first results of PCR analysis showed that 8.7% of plants regenerated from transformed microspores and about 30% of plants regenerated from transformed microspore-derived embryos were putative transformants.

A. tumefaciens survives in plant tissue and occupies intercellular spaces for some time after transformation. This might falsify the results of molecular analysis (PCR). Screening of plant genomic DNA samples for *Agrobacterium* DNA residual contamination was done by PCR analysis. *Agrobacterium* DNA was used in control reactions. Amplification reactions were performed under similar conditions as above, except primers and PCR program. Internal primers of agrobacterial, chromosomal *chvA* gene were designed to amplify the 777bp DNA fragment. For this reason PCR analysis was done with a starter specific to *Agrobacterium* gene *chvA*, which is situated on the bacterial chromosome (Fig. 2).

A further molecular analysis, i.e. Southern blotting and GUS expression of the putative transgenic plants, will be done soon as it can confirm the positive results of the process of transformation.

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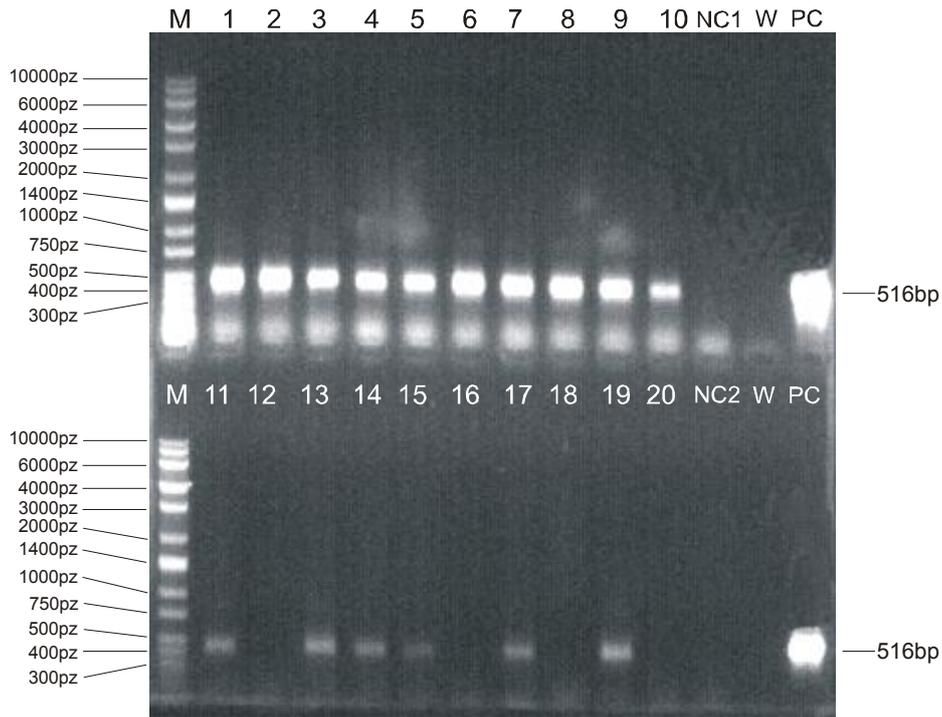
Fig.1.
PCR analysis of putative winter oilseed rape transgenic plants showing amplification of 516bp fragment of *bar* gene.
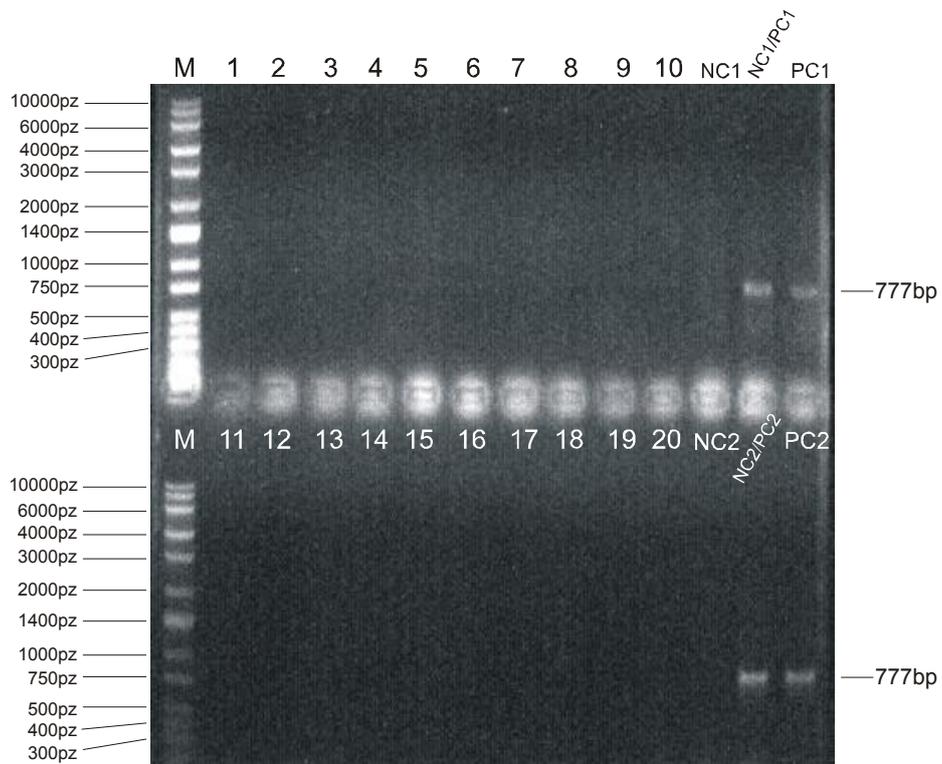

Fig.2.
Screening for *Agrobacterium* DNA traces in genomic DNA samples of putative transgenic winter oilseed rape plants. PCR analysis performed on plant and control DNA templates showing results of amplification of 777bp fragment of agrobacterial chromosomal *chvA* gene

Lanes:

M   – DNA size marker, wide range DNA marker (Sigma)

1-5   – DH line O-120 plants, after MDE transformation using *A. tumefaciens* LBA4404

6-10   – cv. BOR plants, after MDE transformation using *A. tumefaciens* LBA4404

11-12 – DH line O-120 plants, after MDE transformation using *A. tumefaciens* EHA105

13-16 – cv. BOR plants, after MDE transformation using *A. tumefaciens* EHA105

17-18 – cv. BOR plants, after microspore transformation using *A. tumefaciens* LBA4404

19-20 – cv. BOR plants, after microspore transformation using *A. tumefaciens* EHA105

NC1 – negative control 1 - untransformed DH line O-120 plant

NC2 – negative control 2 - untransformed cv. BOR plant

W – water

PC – positive control -pP35SGIB plasmid

PC1 – positive control 1 – DNA of *A. tumefaciens* LBA4404

PC2 – positive control 2 – DNA of *A. tumefaciens* EHA105

NC1/PC1 – plant DNA of DH O-120 line mixed with DNA of *A. tumefaciens* LBA4404

NC2/PC2 – plant DNA of cv. BOR mixed with DNA of *A. tumefaciens* EHA105